P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Review

Advances in gene therapy of liver cirrhosis: a review

Wen Jie Dai and Hong Chi Jiang

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INTRODUCTION

Liver fibrosis or cirrhosis is a common progressively pathological lesion of chronic liver diseases in response to various liver-damaging factors. The main mechanisms of fibrotic or cirrhotic initiation and progression at the level of cellular and molecular events have been elucidated in the past two decades[1,2]. Various causes, including hepatitis virus infections, toxification, ischemia, congestion, parasites infection, abnormal cooper or iron load, etc, result in chronic inflammation and/or wound healing responses, of which the main characteristics manifest in the absolute increase of the excessive extracellular martrix (ECM) synthesis and the relative decrease of them, leading to ECM deposit. With the stimulation of inflammation or toxins, activated hepatic stellate cells(Ito cells), injuried or regenerated hepatocytes, Kupffer cells, sinusoidal cells and natural killer (NK) cells produce certain cytokines or immunoreactive factors, which exert various biological effects on their respective target cells or organs in an autocrine or paracrine manner. These consist of the cellular basis of hepatic fibrosis advances[3,4]. It is the vital molecular event in fibrosis progression that activated hepatic stellate cell exocrine ECM components and fibrosis-implementing factors, for example, the transforming growth factor β (TGF-β), which is considered the key cytokine to accelerate cirrhotic procession[5,6]. Various factors participate in fibrosis or cirrhosis formation. They could be simply divided into the fibrosis-implementing factors, such as TGF-β, platelet-derived growth factor (PDGF), epithelial growth factor (EGF), and antifibrotic factors, such as interferon γ and interleukin-10. These cytokines play important and unique roles in the interactive complicated network. Excessive ECM deposit, disappearance of sinusoidal endothelial fenestra and subsequent capillary vascularization cause the dysfunction of hepatocytes. The unblocked progressively pathological lesions with inevitably result in, lobular reconstruction, pseudolobule formation and nodular regeneration. With the elucidation of vital cellular and molecular events, gene delivery strategies for treatment of liver fibrosis or cirrhosis emerge on the basis of gene manipulations. Our interest focuses on the recent advances of gene therapy for liver fibrosis or cirrhosis.

Effective antifibrotic treatments, including medicinal or gene therapy, should satisfy several essential criteria. First, any therapy should have a sound biological basis. Additionally, the pharmacologically active drugs or compounds should reach or reside in the liver at a high concentration and have little side effect outside the liver. Specific targeting to hepatic stellate cells were preferentially chosen. Finally, in the ideal case, a therapeutic agent should keep the regional target environment for a relatively long period[7].

BLOCKADE OF TGF-β SIGNALING

It has been demonstrated that TGF-β is of great importance in fibrogenesis, and serves as a pivot in regulation of fibrogenic and antifibrogenic mediators. TGF-β exhibits stimulatory or inhibitory properties on cellular proliferation and differentiation, and is considered to represent a fundamental regulatory molecule acting through autocrine and/or paracrine mechanism[8].

TGF-β is clearly associated with hepatic fibrogenesis and cirrhosis in experimental animal models and in human liver diseases[9,10]. It regulates the gene expression of Ito cells, which in the course of liver fibrogenesis, proliferate and transform into the myofibroblast-like cells, synthesizing large amounts of connective tissue components, and also being involved in the ECM degradation by production of matrix metalloproteinases (MMP) inhibitors of MMP (TIMP), etc[11-13]. Because of the key role of TGF-β and Ito cells in the procession of liver fibrogenesis, lots of studies focus on their

Second Department of General Surgery, the First Clinical School, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Dr. Wen Jie Dai, graduated from seven-year system of Harbin Medical University in 1998, and now is a resident surgeon and doctoral student in the Second Department of General Surgery, the First Clinical School, Harbin Medical University, Harbin, China. Hismain research field is hepatobiliary and splenic surgery.
Correspondence to: Dr. Wen Jie Dai, Second Department of General Surgery, the First Clinical School, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Tel. 0086-451-3602829, Fax. 0086-451-3670428
Email. wenjiedai@etang.com
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interactions. In *in vitro* studies, Ito cell cultures were found to be activated by TGF-β to increase the production of collagen type I, II, III, fibronectin, undulin and proteoglycans[14-16]. TGF-β enhanced the Ito cell transformation from resting to activated cells and inhibited Ito cell proliferation[17]. TGF-β signaling was mediated by specific receptors located on the target cell membrane. In non-activated Ito cells, type II and III were present, while in activated Ito cells, there were TGF-β/ activin receptor type I, II and III[18,20].

All the above showed clearly that anti-TGF-β intervention which could block the TGF-β signaling pathway may prevent liver fibrosis. Such interventions have come into being up to date. Qi *et al* have developed adenovirus-mediated gene transfer to rat liver for expressing a truncated type II TGF-β receptor[21]. It inhibits TGF-β activity by competing with binding of the cytokine to endogenous TGF-β receptors. The expression vector, AdCAT β-TR, was administrated via the portal vein for ensured infection and high-level expression in regional liver tissue.

Apparently, there needed a large excess of truncated receptor over the wild type full-length receptor for effective inhibition of TGF-β signaling[22,23]. Three days after AdCAT β-TR administration, two mRNAs corresponding to the truncated human receptor and the rat full-length receptor (5.5kb and 0.9kb) were detected respectively, and the former mRNA was about 20-fold that of the latter. Pathological examination showed that the extent of liver fibrosis, collagen type I, fibronectin, alpha smooth muscle actin (α-SMA), TGF-β 1 and monocytes/macrophages (ED-1) decreased significantly in AdCAT β-TR administration group. This inhibitory effect was observed in all areas of the liver. The content of liver hydroxyproline had no significant difference between AdCAT β-TR group and the intact group, while in the control group of dimethylnitrosamine (DMN)-treated persistent fibrotic rats, the amount of hydroxyproline was 3.4 times higher than that of the above two. It was evident that the activation of Ito cells and Kupffer cells was inhibited, suggesting that truncated type II TGF-β receptor gene transfer could prevent or abolish liver fibrogenesis effectively. More important, liver function of rats with AdCAT β-TR injection recovered with a marked decrease of serum hepatocyte enzyme AST and ALT[21].

Reports from Ueno *et al*[24] supported Qi *et al*'s results as well. They conducted an recombinant adenovirus expressing the entire extracellular domain of the human type ¢ò TGF-â receptor fused to the Fc portion of human immunoglobulin G (AdTβ-ExR) to produce a circulating soluble form of TGF-β receptor with remote vital organ targeting after intramuscular injection of AdT β-ExR. Soluble TGF-β receptor was detectable in circulation and its level peaked at 5-7 days after the injection. AdT β-ExR-infected COS cells secreted fused type II TGF-β receptors, which binded to TGF-β and consequently blocked the TGF-β signaling as the full-length TGF-β receptor did[24]. Injection of AdT β-ExR into the femoral muscle of DMN-induced fibrotic rats prevented hepatic fibrosis, while no side effects or complications were found either macroscopically or microscopically in the main organs including brain, heart, lung, liver and kidney. Histological examination in DMN-treated rats with i.m. injection of AdT β-ExR was essentially as the same as that in Qi *et al*'s report, which locally expressed truncated type II TGF-β receptor in the liver via the administration of AdCAT β-TR into the portal vein[21,24]. This research also disclosed that adenovirus-mediated expression of soluble TGF-β receptors exerted the binding effect to TGF-β in a TGF-β specific manner, that is, others, e.g., PDGF α-receptor and fibroblast growth factor (FGF), could not interfere with TGF-β. Although a large excess of soluble TGF-β receptor seems essential for blocking of TGF-β signaling, there was still apparent difference between specific soluble TGF-β receptor and non-receptor-specific binding proteins, e.g., decorin (would prevent TGF-β induced renal fibrosis), for blockade of TGF-β signaling[25]. Because the latter was lack of receptor specificity and selectivity when it binded to TGF-β, it may bind to other biological molecules, probably resulting in unexpected problems. More exact mechanism was also explored[26-29]. There was evidence that truncated type II TGF-β receptor may form a complex with other type TGF-β receptor and inhibit both the anti-proliferative effect of TGF-β and/or the transcriptional activation by TGF-β[30-31]. Each type of three TGF-β receptor isoforms may have its own distinct signaling pathway, each mediating a separate action setting of TGF-β[31]. Complete inhibition of TGF-β signaling was achieved via local expression of a kind of soluble TGF-β receptor in a remote area from the target organ. The fact suggested that such strategy should be applicable theoretically and therapeutically for other factors belonging to growth factor superfamily.

As a matter of fact, signaling pathways introduced by PDGF, FGF, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin, etc, could be abolished specifically by their corresponding kinase-defective mutated form of receptors[23,32-36]. This kinase-defective or kinase-truncated receptors for signal transduction blockade of their growth factor may be a useful strategy for analysis of the *in vivo* role of these growth factors, because signaling pathway was blocked at the receptor level, not at the cytokine or gene level, without affecting the normal physiological status of these growth factors or signal transfer of other receptors[21,22,37]. Although no side effects were found during the study period, special attention should be paid to the surveillance

of the possible hazards, since Shull *et al* had demonstrated that there would be multifocal inflammation and tissue necrosis in TGF-β gene disrupted mice[38]. Adenovirus displayed the high degree of hepatotrophic activity. It could also mediate the targeted expression even if it was administrated in a remote site[21,24]. Immune responses would be elicited after repeated injection of adenovirus particles, therefore may lower the effectiveness of target gene expression.

STIMULATION OF HEPATOCYTE REGENERATION

In the course of liver fibrosis, the absolute and relative volume of hepatocytes was both reduced under the direct or indirect effect of liver-damaging factors. Stimulation of hepatocyte regeneration is the essential strategy in choice of treatment. Hepatocyte growth activators include an expanding list of complete mitogen and comitogen. Complete mitogen are defined as those that are capable of stimulating DNA synthesis and mitosis of cultured hepatocytes in serum-free media. Hepatocyte growth factor (HGF), EGF, TGF-α, and keratinocyte growth factor are in this category. Comitogens enhance the stimulatory action of complete mitogens and decrease the inhibitory effect of other inhibitors, while they have no direct proliferation-enhancing effect on cultured hepatocytes, including glucagon, insulin, insulin-like growth factors, adrenal cortical hormones, vasopressin, angiotensin, thyroid and parathyroid hormones, norepinephrine, as well as calcium and vitamin D[39]. HGF was regarded as the strongest hepatocyteproliferative agent up to date. It was a pleiotropic factor with potent morphogenic, mitogenic and motogenic effects and has anti-apoptotic action on hepatocytes and other several cells in culture, exhibiting a plethora of effects in many systems and organs[40-49]. The antifibrotic effect of HGF on hepatic fibrosis was first characterized by Yasuda *et al*[50]. In their experiment, the deletion variant of hepatocyte growth factor (dHGF) which was more mitogenic than full-length HGF, was used to examine its role in preventing fibrosis induced with dimethylnitrosamine in rats. Northern hybridization and immunohistochemical staining elucidated clearly the antifibrotic effect of dHGF on DMN-treated rats. The main mechanism appeared to be suppression of Ito cell activation, as demonstrated by the reduction of mRNA of procollagen α2(I), α1(III), α1(IV), TGF-β, desmin and α-SMA. Collagen content, as measured by hydroxyproline, was also decreased. Futhermore, dHGF exerted the mitogenic and antifibrotic activities even after the hepatic fibrosis had been established with DMN[50]. Their study showed that the antifibrotic effect of dHGF is not derived from the possible effect of dHGF on cytochrome P450-dependent metabolic degradation of DMN, but from the ability of dHGF to reduce or inhibit the TGF-β mRNA level[50,51], proliferation and activation of lipocytes and to stimulate liver regeneration[50]. Burr *et al* also demonstrated the vital role of HGF in liver regeneration[52]. After treatment with anti-HGF monoclonal antibody, the serum level of immunodetectable HGF was inhibited, as well as the parenchymal proliferative response to acute CCl_4-induced liver injury. Ueki *et al* developed a novel new gene therapy approach for rat liver cirrhosis by muscle-mediated gene transfer of HGF cDNA[53]. Plasmid containing HGF cDNA was embedded in the liposome, fused to the envelope protein of hemagglutinating virus of Japan (HVJ). This HVJ-liposome complex was intramuscularly injected repeatedly to achieve therapeutically detectable expression level of HGF. Repetitive administration resulted in a sound level of human HGF and increased level of endogenous rat HGF. The number of proliferative cellular nuclear antigen (PCNA) positive cells in HVJ-liposome-HGF injection group was markedly higher than that of control group. Apoptotic hepatocytes decreased significantly as well. The c-met, HGF-specific receptor located on the surface of hepatocytes, increased considerably in HVJ-liposome-HGF treated rats and stayed at this high level even after the repeated DMN administration, meanwhile strong tyrosine phosphorylation of c-met was observed. Immunohistochemistry revealed that with the administration of HVJ-HGF-liposome, staining of TGF-β1, expressed notably in persistent cirrhotic liver, decreased, as well as desmin and α-SMA, which were indicators of activated Ito cells. It suggested that expressed HGF could inhibit activation of Ito cells, decrease the synthesis of collagen type I and TGF-β, and prevent apoptosis of hepatocytes induced by DMN administration. After several injections of HVJ-HGF-liposome, complete remission was achieved, demonstrated by histology[53]. It can be inferred that HGF not only stimulated hepatocyte regeneration, but also remodeled the disorganized cirrhotic tissue. That is the pivotal potential for HGF gene therapy of liver cirrhosis[54]. It is of particular interest that HVJ-liposome was the ideal vehicle for gene transfer, because of the high efficiency, simplicity and lack of toxicity[55]. Another aspect of HGF on promoting neovascularization in certain ischemic diseases was also focused for possible application in the treatment of liver cirrhosis. Aoki *et al* delivered HGF cDNA into the myocardial infarction region in rats[56]. It promoted the neovascularization strongly in infarcted myocardium, including capillary, small- to medium-sized vessels. As an endothelium-specific growth factor with potent motogenic activity to endothelial cells, HGF's angiogenestic activity was also confirmed by the activation and upregulation of a transcription factor-ets, which is essential for angiogenesis[56]. Immunohistochemical staining revealed positive expression of ets 1 in endothelial cells and vascular smooth muscle cells (VSMC)

around the neovascularized vessels. The ets family participated in angiogenesis regulation by activating the transcription of several genes, including collagenase I, stromelysine 1 and urokinase plasminogen activator, which are proteases involved in ECM degradation, as well as migration of endothelial cells[57]. Overexpression of exogenous HGF activated ets family, initiated auto-loop up regulation mechanism of HGF resulting in up-regulation of endogenous HGF expression[53,56], since HGF gene promoter region comprised several putative regulatory elements, such as B cell- and macrophage-specific transcription factor binding sites (PU1/ETS), besides an interleukin-6 response element (IL-6RE), a TGF-β inhibitory element (TIE), and a cAMP response element (CRE)[56,58].

Another candidate for stimulation of hepatocyte proliferation is hepatic stimulatory substance (HSS), which was first described by LaBrecque *et al* in 1975. As a heat-stable stimulator for regenerating liver with partial hepatectomy, it is considered to be the unique liver-specific stimulatory factor and is universally located in mammal embryonic liver, weanling liver and regenerating liver[59]. In 1994, Hagiya *et al* obtained a gene product in rats, called augmenter of liver regeneration (ALR) with a full-length of 1.2kb and open reading frame of 378bp[60]. The molecular weight of ALR was approximately 30kDa, as a homodimeric complex[60,61]. The recombinant ALR expressed from ALR cDNA-transduced COS cells exerted potent and dose-dependent proliferative activity tested in canine Eck fistula (portal caval shunt) model[60]. Subsequent published reports have gradually come to a common view that ALR is actually the HSS. Yang *et al* cloned the human ALR gene in 1996[62,63]. Other groups also successfully cloned the rat and human ALR cDNA subsequently[64,65]. In comparison of the ALR encoding sequences of human and rat, homology of nucleotide sequence is about 87%, and that of protein sequence was 84.8%. Rat, mouse and human ALR genes and protein products were highly conserved and preferentially expressed in the testis and the liver, probably being involved in the synthesis and stability of the nuclear and mitochondrial transcripts, especially in spermatogenesis in actively regenerative tissues. Wang *et al* demonstrated that ALR or hepatopoietin (HPO) exerted the biological activity via the high affinity receptors for HPO on the surface of rat hepatocytes and human hepatoma cells. Each of the above cells has a mean of approximate 10 000 and 55 000 receptor sites respectively, as was identified with the binding of radioisotype ^{125}I-labelled HPO to a 90kDa polypeptide[66]. Updated research by Gandhi *et al* showed a certain new aspect of ALR funtion[67]. Hepatic levels of ALR decreased for 12 hours after 70% hepatectomy in adult rats and rose with no corresponding increase in ALR mRNA transcripts, while serum ALR level increased up to 12 hours. This implied that ALR was constitutively expressed in hepatocytes in an inactive form, and appeared to be an active form after being released outside the hepatocytes in response to partial hepatectomy or other regeneration-promoting circumstances[67]. Liver-derived NK cells are important regulators for liver regeneration[68]. Francavilla *et al*[69] connected the regulation activity of ALR in liver regeneration with the novel role of NK cells. Francavilla *et al* administrated three hepatotrophic factors (ALR, insulin-like growth factor II [IGF-II] and HGF) with the surely inducing sound hepatotrophic activities to assess the *in vivo* effects on NK cells in normal rats. Results showed that NK cell cytotoxic activities were inhibited in the population of mononuclear leukocytes (MNL) in the liver (liver-resident NK cells), but not in the MNL from the spleen or peripleral blood. Results obtained *in vitro* displayed that ALR, IGF-II and HGF had no effect on NK cell function in cultured MNL from the liver, spleen or blood[69]. Results from Polimeno *et al*also verified that ALR plays a pivotal role as growth factor and as immunoregulator by controlling the mitochondrial transcription factor A expression and lytic activity of liver-resident NK cells through IFN-γ levels[70] and regulates hepatocyte proliferation through enhancing cytochrome content and oxidative phosphorylation capacity of liver-derived mitochondia[71]. Liver injuries could also be prevented with recombinant ALR injected intraperitoneally[72-74]. Gene transfer of human ALR gene into cirrhotic rat may be an meaningful tool for reversing liver cirrhosis. Interestingly, no close or stringent homology was found between ALR and other polypeptide growth factor, while ALR has both structural and functional homology compared with yeast scERV1 gene which is involved in biogenesis of mitochondria and regulation of cell cycle, which may imply ALR's evolutional conservation and essentialness to growth and development[60,66].

INHIBITION OF INTERSTITIAL INFLAMMATION

Interstitial inflammation was characterized as the main pathological changes in fibrogenesis. Those inhibiting inflammatory responses would also be the target for gene transfer to block the fibrogenic inflammation. Interleukin-10 (IL-10) is suitable for this strategy. IL-10, originally identified as a cytokine synthesis inhibitory factor, inhibits production of a variety of cytokines in various cell types. It is produced under different conditions of immune activation by the T-H 2 and TH0 subsets of helper T cells, as well as by monocytes, macrophages, B cells, keratinocytes and stromal cells[75]. Sequences of open reading frame, not 5'- or 3'- flanking regions of mouse and human IL-10 cDNA showed high homology to two viruses,

Epstein-Barr virus and equine herpes virus[76]. Acquiring a mammalian IL-10 homology and subsequent inhibitory properties of IL-10 toward macrophages may help the virus escape from the antiviral immune attacks. IL-10 exerted multiple biological activities, including inhibition of T-H1 cells and antigen-presenting cells, stimulation of mast cell proliferation, protease expression, stimulation of B cell proliferation, antibody secretion and major histocompatibility complex II (MHC II) expression[75]. IL-10 takes part in the sophisticated regulation of fibrogenesis. In IL-10 gene knockout (KO) mice treated with tetrachloride (CCl_4), there existed no difference in hepatic toxicity of CCl_4 between KO mice and wild type (WT) mice.

In CCl_4-induced acute liver injury mice, serum TNF-α and TGF-β levels were markedly high in KO mice than that in WT mice. Administration of recombinant IL-10 inhibited cultured Kupffer cells producing superoxide radicals and TNF-α *in vitro*. In CCl_4-induced chronic liver injury model, the degree of hepatic fibrosis was severer and the level of tissue TNF-α was higher than that of WT mice. IL-10 knockout also seemed to enhance monocyte infiltration [77,78]. Up-regulation of IL-10 mRNA was found in both freshly isolated quiescent and activated Ito cells. Co-transfection of Ito cells with an IL-10 expression vector and collagen reporter genes showed a 40% inhibition of α1 (I) collagen promoter activity, suggesting the *in vivo* role of IL-10 in matrix remodeling and the possibility that failure for Ito cell to sustain IL-10 expression underlied pathologic progression to liver cirrhosis[79,80]. Kupffer cell also played a pivotal role in production of IL-10. Reports from Rai *et al*[81] revealed the subtle role of IL-10 in regulating production of TNF-α, a hepatocyte proliferative factor initiated after 70% partial hepatectomy. Kupffer cell depletion induced by gadolinium chloride (GdCl) abolished induction of IL-10, then elongated half-life of TNF-α mRNA. Overexpression of TNF-α promoted liver regeneration potently after partial hepatectomy. Meijer *et al* strengthened the common point of view that Kupffer cell depletion, physically induced with dichloromethylene-diphosphonate, resulted in an imbalanced hepatic cytokine expression, thereby suppressing important growth-stimulating factors, including HGF and TNF-α[82]. Similar results were found in alcoholic cirrhosis[83]. Contraversy existed with respect to effects of IL-10 on extracellular matrix regulation, for IL-10 was likely to have different effects relying upon the differently detailed experimental materials and conditions. In general, IL-10 inhibited α1 (I) collagen gene expression [79,84] stimulated collagenase (MMP-1), stromelysin (MMP-3)[84], gelatinase (MMP-9)[85] expression and elastin promoter activity.

Antifibrotic effect of IL-10 on hepatic fibrosis was also correlated with its inhibition of TGF-β expression in fibrotic tissue, which was verified as the key fibrotic regulator as stated above. This mechanism was also verified in other experiments[82,86]. As an inhibitory regulator with pleiotropic nature, IL-10 may be used as a target gene for gene transfer in the treatment of liver cirrhosis, based on the potent capacity of suppressing production of proinflammatory cytokines[75,77,78,82,83,87-92]. Nelson *et al* showed an exciting report that IL-10 could reduce liver fibrosis and normalize serum ALT levels in IFN-γ unresponsive hepatitis C patients[93].

TELOMERE AND TELOMERASE WITH CIRRHOSIS

Telomere is a special cap-like structure at the end of eukaryotic chromosomes, composed of a tandemly (TTAGGG)-rich repeat DNA sequences and relative catalyzing proteins. Length of telomere within a certain range is essential and vital for normal mitosis, for it enhances the stability of end chromosome, prevents abnormal chromosomal rearrangement and end-end fusion, and protects against the degradation or destruction by nuclease and/or ligase. When telomere was shortened to a checkpoint range of 2-4kb, the stabilization of chromosomes would collapse. Maintenance of telomere length depends on the telomerase, which consists of RNA and two protein subsets and serves as RNA-dependent DNA polymerase to achieve complete and entire replication of chromosome. There are large amounts of evidence to demonstrate that telomerase takes part in complex regulation and plays a crucial role in cell proliferation, aging, immortalization and tumorigenesis[94]. Except for the lymphocytes of peripheral bood, hematopoietic stem cells, germ cells, embryonic somatic cells and those resident in actively proliferating tissues, for example, hair, skin and endometrium, also have telomerase activities[95]. Other tissues exert no telomerase activity.

In cirrhotic liver, evidence verified no or a variety of low activity of telomerase, while telomere length decreased commonly[96-100]. Hytiroglou *et al* found a clear cut difference in telomerase activity levels between hepatocellular carcinoma (HCC) (positive or strongly positive) and cirrhotic liver tissues (weakly positive or negative). They considered that activation of telomerase was an early event in larger nodular cirrhosis formation, and consequently exerted facilitating effect on other factors in the progression of carcinogenesis[101]. A proportion of 86% of large noncancerous nodules exhibited similar telomerase activity to HCCs, and part of it was derived from large regenerative nodules, but not dysplastic nodules[101].

The importance of maintenance of telomere length has been verified in cultured telomerase-negative human retinal pigment epithelial cells and foreskin fibroblasts, transfected with vectors

encoding the human telomerase catalytic subunit[102]. A comparison between human telomerase reverse transcriptase subunit (hTRT) gene transfer group and control group showed that with hTRT gene transfer, the telomerase-negative cells displayed normal phenotype and karyotype, as well as exceeded their normal life-span by at least 20 doublings. The fact was also demonstrated by Kiyono *et al*[103]. Results obtained from telomerase-deficient mice verified the crucial role of telomerase and maintained telomere in development, which owned the nature of progressive telomere shortening from one generation to the next, for lack of active telomerase[104,105].

Based on the elucidation of telomerase's role in senescence and tumorigenesis, Rudolph *et al* brought new insight into the mechanism of telomerase gene transfer (mTR gene) in the treatment of liver injury and cirrhosis. They adopted three systems to gauge how telomere shortening influenced hepatocyte proliferation, survival and ultimately predisposition to cirrhosis[106]. The first was the albumin-directed urokinase plasminogen activator (Alb-uPA) transgenic mouse, in which Alb-uPA expression would cause widespread hepatocytes death and fatal liver failure in newborn mice. Results showed a progressive decline in telomere length of peripheral blood lymphocytes from the first generation (G1) to the third generation (G3), with a 3.6-fold increase in apoptotic hepatocytes and a decrease in proliferative hepatocytes. The second and third system was partial hepatectomy and CCl_4 mediated liver injury, respectively. Flow cytometry verified that telomerase dysfunction inhibited mitosis of mice hepatocytes. Pathology showed abnormal mitosis and formation of anaphase bridges in G6 $mTR^{-/-}$ mice. Induced apoptosis of hepatocytes delayed the volume recovery of hepatectomized liver. Repeated administration of CCl_4, caused liver cirrhosis first in G6 $mTR^{-/-}$ mice, followed by G3 $mTR^{-/-}$ mice and finally by $mTR^{+/+}$. It suggested that with the shortening of telomere, damage-resistant capacity of parenchymal cells decreased and cirrhosis was prone to be induced.

As gene transfer vector, adenovirus revealed good hepatotrophic property (an infection rate of 85%-100%). Telomere was shortened by about 70% in G6 $mTR^{-/-}$ mice, however, after mTR gene transfer, ascites reduced with gain of body weight, and serum levels of ALT and AST improved with active proliferation of hepatocytes. Compared with $mTR^{-/-}$ mice, mTR-transferred G6 $mTR^{-/-}$ mice survived during the experimental stage with 66% decrease of mitosis index and 58% reduction of anaphase chromatin bridges. No TGF-β was detected in mTR-transferred G6 $mTR^{-/-}$ mice, implying that mTR transfer may block TGF-β signaling and/or inhibit activation of Ito cell and TGF-β secretion. Target transfer of telomerase gene into affected organ created a new strategy and also a novel pathway for the treatment of aging and certain telomerase-related chronic disease.

Before actual application, over-high activity of telomerase may induce instablility of genomic structure and tumor formation. From a clinical point of view, telomerase gene therapy may be an ideal choice of treatment for patients awaiting liver transplantation, for it could actually improve liver function and extend survival, and the eventual surgical removal of diseased organ should at most minimize the potential cancer risk [105]. Telomerase gene therapy may be regarded as an effective short-term supporting procedure for patients with end-stage liver disease before carrying out of liver transplantation[107].

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Original Articles

Inhibitory effect and mechanism of acarbose combined with gymnemic acid on maltose absorption in rat intestine

Hong Luo[1], Le Feng Wang[2], Toshiaki Imoto[1] and Yasutake Hiji[1]

Subject headings diabetes mellitus; maltose; gymnemic acid; alpha-glucosidases; intestinal mucosa; rats; nutrition

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Abstract

AIM To compare the combinative and individual effect of acarbose and gymnemic acid (GA) on maltose absorption and hydrolysis in small intestine to determine whether nutrient control in diabetic care can be improved by combination of them.

METHODS The absorption and hydrolysis of maltose were studied by cyclic perfusion of intestinal loops in situ and motility of the intestine was recorded with the intestinal ring *in vitro* using Wistar rats.

RESULTS The total inhibitory rate of maltose absorption was improved by the combination of GA (0.1g/L - 1.0g/L) and acarbose (0.1mmol/L - 2.0mmol/L) throughout their effective duration ($P < 0.05$, *U* test of Mann-Whitney), although the improvement only could be seen at a low dosage during the first hour. With the combination, inhibitory duration of acarbose on maltose absorption was prolonged to 3 h and the inhibitory effect onset of GA was fastened to 15 min. GA suppressed the intestinal mobility with a good correlation ($r = 0.98$) to the inhibitory effect of GA on maltose absorption and the inhibitory effect of 2 mmol/L (high dose) acarbose on maltose hydrolysis was dual modulated by 1 g/L GA in vivo indicating that the combined effects involved the functional alteration of intestinal barriers.

CONCLUSION There are augmented effects of acarbose and GA,which involve pre-cellular and paracellular barriers. Diabetic care can be improved by employing the combination.

[1]Departments of Physiology, and [2]Internal Medicine, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan
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Correspondence to: Dr. Hong Luo, Department of Physiology, the Institute of Basic Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, 5 Dongdansantiao Beijing 100005, China
Tel. 0086-10-65296463, Fax. 0086-10-67611572
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INTRODUCTION

Hyperglycemia and hyperinsulinaemia are characters of non-insulin-dependent diabetes mellitus (NIDDM). Their interaction further impairs the action and secretion of insulin in NIDDM[1]. With the aim of reducing the possibility of developing long-term various complications such as microvascular disorder or neuropathy[2], a good diet regimen and control of nutrient entry are broadly accepted as basic treatment for diabetes mellitus.

Acarbose, an alpha-D-glycosidase inhibitor, was first extracted from the culture broths of actinomycetes by Puls and his colleagues in the 1970s, and was applied in clinical studies for more than 10 years[3-5]. It reversibly inhibits alpha-glycosidases that exist in the brush-border of the small intestinal mucosa[6]. Its efficacy has been reported as a potent inhibitory effect on sucrose hydrolysis, but a weak effect on the maltose. For instance, in humans, even if high doses of 300 mg of acarbose are orally used, there is little effect on the absorption of maltose[7] and no obvious effects on body weight have been observed in most studies possibly due to its short effective duration. During daytime, blood glucose levels were hardly decreased although the postprandial blood glucose amplitudes were reduced, that also due to its short effective duration[8]. In addition, there is no interaction with the intestinal Na^+/glucose- cotransporter[9], although an inhibitory effect on glucose absorption and an osmotic stimulus-mediating glucagon-like peptide-1 secretion has been reported[10,11].

Gymnemic acid (GA)[12], a mixture of triterpene glucuronides, which was found in the leaves of the Indian plant Gymnema sylvestre, not only inhibits glucose absorption in the small intestine[13], but also suppresses hyperglycemia and hyperinsulinaemia in an oral glucose tolerance test[14]. It is especially noteworthy that the body weight gain in fat rats is suppressed by GA treatment [15]. In an ordinary human diet, carbohydrates normally represent the quantitatively greatest part and are the primary source of energy. The typical diet contains far more starch than all other carbohydrates combined. More than 80% final products of intestinal carbohydrate digestion are glucose.

Maltose is a rather important product during starch hydrolysis. In this report, the combined effect of acarbose with GA on maltose absorption in

the rat small intestines is reported. The mechanism and application in perspective are discussed.

METHODS

Animals

Male 8 - 9 weeks old Wistar rats (body weight 300 g ± 25g) obtained from Shimizu (Kyoto), were housed in an air-conditioned room at 22℃ ± 2℃ with a natural lighting schedule for 1 to 3 weeks until the experiment began. They were fed with a standard pellet diet (Oriental Yeast CO., Tokyo) and tap water. Care and treatment of the experimental animals conformed to Tottori University guidelines for the ethical treatment of laboratory animals.

Perfusion of small intestine in vivo

A modified technique as elaborated by Yoshioka[13] was used in the small intestine perfusion experiment. Animals that had fasted overnight with free access to water were anesthetized by an intraperitoneal injection of 50 mg/kg body weight sodium pentobarbital (Dainabot, Osaka). After a catheter was inserted in the trachea, the abdominal cavity was opened. The small intestine of 30 cm length from the 2 cm caudal ward of Treitz's ligament was selected. Two L-shaped cannulae were inserted into each end of the intestine and connected with a peristaltic pump (SJ-1211H, Atto, Tokyo). After the abdominal cavity was closed, the intestinal loop was rinsed with Ringer's solution (145.4 mmol/L NaCl,5.4 mmol/L KCl, 1.8mmol/L $CaCl_2$ and 2.4 mmol/L $NaHCO_3$) for 60 min. Each chemical that was dissolved in Ringer's solution was continuously perfused through the intestine at a constant flow rate of 3 mL/min. The perfusing solution was adjusted to pH 7.5- pH 7.8 with NaOH and used at 37℃.

The animals were randomly grouped and exposed to 20 mL 10 mmol/L maltose (Sigma, ST. Louis) with or without GA (extracted by ourselves from Gymnema sylvestre) and acarbose (Bayer, Osaka) by cyclic perfusion through the loops for 1h (first perfusion), then 10 mmol/L maltose only was perfused for one hour again following an intestinal rinse with Ringer's solution for 30 min (second perfusion). To compare the combined and individual effective duration, 5 groups were chosen, in which the rinse time between the two perfusions was prolonged from 30 min to 60 min, 120 min and 180 min respectively. The perfusates at the first perfusion in the 5 groups were as follows: ① control group,10 mmol/L maltose; ② acarbose group, 2 mmol/L acarbose + 10 mmol/L maltose; ③ GAa2 group, 1 g/L GA + 2 mmol/L acarbose + 10 mmol/L maltose; ④ GAa 0.2 group, 1 g/L GAa + 0.2 mmol/L acarbose +10 mmol/L maltose; and ⑤ GA group, 1 g/L GA +10 mmol/L maltose.

Measurement of maltose absorption and hydrolysis

The samples (20 μL) from perfusion fluid were taken every 15 min during 1 h perfusion period, which were kept at 0℃ until next step to prevent the possibility of further hydrolysis. The maltose hydrolyzed by over dosage of alpha-glucosidase (Funacoshi, Tokyo) and glucose in sample was measured with the glucose-B-test-kit (Wako, Osaka)[16], and by the percentages of absorption and hydrolysis of maltose in perfusate were calculated.

Recording of isometric contraction of the intestinal rings

To explain the combined effect of acarbose and GA, the effect of GA on contraction of the intestinal smooth muscle was investigated *in vitro*. Fasted animals were sacrificed by stunning and exsanguinations. The intestines at the same position as the perfusion experiments were removed, cleaned of adhering fat and connective tissues in the serous membrane, and cut into transverse rings of 1cm wide. The rings were mounted on stainless steel hooks under 5 grams of resting tension and bathed in 25 mL organ bath at 37℃ in Locke's solution containing (mmol/L) NaCl: 154, KCl: 5.6, $CaCl_2$: 2.1, $NaHCO_3$: 2.4 and Glucose: 5.6, which was saturated with 95% O_2 and 5% CO_2. Tension was recorded isometrically using a force transducer (NEC Sanei, Tokyo) through its amplifier on an ink recorder (FBR-252A, TOA, Tokyo). Tissues were allowed to equilibrate at least 90min before the experiments begun. The rings were treated with GA and the alterations of auto-rhythmic contraction were recorded.

GA extraction

Dry Gymnema sylvestre leaves were obtained from Okinawa (Japan), from which GA was extracted with water, ethanol and diethyl carbonate by a slightly modified version of Imoto's method and freeze-dried into GA powder which was confirmed at 230 nm UV with a high-performance liquid chromatography (HPLC, Shimadzu SPD-6AV, Kyoto)[12].

Statistical and pharmacological analyses

Statistical analyses were performed with the *U* test of Mann-Whitney or ANOVA, which was indicated in the result when ANOVA was used, by StatView for a Macintosh computer. $P < 0.05$ was considered to be a significant difference.

The dose-response curves were analyzed by the least-squares fitting method using Cricket Graph for a Macintosh computer. The *r* (correlation coefficient) was tested with the limit table of *r*. If the value of *r* was under $P < 0.05$, the curve and function were accepted, from which the IC_{50} s (50% inhibitory concentrations) were calculated.

RESULTS

Dose-effect relationship and effective onset

The inhibitory effects of GA and acarbose, or both

of them, on 10 mmol/L maltose absorption obtained from the intestinal loop experiments *in situ* are illustrated in Figure 1 as a function of uptake time and compared at different doses of acarbose 0.2 mmol/L (Figure 1A) and 2 mmol/L (Figure 1B), respectively. Both agents inhibited maltose absorption significantly.The first point difference between the treated and control groups in maltose absorption was considered as the onset point of the inhibitory effect on maltose absorption. The duration from GA and/or acarbose perfused to that point was regarded as the onset time. The onset time was 30 min in the GA group and 15 min in both the acarbose and combining groups, in other words the longer onset time of GA was overcome by the combined. The inhibitory rates at 60 min were 50.7% ± 3.68% (1g/L GA), 51.72% ± 2.21% (0.2 mmol/L acarbose) and 68. 97% ± 2.71% (both of them), while at the high dose of acarbose they were increased to 80.68% ± 1.81% (2 mmol/L acarbose) and 72.27% ± 2.57% (both of them) indicating that the inhibitory effects were improved by the combination only in the lower doses during the first perfusion.

Time course of the inhibition

Time-effect curves of the GA and acarbose on absorption of maltose are shown in Figure 2. Even though the intestine was rinsed for 120 min, the altered maltose absorption remained at the inhibitory level in GA, GAa2 and GAa 0.2 groups. In the case of acarbose only application, the absorption returned to almost normal after 30 min rinsing ($P > 0.05$ *vs* control). The present results suggest that the short effective duration of acarbose be overcome by the combination. The peak effect appeared during the first perfusion in GAa2 and acarbose (2 mmol/L) groups and the second perfusion following rinse for 30 min in GAa0.2 and GA groups. The altered absorption of maltose was recovered in each group to the control level after 180 min rinse with Ringer's solution.

The IC_{50}

The acarbose dose dependently inhibited the absorption of maltose with IC_{50s} of 0.65 mmol/L, 0.27 mmol/L, 0. 22 mmol/L and 0.28 mmol/L ($r >$ or $= 0.99$) during 15 min, 30 min, 45 min and 60 min perfusion, respectively. The inhibitory effects were improved when combined with GA ($P < 0.001$ ANOVA). The IC_{50} s in an hour duration decreased to about 73% and 40% of acarbose with 0.1 g/L and 0.25 g/L GA, respectively. The inhibitory effects of GA ($IC_{50} = 0.85$g/L, $r = 0.99$) were also improved by lower doses of acarbose ($P < 0.001$ ANOVA) with IC_{50} s of 0.35 g/L (0.1 mmol/ L acarbose) and 0.04g/L (0.2 mmol/L acarbose) respectively.

Augment of GA and acarbose

The amount of total maltose absorbed during the two perfusions is compared in Figure 3. The first perfusion was performed for 1 h by 10mmol/L maltose containing GA and/or acarbose and the second was performed again for 1 h by 10 mmol/L maltose only, after rinsing for 30 min. The absorption of maltose during 1 h in control group was 42.2 mg and 43.0 mg in the first and second perfusions respectively, which had no significant difference. If the total absorbed maltose in control is taken as 100%, the relative maltose absorption was 64.2% ± 5.0% and 54.0% ± 7.3% in the 0.2 and 2 mmol/L acarbose groups respectively, which was further decreased to only 26.9% ± 2.9% and 31.0% ± 2.7% respectively by the combined 1 g/L GA. In GA (1g/L) group the relative maltose absorption was 38.1% ± 4.3% but still higher than that in combined groups ($P < 0.05$). These values implied that augmented inhibitory effects are highly revealed throughout effective duration in either combined group of GA and acarbose.

Modulation of the acarbose activity by GA

The percentages of maltose hydrolysis during perfusion maltose and acarbose with or without GA are shown in Figure 4A. It is noted that the inhibitory effect of 2 mmol/L acarbose on maltose hydrolysis was time-dependently decreased by combination with 1 g/L GA in the first perfusion. The hydrolysis of maltose in GAa2 group, which was similar to that in 0.2 mmol/L acarbose, was higher than that in 2 mmol/L acarbose group at 30 min ($P < 0.05$), 45 min and 60 min ($P < 0.$ 01) except that at 15 min. On the contrary, the hydrolysis of maltose in the GAa2 group was lower than that in 2 mmol/L acarbose group ($P < 0.05$) in the second perfusion after the intestine was washed out for 120 min (Figure 4B). The hydrolyses at 15 min, 30 min, 45 min and 60 min were 15.77% ± 0.89%, 29. 44% ± 0.18%, 45.18% ± 3.20% and 59.67% ± 5.86% in the former while 21.94% ± 1.36%, 45.38% ± 2.87%, 57.52% ± 1.69% and 73.14% ± 3.92% in the latter. No significant difference was observed in lower dosages of acarbose (0.2 mmol/L) with the presence or absence of GA.

Correlation of GA's inhibitory effects on absorption and mobility of the intestine

To explain mechanism of the combination, the effect of GA on auto-rhythmic contraction of the intestinal ring was recorded isometrically using a force transducer. Although the frequency of auto-rhythmic contraction was 17.2 ± 0.77 times per minute in the intestinal rings, and the tension induced by auto-rhythmic contraction was 1406.67 mg ± 565.33 mg under normal conditions, they were dose-dependently decreased when treated with GA (Figure 5A,B). The inhibitory effects of GA on the absorption of maltose and auto-rhythmic contractions of the ring in the small intestine had a good linear correlation ($r = 0.98$, Figure 5C).

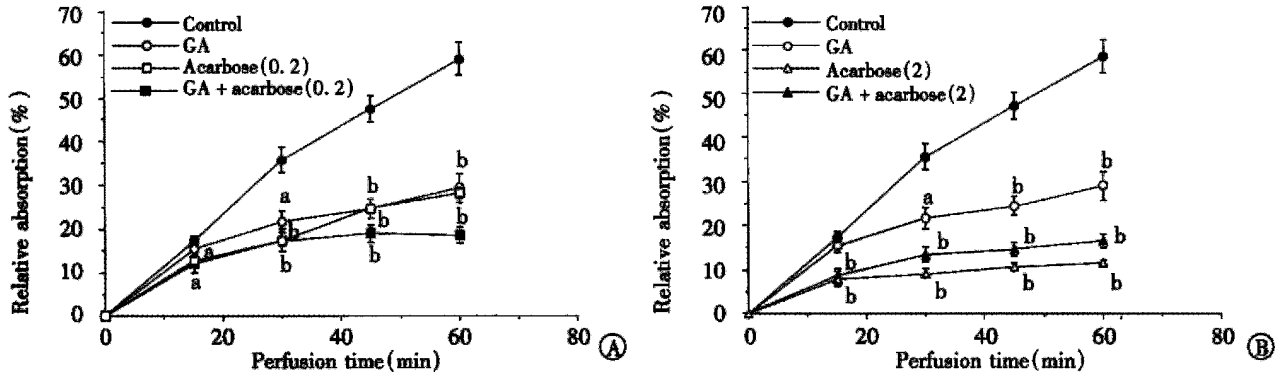

Figure 1 Inhibitory effect of GA and acarbose on maltose absorption. The intestinal loops *in situ* were perfused with 10 mmol/L maltose in the presence or absence of GA (1 g/L) and acarbose (mmol/L). The absorption of maltose is shown as percentage of maltose contained in the beginning of perfusion. Each point is expressed as mean ± SD of 5-10 determinations. ($^aP < 0.05$, $^bP < 0.01$)

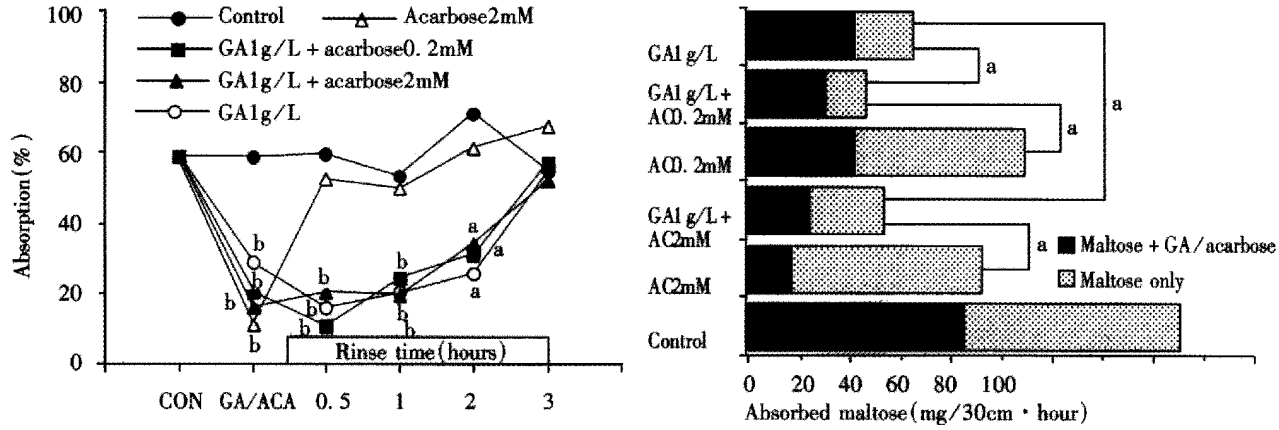

Figure 2 Alteration of maltose (10 mmol/L) absorption following application with GA and/or acarbose. Each point shows the maltose absorption during 60 min perfusion of 10 mmol/L maltose following rise with Ringer's solution after treatment of GA and/or acarbose (the second perfusion) except the points of "CON" or "GA/ACA" which shows the absorption during the first 1 hour perfusion with or without GA and acarbose (the first perfusion). The absorption of maltose is shown as percentage of maltose contained in the beginning of perfustion ($^aP < 0.05$, $^bP < 0.01$).

Figure 3 Maltose absorption during the two perfusions in the first 2.5 hours. Maltose+GA/acarbose: first perfusion, 10 mmol/L maltose containing GA and/or acarbose was perfused for 1 hour. Maltose only: after first perfusion the intestinal loops were rinsed for 30 min, then 10 mmol/L maltose only was perfused for 1 hour again. The absorbed maltose is shown as the absorption in each perfusion. There was significant difference between each treated group versus control. ($^aP < 0.05$, n = 5-10).

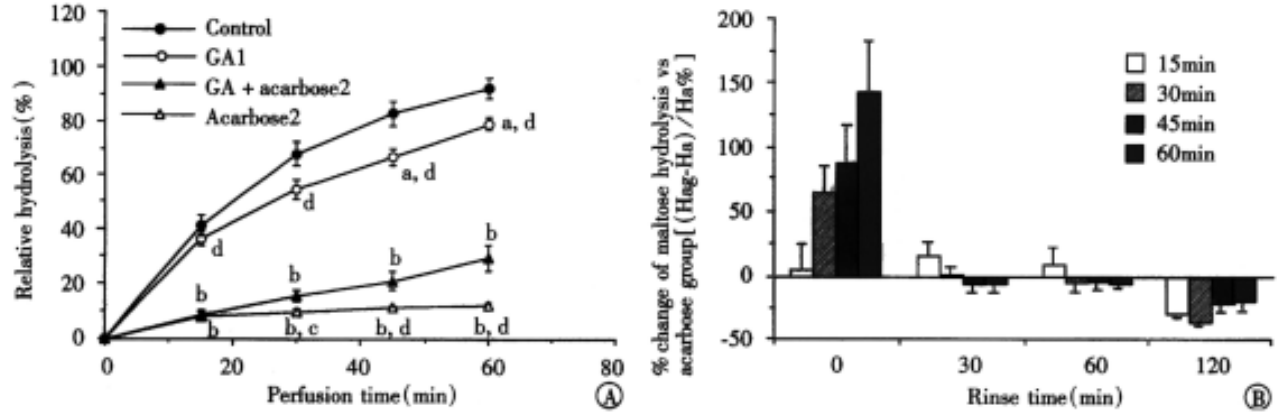

Figure 4 The dual effects of GA (1mg/mL) on the activity of acarbose during the first perfusion (A), maltose with GA and/or acarbose was presented in the perfusates. Maltose contained in the fluid at perfusion starting point was taken as 100%. $^aP < 0.05$ and $^bP < 0.01$ *vs* control; $^cP < 0.05$ and $^dP < 0.01$ *vs* GAa2. (n = 6-10) and second perfusion (B), the intestinal loops were rinsed for 30 min to 120 min, then 10 mmol/L maltose only was perfused for 1 h again. Each bar shows the percentage change of maltose hydrolysis (Hc%) in GAa2 group versus 2 mmol/L acarbose group in different perfusing times, which was calculated by the following equation: Hc% = (Hag-Ha)/Ha × 100%, where Hag and Ha represent, respectively, the hydrolyzed maltose in GAa2 group and in 2 mmol/L acarbose group. Hydrolysis of maltose in the acarbose group is believed as 100% (n = 3-10). A diminished effect in the beginning and improved effect in the end were observed.

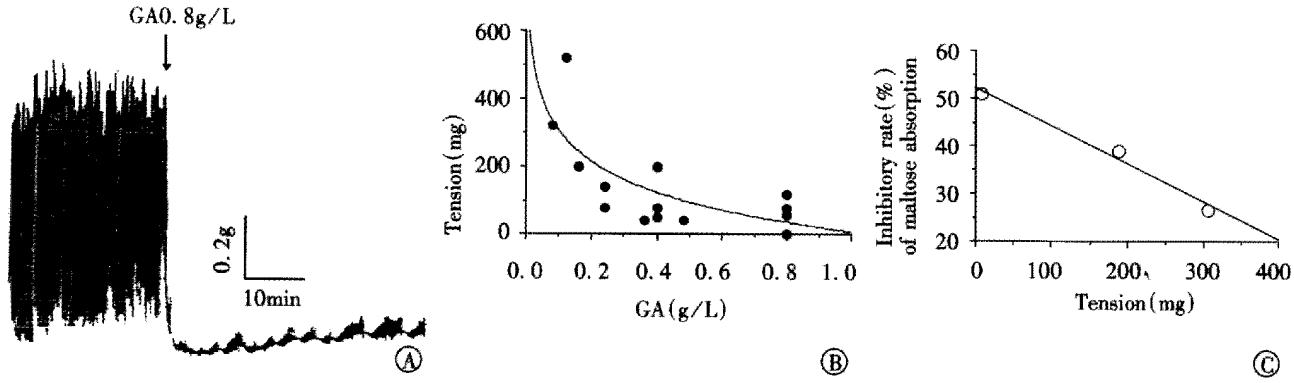

Figure 5 The relationship of GA's inhibitory effects on absorption of the maltose and on mobility of the intestinal ring. A, an example of the inhibitory effect of GA (0.8 g/L) on the intestinal auto-rhythmic contraction. B, dose-dependently inhibitory effect of GA on amplitude of the small intestinal rings which is expressed as the tension induced by the auto-rhythmic contraction in the present different doses of GA. C, correlation of GA's inhibitory effects on absorption of maltose and on tension of the intestinal ring. Each point comes from one dose of GA. The tension is calculated from the function of curve in the Figure B.

DISCUSSION

The combined effects of acarbose and GA are first reported in this paper. With the combination, inhibitory duration of acarbose was prolonged and onset time of GA was shortened, while the total inhibitory rate of maltose absorption was improved each other throughout their effective duration. Subsequently, a more effective reduction of postprandial hyperglycemia and hyperinsulinaemia could be expected by employing the combination in humans.

Acarbose is a pseudo-tetrasaccharide that is structurally similar to the typical oligosaccharides derived from starch digestion and can attach to the carbohydrate binding site of alpha-glycosidases in a competitive manner with a rank order of inhibitory potency of glucoamylase > sucrase > maltase > isomaltase. To alpha-amylase, acarbose has only a weak noncompetitive inhibitory effect[17]. In our experiment, the IC_{50} of acarbose for the 10 mmol/L (3.42 g/L) maltose absorption was 0.65 mmol/L during 15 min perfusion compared with a previous study of Krause *et al*, in which acarbose inhibits the absorption of maltose (1 g/L and 2 g/L) with IC_{50s} of 36 mg/L (0. 06 mmol/L) and 57 mg/L (0.09 mmol/L) respectively during 15 min in perfused loops[18]. The IC_{50s} and concentrations of maltose in the 3 points had a good semilog linear correlation ($r = 0.94$).

The inhibitory effects of GA on intestinal glucose absorption and oral glucose tolerance were first noticed in the 1980s[13,14], although there has been more than a century's history of treating diabetes mellitus with the leaves of Gymnema sylvestre in India [19-24]. Recently, various effects of GA including decreased body weight[15], improved hyperinsulinaemia[14] and inhibited glucose-stimulated gastric inhibitory peptide secretion[25] have been published. In a previous study in our laboratory[13], GA inhibited intestinal glucose absorption consistent with the present results, but with a difference in time (5 mmol/L glucose). Rinsed with Ringer's solution, the loops tended to recover their absorption of glucose. Those contrary results could be coming from the different effects of the unstirred layer and the tight junction on the absorption of maltose and lower concentration of glucose. In a recent study that we performed[26], GA inhibited the absorption of oleic acid and glucose (5 mmol/L) simultaneously with a time course same as the experiment of only 5 mmol/L glucose[13], which implies that the inhibitory effect of GA on the absorption of oleic acid has some relationship with the inhibitory effect of GA on glucose absorption.

Mechanism of the combination

The mechanisms for GA's actions have been considered as participating in the glucose receptor[25], Na^+/glucose cotransporter[13], ATPase[27] and insulin release[28]. In the present experiment, a new mechanism of GA's action was suggested concerned with an influence of the precellular and paracellular barriers of absorption in the intestinal brush borders.

Intestinal absorption of solute requires that the compound cross two barriers, the unstirred layer as an aqueous diffusion barrier, and epithelium which consist of enterocytes and paracellular factors[29-34]. Disaccharides are absorbed at a faster rate than monosaccharides *in vivo*, but inversely *in vitro* for the absorption of disaccharides coupled with the membrane digestion. Oligomers invariably appear in monomeric form at the serosal side of the epithelial cell or in the blood stream[35]. The concentration of glucose hydrolyzed from maltose (10 mmol/L) in the microenvironment of enterocytes and the tight junction is nearly 200 mmol/L-300 mmol/L with membrane

digestion[36], which is 10-15 times of that contained in the perfusate and is a power to drive absorption through paracellular pathway. If the tight junctions were narrowed or the membrane digestion was limited, the effect on the absorption of maltose would be more than that on the absorption of glucose.

"Membrane digestion" was defined by Ugolev and De-Laey[37] as that action of the cellular membranes on enzyme activities combined with the transport of their products of hydrolysis across membrane forms. The structural foundation of the model is presented as enterocytes projecting microvilli and the unstirred layer that is mainly formed by a glycocalyx entrapping water mixture with mucin from nearby goblet cells. Most of the disaccharidases produced by the enterocytes are binding with the membrane (under the unstirred layer) and a small amount of saliva and pancreatic amylase is in the glycocalyx. The maltose is hydrolyzed during it passes through the glycocalyx and enterocytes[38].

On the other hand, thickness of the unstirred layer (glycocalyx) largely depends on motility of the intestine, which becomes relatively thin when the intestine exhibits motility[29,37]. Suppressed by GA, the tension and intestinal auto-rhythmic contraction had un-negligible effects on unstirred layer not only, but also on the tight junction around the enterocytes. When the intestine relaxes, the paracellular pathways become narrow[36,39]. Consequently both hydrolysis and absorption could be inhibited by GA through pre-epithelium and epithelium barriers, in the latter containing transcellular (same as glucose) and paracellular factors (only for maltose or high concentration of glucose), that could be a main reason for the different time courses of GA on maltose and glucose.

It is another evidence altering the function of unstirred layer, that the activity of acarbose in the higher concentration (2 mmol/L) was decreased at the beginning and increased after rinsing by the combination with GA (Figure 4). The unstirred layer limits the diffusion of acarbose entering (at the beginning) and leaving (during rinse) the microenvironment nearest the membrane maltase resulting in the dual effects of GA on the activity of acarbose. It should be due to lower affinity[40] of acarbose with alpha-amylases, that the dual effects only appeared with higher concentration of acarbose. When the activity of alpha-amylases in the unstirred layer (glycocalyx) was inhibited, the glycocalyx digestion itself was decreased. With a combination of high concentration of acarbose and GA, the unstirred layer became thick enough to suppress the diffusion of acarbose. The glycocalyx is estimated to impede solute diffusion to the surface in a manner that is directly proportional to its solubility and inversely proportional to the square root of its molecular weight. The molecular weight of acarbose is nearly 2 times that of maltose, therefor in same condition the barrier of unstirred layer on acarbose is more effective than that on maltose. On the other hand, the solubility of GA (molecular weight about 800) was lower (about 10 g/L) than that of acarbose and maltose, which also is one of the reasons for GA's longer onset time (30 min).

The merits of combination

Tolerability should be considered when a drug is administered to a patient. Acarbose has contained in the drug list of diabetic management either type 1 or type 2 [5,40-46]. The most common adverse effect of acarbose is a gastrointestinal disturbance, which is induced by producing gas with fermentation of the unabsorbed carbohydrates in the bowel[17,43,47-50]. Acarbose has rarely been associated with systemic adverse effects, but in some case acute severe hepatotoxicity has been reported[3,51,52]. These adverse effects tend to increase with higher doses. GA not only improves the effect with a decrease in the IC_{50} of acarbose, but also inhibits the growth of anaerobia[53]. Bacterial overgrowth plays a role in the development of gastrointestinal symptoms[54], therefore the adverse effects of acarbose induced by fermentation are expected to be diminished by the combination of GA.

CONCLUSION

Acarbose inhibits maltose absorption through the inhibition of maltase under the unstirred layer and alpha-amylase in the unstirred layer. The latter is only involved at a higher dose (2 mmol/L) of acarbose. GA inhibits maltose absorption possibly by making the unstirred layer thicker and shutting the paracellular pathway, besides other reported mechanisms that are the same as glucose[13-15,25,27,28]. The effects of GA on maltose absorption and the combined effects of acarbose and GA are first reported in this paper. With the combination, the effective duration of acarbose is prolonged and the effective onset of GA is faster. Improvements in postprandial hyperglycemia, hyperinsulinaemia and insulin resistance, treatments of an overweight condition and diminishing of the adverse effects of acarbose in diabetic control by this combination are also in perspective.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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The development of colon innervation in trisomy 16 mice and Hirschsprungs disease

Ji Cheng Li[1], Kai Hong Mi[1], Ji Lin Zhou[1], LC Busch[2] and W Kuhnel[2]

Subject headings Hirschsprungs disease;colon; down syndrome; immunohistochemistry; nervous system; trisomy 16 mouse

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Abstract

AIM To study the colon innervation of trisomy 16 mouse, an animal model for Down's syndrome, and the expression of protein gene product 9.5 (PGP 9.5) in the stenosed segment of colon in Hirschsprungs disease (HD).

METHODS Trisomy 16 mouse breeding; cytogenetic analysis of trisomy 16 mice;and PG P9.5 immunohistochemistry of colons of trisomy 16 mice and HD were carried out.

RESULTS Compared with their normal littermates, the nervous system of colon in trisomy 16 mice was abnormally developed. There existed developmental delay of muscular plexuses of colon, no submucosal plexus was found in the colon, and there was 5 mm aganglionic bowel aparting from the anus in trisomy 16 mice. The mesentery nerve fibers were as well developed as shown in their normal littermates. Abundant proliferation of PGP 9.5 positive nerve fibers was evealed in the stenosed segment of HD colon.

CONCLUSION Trisomy 16 mice could serve as an animal model for Hirschsprung's disease for aganglionic bowel in the distal part of colon. Abundant proliferation of PGP 9.5 positive fibers resulted from extrinsic nerve compensation, since no ganglionic cells were observed in the stenosed segment of the colon in HD. HD has a genetic tendency.

[1]Department of Lymphology, Department of Histology and Embryology, Zhejiang University Medical School, Hangzhou 310031, China
[2]Institute of Antomy, Medical University of Lubeck, Germany
Dr. Ji Cheng Li, graduated from Zhejiang Medical University in 1983, now professor of histology and embryology, engaged in the research of Hirschsprung's disease, having 50 papers published.
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Correspondence to: Dr. Ji Cheng Li, Department of Lymphology, Department of Histology and Embryology, Zhejiang University Medical School, Hangzhou 310031, Zhejiang Province, China
Tel. 0086-571-7217139, Fax. 0086-571-8855930
Email. lijc@mail.hz.zj.cn
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INTRODUCTION

The most significant pathophysiological change of Hirschsprung's disease is its characteristic abnormal enteric nervous development of colon which resulted in the regional enterospasm and narrowed lumen, loss of intestinal peristalsis and relaxing reflex of the internal enteric sphincter[1]. The physiological peristalsis of the proximal gut pushes the intestinal contents forward, leading to the second megacolon in the proximal part of the narrowed gut. Since the cause of HD is still unknown and the experiment on humans is impossible, we used protein gene product 9.5 to study the enteric nerve development of colon in trisomy 16 mice, an animal model for Down's syndrome[2,3], and the innervation of stenosed segment of the colon in HD so as to reveal the possibility of developing the congenital megacolon in trisomy 16 mice and the genetic tendency of HD in human[4-7].

MATERIALS AND METHODS

Animal stocks and breeding[8-10]

Trisomy 16 mouse embryos examined in this study were produced by crossing NMRI-female mice provided by the Experimental Center of Medical University of Lubeck, with male mice carrying the two Robertsonian (Rb) translocation chromosomes Rb (16,17) 8Lub^{wLub3} perpendicularly and Rb (11,16)2H. The day on which a vaginal plug was observed was considered to be the first embryonic day (ED).

Cytogenetic analysis

The pregnant female mice were sacrificed one hour after intraperitoneal injection of colchicine (0.02 mg/25-35 mg body weight) by dislocation of the neck. The embryos were collected by laparatomy. Small samples of liver were taken from each embryo for direct preparation of karyotypes, by using the standard technique of hypotonic treatment with 0.563% KCl and methanol : acetic acid fixation (3 : 1). Air-dried slides were observed under light microscope. Accurate ascertainment of trisomy 16 was provided by the demonstration of two Rb metecentric chromosomes and a count of 41 chromosome arms. After the defrimy of the chromosomal status, the trisomic and the normal euploid embryos of each litter could be separated (Figure 1).

Immunohistochemical methods[11]

The guts were taken from trisomy 16 embryos and

normal littermates, and fixed in a solution containing 4% paraformaldehyde and 2% picric acid in phosphate-buffered saline (PBS) for approximately 2 hours at room temperature. Fifteen pieces of narrowed HD guts were obtained from the patients aged between 18 months and 9 years. Six segments of normal colons served as controls. The diagnosis of HD was based on the case history, clinical manifestations, examination of barium enema and pressure measurement of rectum and anal. All the cases were confirmed postoperatively by pathological examinations.

Whole mount preparations of the gut were dissected out under stereomicroscopic control[12-14], and freezing sections were made at a thickness of 12 μm, then washed in 0.01 M PBS and 50% ethanol for 2 hours at room temperature. For immunohistochemistry, peroxidase-anti-peroxidase (PAP) was used in the present experiment.

The specimens were treated with 10% normal goat serum (Dakopatts × 907) for 30 min, then incubated in a primary antiserum protein gene product 9.5 (PGP 9.5) (Ultraclone RA 95101, UK) overnight at room temperature, diluted to 1 : 400 in a solution containing 10% normal goat serum, 0.01% sodium azide and 0.05% thimerosal in PBS. After three washes in PBS for 30 min, the specimens were incubated in goat anti-rabbit IgG (Dakopatts Z421) overnight at room temperature as the secondary antiserum, and diluted to 1 : 100 in the above solution. The specimens were then exposed in rabbit PAP (rabbit antibody to horseradish peroxidase and horseradish peroxidase) overnight at room temperature and diluted to 1 : 100 in a solution containing 0.1% bovine serum albumin and 0.05% thimerosal in PBS pH 7.4. 4 Cl1-naphtol or DAB was used as the chromogen for the peroxidase reaction. Control included the omission of the primary antiserum. PGP 9.5 is a cytoplastic protein composed of 212 amino acids with molecular weight of 2.45 kb. This protein only exists in the neurons and neuroendocrine cells. PGP 9.5 is a specific protein with an excellent localizing function for the neurons[15-19].

RESULTS

Development of trisomy 16 mice and their normal littermates

Owing to the abnormality of their chromosomes, the trisomy 16 mice were characterized by their small size, generalized edema and a failure of closed eyelids after embryonic day 16 (ED 16). Both the small size and lid-gap defect were attributed to the generalized growth retardation and developmental delay, leading death at the time of birth in most mice. The trisomy 16 mice in the present study were chosen from the gestational age between ED13 and ED 18. The survival rate of the trisomy 16 mice was lower with the gestational age. The lowest survival rate of trisomy 16 mice was observed at the time of their birth (Figure 2).

Development of colon innervation in trisomy 16 mice and their normal littermates

The nerve development of colon in normal littermates is a continuous process. Based on the morphology and staining degree of the PGP 9.5 positive reactions, the nerve development of colon could be divided into three stages: primary stage (ED14), juvenile stage (ED15 and ED16) and mature stage (ED17 and ED18). At ED14, scantly-distributed PGP 9.5 positive neurons were observed with light staining and round nucleus in the center of the neurons. At ED15, the distribution density of neurons increased greatly with some nervous processes, through which the neurons were connected with each other (Figure 3A). At ED16, muscular plexuses appeared with gathered neurons to form the light-stained ganglion. But the number of neurons in the ganglion was less and sparely arranged. At ED17 and ED18, the precursor cells of muscular plexuses migrated into the submucosal layer to form submucosal plexuses. The arrangement of nervous fibers in submucosal plexus was irregular, accompanied by some small-sized and sparely-distributed ganglions, while muscular plexuses were composed of primary, secondary and tertiary strands displaying a nerve meshwork with regular wide meshes and numerous large ganglia. The muscular and submucosal plexuses were further developed with much stronger PGP 9.5 staining at ED18 (Figure 4A). The increasing number of large-sized ganglions was observed with stronger immunostaining and arranged along the primary nerve strands. Numerous fine nerve strands in the gut villi were observed at ED18 (Figure 5).

When compared with the normal littermates, the developmental delay of the colon- innervation was observed in trisomy 16 mice. Some neurons appeared with a scattered distribution and light staining at ED15 (Figure 3B) and the neurons were found with some nerve processes by which irregular nervous meshwork formed at ED16. In the colon of trisomy 16 mice at ED17 and ED 18, only one plexus was visible, i.e., the myenteric plexus with a few small-sized ganglions (Figure 4B). From ED14 to ED18 there was no submucosal layer in colon, and 5mm aganglionic bowel was found at the distal end of the colon in trisomy 16 mice (Figure 6), which was revealed as a characteristic morphological evidence of the development of congenital megacolon in trisomy 16 mice. This important finding was constantly verified in all the experimental trisomy 16 mice.

The exogenous innervation of colon in trisomy 16 mice and their littermates

As early as ED14, PGP 9.5 positive nerve fibers occurred in the mesentery of both trisomy 16 mice and their normal littermates, which gradually

increased with the embryonic development. At ED17 and ED18, rich mesenteric nerve fibers were detected. In all whole mounts of PGP 9.5 nerve fibers in mesentery, they were observed regularly running in parallel and close to the blood vessels and innervated the colon in both trisomy 16 mice and their normal littermates (Figure 7). Thus, the arcades of the mesenteric vessels were clearly delineated by their immunostained perivascular plexuses.

The distribution of PGP 9.5 positive nerve fibers in the stenosed segment of HD and normal colons

The ganglions of the muscular plexus and submucosal plexus from the normal colon all revealed highly positive reaction to PGP 9.5 (Figure 8). The number of ganglion in muscular plexus was greater than that in the submucosal ones. The neurons presented a dark brown color and gathered as a mass. The adjacent neurons extended processes, by which they were connected with each other. PGP 9.5 positive nerve fibers were distributed in all intestinal layers. The richest part was in the circular muscular layer and parallel to the muscular fibers. In the proper mucous membrane, nerve network was formed around the intestinal glands. In the submucosal layer, the nerve fibers were arranged around the vessels. But in the stenosed segment of HD colon, no ganglionic cell was observed in the muscular and submucosal plexuses. At the distal end of the stenosed segment of the colon, no nerve plexus was found. Occasionally, a few neurons existed, but with abnormal morphology, while PGP9.5 positive nerve fibers were great in number in all intestinal layers. Big, strong, disordered and even twisted nerve tracts could be observed (Figure 9). Such disorderly arranged nerve fibers could also be found in the submucosal layer and proper mucous membrane layer of the colon. Some of the proliferated fibers were twisted along the vessels.

Table 1 The development of the colon and mensenteric innervation of trisomy 16 mice and their normal littermates in chronological sequence

	ED13		ED14		ED15		ED16		ED17		ED18	
	N	T	N	T	N	T	N	T	N	T	N	T
PM	-	-	+	-	++	+	++	+	+++	++	+++	++
PS	-	-	-	-	-	-	+	-	++	-	+++	-
MF	-	-	+	+	++	++	+++	+++	+++	+++	+++	+++

N: normal littermates; T: trisomy 16 mice.
PM: myenteric plexus; PS: submucosal plexus; MF: mesentery nervour fiber
-: no neurons and immunoreactive nerve fibers detected;
+: a few scattering neurons and no enteric plexus or sparse immunoreactive fibers detected;
+: lowly developed enteric plexus or moderate immunoreactive fibers detected;
+++: moderately developed enteric plexus or abundant immunoreactive fibers detected;
++++: fully developed enteric plexus or very abundant immunoreactive fibers detected.

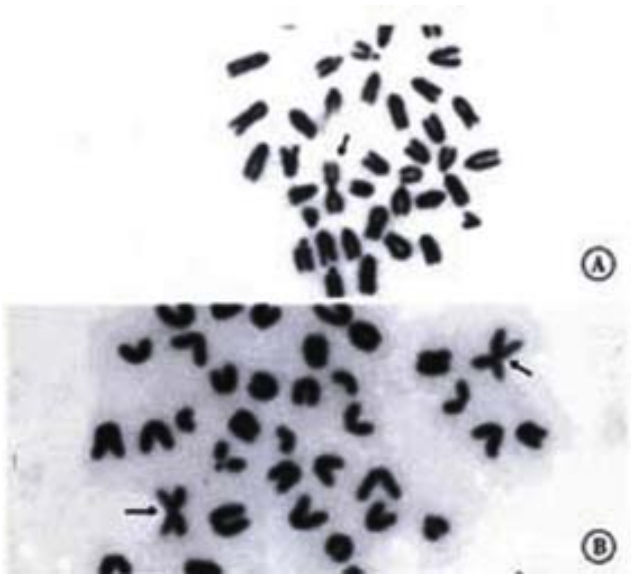


Figure 1 a. The chromosome of normal mice. b. The chromosome of trisomy 16 mice: two Rb metecentric chromosomes (↑) and 41 chromosome arms. × 1000

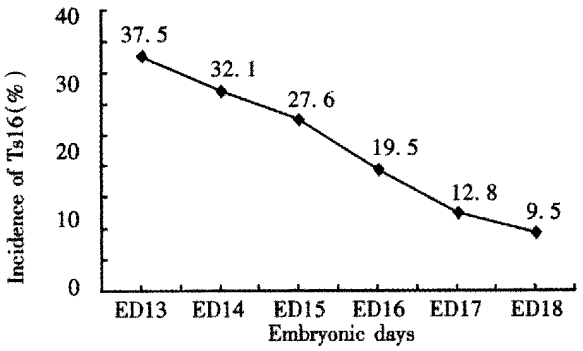


Figure 2 The survival rate of trisomy 16 mice with different embryonic days (ED).

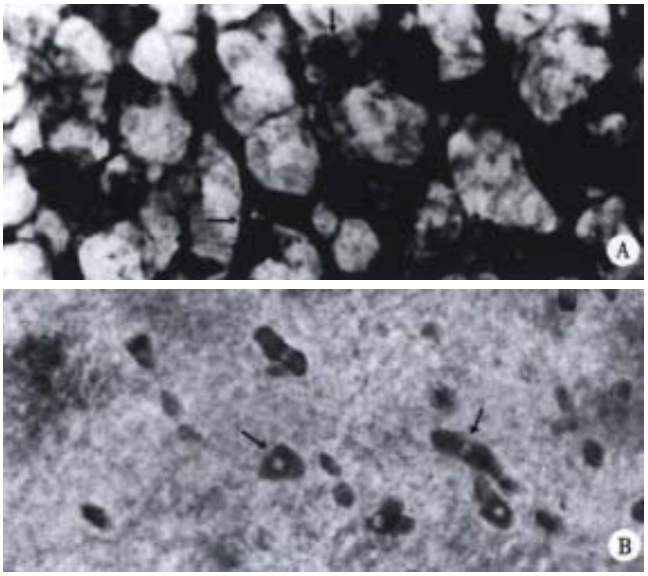
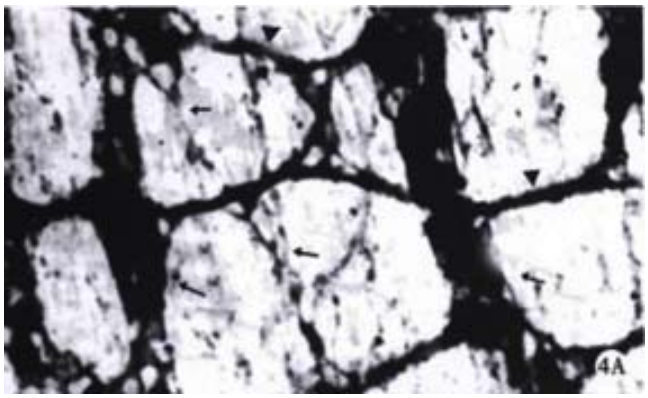
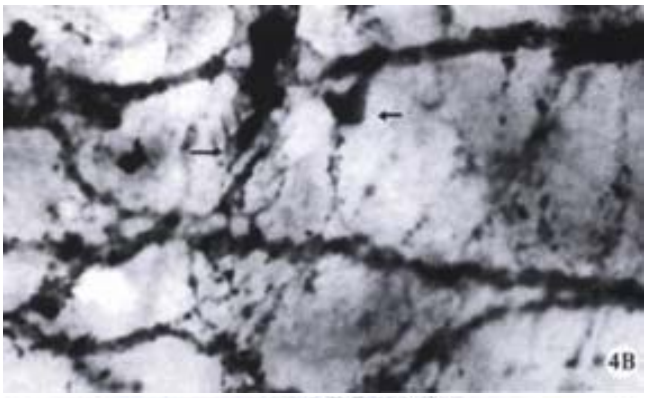
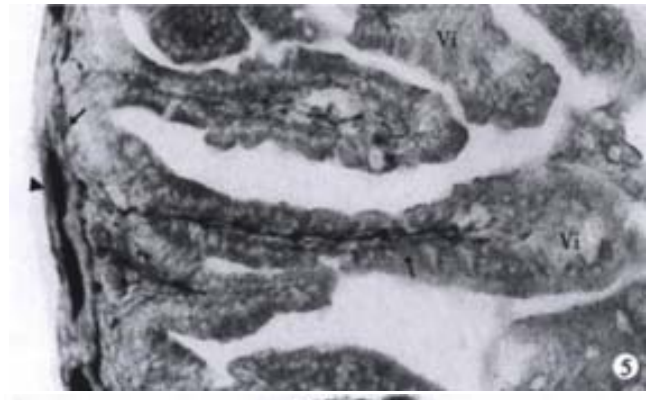
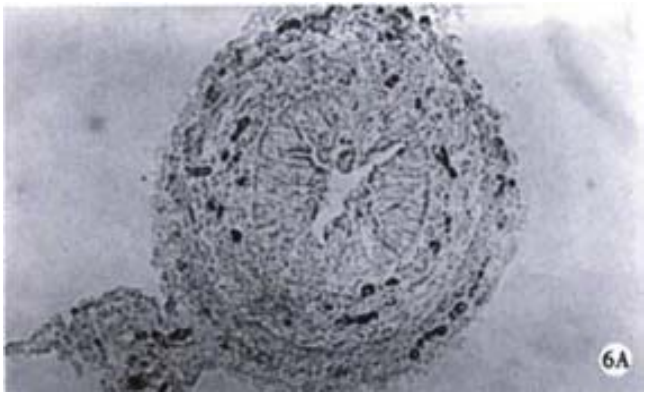


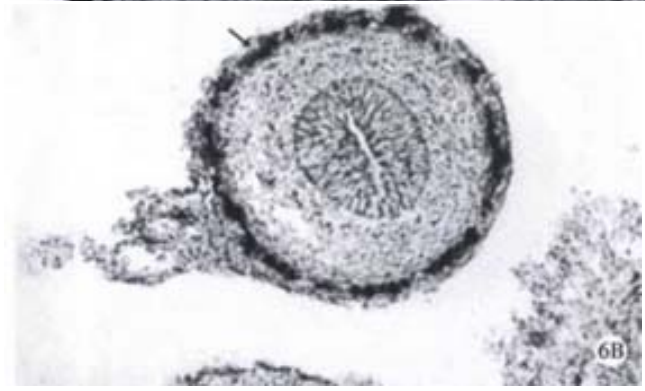
Figure 3 a. Myenteric plexus of normal littermates at ED 15. The nerve meshworks had regular meshes with many neurons (↑). × 400 b. The colon of trisomy 16 mice contained some neurons (arrow) with different staining and size at ED15. × 400

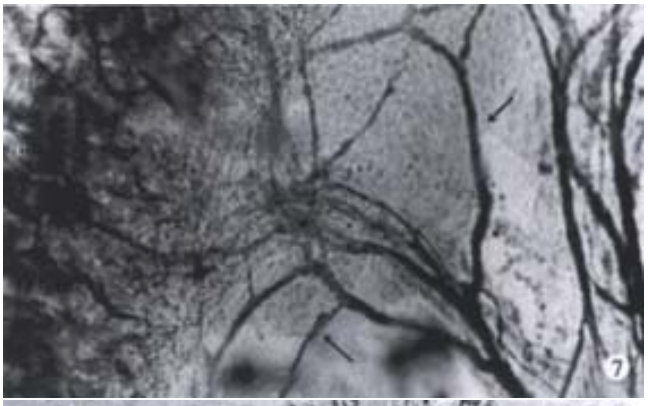


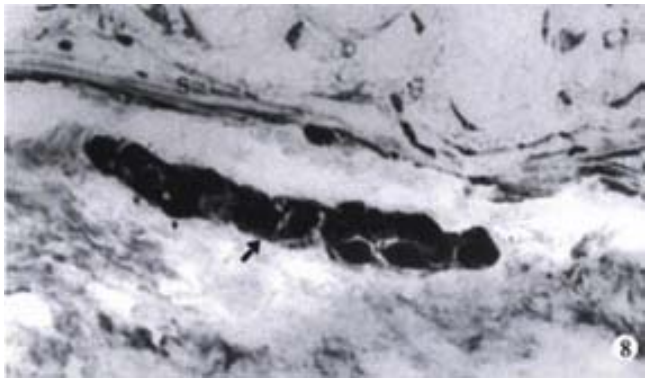












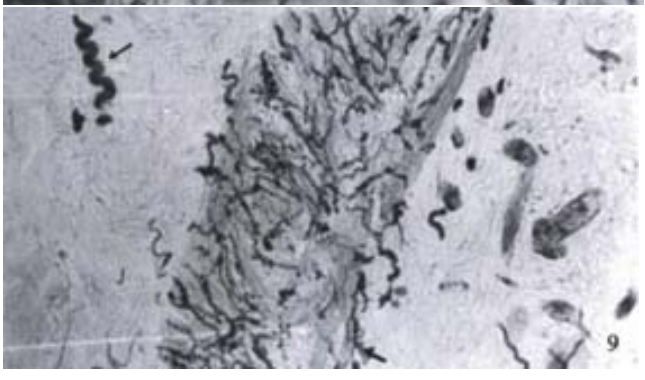


Figure 4 At ED 18, the myenteric plexuses of trisomy 16 mice and their normal littermates in whole mount preparation. × 250
A. The developed myenteric plexus with primary (long arrow), secondary (arrow) and tertiary (arrowhead) strands were displayed in the colon of normal littermates. The primary strands were composed of broad fiber bundles and numerous ganglia with longitudinally arranged meshes. B. The myenteric plexus of trisomy 16mice was poor with less ganglia containing some neurons (arrow) at different staining.
Figure 5 At ED 18, the nerve fibers (long arrow) stretching from the enteric plexuses reached the gut villi (Vi). The myenteric (arrowhead) and submucosal (arrow) plexuses were clear. × 160
Figure 6 Aganglionic segment of trisomy 16 mouse colon. × 160 B. The colon of normal littermate with ganglia (arrow). × 100
Figure 7 The myenteric nervous fibers (arrow) were detected in the colon (C) mesentery of trisomy 16 mice. × 160
Figure 8 The submucosal ganglia (arrow) of normal colon of human. S: submucosal muscular layer. × 100
Figure 9 The aganglionic colon of Hirschprung's disease. Abundant proliferation of nerve fibers (large arrow) was found in the stenosed segment of colon. A wave-like nerve fiber (arrow). × 100

DISCUSSION

The incidence of HD is 1/5000 due to the developmental defect of enteric nervous system[20-23]. Recently, HD incidence is increasing, especially the genetic HD and Down's syndrome with congenital megacolon[24-29], which greatly aroused researchers' interest in investigating the causes. The present study was based on PGP 9.5 as primary antibody to reveal the innervation of the narrowed gut in HD and the nerve development of the colon in trisomy 16 mice.

Enough evidences have been obtained from this study that trisomy 16 mice had a significant anomaly and developmental delay in colon innervation as well as 5 mm aganglionic colon at the distal end, which was the result of the chromosome abnormality. Our present experiment, found that there was a chronological sequence in the nerve development and a clear link between the gestational age and the morphological development in colon innervation of trisomy 16 mice and their normal littermates (Table 1). At ED 14, the precursor cells of ectoderm migrated into the gut along the course of the vagus nerves; at ED 15, nerve processes were observed in the neuron migrated gut; at ED 16, the muscular plexuses and ganglions appeared in the gut; at ED17, the precursor cells in muscular layer migrated into the submucosal layer and developed into submucosal nervous plexuses; at ED 18, the relatively matured muscular and submucosal plexuses occurred, the former controls the intestinal peristalsis, and the latter manages the intestinal secretions and absorption[30, 31]. The lack of submucosal nerve plexuses of trisomy 16 mice accounted for the lower material absorption and poor intestinal secretions while the distal aganglionic colon could result in the neurotrophic and innervating disorders. It is now recognized that the intestinal nerve development begins from the cephalic part to the caudal end[32-41]. But HD was due to the stagnated development of the precursor cells. The earlier the stagnation of the precursor cells, the longer the aganglionic bowel. Since colon is the last segment of gut for the precursor cells to migrate into, colon is most likely to develop HD. The aganglionic colon in trisomy 16 mice strongly confirmed the above findings. The euchromosome 16 of trisomy 16 mice has the same sequence as the 21q22 genes of human euchromosome 21[8]. It is often used as an animal model for Down's syndrome. Besides, trisomy 16 mice have constant genetic tendency and aganglionic colon, being easy to develop congenital megacolon. Epstein and associates believed that the intestinal innervation was composed of intrinsic and extrinsic ones[42]. The well-developed mesenteric nervous system of trisomy 16 mice could be regarded as a compensation for the intrinsic nerves. When there were no ganglionic cells in the intestine, the extrinsic nerve fibers would proliferate greatly. In our experiment, considerable PGP9.5 positive nerve fibers were verified in the stenosed HD colon, which was obviously the compensative nerve proliferation to the innervation of aganglionic HD colon. Since trisomy 16 mice had severe cardiovascular abnormalities, multi-organ deformities[43-45] and could hardly survive after delivery, so in the present study, the distal colon end of trisomy 16 mice showed no such extrinsic compensating nerve proliferations.

Among all clinically verified HD cases, 20% were caused by the lack of genetic factors[46]. Trisomy 16 mice, with its constant chromosome abnormality, is a reliable animal model for studying the genetic tendency of HD. The regional microcircumstance was closely related to the changes of the genetic inheritance. Tennyson and associates have found two striking abnormalities, i.e., an overgrowth of the muscularis mucosa, particularly in the outer longitudinal layer and an extensive thickening of the basal lamina around smooth muscle cell.

These excessive accumulation of basal lamina material and muscularis mucosa interfered with normal migration of precursor cells[47-51]. Payette and associates [52] thought that the excessive accumulation of components of basal lamina material in the aganglionic region of the lethal spotted mutant mouse stopped precursor cells from migrating into the terminal bowel of lethal spotted mutant mice. Our study revealed that 5mm aganglionic bowel in length aparting from anus in trisomy 16 mice, might be caused by the change of regional micro-circumstance due to the abnormal chromosome, finally resulting in the nerve defect.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Reduction of tumorigenicity of SMMCa27721 hepatoma cells by vascular endothelial growth factor antisense gene therapy

Yu Cheng Tang, Yu Li and Guan Xiang Qian

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Abstract

AIM To test the hypothesis to block VEGF expression of SMMC-7721 hepatomacells may inhibit tumor growth using the rat hepatoma model.

METHODS Amplifiy the 200 VEGF cDNA fragment and insert it into human U6 gene cassette in the reverse orientation transcribing small antisense RNA which could specifically interact with VEGF165, and VEGF121 mRNA. Construct the retroviral vector containing this antisense VEGF U6 cassette and package the replication-deficient recombinant retrovirus. SMMC-7721 cells were transduced with these virus and positive clones were selected with G418. PCR and Southern blot analysis were performed to determine if U6 cassette integrated into the genomic DNA of positive clone. Transfected tumor cells were evaluated for RNA expression by ribonuclease protection assays. The VEGF protein in the supernatant of parental tumor cells and genetically modified tumor cells was determined with ELISA. In vitro and in vivo growth properties of antisense VEGF cell clone in nude mice were analyzed.

RESULTS Restriction enzyme digestion and PCR sequencing verified that the antisense VEGF RNA retroviral vector was successfully constructed. After G418 selection, resistant SMMC-7721 cell clone was picked up. PCR and Southern blot analysis suggested that U6 cassette was integrated into the cell genomic DNA. Stable SMMC-7721 cell clone transduced with U6 antisense RNA cassette could express 200 bp small antisense VEGF RNA and secrete reduced levels of VEGF in culture condition. Production of VEGF by antisense transgenea2expressing cells was 65 ± 10 ng/L per 10^6 cells, 420 ± 45 ng/L per 10^6 cells in sense group and 485 ± 30 ng/L per 10^6 cells in the negative control group, ($P<05$). The antisense-VEGF cell clone appeared phenotypically indistinguishable from SMMC-7721 cells and SMMC-7721 cells transfected sense VEGF. The growth rate of the antisense-VEGF cell clone was the same as the control cells. When S.C. was implanted into nude mice, growth of antisense-VEGF cell lines was greatly inhibited compared with control cells.

CONCLUSION Expression of antisense VEGF RNA in SMMC-7721 cells could decrease the tumorigenicity, and antisense-VEGF gene therapy may be an adjuvant treatment for hepatoma.

Department of Biochemistry, Shanghai Second Medical University, Shanghai 200025, China
Dr. Yu Cheng Tang, graduated in 2000 from Shanghai Second Medical University, specialized in tumor biology.
Project supported by National Natural Science Foundation of China, No.863-Z20-01-04
Correspondence to: Yu Cheng Tang, Department of Biochemistry, Shanghai Second Medical University, Shanghai 200025, China
Tel. 0086-21-63846590 Ext. 504, Fax. 0086-21-63842916
Email. tangyc@shsmu.edu.cn
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INTRODUCTION

Neovascularization is critical for supporting the rapid growth of solid tumors[1]. Tumor angiogenesis appears to be achieved by the expression of angiogenic agents within solid tumors that stimulate host vascular endothelial cell mitogenesis and possibly chemotaxis. One such protein, vascular endothelial growth factor (VEGF) or vascular permeability factor[2-5], is a selective endothelial cell mitogen and angiogenic agent .Many tumor cell lines secrete VEGF *in vitro*, suggesting that this diffusible molecule is a mediator of tumor angiogenesis. The clinical results showed high levels of VEGF expression in primary hepatoma, elevated levels of flt-1, the receptors of VEGF in hepatoma blood vessels, and the relationship between VEGF levels and hepatoma invasion and transfer[6]. These data indicated that VEGF and its receptors play important roles in the development of hepatoma vasculature and progressive growth of hepatoma.

In this study, we used the SMMC-7721 hepatoma cell line which has a high expression of

VEGF, as established model for human hepatoma. The strategy of exogenous expression of antisense VEGF transcribed by POL III promoter in the SMMC-7721 cell line was applied to assess the feasibility of disrupting the VEGF/VEGF receptor pathway of angiogenesis and decreasing their tumorigenicity *in vivo*.

MATERIALS AND METHODS

Materials

Bam HI, T4 DNA ligase was purchased from Promega Company. RNase A and RNase T1 were the products of MBI Fermentas, G418 was purchased from Sigma Company and SMMC-7721 cell line from Chinese Academy of Cell Biology.

Methods

Construction of vectors VEGF antisense vector to generate the VEGF anti-sense vector, a DNA fragment containing 250 bp of human VEGF cDNA, was ligated in reverse orientation in the *sal* I, *xho* I sites of the U6 cassette, and subcloned into the *Bam* H I site of pLXSN vector. Expression of the antisense molecule in pLXSN was driven by POLIII promoter of U6 cassette. The pLXSN vector also contained the G418 resistance gene driven by the simian virus (SV40) promoter. To generate infectious virions, PA317 packaging cells were transfected with pLXSN-U6-as-VEGF and selected in the culture medium containing 500 mg/L G418. Virus-containing supernatants were harvested and used to infect SMMC-7721 cells.

Genetic modification of SMMC-7721 cells The SMMC-7721 cells were incubated with the viral stock containing 8 mg/L polybrene. On the following day, the cells were split and selected in 500 mg/L G418. Cultures were added every 3-4 days with the fresh G418 supplemented media for 14 days. Resistant colonies were expanded, and subcloned and the clone which produced the reduced levels of VEGF was selected for further research.

Ribonuclease protection assay A 200bp VEGF PCR product was cloned into T7,T3 vector pBlueScript-SK, the plasmid was linearized by *Eco* R V, treated with proteinase K and purified. The α-^{32}P UTP sense VEGF RNA was generated by addition of T7 polymerase. Ribonuclease protection assays were made as follows, 20 μg of total cellular RNA was hybridized with RNA probes overnight at 45℃. The remaining single-stranded probe RNA and unhybridized RNA were digested with a mixture of RNase A and RNase T1, added yeast RNA, extracted by phenol, precipitated by ethanol, seperated on 7M urea/polyacrylamide gels, and then exposed to X-ray film.

PCR, southern blot analysis PCR was performed on genomic DNA isolated from human SMMC-7721 cells and individual clones of transfected cells using a sense primer corresponding to the U6 promoter (5'-TATACTAAGTCGACTCCTATGTGCTGG-3') and an antisense primer corresponding to the VEGF cDNA (5'-TAGAGAGGGCAGAATCATCACG-AAGTGG-3'). Using the NeoR primer, the sense primer is 5'-CAAGATGGAATTGCACGCAGG-3', the reversal primer is 5'-CCCGCTCAGAAAGAACTCGTC-3'. The PCR was performed using the following protocol: 95℃ 1min, 60℃ 1min, 72℃ 1min 30s; in the last cycle, extend 10 min at 72℃. Southern blot, 20 μg genomic DNA was digested overnight, electrophoresed on 1% agarose gels, transferred onto Hybond N nylon membrane, and hybridized with the DIG labeled NeoR probe at 68℃ for 6 h, the membrane was washed in 2 × SSC for 5 min × 2, and 0.1 × SSC for 15 min. The fragments were visualized by chemiluminescent, and exposed to X-ray film.

Quantitation of VEGF The supernatant of parental or transfected SMMC-7721 cells were measured by ELISA. To generate the conditional medium, the cells were seeded onto 3.0×10^5/well plates. The media was changed next day to MEM /0.5% bovine serum albumin/ 1% dialyzed fetal calf serum for another 24 h. The media was then replaced by the fresh MEM and cells were allowed to grow for another 48 h. The CM was generated by centrifugation at 14 000rpm at 4℃ for 15 min, then for ELISA analysis according to the manufacturer's instructions.

***In vitro* growth rate** SMMC-7721 hepatoma cells and cells transfected with antisense, and sense-VEGF were cultured at 1×10^4 and grown under standard culture conditions. Cell count was made every 24 h for a total of 144 h. The total number of cells from duplicate experiments was determined as a function of time.

Determination of *in vivo* tumor growth Subcutaneous inoculation and tumor growth measurements were carried out, 1×10^6 cells of the parental SMMC-7721 cells or antisense, sense expressing clones were injected into the flank of normal BALB/C nude mice. Tumors were measured in two dimensions every 5 days for 25 days. Tumor volume was calculated using the formula $v = l \times w^2/2$, where v = volume (mm^3), l = long diameter, and w = short diameter.

RESULTS

Construction of the antisense-VEGF RNA expression vector based on U6 POLIII promoter

All of the major transcriptional promoter elements for U6 RNA polymerase III are upstream of the transcription start, which has a potential advantage of the less exogenous RNA coding sequence. Another advantage of the U6 promoter is that U6

gene is heavily expressed in human cells. The U6 cassette contained the first 5' initial 27 nucleotides and 3' stem 19 nucleotides for transcript terminator and the U6+27 transcript was predicted to be most stable because of the γ-phosphomethyl-GTP cap. The fragment of VEGF was cloned into U6 cassette through sense or antisense direction, and verified by DNA sequence. U6 cassette containing sense or antisense VEGF was cleaved by *Hind* III, *Pst* I digestion and cloned into pBlue-SK vector, and subcloned into *Bam* HI restriction site of retroviral expression vector. The positive plasmid was verified by *Bam* HI digestion (Figure 1).

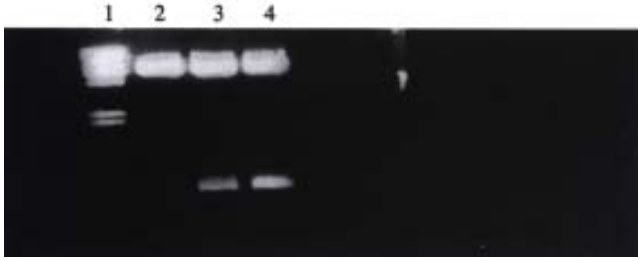

Figure 1 Electrophoresis pattern of pLXSN-U6 sense, antisense VEGF plasmid digested by *Bam* HI.
Lane 1: λ DNA *Hind* III Marker; Lane 2: pLXSN digested by *Bam* HI; Lane 3: pLXSN-U6 sense VEGF digested by *Bam* HI; Lane 4: pLXSN-U6 antisense VEGF digested by *Bam* HI.

SMMC-7721 cells expressing antisense-VEGF

Following transfection by recombinant antisense VEGF or sense VEGF retrovirus, the SMMC-7721 cells were selected by antibiotic G418, the individual clones were isolated and expanded. And the selected clone expressed low VEGF for further analysis and was referred to as anti-1. This clone was evaluated for gene expression in ribonuclease protection assays. In this assay, hybridization of RNA with the complementary RNA probe protects the probe from the subsequent digestion with RNase A and RNase T1. From Figure 2, it can be seen that 200 bp antisense VEGF RNA was only expressed in SMMC-7721 transfected by pLXSN U6 antisense VEGF.

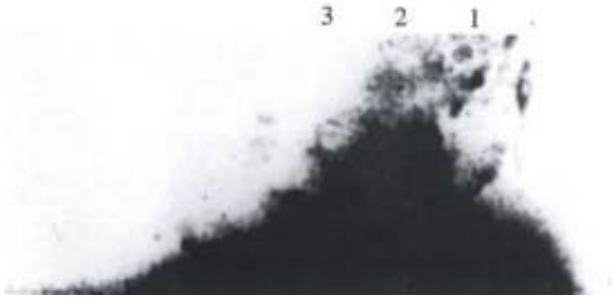

Figure 2 Detection of antisense VEGF RNA expression by RNase protection assay.
Lane 1: Hybridization with total RNA from SMMC-7721 antisense clone showed 200 bp antisenseVEGF RNA; Lane 2: Hybridization with total RNA from SMMC-7721 sense clone showed no positive band; Lane 3: Hybridization with total RNA from SMMC-7721 cells showed no positive band.

PCR analysis of DNA isolated from the antisense-VEGF, sense VEGF clones showed foreign gene integration into the genomic DNA, and the results of PCR using the specific primer, showed that the antisense VEGF U6 gene cassette had inserted the genomic DNA of SMMC-7721 cells. Southern blot analysis was performed on genomic DNA of these antisense VEGF, senseVEGF clones to verify again that there was foreign integrated cDNA (Figures 3, 4).

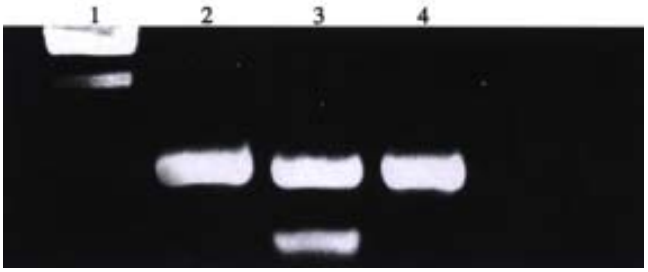

Figure 3 PCR amplification of genomic DNA from SMMC-7721 antisense, sense VEGF clone.
Lane 1: Marker; Lane 2: SMMC-7721 / sense VEGF clone showed of neo gene 790 bp; Lane 3: SMMC-7721 / antisense VEGF clone showed neo gene 790 bp, U6 cassette 260 bp; Lane 4: SMMC-7721 / pLXSN clone showed neo gene 790 bp

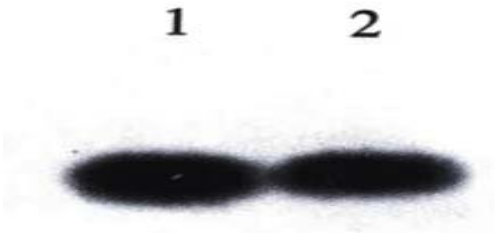

Figure 4 Genomic analysis of SMMC-7721 / U6 antisense VEGF, SMMC-7721 / U6 sense VEGF clones.Lane 1: SMMC-7721 / U6 sense VEGF clone; Lane 2: SMMC-7721 / U6 antisense VEGF clone.

Diminished production of VEGF by SMMC-7721 cells transduced with antisense VEGF cDNA

In order to determine if the expression of the antisense VEGF transgene reduced production of secreted protein, supernatant from control-transduced (SMMC-7721 sense VEGF) and antisense VEGF transduced cells were assayed for VEGF by ELISA. Production of VEGF by antisense transgene-expressing cells was 65 + 10ng/L per 10^6 cells, as compared with 420 + 45 ng/L per 10^6 cells in sense group and 485 + 30 ng/L per 10^6 cells in negative control group, $P < 0.05$.

In vitro growth rate of antisense-VEGF cell lines

The antisense-VEGF cell lines appeared phenotypically indistinguishable from normal SMC-7721 cells and SMMC-7721 transfected sense VEGF cells. And growth rates of antisense-VEGF cell lines were the same as the control cells (Figure 5).

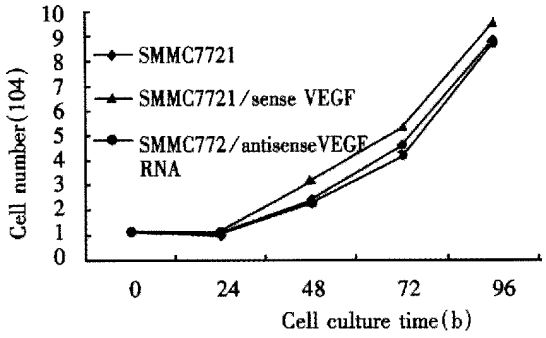

Figure 5 Proliferation curves of parental SMMC-7721 cells and antisense-VEGF, sense-VEGF cell lines.

In vivo growth of the antisense-VEGF cell lines

Control SMMC-7721 cells and antisense-VEGF SMMC-7721 cells were s.c. injected into nude mice, tumor volumes were measured every 5 days. Tumor growth was detectable and measurable for control SMMC-7721 cells 5 days post-implantation, while the antisense VEGF cell lines gave rise to tumors. Examination of mice at 25 days post-implantation revealed that the negative control SMMC-7721 group produced tumors of 630.92 ± 85 mm^3, sense-VEGF SMMC-7721 group produced tumors of 601.07 ± 52 mm^3, while the antisense VEGF SMMC-7721 group produced tumors of 76.33 ± 20 mm^3. This experiment demonstrates that the reduced tumorigenicity of antisense-VEGF SMMC-7721 cells in nude mice may be attributed to the reduced expression of VEGF (Figure 6).

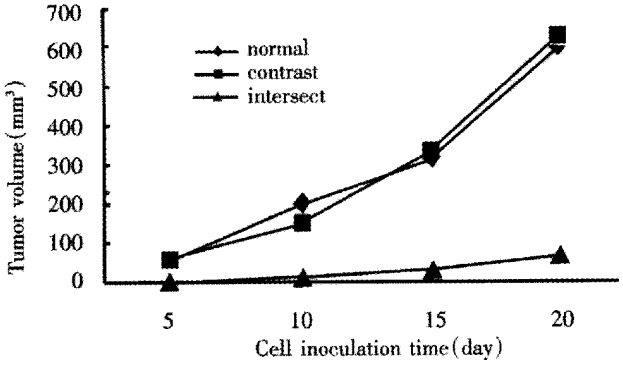

Figure 6 Tumorgenicity of antisense VEGF SMMC-7721 in nude mice.

DISCUSSION

Angiogenesis, the formation of new blood vessels , is essential for both tumor growth and metastasis [7-10]. Tumor angiogensis is a process controlled by certain chemicals produced in cancer cells. These chemicals stimulate endothelail cells to form new blood vessels. Candidates as major physiological stimulators include VEGF[11], bFGF. VEGF, and its receptors play critical roles in tumor-associated angiogenesis and represent good targets for therapeutic intervention[12-15]. VEGF was initially termed vascular permeability factor, its first function was discovered by Dvorak and colleagues[16]. There are several VEGF isoforms, in which VEGF121 and VEGF165 are readily secreted. Unlike bFGF, VEGF is a very specific mitogen for vascular endothelial cells. It also functions as a potent pro-survival factor for endothelial cells in nearby formed vessels and this may be one of its most important functions[17,18].

It is reported that VEGF is an angiogenic factor most closely associated with the neovascularization in solid tumors. VEGF is expressed by vast majority of cancers at elevated levels and blocks its activity by specific neutralizing antibodies to VEGF[19,20]. VEGF-toxin conjugates[21], aptamers[21] and small molecule VEGF receptor antagonists[22] could inhibit the growth of cancer in animal models. In human hepatocellular carcinoma, abundant tumor vascularity was observed. And vascular endothelial growth factor gene and protein expression was analyzed by means of Northern hybridization and immunohistochemistry, increased expression of VEGF has been reported in hepatocellular carcinoma cells (HCC)[23-26]. So VEGF gene expression is significantly associated with angiogenesis of HCC. Tang Zhao You *et al* studied the angiogenesis induced by liver cancer with different metastatic potentials using corneal micropocket model in nude mice. It was suggested that highly metastatic liver cancer was more angiogenic than low metastatic cancer and liver tissue[26]. In HCC with metastasis, mRNA of VEGF is closely related to the growth of HCC as well as its metastasis[27].

In China, the hepatocarcinogenesis is closely related with the hepatitis virus[28], the results of the researches showed that, after viral infection,there is abnormal expression of oncogene such as ras, bcl-2, especially P53[29-33], and there is also a possible link between oncogenes and tumor angiogenesis. Expression of mutant ras can lead to a marked induction of a potent paracrine stimulator of angiogenesis. In addition, hypoxia stimulates expression of VEGF and tumor angiogenesis[34-40]. The results of therapeutic experiments showed that the chimeric protein consisting of DT390-VEGF165 or DT390-VEGF exon7 can efficiently kill the HepG2 and gastric carcinoma cells and may kill vascular endothelial cells in the cancer[41, 42] and antiangiogenesis inhibitor TNP-470 plus lipiodol greatly decreased the hepatoma growth in animal models which depend on the reduction of microvessel density[43].

Blocking the interaction between the VEGF and its receptor can inhibit the growth of tumor through the antiangiogenesis effect[44-47]. From our

previous experiment, we found VEGF expression in hepatoma cell line SMMC-7721 cells. We there fore sought to determine if inhibition of secretion of VEGF in SMMC-7721 tumor cells would inhibit the growth of this tumor in animal model.

In order to improve the expression of the antisense VEGF RNA in the target cell, we constructed the retrovirus vector containing the human U6 promoter cassette that had the POL III promoter to transcribe the small therapeutic RNA in the nuclei of cells. Compared with other transcriptional promoters such as POL II, tRNA, there are two advantages of U6 promoter: ① high expression in human cells, and ② the therapeutic RNA contains less unnecessary RNA encoding the intragenic ptomoter. In the same time, we amplified a common VEGF cDNA and inserted reversely into U6 cassette[48-52]. U6 promoter transcribed a small antisense VEGF RNA fragment that could specifically interacted with VEGF165 and VEGF121 mRNA. Our previous results, verified that U6 cassette could effectively express antisense VEGF RNA molecules and decreased the expression of mRNA VEGF165, and VEGF121. Then U6 cassette that expressed antisense VEGF RNA was inserted into the retroviral construct. After packaging the recombinant retrovirus, this cassette was introduced into the SMMC-7721 cells. Ribonuclease protection analysis using the RNA probe specific for antisense VEGF demonstrated that there was antisense VEGF RNA expression in the SMMC-7721 cells genetically modified by antisense U6 cassette. The antisense clone selected for further study showed radical decrease in VEGF protein in supernatant compared with the sense and negative SMMC-7721 cell group. Inhibition of VEGF expression in SMMC-7721 cells resulted in severely impaired growth of this tumor *in vivo*. This may be related with the reduced levels of VEGF produced by the antisense-VEGF-transfected SMMC-7721 cell clone, and this resulted in a decrease of number of tumor blood vessels. Our findings demonstrate that the inhibition of VEGF is sufficient to control the tumor growth *in vivo*. The antisense VEGF srategy offers a way for gene therapy as an adjuvant treatment for hepatoma.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Multimodality treatment in hepatocellular carcinoma patients with tumor thrombi in portal vein

Jia Fan, Zhi Quan Wu, Zhao You Tang, Jian Zhou, Shuang Jian Qiu, Zeng Chen Ma, Xin Da Zhou and Sheng Long Ye

Subject headings carcinoma, hepatocellular/therapy; neoplasm circulating cells; portal vein; antineoplastic agents; combined modality therapy; chemoembolization, therapeutic

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Abstract

AIM To compare the therapeutic effect and significances of multimodality treatment for hepatocellular carcinoma (HCC) with tumor thrombi in portal vein (PVTT).

METHODS HCC patients (*n* = 147) with tumor thrombi in the main portal vein or the first branch of portal vein were divided into four groups by the several therapeutic methods. There were conservative treatment group in 18 out of patients (group A); and hepatic artery ligation (HAL) and/or hepatic artery infusion (HAI) group in 18 patients (group B), in whom postoberative chemoembolization was done periodically; group of removal of HCC with PVTT in 79 (group C) and group of transcatheter hepatic arterial chemoembolization (TACE) or HAI and/or portal vein infusion (PVI) after operation in 32 (group D).

RESULTS The median survival period was 12 months in our series and the 1-, 3-, and 5-year survival rates were 44.3%, 24.5% and 15.2%, respectively. The median survival times were 2, 5, 12 and 16 months in group A, B, C and D, respectively. The 1-, 3- and 5-year survival rates were 5.6%, 0% and 0% in group A; 22.2%, 5. 6% and 0% in group B; 53.9%, 26.9% and 16.6% in group C; 79.3%, 38.9% and 26.8% in group D, respectively. Significant difference appeared in the survival rates among the groups (*P*<05).

CONCLUSION Hepatic resection with removal of tumor thrombi and HCC should increase the curative effects and be encouraged for the prolongation of life span and quality of life for HCC patients with PVTT, whereas the best therapeutic method for HCC with PVTT is with regional hepatic chemotherapy or chemoemblization after hepatic resection with removal of tumor thrombi.

Liver Cancer Institute, Zhongshan Hospital, Fudan University Medical Center (Former Shanghai University), 136 Yixueyuan Road, Shanghai 200032, China

Dr. Jia Fan, doctor degree from Shanghai Medical University in 1995, professor of surgery, major in surgical oncology of liver, having more than 60 papers published.

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Correspondence to: Jia Fan, M.D., Ph.D. Liver Cancer Institute, Zhongshan Hospital, Fudan University Medical Center (Former Shanghai University), 136 Yixueyuan Road, Shanghai 200032, China
Tel/Fax. +86-21-64037181
Email. jiafan 99 @ yahoo.com

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INTRODUCTION

Hepatocellular carcinoma (HCC) with tumor thrombi in the main trunk or the first branch of the portal vein would be considered to be advanced stage of liver cancer[1], which often results in intrahepatic metastasis and can only be treated with conservative or non-operative methods such as per oral chemotherapy, biotherapy, traditional Chinese medicine, etc. In China, some patients even give up all the therapeutic methods for HCC. The majority of those patients usually die of liver failure or bleeding of the upper digestive tract within several months[2,3]. In recent decade, the active therapeutic measure for HCC with tumor thrombi in portal vein (PVTT) was performed at the Liver Cancer Institute of Shanghai Medical University, i. e., hepatic resection with removal of PVTT. After the operation, some patients obtained satisfactory curative effects through transcatheter chemotherapy or transcatheter hepatic arterial chemoembolization (TACE), or hepatic artery infusion (HAI) and/or portal vein infusion of chemotherapeutic agents (PVI).

MATERIALS AND METHODS

Patients

By December 1996, 147 patients with pathologically proven HCC with tumor thrombi in the main trunk or the first branch of the portal vein had been treated at the Liver Cancer Institute of Shanghai Medical University. Among them, 144 were males and 3 females, the median age was 48. 2 years (ranged from 20 to 70 years). Serum hepatitis B surface antigen was found positive in 123 cases (83.7%). Coexisting cirrhosis was found in 138

cases (93.9%): macronodular cirrhosis (cirrhotic nodule >3 mm) in 81 patients and micronodular cirrhosis (cirrhotic nodule ⩽3 mm) in 57 patients. Alpha-fetoprotein (AFP) levels were elevated (21-16 000 μg·L^{-1}) in 120 cases (81.6%). The tumors were situated in the left lobe in 53 cases, the right lobe in 61 and both lobes in 33.

Groups of patients

One hundred and forty-seven patients were divided into 4 groups. Group A: conservative treatment group, receiving only oral medications of chemotherapy or Chinese herbal decoction and supporting treatment, 18 cases; Group B: hepatic artery ligation (HAL) and/or HAI or PVI (postoperative chemotherapy through hepatic artery or portal vein, and periodical chemoembolization through hepatic artery), 18 cases; Group C: removal of HCC with PVTT in 79 cases; among them, 5 patients were lost to follow-up. The tumors and tumor thrombi were resected simultaneously or tumor thrombi were extracted from the portal vein after removal of the tumors for the HCC patients with PVTT; Group D: Intrahepatic arterial chemoembolization or chemotherapy and/or intraportal chemotherapy by catheterization or TACE were periodically performed after resection of HCC with PVTT in 32 cases; among them, 3 cases were lost to follow-up.

Location of tumor thrombi

Fifty-one cases had tumor thrombi in left branch of portal vein, 47 in right branch, 10 in both branches, 14 in left branch extending to the main portal vein, 9 in right branch extending to the main trunk of portal vein, and 16 in both branches and the main portal vein.

Comparison of clinical data in different groups in HCC patients with PVTT

These data are shown in Table 1, indicating that there were no statistical differences between groups.

Table 1 Comparison of several treatments groups for HCC with PVTT

	Group A (n = 18)	Group B (n = 18)	Group C (n = 74)	Group D (n = 29)	P
Tumor diameter					
<10 cm	2	5	39	14	NS
⩾10 cm	16	13	35	15	
Tumor number					
Single	7	13	46	18	NS
⩾2	11	5	28	11	
Encapsulation					
Well	1	3	16	5	NS
Poor	17	15	58	24	
PVTT in main trunk					
-	6	12	58	24	NS
+	12	6	16	5	

NS: no significance statistically.

Resection of HCC and catheterization after resection

Resection of left lateral lobe was performed in 18 cases, resection of left lateral lobe and partial right hepatic resection in 3, left hemihepatectomy in 40, left trilobectomy in 9, resection of hepatic medial lobe in 3, right hemihepatectomy in 4 and right partial hepatectomy in 34. PVI was followed by resection in 5 cases, HAI in 7 and HAI + PVI in 9. The catheters were cannulated into the hepatic artery and portal vein, and the ports were completely implanted beneath the abdominal wall.

Treatment methods for PVTT

For the tumor thrombi in left branch of portal vein, the right branch of portal vein was detached at first and then ligated by using a cotton ribbon or a fine rubber tube. The purpose was to prevent spreading of cancer cells of tumor thrombi from the left branch of portal vein to the right lobe during the resection or PVTT removal. After resection of left lateral lobe or left hemihepatectomy, the tumor thrombi were taken out from the stump of left branch of portal vein and irrigation was done with physiological saline, then the stump of portal vein was sutured and the ligature of the right branch of portal vein was released.

For tumor thrombi in the right branch of portal vein, the treatment for HCC with PVTT was similar to that in the left branch.

For the tumor thrombi in both branches of portal vein, the stump of one branch of portal vein was exposed after resection of liver cancer in left or right lobe, the hepatoduodenal ligament was ligated to block the blood flow entering the liver, and then the tumor thrombi were sucked or taken out through the stump.

For the tumor thrombi in the left or right branch of portal vein extending to the main trunk, the liver cancer in left or right lobe was removed first after detaching and ligating the right or left branch of portal vein, the stump of portal vein was clipped, then the main portal vein over the duodenum was gently held by the thumb and index finger. While the stump was being loosened, the tumor thrombi would flow out with the on-going portal blood flow.

For the tumor thrombi in both branches and the main trunk of portal vein, the tumor thrombi were sucked through the stump of one branch of portal vein, while pressing the opposite branch with an index finger for preventing dissemination of tumor cells. If the tumor thrombi were difficult to be sucked or taken out, the main portal vein should be detached. A longitudinal incision of 1.5 to 2.0 cm was made on anterior or right vascular wall of the main trunk of portal vein, and then the tumor thrombi were taken out directly. After the operation, B-mode ultrasonography was performed to make out whether the tumor thrombi had been

removed completely from the portal vein.

Chemotherapy after operation

Group D: After operation, 11 patients received 1 to 3 times of TAI or TACE (median 1.5 times) and other 21 patients received 2 to 4 times (median 2.5 times) of HAI and/or PVI or hepatic arterial chemoembolization (HAE). Group B: All patients were treated by HAI or HAE and/or PVI 1 to 4 times (median 2 times). The dosages of chemotherapy in HAI or TAI and/or PVI were 5-fluorouracil (5-Fu) 1 000 mg, mitomycin (MMC) 12 to 20 mg and cisplatin or carborlatinum 80 mg, and lipoidal 5 mL in group D to 20 mL in group B of lipiodol was used in the TACE or HAE.

Statistical method

All the data were calculated with the digital Cox model and the limit of significant difference was $P<0.05$.

RESULTS

Curative effects of several therapeutic methods for HCC patients with PVTT

Among the series, the follow-up survey was lost in 8 cases. The median survival period of the 139 patients was 12 months, and the 1-, 3 and 5- year survival rates were 44.3%, 24.5% and 15.3% respectively. The median survival period and the 1, 3 and 5- year survival rates in different groups are shown in Table 2.

Table 2 Survival time and rates of patients with PVTT

	Group A (n = 18)	Group B (n = 18)	Group C (n = 74)	Group D (n = 29)
Median survival periods (months)	2.0	5.0	12.0	16.0
Survival rates				
1-year(%)	5.6	22.2	53.9[a2]	79.3[a1]
3-year (%)	0	5.6	26.9[a2]	38.9[a1]
5-year(%)	0	0	16.6[a2]	26.8[a1]

[a1]$P<0.05$, *vs* Group C, B and A; [a2]$P<0.05$, *vs* Group B and C.

Table 3 Survival time and rates of patients with the tumor thrombi in the main trunk and the first branch of portal vein

	Tumor thrombi in the main trunk				Tumor thrombi in the first branch			
	Gr.A (n = 12)	Gr.B (n = 6)	Gr.C (n = 16)	Gr.D (n = 5)	Gr.A (n = 6)	Gr.B (n = 12)	Gr.C (n = 58)	Gr.D (n = 24)
MST* (months)	2.0	5.5	8.0	16.0	2.0	5.0	13.0	16.5
Survival rate								
1-year(%)	8.3	33.3	29.4	80.0	0	16.7	59.7	79.2
3-year (%)	0	0	14.3	20.0	0	8.3	27.4	54.6
5-year(%)	0	0	11.1	0	0	0	8.8	42.0

* MST: median survival time.

Curative effects between PVTT in the main trunk and the first branch

Table 3 shows the results of multimodality treatment in patients with tumor thrombi in the main trunk and the first branch of portal vein. It indicates that the results of treatment in patients with PVTT in the first branch were better than that in patients with PVTT in the main trunk in groups C and D.

Curative effects and factors influencing the prognosis of the patients

after resection of HCC with PVTT (Table 4) The results of resection followed by chemotherapy or chemoembolization in patients with a diameter of tumor mass less than 10 cm, without tumor thrombi in the main portal vein were better than those in patients with diameter of more than 10 cm, with tumor thombi in the main portal vein and without chemotherapy or chemoembolization after resection.

Table 4 Curative effects and factors influencing the prognosis of patients after removal of HCC with PVTT

	Patients	1-year(%)	3-year(%)	5-year(%)	P
Removal of PVTT	103	61.7	32.3	22.4	
Tumor diameter					
<10 cm	53	64.2	41.5	27.0	0.014[a1]
⩾10 cm	50	59.7	25.2	19.3	
PVTT in main trunk					
-	82	66.3	36.9	26.1	0.027[a2]
+	21	42.9	14.3	8.6	
Encapsulation					
Well	21	75.6	30.9	8.6	0.928
Poor	82	58.4	32.9	26.7	
Chemotherapy or chemoembolization after operation					
No	74	53.9	26.9	16.6	0.012[a3]
Yes	29	79.3	38.9	26.8	
Tumor number					
Single	64	59.4	27.7	18.7	0.561
⩾2	39	65.4	38.7	27.7	

[a1]$P<0.05$ *vs* ⩾10 cm group; [a2]$P<0.05$ *vs* PVTT in main trunk (+) group; [a3]$P<0.05$ *vs* chemotherapy or chemoembolization after operation (yes) group.

DISCUSSION

HCC with tumor thrombi in the main trunk or the first branch of the portal vein is considered to be a late stage of liver cancer, lacking in ideal therapeutic measures. The treatment was often given up in former times. As the majority of the patients with HCC have been in advanced stage when first seen, a large probortion of the patients are associated with tumor thrombi in the main trunk or the first branch of the portal vein. Therefore, it is very important to explore effective therapeutic methods for HCC with PVTT and to raise the survival rates of these patients.

Until now, the treatments for HCC with PVTT are limited. In the medical literaures about the treatments of HCC with PVTT, there were the following methods: TACE, PVI, percutaneous ethanol injection in tumor thrombi, TACE + radiotherapy and surgical removal of tumor thrombi etc. Chung *et al*[4] reported that the 1 and 2-year survival rates were 30% and 18% respectively in 110 HCC patients with PVTT by TACE. Katsumori *et*

al[5] reported that the 3-year survival rate was 44% in 9 cases of tumor thrombi in the main portal vein by hepatic arterial chemotherapy through the implanted ports beneath the abdominal wall. It was also reported that the percutaneous ethanol injection in PVTT and TACE + radiotherapy gave satisfactory curative effects for PVTT[6-8]. There were only a few papers about the resection of HCC with removal of tumor thrombi in the main trunk or the first branch of the portal vein[9,10]. Yamaoka *et al*[9] adopted 5 resection modes for PVTT. The operative mortality of 29 patients with PVTT was 11%, and the 1, 2 and 3-year survival rates after operation were 52.2%, 23. 2%, and 11.6%, respectively. Four different procedures were used for the patients with PVTT in our series, and the results revealed poor efficacy in the conservative treatment group. The median survival time was only 2 months (group A). The best curative effect was in group of removal of HCC with PVTT + HAI and/or PVI or TACE (group D). The median survival time was 16 months and the 5-year survival rate was 26.8%. For this reason, if the tumors with PVTT are limited in the left or right lobe of liver and liver function as evaluated is able to tolerate the operation, we should adopt active therapeutic procedure. The best try should be made for removal of HCC with PVTT and hepatic regional chemotherapy or chemoembolization are periodically performed after operation. This procedure can further raise the treatment efficacy and prolong the survival for the HCC patients with PVTT.

The conservative treatment (only per oral chemotherapy or Chinese herbal decoctions) can not inhibit or kill cancer cells in the HCC patients with PVTT. Rapid progression of liver cancer will result in death in a short time due to liver failure or portal hypertension, with vigorous variceal bleeding. Among 18 patients undergoing conservative treatment, 12 cases died of vigorous bleeding of upper digestive tract within 3 months and only one case had the longest survival of 13 months. Although the survival time of 18 patients treated with HAL and/or HAI were shorter, the curative efficacy was better than that of conservative treatment group. The 1-year survival rate was 22.2%. Perhaps the periodical hepatic artery chemotherapy or chemoembolization can inhibit the tumor growth in various degrees and result in partial necrosis of tumor thrombi. The results of Katsumori[5] and Ando[11] also support this viewpoint. The result in the group of removal of HCC and PVTT (Group C) was evidently better than those of Groups A and B, the median survival time being 12 months and the 1, 3 and 5-year survival rates being 53.9%, 26.9%, 16.6% respectively. If HCC is limited to one lobe and the residual liver is estimated to be functionally efficient, we should first choose surgical resections for the patients with PVTT. The residual cancer and residual tumor thrombi are primary factors influencing the survival of the HCC patients with PVTT. According to patient's conditions, periodical HAI/PVI or TACE are effective methods to improve the survival rate after operation. In the group of HAI/ PVI or TACE after resection of HCC with PVTT (group D), the median survival time was 16 months and the 1, 3, and 5-year survival rates were 79.3%, 38.9%, 26.8% respectively. The result was better than the other three groups. These results indicate that the best therapeutic method is sequential regional hepatic chemotherapy or TACE after surgical removal of HCC and PVTT. Table 3 shows curative effects in HCC patients with tumor thrombi in the main trunk of portal vein were inferior to those in patients with tumor thrombi in the first branch. The tumor thrombi in the main trunk represent the disease in advanced stage, which is often accompanied with tumor thrombi in both branches and disseminated foci in the opposite side of liver. Moreover, the resection of thrombi in the main trunk of portal vein may lead to cancer cell dissemination or incomplete extermination and rapid formation of thrombi in the main trunk after operation. The above factors cause the poor efficacy for HCC patients with tumor thrombi in the main trunk of portal vein, and poor quality of life than those with tumor thrombi in the first branch.

It has been convinced that the factors influencing curative effects for HCC with PVTT such as location of tumor thrombi (in the main trunk or the first branch of portal vein), tumor size and regional hepatic chemotherapy, etc. To our experience, the treatment methods for removal of tumor thrombi are closely related to the efficacy. Before operation, we must find out the scope of tumorthrombi with ultrasonography, CT or magnetic resonance angiography (MRA). During resection, we should block the blood flow of portal vein into liver or blood flow of the opposite branch, suck the tumor thrombi completely as far as possible and not break it. Then, we scrape off with curet the residual tumor thrombi attached on the vascular wall, and perform repeated irrigation and exsuction with physiological saline. Furthermore, anticoagulant injections through the portal vein during several postoperative days may also be an effective procedure to prevent and reduce the rapid recurrence of thrombi in the portal vein[12,13].

The therapeutic methods for HCC with PVTT are still at the investigative stage. Such patients occupy a considerable proportion in the liver cancer clinic. As PVTT is an important factor resulting in intrahepatic metastasis and recurrence after operation, it will be very valuable to study the mechanism of the PVTT formation in HCC patients and to explore more effective treatment measures for prolonging survival and improving their quality of life.

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Edited by Pan BR
proofread by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance

De Yun Feng, Hui Zheng, Yi Tan and Rui Xue Cheng

Subject headings liver neoplasms; mitogen-activated protein kinases; signal transduction; trans-activators; oncogenes; immunohistochemistry; precancerous conditions

Feng DY, Zheng H, Tan Y, Cheng RX. Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance. ***World J Gastroenterol***, 2001;7(1):33-36

Abstract

AIM To study the effect of phosphorylation of MAPK and Stat3 and the expression of c-fos and c-jun proteins on hepatocellular carcinogenesis and their clinical significance.

METHODS SP immunohistochemistry was used to detect the expression of p42/44^{MAPK}, p-Stat3, ca2fos and c-jun proteins in 55 hepatocellular carcinomas (HCC) and their surrounding liver tissues.

RESULTS The positive rates and expression levels of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs were significantly higher than those in pericarcinomatous liver tissues (PCLT). A positive correlation was observed between the expression of p42/44^{MAPK} and c-fos proteins, and between pa2Stat3 and c-jun, but there was no significant correlation between p42/44^{MAPK} and p-Stat3 in HCCs and their surrounding liver tissues.

CONCLUSION The abnormalities of Ras/Raf/MAPK and JAKs/Stat3 cascade reaction may contribute to malignant transformation of hepatocytes.Hepatocytes which are positive for p42/44^{MAPK}, c-fos or c-jun proteins may be potential malignant pre-cancerous cells. Activation of MAPK and Stat3 proteins may be an early event in hepatocellular carcinogenesis.

Department of Pathology, Hunan Medical University, Changsha 410078, Hunan Province, China
New England Biolab, MA, USA
Dr. De Yun Feng, graduated from Hunan Medical University as postgraduate in 1991, now associate professor of pathology, specialized in hepatic pathology, having 21 papers published.
Supported by New England Biolab, MA, USA.
Correspondence to: Dr. De Yun Feng, Department of Pathology, Hunan Medical University, Changsha 410078, Hunan Province, China
Tel. 0086-731-4805407
Email. cslj4841401@public.cs.hn.cn
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INTRODUCTION

The regulation of cellular mitogenesis and proliferation under normal circumstances are dependent on a number of separate, yet integrated, signaling pathways responsible for the transduction of extracellular signals to the cell nucleus[1-3]. Similar to other tumorigenic cells, hepatocellular carcinoma (HCC) is characterized by the imbalance of the normal growth-promoting and growth-arresting signal transduction cascade reaction, the net result of which leads to uncontrolled hepatocyte growth [1,2]. Mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (Stat3) are important molecules in signal transduction cascades, which take part in cellular physiological growth, development, mitogenesis and differentiation, and play key roles in cellular malignant transformation[4,5]. In order to study the function of signal transduction cascade in hepatocarcinogenesis, the phosphorylation of MAPK (p42/44 MAPK, Erk1/Erk2) and Stat3 (p-Stat3), and the expression of c-fos and c-jun proteins were investigated by immunohistochemical method in HCCs and their surrounding liver tissues.

MATERIALS AND METHODS

Tissue samples

Fifty-five HCCs with pericarcinomatous tissue (PCLT) and 5 normal liver tissues were obtained by surgical resection in Xiang Ya Hospital and the Affiliated Second Hospital of Hunan Medical University, Changsha, People's Republic of China. Of 55 HCC patients, 43 were men and 12 women. The age ranged from 36 to 69 years (mean, 51.8 years). The tumor sizes were less than 3cm in diameters in 3 cases, 2.1 cm-5 cm in 32 cases, 5.1 cm-10 cm in 18, and larger than 10 cm in 2 cases. All patients were HBsAg negative. Remote metastasis was not found, and preoperative radiological or chemical therapy was nor performed in all patients. All HCCs and PCLTs were fixed in 10% formalin and embedded in paraffin. Five μm serial sections were cut. According to Edmondson's criteria[6], 10 HCCs were grade I, 21 grade II, 17 grade III, and 7 grade IV. Thirty-eight atypical hyperplasia cases were observed in 55 PCLTs.

Reagents

p42/44 MAPK(Erk1/Erk2, Thr202/Tyr204) and p-Stat3(Ser727) MAbs, were used to detect the

phosphorylation of MAPK and Stat3, and S-P detection kit were kindly donated by New England Biolab, USA. Anti-c-fos and anti-c-jun protein MAbs were purchased from GIB Com (Beijing, China).

Methods

Immunohistochemical staining Five μm tissue sections were deparaffinized, washed in 0.05 M PBS, and treated with 2% H_2O_2 for 20 min. According to the SP method, the tissues were detected with immunohistochemical technique. PBS was used as substitutes of MAbs for negative control groups[7].

Histological assessment Semi-quantity analysis was made as Formowitz described[8].

Statistical analysis

The difference between each group was analyzed by Chi-Square test and correlativity.

RESULTS

Expression of p42/44^{MAPK} and p-Stat3 proteins in HCCs, PCLTs and normal liver tissues

p42/44^{MAPK} was negative in normal liver tissues. The positive rate of p42/44^{MAPK} in HCCs (87.3%, 48/55) was significantly higher than that in PCLTs (32.7%, 18/55, $P<0.01$) (Figure 1). Positive signals were mainly located in the nucleus. The distribution of positive cells for p42/44^{MAPK} was clustered and/or diffused in HCCs (Figure 2), and scattered in PCLTs, but also clustered in atypical hyperplasia regions of some PCLTs near cancer tissues. The expression level of p42/44^{MAPK} in HCCs was higher than that in PCLTs ($P<0.01$), but it was not correlated with the differentiation degree of cancer cells (Table 1). Positive signals of p-Stat3 were of nuclear-type. Of 9 normal liver tissues, 1 case was p-Stat3 positive. The positive rate of p-Stat3 in HCC (74.5%, 41/55) was significantly higher than that in PCLTs (23.6%, 13/55) ($P<0.01$). The p-Stat3 positive cells were clustered in HCC (Figure 3), and scattered in PCLTs. The expression intensity of p-Stat3 in HCCs was also higher than that in PCLTs ($P<0.01$), but it was not correlated with differentiation degree of cancer cells ($P>0.05$) (Table 1).

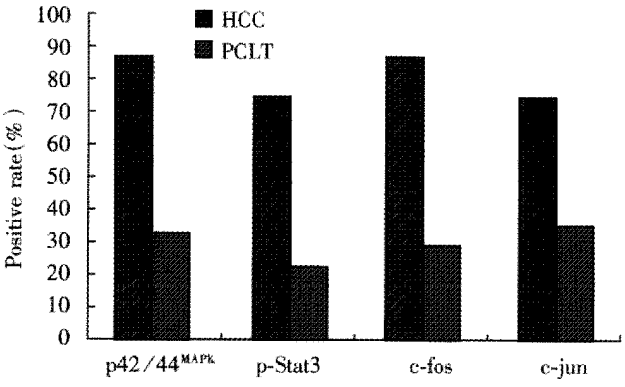


Figure 1 The positive rates of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs and PCLTs.

Table 1 Expressions of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs, PCLTs and normal liver tissues

Groups	No	p42/44^{MAPK}				p-Stat3				c-fos				c-jun			
		-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Normal	9	9	0	0	0	8	1	0	0	9	0	0	0	9	0	0	0
PCLT	55	37	9	7	2	42	4	7	2	36	12	7	0	39	13	3	0
HCC																	
Grade I	10	1	4	4	1	5	1	2	2	8	1	1	0	4	4	1	1
Grade II	21	3	6	12	0	2	12	5	2	3	12	4	2	1	15	4	1
Grade III	17	1	9	2	5	4	3	6	4	2	4	8	3	2	8	5	2
Grade IV	7	2	1	3	1	3	1	3	0	0	1	2	4	0	0	2	5

Expression of c-fos and c-jun proteins in HCCs, PCLTs and normal liver tissues

The expression of c-fos and c-jun proteins was not found in normal liver tissues. The rates of c-fos and c-jun protein expression in HCCs (76.4%, 87.3%) were significantly higher than those in PCLTs (34.5%, 29.1%) ($P<0.01$). Their positive signals were mainly of nuclear type in HCCs (Figures 4, 5) and nuclear-plasmic type in PCLTs. The positive intensity in HCCs was distinctly higher than that in PCLTs, and related to the differentiation degree of HCC cells. The poorer the differentiation of HCC cells, the stronger the expression of c-fos and c-jun proteins. The distribution of positive cells for c-fos or c-jun protein in HCCs was diffuse, and scattered in PCLTs.

Relativity of expression intensities of p42/44^{MAPK} and p-Stat3 in HCCs and PCLTs

No relationship was found between p42/44^{MAPK} and p-Stat3 signal intensities in HCCs and PCLTs (r = -0.167 and 0.0844, $P>0.05$).

Relationship between expression strength of p42/44^{MAPK} and c-fos proteins, between p42/44^{MAPK} and c-jun in HCCs and PCLTs (Table 2)

In HCCs, the expression of p42/44^{MAPK} was positively related to the expression intensities of c-fos and c-jun proteins. The stronger the expression of p42/44^{MAPK}, the higher the signal intensities of c-fos and c-jun proteins. In PCLTs, there was a positive relationship between the expression of p42/

44^{MAPK} and c-fos proteins, and no relationship between the expression of p42/44^{MAPK} and c-jun proteins.

Table 2 Relationship in expressive strengthes of p42/44^{MAPK}, c-fos and c-jun proteins between HCCs and PCLTs

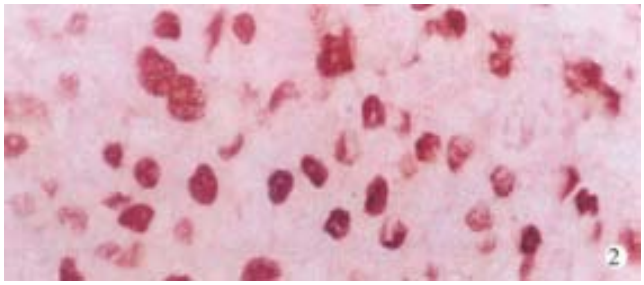
Groups	*r*	*P*
HCC		
p42/44^{MAPK}/c-fos	0.44835	<0.01
p42/44^{MAPK}/c-jun	0.3054	<0.01
PCLT		
p-MAPK/c-fos	0.9433	<0.01
p-MAPK/c-jun	0.1472	>0.05

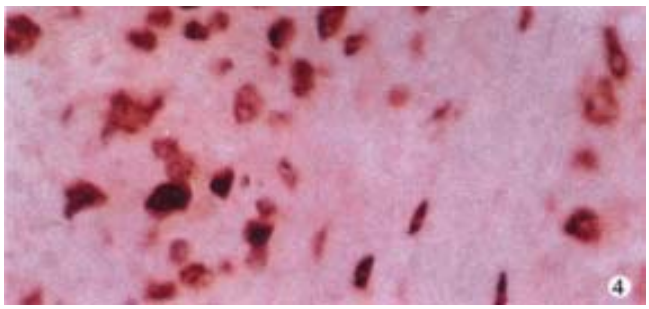
Relationship between expression strength of p-Stat3 and c-fos proteins, between p-Stat3 and c-jun in HCCs and PCLTs (Table 3)

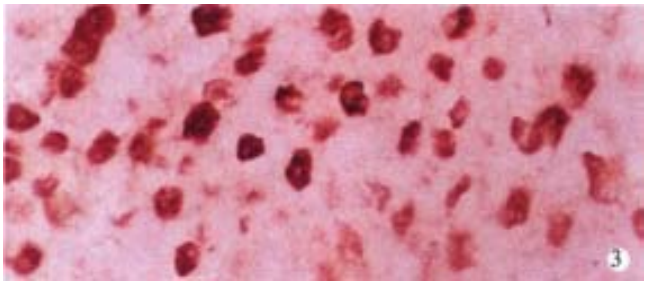
In HCCs, the expression of p42/44^{MAPK} was significantly positively related to expression intensities of c-fos and c-jun proteins. In PCLTs, there was a positive relationship between the expression of p-Stat3 and c-jun proteins, whereas no relationship between the expression of p-Stat3 and c-fos proteins was observed.

Table 3 Relationship in expression strength of p-Stat3, c-fos and c-jun proteins between HCCs and PCLTs

Groups	*r*	*P*
HCC		
p-Stat3/c-fos	0.3967	<0.01
p-Stat3/c-jun	0.5444	<0.01
PCLT		
p-Stat3/c-fos	0.0499	>0.05
p-MAPK/c-jun	0.3596	<0.01







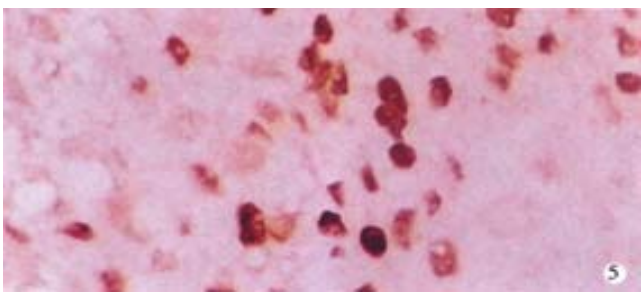


Figure 2 Localization of positive signal and distribution of positive cells for p42/44^{MAPK} protein in HCC. SP×400
Figure 3 Localization of positive signal and distribution of positive cells for Stat3 protein in HCC. SP×400
Figure 4 Positive expression of c-fos protein in HCC. SP×400
Figure 5 Positive expression of c-jun protein in HCC. SP×400

DISCUSSION

Ras/Raf/MAPK and Janus protein tyrosine kinases (JAKs)/Stat3 cascades are two major signaling pathways of growth factors and cytokines[1,2,4,5,9,10]. The former involves transient formation of ras-GTP and activation of raf kinase at the cell membrane to activate MAPK kinase which in turn activates MAPK through dual phosphorylation on threonine and tyrosine residues[11-14]. Activated MAPK then transduces into the nucleus and phosphorylates the ternary complex factor TCF which activates the expression of immediate-early genes, such as c-fos and egr-1[14,15]. There have been a few reports concerning MAPK activation in human cancers[1,2,16-21]. It was reported that MAPK activity level in HCC was significantly higher than that in the adjacent non-cancerous lesions[1,2,18]. Our results showed that the positive signal of p42/44^{MAPK} was almost located in the nucleus, indicating that the detected MAPK is activated MAPK. The positive rate and intensity of p42/44^{MAPK} in HCCs were all distinctly higher than those in PCLTs, which is consistent with the results reported in literature. Statistical analysis found that the expression intensity of p42/44^{MAPK} in HCCs was significantly positively correlated with the expression strength of immediate-early genes (c-fos and c-jun), and the expressive intensity of p42/44^{MAPK} in PCLTs was also significantly positively correlated with the expression strength of c-fos. These findings further suggest that the imbalance of Ras/Raf/MAPK cascade components may play a key role in hepatocarcinogenesis.

JAKs associate with cytokine receptors which lack intrinsic tyrosine kinase activity and become activated upon cytokine stimulation. Activated JAKs activate Stat3 through phosphorylation of Tyr-705[22]. Phosphorylated Stat3 (p-Stat3) then

translocates into the nucleus and binds to DNA response elements and transactivates gene expression [14,23,24]. The data presented here showed that the positive signals of p-Stat3 were mainly of nuclear type, and the positive rate and strength of p-Stat3 in HCCs were significantly higher than those in PCLTs, and the signal intensity of p-Stat3 was positively correlated with the expression level of the c-jun protein in HCCs and PCLTs. These results suggest that JAKs signaling cascade may also contribute to malignant transformation of hepatocytes besides Ras/Raf/MAPK signaling pathway in hepatocellular carcinogenesis.

Results in literature were inconsistent on the correlation between Ras/Raf/MAPK and JAKs/Stat3 pathways in tumor genesis and development[14,25,26]. Chung *et al*[25] reported that MAPK activated Stat3 through phosphorylation of Ser-727. Leaman *et al*[26] found that JAKs may not be required for the activation of Stat3 by some growth factors such as EGF and PDGF. As the MAPK and Stat3 become activated upon many growth factors and cytokines, it was considered that MAPK may promote activation of Stat3, but in Jain's results MAPK may inhibit transcription activity of Stat3[14]. Our data indicated that there was no correlation between expression intensities of $p42/44^{MAPK}$ and p-Stat3 in HCCs and PCLTs. The Stat3 phosphorylation is a complex event. Because the Stat3 can be phosphorylated through different protein kinases and pathways, detecting Stat3 at different phases of signaling cascades may produce entirely contradictory results about Stat3 activity. We think that both Ras/Raf/MAPK and JAKs/Stat3 pathways may play important roles in hepatocellular carcinogenesis, but their detailed mechanisms need to be further studied.

Up to now, the characteristics of pericar-cinomatous hepatocytes has not been clear[27]. The results showed that pericarcinomatous hepatocytes, especially proliferative or atypical hyperplasia hepatocytes, strongly express $p42/44^{MAPK}$, p-Stat3, c-fos and c-jun proteins, which suggest that activation of MAPK and Stat3 may be an early event in hepatocellular carcinogenesis. It also implys that the hepatocytes which express $p42/44^{MAPK}$ and/or p-Stat3 may be potentially malignant precancerous cells. Therefore, in order to avoid local recurrence, the operative range should be as extended as possible in the resection of HCCs.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Expression changes of activin A in the development of hepatic fibrosis

Xin Huang[1], Ding Guo Li[1], Zhi Rong Wang[1], Hong Shan Wei[1], Ji Lin Cheng[1], Yu Tao Zhan[1], Xin Zhou[1], Qin Fang Xu[1], Xin Li[2] and Han Ming Lu[1]

Subject headings liver cirrhosis; transforming growth factor beta; carbon tetrachloride; polymerase chain reaction; immunohistochemistry; RNA, messenger

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Abstract

AIM To examine the expression of activin A, a member of the transforming growth factor (TGF-β) superfamily, recently has been reported to beoverexpressed in liver cirrhosis, in the course of carbon tetrachloride-induced rat hepatic fibrosis.

METHODS Hepatic fibrosis was induced in rats by subcutaneous injections of 40% carbon tetrachloride oily solution for a period of 1 to 7 weeks. At the end of 1, 2, 3, 4, 5, 6 and 7 weeks after carbon tetrachloride injections, the rats were killed in group (6-10 rats each time) for study. The activin A messenger RNA expression and its protein localization were assessed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry.

RESULTS The normal rat liver expressed activin A mRNA and protein, and its expression was transiently decreased and became undetectable after carbon tetrachloride injections for 2 or 3 weeks and then increased gradually. After injection of carbon tetrachloride for 6 and 7 weeks, activin A mRNA and protein expressions were significantly enchanced in rat liver. Compared with that of the normal rat liver. Activin A mRNA expression levels in rats receiving carbon tetrachloride injections for 6 and 7 weeks were 1.6 and 2.2 times that of those in normal rat liver respectively (0.456 ± 0.094 *vs* 0.286 ± 0.0670, *P*<0.01; 0.620 ± 0.134 *vs* 0.286 ± 0.0670, *P*<0.01). Immunohistochemistry showed that activin A expressed in hepatocytes of normal liver, and its expression was decreased in rats receiving carbon tetrachloride for 2 or 3 weeks. Compared with normal liver, activin A expression distribution mode changed in fibrotic liver, being increased significantly in hepatocytes around fibrotic areas.

CONCLUSION Activin A expression was increased in late stage of hepatic fibrosis, and this may be involved in hepatic fibrosis formation in this period.

INTRODUCTION

Many researchers have tried to elucidate the pathogenesis of hepatic fibrosis[1-11]. Multiple studies have proved that cytokines play a vital role in this process, especially transforming growth factor β (TGF-β) superfamily[12-21]. Activins are newly found multifunctional proteins that belong to the TGF-β superfamily[22]. There are three kinds of activins, A(βAβA), B(βBβB) and AB(βAβB), which are hetero/homodimers closely related β-subunits of inhibin (βA and βB)[23]. Discovered by virtue of their capacity to stimulate production of follicle-stimulated hormone (FSH) from pituitary gland, activins have widespread anatomical distribution and are implicated in the regulation of many biological processes, including proliferation and differentiation of various types of cells[24]. In liver, ACT A inhibits growth of heptocytes and induces apoptosis, but promotes mesenchymal proliferation and extracellular matrix (ECM) production, and these effects are similar to those of TGF-β[25-27]. Activins and TGF-β may implicate in development of hepatic fibrosis jointly. Sugiyama *et al*[27] have demonstrated that activin A expression was significantly enhanced in cirrhotic and fibrotic rat livers induced by dimethylnitrosamine (DMN) and porcine serum. However, there has been no consensus of opinion about activin expression changing mode and its cell origins in fibrotic rats liver yet, for some researchers have got different results from those of Sugiyama. By means of RT-PCR and immunohistochemstry, we have examined the activin A expression mode in carbon tetrachloride (CCl_4) induced fibrotic rat liver.

[1]Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
[2]Ninth Hospital, Shanghai Second Medical University, Shanghai 200011, China
Dr. Xin Huang, is studying in Shanghai Second Medical University Affiliated Xinhua Hospital for doctor degree, specialized in hepatic pathophysiology, having 3 papers published.
Correspondence to: Dr. Xin Huang, Gastroenterology Department, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Tel. 0086-21-65790000 Ext. 5319, Fax. 0086-21-55571294
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MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats, weighing 180 g-210 g, were obtained from Shanghai Experimental Animal Institute of the Chinese Academy of Sciences. Hepatic fibrosis rat model was created by subcutaneous injections of CCl_4[28-36]. Altogether 72 rats were used. They were randomly divided into the normal control group (6 rats) and model group (64 rats). For the model group rats, CCl_4 olive solution (40% CCl_4) was injected subcutaneously at a dose of 0.4 mL/100 g twice a week. A double dose was used for the first injection. A same volume of saline was injected in normal control group animals. For study by lots every week (from the 1st to 7 th week), 72 hours after last injection of CCl_4, 6-10 model group rats were sacrificed. One portion of each liver was immediately frozen in liquid nitrogen for RNA extraction, and the remainder was fixed with 10%(vol/vol) formalin for van Gieson (VG) staining or immunostaining.

Immunohistochemistry

Deparaffinized 5 μm thick liver sections were washed three times with phosphate-buffered saline (PBS; pH 7.4), incubated in endogenous peroxidase blocking solution (Immunostain EliVision Kit, Maxim Biotech, Inc), and then treated with 0.01 M citrate buffer pH 6.0 for 10 minutes in a microwave oven at 650W. Non-specific-antibody binding was blocked by pretreatment with PBS containing 0.5% bovine serum albumin (fraction V powder, Sigma). Sections were then rinsed in PBS and incubated overnight at 4℃ with diluted monoclonal antibody against activin βA subunit (Serotic, UK) at 1:100 in PBS followed by three washes in PBS containing 0.05% Tween-20. The steps were performed using Immunostain EliVision kit according to the manufacturer's instructions. Sections were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. The slide was rinsed with distilled water, counterstained with hematoxylin, dehydrated, air dried, and mounted. The negative control slides were treated with nonspecific mouse IgG. The sections were examined under light microscopy.

Preparation of RNA

Total RNA was extracted from liver tissues according to the guanidinium isothiocyanate single-step methods (Kit from Watson Biological Inc, Shanghai).The RNA concentration of each sample was estimated by measuring the absorbance at 260 nm.

Reverse transcription and polymerase chain reaction (PCR)

Reverse transcription (RT) was performed using RT kit from Bioneer. The 20 μL reaction contains total RNA 2 μg, and oligo$(dT)_6$ 12.5pmol (Sangon, Shanghai). The first-strand cDNA sample (2 μL) was added to 25 μL of a PCR reaction mixture containing 0.5 μM gene-specific primers, 2 mM $MgCl_2$, 0.2 mM dNTP, and 1.5 units Taq polymerase, 2.5 μL 10 × buffer. Activin βA[37] sense primer: 5'-cagtcgtggacggtgcagaagt-3'; antisense primer: 5'-gcctgcggtgaggatggtctt-3'; the predicted size of the amplification products was 474 bp. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (house-keeper gene) sense primer [38]: 5'-catggtctacacgttccagt-3'; antisense primer: 5'-ggctaagcagttggtggtgc-3'; the 329 bp fragment amplification product was used as a positive control of the reverse-transcriptase receation and tissue mRNA integrity. Amplification conditions included initial denaturation for 5 minutes at 94℃, 30 cycles of amplification for activinβA and 28 cycles for GAPDH, with denaturation at 94℃ for 45 seconds, annealing at 60℃ for 45 seconds, and extension at 72℃ for 1 minute. PCR products were analyzed by electrophoresis through agarose gel(2% wt/vol) and visualized by ethidium bromide staining and ultraviolet illumination. The length of PCR products was measured by comparison with standard molecular-weight marker. The signal intensity of each band was normalized by comparing it with the intensity of GAPDH.

Statistical analysis

Results were expressed as mean ± SE. To compare the mean values, ANOVA analysis was applied. Differences were considered significant if $P<0.05$.

RESULTS

Van Gieson staining

Hepatic fibrosis gradually developed with increasing CCl_4 injections. After 6-7 weeks, VG staining showed that liver from most of model group animals were forming fake liver lobuli with reticulin fibers spreading radially between the portal tracts and central veins (Figure 1).

Immunohistochemistry

Activin βA subunit was detected in normal liver. Figure 2 shows that activinβA immunoreactivity was distributed within the lobuli and localized in parenchymal cells. More staining was found in hepatocytes around central veins. No staining was found in hepatocytes around portal tracts. After CCl_4 injection for 2-3 weeks, activin βA immunostaining was decreased but still detectable, and its distribution was changed, localizing mainly in hepatocytes around fibrotic areas (Figure 3). After CCl_4 injection for6-7 weeks, activinβA immunostaining was enhanced. Many hepatocytes around fibrotic areas (around portal tracts, fibrotic septa and central veins areas) were stained positive (Figures 4,5).

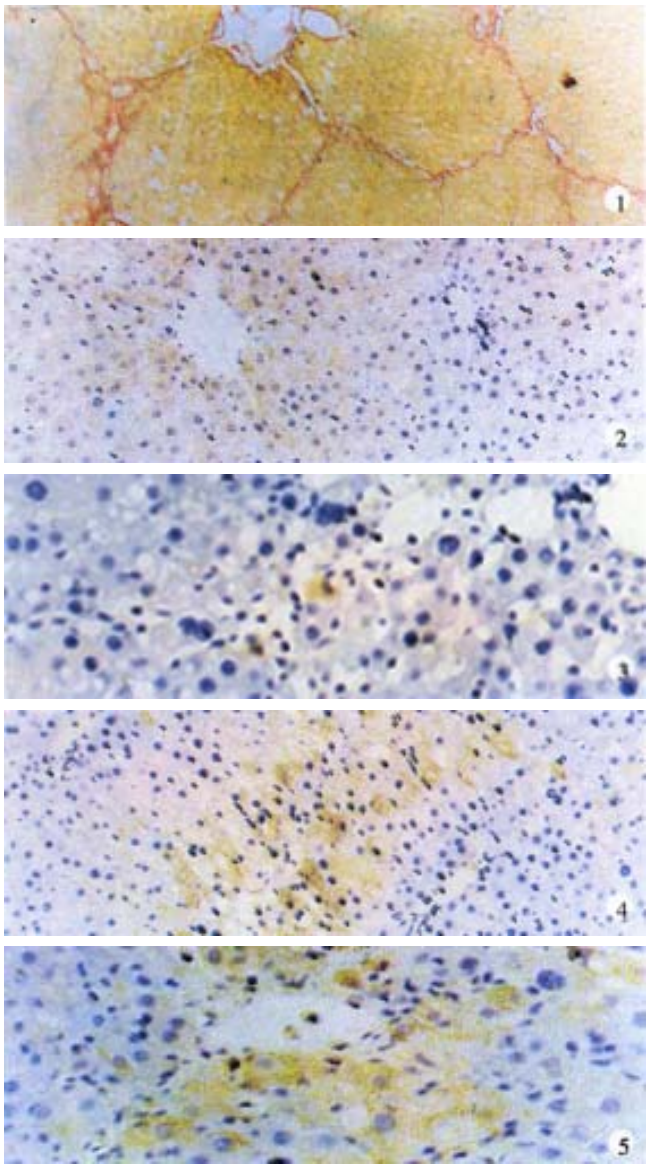

Figure 1 Fake lobuli formed after CCl_4 was injected for 7 weeks. VG staining, ×100

Figure 2 Normal rat liver activin βA immunostaining, immunoreactivity was detected in hepatocytes. DAB, ×100

Figure 3 Activin βA immunostaining after 3-week CCl_4 injections. DAB, ×400

Figure 4 Hepatocytes around connective septa were detected by immunostaining after 7 weeks' CCl_4 treatment. DAB, ×200

Figure 5 Hepatocytes around central veins by activin β immunostaining after 7-weeks CCl_4 treatment. DAB, ×400

PCR

ActivinβA mRNA subunit was detected in normal rat liver, but it became undetectable after CCl_4 was injected for 2-3 weeks. Four weeks after CCl_4 injections, activin βA mRNA levels increased gradually, and were significantly enhanced after 6 or 7 weeks when compared with normal rat liver (P<0.01) (Figures 6,7). Activin βA mRNA levels in rats treated with CCl_4 for 6 and 7 weeks were 1.6 and 2.1 times of those in normal rat liver respectively.

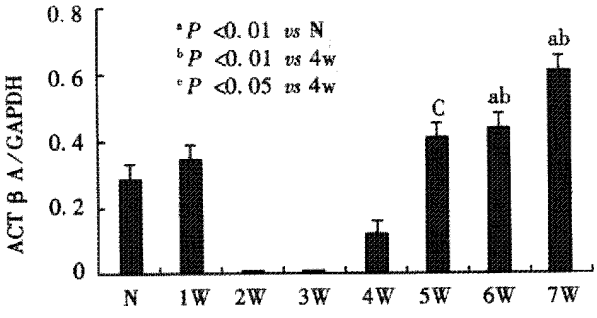

Figure 6 The mRNA expression of activin A at the different stages of CCl_4 induced hepatic fibrosis. N: normal, W: week, M: marker

Figure 7 A relative amount of activin βA mRNA were quantified using an image analyzer.
^aP<0.01 *vs* N; ^bP<0.01 *vs* 4w; ^cP<0.05 *vs* 4w.
N: normal group, W: week

DISCUSSION

Liver cirrhosis is a final stage of various chronic liver diseases and is characterized by a marked deposition of extracellular matrix, combined with impared hepatic regeneration. Several cytokines are involved in the pathogenesis of this disease[39]. In particular, TGF-β1 is overexpressed in nonparenchymal cells of fibrotic liver of human or animals, and plays a pivotal role in the development of cirrhosis by stimulating fibroblast proliferation and ECM secretion[40-44]. Recent studies showed that activin may also be involved in the development of cirrhosis.

Activin A has mutiple effects including proliferation and differentiation of various types of cells[24]. In terms of its effects on hepatocytes, activin A inhibits DNA synthesis and induces apoptosis of hepatocytes[25,26,45]. In fibrotic or cirrhotic rat liver induced by CCl_4, De Bleser found that activin A disappeared in parenchymal cells, and it was expressed when the liver was normal. The normal hepatic stellate cells showed no activin A expression, but its expression was significantly enhanced when hepatic fibrosis had formed[17,46]. Subsequently, Sugiyama[27] found activin A expression was greatly increased in fibrotic rat liver induced by DMN and porcine serum, but it was in hepatocytes but not hepatic stellate cell that activin

A expression was significantly increased. Obviously, results from De Bleser and Sugiyama were not completely consistent with each other. Our research showed that activin A expression was increased in CCl_4-induced fibrotic rat liver, being localized in hepatocytes. These results were consistent with those of Sugiyama.

We had used the same hepatic fibrosis model as De Bleser's, but the results were different. This may be due to a difference in the period of model-making. We found that normal liver expressed activin A, but after injection of CCl_4 for 2-3 weeks, activin A mRNA became undetectable, although its protein could still be found. Four weeks later, activin A mRNA increased gradually. But it was not significantly increased until CCl_4 was injected for 6 or 7 weeks when hepatic fibrosis had formed completely. This means that most of livers have formed pseudo lobuli. Sugiyama also found that in the early period of hepatic fibrosis induced by DMN, the expression of activin βA mRNA was discreased transiently, and then increased gradually with lapse of time. However, the expression was enhanced continuously in porcine serum-induced cirrhosis. This difference may be due to serious hepatocytic damage caused by CCl_4, which is also found in the early stage of the development of DMN induced liver cirrhosis but not in porcine serum induced cirrhosis[47-50]. As activin A acts as an autocrine growth inhibitor of hepatocytes, this transient decrease in expression may promote hepatocytic regeneration. De Bleser reported that activin βA expression disappeared in parenchymal cells of CCl_4 induced cirrhosis, but we think that it might be a transient disappearance, for liver cirrhosis was not formed completely. In addition, De Bleser found that activin A expressed in activated hepatic stellate cells (HSC) of fibrotic liver, but according to our result, activin A immunostaining was mainly distributed in hepatocytes around fibrotic areas (Figures 4,5), a result also consistent with Sugiyama's reports. So it is worthy of further study to confirm whether HSC expresses activin A or not.

The expression of activin A protein was not complete in conformity with its mRNA. Its mRNA became undetectable after CCl_4 injections for 2 to 3 weeks, but activin A protein could be detected by immunohistochemistry. Sugiyama described similar phenomenon in DMN-induced cirrhosis. This may be because of the fact that the mRNA level was too low to be detected by our method. Of course, other reasons may exist and worth further studying.

In summary, our study shows that in CCl_4-induced rat cirrhosis, the expression of activin A is decreased transiently at first, and then increased gradually. Activin A may be involved in the late stage of the development of liver cirrhosis. Distribution of activin A also changes with the development of fibrosis, that is from hepatocytes around central veins to hepatocytes around fibrotic areas including central vein, connective septa and portal tracts.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Animal experiment and clinical study of effect of gamma-interferon on hepatic fibrosis

Hong Lei Weng, Wei Min Cai and Rong Hua Liu

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Abstract

AIM To evaluate the antifibrotic effect of different doses of recombinant human Gamma-Interferon (IFN-γ) in two rat models of hepatic fibrosis, and to observe its effect on moderate chronic hepatitis B virus fibrosis.

METHODS Hepatic fibrosis was successfully induced in 150 and 196 rats by subcutaneous injection of carbon tetrachloride (CCl_4) and intraperitoneal injection of dimethylnitrosamine (DMN), respectively. Each of the two model groups was divided into: ① fibrotic model group; ② colchicine treatment group (0.1 mg/kg/day, gastrogavage for 8 weeks); ③ high-dose IFN-γ group (15 MU/kg per day, i.m. for 8 weeks); ④ medium-dose IFN-γ group (5MU/kg daily, i.m. for 8 weeks); and ⑤ low-dose IFN-γ group (1.67 MU/kg daily, i.m. for 8 weeks). Another group of 10 rats without any treatment was used as normal controls. At the end of the experiment, semi-quantitative histopathological scores of inflammation and fibrosis, liver α smooth muscle actin (α-SMA) expression level, liver hydroxyl proline content and serum hyaluronic acid levels were compared. And 47 medium chronic hepatitis B viral fibrosis patients were studied. They were given IFN-γ treatment, 100 MU/day i.m. for the first three months and 100 MU qod i.m. for the next six months. Semi-quantitative pathological scores of inflammation and fibrosis and serum hepatic fibrosis indices were compared within the 9 months.

RESULTS In animal experiment, the pathological fibrosis scores and liver hydroxyl proline content were found to be significantly lower in rats treated with different doses of IFN-γ as compared with rats in fibrotic model group induced by either CCl_4 or DMN, in a dose-dependent manner. For CCl_4-induced model, pathological fibrosis scores in high, medium and low doses IFN-γ groups were 5.10 ± 2.88, 7.70 ± 3.53 and 8.00 ± 3.30, respectively, but the score was 14.60 ± 7.82 in fibrotic model group. Hydroxyl proline contents were 2.83 ± 1.18, 3.59 ± 1.22 and 4.80 ± 1.62, in the three IFN-γ groups, and 10.01 ± 3.23 in fibrotic model group. The difference was statistically significant ($P<0.01$). Similar results were found in DMN-induced model. Pathological fibrosis scores were 6.30 ± 0.48, 8.10 ± 2.72 and 8.30 ± 2.58, in high, medium and low doses IFN-γ groups, and 12.60 ± 3.57 in fibrotic model group. Hydroxyl proline contents were 2.72 ± 0.58, 3.14 ± 0.71 and 3.62 ± 1.02, in the three IFN-γ groups, and 12.79 ± 1.54 in fibrotic model group. The difference was statistically significant ($P<0.01$). Serum hepatic fibrosis indices decreased significantly in the 47 patients after IFN-γ treatment (HA: 433.38 ± 373.00 *vs* 281.57 ± 220.48; LN: 161.22 ± 41.02 *vs* 146.35 ± 44.67; PC(r): 192.59 ± 89.95 *vs* 156.98 ± 49.22; C-I: 156.30 ± 44.01 *vs* 139.14 ± 34.47) and the differences between the four indices were significant ($P<0.05$). Thirty-three patients received two liver biopsies, one before and one after IFN-γ treatment. In thirty of 33 patients IFN-γ had better effects according to semi-quantitative pathological scores (8.40 ± 5.83 *vs* 5.30 ± 4.05, $P<0.05$).

CONCLUSION All the three doses of IFN-γ are effective in treating rat liver fibrosis induced by either CCl_4 or DMN, the higher the dose, the better the effect. And IFN-γ is effective for patients with moderate chronic hepatitis B viral fibrosis.

INTRODUCTON

Hepatic fibrosis can cause liver cirrhosis in chronic liver diseases (viral, schistosomal, alcoholic). Regardless of causes, hepatic fibrosis is characterized by a large deposition of extracellular matrix, collagen being a major component. Studies on interferon-gamma (IFN-γ) *in vitro* or *in vivo* are increasing[1-7]. Although the interferons were initially described for their antiviral properties, IFN-γ has also been found to have significant effects against hepatic fibrosis and to inhibit the synthesis

Institute of Infectious Diseases, First Affiliated Hospital, Medical School, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Dr. Hong Lei Weng, graduted from Medical School of Zhejiang University as a postgraduate in 1999, now associate professor of Institute of Infectious Diseases, First Affiliated Hospital, Medical School, Zhejiang University.
Correspondence to: Wei Min Cai, Institute of Infectious Diseases, First Affiliated Hospital, Medical School, Zhejiang University, 261 Qingchun Lu, Hangzhou 310003, Zhejiang Province, China
Tel. 0086-571-7236580, Fax. 0086-571-7086731
Email. syliao@netease. com
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of extracellular matrix[8-14]. However, there have been few studies on the relationship between different doses and effects of IFN-γ for hepatic fibrosis. Our previous study showed that IFN-γ could lower serum hyaluronic acid levels in chronic hepatitis patients with hepatic fibrosis[15]. But no reports concerning histological changes after IFN-γ treatment have ever been found. We were prompted to systematically observe the effect of IFN-γ and investigate its effective doses on rat hepatic fibrosis induced by intraperitoneal injection of two different toxins, carbon tetrachloride (CCl_4) and dimethylnitrosamine (DMN). Once hepatic fibrosis emerged in two rat models, different doses of IFN-γ were injected intramuscularly to observe the following changes after IFN-γ treatment: pathological fibrosis scores, liver hydroxylproline content and serum hyaluronic acid levels. In the meantime, we observated 47 chronic hepatitis B patients with hepatic fibrosis who received IFN-γ treatment for 9 months. Liver biopsy and other laboratory tests were performed within the 9 months, the effect of IFN-γ was assessed.

MATERIALS AND METHODS

Animal experimental design

Sprague-Dawley rats (n = 483) weighing 200 gm-250 gm were used. They were fed with Good Laboratory Practice diet in pellets (provided by Zhejiang University Animal Study Center). Rats were maintained under 12 h light/dark cycles and allowed free access to food and water. Experiments were performed in accordance with the institutional ethical guidelines.

Hepatic fibrosis was induced by intraperitoneal injections of CCl_4 or DMN. Recombinant human IFN-γ was kindly provided by Shanghai Clonbiotech Co., Ltd (Shanghai, China, Batch Number 970521).

Rats (n = 205) were subcutaneously administered dissolved CCl_4 in olive oil (a proportion of 4:6) at 0.3 mL/kg of body weight, i.p., for 2 consecutive days a week for 16 weeks. Five rats were killed at the end of each week to examine their pathological changes.

For DMN-induced fibrosis, 278 rats were injected i.p. repeatedly, for 3 consecutive days at the first week and 2 consecutive days for the next 5 weeks. Five rats were killed at the end of each week to examine their pathological changes.

According to histological sections, hepatic fibrosis appeared at the end of the fourth week from the start of the study. One hundred and fifty rats in CCl_4 induced model and 196 rats in DMN-induced model could be used for the next study. Rats in the two models were divided into five groups: ① fibrotic model group; ② colchicine treatment group ($0.1 mg \cdot kg^{-1} \cdot day^{-1}$, gastrogavage); ③ high-dose IFN-γ group ($15 MU \cdot kg^{-1} \cdot day^{-1}$, i.m.); ④ medium-dose IFN-γ group ($5 MU \cdot kg^{-1} \cdot day^{-1}$, i.m.); and ⑤ low-dose IFN-γ group ($1.67 MU \cdot kg^{-1} \cdot day^{-1}$, i.m.). In addition, 10 rats were chosen as the normal control group. Rats in the normal control and fibrotic model groups were given 0.9% sodium chloride by intramuscular injection instead of IFN-γ.

Rats of CCl_4 induced model received 8-week treatment. At the end of the eighth week, 10 rats of each group were killed, and histological sections of their livers were evaluated by hematoxylin and eosin and Sirius red stains. α-smooth muscle actin (α-SMA) was examined with immunohistochemical technique. Liver hydroxylproline content and serum hyaluronic acid level were also detected. In the next 4 weeks, all treatments stopped. The rest of the rats were killed at the end of the sixteenth week from the start of the experiment.

Rats of DMN-induced model received 4-week treatment. At the end of the fourth week, 10 rats of each group were killed. All items performed in CCl_4-induced model were detected, too. The other rats were killed at the end of the 12 th week from the start of the study.

Histological study

Excised liver tissues from rats were fixed in 10% neutralized formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin. Alternatively, the Sirius red stain method was used to specifically stain fibrous tissue components. These sections were observed under polarization microscopy (Leica DMLB, Leica Wetalar, Germany.) to distinguish type I and III collagen[16]. Specimens were reviewed under code by a single pathologist who graded and scored the specimens according to the criteria of Scheuer and Chevallier *et al*[17,18]. In Scheuer criteria, the transformation of grade and stage was as follows: stage 0, = 2°; grade or stage 1, = 2^1; grade or stage 2, = 2^2; grade or stage 3, = 2^3; grade or stage 4, = 2^4.

Identification of activated hepatic stellate

α-SMA, a marker of activated hepatic stellate (HSC) [19,20], was detected in formaldehyde-fixed paraffin-embedded section. Briefly, liver tissue was incubated with a monoclonal IgG_2a recognizing α-SMA (Dako M851) and reactive sites were detected using the labeled streptavidin-biotin technique (Dako M680), resulting in a brown staining after incubation with Tris-buffered saline containing diammobenzidine and H_2O_2. In control slides, the primary antibody was substituted by the Tris-buffers.

Biochemical measurements

The liver was homogenated into powder and hydrolyzed with 6 mol/L HCl. The hydroxyl proline content was measured by the means of Kivirikko *et al*[21]. The routine serum biochemical tests, including serum aspartate transaminase and alanine transaminase, were performed.

Examination of serum hyaluronic acid level

Serum hyaluronic acid level was detected by radioimmunoassay technique. The kit was provided by the Shanghai Navy Medical Institute. The operations were performed according to the manufacturer's instructions.

Patients

Fourty-seven hepatic fibrosis patients with chronic hepatitis B, 37 males and 10 females, were chosen for the study. They were confirmed to have moderate hepatic fibrosis pathologically according to Scheuer and Chevallier scoring system[17,18] and had chronic hepatitis B for 6 months to 30 years. Additional criteria included: age between 18 and 65 years; presence of HBsAg in serum for at least 6 months; positive serum tests for anti-HBe documented on four occasions, at least 1 month apart, during the 6 months before entry into the study. Among four serum hepatic fibrosis indices, including hyaluronic acid (HA, upper nomal limit <110 μg/L), laminin (LN, upper normal limit <150 μg/L), type III procollagen (PCIII, upper normal limit <120 μg/L) and type IV collagen (C-IV, upper normal limit <80 μg/L), two indices were higher than upper normal limit. Patients were excluded when the haemoglobin ≤10 g/dL, platelet count ≤50 000/mL, and white cell count ≤4 000/mL. Other exclusion criteria were patients with decompensated liver disease (serum albumin <3.5 g/dL; serum total bilirubin ≥85.5 μmol/L; prothrombin time prolongation >3sec), histories of hepatic encephalopathy or ascites, esophageal or gastric varices at risk of bleeding, hepatitis delta virus, hepatitis C virus infections, causes of liver disease other than HBV, intravenous drug abuse, pregnancy, malignancy, chronic heart and/or renal failure, anti-virus therapy (such as IFN-α, lamivudine, Famciclovir and Penciolovir therapy) and immune modulation therapy (such as Thymosin α_1) within a period of 3 months, and anti-hepatic fibrosis drugs (such as colchicines, herbal recipe 861 and Extractum Semen Percise).

Study protocol

In this open label study, all the patients were given routine therapy and received rhIFN-γ (kindly provided by Shanghai Clonbiotech Co., Ltd, Shanghai, China, Batch Number 981210) at a dose of 1 MU intramuscularly daily for the first three months and every other day for the following six months. They were asked to take rhIFN-γ in local hospitals near their homes. They were visited every three months during the treatment and thereafter followed up for 3 months after the treatment stopped. At each visit, clinical and laboratory assessments were made, including serum biochemical tests, such as serum hepatic fibrosis indices, routine serum biochemical tests, such as ALT, AST and γ-glutamyl transpeptidase, alkaline phosphatase, albumin, total bilirubin and alfha fetoprotein (AFP), routine blood tests, and HBV serology. Ethical Committee of the Centers involved approved the study. All the patients were volunteers.

Histology

Liver biopsies were performed twice within the 9 months, one before the treatment and one after it. All liver biopsy specimens were stained with hematoxylin-eosin and Sirius red. A single pathologist who was blinded with respect to treatment regimen evaluated specimens according to the criteria of Scheuer and Chevallier *et al*[9,10].

Statistical analysis

Results were expressed as mean ± standard deviation and compared when appropriate, by Student's two-tailed paired and unpaired *t* test and Kruskall Wallis nonparametric one-way ANOVA.

RESULTS

Animal experiment

In normal control group, Sirius red staining showed a normal distribution of collagen in a small amount in the portal tracts and around the terminal hepatic veins.

CCl_4- induced model showed different pathological changes from DMN-induced model. In the former, besides hepatocyte necrosis, fatty degeneration was the major feature[22] while in the latter, lymphomonocyte infiltration and local hepatocyte necrosis were the main characteristics[23]. However, after 12 weeks of CCl_4 and 8 weeks of DMN treatment, all the rats in fibrotic model groups showed complete fibrotic septum, forming a pattern of micronodular cirrhosis to the parenchyma (Figure 1).

In animal treated with high-dose of IFN-γ, Sirius red staining showed a small collagen deposition in the portal tracts and lobules. Only collagen fibers around the terminal hepatic veins were observed (Figure 2). Collagen depositions in liver sections taken from animals treated with medium or low dose of IFN-γ were particularly evident but only thin bands of collagen which formed short, incomplete septum could be seen (Figures 3, 4). In colchcine treated animals, complete fibrotic septum departing from the central vein were present, and no cirrhosis could be observed (Figure 5). Tables 1 and 2 show the histological stage and grade of different groups.

Table 1 Histological grading and staging in CCl_4-induced specimens after 8 week treatment

Groups	2^{g*}	2^{s*}	SSS△
Normal control group	1.00 ± 0.00	1.00 ± 0.00	0
Fibrotic model group	9.20 ± 5.01	10.40 ± 5.06	14.60 ± 7.82
Colchicine treatment group	10.40 ± 5.06	7.40 ± 4.99[a]	10.60 ± 3.34[a]
High-dose IFN-γ group	9.60 ± 3.37	3.00 ± 1.05[bc]	5.10 ± 2.88[bc]
Medium-dose IFN-γ group	6.00 ± 4.22	5.60 ± 4.20[bc]	7.70 ± 3.53[bc]
Low-dose IFN-γ group	7.60 ± 4.79	5.40 ± 2.32[bc]	8.00 ± 3.30[bc]

* Scheuer's criteria; △ Chevallier's criteria

[a]$P<0.05$ *vs* fibrotic model group; [b]$P<0.01$ *vs* fibrotic model group; [c]$P<0.05$ *vs* colchicines treatment group.

Table 2 Histological grading and staging in DMN-induced specimens after 4 week treatment

Groups	2^{g*}	2^{s*}	SSS△
Normal control group	1.00 ± 0.00	1.00 ± 0.00	0
Fibrotic model group	7.00 ± 2.16	9.60 ± 4.70	12.60 ± 3.57
Colchicine treatment group	6.80 ± 4.13	7.00 ± 3.92[a]	11.3 0 ± 2.37[a]
High-dose IFN-γ group	6.40 ± 2.07	2.60 ± 0.97[bc]	6.3 0 ± 0.48[bc]
Medium-dose IFN-γ group	4.80 ± 2.35	4.00 ± 2.32[bc]	8. 10 ± 2.72[bc]
Low-dose IFN-γ group	5.00 ± 2.71	4.40 ± 2.07[bc]	8.30 ± 2.58[bc]

*Scheuer's criteria; △ Chevallier's criteria
[a]P<0.05 *vs* fibrotic model group; [b]P<0.01 *vs* fibrotic model group; [c]P<0.05 *vs* colchicines treatment group.

In the normal control group, α-SMA positive cells were detected mainly in the portal space as elements of vascular walls or as fibroblastlike cells scattering in the connective tissue or near bile ductules. In lobule, α-SMA positive cells were present on the walls of large and medium sized terminal hepatic veins. The pattern of distribution of α-SMA positive cells was modified in CCl_4 or DMN treated animals as compared with the normal control rats. In fibrotic model groups, α-SMA positive cells were detected in portal space, sinusoid, lobule and areas where fibrotic septum appeared. Different doses of IFN-γ appeared to strikingly decrease the activation of HSC. After 8 weeks of IFN-γ treatment in CCl_4 model or 4 weeks in DMN model, only thin and incomplete parenchymal α-SMA positive septum joining thickened centrilobular veins were observed. α-SMA positive cells were mainly found in portal space and areas around fibrotic septum. Few α-SMA positive cells were present in sinusoid and lobule.

Analysis of total collagen content in liver substantiated the histological impression. Hydroxylproline evaluation in fibrotic model groups showed that the amount of Hydroxyl proline content increased approximately 5-fold (in CCl_4 model) or 6-fold (in DMN model) as compared with the normal control group, whereas, figures of different doses of IFN-γ groups were lower (<2-fold). The results in Tables 3 and 4 indicate the serum hyaluronic acid level of different groups. Except for low-dose IFN-γ group in CCl_4-induced model, other IFN-γ treatment groups had obvious decrease of serum hyaluronic acid level in both models.

Table 3 Serum hyaluronic acid level and liver hydroxylproline content in CCl_4-induced specimens after 8 weeks treatment

Groups	HA (μg/L)	Hyp (mg/g liver dry weight)
Normal control group	147.64 ± 19.28	2.43 ± 0.33
Fibrotic model group	900.04 ± 508.84	10.01 ± 3.23
Colchicine treatment group	745.57 ± 170.44[a]	6.90 ± 1.70
High-dose IFN-γ group	482.05 ± 210.57	2.83 ± 1.18
Medium-dose IFN-γ group	765.51 ± 586.65[a]	3.59 ± 1.22[bd]
Low-dose IFN-γ group	981.52 ± 509.95	4.80 ± 1.62

[a]P<0.05 *vs* fibrotic model group; [b]P<0.01 *vs* fibrotic model group; [c]P<0.05 *vs* colchicines treatment group; [d]P<0.01 *vs* colchicines treatment group.

Table 4 Serum hyaluronic acid level and liver hydroxylproline content in DMN-induced specimens after 4-weeks treatment

Groups	HA (μg/L)	Hyp (mg/g liver dry weight)
Normal control group	147.64 ± 19.28	2.43 ± 0.33
Fibrotic model group	969.52 ± 257.80	12.79 ± 1.54
Colchicine treatment group	323.72 ± 388.45[a]	6.40 ± 1.84[a]
High-dose IFN-γ group	144.25 ± 105.66	2.72 ± 0.58
Medium-dose IFN-γ group	334.10 ± 420.06	3.14 ± 0.71
Low-dose IFN-γ group	407.95 ± 386.61[a]	3.62 ± 1.02[bd]

[a]P<0.05 *vs* fibrotic model group; [b]P<0.01 *vs* fibrotic model group; [c]P<0.05 *vs* colchicines treatment group; [d]P<0.01 *vs* colchicines treatment group.

Clinical study

All the 47 patients completed the 9-month treatment. Table 5 shows the changes of symptoms after the 9-month IFN-γ treatment. Except for these changes, 12 patients (12/47, 25.5%) had a better appetite than before the treatment.

Thirty-three patients were given a second biopsy after the 9-month treatment (33/47, 70.2%). According to Chevallier's criteria, semi-quantitative system score (SSS) was calculated. After the 9-month IFN-γ treatment, the SSS decreased significantly in 14 patients (14/33, 42.4%) and the SSS dropped over 2 points, increased in 3 patients (3/33, 9.1%), and remained unchanged or dropped 1 point in the remaining 16 patients (16/33, 48.5%). Table 6 shows the mean SSS change during the treatment. A significant decrease of hepatic fibrosis score in accordance with the SSS was seen at the end of the IFN-γ treatment (8.40 ± 5.83 *vs* 5.30 ± 4. 05, P<0.05). In the meantime, a decrease of inflammation score in accorda nce with the SSS was also found although the difference was not statistically significant (P>0.05). Figures 6-9 display the collagen deposition changes within the IFN-γ treatment.

Table 7 shows the mean serum hepatic fibrosis indices, including HA, LN, PCIII and C-IV, and changes during the entire study. Decreases were present in the four indices (HA: 433.38 ± 373.00 *vs* 281.57 ± 220.48; LN: 161.22 ± 41.02 *vs* 146.35 ± 44.67; PCIII: 192.59 ± 89.95 *vs*. 156.98 ± 49.22; C-IV: 156.30 ± 44.01 *vs*. 139. 14 ± 34.47) and the differences between the four indices were significant (P<0.05).

As described by Cannon *et al*[24], the main side-effects in our study during the IFN-γ treatment were: fever (25) , joint pain (13), nausea (1), myagias (18), headache (10), leukopenia (5) and platelet decreases (7). Fever presenting in some patients was temporary and only 1 person had persistent fever (about 38℃), which disappeared when the treatmentended.

We also performed other routine serum biochemical tests, including ALT, AST and γ-GT, AFP. The results showed that the mean serum ALT, AST, γ-GT levels lowered in those patients as compared with those of IFN-γ treatment. No obvious change in serum AFP level was present within the 9 months (data not shown).

Table 5 Symptom change after IFN-γ treatment

	Fatigue	Abdominal distention	Nausea	Liver area pain
Before treatment	33	31	12	22
After treatment	4	4	0	4

Table 6 Histological grading and staging of liver biopsy specimens before and after 9-month treatment

	Grade*	Score of grade△	Stage*	Score of stage△
Before treatment	2.30 ± 0.98	7.30 ± 5.39	2.60 ± 0.88	8.40 ± 5.83
After 9-month treatment	1.95 ± 0.69	4.90 ± 2.27	2.05 ± 0.76	5.30 ± 4.05
t		2.719		2.719
P		0.051		0.014[a]

* Scheuer's criteria; △ Chevallier's criteria; [a]*P*<0.05 *vs* before treatment

Table 7 Serum hepatic fibrosis indices before and after 9-month treatment

Hepatic fibrosis indices	HA (μg/L)	LN (μg/L)	PCIII (μg/L)	C-IV (μg/L)
Before treatment	435.38 ± 373.00	161.22 ± 41.02	192.59 ± 89.95	156.30 ± 44.01
After 9-month treatment	281.57 ± 220.48	146.35 ± 44.67	156.98 ± 49.22	139.14 ± 34.47
t	2.341	2.434	2.381	2.104
P	0.017[a]	0.021[a]	0.020[a]	0.038[a]

[a]*P*<0.05 *vs* before treatment.

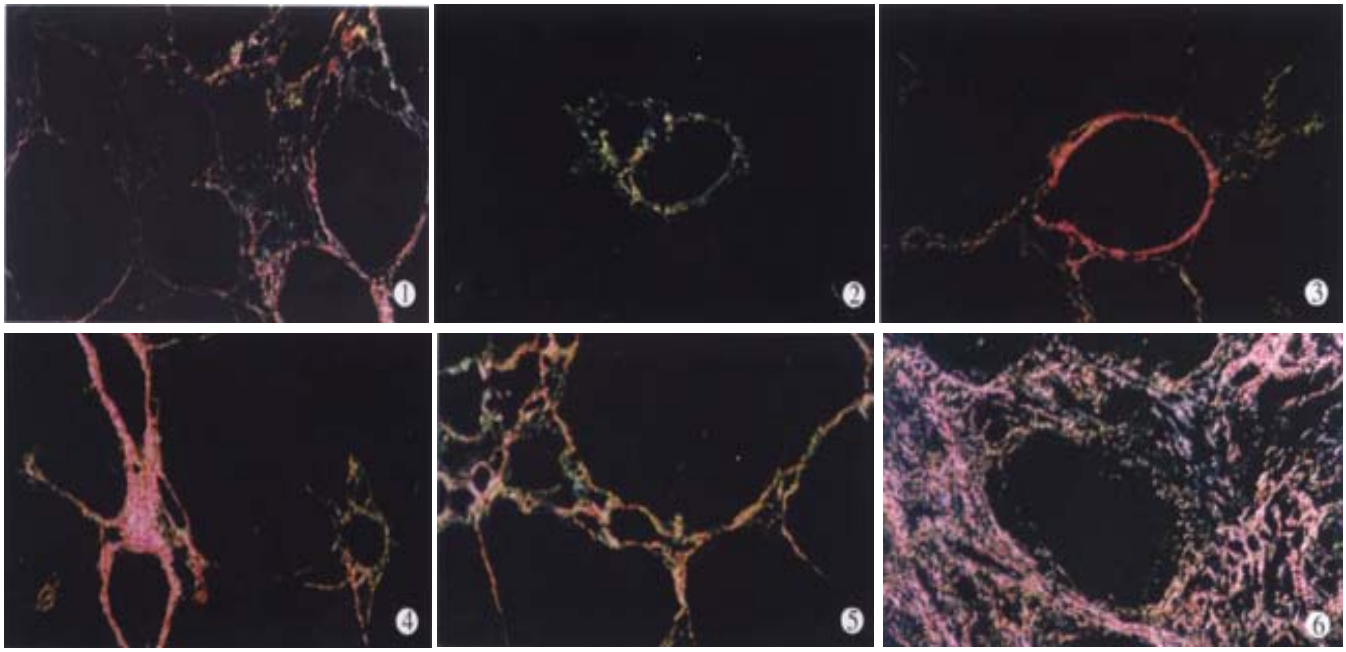

Figure 1 Histological section of rat liver after 12- weeks of CCl_4 treatment under polarizes after staining with Sirius Red. Red and yellow represented collagen type I and green collagen type III (the same below). The pattern of micronodular cirrhosis is evident. ×100

Figure 2 Histological section of rat liver after 8- weeks of treatment with high-dose IFN-γ and 12 weeks of CCl_4 under polarizes after staining with Sirius Red. ×100

Figure 3 Histological section of rat liver after 8-weeks of treatment with medium-dose IFN-γ and 12 weeks of CCl_4 under polarizes after staining with Sirius Red. ×100

Figure 4 Histological section of rat liver after 8- weeks of treatment with low-dose IFN-γ and 12 weeks of CCl_4 under polarizes after staining with Sirius Red. ×100

Figure 5 Histological section of rat liver after 8- weeks of treatment with colchicine and 12 weeks of CCl_4 under polarizes after staining with Sirius Red. ×100

Figure 6 and 7 Histological section of liver biopsy of a patient with chronic hepatitis B before IFN-γ treatment under polarizes after staining with Sirius Red. Figures 6 and 7 show two different fields. ×100

Figure 8 and 9 Histological section of liver biopsy of a patient with chronic hepatitis B after 9-month treatment with IFN-γ under polarizes after staining with Sirius Red. Figures 8 and 9 show two different fields. ×100

DISCUSSION

CCl_4 is one of the most widely used hepatic toxins for experimental induction of liver fibrosis cirrhosis in laboratory[22,25,26]. The model was used even in 1936. But, disadvantages of the model were apparent, such as higher mort ality rate. For this reason, 205 rats were used to produce CCl_4-iduced hepatic fibrosis model in our study. By killing some animals at the end of each week, we successfully got the hepatic fibrosis model at the fourth week. A total of 150 rats were used for the following study. We used DMN-induced hepatic fibrosis model. The characteristics of this model had been described by Madden *et al* [23]. Low mortality rate, shortproduction period and easy reproduction were the main advantages. We could investigate the effect of IFN-γ on hepatic fibrosis caused by different toxins because the two models had different mechanisms of inducing hepatic fibrosis.

Earlier report on the effect of IFN-γ anti-fibrosis could be found in 1984[8]. Studies *in vitro* or *in vivo* later proved that IFN-γ had a better effect on hepatic fibrosis[9-14]. However, IFN-γ and toxins were used at the same time in these studies. In the strict sense, their results only demonstrated that IFN-γ could prevent hepatic fibrosis, but failed to prove the effect of IFN-γ. In this study, IFN-γ was used on the first day of the fifth week when hepatic fibrosis had appeared so as to observe its effect on hepatic fibrosis.

In our study, we substantiated the impression that pathological changes taking place in CCl_4-induced model were different from those in DMN-induced model[22,26]. But, all the animals in fibrotic model groups showed complete fibrotic septum, forming a pattern of micronodular cirrhosis. Treatments with different doses of IFN-γ indicated significant decreases in the SSS, liver hydroxylproline content and serum hyarunic acid level. Among the three IFN-γ treatment groups, the best effect was present in the high-dose IFN-γ group.

To our knowledge, this is the first study on the effect of IFN-γ using different doses in animals. Our results showed that the effect of IFN-γ was associated significantly with doses. The higher the dose, the better the effect (Tables 1-4). The dose of IFN-γ used for virus is as high as 600MU/day. However, in our study, the medium dose of IFN-γ (5 MU/kg/day) used in rats is equal to 100 MU/day used in human. Whether the dose of IFN-γ could be increased clinically needs further studies.

Colchicine was used as the control drug in the experiment. Its effect on hepatic fibrosis in rats was not as good as that of IFN-γ according to the pathological and biochemical examinations (Tables 1-4).

In order to complete the animal experiment, we substantiated the effect of IFN-γ on chronic hepatitis B patients with hepatic fibrosis. Fourty-seven patients conforming to the study criteria were chosen in our study. After a 9-month IFN-γ treatment, 33 patients underwent a second liver biopsy. The results showed that the SSS decreased or unchanged in 30 patients (Table 5). The mean fibrosis score in accordance with SSS decreas ed from 8.40 ± 5.83 to 5.30 ± 4.05 (Table 6). And serum indices (including HA, C-IV, PCIII and LN) strengthened the pathological impression. All the four indices decreased significantly after the IFN-γ treatment (Table 7).

It was concerned that IFN-γ would increase inflammation[27]. However, our study showed a decrease of inflammation as compared with the previous IFN-γ treatments although the difference was not statistically significant (Table 6). We chose chronic hepatitis patients for the experiment so as not to cause patients any worries at the stage of acute inflammation. The results showed that IFN-γ treatment was safe for chronic hepatic patients. In the study, we also performed routine serum biochemical tests, including ALT, AST, and γ-GT, AFP. The mean serum ALT, AST, and γ-GT level lowered in those patients as compared with those of IFN-γ treatment. No obvious change in serum AFP level was found within the 9 months.

It is well known that collagen type I is the predominant extracellular matrix protein in fibrosis and cirrhosis but few techniques could discriminate different collagens in the same section[28,29]. We used Sirius red stain to resolve the problem as described by Zhang *et al*[16]. Under Polarization microscopy, sections stained with Sirius red showed different colors, red and yellow representing collagen type I and green collagen type III.

In our previous report, decrease of serum hepatic fibrosis indices was observed[15] after 3 months of IFN-γ treatment. So, at the earlier stage of the experiment, we adopted a 6-month treatment. There were 2 patients who received a second liver biopsy. No obvious changes were found in liver sections. We considered that a 9-month IFN-γ treatment was appropriate.

In this study, we paid more attention to side-effects. Symptoms including fever, joint pain, myagias, headache, nausea, leukopenia and platelet decrease were found in the experiment. IFN-γ was first used to treatrheumatoid [30,31], for as long as 24- months[24]. No serious side-effects were reported. However, whether IFN-γ may cause any side effects when it is used to treat hepatic fibrosis needs further observations.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Inhibition of hepatitis B virus by oxymatrine *in vivo*

Xiao Song Chen, Guo Jun Wang, Xiong Cai, Hong Yu Yu and Yi Ping Hu

Subject headings hepatitis B virus; antiviral agents; oxymatrine; hepatitis B surface antigens; hepatitis B core antigens; immunohistochemistry

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Abstract

AIM To investigate the anti-HBV effect of oxymatrine (oxy) *in vivo*.

METHODS HBV transgenic mice were produced by micro-injection of a 4.2 kb fragment containing the complete HBV genomes. Expression level of HBsAg and HBcAg in the transgenic mice liver was determined by immunohistochemical assay.

RESULTS Four groups (6 mice in each group) were injected intraperitoneally with oxy at the dosage of 100, 200, and 300 mg/kg or with saline once a day for 30 days. Both HBsAg and HBcAg were positive in livers of all the six mice in the control group (injected with saline), and were positive in livers of two mice in 100 mg/kg group and 300 mg/kg group. In 200 mg/kg group, HBsAg and HBcAg were negative in livers of all the six mice. Based on the results, 200 mg/kg is the ideal dosage to explore the effect of oxy at different time points. According to the oxy treatment time, mice were divided into four groups: 10 d, 20 d, 30d and 60 d (4 mice in each group). Each mouse underwent liver biopsy two weeks before the treatment of oxy. Down-regulation of HBsAg and HBcAg appeared after treatment of oxymatrine for 10 d and 20 d, Dane-like particles disappeared after the treatment of oxy for 20 d under electron microscopy, however, the expression level of HBsAg and HBcAg returned to normal 60 d later after oxy treatment.

CONCLUSION oxymatrine can reduce the contents of HBsAg and HBcAg in transgenic mice liver,longer treatment time and larger dosage do not yield better effects.

[1]Department of Infectious Diseases, Changzheng Hospital, the Second Military Medical University, Shanghai 200003, China
[2]Department of Pathology, [3]Department of Cell Biology, Department of Basic Medicine, the Second Military Medical University, Shanghai 200433, China
Xiao Song Chen, graduated from Second Military Medical University as a postgraduate in 1999, specialized in viral hepatitis B.
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Correspondence to: Yi Ping Hu, Department of Cell Biology, Department of Basic Medicine, the Second Military Medical University, Shanghai 200433, China
Tel. 0086-21-25070240
Email. Yphu@smmu.edu.cn
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INTRODUCTION

There are about 30 million patients suffering from chronic hepatitis B in China. Patients with active liver disease carry a high risk of developing cirrhosis and hepatocellular carcinoma. At present, the most effective medicine is IFN-α, but it is too expensive for most patients. Even in the patients treated by IFN-α, the sera negative-convertion rate of HBeAg and HBVDNA is about 40%[1-3]. Previous works in our department by Cai X *et al* showed that the sera negative-convertion rates of HBeAg in patients with chronic active hepatitis B treated with oxymatrine was 61%[4]. To investigate the inhibiting mechanism of oxymatrine (oxy) on HBV replication, transgenic mice were employed as animal model to observe the changes of HBsAg and HBcAg expression in liver tissues.

MATERIALS AND METHODS

Materials

Matrine injection is the product of Ningxia Pharmaceutical Company Ltd., containing 98% oxymatrine. The restriction enzymes were purchased from PROMEGA. Taq enzyme purchased from SANGON. Immunohistochemical kits were purchased from DAKO. Primers of PCR were designed by ourselves and synthesized by GIBCO. ^{32}P labeled kit was the product of PROMEGA. Other reagents were purchased from WASON, HUAMEI and so on.

Methods

Preparation of animals HBV transgenic mice (official designation: ICR-TgN HBV adr1.2 SMMU) were produced by micro-injection of a 4.2 kb fragment containing the complete HBV genome (adr subtype)[5]. Structural analysis of the transgene revealed that at least one complete uninterrupted HBV genome was present. HBsAg and HBeAg were not detectable in the sera of the mice, but can be detected in livers by immunohistochemical assay (ABC), which was used to determine the expression level of HBV.

PCR and Southern-blot analysis Total tail genome DNA was analyzed by PCR using HBV-specific primers 5'CCCAATGGAACACTCACC[sense], 5' AGGAACCACTGAACAAATGGC [antisense]), generating a 380 bp fragment. Twenty mililiter of PCR products were analyzed by electrophoresis on a 1% agarose gel in the presence of 0.5 mg of ethidium bromide per mililiter. DNA bands were visualized by UV fluorescence. Southern-blot analysis was performed on total genomic DNA by agarose gel electrophoresis of 30 mg restricted

genomic DNA. Samples added on nylon filters were hybridized with HBV specific ^{32}P labeled DNA probes.

Histological analysis and electron microscopy were carried out as routine methods. The expression of HBsAg and HBcAg in liver tissues were assessed by immunohistochemical analysis according to Guidotti *et al*[6].

RESULTS

Effect of oxy at different doses on the expression of HBsAg and HBcAg Twenty-four age and sex-matched mice were divided into four groups. Each group was injected intraperitoneally with oxy at the dosage of 100, 200 and 300 mg/kg or with saline separately once a day for 30 days. Livers were harvested 2 hours after the last injection for immunohistochemical assay. Both HBsAg and HBcAg were positive in livers of all the six mice in the control group (injected with saline). In 100 mg/kg group, HBsAg and HBcAg were positive in two mice, while HBsAg and HBcAg were negative in the other four mice. In the 200 mg/kg group, both HBsAg and HBcAg were negative in all the six mice, none of the six mice had detectable HBV antigen in the livers. In the 300 mg/kg group, HBsAg and HBcAg were positive in two mice, and negative in the other four mice. No pathological changes were found in the transgenic mice. Based on the results, we considered that 200 mg/kg is the ideal dosage of oxy for further study.

The effect of oxy at different time on the expression of HBsAg and HBcAg

Mice were divided into four groups according to the oxy treatment time, 10d (group 1), 20 d (group 2), 30 d and 60 d (group 3 and 4). In each group, 4 mice were randomly entered. Each mouse underwent liver biopsy two weeks before the treatment of oxy (200 mg/kg). The liver samples before and after oxy treatment were collected, and immunohistochemical analysis was performed to determine the expression level of HBsAg and HBcAg. All the samples contained HBsAg and HBcAg positive cells, and the positive and negative cells were counted in 5 randomly selected high field vision, and χ^2 test was made to compare the HBV expression level before and after oxy treatment. In group 1, the number of HBsAg and HBcAg positive cells was significantly lower than before treatment of oxy in all the four mice livers. In group 2, the similar results were observed (Figures 1 and 2), and Dane-like particles could be found in the livers before oxy treatment under electron microscope and such particles could not be found after the treatment of oxy for 20 d (Figure 3). In group 3, the expression of HBV was decreased only in two of four mice. No difference was observed on the expression of HBsAg and HBcAg between the two liver samples harvested before and after the treatment of oxy in group 4.

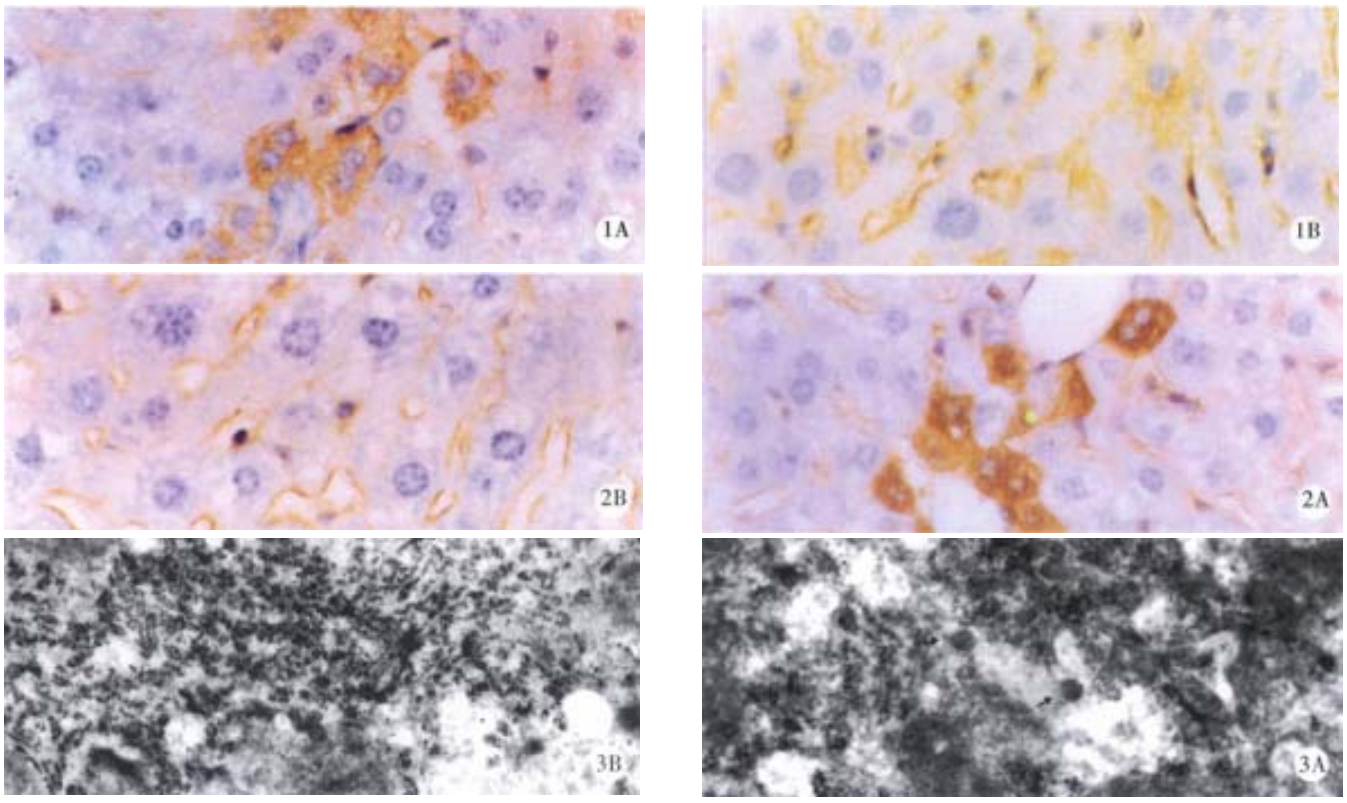

Figure 1 HBsAg in liver of the mice before (A) and after (B) treatment with 200 mg/kg oxy for 20 d.
Figure 2 HBcAg in liver of the mice before (A) and after (B) treatment with 200 mg/kg oxy for 20 d.
Figure 3 Dane's like particles can be seen in the liver of untreated mice (A) and disappeared after treatment with 200 mg/kg oxy for 20 d (B).

DISCUSSION

As a traditional Chinese medicine, *Sophra Flavescens Ait* has been used for the treatment of many diseases for thousands of years. Its extract, oxy, has long been extensively used in China. It is reported that oxy has a lot of pharmacological functions which can be divided into four classes: ① Anti-bacterial and anti-parasitic actions. It has been reported that oxy can cure acute dysentery, *Trichomonas vaginalis* and Giardia lamblia infection [7-9], but the mechanism is still unclear. ② Regulating immune reaction. Oxy can stimulate immune response at a low concentration while inhibiting immune response at a high concentration[10]. Recently, more researchers have paid attentions to the immune inhibitory effect of oxy. It has been reported that oxy has many functions such as anti-inflammation, anti-hypersensitive reactions, inhibiting histamine releasing[11-14]. The mechanism may be related to the changes of cAMP in the cell[15] and inhibiting production of cytokine[16]. ③ Inducing production of cytochrome P450. Oxy can increase the content of P450 in the rat liver significantly after treatment of oxy at the dosage of 200 mg/kg for 4d [17]. ④ Anti-virus actions. Liu JX reported that oxy could inhibit coxsackie virus B3 *in vivo* and *in vitro* [18,19]. Cai X found that the sera negative convertion rates of HBVDNA and HBeAg were 61.9% and 61.0% in chronic active hepatitis treated by oxy, while such rates in the therapy of IFN-α were 57.9% and 55.3%[4]. In our study, the content of HBV antigen in livers of transgenic mice decreased significantly after treatment of oxy for 10 and 20 d. Dane-like particles disappeared in the liver of transgenic mice after oxy treatment for 20 d. However, HBV expression level returned to normal after treatment by oxy for 60 d. We concluded that oxy can be used as an effective drug in managing HBV infection. There are two features of oxy on HBV: ① HBsAg and HBcAg was down regulated at the same time, ② longer time and larger dose did not yield better effect.

Our results strongly suggested that oxy can significantly inhibit the expression of HBV antigen in transgenic mice and the replication of HBV as well[20]. But how oxy give play to its effect can not be concluded from our experiments. However, based on the previous researches, it seems that oxy may act by two ways: ① oxy acts as an immune reaction regulator: Since HBV transgenic mice were first found by Chisari in 1985[21], *in vivo* study of HBV has become convenient and objective. Different lineage of HBV transgenic mice has also been found in our country[5,22]. A serial studies by Chisari *et al* have shown that certain soluble products of the immune response, especially IFN-γ, TNF-α, IFN-α, IL-2 and IL-12[23-28] could suppress the steady-state content of HBV messenger RNA in the hepatocytes of transgenic mice. Furthermore, these effects were found to be mediated by a post-transcriptional mechanism that selectively accelerates the degradation of cytoplasmic HBV mRNA[27]. The same events were set in motion when HBsAg-specific CTL secreted IFN-γ and induced TNF-α after antigen recognition[23,29]. The interhepatic nucleocapsid particles and replicative intermediates were also eliminated during unrelated virus infection[30,31] or during hepatocellular regeneration after partial hepatectomy[32]. Oxy is a strong immune regulator, Wang HX has reported that oxy can inhibit the competence of LAK cell killing P815 cell by about 70%-80%[33], and Shang HS reported that oxy has the same effect of marcophage on P815 cell[34], which proved that oxy may be an agonist of IL-2. Thus, IL-2 could not be the mediator of oxy inhibiting HBV. Whether other cytokines may be the mediator remains unclear. ② Oxy acts as an inducer of cytochrome P450. HBV antigen is exogenous proteins in mice hepatocytes. mRNA of HBV in hepatocytes may be degraded by cytochrome P450. Therefore, oxy can induce the production and enhance the activity of cytochrome P450[17], hence accelerating the degradation of HBV mRNA and inhibiting HBV replication. Further study is needed.

Oxy is a broad-spectrum anti virus drug, at least to HBV and coxsackie B virus 3 so far. This may give us new hope for the treatment of chronic hepatitis HBV infection including other viral infection such as HCV and HIV infection.

Cirrhosis is a servere consequence of chronic HBV infection and preventing the development of cirrhosis is very difficult[35]. Gan LW *et al* reported that oxy can inhibit the liver fibrosis induced by CCl_4 in rats[36]. Oxy can not only down-regulate HBV expression but also inhibit the liver fibrosis. Based on the two points, we concluded that oxy can be used as an effective drug in managing HBV infection. However, the exact mechanism of oxy inhibiting expression of HBV and liver fibrosis has not yet been fully understood. Further studies both basicaly and clinically are needed.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Relationship between phenotypes of cella2function differentiation and pathobiological behavior of gastric carcinomas

Yan Xin, Xiao Ling Li, Yan Ping Wang, Su Min Zhang, Hua Chuan Zheng, Dong Ying Wu and Yin Chang Zhang

Subject headings stomach neoplasms; cell differentiation; lymphatic metastasis; prognosis; cell-function classification; immunohistochemistry; absorptive function differentiation type

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Abstract

AIM To reveal the correlation between the functional differentiation phenotypes of gastric carcinoma cells and the invasion and metastasis by a new way of cell-function classification.

METHODS Surgically resected specimens of 361 gastric carcinomas(GC) were investigated with enzyme-, mucin-, and tumor-related marker immunohistochemistry. According to the direction of cell-function differentiation, stomach carcinomas were divided into five functionally differentiated types.

RESULTS ① Absorptive function differentiation type (AFDT): there were 82 (22.7%) patients including 76 (92.7%) aged 45 years. Sixty-nine (84.1%) cases belonged to the intestinal type. Thirty-eight (46.3%) expressed CD44v6 and 9 (13. 6%) of 66 male patients developed liver metastasis. The 5-year survival rate of patients in this group (58.5%) was higher than those with the other types ($P<0.01$). ② Mucin secreting function differentiation type (MSFDT): 54 (15%) cases. Fifty-three (98.1%) tumors had penetrated the serosa, 12 (22.2%) expressed ER and 22 (40.7%) expressed CD44v6. The postoperative 5-year survival rate was 28.6%. ③ Absorptive and mucin-producing function differentiation type (AMPFDT): there were 180 (49.9%) cases, including 31 (17.2%) aged yanger than 45 years. The tumor was more common in women (62, 34.4%,) and expressed more frequently estrogen receptors (ER)(129, 81. 7%) than other types ($P<0.01$). Ovary metastasis was found in 12 (19.4%) out of 62 female subjects. The patients with this type GC had the lowest 5-year survival rate (24.7%) among all types. ④ Specific function differentiation type (SFDT): 13 (3. 6%) cases. Nine (69.2%) tumors of this type derived from APUD system, the other 4 (30.7%) were of different histologicaldifferentiation. Sixty per cent of the patients survived at least five years. ⑤ Non-function differentiation type (NFDT): 32 (8.9%) cases. Nineteen (59.%) cases had lymph node metastases but no one with liver or ovary metastasis. The 5-year survival rate was 28.1%.

CONCLUSION This new cell-function classification of GC is helpful in indicating the characteristics of invasion and metastasis of GC with different cell-function differentiation phenotypes. Further study is needed to disclose the correlation between the cell-functional differentiation phenotypes and the relevant genotypes and the biological behavior of gastric carcinoma.

The Fourth Laboratory of Cancer Institute, China Medical University, Shenyang 110001 ,Liaoning Province, China
Yan Xin, graduated from China Medical University in 1993 with M.D, Ph.D, now Professor, Director of the Fourth Laboratory of Cancer Institute of China Medical University, specialized in tumor pathology, focusing on the pathobiological behavior of gastrointestinal carcinoma and the mechanism of metastasis, having more than 60 papers published.
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Correspondence to: Prof. Yan Xin, The Fourth Laboratory of Cancer Institute, China Medical University, Shenyang 110001, Liaoning Province, China
Tel. 0086-24-23256666 Ext.6351, Fax. 0086-24-23252377
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INTRODUCTION

Gastric carcinoma is one of the most common malignancies and has the highest mortality in China [1,2]. The most important reason causing patients' death is the metastasis to distant important organs. It was reported that 64.2% of gastric carcinomas developed distant organ metastases, among which the rate of liver metastasis was 31.8% of all patients, and ovary metastasis rate was 43.6% of the female patients[3]. As liver is the biggest parenchymatous organ in the body, the micrometastasis of it is very difficult to be diagnosed early. The same is true in ovary metastasis because ovaries lie in the bottom of abdominal cavity. There has been no good indicator so far to objectively predict the risk of liver and ovary metastases, making the diagnosis for micrometastases of these two organs very difficult. The present study put

forward a new classification of gastric carcinoma based on the cell-function differentiation features, in an attempt to reveal the relationship between the characteristics of cell-function differentiation and the local invasion and distant organ metastases of gastric carcinomas, and to clarify the causes and molecular mechanism of metastasis to the liver or ovary.

MATERIALS AND METHODS

Subjects

Three hundred and sixty-one cases of surgically resected gastric carcinomas (from Cancer Institute, China Medical University) were involved in the study, consisting of 258 men and 103 women, including 11 without metastasis, 224 with lymph node metastases, 12 with liver metastases, and 14 with ovary metastases. At least 2 blocks were cut from each primary tumor and 1 block from each metastatic tumor of any organ involved respectively.

Principle and standards of cell-functional differentiation classification

Glandular epithelial cells of gastrointestinal tract can be divided into three main groups: ① absorptive cells of the small intestine, ② mucinous cells including goblet and columnar mucous cells; and ③ cells with specific secretary function, such as parietal, chief, paneath, and APUD system cells. According to the principle that cancer cells maintain more or less the functional differentiation potential of their ancestors, a group of comprehensive indicators that could objectively reflect absorptive, mucin-secreting, and specific function differentiation were selected: indicators of absorptive function differentiation were brush border enzymes, such as alkaline phosphatase (AKP) and L-aminopeptidase (LAP); indicators of mucin secreting function differentiation were sulfomucin (HID+), sialomucin (ABpH2.5+) and neutral mucin (PAS+), and indicators of specific function differentiation were antibodies to hormones produced by APUD cells, cellular keratin and others. According to the expressions of these indicators, the gastric carcinomas were divided into five cell-function differentiation types: ① gastric carcinomas, in which more than two thirds of cancer cells expressed AKP and LAP but did not secrete mucin or hormone, were defined as absorptive function differentiation type (AFDT); ② those in which more than two thirds of the cancer cells secreted only mucin but not AKP or LAP, were defined as mucin secreting function differentiation type (MSFDT); ③ those in which more than two thirds of the cancer cells not only expressed AKP and LAP but also produced mucin, were defined as absorptive and mucin-producing function differentiation type (AMPFDT); ④ those which neither expressed AKP and LAP nor produced mucin, were defined as non-function differentiation type (NFDT); and ⑤ specific function differentiation type (SFDT) was defined as that more than two thirds of cancer cells possessed some special functions, such as the tumors from APUD system and squamous cell cancer (Table 1 and Figures 1-6).

Table 1 Standards of cell-function classification of gastric carcinoma

Type	Brush border enzyme		Mucin			Immunohistochemistry	
	AKP	LAP	HID	ABpH2.5	PAS	Ch-A	Keratin
AFDT	+	+	-	-	-	-	-
MSFDT	-	-	+	+	+	-	-
ANOFDT	+	+	+	+	+	-	-
SFDT	-	-	-	-	-	+	+*
NFDT	-	-	-	-	-	-	-

*Chosen according to practice.

Detection of cell-functional differentiations

Enzyme histochemistry[4]: alkaline phosphatase (AKP) was detected by Gomori's method, and L-aminopeptidase (LAP) by Nachal's method. Mucin histochemistry was done by the HID/ABpH2.5 / PAS method[5]; endocrine histochemistry was performed by ABC immunostaining with antibodies to hormones produced by APUD cells. The results were evaluated using the standards given in references[6,7].

Detection of tumor associated markers[8-13]

The sections from all cases were stained by ABC histochemistry using antibodies to ER and CD44v6 respectively. The ploidy of DNA patterns of the cases with liver or ovary metastasis was examined by flow cytometry (FCM). p53 and laminin expressions in the gastric carcinomas with liver or ovary metastasis were also detected immunohistochemically.

Statistics

χ^2 test was employed in this study to analyze the results of histochemistry and survival data.

RESULTS

The carcinomas were divided into five types: 82 cases classified as AFDT (22.7%); 54 MSFDT (15%); 180 AMPFDT (49.9%); 32 NFDT (8.9%) and 13 SFDT (3.6%). Tables 2 and 3 illustrate the relationship between the cell function classification and the patients' age and sex. The relationship between the cell-function classification and histological types, growth patterns, clinicopathological stages, ER expression, CD44v6 expression, ability forming laminin-positive basement membraneous structure, lymph node metastasis, liver and ovary metastases, and postoperative 5-year survival rates are shown in Tables 4-13 and Figures 1-12.

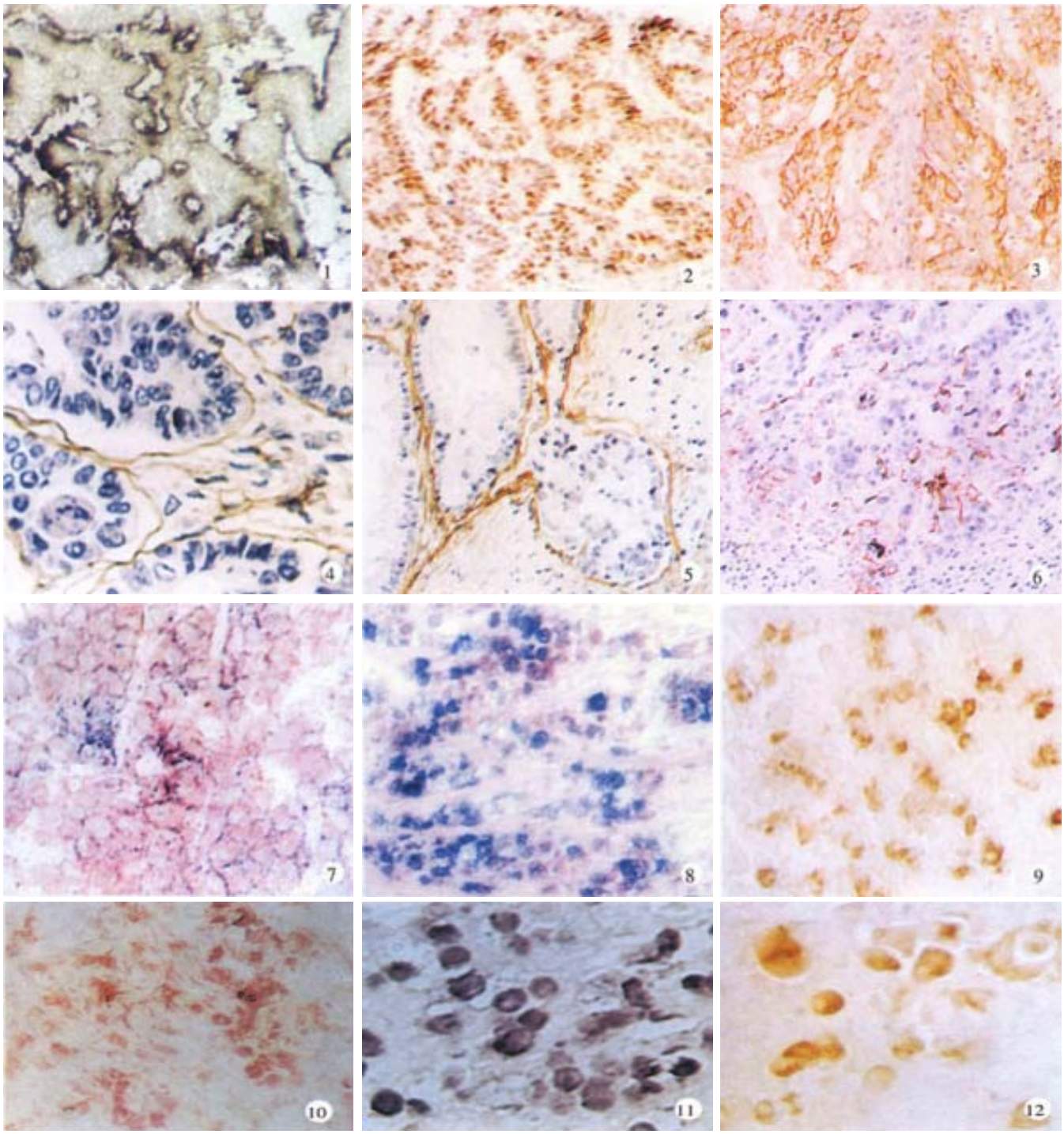

Figure 1 Primary stomach cancer of AFDT with liver metastasis. AKP was moderately positive and distributed along the free edge of cancerous papillary structure. Frozen section. ×20

Figure 2 The same case in Figure 1, Tp53 protein was expressed in most of primary cancer cells. Immunostain, ×16

Figure 3 The same case of Figure 1. CD44v6 was expressed in most of primary cancer cells. Immunostain, ×20

Figure 4 The same case of Figure 1. There was obvious basement membrane like structure with Laminin positive in the primary tumor. Immunostain, ×40

Figure 5 The same case of Figure 1. There was also obvious basement membrane like structure with laminin positive in the liver metastatic tumor. Immunostain, ×20

Figure 6 The same case of Figure 1. CD44v6 was also expressed in the liver metastatic cancer cells. Immunostain, ×20

Figure 7 Primary gastric carcinoma of AMPFDT with ovary metastasis. LAP was moderately positive and distributed in the membrane and cytoplasm of cancer cells. Frozen section, ×40

Figure 8 The same case of Figure 7. Sialomucin and neutral mucin were positive in the primary cancer cells. Mucin histochemistry, ×20

Figure 9 The same case of Figure 7. Most of primary cancer cells expressed ER, which was distributed in the nuclei and cytoplasm. Immunostain, ×20

Figure 10 The same case of Figure 7. LAP was moderately positive in the ovary metastatic cancer cells. Frozen section, ×20

Figure 11 The same case of Figure 7. Sulfomucin was positive in the ovary metastatic cancer cells. Mucin histochemistry, ×40

Figure 12 The same case of Figure 7. Most of the ovary metastatic cancer cells expressed ER. Immunostain, ×40

Table 2 Relationship between cell function classification and age of patients with gastric carcinoma

Type	*n*	Age		
		<45 yrs(%)	45-65 yrs(%)	>65 yrs(%)
AFDT	82	6 (7.3)	59 (72.0)	17 (20.7)
MSFDT	54	2 (3.7)	43 (79.6)	9 (14.7)
AMPFDT	180	31 (17.2)[a]	118 (65.5)	31 (17.2)
SFDT	13	1 (7.7)	9 (69.2)	3 (23.1)
NFDT	32	6 (18.8)	17 (53.1)	9 (28.1)
Total	361	46 (12.7)	246 (68.1)	69 (19.1)

[a]$P<0.05$ ($\chi^2 = 4.5575$), *vs* AFDT of the patients below 45 years old.

Table 3 Relationship between cell-function classification and sex of patients with gastric carcinoma

Type	*n*	Female (%)		Male (%)	
		n	%	*n*	%
AFDT	82	16	(19.5)	66	(80.5)
MSFDT	54	15	(27.8)	39	(72.2)
AMPFDT	180	62	(34.4)[a]	118	(65.6)
SFDT	13	3	(23.1)	10	(76.9)
NFDT	32	7	(21.9)	25	(78.1)
Total	361	103	(28.5)	258	(71.5)

[a]$P<0.05$ ($\chi^2 = 6.0079$), *vs* AFDT of female patients.

Table 4 Relationship between cell-function and WHO's histological classifications of gastric carcinoma

Types	*n*	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
Papillary adenocarcinoma	37	29 (78.4)[a]	2 (5.4)	6 (16.2)	0	0
Tubular adenocarcinoma						
Well-diff. ade.	20	17 (85.0)	0	2 (10.0)	0	1 (5.0)
Mode. diff. ade.	59	20 (33.9)	5 (8.5)	27 (45.8)	1 (1.7)	6 (10.2)
Poor. diff. ade.	126	15 (11.9)	14 (11.1)	79 (62.7)	2 (1.6)	16 (12.7)
Undiff. car.	30	1 (3.3)	7 (23.3)	13 (43.3)	0	9 (30.0)
SRC	41	0	5 (12.2)	6 (87.8)[b]	0	0
Mucous ade.	38	0	21 (55.3)	17 (44.7)	0	0
Carcinoid	9	0	0	0	9 (100.0)	0
Squamous car.	1	0	0	0	1 (100.0)	0
Total	361	82 (22.7)	54 (15.0)	180 (49.9)	13 (3.6)	32 (8.9)

[a]$P<0.01$ ($\chi^2 = 28.6784$), *vs* others of the papillary adenocarcinomas; [b]$P<0.01$ ($\chi^2 = 46.8780$), *vs* others of signetring cell carcinomas. ade: adenocarcinoma; diff.: differentiated; mode.: moderately; undiff.: undifferentiated; SRC: signet ring cell carcinoma

Table 5 Relationship between cell-function and Lauren£§s classifications of gastric carcinoma

Types	*n* (%)	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
Intestinal	124(36.4)	69(84.1)[a]	11(20.4)	33(18.3)	11(84.6)[b]	0
Diffuse	237(63.6)	13(15.9)	43(79.6)	147(81.7)	2(15.4)	32(100.0)
Total	361	82	54	180	13	32

[a]$P<0.01$ ($\chi^2 = 102.6339$), *vs* AMPFDT; [b]$P<0.01$ ($\chi^2 = 15.11320$),*vs* AMPFDT.

Table 6 Relationship between cell-function classification and histological growth patterns of gastric carcinomas

Patterns	*n*(%)	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
Mass	19(6.1)	12(19.4)	0	0	7(53.8)	0
Nest	84(27.0)	37(59.7)	12(22.2)	23(14.8)	6(46.2)	6(22.2)
Diffuse	208(66.9)	13(21.0)[a]	42(77.8)	132(85.2)	0	21(77.8)
Total	311	62(19.9)	54(17.4)	155(49.8)	13(4.2)	27(8.7)

[a]$P<0.01$(Exact test: $\chi^2 = 12.2793$), *vs* MSFDT, AMPFDT and NFDT.

Table 7 Relationship between cell-function classification and clinicopathological stages of gastric carcinomas

Stages	*n*(%)	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
EGC	50(13.9)	20(24.4)	0	25(13.9)	0	5(15.6)
AGC						
Subserosa	42(11.6)	8(9.8)	1(1.9)	28(25.6)	1(7.7)	4(12.5)
Through serosa	269(74.5)	54(65.9)	53(98.1)[a]	127(70.6)	12(92.3)	23(71.9)
Total	361	82	54	180	13	32

[a]$P<0.01$($\chi^2 = 10.1203$, 8.9078 and 6.7487 respectively), *vs* AFDT, AMPFDT and NFDT.

Table 8 Relationship between cell-function classification and ER expression of gastric carcinomas

ER expression	AFDT	MSFDT	AMPFDT	SFDT	NFDT	Total
Positive / examined	7 / 82	12 / 54	129 / 180	0 / 13	7 / 32	155 / 361
Positive rate(%)	8.5	22.2	71.7[a]	21.9	42.9	

[a]$P<0.01$($\chi^2 = 45.6418$), *vs* others.

Table 9 Relationship between cell-function classification and CD44v6 expression in gastric carcinoma tissues

CD44v6	*n*	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
-	241	44	32	132	9	24
+ (%)	120(33.2)	38(46.3)[a]	22(40.7)[b]	48(26.7)	4(30.8)	8(25.0)
Total	361	82	54	180	13	32

[a]$P<0.01$ ($\chi^2 = 9.8900$), *vs* AMPFDT; [b]$P<0.05$ ($\chi^2 = 3.9245$), *vs* AMPFDT.

Table 10 Relationship between cell-function classification and metastases of gastric carcinomas

Types	*n*(%)	AFDT(%)	MSFDT(%)	AMPFDT(%)	SFDT(%)	NFDT(%)
No mets	111(30.7)	28(34.1)	13(24.1)	51(28.3)	6(46.2)	13(40.6)
LN mets	224(62.0)	45(54.9)	38(70.4)	116(64.4)	6(46.2)	19(59.4)
Liver mets	12(3.3)	9(11.0)[a]	1(1.9)	1(0.6)	1(7.7)	0
Ovary mets	14(3.9)	0	2(3.7)	12(6.7)	0	0
Total 361	82(22.7)	54(15.0)	180(49.9)	13(3.6)	32(8.9)	

[a]$P<0.05$ (Exact test: $\chi^2 = 5.4385$), *vs* AMPFDT.Mets: metastasis; LN: lymph node.

Table 11 Relationship among cell-function classification, metastasis and sex of patients with gastric carcinoma

Types	Liver metastasis		Ovary metastasis
	Male (%)	Female (%)	
AFDT	9/ 66 (13.6)[a]	0/ 16	0/ 16
MSFDT	0/ 39	1/ 15 (6.7)	2/ 15 (13.3)
AMPFDT	1/ 118 (0.8)	0/ 62	12/ 62 (19.4)[b]
SFDT	1/ 10 (10.0)	0/ 3	0/ 3
NFDT	0/ 25	0/ 7	0/ 7
Total	11/258 (4.3)	1/103 (1.0)	14/103 (13.6)

[a]$P<0.01$ (Exact test $\chi^2 = 8.9527$), *vs* AMPFDT of the male with liver metastasis;[b]$P<0.05$ (Exact test $\chi^2 = 4.2992$), *vs* AFDT, SFDT and NFDT of the female with ovary metastasis.

Table 13 Relationship between postoperative survival rates and cell function classification of gastric carcinomas

Types	*n*	Cases survived over 5 years (%)	
AFDT	82	48	(58.5)[a]
MSFDT	49	14	(28.6)
AMPFDT	174	43	(24.7)
SFDT	5	3	(60.0)
NFDT	32	9	(28.1)
Total	342	117 (34.2)	

[a]$P<0.01$($\chi^2 = 11.0478$, 28.7138 and 10.3250), *vs* MSFDT, AMPFDT and NFDT, respectively.

Table 12 Comparison of cell-function differentiation classification and molecular biological features of gastric carcinoma with liver or ovary metastases

Comparison	GC with liver mets.	GC with ovary mets.
	Positive / 12 cases(%)	Positive / 14 cases(%)
Cell-function differentiation types		
AFDT	9 (75.0)[a]	0
MSFDT	1 (8.3)	2 (14.3)
AMPFDT	1 (8.3)	12(85.7)[b]
SFDT	1 (8.3)	0
NFDT	0	0
Comparison of molecular biological features		
Estrogen-dependent ER(+)	0	12(85.7)[c]
LN (+) BM structure (+)	12 (100.0)	0
Mutant p53 protein (+)	10 (83.3)[d]	3 (21.4)
DNA ploidy:Diploid	3 (25.0)	10 (71.4)
Tetraploid	0	2 (14.3)
Aneuploid	9 (75.0)[e]	2 (14.3)
CD44v6 expression (+)	10 (83.3)[f]	0

LN: laminin; BM: basement membrane; Mets: metastasis
[a]$P<0.01$ (Exact test $\chi^2 = 12.9160$), *vs* AFDT with ovary mets;
[b]$P<0.01$ (Exact test $\chi^2 = 12.5357$), *vs* AMPFDT with liver mets;
[c]$P<0.01$ (Exact test $\chi^2 = 15.8087$), *vs*GC with liver mets;
[d]$P<0.01$ (Exact test $\chi^2 = 7.5833$), *vs* GC with ovary mets;
[e]$P<0.01$ (Exact test $\chi^2 = 6.0420$), *vs* GC with ovary mets;
[f]$P<0.05$ (Exact test $\chi^2 = 15.6010$), *vs* GC with ovary mets.

DISCUSSION

In the light of differentiation degree, malignant tumors from epithelial cells of gastrointestinal tract may retain more or less the functional differentiation potential of their ancestors, which has effects on their biological behavior. Previous studies revealed the relationship between the morphological differentiation and the pathobiological behavior of gastric cancer[14-22], but so far there have been very few studies on the phenotypes of functional differentiation of gastric cancer cells[23-25]. We have studied simple morphological and the functional differentiation indicators of gastric carcinoma to infer its biological behavior during last fifteen years, and found that there was a correlation between the functional differentiation of gastric carcinoma cells and their biological behavior[26-32]. In this study, we investigated more cases and compared the degree of functional differentiation and morphological differentiation features of gastric carcinomas, and found no definite correlation between them. Although most well-differentiated papillary and tubular adenocarcinomas possessed absorptive functions, all signet ring cell cancers were classified as AMPFDT and carcinoid tumors as SFDT, the direction of functional differentiation of most moderately and poorly differentiated adenocarcinomas were undetermined. Most so-called undifferentiated cancers determined histologically displayed some functional differentiation, and were mostly MSFDT or AMPFDT gastric carcinomas; truly undifferentiated cancers made up only 8.3% in our study. Interestingly, 55.3% of mucinous adenocarcinomas classified by histology had mucin-secreting function (MSFDT) but the other 44.7% had absorptive and mucin-producing function (AMPFDT). Furthermore, Tumors of MSFDT were significantly different from those of AMPFDT in patient age, the serosa involvement, estrogen dependence and the CD44v6 expression. The results indicated that the cancer cells of mucinous adenocarcinoma possessed obvious heterogenicity in cell function differentiation and biological behavior, which should be paid special attention to predict the invasive and metastatic features.

The results also indicated that stomach cancers with different functional characteristics often possessed different pathobiological behavior. For example, the MSFDT tumors, constantly growing invasively, were mostly accompanied by the

serosa involvement, and were not obviously dependent on estrogen. NFDT independent of estrogen invades weakly, of which about 60% metastasized to lymph node. Among different functionally differentiated gastric carcinomas, AFDT and AMPFDT had specific clinicopathologically biological features as follows. Gastric carcinomas of ATDT were most common in the middle aged and senile (92.7%), only 7.3% were found in the young; with men (80.5%) surpassing women (19.5%), and intestinal type gastric carcinoma (84.1%) exceeding diffuse ones; and exhibited mainly "mass" or "nest" styles of growth. The invasion of this type of tumor was often beneath the serosa, which resisted local infiltration as a barrier. AFDT, whose growth was not very much dependent on estrogen, expressed metastasis-associated cell adhesion molecule CD44v6 at a higher rate than AMPFDT, and its postoperative 5-year survival rate was 58.5% revealed its best prognosis. Liver metastasis was frequently observed in patients with AFDT tumor whose biological behavior was as follows: ① mutant p53 protein positively expressed at a rate of 83.3%; ② among 75.0% of cases, DNA showed aneuploid; ③ 83.3% of this type presented positive expression of CD44v6; and ④ the thread-like basement membraneous structure containing LN was often formed. Special attention should be paid clinically to the characteristics of AFDT stomach cancer mentioned above. AMPFDT gastric carcinoma often occurried in the young aged below 45 years (17.2%) with more female patients (34.3%) than in other types, and histologically, 81.7% were diffusing invasive type and 71.7% were estrogen-dependent type, and there were more ovary metastases (19.4%) than in the other types. The 5-year survival rate of 24.7% embodied the worst prognosis. AMPFDT gastric carcinoma that grew in women with ovary metastasis had the following biological behavior: 85.7% of cases had high ER expression; mutant P53 protein displayed low expression at a rate of 21.4%; no expression of metastasis-associated adhesion molecule CD44v6; and diploid DNA occurred in 71.4% of tumors. Although the molecular mechanism of liver and ovary metastases was not clear, we should pay much more attention to the cell-function differentiation and biological behavior of gastric carcinomas mentioned above in order to help with the early diagnosis and treatment of the micrometastasis in the liver and ovary.

Much attention has been paid to the mechanism of cancer invasion and metastasis[33-42], especially to the mechanism of the liver and ovary metastases from gastric carcinomas. Several hypotheses have been proposed but cannot explain the specific organic affinity satisfactorily[43-50]. Our study found that the gastric carcinomas with liver metastasis possessed different cellular biological behaviors from those with ovary metastasis (Tables 10-12). It is thus possible to consider a new explanation of the mechanisms of the organic affinity of liver and ovary metastases: the fact that the gastric carcinoma with liver metastasis displayed absorptive function differentiation and expressed LN positive basement membrane-like structure suggested that these cancer cells may have increased the number of exposed laminin receptors. When the cells with exposed laminin receptors (LR) encounter the basement membrane of capillaries, the cancer cells can combine with the basement membrane by the specific affinity between LN and LR, making it possible for the cancer cells to invade the blood vessels. Therefore, we can hypothesize that the presence of exposed LR on the surface of cancer cells is an important precondition for the metastasis through the blood vessels. Most gastric cancers with ovary metastasis displayed disordered functional differentiation in the directions of absorption and mucin-production, and all these cancers expressed estrogen receptors (ER). This finding suggests that the specific organic affinity between the ovary and the specific type of gastric carcinomas may be related to an estrogen-estrogen receptor (E-ER) link.

In addition, our study found that gastric carcinomas exhibiting "mass" or "nest" growth pattern, all expressed thread-like structure containing LN. The reason for this phenomenon is probably that LR is polarized on the basal side of the cancer cells which grow in masses or nests, so these cancer cells can arrange in a row along basement membranes containing LN. On the contrary, the laminin receptors were absent or disordered on those cancer cells which grow diffusely, so that they cannot form a regular structure[51-53].

In summary, the findings of the study indicate that there was some correlation between the cell-function differentiation and invasion and metastasis of gastric carcinomas. A further study is necessary to make clear the genotypes of different functional phenotypes of gastric carcinomas and find out new molecular biological markers for the early diagnosis and treatment of the metastasis. Additionally, the remarkable difference between liver and ovary metastasis of gastric carcinoma in cell-function differentiation, P53 gene mutation, ploidy of DNA, CD44v6 expression and laminin expression indicates their different pathways of gene regulation.

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P.O.Box 2345 Beijing 100023, China

In a-tocopheryl succinate on)-induced forestomach ca nice

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Kun Wu[1], Yu Juan Shan[2], Yan Zhao[1], Jian Wu Yu[1] and Bai He Liu[1]

Fax: 0086-10-65891893 Tel: 0086-10-65897901
E-mail:wcjd@public.bta.net.cn www.wjg.org.cn

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Abstract

AIM To study the inhibitory effects of VES (RRR-α-tocopheryl Succinate, VES), a derivative of natural Vitamin E, on benzo(a)pyrene(B(a)P)-induced forestomach tumor in female mice.

METHODS The model of B(a)P-induced forestomach tumor was established according to the methods of Wattenberg with slight modifications. One hundred and eighty female mice (6 weeks old) were divided into six groups equally; negative control (Succinic acid), vehicle control (Succinate+B(a)P),positive control(B(a)P), high VES(2.5 g/kg.b.w+B(a)P), low VES(1.25 g/kg.b.w+B(a)P) ig as well as VES by ip (20 mg/kg.b.w + B(a)P). Except the negative control group, the mice were administrated with B(a)P ig. and corresponding treatments for 4 weeks to study the anti-carcinogenetic effect of VES during the initiation period. The experiment lasted 29 weeks, in which the inhibitory effects of VES both on tumor incidence and tumor size were tested.

RESULTS The models of B(a)P-induced forestomach tumor in female mice were established successfully. Some were cauliflower-like, others looked like papilla, even a few were formed into the ulcer cavities. VES at 1.25 g/kg.b.w, 2.5 g/kg.b.w. by ig and 20 mg/kg.b.w. via ip could decrease the number of tumors per mouse (1.7.±0.41, 1.6.±0.34 and 1.1.±0.43), being lower than that of B(a)P group (5.4.±0.32, *P*<0.05). The tumor incidence was inhibited by 18.2%, 23.1% and 50.0%. VES at 1.25 g/kg.b.w., 2.5 g/kg.b.w. by ig and 20 mg/kg.b.w. via ip reduced the total volume of tumors per mouse (54.8 ± 8.84, 28.4 ± 8.32 and 23.9 ± 16.05), being significantly lower than that of B(a)P group (150.2 ± 20.93, *P*<0.01). The inhibitory rates were 63.5%, 81.1% and 84.1%, respectively.

CONCLUSION VES has inhibitory effects on B(a)P-induced forestomach carcinogenesis in female mice, especially by ip and it may be a potential anti-cancer agent *in vivo*.

[1]Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
[2]The Second Affiliated Hospital, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Kun Wu, Professor, engaged in the study on anti-carcinogenetic nutrients in food, having more than 40 papers published.
Project Supported by National Natural Science Foundation of China, No.39870662
Correspondence to: Prof. Kun Wu, Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Tel. 0086-451-3648665, Fax. 0086-451-3648665
Email. wukun@public.hr.hl.cn
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INTRODUCTION

RRR-α-Tocopheryl Succinate (referred to Vitamin E Succinate, VES) is a derivative of natural vitamin E (RRR-α-tocopheryl)[1]. The interest in this derivative of vitamin E is the fact that it can inhibit the proliferation of a variety of tumor cells *in vitro*[2]. Tumor cells responsive to VES antiproliferative effects include human monoblastic leukemia cells[3], murine B-16 melanoma cells[4], human prostatic adenocarcinoma cells[5], avian lymphoid cells[6,7], human promyelocytic cells[8], human breast cancer cells[9,10]and murine EL4 T lymphoma cells[11,12].

Although the mechanisms whereby VES inhibits the proliferation of rapidly dividing cells are not well understood, induction of cell cycle blockage[13], increasing secretion and activation of potent negative growth factors, i.e., transforming growth factor-βs and their type II- cell surface receptors [2,7,11,14], and induction of apoptosis[15-17] have been observed in VES-treated cells. Earlier studies in our laboratory using the SGC-7901 cell lines (human gastric cancer cells) as model indicated that VES has antagonistic effects on cell growth and DNA synthesis[18], and can obviously induce apoptosis of SGC-7901 cells[19,20].

To date, VES has not been extensively studied on its antitumor properties as well as its mechanism of action *in vivo*. Schwartz J and co-workers[21] found that injection of VES directly into the tumor-bearing buccal pouch of hamsters (twice a week for

four weeks at a dose of 250 μg/injection) caused chemically induced oral epidermoid carcinomas to regress. As VES is found to be a potential chemopreventive and chemotherapeutic agent, it is of interest to ascertain the manner in which tumor growth is inhibited in VES-treated mice and to better understand the relationship between the structure and the function involved. This study characterizes the ability of VES to inhibit B(a)P-induced forestomach carcinogenesis in mice, addresses the involvement of different administration ways in this experiment, and shows that VES is capable of lowering both the tumor incidence and the total tumor size. The inhibitory effects of VES may be attributed to its intact compound.

MATERIALS AND METHODS

Materials

Reagents VES (purity>98%) was obtained from Sigma Co. Ltd, 10% buffered formalin phosphate (10% formalin in neutral phosphate buffer) was purchased from Shanghai Chemical Co. (China), and B(a) P (purity>98%) from Fluka Chemical Co. Ltd (Switzerland).

Animals Female Kunming mice (4-5 weeks old) were purchased from the Animal Center of the Cancer Institute in Heilongjiang Province, China. Fifteen mice were placed in a plastic cage and the animals were maintained under the following standard conditions: 22℃ ± 2℃, 45% ± 10% relative humidity, and 12 h light/12 h dark cycles each day. All animals were fed with basic diet (purchased from the Center of Animal Experiment, Heilongjiang, China) and water.

Methods

B(a)P-induced forestomach tumorgenesis Female Kunming mice (6 weeks old) were divided into six groups, each group was composed of 30 mice. Succinic acid, B(a)P and VES were dissolved in corn oil. In group 1 (negative control) and group 2 (vehicle control), the mice were intubated with 1 g/(kg b.w.) succinic acid 4 times per week for 4 weeks. In all groups except group 1, the mice were intubated with 1mg per mouse B(a)P twice a week for 4 weeks. The mice in group 3 (positive control) were given nothing except B(a)P. Groups 4 and 5 were given 2. 5 g/(kg b.w.) or 1.25g/(kg b.w.) VES by the same way 4 times/week for 4 weeks. Group 6 was given 20 mg/(kg b.w.) VES via ip twice a week for 4 weeks. The mice were then sacrificed at week 11, 16 and 29 after the first administration of B(a) P. Buffered formalin-phosphate (10%) was immediately injected into the stomach by intubation into the mouth so that the stomach was distended and fixed. Each stomach was removed and placed on a plastic sheet and the number of tumors in each forestomach was counted. The samples were stored in 10% buffered formalin-phosphate for histological examination.

Tumor volume All tumors were examined with the aid of a magnifying lens, and tumor size was measured. As described previously[22], tumor volume was determined by measuring the three dimension size of all tumors using the average of the three measurements to calculate radium. Tumor volume was calculated with the formula: volume = $4/3\pi R^3$

Histological examination of tumors Tumors found by visual examinations were further confirmed histologically. The stomach samples were excised, fixed in 10% buffered formalin-phosphate, embedded in paraffin and processed for histologic slides with hematoxylin and eosin (HE) staining. Slides were read blindly by a pathologist and the tumors were classified according to the pathological principle.

Statistical analysis

The significance of data was determined by *t* test.

RESULTS

Establishment of B(a) P-induced forestomach tumor model On the basis of previous method with some slight modifications, the model of B(a) P-induced forestomach tumor was successfully established. Treatment of Kunming mice with 1 mg/mouse of B(a) P by ig twice a week for 4 weeks mainly resulted in four forms of tumors (Figure 1). Some bigger ones appeared cauliflower-like, the moderate tumors looked like papilloma, and the smaller ones were usually grain-like. A few tumors had broken into ulcer-like cavities.

The dynamic processes of tumorigenesis in pathology at the week 11, 16 and finally 29 were observed. Before the treatment with B(a) P, the thickness of normal gastric mucosa was uniform with all characterizstics of gastric mucosa in previous studies[23-25]. The pathological changes at week 11 after the first administration of B(a)P were mainly the hyperplasia of the gastric mucosa, just as what we said "precancerous lesions"[26], and only a few papillomas could be seen. Under this condition, the thickness of epidermins was not uniform, some positions were very thin, while others were very thick. The nuclei of hyperplastic neoplastic cells were enlarged, round or ovoid with prominent necleoli. At week 16, the dominant features were papillomas, being more serious than those at week 11, and the proportion of squamous cell carcinomas in the positive group (B (a)P) was 20%, which was more malignant than before. Tumor cells proliferated lumpy into the connective tissues and were polyhedral, with abundant cytoplasms and large nuclei, in which nucleolus was prominent and some were polynucleolar. The desmosomes were clear, the mitotic figures and a few keratinized cells could be seen. The typical pathological alterations

of B(a)P-induced forestomach tumor at the week 29 were that the number of papilloma and squamous cell carcinoma were both increased. The pathological changes of B(a)P-induced forestomach tumor are illustrated in Figure 2 and the dynamic processes of tumorigenesis in each period are shown in Table 1.

Inhibitory effects of VES on B(a)P-induced forestomach tumorigenesis The treatment with 1mg/mouse- B(a)P twice a week for 4 weeks by ig in group 3 resulted in 100% incidence of forestomach tumors. There was an average of 5.4 tumors/mouse at week 29 of the experiment (Table 2, group 3). Similar results were obtained from group 2. The results in both groups showed that the succinic acid alone had no anticarcinogenic effect. Administration of 1.25 g/(kg b.w.) and 2.5 g/(kg b.w.) VES by ig 4 times/week for 4 weeks inhibited the number of B(a) P-induced forestomach tumors per mouse by 68.5% and 70.4%, respectively (Table 2, groups 4 and 5). When 20 mg/(kg b.w.) VES was injected intraperitoneally into the mice twice a week for 4 weeks, the number of B(a)P-induced forestomach tumors was inhibited by 79.6% (Table 2, group 6). These results suggested that VES could significantly decrease the number of B(a)P-induced tumors per mouse with a dose-dependent manner.

Inhibitory effects of VES on the size of B(a)P-induced forestomach tumor Administration of 1.25g/(kg b.w.) and 2.5 g/(kg b.w.) VES by ig significantly decreased the total tumor volume per mouse by 63.5% and 81.1%, respectively. Notably, the treatment with VES via ip at a much lower dose of 20 mg/(kg b.w.) and shorter time of twice a week for 4 weeks showed even higher rate of decreasing the total tumor volume per mouse (84.1%) (Table 3).

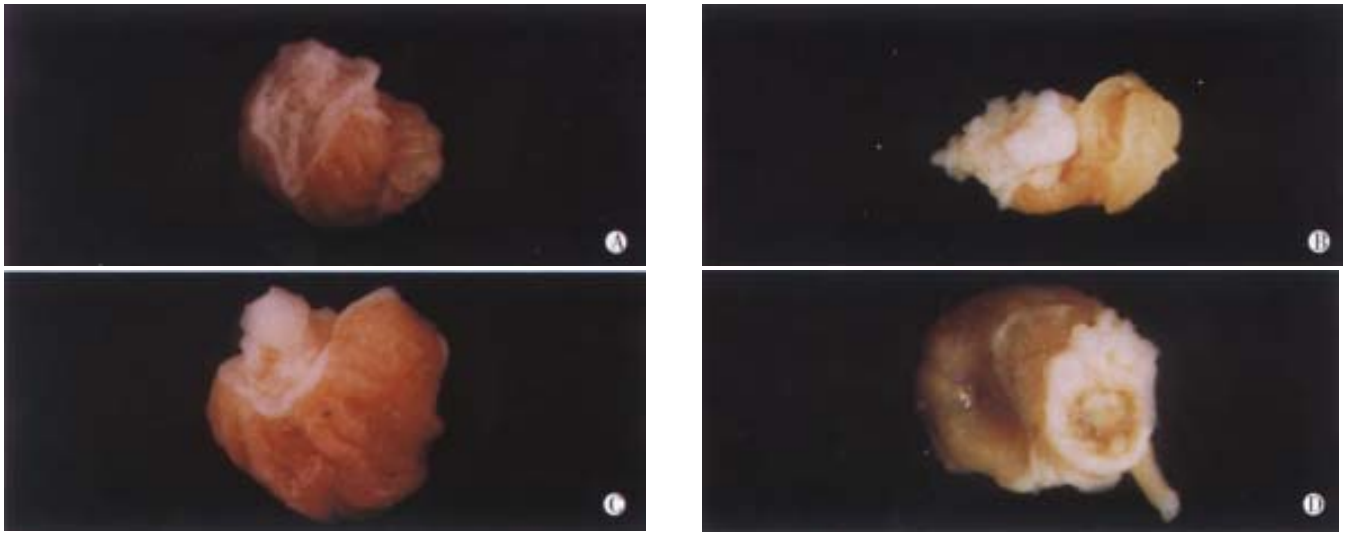

Figure 1 The model of B(a)P-induced forestomach tumor.
A. Normal mucosa of forestomach; B. Cauliflower-like tumor; C. Papilla-like tumor; D. Ulcer cavities-like tumor.

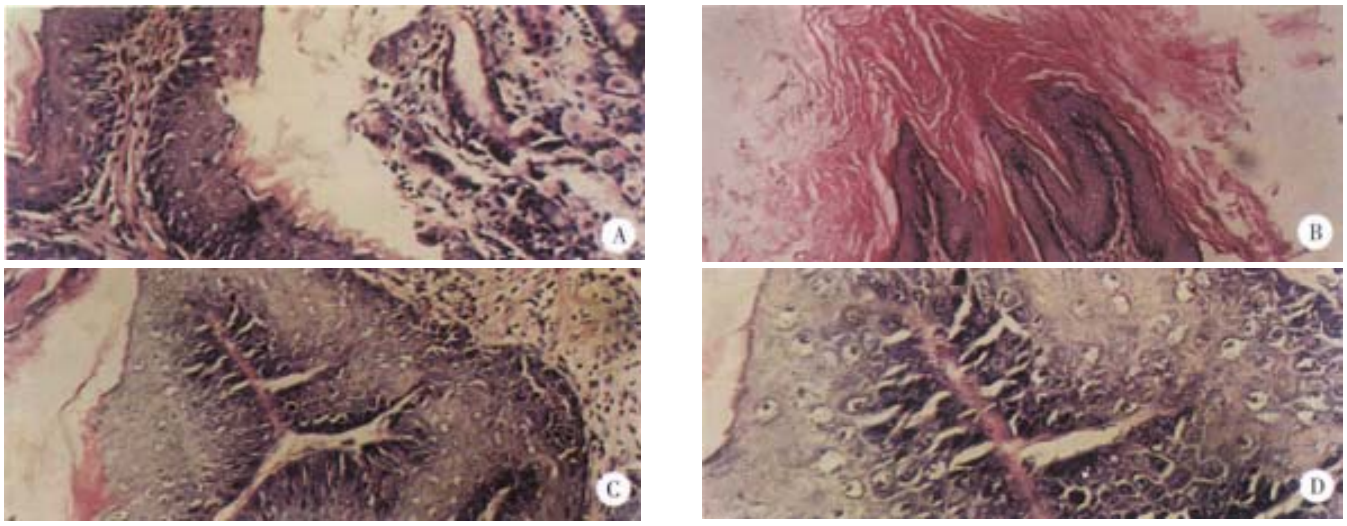

Figure 2 Pathological observation of B(a)P-induced forestomach tumor (HE staining) in mice.
A. The normal gastric mucosa ×200; B. The epithelium of gastric mucosa formed papillary projection ×100; C. Squamous tumor cells proliferated into connective tissues and they were polyhedral ×100; D. The mitotic figures (white arrow) in the squamous cell carcinoma ×400

Table 1 Pathological alterations in each group at different periods of the experiment

Groups	Week 11			Week 16			Week 29		
	H(%)	P(%)	S(%)	H(%)	P(%)	S(%)	H(%)	P(%)	S(%)
1. Succinic acid	0	0	0	0	0	0	0	0	0
2. Succinic acid+B(a)P	100	17.9	0	100	100	18.3	100	100	76.8
3. B(a)P	100	9.6	0	100	100	19.9	100	100	70.4
4. 1.25g/kgVES+B(a)P	80.3	0	0	100	80.1	0	100	91.8	56.9
5. 2.5g/kgVES+B(a)P	79.7	0	0	100	60.4	0	100	90.3	49.8
6. 20mg/kgVES(ip)+B(a)P	69.7	0	0	100	49.7	0	100	80.7	18.4

H: Hyperplasia, P: Papillomas, S:Squamous cell carcinoma

Table 2 Inhibitory effects of VES on B(a)P induced forestomach tumorgenesis ($\bar{x} \pm s_{\bar{x}}$)

Groups	No.of mice	Body weight (g)	Total tumors	
			Tumors/mouse	% of mice with tumors
1. Succinic acid	12	39.5±1.58	0	0
2. Succinic acid+B(a)P	9	40.2±1.12	5.3±0.48	100
3. B(a)P	10	40.6±1.14	5.4±0.32	100
4. 1.25g/kgVES+B(a)P	11	40.1±1.22	1.7±0.41[a](68.5)	81.8 (18.2)
5. 2.5g/kgVES+B(a)P	13	39.9±1.66	1.6±0.34[a]	76.9 (23.1)
6. 20mg/kgVES(ip)+B(a)P	10	38.7±1.06	1.1±0.43[a] (79.6)	50.0 (50.0)

[a]Compared with B(a)P group (group 3), $P<0.05$ (using Student *t* test)
Numbers in parentheses are % of inhibition compared to group 3.

Table 3 Inhibitory effects of VES on the size of B(a)P-induced forestomach tumors ($\bar{x} \pm s_{\bar{x}}$)

Groups	No.of mice	Vol/tumor(mm^3)	Total tumor Vol/mouse(mm^3)
1. Succinic acid	12	0	0
2. Succinic acid+B(a)P	9	31.5±4.51	169.5±33.42
3. B(a)P	10	27.8±8.52	150.2±20.93
4. 1.25g/kgVES+B(a)P	11	31.7±8.62 (0)	54.8±8.84[b] (63.5)
5. 2.5g/kgVES+B(a)P	13	17.6±5.54 (36.7)	28.4±8.32[b]
6. 20mg/kgVES(ip)+B(a)P	10	21.7±2.13 (21.9)	23.9±16.05[b] (84.1)

[b]Compared with the B(a)P group (group 3), $P<0.01$ (using Student's *t* test)[a]¤Numbers in parentheses are % of inhibition of tumors compared to group 3.

DISCUSSION

The results of our study demonstrated that administration of VES to mice, by both ig. and ip., inhibited B(a)P-induced forestomach tumorigenesis during the initiation period. VES may be useful in the future as a chemopreventive agent of carcinogenesis. A study *in vitro* indicated that VES is able to inhibit the proliferation of tumor cells without adverse effects on non-tumor cells[27]. Kline *et al*[2] showed that VES did not inhibit the proliferation of normal murine bone marrow cells and the non-mitogen-stimulated normal avian B and T cells. They also reported that VES can retard the growth of MCF-7, a kind of human breast cancer cell, while it has no inhibitory effects on MCF-10A, a non-tumorigenic mammary cell. Since 1997, we have studied the antagonistic effects of VES on gastric cancer, and found the inhibitory effects of VES on the growth of human gastric cancer cell (SGC-7901), DNA synthesis arrest and induction of apoptosis[18,19].These findings also supported the possibilities of VES as prospective chemopreventive and chemotherapeutic agent of tumors in the future. VES given intraperitoneally was more effective than given by ig. in lowering both the number and size of B(a)P-induced forestomach tumors even at a much lower dose. Therefore, it seems that VES administrated by ig. may be decomposed by some non-specific esterases which may normally exist in stomach, thus losing its original intact structure. VES contains a succinic acid moiety attached to the chroman head structure of RRR-α-tocopherol via an ester linkage. Esterification eliminates the hydroxy moiety that mediates RRR-α-tocopherol's classical antioxidant properties that prevents VES from acting as an antioxidant, unless the esterified succinic acid moiety is removed by cellular esterases, thereby generating free RRR-α-tocopherol. After VES was proteolyzed into succinic acid and Vitamin E, succinic acid had no inhibitory effects on B(a)P-induced forestomach tumors, as shown in group 2. Previous studies have proved that the inhibitory effect of Vitamin E on

tumors was performed through its antioxidant functions, and the inhibitory effects of natural vitamin E on human promyelocytic leukemia (HL-60) cell proliferation were much weaker than that of VES *in vitro*. All these data suggest that the inhibitory effects of VES may be attributed to its intact structure, as suggested by Fariss and co-workers[27].

The mechanisms by which VES has the inhibitory effects on chemically induced forestomach tumorigenesis in mice are still unknown. VES may influence the metabolic activation and detoxification of carcinogens as well as the postinitiation phase of carcinogenesis[28-30]. B(a)P is a suspected human carcinogen and is known to cause tumors in the forestomach of mice. Singh and Hu *et al*[31-33] reported that GST plays a major role in the detoxification of the ultimate carcinogen of B(a)P (+) anti-BPDE. In our lab, VES has been found to increase the activity of glutathione S transferase (GST) which is phase II metabolic enzyme and inhibits the activity of ethoxyresorufin O-deethylase (EROD) of liver S-9 fraction in mice, a phaseIxenobiotic-metabolizing enzyme. This was the same as the results of Wu[34,35] and Seng[36]. During the developing process of cancer, the immune functions will be decreased[37-39]. Of course, this is a very complex process and it may be involved in many factors[40-42], such as IL-2, IL-4[43,44], TNF[45, 46], TGF-β[47-52] and so on. In 1993, Kline *et al*[52] found that RRR-α-tocopheryl succinate could induce IL-2 production by avian splenic T lymphocytes and murine EL-4 thymic lymphoma cells. And they suggested that VES-induced IL-2 production may involve a mechanism other than antioxidant effects of Vitamin E. In addition, administration of VES to mice increased the serum levels of IL-2 and IL-4 (unpublished data). The information indicates that the mechanisms of VES *in vivo* may be related to the activation pathway of B(a) P metabolism in liver and regulation of the immune system.

Our findings that VES can inhibit chemically induced carcinogenesis in the forestomach of mice suggest a need for further studies on pharmacology and toxicology to determine whether VES may be a useful chemopreventive agent against human gastric carcinogenesis.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effect of bowel rehabilitative therapy on structural adaptation of remnant small intestine: animal experiment

Xin Zhou, Yuan Xin Li, Ning Li and Jie Shou Li

Subject headings short bowel syndrome; intestinal mucosa; somatotropin; glutamine; dietary fiber; parenteral nutrition, total; rats

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Abstract

AIM To investigate the individual and the combined effects of glutamine, dietary fiber, and growth hormone on the structural adaptation of the remnant small bowel.

METHODS Forty-two adult male Sprague-Dawley rats underwent 85% mid-small bowel resection and received total parenteral nutrition (TPN) support during the first three postoperational days. From the 4 th postoperational day, animals were randomly assigned to receive 7 different treatments for 8 days: TPNcon group, receiving TPN and enteral 20 g·L^{-1} glycine perfusion; TPN+Gln group, receiving TPN and enteral 20 g·L^{-1} glutamine perfusion; ENcon group, receiving enteral nutrition (EN) fortified with 20 g·L^{-1} glycine; EN+Gln group, enteral nutrition fortified with 20 g·L^{-1} glutamine; EN+Fib group, enteral nutrition and 2 g·L^{-1} oral soybean fiber; EN+GH group, enteral nutrition and subcutaneous growth hormone (GH) (0.3IU) injection twice daily; and ENint group, glutamine-enriched EN, oral soybean fiber, and subcutaneous GH injection.

RESULTS Enteral glutamine perfusion during TPN increased the small intestinal villus height (jejunal villus height 250 μm ± 29 μm in TPNcon *vs* 330 μm ± 54 μm in TPN+Gln, ileal villus height 260 μm ± 28 μm in TPNcon *vs* 330 μm ± 22 μm in TPN+Gln, $P<0.05$) and mucosa thickness (jejunal mucosa thickness 360 μm ± 32 μm in TPNcon *vs* 460 μm ± 65 μm in TPN+Gln, ileal mucosa thickness 400μm ± 25 μm in TPNcon *vs* 490 μm ± 11 μm in TPN+Gln, $P<0.05$) in comparison with the TPNcon group. Either fiber supplementation or GH administration improved body mass gain (end body weight 270 g ± 3.6 g in EN+Fib, 265.7 g ± 3.3 g in EN+GH, *vs* 257 g ± 3.3 g in ENcon, $P<0.05$), elevated plasma insulin-like growth factor (IGF-I) level (880 μg·L^{-1} ± 52 μg·L^{-1} in EN+Fib, 1 200 μg·L^{-1} ± 96 μg·L^{-1} in EN ± GH, *vs* 620 μg·L^{-1} ± 43 μg·L^{-1} in ENcon, $P<0.05$), and increased the villus height (jejunum 560 μm ± 44 μm in EN ± Fib, 530 μm ± 30 μm in EN ± GH, *vs* 450 μm ± 44 μm in ENcon, ileum 400 μm ± 30 μm in EN+Fib, 380 μm ± 49 μm in EN ± GH, *vs* 320 μm ± 16 μm in ENcon, $P<0.05$) and the mucosa thickness (jejunum 740 μm ± 66 μm in EN ± Fib, 705 μm ± 27 μm in ENGH, *vs* 608 μm ± 58 μm in ENcon, ileum 570 μm ± 27 μm in EN ± Fib, 560 μm ± 56 μm in EN ± GH, *vs* 480 μm ± 40 μm in ENcon, $P<0.05$) in remnant jejunum and ileum. Glutamine-enriched EN produced little effect in body mass, plasma IGF-I level, and remnant small bowel mucosal structure. The ENint group had greater body mass (280 g ± 2. 2 g), plasma IGF-I level (1450 μg·L^{-1} ± 137 μg·L^{-1}), and villus height (jejunum 620 μm ± 56 μm, ileum 450 μm ± 31 μm) and mucosal thickness (jejunum 800 μm ± 52 μm, ileum 633 μm ± 33 μm) than those in ENcon,EN+Gln (jejunum villus height and mucosa thickness 450 μm ± 47 μm and 610 μm ± 63 μm, ileum villus height and mucosa thickness 330 μm ± 39 μm and 500 μm ± 52 μm), EN+GH groups ($P<0.05$), and than those in EN+Fib group although no statistical significance was attained.

CONCLUSION Both dietary fiber and GH when used separately can enhance the postresectional small bowel structural adaptation. Simultaneous use of these two gut-trophic factors can produce synergistic effects on small bowel structural adaptation. Enteral glutamine perfusion is beneficial in preserving small bowel mucosal structure during TPN, but has little beneficial effect during EN.

[1]Department of General Surgery, Medical School, Nanjing University, Nanjing 210093, Jiangsu Province, China
[2]Research Institute of General Hospital, Chinese PLA General Hospital of Nanjing Military Area, Nanjing 210002, Jiangsu Province, China
Xin Zhou, graduated from Medical School of Nanjing University in 1998, now a doctoral candidate of Nanjing University, majoring general surgery.

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Correspondence to: Xin Zhou, Research Institute of General Surgery, Chinese PLA General Hospital of Nanjing Military Area, 305 East Zhongshan Road, Nanjing 210002, Jiangsu Province, China
Tel. 0086-25-3387871 Ext.58088, Fax. 0086-25-4803956

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INTRODUCTION

Various clinical conditions necessitate therapeutic massive small bowel resection, which is often followed by serious malabsorption, characterized by intractable diarrhea, steatorrhea, and weight loss. This malabsorptive state is defined as short bowel syndrome. Traditionally, parental nutrition (PN) is prescribed for these patients to assure adequate nutritional status and obtain enough time to wait for the remnant small bowel to undergo adaptation, with the hope of eventual transition from parental nutrition support to volitional enteral nutrition. Under this supportive therapeutic strategy, however, some patients may never achieve complete adaptation, i.e., sustaining completely on enteral nutrition, and life-long total parental nutrition (TPN) become lifesaving unless successful small bowel transplantation is conducted[1-3]. Unfortunately, long-term TPN is associated with great expense, vitamin or trace element malnutrition, recurrent catheter sepsis, and progressive cholestatic liver disease[4], whereas small bowel transplantation is still used currently as a salvage therapy for patients with severe metabolic complications, hepatic failure, or lack of venous access due to imperfect management of post-transplantational rejection[5]. Therefore, any therapy aimed at actively accelerating or enhancing the adaptive process in the residual bowel is likely to impact greatly on the patients' life style and the costs of medical care. Since 1995 Byrne *et al* have published a series of studies, which demonstrated that a combination of growth hormone, glutamine, and modified fiber-enriched diet can improve nutrient absorption, decrease stool output, and reducte TPN requirement[6-8]. These reports are the first to introduce the concept that further bowel adaptation can occur with the use of specialized nutrients and growth factors, and ushered in a new era in the treatment of short bowel syndrome. However, many questions about this therapy are to be answered, e.g., whether this therapy has enhanced the structural adaptation in the remnant small bowel and how each component of the remedy contributes to bowel adaptation.

Due to the heterogeneity in patient age, underlying diseases, and anatomy of the remnant bowel as well as ethic concerns, it is rather difficult to answer these questions directly from the patients. Consequently, we conducted this animal experiment in an attempt to assess the individual and combined effect of glutamine, dietary fiber and growth factor on the structural small bowel adaptation immediately after massive small bowel resection.

MATERIALS AND METHODS

Animals and surgical procedures

The animal protocols and procedures were approved by the Laboratory Animal Medicine Ethics Committee of Nanjing Military Area General Hospital of Chinese PLA. Male Sprague Dawley rats weighing between 220 g and 250 g (Shanghai Laboratory Animals Center, Chinese Academy of Sciences) were allowed 1 week to get acclimatized to our laboratory conditions before surgery. They were kept in individual stainless steel cages and fed a standard rat chow with free access to tap water in a room maintained at 22℃ on a 12 h day/night cycle (06:00/18:00).

The animals weighed about 260 g to 290 g at the end of acclimation period, and were fasted for 12 hours before surgery. Surgical procedures were performed using aseptic technique under anesthesia by intramuscular ketamine cocktail (ketamine, 100 $mg \cdot kg^{-1}$; and xylazine 8 $mg \cdot kg^{-1}$). Three surgeries were performed on each animal in the following sequence: placement of TPN catheter in the superior vena cava via the external jugular vein, installation of gastrotomy tube for liquid diet delivery, and 85% mid-small bowel resection. Both tubes were tunneled subcutaneously, and the dorsal cervical region exited through a spring-swivel apparatus. Normal saline was administered at 1 $mL \cdot h^{-1}$ through the TPN catheter with a minipump. Small bowel resection left the first 6 cm jejunum from the Treitz ligament and the terminal 6 cm ileum. The day when operations were performed was dated as day 0. The postoperative days were dated as day *n*.

Experimental design

After the operation, animals were placed back to stainless steel cages and supported with total parenteral nutrition (TPN), the composition of which is indicated in Table 1. TPN was administered at a half-rate of 1.25 $mL \cdot h^{-1}$ on day 1, and full-rate of 2.5 $mL \cdot h^{-1}$ on day 2 and 3. From day 4, animals were randomly allocated to seven experimental groups: ENcon group, receiving control liquid enteral nutrition (EN); EN+Gln group, receiving EN enriched with 20 $g \cdot L^{-1}$ glutamine; EN+Fib group, receiving control EN and fed with 2 g/d soybean fiber, containing 70% total dietary fiber (provided by Nanjing Military Area General Hospital of Chinese PLA, Nanjing, China), and was mixed with water to the consistency of porridge; EN+GH group, injected with control EN and 0.3IU recombinant human growth hormone (rhGH) (Saizen, provided by Laboratories Serona S.A., 1170 Aubonne, Switzerland) subcutaneously twice a day; ENint group, receiving subcutaneous injection of EN enriched with 20 $g \cdot L^{-1}$glutamine, 2 $g \cdot d^{-1}$ soy bean fiber, and 0.3IU rhGH twice a day; TPNcon group, receiving TPN, and 20 $g \cdot L^{-1}$glycine perfusion *via* gastrotomy tube; and TPN+Gln group, receiving TPN, and 20 $g \cdot L^{-1}$ glutamine perfusion *via* gastrotomy tube. Each experimental group contained 6 animals. The liquid EN was fiber-free with the compositions as indicated in Table 2, and was reconstituted with sterile water before

perfusion and used within 12 hours to avoid bacteria growth. Gastrotomy tube feeding of EN or amino acid solution was introduced gradually by the following schedule: half-strength solution at 1.25 mL·h^{-1} on day 4, full-strength solution at 1.25 mL·h^{-1} on day 5, and full-strength solution at 2.5 mL·h^{-1} from day 6-until day 12. PN was continued until full-strength EN was administered at full-rate, thus combined EN and PN to supply 251kJ calorie to the animals. Our formulas for TPN and EN supplied isocaloric and isonitrogenous nutrition to all animals, i.e., 251Kj nonprotein calories, 0. 414 g nitrogen from PN or Pepti-2000 variant, and 0. 272 g nitrogen from glutamine enrichment or glycine for control per day for each animal. Glutamine enrichment constituted 39.5% of the total nitrogen. Water was provided *ad lib* throughout the study.

Table 1 Total parenteral nutrition composition (400 mL)

Ingredients	mL
500 g·L^{-1} glucose	100.0
Fat (300 g·L^{-1} intralipid *)	67.5
Amino acid (114g·L^{-1} Novamin *)	150.0
100 g·L^{-1} NaCl	10.0
100 g·L^{-1} KCl	10.0
100 g·L^{-1} calcium gluconate	5.0
Multi-electrolytes (Addemel *)	2.5
Water-soluble vitamins (Soluvit *)	2.5
Lipid-soluble vitamins (Vitalipid *)	2.5
Nonprotein energy (kJ·mL^{-1})	4.2
Total nitrogen (g)	2.7
NPC / N (kJ·g^{-1})	620

*Provided by Sino-Swed Pharmaceutical Corp. Ltd., Wuxi, China. NPC / C: nonprotein energy per gram of nitrogen.

Table 2 Enteral nutrition composition (420 mL)

Ingredients	Control EN	Glutamine enriched EN
Pepti-2000 variant * (g)	126.0	126.0
Protein hydrolysate (g)	19.9	19.9
Nitrogen (g)	2.9	2.9
Fat (g)	4.9	4.9
Vegetable (g)	2.45	2.45
MCT (g)	2.45	2.45
Linoleic acid	1.12	1.12
Carbohydrates (g)	93.1	93.1
Malto^{a2}dextrin (g)	91.7	91.7
Lactose (g)	<1.12	<1.12
Organic acid (g)	0.01	0.01
Minerals (g)	2.6	2.6
Vitamins (g)	0.4	0.4
Glycine (g)	10.0	0
Glutamine (g)	0	10.0
Nonprotein energy (kJ·mL^{-1})	4.2	4.2
NPC / N	610	610

*A commercial, nutritional complete, short-chain peptide based elemental diet (Nutricia, the Netherland). NPC / N: nonprotein energy per gram of nitrogen from Pepti-2000 variant.

Plasma and tissue isolation

Body mass was monitored every three days after surgery as an index of nutritional status. At about 12:00 am on the 12 th postoperative day, laparotomy was performed on all animals under anesthesia with ketamine hydrochloride (100 mg·kg^{-1}). Blood was obtained from the inferior vena cava and placed on ice in a heparin-pretreated tube. Plasma was then isolated by centrifugation at 4℃ and stored at -20℃ for later analysis.

The residual small intestine was rapidly resected from peritoneal and vascular connections, and the luminal content was removed. After the intestine was flushed with ice-cold normal saline, 2 cm segments from both jejunum and ileum located between 2 and 4 cm from the anastomosis were removed and fixed in phosphate-buffered neutral formalin. The animals were then killed by exsanguination.

Plasma insulin-like growth factor (IGF-I) assay and histological image analysis

Total plasma IGF-I concentrations were measured after acid-ethanol extraction with the rat IGF-I RIA DSL-2900 kit (Diagnostic Systems Laboratories, Inc., Webster, USA) in duplication, following the instruction of the manufacturer. This measurement was taken both as indicator of nutritional status and evidence of rhGH treatment.

Villus height (from villus base to villus tip), crypt depth (from crypt base to villus base), and mucosal thickness (from crypt base to villus tip) were taken as indicators of structural adaptation. Routine hematoxylin and eosin stained sections were prepared, and HPIAS-1000 True Color Image Analysis System (Tongji Qianping Image Engineering Corp., Wuhan, China) was employed to analyze the image. Fifteen intact axially oriented villi and crypts selected from each specimen were measured.

Statistical analysis

Data were expressed as $\bar{x} \pm s$. Multiple comparison tests were performed after analysis of variance with the Student-Newman-Keuls test. Differences with P value less than 0.05 were considered to be statistically significant.

RESULTS

Body mass

As shown in Table 3 and Figure 1, the initial body mass on day 0 was similar among the 7 experimental groups. The body mass on day 3 was not significantly different among the 7 groups, which reflected similar operative stress and postoperative nutritional support remedy. The overall body mass loss during the first three postoperative days averaged 26.3 g. Marked body mass difference was evident from day 6. The two TPN-supported groups had gained remarkable body mass by day 6 which was kept steady during the remainder of the experimental period, suggesting the ebbing of stress and fixed nutrition supply. No significant body mass difference was observed between the two TPN groups throughout the experiment. In ENcon, EN+Gln, and EN+GH groups, the introduction of EN resulted in further body mass loss, which was accompanied by a moderate to a large amount of liquid stool production, indicating insufficient

nutrition assimilation from the remnant small bowel. From day 6, diarrhea in these groups became increasingly milder and almost disappeared after day 9, which was associated with gradual body mass gain. Each animal in two fiber-supplied groups ate up daily fiber supply, and fiber-containing solid stool instead of large liquid stool was observed after EN introduction. Continuous body mass gain began from day 3 in ENint group, and after day 6 in EN+Fib group. From day 3, the mean body mass of ENint group maintained higher than those of the other groups, which bore statistical significance on day 6 when compared with ENcon, EN+Fib, and EN+GH groups, and on day 9 and day 12 when compared with TPNcon, ENcon, EN+Fib, and EN+GH groups. Throughout the study, ENcon and EN+Gln groups had similar body mass, which remained significantly lower than that of TPNcon on day 6 and day 9 and rose to the similar level as TPNcon on day 12. From day 6, mean body mass of EN+Fib groups began to distinguish itself significantly from ENcon, and became markedly higher than both ENcon and TPNcon on day 12. On day 9, the mean body mass of EN+GH groups was significantly higher than that in ENcon group, and on day 12 significantly higher than both ENcon and TPNcon groups.

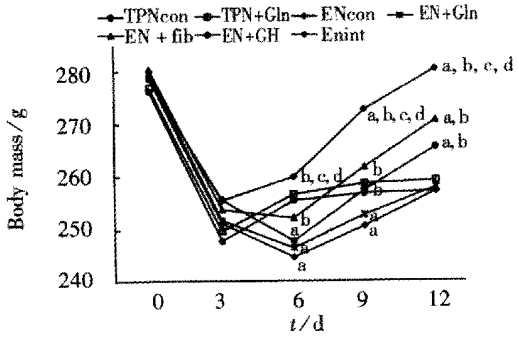

Figure 1 Body mass before and after surgery.
[a]*P*<0.05, *vs* TPNcon; [b]*P*<0.05, *vs* ENcon; [c]*P*<0.05, *vs* EN+GH; [d]*P*<0.05, *vs* EN+Fib. Day 0, refers to the day operation was performed, from which the time was dated.

Plasma IGF -I concentrations

Plasma IGF-I concentrations were similar among TPNcon, TPN+Gln, ENcon, and EN+Gln groups. Plasma IGF-I level in EN+fib group was significantly higher than that in ENcon group. GH treatment resulted in significant increase in plasma IGF-I concentration as compared with ENcon group. ENint group had the highest level of plasma IGF-I (Figure 2).

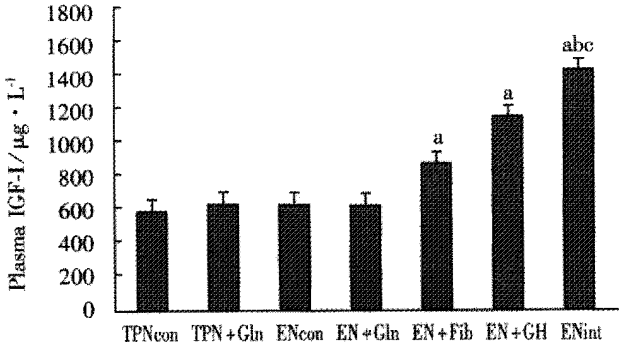

Figure 2 Plasma insulin-like growth factor level on day 12.
[a]*P*<0.05, *vs* Encon; [b]*P*<0.05, *vs* EN+Fib; [c]*P*<0.05, *vs* EN+GH.

Remnant intestinal mucosal structure

Compared with ENcon group, TPNcon group had significantly lower mean villus height and mucosal thickness in both remnant jejunum and ileum, and significantly shallower jejunal crypt (Figures 3 and 4). Luminal glutamine perfusion completely restored the villus height and mucosal thickness in ileum and partially restored the villus height, crypt depth, and mucosal thickness in jejunum. However, glutamine-enriched EN had a little impact on mucosal structural parameters whether in remnant jejunum or ileum when compared with ENcon group. The villus height and mucosal thickness in both remnant jejunum and ileum were more significantly increased by fiber supplementation than in ENcon group. GH treatment produced significant increase in villus height in both remnant jejunum and ileum, and mucosal thickness in remnant ileum when compared with ENcon group. Combined treatment with glutamine, fiber, and GH significantly deepened the crypt in remnant ileum as against TPNcon group, and further increased villus height and mucosal thickness in both remnant jejunum and ileum, which, when compared with EN+GH group, achieved statistical significance in villus height of both jejunum and ileum, and in mucosal thickness of ileum.

Table 3 Comparison of body mass before and after operation between each experiment groups (n = 6, $\bar{x} \pm s$, g)

Groups	Day 0	Day 3	Day 6	Day 9	Day 12
TPNcon	276.3 ± 9.0	247.8 ± 7.4	255.5 ± 3.6	256.8 ± 3.4	257.3 ± 2.9
TPN+Gln	277.0 ± 6.1	249.7 ± 6.5	256.7 ± 4.0	258.7 ± 3.7	259.3 ± 2.9
Encon	278.8 ± 7.8	251.2 ± 8.0	244.7 ± 3.2[a]	250.7 ± 3.5[a]	257.3 ± 3.3
EN+Gln	279.8 ± 8.9	251.8 ± 7.1	246.5 ± 3.4[a]	252.8 ± 2.7	258.0 ± 2.5
EN+fib	280.5 ± 8.0	253.8 ± 6.6	252.2 ± 2.0[b]	261.8 ± 3.4[b]	270.7 ± 3.6[a,b]
EN+GH	278.7 ± 12.9	255.7 ± 6.2	247.8 ± 2.3[a]	257.5 ± 3.3[b]	265.7 ± 3.3[a,b]
ENint	278.8 ± 8.1	255.5 ± 6.2	260.5 ± 2.4[b,c,d]	272.7 ± 2.3[a,b,c,d]	280.5 ± 2.2[a,b,c,d]

[a]*P*<0.05, *vs* TPNcon; [b]*P*<0.05, *vs* ENcon; [c]*P*<0.05, *vs* EN+Fib; [d]*P*<0.05, *vs* EN+GH.
Day 0, refers to the day operation was performed, from which the time was dated.

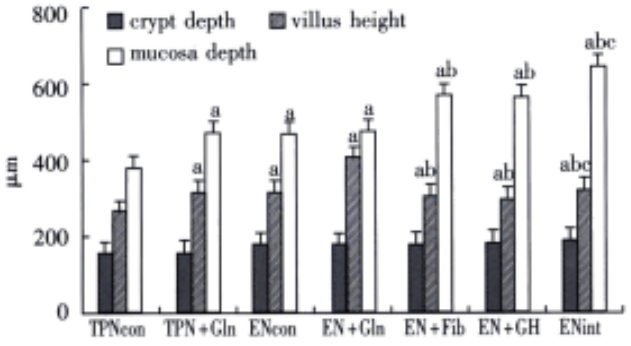

Figure 3 Crypt depth, villus height and mucosal thickness of the remnant ileum.
[a]*P*<0.05, *vs* TPNcon; [b]*P*<0.05, *vs* ENcon; [c]*P*<0.05, *vs* EN+GH.

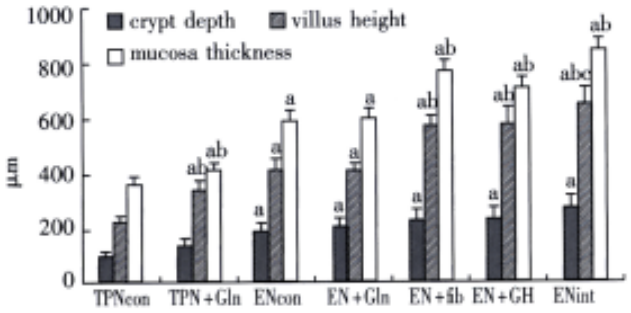

Figure 4 Crypt depth, villus height and mucosal thickness of the remnant jejunum.
[a]*P*<0.05, *vs* TPNcon; [b]*P*<0.05, *vs* ENcon; [c]*P*<0.05, *vs* EN+GH.

DISCUSSION

After massive small bowel resection, the structural adaptation of the remnant small bowel was characterized by mucosal hyperplasia. The villus height and crypt depth are both increased, while cell hypertrophy is considered unimportant[9]. This process is influenced by many factors, among which, both the quantity and route of nutrient intake are important regulators. Luminal nutrient supply, which is essential for structural adaptation, serves as not only energy source, but also signal for endogenous secretions and the release of various gut-trophic hormones and growth factors. On the other hand, normal nutritional status, which usually needs the facilitation of TPN to maintain, favors bowel adaptation[1]. In this study, great efforts were made to ensure strict control on the quantity and route of nutritent supply, and TPN was used immediately after bowel resection and during the period of reintroduction of enteral nutrition to improve the nutritional status. The compositions and delivery schedules for TPN and EN were set to provide isocaloric and isonitrogenus nutrition and to produce comparable overall nutritional status in animals of each experimental group. And the goal of producing comparable nutritional status was attained, which was attested by similar body mass and plasma total IGF-I concentration among TPNcon, TPN+Gln, ENcon, and EN+Gln groups. The utilization of plasma IGF-I as index of nutritional status is justified by sensitive IGF-I response to dietary intake, close correlation with body composition, short half-life, and its nycthermeral stability[10]. Thus the risk of confounding effects of variation in nutritional supplementation had been minimized.

Substantial researches have been done to modify the formulation of enteral nutrition to optimize the postresectional small bowel adaptation. Pepti-2000 variant has often been recommended as fiber-free EN to patients with short bowel syndrome in Nanjing Military Area General Hospital of Chinese PLA and has been chosen in this experiment as the EN formulation. This is justified by its compositional feature as peptide-based, polymeric nutrient-complete diet, which provide equal energy from medium-chain triglycerides and long-chain triglycerides. Polymeric diets are more trophic for intestinal adaptive hyperplasia compared with monomeric diets. Partial hydrolysation or protein facilitates amino acid absorption from peptide transporters while preserves gut trophic effect. Medium-chain triglycerides are water-soluble and are better absorbed in the presence of bile acid or pancreatic insufficiency, while long-chain triglycerides are more effective in inducing adaptation[11].

Glutamine is an essential nutrient for intestinal mucosa. Besides serving as the structural unit of protein synthesis, a precursor for synthesis of nucleotides and other micromolecules, it is the major respiratory fuel for intestinal mucosa[12]. *in vitro* studies have indicated that intracellular mitogenic signal transduction can be modified by glutamine supply and metabolism[13]. Animal studies have consistently shown that total parenteral nutrition (TPN)-induced intestinal hypoplasia can be attenuated by parenteral glutamine supplementation[14-16]. Glutamine-fortified-parent-eral or enteral nutrition has also been demonstrated to accelerate small intestinal healing and improves survival outcome after chemotherapy and radiation[17,18].

Following massive bowel resection, malabsorption occurs and patients may be intolerable to enteral nutrition and have to sustain on TPN for a certain period. Efforts made to preserve intestinal mucosal mass and absorption area during TPN will assist early reintroduction of enteral nutrition, which is essential for the initiation of intestinal adaptation. Parenteral glutamine supplementation has been shown to prevent TPN induced mucosal atrophy in the remnant small intestine after 85% resection[19]. Since enteral glutamine can be readily absorbed by small intestinal epithelium, and glutamine oxidation

stimulates enterocyte Na^+/H^+ exchange, leading to a high rate of electroneutral NaCl absorption in healthy and diseased jejunum[20], we studied the effect of enteral glutamine perfusion on the remnant intestinal mucosal structure during TPN. Because previous studies have not confirmed the beneficial effect of enteral glutmine supplementation during EN[21-25], we also studied the effect of adding extra glutamine to nutrition-complete enteral diet. Our results confirmed the existence of TPN induced intestinal hypoplasia, which was indicated by significantly lower villi in both jejunum and ileum and markedly shallower crypts in jejunum in TPNcon group compared with ENcon group. Luminal glutamine perfusion was effective to reverse TPN-induced hypoplasia in ileum and partially reverse TPN-induced hypoplasia in jejunum. In contrast, glutamine-supplemented enteral nutrition has little impact on small intestinal structural parameters. This finding is surprising, yet is similar to the results of several other studies[19-23]. The dosage ranged approximately from 2 $g \cdot kg^{-1} \cdot day^{-1}$ to 5.6 $g \cdot kg^{-1} \cdot day^{-1}$. And a dosage of more than 4 $g \cdot kg^{-1} \cdot day^{-1}$ was found effective in preventing TPN-induced intestinal hypoplasia in rats[12]. In all these studies, glutamine-fortified enteral nutrition was compared with standard rat chow or non-glutamine-containing elementary diet, or EN with insufficient glutamine due to partial hydrolysation of protein. Therefore, the lack of effect cannot be interpreted as insufficient glutamine delivered to intestine, or overload of glutamine. Different routes of administration were tried. Extra glutamine was mixed with enteral diet to form *ad lib* diet, or was administrated separately in bolus form, or administered as 24 h continuous enteral perfusion. The remnant intestines were analyzed 2, 7, 14 or 21 days after small bowel resection. The time points represent the beginning, active, and maximal remnant intestinal hyperplasia. In most of those studies, the animals gained body mass. In our study, the animals maintained the body mass lower than that of preoperation, reflecting our restrict nutrition supply. Nevertheless, despite different experimental protocols, similar results were obtained that during enteral nutrition, enteral glutamine supplementation produced little effect on remnant small intestinal morphological parameters. Therefore, the current available data raise the hypothesis that for otherwise healthy small intestine, with adequate enteral nutrition stimulation, glutamine delivered by arterial blood is sufficient for optimal intestinal growth. In consistent with this hypothesis, *in vitro* studies have shown that in a number of cell lines, maximal proliferation occurs when glutamine concentrations are maintained at 0.5 $mmol \cdot L^{-1}$ or above, a concentration approximates normal plasma concentration[26]. With the maximal mitogenic stimulation of epidermal growth factor, the optimal proliferation of IEC-6, a rat jejunum cell line, occurred at a glutamine concentration of 1.0 $mmol \cdot L^{-1}$ in the cultural medium, a concentration within the physiologic ranges of glutamine found in rat plasma[27]. Under normal nutritional status, plasma glutamine homeostasis can be maintained without nutritional glutamine supply[28]. However, in the absence of luminal nutrition stimulation during TPN, glutamine delivered by blood flow may be insufficient for optimal intestinal growth, as is attested by the fact that parenteral or enteral glutamine supplementation is effective in reducing TPN-induced intestinal hypoplasia. This insufficiency may be caused by decreased blood flow induced by TPN, or enteral nutrition can stimulate the uptake of glutamine through the basolateral membrane by modifying the activity of the transporters. However, our data do not exclude the possibility that when inadequate enteral nutrition is received, enteral glutamine may exert a trophic effect on small intestine, since minimum luminal nutrition has been found indifferent in stimulating small intestinal mucosal growth as compared with TPN[29].

Dietary fiber includes a wide variety of carbohydrates that, as a group, are resistant to enzymatic hydrolysis within the human gastrointestinal tract. The principal physiologic function of dietary fiber are regulating gastric emptying and intestinal transit time, based on the bulking action of the fiber. Insoluble fibers are minimally fermented and function almost solely as bulking agents that decrease colonic transit time and increase fecal mass. Soluble fibers are largely fermented by anaerobic gut flora, resulting in increase of the bacterial quantity, and fecal mass, and production of short-chain fatty acids (SCFAs), which are quickly absorbed by the colonic mucosa. The importance of dietary fiber in maintenance of normal colonic morphology and function has been widely acknowledged. However, its role in short-bowel syndrome has not been investigated thoroughly. Previous studies on the effect of dietary fiber on colon were mostly carried out in normal animals with intact small and large intestine. In contrast, after massive small bowel resection, the colon in continuity with the remnant small bowel will receive substantial unabsorbed carbohy drates and protein, which will undergo fermentation in colon by various anaerobic bacteria and produce SCFAs. This represents an important mechanism whereby the colon compensates some energy absorption function of the lost small intestine[30,31]. How colon with this altered environment responses to additional dietary fiber loading is not clear. Although there is the speculation that in carbohydrates malabsorption, dietary fiber supplementation seemed to be redundant in

enhancing the SCFAs production[11], available data from massive small bowel resection models showed the beneficial effect of dietary fiber supplementation on residual small bowel as well as colon adaptation[32-34]. In contrast, dietary fiber had little effect on intact intestinal function[35]. Therefore, it seems that the effect of dietary fiber is modified by the absorptive function of intestine.

We fed the animals with fiber-free enteral nutrition of 2 g soy bean fiber daily, containing about 70% total dietary fiber consisting predominantly of insoluble fiber, and found that soy bean fiber supply led to large solid fiber-containing stool, instead of large liquid stool as seen in non-fiber supplied EN groups. This indicates that fiber supply may alter the colon absorptive and moving function. Significantly improved body mass recovery was associated with this improved stool consistency. In addition, the plasma IGF-I concentration was simultaneously elevated, which implied that the improved body mass was not solely caused by colonic fiber retention. Both the residual jejunum and ileum displayed significantly greater mean villus height and mucosal thickness. The exact mechanism that soybean fiber enhanced the postresectional adaptation was not investigated in this study. Since the soy bean fiber consisted predominantly of insoluble fiber which mainly influences the intestinal transit, it is most likely that the presence of fiber in combination with unabsorbed carbohydrates and proteins in the colon had delayed the intestinal transit through direct bulk effect, hence promoting the colonic fermentation of unabsorbed carbohydrate and protein. Consequently, the secretion of colon-derived enteric hormones, such as enteroglucagon and peptide YY, might be augmented, which in turn exerted their small bowel trophic effect and stimulated further structural adaptation in small bowel.

Besides gut-special nutrients such as glutamine and dietary fiber, the possible role of many growth factors has met with intense research interest. Strong evidence has demonstrated that growth hormone (GH) is an important growth factor for intestine. Complete GH depletion due to hypophysectomy caused pronounced hypoplasia of small intestinal mucosa with decreased villus height and reduced crypt cell proliferation[36]. Simple replacement of GH can restore mucosal proliferative activity[37]. The study of the transgenic mice overexpressing the bovine growth hormone demonstrated that chronic excessive GH can produce hyperplasia in small intestinal mucosa whether food intake was *ad lib* or restricted[38]. Hypophysectomy could impair the adaptive hyperplasia in response to small bowel resection[39]. In an unpaired-fed study, exogenous GH was found to enhance mucosal hyperplasia after extensive small bowel resection[40]. Our pair-fed study revealed that exogenous GH administration significantly elevated the plasma IGF-I level, increased villus height in both remnant jejunum and ileum, and significantly increased ileum mucosal thickness. Postresectional body mass recovery was also accelerated by GH administration, suggesting the enhanced nutrient absorption. Our data further confirmed the gut-trophic property of GH. The more important findings of our study are that concomitant fiber supplementation further enlarged the villus height and mucosal thickness in both remnant ileum and jejunum, enhanced body mass gain, and increased the plasma IGF-I concentration in ENint group. These results suggest that gut-trophic growth factor, GH, can be used in combination with gut-special nutrient, dietary fiber, to bring about synergistic effect on postresectional remnant small bowel structural adaptation.

In summary, our study demonstrated that both dietary fiber and growth hormone can enhance the postresectional small bowel structural adaptation, promote bodymass-gain, and increase plasma IGF-I level when used separately. Simultaneous use of these two gut-trophic factors produced synergistic effect on structural adaptation parameters, body mass, and plasma IGF-I concentration. In contrast, the gut essential nutrient, glutamine when enterally administered with enteral nutrition, showed little beneficial effect on remnant small bowel structural adaptation, and the body mass did not alter in comparison with the control enteral nutrition. However, enteral glutamine is effective in preserving intestinal mucosal structure during TPN. These findings raise the doubt about the necessity of enteral glutamine supplementation during enteral nutrition. On the other hand, they provide the evidence favoring the combined utilization of GH and dietary fiber with enteral nutrition in patients with short bowel syndrome.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Comparison of a new stent and Wallstent for transjugular intrahepatic portosystemic shunt in a porcine model

Gao Jun Teng[1], Michael A. Bettmann[2], P. Jack Hoopes[2] and Li Yang[3]

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Abstract

AIM To evaluate a new balloon-expandable stainless steel stent (Cordis stent) in a transjugular intrahepatic portosystemic shunt (TIPS) porcine model and compared with Wallstent.

METHODS TIPS was performed in 26 normal domestic pigs weighing 20 kg-30 kg using a Cordis stent or Wallstent (13 pigs in each stent). All pigs were sacrificed at the 14 th day after TIPS. The stent deployment delivery system, stent patency, and stent recoil after placement were evaluated.Proliferative response in representative histological sections from the center,hepatic and portal regions of the two stent designs were quantified.

RESULTS The shunt was widely patent in 4 pigs in the Cordis stent group (4/12, premature dead in 1 pig), and in 5 pigs in the Wallstent group (5/13). All remaining stents of both designs were occluded or stenotic. The mean quantified proliferation including thickness of the proliferation and the ratio of proliferation: total area in three assayed regions in Cordis stent and Wallstent was 2.18 mm² 2:00 mm, and 59.18 mm². 51.66 mm², respectively ($P<0.05$). The delivery system and mechanical properties of the Cordis stent fuctioned well.

CONCLUSION The new Cordis stent is appropriate for TIPS procedure.

[1]Department of Radiology, Zhong-Da Hospital, Southeast University, Nanjing 210009, China
[2]Department of Radiology, Dartmouth-Hitchcock Medical Center, USA
[3]Department of Radiology, PLA General Hospital, Beijing
Corresponding to: Gao Jun Teng, M.D., Department of Radiology, Zhong-Da Hospital, Southeast University, 87 Dingjiaqiao Road, Nanjing 210009, China
Tel/Fax. 0086-25-3311083
Email. gjteng@public1.ptt.js.cn
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INTRODUCTION

The high incidence of stenosis following transjugular intrahepatic portosystemic shunt (TIPS) remains the major barrier in this relatively new technique which is effective in controlling variceal hemorrhage secondary to portal hypertension and utilized in refractory ascites[1,2]. The exact mechanism for TIPS restenosis is not well understood and seems multifactorial. One relevant factor may be stent design. Wallstent is the most common stent currently used for TIPS procedures, but various stent designs may be effective. To evaluate a new balloon-expandable stent (Cordis stent) in TIPS, a comparative study of the Cordis stent and Wallstent was performed in a TIPS porcine model.

MATERIALS AND METHODS

TIPS was performed in 26 domestic swine weighing 20 kg-30 kg, with two stent designs: balloon-expandable stainless steel stents (Cordis Co., Miami, FL) in 13 pigs and Wallstents (Schneider, Minneapolis, MN) in 13 pigs, respectively. The Cordis stent is made up of one stainless steel wire, which is formed into a sinusoidal wave pattern, wrapped in a tubular helical fashion and held in place by a series of welded joints (Figure 1). Either Cordis stent or Wallstent with an diameter of 8 mm and 4 cm in length was used in each pig.

TIPS was performed with a 22 G, 80 cm long puncture needle with a 5-F,70 cm long coaxial Teflon catheter (AngioDynamic, Queenshury, NY), a 9 F sheath set and a 14 G metal cannula with a curved tip (Cook, Bloomington, IN).

The animals in this study had normal livers and did not have portal hypertension. All protocols were approved by the Dartmouth Animal Care and Use Committee. Animals were sedated with intramuscular Ketamine (20 mg/kg) injection, intubated and maintained with 1%-3% halothane and oxygen.

The sheath was assembled with a 9-F Teflon catheter with a tapered tip and was introduced into the IVC by a standard percutaneous Seldinger method from the right jugular vein. A 7-F NIH catheter was advanced into the right lateral hepatic vein. The sheath was then placed into the hepatic vein and followed by the metal cannula containing the coaxial puncture needle. Through the metal cannula, the puncture needle together with the coaxial catheter was advanced into the hepatic parenchyma in an anterior direction targeted toward

the right branch of portal vein. Following confirmation of suitable portal vein puncture by contrast injection, a 0.035 inch steerable guidewire (Cordis Co., Miami, FL) was advanced into the portal vein and followed by the 5-F catheter. Following portography, the floppy-tip guide wire was exchanged for an Amplatz super stiff guide wire, and a hepatic parenchymal tract was dilated using an 8 mm balloon catheter. The Cordis balloon catheter-stent delivery system was introduced into the sheath by advancing the insertion tool over the wire through the hemostasis valve, until a hand stop was reached. A stent mounted balloon was introduced through the insertion tool and advanced over the guidewire until the stent was in the desired location for deployment. The stent was deployed by inflating the balloon with a 50% saline and 50% Renografin76 solution using a standard manual technique (Le Veen balloon inflator). A 10 mm diameter balloon catheter was used with 6 atm pressure for 2 minutes with 3 repetitions. The Wallstent was deployed in a standard fashion and inflated using equivalent inflation pressures and technique for Cordis stent deployment.

Since the coagulation process is much faster in pigs than in humans based on our previous experience, 500U/kg heparin was routinely administered in each procedure. No anticoagulants or other medicines were used after the procedure.

A numerical rating system was developed in the evaluation of the following deployment factors at implantation: stent insertion through the sheath, insertion tool function, stent tracking, balloon inflation and deflation, stent expansion, ease of positioning, balloon catheter removal, and stent architecture and visibility under fluoroscopy. The rating system was scored as follows: excellent = 1; satisfactory = 2; poor = 3; and not achieved = 4.

Portal venography was performed following TIPS placement and prior to sacrifice. Plain radiography was taken and the following factors were assessed: stent migration, distortion of stent architecture and changes of diameter and length of the stent.

Animals were euthanized 2 weeks after TIPS placement by intravenous injection of 15 mL saturated KCL. All of the shunts were examined by portography via transjugular i mmediately prior to euthanasia.

Necropsy was performed i mmediately following euthanasia. The TIPS shunt and involved adjacent tissues were evaluated by gross examination at necropsy and preserved for histologic assessment. Representative crossections from the central, hepatic and portal vein regions of the stent were cut after fixation in buffered 4% formalin for at least 24 hours. The metal of Wallstent was removed before embedding while the metal of Cordis stent had to be removed using a hand saw after embedding and then reembedded in JB-4. The tissues were then cut at 5 microns and stained with modified Giemsa and basic fuchsin.

At least 3 sections in each region of stent were prepared and reviewed. Histologic quantification including thickness, the area of the proliferative response and the ratio of the proliferative area/total area of the stent was achieved using standard planimetry techniques and Nikon microscopy and Camera Lucita with Micro-Plan II Image Analysis (Laboratory Computer Systems, INC).

All quantitated data were expressed as the mean value ± SD. Stent patency and quantified proliferative reaction within the stent were compared using Chi-Square test and Student's *t* test, respectively. Statistical significance was defined as $P<0.05$.

RESULTS

TIPS was successfully performed in all pigs with both stent designs (100%). No difficulties were encountered in delivering and deploying the Cordis stents. All pigs remained healthy until the scheduled date of euthanasia except one. This pig died 2 days following Cordis stent placement from introgenic hemorrhage secondary to inadvertent gastric puncture during the TIPS procedure. This early death was excluded from the numerical results.

Patency

Stent patency was determined by venography and pathohistological examinations, 33.3% (4/12) of the Cordis stent group and 38.5% (5/13) of the Wallstent group were patent at 2 weeks post-deployment (Figure 2). This difference did not reach a statistical significance ($P>0.05$, Chi-Square test). One pig had an occluded Cordis stent because its proximal end wedged into the liver parenchyma due to its stiff property (Figure 3). Likewise, one pig had an occluded Wallstent due to longitudinal recoiling of the stent causing the distal end to withdraw from the portal vein into the liver parenchyma.

Stent deployment factors

The mean score for stent deployment factors are listed in Table 1. Stent insertion through the sheath and stent expansion were scored excellent in all procedures performed with both Cordis stent and Wallstent. Cordis stent had a higher score than Wallstent in stent tracking, positioning, and visibility by fluoroscopy. The factors of tool function, balloon in/de-flation and catheter removal which are unique to Cordis stent, were scored excellent for all procedures.

Recoiling of stents

No stent migration or stent distortion was demonstrated in either Cordis stent or Wallstent groups. Stent diameter and length i mmediately after TIPS placement and 2 weeks after TIPS are shown in Tables 2 and 3. The interval change in the Wallstent group at 2 weeks after TIPS is larger than

in the Cordis stent group. The Wallstent demonstrated an average shortening of 5.18% as compared with 0. 69% with Cordis stent.

Histopathologic characteristics

Pseudointimal proliferation (PIH) formed within the shunt with both stent designs, and histology was similar in all three assayed areas. PIH was primarily composed of myofibroblasts (Figures 2,4), organized thrombus, and collagen. Infla mmatory cells were mainly lymphocytes and mononuclear cells co mmonly located around the stent wires. Organized thrombus was characterized by palisading and herringbone deposition of the fibrin and the invasion of blood vessels (neovascularization). A single-cell lining of endothelial-like cells covering the lumen was observed only in specimens with a patent shunt. The liver tissue adjacent to the stent showed minimal injury characterized by sinusoidal dilatation, hemorrhage, focal necrosis, mononuclear infla mmation and fibrosis. There was no major difference in histological characteristics between the two groups with the different stent designs.

In 10 specimens (5 in each stent group), bile leak was identified by bile staining of thrombus. Histologic sections demonstrated extensive neo-bile duct proliferation within the PIH or on the surface of the lumen in some specimens with bile leak (Figure 4). This partially contributed to occlusion of the stent.

Quantified histological results

PIH thickness and the ratio of proliferative area/total area within both stent designs are shown in Table 4. The portal vein aspect of the stents had the highest proliferative response, the hepatic region the second highest and the middle region of the stents the least proliferative. There was no statistically significant difference among the three assayed regions between Cordis stent and Wallstent specimens (P>0.05).

DISCUSSION

Various stent designs including Palmaz stent, Gianturco-Rosch Z stent, Strecker stent and Memotherm stent have been utilized in TIPS procedures[3-6]. Wallstent is the most co mmon stent used for TIPS currently within the United States due to its flexibility which allows easy negotiation of the angles bridging a hepatic vein and a portal vein[7]. Unfortunately, TIPS stent stenosis rates are high with all stent designs, although PTFE covered stent seems to decrease the stenosis of TIPS shunt in pigs[8,9]. The mechanism and process of restenosis in TIPS remains poorly understood. However, it is well accepted that stent design including mechanical properties are important factors influencing stent patency.

Table 1 Scores of deployment factors

Stent types	Insert thru sheath	Tool function	Stent tracking	Balloon in / de flation	Stent expansion	Position	Catheter removal	Architecture & visibility
Cordis	1 ± 0.0	1 ± 0.0	1.07 ± 0.27	1 ± 0.0	1.14 ± .53	1.29 ± .61	1 ± 0.0	1.86 ± .54
Wallstent	1 ± 0.0	*	2.17 ± .84	*	1.00 ± 0.00	*	1.75 ± .87	2.42 ± .52

*Not applicable to the device.

Table 2 Recoil data in Cordis stent

Interval	Diameter (mm)				Length (CM)
	Proximal	Middle	Distal	Overall	
I mm post-TIPS	7.46 ± .80	7.32 ± .70	7.43 ± .73	7.40 ± .73	4.35 ± .33
2 weeks	7.51 ± .79	7.25 ± .73	7.50 ± .74	7.42 ± .74	4.32 ± .32
Change (%)	+0.67	-0.96	+0.94	+0.27	-0.69

I mm: I mmediately

Table 3 Recoil data in Wallstent

Interval	Diameter (mm)				Length (CM)
	Proximal	Middle	Distal	Overall	
I mm post-TIPS	9.17 ± .94	7.42 ± .60	7.72 ± .71	7.98 ± 1.05	4.83 ± .42
2 weeks	9.28 ± .83	7.67 ± .53	7.94 ± .52	8.22 ± 0.90	4.58 ± .48
Change (%)	+1.2	+3.37	+2.77	+3.01	-5.18

I mm: I mmediately

Table 4 Quantified histological proliferation in specimens with Cordis stent *vs*. Wallstent

Region	Thickness (mm)			Proliferation area / total area (%)		
	Cordis stent	Wallstent	*P* value*	Cordis stent	Wallstent	*P* value*
Hepatic	2.04 ± 1.28	1.85 ± .96	0.66	57.49 ± 23.60	51.43 ± 24.61	0.74
Middle	1.42 ± 0.82	1.31 ± .87	0.63	49.89 ± 17.31	47.91 ± 20.12	0.60
Portal	2.46 ± 0.98	2.38 ± .23	0.61	71.88 ± 21.18	69.97 ± 29.45	0.57
Average	2.18 ± 1.07	2.00 ± .02	0.73	59.18 ± 23.06	51.66 ± 25.11	0.78

*Two-tail *t* test.

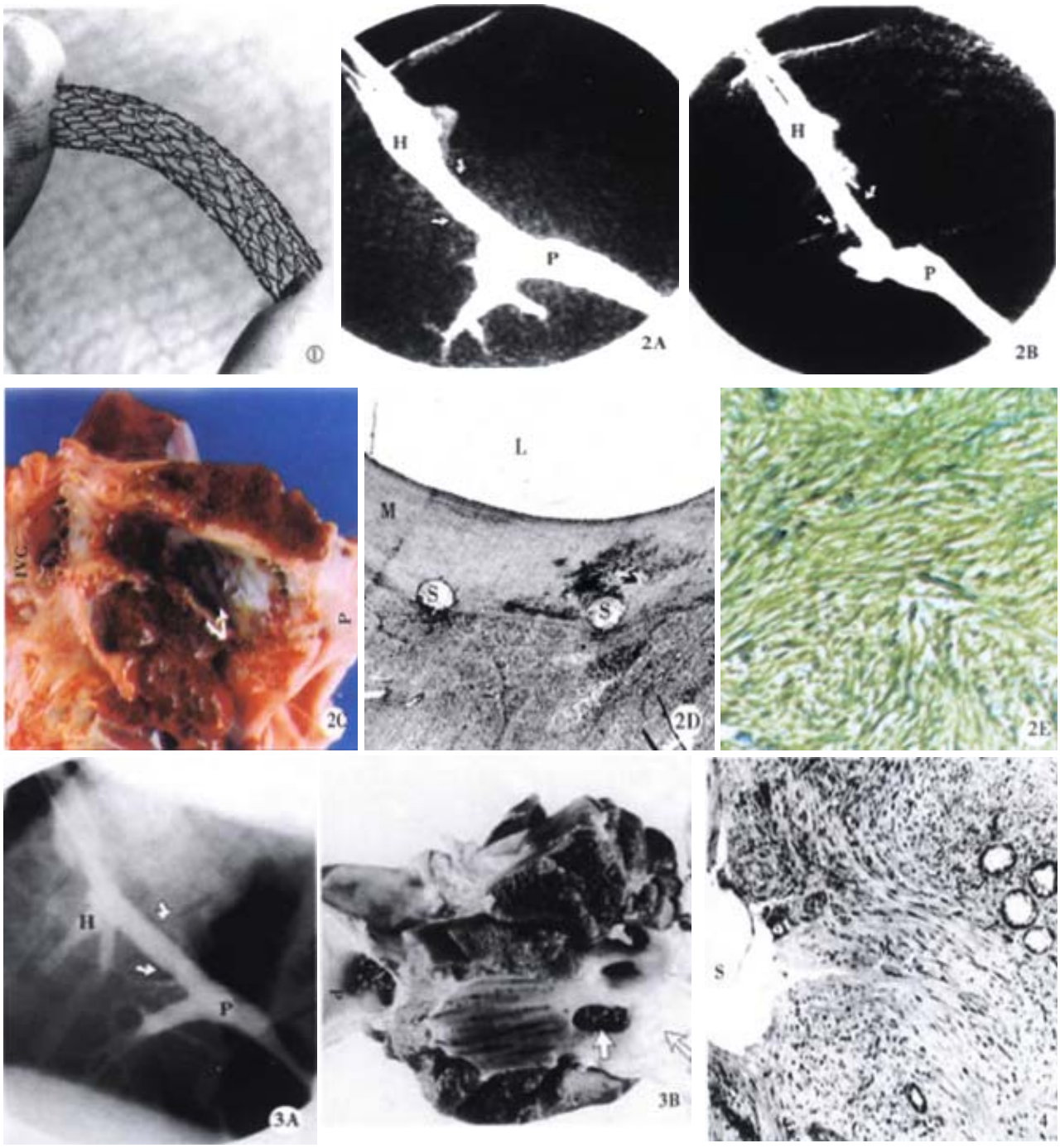

Figure 1 The Cordis balloon-expandable stent, which is constructed as a sinusoidal wave pattern by one stainless steel wire, with high rigidity.

Figure 2 a. The portal venography obtained i mmediately shows that the TIPS shunt (arrows) is well created between the hepatic vein (H) and the portal vein (P). **b.** The shunt remains patent in the portography taken i mmediately before the euthanasia at 2 weeks after stent placement. **c.** The gross examination of the specimen shows that the surface of the stent (straight arrows) is covered by a smooth and thin layer endothelia-like tissue (curved arrows) which does not extend to the ends of the stent at the IVC and the portal vein (P). **d.** Low magnification photomicrography of the TIPS reaction shows a mild myofibroblastic cell proliferation (M) under stent meshes (S) (Modified Giemsa and basic Fuchsin stain, ×3.12). **e.** The myofibroblastic cells are primarily composed of smooth muscle cells. (Anti-SMCα actin stain, ×125).

Figure 3 a. An occluded TIPS shunt from a pig using a Cordis stent. The i mmediate portal venography after TIPS shows a patent TIPS shunt (arrows). **b.** The occluded shunt was confirmed by the portography obtained i mmediately before the euthanasia in two weeks after the stent placement. The gross examination of the specimen demonstrates that the hepatic end of the stent wedges into the liver parenchyma and the proximal end of the shunt is totally occluded by the healed hepatic vein (arrows), while the portal end (P) of the stent keeps patent.

Figure 4 Photomicrography from an occluded TIPS shunt using a Wallstent shows that a massive pseudointimal proliferative tissue under the stent (S), which primarily composed of myofibroblastic cells (M). Neo-bile duct proliferation within the proliferation is also noted (arrows). Modified Giemsa and basic Fuchsin stain, ×125

Since multiple interdependent mechanical characteristics of stents may play a role in stent restenosis [10-19], the ideal stent appears to be non-existent. To produce a satisfactory stent, the following factors should be considered: stent wire material and diameter, longitudinal flexibility, hoop strength (circumferential strength), stent texture and stent surface area.

A layer of metal oxide provides the ultimate interface between stent and host following stent implantation. This is independent of metal content[10]. Similar thrombogenicity and patency rate have been observed in stented arteries using different stent metals[16,20]. Hehrlein *et al*[18] evaluated stent biocompatibility as related to surface texture and charge. Palmaz-Schatz stents were coated either by electrochemical metal deposition (platinum, gold, copper) or coated with a metallic film implanted onto the stent surface by argon ion bombardment. These stents were implanted and evaluated in a rabbit iliac arterial model. The results demonstrated that the most electropositive coating (platinum or gold) induced markedly less neointima formation than the least electropositive (copper). The authors therefore concluded that stent surface texture was the most important factor determining biocompatibility of the stent while the charge on stents appeared to be less important[18]. Strength, elasticity, and plasticity of various stents co mmonly used were recently investigated in an *in vitro* model[12,13]. The results suggested that the Palmaz stent is appropriate for insertion into highly resistant obstructions due to its superior resistance to deformation. Strecker stents and Wallstents required good wall contact to achieve adequate strength and Wallstent may not function well if implanted into eccentric stenoses of tough consistency due to its unique deformation. The Gianturco stent showed the lowest resistance and deformed more readily. We did not evaluate such criteria regarding the Cordis stent in this study. However, the higher longitudinal rigidity and hoop strength of this new stent may have merit for placement within tough and highly resistant cirrhotic liver tissue. Strong mechanical support by a stent is important to prevent proliferation as demonstrated by Ikari *et al*[17]. They demonstrated significantly higher proliferation at the articulation of Palmaz-Schats stents in stented conorary arteries and partially attributed this to lack of mechanical support in the articulation area of the stent. Surface area occupied by the metal stent mesh also influences restenosis.

A predominant difference between Cordis stent and Wallstent evaluated in this study is the rigid characteristic of Cordis stent and the flexible characteristic of Wallstent. The benefits of the Palmaz stent and Wallstent in TIPS were compared in a prospective randomized study[21]. The authors illustrated that early shunt thrombosis was more frequent with Wallstent (4/45 *vs* 0/45). Palmaz suggests that the relatively more rigid Palmaz stent could provide a stable, nonshifting surface for endothelial growth, whereas dimensional change in a prosthetic surface causes increased endothelial slough and platelet proliferation[10]. However, late observation in the same study[21] showed more frequent shunt insufficiency with Palmaz stent than Wallstent (6/45 *vs* 2/45). This indicates that the process of TIPS stenosis should be multifactorial.

Wallstent is frequently associated with unpredictable shortening in TIPS at follow-up due to the self-expanding force from elasticity[6,22]. Wallstent shortened 5.18% in our study at 2 weeks as compared with 0. 69% shortening of the Cordis stent. Stent flexibility is required to negotiate curves when delivering the stent by catheter through tortuous vessels leading to the target area. Is it possible to compromise flexibility and rigidity without the drawback of shortening like a Wallstent in TIPS Several other stent designs have been utilized in TIPS. Gianturco-Rosch stent was the first stent designed in experimental TIPS, but it seems to be unco mmonly used. Strecker stent is highly radiopaque, flexible and shortens minimally but tends to dislodge and is difficult to be recatheterized[23]. Preliminary experience in TIPS using the Memotherm stent (Angiomed, Germany) showed promising results, but the number of samples was small[6]. The new Cordis stent used in this study is relatively more flexible than the Palmaz stent, and has the added advantage of being highly radiopaque with minimal shortening. From a technical perspective, the Cordis stent deployment system consistently works well and is easy to use, compared with Wallstent. In our opinion, the Cordis stent is an appropriated compromise between the rigid Palmaz and the flexible but foreshortening Wallstent.

The similar histologic characteristics of proliferative response were observed in the two groups using different designs of stent, which is also very similar to the findings described previously by us and other authors[24-26].

To date, there are few experimental studies or clinical trials available to compare different stent designs in TIPS. It is difficult to directly compare different stent designs following deployment. For instance, although equal stent diameters and balloons with equal inflation pressures were used in our study, TIPS stent length was not equivalent following deployment. Stent diameter following TIPS completion in the Wallstent group was larger than the Cordis stent group (7.98 mm *vs* 7.40 mm). This unexpected size variation may contribute to the relative rigidity of Cordis stents compared with the relative flexibility of Wallstents and the stronger circumferential strength of Cordis stents compared with the relatively decreased hoop strength of Wallstents. Perhaps the difference in luminal diameter contributed to the increased PIH in the pigs with Cordis stents (not statistically

significant). Such an explanation is favored by our recent study (unpublished data, 1996) which demonstrated significantly thicker pseduointimal formation within the lumen (2.07 mm *vs* 1.25 mm, $P = 0.006$) when comparing a smaller diameter Wallstent (6.50 mm) to larger Wallstent (7.25 mm) in a TIPS porcine model. Nevertheless, the short-term patency rate using the Cordis balloon-expandable stainless steel stent in a TIPS porcine model in this study is comparable to that found using Wallstent.

There were interesting findings in two occluded stents in this study: one stent probably occluded because of Cordis stent rigidity, and the other probably occluded because of Wallstent longitudinal recoil. This probably reflects the inherent drawbacks of Cordis stent and Wallstent respectively. However, Cordis stent rigidity may be an advantage when deployed in stiff cirrhotic liver parenchyma in TIPS rather than in healthy, compliant normal pig livers, although Cordis stent should not be deployed where sharp curves are required to bridge the portal and hepatic veins. Use of a longer Wallstent than the exact length of the tract required in TIPS may overcome the shortening disavantage of Wallstent[22]. However, it is important to accurately place the stent in the hepatic vein to ensure patency and not to compromise future opportunity for liver transplantation[27]. The easy to use, reliable Cordis deployment system and increased radiopacity allow for accurate manipulation and precise positioning under fluoroscopy.

Therefore, our study suggests that the Cordis stent merits further investigation for potential use in TIPS patients.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Brief Reports

Intensify standardized therapy for esophageal and stomach cancer in tumor hospitals

Shi Jie Wang, Deng Gui Wen, Jing Zhang, Xin Man and Hui Liu

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INTRODUCTION

Cancer treatment situation in tumor hospitals in China has its own unique characteristics which are not found in other parts of the world. Because of the huge population and high incidence rates of esophageal and stomach cancer[1-5], the number of cancer patients waiting for admission is inconceivably large. It is estimated that there are about 1.6-2.0 million new cases per year in China. In 1999, the Hebei Tumor Hospital had 6884 carcinoma patients hospitalized, among them there were 1035 esophageal cancer and 1057 stomach cancer patients. Because of the financial problem and most peasants have not joined the medical care insurance plan. Therefore, they are not benefited by the ideal treatment regimen after being diagnosed with TNM staging.

Hebei Tumor Hospital was established in 1958 to provide medical care to cancer patients in the central and southern parts of Hebei Province. It has 621 beds. The southern part of Hebei Province around Chixian is adjacent to Linxian of Henan Province, both Chixian and Linxian counties have the highest incidence rates of esophageal cancer which are 135/100 000. Besides, stomach carcinoma is another major cancer in this region.

With a view to standardize the therapy of esophagus and stomach cancer, the authors reviewed the national and international standard treatment and recent progress in this field, and the therapeutic modalities given in this hospital from 1981 to 1997, and hope this analysis may benefit the development of standardized therapy for the above two malignances in clinical management.

MATERIALS AND METHODS

A database was constructed for esophageal and stomach cancer patients who had been hospitalized in Hebei Tumor Hospital from 1981 to 1997 by the Foxprow database management techniques. Each hospitalized patient had his or her own record in the dataset. Percentages of therapeutic modalities as surgery, radiotherapy, chemotherapy, separate or in combination were calculated by the programs written in the Foxprow language. Finally, the results were analyzed according to *Diagnosis and Treatment Criteria for Common Malignances in China* by Ministry of Health of China and *Manual of Clinical Oncology* by UICC, and recent advances in these fields so as to find out the most rational suggestions for esophageal and gastric cancer.

RESULTS AND DISCUSSION

Esophageal carcinoma

As seen in Table 1, surgery and radiotherapy are two major options for esophageal cancer. Only 2.76% of the patients were treated by chemotherapy alone, and even fewer cases had been treated by multimodality treatment regimens such as surgery + radiotherapy, surgery + chemotherapy, chemoradiotherapy, and surgery + chemoradio-therapy. Radiotherapy as a preoperative option is eligible for esophageal cancer of various locations[6]. Preor postoperative radiotherapy in addition to surgery could reduce tumor recurrence and metastasis[7,8]. Either surgery or radiotherapy alone is not satisfactory because of the high rate of recurrence and metastasis[9,10]. The rationale for preoperative radiotherapy includes cytoreduction, improved resectability and reduced chance of marginal dissemination of tumor during the surgical procedure[11]. Therefore, preoperative radiotherapy is especially beneficial to patients with middle or late stage esophageal cancer[12].

Postoperative radiotherapy had been proved to be useful in reducing recurrence and metastasis[11]. Recent trials showed that although the 5-year survival rate of esophageal cancer was still poor, it had improved somewhat from 3% 30 years ago to 10%-15% today[13]. It was reported that multimodality approaches combining surgery, radiotherapy, and chemotherapy had resulted in 5-year survival rate of 40%-75% for advanced stage patients with a complete histological response to

Dr. Shi Jie Wang, graduated from Hebei Medical University in 1973, Professor of Cancer Surgery, President of Hebei Tumor Hospital (Fourth Affiliated Hospital of Hebei Medical University) and Hebei Cancer Institute, having 27 papers published.
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Correspondence to: Dr.Shi Jie Wang, Hebei Tumor Hospital, 5 Jiankanglu, Shijiazhuang 050011, Hebei Province, China
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preoperative chemotherapy[14]. These results suggest that further improvement in multimodality treatment regimens may allow a significant increase in survival period for esophageal carcinoma patients in the future [15].

Preoperative chemotherapy has been shown in nonrandomized trials to have similar side effects and mortality rate with surgery alone. Furthermore, phase two trials have suggested higher survival rates for combination of preoperative chemotherapy and surgery than that seen in surgery alone[16]. Another randomized study of 113 patients with esophageal carcinoma found that preoperative Cisplatin and 5-Fu given concurrently with 40 Gy of radiation significantly improved 3-year survival rate (32% vs 6%)[17]. Criticism of the study included poor preoperative staging and small sample size (113 patients). However, this trial offered hope that multimodality treatment regimens may ultimately prove beneficial in improving the overall survival period in esophageal cancer.

As seen in Table 1, although the cured and improved rate on discharge was highest in patients treated by surgery + radiotherapy, the percentage was only 3.58%, far less than those with surgery alone (48.93%) or radiotherapy alone (32.87%). The percentage of multimodality treatment regimens was only 4.32%, this was because in the past, we adopted the policy of elimination of symptoms rather than eradication of the disease. Therefore, in the future, we should focus our attention on the multimodality treatment regimens and to search for the most eligible combinations.

Table 1 Treatment modalities of esophageal and stomach cancer patients from 1981 to 1997 in Hebei Tumor Hospital

Treatment modality	Esopageal cancer			Stomach cancer		
	n	%	Cured & improved(%)	*n*	%	Cured & improved(%)
Surgery	4683	48.93	92.70	5328	67.48	59.51
Radiotherapy	3146	32.87	93.01	1758	22.26	56.66
Chemotherapy	264	2.76	91.67	117	1.48	84.67
Surgery+radiotherapy	343	3.58	95.04	260	3.29	93.83
Surgery+chemotherapy	41	0.43		114	1.44	88.60
Chemo-radiotherapy	24	0.25	87.50	8		87.50
Surgery+radio+chemotherapy	5			4		75.00
Other therapy	1064	11.12	53.20	307	3.89	83.71
Total	9570	100.00	88.46	7896	100.00	84.04

Gastric carcinoma

As seen in Table 1, surgery remains the first choice as curative treatment for patients with gastric cancer, especially at the early stages[18-20]. Early intraepithelial gastric cancer without nodal metastasis by surgery alone can yield a 5-year survival rate as high as 90%-95%. These early carcinomas may also be treated by endoscopic resection or endoscopic mucosectomy[21-24]. Gastric cancer survival is clearly stage dependent and is significantly affected by lymph node involvement[25]. The 5-year survival rate for patients with early gastric carcinoma after treatment approaches 90% but only less than 5% for cancer that involves the serosa[26]. The better survival statistics among Japanese are mainly due to greater frequency of early gastric cancer. Early detection of gastric carcinoma relies upon advanced development of X-ray and endoscopies[27,28], and also upon massive screening in high risk populations.

Although surgery has been proven to be the curative therapy for early gastric cancer, unfortunately, only 10% are early cancers[29]. In fact, locoregional recurrence is also common even after curative resection[30,31], therefore adjuvant chemo and radiotherapies are absolutely necessary[32-34].

Currently, preoperative chemotherapy and intraoperative radiotherapy are used as adjuvant therapies.

The operative morbidity of gastrectomy is high. It frequently precludes patients from entering into adjuvant therapy in a timely manner. Therefore, many studies are investigating the use of preoperative therapy. Wilke *et al*[35] described 34 patients who were demonstrated at laparotomy to have locally advanced unresectable cancer, these patients received two to five cycles of etoposide, doxorubicin, and cisplatin (EAP), 70% responders were offered a second laparotomy, and 15 were without clinical evidence of local extension. Kelsen *et al*[36] reported 56 patients who underwent preoperative therapy with FAMTX combined with postoperative intraperitoneal 5-FU and Cisplatin, among whom 34 patients received a potentially curative resection. For the entire group, the median survival period was 15 months; however, for those who underwent the above preoperation chemothe rapy and surgical resection, the median survival period was 31 months. Lowy *et al*[37] reported 24 patients who responded to similar- therapy, 84% had a 5-year disease-free survival, while 30% of those who did not respond had a 5-year disease-free survival. These studies suggest an appealing potential benefit of preoperative chemotherapy by inducing positive response in some patients[38].

The largest experience with intraoperative radiotherapy (IORT) came from Japan, where it had been studied for over 20 years. Abe *et al*[39] performed a prospective randomized trial of IORT in over 200 gastric cancer patients, and a more significant survival advantage was found for patients treated with IORT and surgery over those treated by surgery alone. This 5-year survival advantage was seen in patients with stage two (84% vs 62%) and stage three (62% vs 37%). Patients with stage one disease received no benefit from IORT. In addition, the only 5-year survivors in stage four were those who received IORT. This study suggests that IORT may be able to control locoregional recurrence after resection, which alone is often a major cause of treatment failure.

According to the criteria for gastric cancer

therapy modality planning of the Diagnosis and Treatment Criteria for Common Malignances in China for early gastric carcinomas with no nodal metastasis, no adjuvant therapy is necessary following curative resection depending on whether the disease is restricted to the mucosa or submucosa, but if there is nodal metastasis, adjuvant chemotherapy should be considered. For stage two and/or stage three progressive gastric cancer patients, preoperative radiotherapy is helpful to improve resectability. Postoperative chemotherapy should be considered for all progressive gastric cancer patients whether they have undergone curative or noncurative resection, and whether there is nodal metastasis or not. Intraoperative radiotherapy should be applied for stage two or stage three patients who are undergoing curative resection[40].

As seen in Table 1, the percent of surgery alone for treatment of gastric cancer is 67.48%, surgery plus chemotherapy and surgery plus radiotherapy account for only 1.44% and 3.29% respectively. Considering only 10% of gastric cancer patients diagnosed in hospitals are early cancer and may be treated by surgery alone, all other patients present with advanced disease should be treated by multimodality regimens such as preoperative chemotherapy, pre, intra or postoperative radiotherapy etc.

CONCLUSIONS

The advent of multimodality treatment is introduced in the last decade. Pre-operational chemotherapy can eliminate micro-metastasis and post-operational radiotherapy can kill the residual cancer cells, therefore in the future surgical resection with preoperational chemotherapy and postoperational radiotherapy should be considered to achieve better results and longer-survival periods.

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proofread by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effect of vitamin E succinate on expression of TGF-β_1, c-Jun and JNK1 in human gastric cancer SGC-7901 cells

Kun Wu, Bai He Liu, Dan Yang Zhao and Yan Zhao

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INTRODUCTION

Vita min E succinate (RRR-α-Tocopheryl Succinate, VES), a derivative of natural vita min E, is a compound esterified by succinic acid and 6-hydroxyl-α-tocopheryl. VES can increase the stability of á-tocopheryl and protect 6-hydroxyl from oxidation. Nowadays, this kind of vita min E has been on sale on the markets. Previous researches indicate that VES has an evident inhibitory effect on the growth of various tumor cells, but has no harm to the growth of normal cells[1-7]. The experiment *in vivo* has demonstrated that VES can cause the tumor to retrogress by regional injection[8,9]. Thus it is presumed that VES may be a potential tumor chemopreventive and chemotherapeutic agent.

Gastric cancer is one of the most common tumors in China. Up to now, most studies have focused on the preventive measures for tumors[10-13], but there has been few reports that VES inhibits the growth of gastric cancer. Our study showed that VES has an obviously inhibitory effect in the growth and DNA synthesis of human gastric cancer SGC-7901 cells[14]. The observation under electron microscope and the detection by flow cytometry and *in situ* end-labeling methods implicated that VES can induce gastric cancer cells to undergo apoptosis[15]. The exact mechanism of VES-induced growth inhibition in tumor- cells has still been unclear, but it has been reported that transforming growth factor beta (TGF-β) played a critical role in VES-induced apoptosis in tumor cells[16,17]. In addition, VES-triggered apoptosis was inhibited when c-Jun mutant was transfected into breast cancer cells[18]. The data above show that both TGF-β and c-Jun may be important factors for VES-induced apoptosis. In this study, the expression of TGF-β_1, c-Jun and c-Jun NH_2-ter minal kinase (JNK) was deter mined in SGC-7901 cells treated with VES so as to explore the mechanisms of VES-triggered apoptosis in gastric cancer cells.

MATERIALS AND METHODS

Materials

Human gastric cancer SGC-7901 cell line was obtained from the Beijing Cancer Institute and cultured in RPMI1640 medium plus 10% fetal bovine serum and routinely incubated with 5% carbon dioxide at 37℃. VES was purchased from the Sigma Corporation. Quantikine human TGF-β_1 ELISA kit was purchased from R&D Systems Company. Rabbit JNK1 polyclonal antibodies were kindly presented as a gift by Dr. Rui Hai Liu of Cornell University, USA. Rabbit c-Jun/AP_1 polyclonal antibodies were purchased from Beijing Zhongshan Biotechnical Company.

Drug treatment

VES was prepared at 5, 10 and 20mg/L by 100% ethanol. The concentration of ethanol in VES solution was adjusted to 0.1% (v/v) by 100% ethanol. Exponentially growing SGC-7901 cells were treated with VES. RPMI1640 complete condition media containing 1 μL/mL ethanol served as control.

Methods

ELISA When the cells were cultured for 12 h,18 h, 24 h and 48 h respectively, condition media was aspirated and centrifuged. The supernatant was prepared for the detection of activated TGF-β_1. Next, 500 μL culture supernatant was added into 0.1 mL 1N HCl and centrifuged for 10 min at room temperature. Then 0.1 mL 1.2N NaOH was used to neutralize the acidified samples for the detection of total TGF-β_1. Standard solution was prepared at different concentrations according to the instructions in the kit. Standard solution (200 μL per well) or activated samples were added into the 96-well plates bound with human recombinant TGF-

Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Kun Wu, a professor, engaged in the study on anti-carcinogenetic nutrients in food, having more than 40 papers published.
Project supported by National Natural Science Foundation of China, No. 39870662
Correspondence to: Prof. Kun Wu, Department of Nutrition and Food Hygiene, School of Public Health, Harbin Medical University, Harbin 150001, Heilongjiang Province, China
Tel. 0086-451-3648665
Email. wukun@public.hr.hl.cn
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βs RII. Then the steps proceeded as instructed. Finally, the value of optical density (OD) of each well was deter mined at 450 nm within 30 min, using a microplate reader set to 450 nm, and corrected at 540 nm.

Western blot SGC-7901 cells were harvested, decomposed on ice and centrifuged. Total protein contents in the supernatant were detected by nucleic acid and protein analyzer. Total protein (100 μg) was used for 12% SDS-PAGE electrophoresis and transferred onto nitrocellulose membrane, which was hybridized with rabbit c-Jun/AP-1 or JNK1 polyclonal antibodies (1:1000 dilution) and then with the secondary horseradish peroxidase-labeled antibody to detect the expression of c-Jun and JNK1.

RESULTS

Effects of VES on the expression of TGF-â₁ protein

Standard curve Standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration of TGF-β_1 on the x-axis. The linear regression equation was as follows:

$$Y = -0.037+8.559\times10^{-4}\, x \qquad r = 0.9991$$

Detection of total TGF-β_1 When SGC-7901 cells were collected following VES treatment for varying time periods, the OD value of TGF-β_1 was measured by microplate reader and converted into the concentration of total TGF-β_1 according to the regression equation above. Compared with the control, the expression of total TGF-β_1 in VES-treated cells was enhanced with a dose-time effect. After 48h treatment of VES at 5, 10, and 20 mg/L, the level of total TGF-β_1 was increased by 44.8%, 92.4% and 182. 3%, respectively (Figure 1).

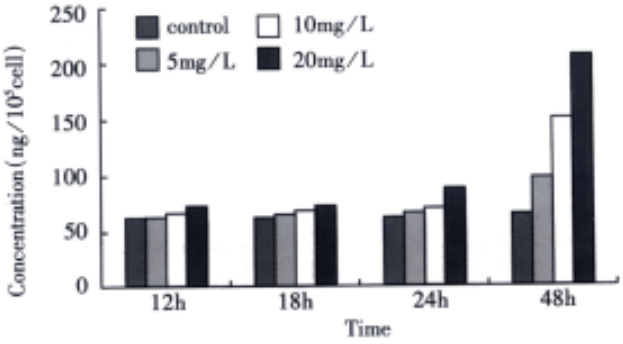

Figure 1 The effects of VES on total TGF-β_1 in SGC-7901 cells.

Detection of activated TGF-β_1 The OD value was directly measured in SGC-7901 cells harvested without acidification, and converted into the concentration of activated TGF-β_1 by means of the equation above. In comparison with the control, VES-treated cells exhibited increased level of activated TGF-β_1 in a dose-dependent manner. When the cells were treated with VES at 10 mg/L for 48 h and at 20 mg/L for 24 h and 48 h, the expression of activated TGF-β_1 was increased by 130.9%, 115.8% and 559.6%, respectively (Figure 2).

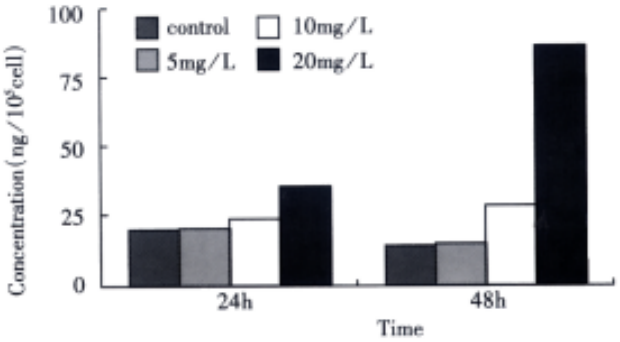

Figure 2 The effects of VES on the expression of active TGF-β_1 in SGC-7901 cells.

Effects of VES on c-Jun expression

The transferred filter membrane was hybridized with rabbit c-Jun/AP-1 polyclonal antibodies, then with the secondary antibody, colorated with DAB and imaged by digital imaging maker (Figures 3 and 4). From Figure 3, it can be seen that VES-treated SGC-7901 cells at 10 mg/L for 3 h exhibited evidently increased level of c-Jun protein. It was in a high state within 24 h and began to decrease after 24 h. Figure 4 indicates that the expression of c-Jun in VES-treated SGC-7901 cells at different concentrations at the same time was increased with a significant dose-effect relationship.

Effects of VES on JNK1 expression

The procedure was similar to that for c-Jun (Figures 5 and 6). Figure 5 shows that when SGC-7901 cells were treated with VES at 10 mg/L for 3 h, the expression of JNK1 was obviously promoted and further promotion occurred with the lapse of time within 24 h. Figure 6 implicates that the level of JNK1 expression was different in VES treated SGC-7901 cells at different concentrations at the same time. Compared with the control, the expression of JNK1 was evidently elevated in a dose-dependent manner.

Figure 3 The expression of c-Jun in VES treated SGC-7901 cells at 10 mg/L for different time.
A. Protein marker; B. VES treatment for 0 h; C. VES treatment for 3 h; D. VES treatment for 6 h; E. VES-treatment for 12 h; F. VES treatment for 24 h; G. VES- treatment for 48 h.

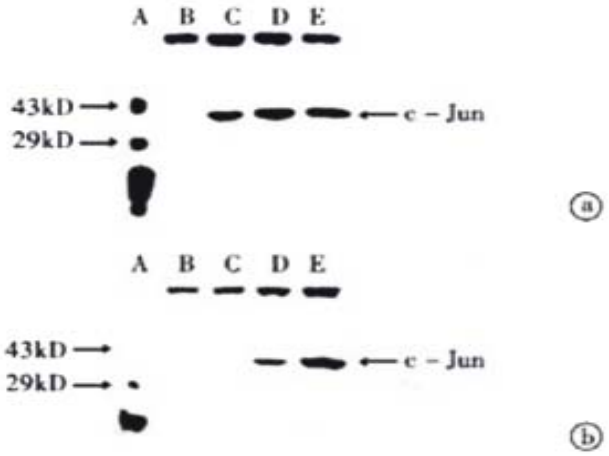

Figure 4 The expression of c-Jun in VES-treated SGC-7901 cells.
(**a**) VES-treated SGC-7901 cells for 24 h; (b) VES-treated SGC-7901 cells for 48 h.
A. Protein marker; B. Control; C. VES at 5 mg/L; D. VES at 10 mg/L; E. VES at 20 mg/L.

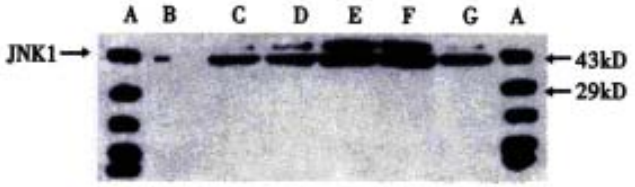

Figure 5 The expression of JNK1 in VES-treated SGC-7901 cells at 10 mg/L for different time points.
A. Protein marker; B. VES- treatment for 0 h; C. VES treatment for 3 h; D. VES treatment for 6 h; E. VES treatment for 12 h; F. VES treatment for 24 h; G. VES treatment for 48 h.

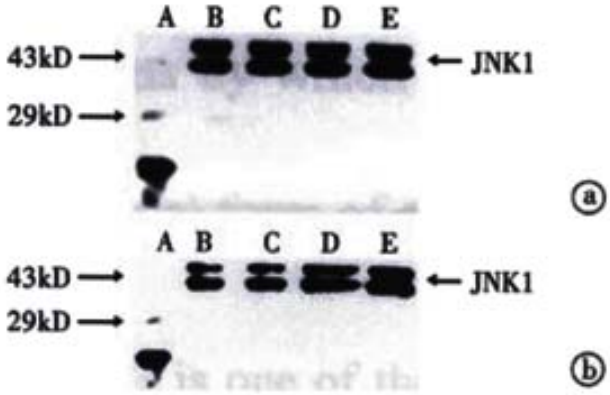

Figure 6 The expression of JNK1 in VES-treated SGC-7901 cells.
(**a**) VES-treated SGC-7901 cells for 24 h; (b) VES-treated SGC-7901 cells for 48 h.
A. Protein marker; B. Control; C. VES at 5 mg/L; D. VES at 10 mg/L; E. VES at 20 mg/L.

DISCUSSION

Previous researches showed that induction of apoptosis in tumor cells is one of the important mechanisms by which VES inhibits tumor cell growth[15,19]. Apoptosis is the most common physiological form of cell death and occurs-during embryonic development, tissue remodeling, immune regulation and tumor regression. Thus, initiation of apoptotic signal pathway and induction of apoptosis in tumor cells will be an effective approach for treating tumors[20-23]. Apoptosis is controlled by genes. Now hundreds of regulatory factors have been identified. These factors form many apoptotic signal transduction pathways which are complex[24-28]. In this study, several apoptosis-related proteins were deter mined to investigate the possible pathways involved in VES-induced apoptosis of human gastric cancer SGC-7901 cells.

Transfor ming growth factor (TGF) is a large family of multifunctional cytokines which can regulate cell proliferation, differentiation and death[29-31]. It has been proved that TGF-β, a latent inhibitor of epidermal proliferation, plays a role in the inhibition of more than three quarters of human primary tumors (such as breast cancer[32,33], prostatic carcinoma[34], lung cancer[35,36], colic carcinoma[37] and liver cancer[38,39]). Inhibition of the activation of latent TGF-β, down-regulation or loss of the expression of TGF-β receptors and deficiency of important elements responsible for cellular message transmitting pathway cause tumor cells to escape the growth inhibition of TGF-β, resulting in cancer [40-42]. Yu *et al*[43] found that VES can improve the secretion of activated TGF-β_1 and induce apoptosis in human breast cancer MDA-MB-435 cells. When TGF-β antibody neutralization assay was applied or antisense TGF-βRII oligonucleotide was transfected into breast cancer cells, the ability of VES-induced apoptosis was blocked by 50%, which implicated that the increase of TGF-β expression was related to induction of tumor cell-undergoing apoptosis. In our study, activated TGF-β_1 was directly measured with the kit in VES-treated SGC-7901 cells and its expression was distinctly increased; yet total TGF-β_1 was detected when the samples were acidified and its expression was also clearly promoted. These facts suggested that VES can induce not only the activation of active TGF-β_1 but also an increased secretion of latent TGF-β_1. Latent TGF-β_1 is released from cells into media and activated by some enzyme outside the cell membrane.

c-Jun is an early and transient gene[44] and its response to many kinds of stimuli is rapid and transient. The expression of c-Jun can be enhanced and prolonged by many stress stimuli such as ultraviolet, irradiation, hydrogen peroxide, tumor necrosis factor α and other apoptosis-triggering factors[45,46]. As mentioned above, VES-induced apoptosis rate was obviously decreased when human breast cancer cells were transfected with antisense c-Jun oligonucleotide or c-Jun mutant[18]. Our results showed that the expression of c-Jun was elevated and prolonged in VES-treated SGC-7901 cells (usually the expression of c-Jun only lasted 3 h-4 h or even shorter), indicating that c-Jun was also involved in apoptosis of VES-treated SGC-7901

cells.

It is reported that the mainly biological roles of c-Jun are the induction of cell cycle arrest and apoptosis [47-49]. JNK can directly bind with transcription factor c-Jun and activate c-Jun through phosphorylation of serine 63/73, suggesting that the activation of JNK is the upstream events involved in phosphorylation of c-Jun. It is also known that JNK belongs to mitogen-activated protein kinases (MAPK) family whose activation can be evidently affected by TGF-β, which shows that the activation of JNK can be possibly influenced by TGF-β[50-52]. These data suggest that JNK may be involved in VES-mediated apoptosis in tumor cells. In our study, the expression of JNK protein in VES-treated SGC-7901 cells was obviously increased with a dose-dependent relationship. Therefore, we suspect that JNK1 may be involved in apoptotic signal transduction similar to TGF-β_1 and c-Jun. When SGC-7901 cells were treated with VES, the secretion and activation of TGF-β were improved, TGF-β increased the activity of JNK, JNK caused the phosphorylation of c-Jun, and finally activated c-Jun triggered apoptosis in tumor cells.

Although we presumed a signal pathway responsible for VES-induced apoptosis in SGC-7901 cells, several interrelated pathways may trigger apoptosis simultaneously owing to the fact that the mechanisms of apoptosis are complicated and precisely regulated. So the observation that the expression of these three proteins is increased is only the preli minary survey on VES. With the development of molecular biological technology, if the strategies that a point in designed signal pathway is "knockout" by different means and then the blockage of downstream events is observed can be carried out, it will be critical for further studies on the mechanisms of VES-mediated growth inhibition in tumor cells.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Relationship between Fas/FasL expression and apoptosis of colon adenocarcinoma cell lines

Zhi Hai Peng, Tong Hai Xing, Guo Qiang Qiu and Hua Mei Tang

Subject headings colonic neoplasms/drug therapy; apoptosis; cisplatin/therapeutic use; mitomycins/therapeutic use; fluorouracil/therapeutic use; epirubicin/therapeutic use; flow cytometry

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INTRODUCTION

Fas/FasL system has been identified as a key mediator of apoptosis in tumor cells[1-4]. The occurrence and development of neoplasm are closely related to apoptosis [5-7]. Most chemotherapeutic drugs kill cancer cells mainly by inducing apoptosis[8-14]. We determined the Fas/FasL expression and apoptosis of colon adenocarcinoma cell lines induced by chemotherapeutic drugs with flow cytometry (FACScan), and studied the relation ship between Fas/FasL expression and apoptosis of colon adenocarcinoma cell lines for selection of sensitive chemotherapeutic drugs and appropriate dosage.

MATERIAL AND METHODS

Cell lines and cell culture

Colon adenocarcinoma cell lines (LS174-T cells and LoVo cells, provided by the Cell Bank of Chinese Academy of Sciences) were cultured in RPMI 1640 medium at 37℃ in a humidified 5% CO_2 atmosphere. Cells were seeded at a concentration of 1×10^6/well in 6-well plates and incubated at 37℃ in a humidified 5% CO_2 atmosphere overnight to ensure that the cells adhere to the walls, then chemotherapeutic drug of various concentrations was added and incubated again for 24 h or 48h.

Treatment with chemotherapeutic drugs

Different cell lines were treated with Cisplatin (CDDP), Mitomycin (MMC), 5-fluorouracil (5-FU) and epirubicin Hydrochloride (EPH) at concentrations of plasma peak concentration (PPC), 1/10 PPC, 1/5 PPC, 5 PPC and 10 PPC for 24 h and 48 h. The PPC of CDDP, MMC, 5-FU, EPH was 3 mg/L, 3 mg/L, 10 mg/L and 0.6 mg/L[5].

Apparatus and reagent

EPHCS-XL FACScan was purchased from Coulter Co. of America. Annexin-V-Fluos labeling reagent for identifying apoptosis was purchased from Boehringer Mannheim Co., and reagent for identifying Fas/FasL expression was purchased from Pharmingen Co..

Determination of apoptosis and Fas/FasL expression

Early apoptotic changes were identified using Annexin-V-Fluos, which binds to phosphati dylserine on the outer leaflet of apoptotic cell membranes. Propidium iodide was used for the discrimination of necrotic cells from the annexin V positively stained cell cluster. Cells were trypsinized, washed with PBS twice, centrifuged at 200×g for 5 min, and resuspended in 100mL Annexin-V-Fluos labeling solution containing Annexin-V-Fluos reagent and propidium iodide. Cells were incubated for 10-15 min and analyzed on FACScan. Percentage of apoptosis = 100% [experimental apoptosis (%)-spontaneous apoptosis in medium (%)]/[100%-spontaneous apoptosis in medium (%)][16]. For determination of Fas expression, cells were stained with anti-Fas IgG1 monoclonal antibody (mAb 1 g/mL) for 45 min at 4℃ followed by rat anti-mouse IgG1-FITC for 30min at 4℃. FII23 IgG3 antibody was used as isotype-matched antibody to control non-specific binding. For determination of FasL expression, cells were stained with FITC-conjugated rat anti-FasL mAb for 45 min at 4℃ followed by goat anti-mouse Igs-biotinylated. Rat IgG2a antibody was used as isotype-matched control antibody. Fas/FasL expression was assessed by FACScan. Fas expression = (%Fas + treated cells - %IgG1 - FITC + treated cells) - (%Fas + control cells - %IgG1 - FITC + control cells)[16]. FasL expression was calculated according to the formula as that of Fas expression. Cells were analyzed by FACScan using Cell Quest software.

DNA extration and gel electrophoresis

LS174T cells and LoVo cells of colon adenocarcinoma cell lines were added to 6-well culture plates at a concentration of 1×10^6/mL.

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Correspondence to: Dr. Zhi Hai Peng, Shanghai No.1 People's Hospital, 85 Wujin Road, Shanghai 200080, China
Tel. 0086-21-63240090 Ext.3102, Fax. 0086-21-63240825
Email. pengpzhb@online.sh.cn
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Cell cultures without treatment, or with treatment at different PPC of above chemotherapeutic drugs were harvested from the culture plates after 24 h or 48 h, washed twice with PBS (pH 7.4), and moved to the micro-centrifuging tube. Cells were pelleted at 300×*g* and the medium was removed by aspiration. Cell pellets were resuspended in 100 mL lysis buffer and incubated at 50℃ for 1 h, then mixed with 10 μL RNase and incubated at 50℃ for another 1 h. The DNA was resuspended in sample buffer and quantified by absorbance at 260 nm. Ten micrograms of DNA was applied to a 1.5% agarose gel in TBE buffer and resolved at 75 mA constant current for 2 h. The DNA was then visualized by ethidium bromide staining for 5 min and washed twice with distilled water. The result was observed under ultraviolet radiation (Figures 5 and 6).

Statistical analysis

Fas expression FasL expression and apoptosis percentage of each treated group were compared and checked with *F* test, and their relationship was analyzed.

RESULTS

The drug concentrations of CDDP, MMC, 5-Fu and EPH ranged between 1/10 PPC and 10 PPC[15], among which we set 1/5 PPC, PPC, and 5 PPC[17]. We treated LS174T cells and LoVo cells with chemotherapeutic drugs fo 24 h and 48 h respectively, and detected apoptosis and Fas/FasL expression with FACScan.

In untreated control group of LS174T cells, Fas expression, FasL expression and apoptosis percentage was 0.5%, 1.5% and 2.8% separately. The percentage in those treated with chemotherapeutic drug for 24 h was similar in that treated for 48 h, but figures were lower. LS174T cells treated with chemotherapeutic drugs for 48 h showed that Fas expression was upregulated when drug concentration was below 1/5 PPC or at PPC, and downregulated when drug concentration was above 1/5 PPC or at PPC (Figure 1). FasL expression was upregulated obviously when drug concentration was above PPC (Figure 2). When drug concentration of CDDP, MMC and EPH was below 1/5 PPC or PPC, apoptosis was upregulated, and downregulated at a concentration above 1/5 PPC or PPC. Apoptosis of 5-Fu group cells was upregulated with increase of drug concentration. Apoptosis in various drug concentrations in each group is shown in Figure 3.

We did not detect Fas expression of LoVo cells treated with chemotherapeutic drugs for 24 h. FasL expression was lower than that of LS174T cells. Apoptosis of 5-Fu group was upregulated with ascending drug concentration which was 54.8% when drug concentration was 10PPC. The effect of apoptosis induced by 5-Fu was the best while that induced by EPH was the worst. The results were just opposite to the results in LS174T cells. Apoptosis of CDDP group and MMC group were downregulated with ascending drug concentration (Figure 4). We could not detect Fas expression or FasL expression of LoVo cells treated with chemotherapeutic drugs for 48 h. Necrosis was found mainly in LoVo cells, apoptosis of which was lower than that of the control group.

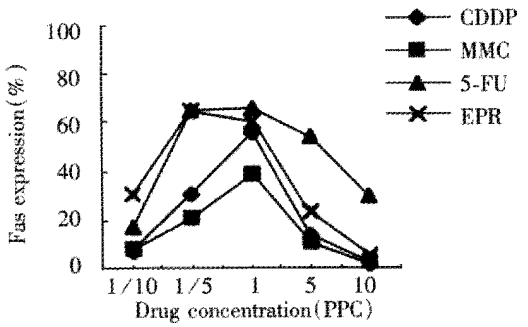

Figure 1 Fas expression of LS174T cells treated with chemotherapy drugs for 48 h.

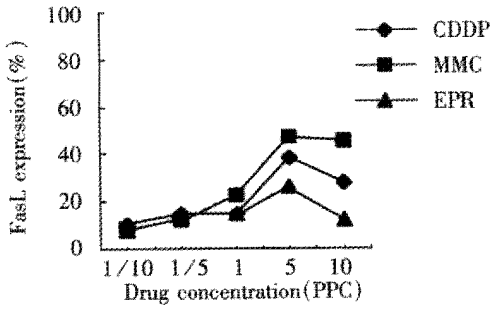

Figure 2 FasL expression of LS174T cells treated with chemotherapy drugs for 48 h.

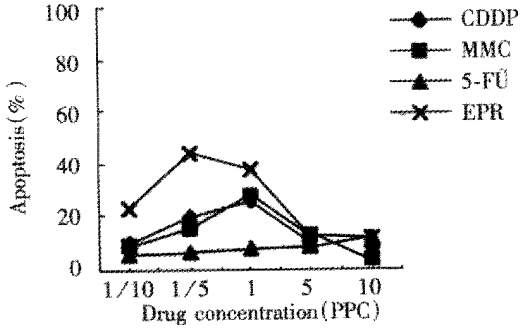

Figure 3 Apoptosis of LS174T cells treated with chemotherapy drugs for 48 h.

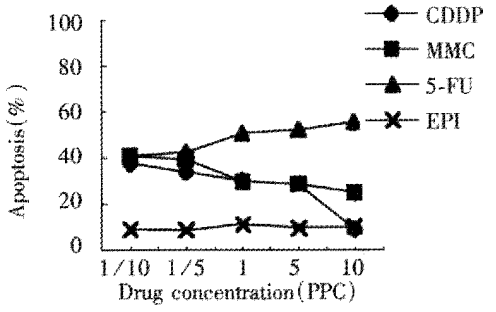

Figure 4 Apoptosis of LoVo cells treated with chemotherapy drugs for 24 h.

We abstracted DNA from the control group of cell lines and the group treated with chemotherapeutic drugs of various concentrations for 24 h and 48 h, and examined DNA ladder by agarose gel electrophoresis. The result showed that DNA ladder was remarkable in the treated group of LS174T cells when drug concentration of CDDP, MMC and EPH was 1/5 PPC or PPC, and 5-Fu concentration was 10 PPC. DNA ladder was remarkable in the treated LoVo cells when CDDP, and MMC concentration was 1/10 PPC, and 5-Fu concentration was 10PPC.There was no obvious difference among the DNA ladders at any concentration of EPH (Figures 5, 6).

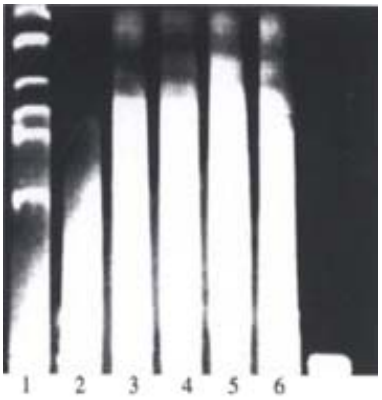

Figure 5 DNA ladder LS174T cells treated with chemotherapy drugs for 48h (1. Markers; 2. The control group; 3. 5-Fu; 4. DDP; 5. EPI; 6. MMC).

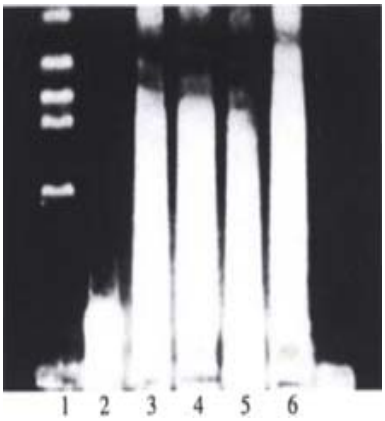

Figure 6 DNA ladder of LoVo cells treated with chemotherapy drugs for 24 h (1. Markers; 2. The control group; 3. 5-Fu; 4. DDP; 5. EPI; 6. MMC).

DISCUSSION

Fas is a 45 kDa type I transmembrane receptor expressed by a variety of normal and neoplastic cells. It belongs to the tumor necrosis factor (TNF) receptor family. When there is no antagonistic factor in the induction of apoptosis, apoptosis of chemosensitive tumor cells is induced following cross-linking of agonistic anti-Fas antibody or FasL with functional Fas expressed by tumor cells. Chemotherapeutic drug could induce tumor cells, which did not express or weakly expressed Fas, to strongly express functional Fas. Our data by FACScan showed that the Fas expression of LS174T cells untreated with chemotherapeutic drugs was only 0.5%, but compared with the Fas expression of the control cells, the Fas expression of LS174T cells treated with CDDP, MMC, 5-Fu and EPH for 48 h was notably upregulated. Fas expression of each treated group reached its peak at 1/5 PPC or PPC, which proved that the appropriate selection of chemotherapeutic drug concentration could effectively induce functional Fas to be strongly expressed on the surface of tumor cells.

In LS174T cells, apoptosis induced by CDDP, MMC and EPH was closely related to Fas-expression. Fas expression of the four LS174T cell groups treated with chemotherapeutic drugs for 48 h and apoptosis reached their peak at 1/5PPC or PPC. Fas expression was downregulated with chemotherapeutic ascending, drug concentration, so was apoptosis. The results of DNA fragmentation studies showed that the DNA ladder was most visible when the concentration of CDDP, MMC and EPH was 1/5 PPC or PPC. Fas expression and apoptosis of CDDP, MMC and EPH groups were positively correlated by relativity analysis ($P<0.05$). In LS174T cells, Fas expression induced by EPH was most notable and apoptosis of this group was the highest of all. There was statistical significance in Fas expression and apoptosis between EPH and CDDP or MMC groups ($P<0.05$). The above data and analysis suggest that kinetics of cell apoptosis correspond to kinetics of Fas induction. We could find the hypersensitive drugs and their appropriate dosage according to apoptosis and functional Fas expression. The different apoptosis paths between LS174T cells and LoVo cells demonstrated the importance of individualized selection of chemotherapeutic drugs.

After treatment with 5-Fu, Fas expression of LS174T cells was remarkablely upregulated while apoptosis was the lowest of all. On the contrary, the anti-tumor effect of 5-Fu on digestive tract tumor is the best among the clinical chemotherapeutic drugs. This contradiction, we believed, may be caused by the different effects between *in vitro* experiment and *in vivo* treatment. *in vitro*, FasL expression of LS174T cells induced by 5-Fu was too low to effectively activate the Fas system and trigger the apoptosis cascade; *in vivo*, FasL expressed strongly on the surface of CTL or NK could mediate autocrine or paracrine chemosensitive cell death by crossing its cognate receptor. This may be the reason why the 5-Fu had best anti-tumor effect in digestive tract tumor. Our study also showed that apoptosis induced by 5-Fu increased with the concentration, which was different from other drugs used in this research.

In LS174T cells, the FasL expression of CDDP,

MMC and EPH groups reached its peak at 5PPC, and there was no significant correlation between FasL expression and apoptosis of each group (P>0.05). FasL was upregulated on the tumor surface upon drug incubation in chemosensitive tumor cells and tumor cells could mediate autocrine or paracrine to release soluble FasL (sFasL). Apoptosis could occur in tumor cells expressing FasL through suicide or fratricide, inducing Fas-mediated apoptosis of peripheral normal cells, CTL and NK which express Fas, to promote local tumor invasion or metastases [18]. Therefore, drug-induced expression of FasL in Fas-negative tumors is not effective and, could result in selective elimination of antitumor lymphocytes. The incorrect chemotherapy could cause drug resistance or immune privilege of tumor cells[19]. Therefore, the induction of FasL by chemotherapeutic drug has dual effects on tumor therapy.

FACScan determination showed that LoVo cells treated with chemotherapeutic drug did not express Fas, weakly expressed FasL, and there was no significant correlation between Fas expression and apoptosis in LoVo cells (P>0.05). However, DNA ladder of LoVo cells treated with chemotherapeutic drug was similar to that of LS174T cells, suggesting that drug-induced apoptosis of LoVo cells did not depend on FasL/Fas interaction. Drug might kill tumor cells through activating other death receptors or directly acting on the downstream factor of Fas system (such as caspases family, etc.), also might trigger apoptosis in another way[20-28]. Nita *et al*[29] used equitoxic (IC50%) doses of 5-Fu to induce apoptosis in LoVo cells and analysed Bcl-2, Bcl-XL, Bax, Bad, Bak and p53 protein expression of LoVo cells by Western blotting, and found that Bcl-XL was expressed in all the cell lines and accompanied by increased expression of Bax and Bak. We are doing further studies to find out whether the ratio of Bcl-XL to Bax is correlated with chemosensitivity of LoVo cells treated with 5-Fu.

The determination of Fas expression, FasL expression and apoptosis of tumor cells treated with chemotherapeutic drug by FACScan indicated that the induction of Fas expression and apoptosis by major chemotherapeutic drugs was the best at the concentration of PPC or below PPC. DNA ladder also proved that the selection of appropriate dosage was of great importance in chemotherapy [30-41]. We could find hypersensitive drugs and their appropriate dosage according to apoptosis determined by FACScan, partly according to Fas expression. The different apoptosis paths between LS174T cells and LoVo cells demonstrated the importance of individualized selection of chemotherapeutic drugs.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Activity of boanmycin against colorectal cancer

Yong Chuan Deng[1], Yong Su Zhen[2], Shu Zheng[1] and Yu Chuan Xue[2]

Subject headings colorectal neoplasms; boanmycin; antineoplastic agents; neoplasm transplantation; mitomycin C; fluorouracil; mice, nude

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INTRODUCTION

Boanmycin (Bleomycin A6, BAM), a new antitumor antibiotic, was isolated from many components of bleomycin (BLM) produced by streptomyces pingyangensis which were obtained from a soil sample collected in Pingyang County, Zhejiang Province, China. Boanmycin has a similar chemical structure to that of BLM, but the terminal amine moiety is different [1]. Pingyangmycin (bleomycin A5), one of multicomponent bleomycin complex produced by the strain, was found to have a high activity against a wide spectrum of murine transplantable tumors, but have a relatively low pulmonary toxicity in mice[2-4]. Pingyangmycin, as a single agent, shows marked inhibition on the growth of human colon cancer, stomach cancer and nasopharyngeal cancer xenografts in nude mice[5-7]. It has been widely used clinically in the treatment of tumors since 1979 in China[8]. Because of a good antitumor efficacy of pingyangmycin as a single agent and low marrow toxicity, attempts have been made to develop new superior bleomycin derivatives[9]. BAM, a minor component of bleomycin complex, was also found to be highly active against murine tumors, human liver cancer and colorectal xenografts in nude mice, and markedly inhibit the spontaneous pulmonary metastasis of Lewis carcinoma in mice[10-14]. BAM-monoclonal antibody immunoconjugates were highly effective against related human tumor *in vivo* and *in vitro*[15-19]. BAM also reached a higher concentration and remained for a longer time in murine transplantable carcinomas as compared with other bleomycin components[3]. As observed under electron microscopy, the pulmonary toxic damage caused by BAM was less than that induced by bleomycin[20]. Phase I clinical study of BAM showed no myelosuppression and cardiac toxicity, and its major adverse reactions were fever, gastrointestinal reactions and hardening at the site of i.m. injection. All adverse effects disappeared after discontinuation of the therapy[21].

An ideal animal model for cancer is one that mimics human disease in every respect. Most tumor xenograft studies, including colorectal tumor, for the evaluation of antitumor activity of drugs used subcutaneous implantation system due to its convenience and access to direct detection and therapeutic effect. However, those models have limitations for the study of interaction of tumor cells with their relevant organ environment or organ distribution of drugs. Alteration of microenvironment surrounding tumor tissue will not only influence growth and spread of tumor but also is important for drug delivery[22-24]. Recently, use of orthotopic models for the growth of tumors in mice or rat has aroused more interest, including lung cancer, colorectal cancer, breast cancer, pancreatic cancer, etc[25-30]. However, whether such orthotopic colorectal tumor models apply to the evaluation of new anticancer agents remains unknown.

In the present study, human colorectal tumor xenograft model in nude mice and the orthotopic model of murine colon cancer were used to clarify the antitumor effect of BAM in comparison with that of mitomycin C and 5-fluorouracil, drugs commonly used in clinics against colorectal cancer. We attempted to determine the effect of BAM against colorectal cancer and whether the organ microenvironment could influence the response of a murine colon cancer to systemic therapy with BAM.

MATERIALS AND METHODS

Mice

Nude mice with a BALB/c genetic background were bred under specific pathogen-free conditions at the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (CAMS). All experiments were carried out in the Institute of Laboratory Animal Sciences under specific pathogen-free conditions using laminar airflow racks. Six to seven week old male or female nude

[1]Cancer Institute, Medical School, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
[2]Institute of Medicinal Biotechnology, CAMS & PUMC, Beijing 100050, China
Dr. Yong Chuan Deng, graduated from Zhejiang Medical University as a Ph.D. of Oncology in 1991, now associated professor of surgery, specialized in oncological surgery, having 20 papers published.
Supported by the National Natural Science Foundation of China, No. 93090012-03
Correspondence to: Yong Chuan Deng, 68 Jiefang Road, Cancer Institute, Medical School, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
Tel. 0086-571-7783572, Fax 0086-571-7214404
Email. dengyc@mail.hz.zj.cn
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mice weighing 18 g-22 g were used for the experiment. All food, water and bedding were sterilized.

Tumors

Two human cell lines of colorectal cancer, HT-29 and Hce-8693, transplanted into nude mice, were used. HT-29 was an adenocarcinoma of the colon established in the Memorial Sloan-Kettering Cancer Center, New York. Hce-8693 was a poorly differentiated adenocarcinoma of the cecum established in the Zhejiang Tumor Hospital, Hangzhou.

Colon tumor NO. 26 (CT-26), a murine colon adenocarcinoma, was induced in a BALB/c mouse by chemical carcinogens. Initially, CT-26 cells were serially transplanted and then established as a cell line and used in a large number of chemotherapy studies.

Drugs

Commercially available mitomycin C (MMC) and 5-fluorouracil (5-FU) were used for the experiment. BAM, a single component of bleomycin A6, was purified from bleomycin complex at Institute of Medicinal Biotechnology, CAMS & PUMC, Beijing. A 1/9 LD50 equitoxic dose for each drug, MMC 1.0 mg/kg per injection, 5-FU 27 mg/kg per injection and BAM 10 mg/kg per injection, was used for *in vivo* comparative studies[12].

Chemotherapy of human colorectal cancer xenografted in nude mice

Two human tumor fragments of about 2 mm^3 were inoculated subcutaneously into the right axillary region of nude mice. When diameter of tumor reached 3 mm-4 mm, tumor-bearing mice were randomized into test groups of 5-6 each and drug treatment was initiated. Test mouse was weighed and size of tumor was measured with sliding calipers two times weekly, and tumor weight (W) was calculated by the formula W (mg) = a(mm) × b^2(mm)/2 where a was the width and b is the length. The drugs were administered i.p., *b.i.w.*, a total of 10 injections. Next day after last treatment, mice were killed and the tumors were removed and weighed. The antitumor effect of drug was evaluated in terms of inhibitory ratio [1-(mean tumor weight of treated group - mean tumor weight of control group)] × 100%[12].

Chemotherapy of murine CT-26

Orthotopic tumor implantation Six-week-old (18 g-20 g) female BALB/c mice were anesthetized with diethylether, and the abdomen was prepared for sterile surgery. A small incision was made, the cecum was exteriorized and CT-26 cells (1×10^6/0.02 mL) were injected with a 30-G needle between the submucosa and the subserosa. The lack of extracecal leakage was the criterion for a successful injection. The cecum was returned to the abdominal cavity, and the wound was closed in one layer[7].

Subcutaneous tumor implantation CT-26 cells (1×10^6/0.02 mL) were injected into *s.c* in right axilla region. Mice were randomized into treatment and control groups based on the body weight next day after tumor cell injection. The drugs were administered *i.m.* (hindlimb), *q.o.d.*, for a total of 10 injections. Mice with *s.c* tumors and cecal tumors were killed on day 21 after tumor cell injection, and tumors were removed and weighed. The antitumor effect of drug was also evaluated in terms of inhibitory ratio [1-(mean tumor weight of treated group - mean tumor weight of control group)] × 100%[7].

Pathologic examination

Samples from untreated and drug-treated tumors were collected from subcutis or cecum and fixed in Bouin's solution, and embedded in paraffin. Sections were made, stained with H & E and observed under microscopy with a rectangular net-like micrometer. Whole area and necrosis area in tumor section was measured, and the necrosis ratio of tumors was calculated. Ten optical fields were examined along the peripheral area of the tumor and the mitotic figures were recorded[12].

Examination of nucleated cells in bone marrow

Femurs were removed and the bone marrow cavity was washed out with 3 mL white-blood-cell diluting solution. The nucleated cells per femur were counted under microscopy[12].

RESULTS

Response of colon cancer to BAM in nude mice

The results shown in Table 1 reveals the same response of the two types of human colon cancer to intraperitoneal BAM in both 10 and 15 mg/kg dose groups. Both doses exerted marked inhibition on the growth of HT-29 and Hce-8693 xenografts in nude mice.

Table 1 Inhibitory effect of BAM on the growth of human colorectal xenografts in nude mice*

Drug	Tumor	No. of mice	Dose (mg/kg)	Tumor weight (g) $\bar{x} \pm s$	Tumor growth inhibition (%)
BAM	HT-29	6		1.300 ± 0.620	
		6	10	0.138 ± 0.064	89[b]
		6	15	0.098 ± 0.074	92[b]
	Hce-8693	5		1.501 ± 1.445	
		5	10	0.154 ± 0.125	90[b]
		5	15	0.122 ± 0.154	92[b]

*Treatment was started on day 3 and day 14 after HT-29 and Hce-8693 tumor transplantation, *ip.*, twice a week, with a total of 10 injections. [b]$P<0.01$, *vs* control group.

Comparative response of colon cancer to BAM, 5-FU and MMC in nude mice

On the basis of equitoxic doses (1/9 LD50), BAM and two clinically active drugs (5-FU and MMC) were evaluated against human colon cancer HT-29 xenografts in nude mice. Table 2 shows the response of the tumor to two weekly treatments with three drugs. BAM is the most active single agent with an inhibition rate of 82% and exerted much stronger growth inhibition against HT-29 xenografts than 5-FU(P<0.01) and MMC(P<0.05). MMC had moderate activity against HT-29 xenografts with an inhibition rate of 53%. 5-FU did not arrest tumor growth throughout the experiment, and tumor increased in size at the same rate as the untreated control with an inhibition rate of 12%.

Comparative response of CT-26 growing at subcutis and cecum to BAM 5-FU and MMC

In the experiment, BALB/c mice were given injections of CT-26 cells into subcutis and cecum, which produced s.c tumor and cecum tumor. In view of the drug distribution, BAM, 5-FU and MMC were administered *i.m.* rather than *i.p.* at an equitoxic dose. In the treatment, the equitoxic dose was administered, and the antitumor activity of the three agents was compared. Table 3 shows the antitumor activity of BAM on the intracecal and *s.c* CT-26 tumor in comparison with that of 5-FU and MMC. BAM displayed a striking activity against intracecal CT-26 tumor; the inhibition of tumor growth was higher at the cecum than at the s.c site (P<0.05). The mean tumor weight after BAM treatment was less than that in the control, 5-FU and MMC groups, and the difference was statistically significant (P<0.01). On the other hand, 5-FU did not effectively suppress the tumor growth, and MMC showed mild activity.

Changes in tumor necrosis and mitosis

In contrast to the control HT-29 tumors that maintained their feature of poorly differentiated adenocarcinoma, the BAM-treated HT-29 xenografts in athymic mice presented extensive tumor necrosis and fibrosis. In areas of residual tumor, tumor cells were frequently found to have giant, bizarre-shaped pyknotic nucleoli, or prominent inclusion-type nucleoli. Xenografts treated with 5-FU and MMC showed scattered giant, bizarre-shaped nuclei and nucleolar prominence but these changes were focal and less apparent than in BAM-treated tumors. More extensive necrosis was found in tumors treated with BAM than in those treated with 5-FU or MMC. The necrotic ratio of tumors (whole necrotic area/whole tumor area) in BAM-treated group (67%) was much higher than that in 5-FU-treated (35%), MMC-treated (43%) and control groups (35%). In contrast to the tumor of the control group, the pathologic mitosis figures in BAM-treated group were reduced by 69%, which were fewer than those in 5-FU-treated, MMC-treated and control group (Table 3).

Table 2 Inhibitory effect of BAM, MMC and 5-Fu on the growth and tumor cell mitosis of human colon cancer HT-29 xenografts in nude mice*

Drug	Dose** (mg/kg)	No. of mice	Tumor weight (g) $\bar{x} \pm s$	Inhibitory rate (%)	Necrotic ratio of tumors△ (%)	Mitotic figures△△	
						$\bar{x} \pm s$	(%)
Control		5	0.804±0.173		35[b]	104±12	100
BAM	10	5	0.148±0.059	82	67	41±4	39[b]
MMC	1	5	0.376±0.174	53[a]	43[b]	107±12	103
5-FU	27	5	0.707±0.168	12[b]	35[b]	130±17	130

*Treatment was started on day 9 after tumor transplantation, *ip.*, twice a week, with a total of 10 injections;
**Drugs were administered at equitoxic doses (1/9 LD50); △Ratio of necrotic areas in whole section of the tumor.
△△Number of mitotic figures in 10 optical fields of the section of tumor.
[a]P<0.05, *vs* BAM group; [b]P<0.01, *vs* BAM group.

Table 3 Inhibitory effect of BAM, MMC and 5-FU on the growth of CT-26 tumor at the cecum and *s.c* site in mice*

Exp.	Drug	Dose** (mg/kg)	No. of mice	*s.c.* tumor		Cecal tumor	
				Tumor weight (g) $\bar{x} \pm s$	Inhibitory rate (%)	Tumor weight (g) $\bar{x} \pm s$	Inhibitory rate (%)
I	Control		9	0.854±0.151		0.557±0.112	
	BAM	10	9	0.101±0.054	88[b]	0.005±0.010	99[b]
	MMC	1	8	0.311±0.105	64	0.159±0.043	71
	5-FU	27	8	0.459±0.118	46	0.310±0.148	44
II	Control		6	0.900±0.396		0.740±0.446	
	BAM	10	6	0.105±0.088	88[b]	0.011±0.019	99[b]
	MMC	1	5	0.674±0.270	25	0.159±0.510	78
	5-FU	27	6	0.657±0.322	27	0.434±0.255	41

*Treatment was started on next day after tumor cell injection, *im.*, *qod.*, with a total of 10 injections.
**Drugs were administered at equitoxic doses (1/9 LD50).
[b]P<0.01, *vs* any other group.

Toxicity

No death or body weight loss of more than 20% was seen in the control or treated mice during the experiment. No inhibition on bone marrow cellularity was found in HT-29-bearing nude mice and CT-26-bearing mice at therapeutic doses of BAM. There was no significant difference in nucleated cell counts of marrow between the control and BAM group. In the CT-26-bearing mice, 5-FU and MMC caused significant decrease in bone marrow cellularity (Table 4). At therapeutic doses, no pathologic changes were found in the heart, lung, liver, spleen, kidney and brain of BAM-treated mice.

Table 4 Bone marrow nucleated cells in mice bearing CT-26 treated by BAM, 5-FU and MMC

Exp.	Drug	Dose** (mg/kg)	No. of mice	Nucleated cells (10^6/femur) $\bar{x} \pm s$	%
I	Control		6	13±3	100
	BAM	10	6	13±3	100
	MMC	1	5	7±2	54[b]
	5-FU	27	6	7±2	54[b]
II	Control	9		12±3	100
	BAM	10	9	12±2	100
	MMC	1	8	3±1	25[b]
	5-FU	27	8	4±1	33[b]

*Drugs were administered at equitoxic doses (1/9 LD50).
[b]$P<0.01$, *vs* control group.

DISCUSSION

Colorectal carcinomas are generally not very sensitive to the established chemotherapeutic agents; only 5-FU and MMC have shown some activity against colon carcinomas; the effects achieved, however, are of only a little value with respect to patient survival[31-41]. In the present study, we investigated the antitumor effectiveness of BAM, a new antitumor antibiotic, against human colorectal adenocarcinoma heterotransplanted to nude mice and murine colon adenocarcinoma. On the other hand, BAM, an analog of BLM that is clinically characterized by marked antineoplastic activity against carcinoma of the head and neck[42,43], showed a pronounced antitumor effect against the colorectal carcinoma used in the present study. At a 1/9 LD50 equitoxic dose, BAM exerted much stronger growth inhibition against human colon cancer HT-29 xenograft in nude mice than MMC and 5-FU. However, the tumor that remained after treatment consisted of viable cells with no degenerative changes, which were the source of early recurrence[44]. These results indicated that the effect of cancer chemotherapy should be judged not only by tumor reduction rate but also by histological changes, such as the necrotic ratio of tumors. In the present study, more extensive necrosis and fewer mitosis figures were found in tumors treated with BAM than those with MMC and 5-FU, indicating that BAM was more active against colon cancer HT-29 xenografts among three agents.

Most patients with colorectal carcinoma will die from distant metastases that are not detectable at the initiation of treatment[45,46]. Two major factors that influence the outcome of systemic therapy of cancer are heterogeneity of malignant neoplasms and *in vivo* conditions. Organ environment effects on the response of tumor to systemic chemotherapy are multifactorial, including the nutritional status of cells, the presence of organ-specific growth factors and other single-transducing agents, the degree of oxygenation, pH, extent of the vascular network and its functionality, local immunity, extracellular matrix components and drug metabolism[22-24]. The current model of orthotopic implantation of a colon carcinoma provides a unique opportunity to study a human malignancy in a context that is as close as possible to the clinical condition. Since intracecal tumors were much closer to clinical tumors than *s.c* tumors from the view of the histology of tumor growth or metastasis, this system was applicable to the evaluation of the tumor growth inhibitory effect by BAM. The present study demonstrated that murine adenocarcinoma CT-26 can successfully, using the orthotopic implantation technique, produce an aggressive tumor which retained the morphological biological characteristics of the donor tumor and metastasized to the mesenteric glands. BAM inhibited tumor growth on CT-26 implanted into the cecum and *s.c* more than 5-FU and MMC at the equitoxic dose. Moreover, the inhibitory effect of BAM on the growth of CT-26 tumor was higher at the cecum than at the *s.c* site in mice, which implicates that BAM may have the organ-specific effect. Organ-specific differences in the chemosensitivity of tumor cells have been reported by a few authors. Staroselsky et al have reported that a murine fibrosarcoma growing s.c in syngeneic mice is more sensitive to DXR than the same tumor growing as lung metastases[24]. Pratesi *et al* investigated the antitumor efficacy of flavone acetic acid against human ovarian carcinoma cells xenografted into different organ sites in nude mice, while tumors in the liver and subcutis were sensitive to the flavone, and ascites and lung tumors were resistant[22].

Since current clinical chemotherapy of colorectal cancer generally gives poor results, the finding of the present study is of interest with respect to the growth inhibiting activity of BAM against human colon carcinoma xenograft and murine colon carcinoma. However, further and extensive studies are necessary to confirm this finding and to evaluate the actual antineoplastic effectiveness of BAM against colon carcinomas.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Immunogenicity of HGV NS5 protein expressed from Sf9 insect cells

Hao Ren, Fen Lu Zhu, Shi Ying Zhu, Yan Bin Song and Zhong Tian Qi

Subject headings hepatitis agents, GB/immunology; recombinant proteins; electrophoresis, polyacrylamide gel; flaviviridae infections; Blotting, western; insect vectors; polymerase chain reaction

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INTRODUCTION

Although reliable assays for the detection of hepatitis C virus and E virus became available, still 10%-20% hepatitis are not caused by hepatitisA-E virus[1-3]. In 1996, two research groups isolated this agent independently and almost simultaneously and named hepatitis G virus and GB virus C, respectively[4-7]. The nucleotide and amino acid homologies between GBV-C and hepatitis G virus (HGV) were 85% and 95%[5-7]. Therefore, GBV-C and HGV were considered as two different isolates of the same virus, referred to as HGV in this paper. HGV is a single-strand, positive sense RNA virus with approximately 9.4kb in length, and classified as a member of *Flaviviridae*. HGV is mainly transmitted through transfusion and could be responsible for chronic liver infection. HGV RNA has been detected in the serum of intravenous drug users (IVDUs), volunteer and commercial blood donors, and patients with cryptogenic hepatitis[8-10]. Until now, RT-PCR is the most commonly used method for the diagnosis of HGV infection. It is necessary to develop a more convenient antibody detection assay. The baculovirus expression system is of a strong polyhedrin promoter[11], and can carry out many types of postranslation modification for a variety of proteins. Most of the expressed proteins were usually shown to be antigenic, immunological, and functionally similar to their authentic counterparts[12-16]. In this study, we used the baculovirus expression system to express HGV NS5 protein in Sf9 cells, and studied its immunogenecity.

Department of Microbiology, Second Military Medical University, Shanghai 200433, China
Dr. Hao Ren, graduated from Shandong Medical University in 1996, got master degree in 1998 from Second Military Medical University, and now is a Ph.D. student, majoring molecular virology.
Supported by the National Natural Science Foundation of China, No.39825116, 39970394.
Correspondence to: Prof. Zhong Tian Qi, Department of Microbiology, Second Military Medical University, Shanghai 200433, China
Tel. 0086-21-25070267, Fax. 0086-21-25070265
Email: qizt@smmu.edu.cn
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MATERIALS AND METHODS

Materials

HGV positive sera were collected from HGV RNA positive hemodialyzed patients. The plasmid pFastBac HTa, *E.coli* DH10 Bac cell, *Spodoptera frugiperda* (Sf9) cell and recombinant plasmid HGV Iwh6 were prepared previously in this laboratory[17]. The pPROEX HTa, Lipofectin and Grace's medium were purchased from GIBCO/BRL; and expand™ Long Template PCR System was purchased from Boehringer Mannherm Company. PCR primers were designed according to HGV-Iwh6 and synthesized by Sangon Biotechnology Company. Two restriction enzyme sites *Bam* H I and *Kpn* Iwere added to the 5' end of sense and antisense primers separately. The primer sequences are sense: 5'-GCG GAT CCC TAT CGG CTG CTG TAG CTA AG-3'; antisense: 5'-GCG GTA CCT TAT TGA GCG GCC CTC TTA GC-3'.

Amplification and sequence analysis of HGV NS5 fragment

HGV NS5 fragment was amplified using HGV-Iwh6 clone as the template (PCR condition: predenature 94℃ 2 min, followed by 94℃ 30 s, 60℃ 1 min, 68℃ 2 min, 35 cycles, and extension 10 min before the ending of the reaction). The amplified fragments and pPROEX HTa were digested with *Bam* H I and *Kpn* I. Fragment and vector were recovered respectively and ligated by T4 DNA ligase to obtain recombinant plasmid pHTNS5. Sequence analysis was carried out using ABI PRISM 377 DNA sequencer (PE Company) with M13/pUC primer.

Cloning into transposing vector pFastBac HTa

pHTNS5 and transposing vector pFastBac HTa were digested with *Bam* H I and *Kpn* I, and were ligated by T4 DNA ligase. The ligation mixture was transformed into DH5α competent cell, the positive colonies were chosen on selecting agar plate (ampicillin 100 μg/mL) and identified with endonuclease digestion to obtain the recombinant plasmid pFHTNS5.

Transposon between pFHTNS5 and bacmid

Plasmid pFHTNS5 was transformed into DH10Bac competent cells containing bacmid with a mini-att Tn7 site and helper plasmid. Following hot-shock at 42℃ for 45 s, the transformation mixture was placed in a shaking incubator at 37℃ for 4 h. Recombinant

bacmid was selected on selecting plate agar containing kanamycin 50 μg/mL, gentamicin 7 μg/mL, tetracyline 10 μg/mL, X-gal 200 μg/mL, and IPTG 40 μg/mL after 24 h-48 h incubation at 37℃.

Transfection of Sf9 cells

Recombinant bacmid was extracted according to the procedure of Bac-to-Bac system. For transfection, Sf9 insect cells were grown to 60%-70% confluence. The recombinant bacmid DNA 2 μg was transfected into insect cells Sf9 with Lipofectin. After 5 d-6 d incubation at 27℃ until the morphology of the cells had obvious changes, Sf9 cells and viral supernatant were harvested respectively.

Expression of recombinant protein in insect cells and SDS-PAGE, Western-blot analysis

Twenty μL viral supernatant harvested from the transfected cells was used to infect fresh insect cells. After 5 d-6 d incubation at 27℃, the cells were harvested for protein expression analysis. The cells were washed twice with PBS and analyzed by SDS-PAGE according to the standard procedure. Western-blot was performed using HGV RNA positive sera (1:40 dilution).

RESULTS

Amplification of HGV NS5 fragment and sequence analysis

PCR product was analyzed by agrose gel electrophoresis and the length was the same as expected (Figure 1). Sequence analysis showed that the HGV NS5 fragment was cloned into the vector with correct orientation (data not shown).

Construction of recombinant transposing plasmid pFHTNS5

Figure 2 shows the construction of recombinant transposing plasmid pFHTNS5. Figure 1 shows the analysis of recombinant plasmid on agarose gel by restriction endonuclease digestion which verified that target fragment was correctly cloned into the transposing vector. The results demonstrated a successful construction of recombinant transposing plasmid pFHTNS5.

Figure 1 Analysis of recombinant plasmid by restriction endonuclease digestion.
1. λDNA / *Eco* R I + *Hind* III; 2. PCR product; 3. pHTNS5 / *Bam* H I + Kpn I; 4. pFHTNS5 / *Bam* HI + *Kpn* I.

Screening of recombinant bacmid

After transforming competent cell DH10Bac with transposing plasmid pFHTNS, the recombinant bacmid was screened by colour selection. White clones (*lacZ*$^-$) were selected as positive recombinant bacmid in a background of blue colonies (*lacZ*$^+$). The recombinant bacmid was extracted according to the procedures described in the manual of Bac-to-Bac system.

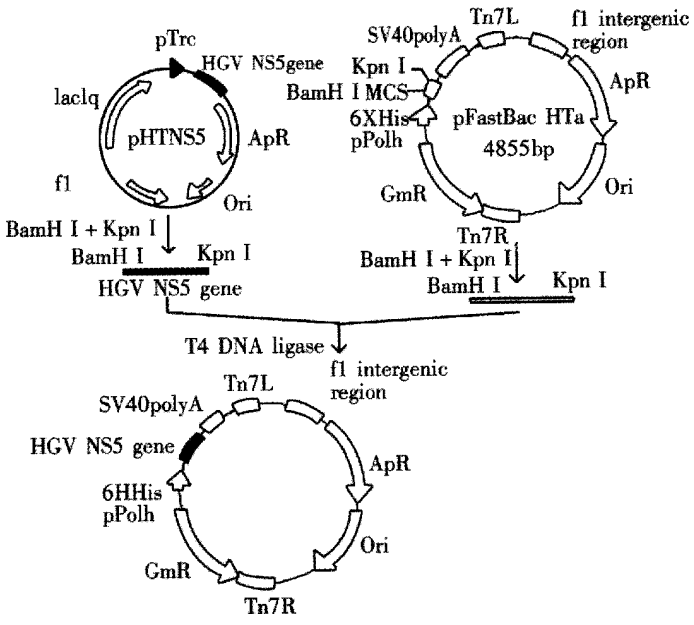

Figure 2 Construction of recombinant plasmid pFHTNS5.

Morphology of transfected or infected Sf9 cells

The morphology of Sf9 cells changed gradually after transfection or infection. The cells became big and round obviously at 4 d-5 d after transfection or infection. Cytopathic effects (CPE) were seen whereas no pathological effects were observed in normal cells (Figure 3).

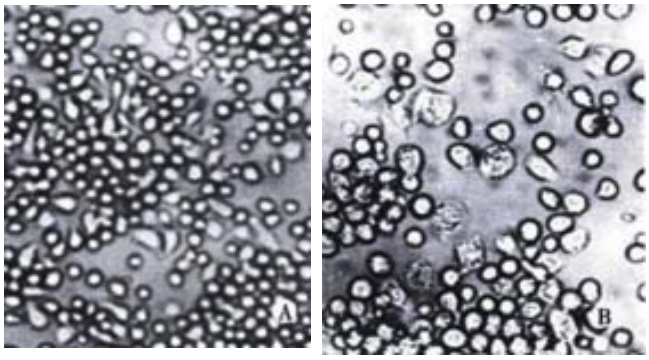

Figure 3 Morphology of uninfected, transfected and infected Sf9 cells (100×). A: Uninfected cells; B: Transfected and infected cells

SDS-PAGE and Western blot analysis of the recombinant protein

Transfected or infected Sf9 cells were harvested and analyzed on 12.5% polyacrylamide gels. Figure 4 shows the result of expressed target HGV NS5 protein with a molecular weight of M_r 41 500. Scanning results indicated that the recombinant protein amounted to 11.7% of the total proteins. Western blot results implied that the recombinant protein could react with HGV RNA positive sera (Figure 5).

Figure 4 SDS-PAGE analysis of expressed HGV NS5 protein. 1. Uninfected sf9 cells; 2. sf9 cells infected with recombinant viruses; 3. Protein relative molecular mass standards. Arrow indicates the position of recombinant protein.

Figure 5 Western-blot analysis of recombinant protein HGV NS5. 1. Uninfected sf9 cells; 2. sf9 cells infected with recombinant viruses; 3. Protein relative molecular mass standards.

DISCUSSION

Although easy and reliable assays for the clinical diagnosis of HBV and HCV infection have been established[18-26], there still existed 10%-20% parenterally and community acquired hepatitis cases of unknown cause[4,5,7]. Transmission and molecular biology of these viruses have been studied thoroughly [27-34]. Clinical studies suggest that some of these may be of viral origin. HGV is a potential aetiological agent for viral hepatitis. As a member of Flaviviridae, HGV is a single-stranded RNA virus with a genome of 9 400 bp in length which includes 5' non-coding region, structural gene region C, E1, E2, non-structural gene region NS2, NS3, NS4, NS5a, NS5b and 3' non-coding region. The genome contains a single open reading frame (ORF) which encodes a 2 900 amino acid polyprotein precursor. Many researches have been carried out since the discovery of HGV,the studies of its antigencity is one of them [17,35-39]. HGV NS5B protein functions as RNA-dependent RNA polymerase]. In addition, Pilot-Matias *et al*[40] also found that C26,C27,C28 (2047-2376 aa) of HGV NS5 gene had potential antigen epitopes. Wang *et al*[41] reported that two linear epitopes (P22, P6) might exist in HGV NS5 gene. The obtained HGV NS5 recombinant protein will provide important materials for studying its structure and function.

The Bac-to-Bac system was established by Luckow[11] in 1993, and a variety of proteins have been expressed with the control of a strong polyhedin promoter since then. It is based on site-specific transposition (transposon Tn7) of an

expression cassette into baculovirus shuttle vector (bacmid) propagated in *Escherichia coli*. After selection of blue-white clonies, recombinant bacmid DNA was extracted for the transfection of Sf9 cells. Insect cells can identify and run many modifications of post-translation and make the expressed protein close to natural protein.

In this study, HGV NS5 gene fragment amplified by PCR was confirmed by restriction enzyme and sequence analysis, and cloned into baculovirus transposing vector pFastBacHTa. Recombinant bacmid was obtained with site-specific transposition, Sf9 cell was transfected with recombinant bacmid or infected with viral supernatant. On the polyacrylamine gel, an expected protein band was seen at M_r 41 500. Western blot found that HGV NS5 recombinant protein could react strongly with HGV RNA positive sera, which implied that recombinant HGV NS5 protein could be used as antigen to detect HGV infection.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Humoral and cellular immunogenecity of DNA vaccine based on hepatitis B core gene in rhesus monkeys

Zu Hu Huang[1], Hui Zhuang[2], Shan Lu[3], Ren Hua Guo[1], Guo Min Xu[2], Jie Cai[1] and Wan Fu Zhu[2]

Subject headings hepatitis B virus; DNA vaccine; hepatitis B core antigens; nucleic acid vaccine; enzyme-linked immunosorbent assay; macaca mulatta

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INTRODUCTION

Hepatitis B virus (HBV) is the most common etiologic agent for infectious liver diseases. It is estimated that there are more than 250 million chronic HBV carriers in the world today and there is a significant association among persistent infection, liver cirrhosis and hepatocellular carcinoma[1-3]. The control of HBV infections is thought to be mediated by both humoral and cellular immune responses involving neutralizing antibodies as well as class I and class II major histocompatibility complex (MHC)-restricted T- cells[4,5]. Among the HBV antigens, a number of studies have highlighted the importance of the human immune response against the HBcAg and HBeAg during HBV infections. During acute HBV infection, cytotoxicity T lymphocyte (CTL) specific for HBcAg and HBeAg can be detected in the circulation of the infected host. In contrast, in chronic HBV infection, HBcAg and HBeAg-specific CTL and T-helper cell activity are not readily detected. The cumulated data suggest that CTL activity may play an important role in resolving HBV infection[6-11].

DNA mediated immunization has been shown to be an novel method to induce both humoral and cell-mediated immune responses against many different antigens including HBV antigens[12,13]. We have demonstrated that the DNA vaccine based on HBV core gene has strong humoral and cellular immunogenecity in different species of mice[14,15]. In our experiments, we have further investigated the immunogenecity of this DNA vaccine in rhesus monkeys. The results show that the DNA vaccine of HBV core gene can prime obvious antigen-specific antibody and cell mediated immune responses.

[1]Department of Infectious Diseases, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
[2]Faculty of Microbiology, Beijing University, Beijing 100000, China
[3]University of Massachusetts Medical Center
Dr. Zu Hu Huang, graduated from Nanjing Medical University in 1982 and earned his Master Degree of Medicine from NMU in 1988, trained in molecular biology and virology in Australia and United States, now Professor and Director of Department of Infectious Diseases, specialized in clinical molecular biology for diagnosis and treatment of viral hepatitis, having 35 papers published.
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Correspondence to: Zu Hu Huang, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, China
Tel. 0086-25-3737024, Fax. 0086-25-3724440
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MATERIALS AND METHODS

Preparation of DNA vaccine of HBV core gene

The control plasmid (pJW4303) and DNA vaccine of HBV core gene (pJW4303/HBc) were propagated by a large amount of culture of the transformed *E.coli* strain of HB101. Plasmid DNA was purified with QIAGEN Plasmid Mega Kit (QIAGEN, Germany).

Rhesus monkeys

Four rhesus monkeys (2 male, 2 female, 3 years of age) were purchased from Special Animal Breeding and Raising Center, Xingye, Henan Province, China and maintained at the animal house in Beijing Medical University. The monkeys were divided into experimental group and control group (2 monkeys in each group).

Protocols of DNA immunization

The monkeys in the experimental group were immunized with plasmid pJW4303/HBc and that in the control group were immunized with plasmid pJW4303. The plasmids were dissolved in normal saline to a final concentration of 1 g/L. Each time one monkey received 4-site intramuscular injections with a total volume of 2 mL plasmid solution containing 2 mg plasmid DNA. Three boosts with same dose were given at an interval of 2 months. The monkeys' sera before and after immunizations were collected and stored at -30℃.

Detection of anti-HBc antibody

Anti-HBc antibodies in monkeys' sera were first detected by Abbott Imx System (Abbott, USA) according to the manufacturer's instructions and end-point titers of anti-HBc antibody were then detected by an enzyme linked immunosorbent assay (ELISA). The procedures were as follows: ① The 96-well microplates were coated with recombinant HBcAg (1 mg/L) and blocked with PBS containing

10% FCS. ② Three-fold dilutions of monkeys' sera (1: 50, 1:150, 1:450 …… 1:984150) were added to triplicate wells. ③ HRP labeled rabbit anti-human IgG (Sino-American Biotechnology Co.) at the dilution of 1:3000 was used as second antibody. ④ The substrate solution (TMB) was then added to each well and reaction was stopped by 2M H_2SO_4. ⑤ The absorbence value was measured at wavelength of 450 nm by an ELISA reader. Microplate washings were performed between each step with PBST solution. The end-point of anti-HBc titer was defined as the highest serum dilution that resulted in an absorbence value two times that of non-immune or control serum.

Detection of IgG subclasses of anti-HBc

Subclasses of anti-HBc antibodies were detected in the sera of the monkeys positive for anti-HBc. The procedures were similar to the ELISA method mentioned above for detecting anti-HBc, except that serum was diluted to 1:30. 1:500 diluted sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Nordic Immunological Laboratories, Tilburg, the Netherlands) were used as the second antibody, and 1:5000 diluted HRP labeled rabbit anti-sheep IgG (Jackson Immuno-Research Laboratories Inc, PA, USA) was used as the third antibody.

Detection of IFN-γ and IL-4 in PBMC culture supernatant

The procedures were as follows: ① PBMCs were separated from heparinized monkey blood by Ficoll gradient sedimentation method. ② PBMCs were resuspended with RPMI-1640 containing 10% FCS to a final concentration of 2×10^6 cells/ mL. ③ PBMC suspension 250 μL (5×10^5 cells) was added to triplicate wells in a 24-well cell culture plate, and recombinant human IL-2 (500U/well) was added as well. ④ Except for control wells, PBMCs in each triplicate wells were restimulated with recombinant HBcAg at different doses of 5 μg/well, 10 μg/well and 12.5 μg/well. ⑤ After 48 h incubation under the condition of 37℃, 5% CO_2, the supernatant was collected from each well and stored at once at -70℃. ⑥ IFN-γ and IL-4 concentrations were detected by the ELISA kits (Jinmei Biotechnology Co., Shenzheng, China).

PBMC proliferation assay

The procedures were similar to that for detecting IFN-γ and IL-4 in PBMC culture supernatant, except that ① PBMCs were incubated for 72 h; ② 0.5 μCi ^{3}H-TdR was added to each well and followed by another 4h incubation; PBMCs were then collected onto filter membrane which were then backed 2 h at 80℃; and the radioactivity (CPM) was determined by a beta-scintillation counter (Beckman). The PBMC proliferation activity was expressed by Stimulation Index (SI), which was calculated according to the following formula: (SI = CPM of HBcAg stimulated well/CPM of non HBcAg stimulated well). SI value greater than 2 was generally considered as having antigen specific PBMC proliferation.

RESULTS

Anti-HBc IgG and its end-point titer in monkey's sera

The results of anti-HBc IgG and its end-point titer in monkey's sera are shown in Table 1.

Table 1 Anti-HBc in sera of experimental and control monkeys

Monkey No.	Group	Anti-HBc antibody					End-point
		0 month	2 month	4 month	6 month	8 month	
1	Experimental	N[a]	P[b]	N/D[c]	N/D	N/D	1:36450
2	Experimental	N	N	P	P	P	1:109350
3	Control	N	N	N	N	N	1:150
4	Control	N	N	N	N	N	1:150

[a] N: negative

[b] P: positive (in Abbott Imx System, the detected value less than 1. 00 was considered positive for anti-HBc).

[c] not detected because of death.

Subclasses of anti-HBc IgG in sera of experimental group of rhesus monkeys

Subclasses of anti-HBc IgG (IgG1, IgG2, IgG3 and IgG4) were detected in the experimental monkeys (No.1 and No.2), which were found to be positive for anti-HBc in the previous tests. The results are shown in Table 2.

Table 2 Subclasses of anti-HBc IgG and IgG1/IgG2 ratio in rhesus monkeys' sera

Monkey No.	IgG1	IgG2	IgG3	IgG4	IgG1/IgG2
1	0.61 + 0.01[a]	1.02 + 0.08	0.32 + 0.02	0.12 + 0.01	0.60
2	0.61 + 0.04	1.05 + 0.04	0.40 + 0.01	0.18 + 0.03	0.58

[a] The values indicated $\bar{x} \pm s$ of triplicate wells.

IFN-γ and IL-4 levels in culture supernatant of PBMCs stimulated with recombinant HBcAg

IFN-γ and IL-4 levels were detected in monkey No.2 (experimental group) and monkey No.3 (control group). Monkey No.1 and No.4 died before this test was performed. The results are shown in Table 3.

Table 3 IFN-γ and IL-4 values in culture supernatant of PBMCs

Monkey No.	Group	IFN-γ (ng/L)	IL-4 (ng/L)
2	Experimental	15.63	6.25
3	Control	<3.13	6.25

HBcAg specific PBMCs proliferation activity in experimental and control groups of rhesus monkeys

HBcAg specific PBMCs proliferation activities were measured in monkey No.2 and No.3 by the time of 12 months after first immunization. The results are listed in Table 4.

Table 4 HBcAg specific PBMC proliferation activity*

Monkey No.	Group	HBcAg dose for stimulation (μg/well)			
		0	5	10	12.5
2	Experimental	354.4 ± 64.5	984.9 ± 105.4[a] (2.74)	1364.9 ± 47.9[a] (3.83)	890 ± 155.6[a] (2.12)
3	Control	198.4 ± 3.9	274.5 ± 33.2 (1.37)	261.5 ± 28.2 (1.32)	250 ± 70.0 (1.24)

*The values in the table refer to CPM ($x \pm s$ from each triplicate well), the values in the brackets indicate stimulation index (SI). [a]$P<0.05$ *vs* control monkey.

DISCUSSION

DNA-mediated immunization refers to the induction of an immune response to antigen expressed *in vivo* subsequent to the introduction of DNA carrying the protein coding sequences and the regulatory elements needed to express them[16,17]. An important feature of DNA-based immunization is the *in situ* production of the expressed protein (s), mimicking a viral infection. The endogenous synthesis should allow presentation of antigens by class I molecules of MHC, resulting in the induction of CD8 + cytotoxic T lymphocytes (CTL)[18]. There have been several experimented reports in which recombinant plasmid DNA was used to induce immune responses to particular pathogens, including malaria[19], herpes simplex virus (HSV)[20], influenza A[21], rabies virus[22], simian immunodeficiency virus (SIV)[23], human immunodeficiency virus type I (HIV)[24] and hepatitis B virus (HBV)[25-32].

In our earlier work, the HBV core gene fragment, which was modified to assure the high level expression of HBcAg[33], was successfully cloned into the plasmid pJW4303, the vector containing CMV immediate early promotor. This recombinant plasmid was named pJW4303/HBc. The DNA immunization using pJW4303/HBc among Balb/c (H_2d) and C57BL/6 (H_2b) mice showed that this recombinant could induce strong humoral (antibody) and cellular (CTL) immune responses[34].

When evaluating the immunogenecity and safety of potential DNA vaccine for eventual use in humans, the nonhuman primate models should be considered. The best nonhuman primate candidate would be those closest to humans on a phylogenetic basis. However, cost and other considerations may preclude studies in hominoid species, such as chimpanzee, orang utans, gorillas, and gibbons. Based on the cost and availability, nonhominoid primate species including rhesus monkeys, represent the alternative candidate nonhuman primate species for pre-clinical immunogenecity studies[35,36].

Townsend *et al*[37] observed the specific immune responses in mouse and rhesus monkeys after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. Their results showed that intramuscular injections with 10^8 CFU of the the LHBc-Neo retrovirus vector into rhesus monkeys induced HBc/eAg-specific antibody production and CD8 + CTLs. The CTL response is long-lasting, and being detectable as late as 16 weeks after immunization.

We used the plasmid as the vector to carry HBV core gene for DNA immunization in rhesus monkeys, which was different from the observation above the reason for that is that the safety of the vector for retrovirus vector was integratable to the host genome.

In our experiments, all 4 monkeys were negative for anti-HBc before DNA immunization. After intramuscular immunization of pJW4303/HBc and pJW4303, the monkeys in the experimental group all developed anti-HBc antibody while the monkeys in the control group all negative for this antibody, indicating that this DNA vaccine could induce antigen specific humoral response in rhesus monkeys. We also found that the monkeys in the experimental group could show different antibody response profiles. Monkey No.1 became positive for anti-HBc (1:36450) after the first immunization while monkey No.2 was not negative for anti-HBc until the second immunization and the antibody titer became higher (1: 103 950) as late as the total four immunizations were accomplished. This different antibody production profiles might indicate the individual difference in response to DNA immunization.

In human and other hominoid primates, the serum IgG exhibited four subclasses, i.e., IgG1, IgG2, IgG3 and IgG4. The relative concentrations of these IgG subclasses were 60%-70% for IgG1, 15%-20% for IgG2, 5%-10% for IgG3, and 1%-7% for IgG4. When looked into the antigen specific IgG antibodies the concentration of IgG1 and IgG2 and its ratio IgG1/IgG2 could reflect the response profiles of helper T cells (T-H1 type or T-H2 type) to some extent. Generally speaking, IgG1/IgG2<1 or IgG1/IgG2>1 reflected T-H1 type or T-H2 type immune responses. The previous data showed that T-H1 type response was beneficial for the clearance or eradication of chronic infected viruses while the T-H2 type- response was usually correlated to the exacerbation of immunopathogenic damage of host tissues[38]. Feltquate *et al* had found that intramuscular immunization of DNA vaccines was prone to induce T-H1 type of immune response, thereby facilitating the recovery of the host from chronic viral infection[39]. Our results also

demonstrated that two monkeys intramuscularly immunized with HBV core DNA vaccine all exhibited T-H1 type of immune response based on the fact that their IgG1/IgG2 ratios were all less than 1 (0.60 and 0.58, respectively).

The profiles of cytokine production were another indicators of helper T cell responses[40-44]. IFN-γ, IL-2, TNF-α and GM-CSF were usually considered as T-H1 type cytokines, while IL-4, IL-5 and IL-10 were T-H2 type cytokines. IFN-γ and IL-4 were chosen in this experiment to observe helper T cell responses after DNA immunization of HBV core gene in rhesus monkeys. IFN-γ level was significantly higher in the culture supernatant of PBMC from the monkeys immunized with HBV core DNA vaccine than that from monkeys injected only with control plasmid (15.63 ng/L *vs* <3.13 ng/L). At the same time, IL-4 levels in both monkeys with injections of pJW4303/HBc or PJW4303 were similar (6.25 ng/L *vs* 6.25 ng/L). The results indicated IFN-γ prominent cytokine profile in the monkey immunized with HBV core DNA vaccine. This result combined with the result of IgG1/IgG2 ratios mentioned above further confirmed the T-H1 type immune responses in the monkeys of the experimental group.

Cell-mediated immune response is critical for the termination of chronic HBV infections[45-47]. Antigen specific lymphocyte proliferation assay is an alternative for the CTL assay to evaluate the cell-mediated immune response[48]. In this experiment, the HBcAg specific PBMC proliferation activity was seen in the monkey immunized with pJW4303/HBc but not in the monkey injected with pJW4303 ($P<0.05$). After stimulation with three different doses of HBcAg, the stimulation index (SI) was all >2 in the experimental monkeys but all <2 in the control monkeys, which strongly indicated that DNA vaccine of pJW4303/HBc could induce antigen-specific cell-mediated immune response in rhesus monkeys.

Sallberg *et al* reported that DNA immunization of HBV core gene using retrovirus as vector could markedly decrease the HBV DNA level in the sera of experimental chimpanzees, and even induce the seroconversion of HBeAg to anti-HBe[49]. Our results showed that using plasmid as vector the DNA vaccine could also stimulate the immune responses in nonhuman primate rhesus monkeys, which was obviously helpful and beneficial for the host to inhibit and eventually eradicate chronically infected virus, including hepatitis B virus. As the designer vaccines for the 21st century, DNA vaccines demonstrated its feasibility of inducing specific cellular immunity in humans[50]. We believed that DNA vaccine of HBV core gene may become a potential therapeutics for the treatment of chronic HBV infection in humans in the near future.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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A comparative study on serologic profiles of virus hepatitis B

Jin Nü Fang[1], Chang Ji Jin[1], Lian Hua Cui[1], Zhen Yu Quan[1], BoYoul Choi[2], Moran Ki[3] and Hung Bae Park[2]

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INTRODUCTION

Hepatitis B viral infection, one of the most-prevalent liver disorders in China and Korea, is a serious infectious disease as it has the potential of progressing into liver cirrhosis and primary hepatic carcinoma. China and Korea both belong to high-risk endemic regions of viral hepatitis[1]. The HBsAg positive rates in China ranged from 6.9%-17.9% by age, race and test methods[2-5]. In Korea, they were 6.5%-13.3% in all age groups[6-9], and 3.9%-5.9% in childhood groups[10-11]. There have been few comparative studies on Korean-Chinese and other Chinese. Considering the high mortality rates of liver cirrhosis or hepatoma among Korean-Chinese, HBsAg positive rate of Korean-Chinese must be higher than that of other Chinese. The positive rates of Korean-Chinese in Yanji and Longjing cities of Yanbian area by RPHA method were 7.5% and 7.1% respectively, which were higher than 6.2% and 4.7% in Han-Chinese respectively[12].

In regard to the possible reasons of such differences, some studies have laid special emphasis on social, economic and demographic variables such as age, sex, life style, and environment.

This study was conducted in order to assess the pattern of hepatitis B infection prevailing among Han-Chinese, Korean-Chinese, and Koreans. For collection of data, two serological surveys were carried out in 1996 in Korea and China respectively.

MATERIALS AND METHODS

Study areas

Study areas were Yangpyung County of Kyonggi Province in Korea and Helong County of Yanbian, a Korean Autonomous Prefecture in China. In Helong County, the proportion of residents by ethnicity were 55% for Korean-Chinese and 44% for Han-Chinese[13]. To compare the prevalence of hepatitis B between Korea and China, we carefully considered the characteristics of selected areas. In both areas, 70% of the residents were farmers. But the pattern of age distribution was different; the majority residing in Yangpyung County in Korea were more than 50 years old, while those in Helong County in China were over 40 years old.

Study subjects

Study subjects among ethnic groups were 556 Korean (male 41.7%, female 58.3%, $P<0.05$), 541 Korean-Chinese (male 51.6%, female 48.4%) and 261 Han-Chinese (male 39.5%, female 60.5%, $P<0.05$). These distributions by gender were statistically significant in Korean and Han-Chinese. Age distributions by ethnic groups were also significantly different; and the Koreans had older age and the Chinese had younger age. Age distributions by gender were not different between Korean and Korean-Chinese, but they were significantly different in Han-Chinese (Table 1). Therefore, this study showed the results with age-adjusted rates by gender.

Table 1 Characteristics of subject by ethnic groups[d]

Ethnic groups	Age (years)	Gender		Total
		Male	Female	
Korean-Chinese[b,c]	20-39	103	117	220
	40-49	89	77	166
	50-	87	68	155
Total		279	262	541
Han-Chinese[a,b,c]	20-39	42	103	145
	40-49	30	44	74
	50-	26	16	42
Total		98	163	261
Koreans[a,b]	20-39	43	70	113
	40-49	37	60	97
	50-	152	194	346
Total		232	324	556

[a]$P<0.05$ between sex, by χ^2 test; [b]$P<0.05$ among age groups, by χ^2 test; [c]$P<0.05$ among age groups by sex, by χ^2 test;
[d]Koreans-Chinese: Koreans in Yanbian in China, Han-Chinese: Chinese in Yanbian in China, Koreans: Koreans in Yangpyung in Korea.

[1]Department of Epidemiology, Yanbian University College of Medicine, Yanji 133000, Jilin Province, China
[2]Department of Preventive Medicine, Hanyang University College of Medicine, Seoul 133791, Korea
[3]Department of Preventive Medicine, Eulji University School of Medicine, Taejon 301110, Korea
Dr. Jin Nü Fang, graduated from Norman Bethune University of Medical Sciences in 1985, Hanyang University College of Medicine with a doctoral degree in 1999, now associate professor of epidemiology, specialized in infectious disease epidemiology and cardiovascular epidemiology, having 10 papers published.
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Correspondence to: Dr. Jin Nü Fang, Department of Epidemiology, Yanbian University College of Medicine, Yanji 133000, Jilin Province, China
Tel. 0086-433-2660621
Email. epidem@public.yj.jl.cn
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Questionnaires

The questionnaire survey gave direct interviews, including the relative factors on hepatitis B infection such as demographic characteristics, life style, vaccination and disease history.

Serologic tests

Serological markers on hepatitis B virus (HBsAg, anti-HBs and anti-HBc) were tested by EIA (enzyme immunoassays). Sera were stored in a deep freezer, at -30℃ until this test. The serologic tests were all done in Korea.

Statistical analysis

For comparison by ethnic groups, age-adjusted rates by direct method were used. The statistical significance was determined using χ^2 test or Mantel-Haenszel's χ^2-test on SPSS or EPISTAT.

RESULTS

There were no vaccinees on hepatitis B among Chinese, but the vaccination rate among Koreans was 32.4% (36.5% for males and 29.0% for females). The vaccination rate was 44.2% for the age group of 20-39 years, 45.4% for the age group of 40-49 years, and 24. 4% among those aged 50 and over (Table 2). In order to compare hepatitis B virus markers among ethnic groups, the vaccinees were excluded.

HBsAg positive rate of males was higher than that of females in the three ethnic groups. Age-adjusted HBsAg positive rates were 7.2%, 12.0% and 4.1% in Han-Chinese, Korean-Chinese, and Korean respectively (P<0.05). This order was same in both genders. But, the difference was statistically significant only in males (P<0.05). Anti-HBs positive rates of males were higher than those of females in Korean-Chinese and Korean, but the differences were not statistically significant. There was also no difference by gender in the Han-Chinese. The age-adjusted anti-HBs positive rate of Korean (62.5%) was the highest, of Korean-Chinese was 57.6%, and of Han-Chinese 49.2%. This order was true in males and females, but the differences among the three ethnic groups were not significant. Anti-HBc positive rates of males were higher than those of females in all three groups, but these differences were not significant. Age-adjusted anti-HBc positive rate was 69.7%, the highest in Korean-Chinese, 60.9% in Korean and 54.0% in Han-Chinese. This order was also found by gender, and these differences were all statistically significant (P<0.05).

The definition of hepatitis B infection was determined as cases that have any hepatitis B virus markers among HBsAg, anti-HBs and anti-HBc. The infection rate was higher in males than in females among the three ethnic groups. But the difference was significant only in Koreans (P<0.05). Age-adjusted infection rates were 78.6% in Korean, 77.0% in Korean-Chinese and 60.7% in Han-Chinese. These differences among the three ethnic groups were significant (P<0.05) in males, females and the total.

In Koreans, the HBsAg positive rate was lower than that of Korean-Chinese, but the HBV infection rate was not different from Korean-Chinese, and was higher than that of the Han-Chinese. In Korean-Chinese, the HBsAg positive rate and HBV infection rate were higher than those of the Han-Chinese. In the Han-Chinese, the HBV infection rate was the lowest and the percentage of those who are susceptible was the highest (Table 3).

We classified the serologic profiles into 8 types by 3 HBV markers, which are HBsAg, anti-HBc and anti-HBs. Mushahwar *et al* (1981)[21] used 15 classifications by 5 HBV markers including HBeAg and anti-HBe, to determine the HBV infectivity. We used 8 types for the description of HBV serologic profiles in the cross-sectional study. Type I of our classification means those susceptible who have all three negative markers. These percentages of those susceptibles were higher in females than in males in all three ethnic groups. The percentage was 18.6% for Koreans or Korean-Chinese, and 36.7% for Han-Chinese. In females, the percentage was 40.5% for Han-Chinese, 29.6% for Korean, and 23.7% for Korean-Chinese. Among HBsAg positive serologic profiles, type VII was dominant. But, varied types such as V, VI and VIII were found only in Korean-Chinese and Han-Chinese excluding Koreans. Koreans had only one type VII, among HBsAg positive profiles. The percentage of HBsAg negative combination (HBsAg- and anti-HBs+ and/or anti-HBc+), were 78.5% and 65.5% for Korean, 67.5% and 67.6% for Korean-Chinese and 53.1% and 52.1% for Han-Chinese. Among these profiles, type IV was dominant in all three ethnic groups (Table 4).

Table 2 Rate of vaccination by sex and age in Koreans[b]

Age (yrs)	Male[a]			Female[a]			Total		
	No. of respondents	No. of vaccinces	%	No. of respondents	No. of vaccinces	%	No. of respondents	No. of vaccinces	%
20-39	43	19	44.2	70	31	44.3	113	50	44.2
40-49	37	23	62.2	60	21	35.0	97	44	45.4
50-	152	44	28.9	194	42	21.6	346	86	24.9
Total	232	86	37.1	324	94	29.0	556	180	32.4

[a]P<0.05 among age groups, by χ^2 test; bKoreans: Koreans in Yangpung in Korea.

Table 3 Positive rates and infection rate of Hepatitis B virus by sex and ethnic groups[e]

	Male			Female			Total		
	No. of tested	Crude rate(%)	Age-adjusted rate[d](%)	No. of tested	Crude rate(%)	Age-adjusted rate[d](%)	No. of tested	Crude rate(%)	A ge-adjusted rate[d](%)
HBsAg positive rate[a,c]									
Korean-Chinese	279	14.0	14.8	262	8.8	8.8	541	11.5	12.0
Han-Chinese	98	10.2	9.4	163	7.4	6.0	261	8.4	7.2
Korean	146	2.7	6.1	230	4.8	3.0	376	4.0	4.1
Anti-HBc positive rate[a,b,c]									
Korean-Chinese	279	74.6	73.2	262	68.3	66.0	541	71.5	69.7
Han-Chinese	98	58.2	59.1	163	50.9	50.4	261	53.6	54.0
Korean	140	69.3	61.3	226	58.0	60.0	366	62.3	60.9
Anti-HBc positive rate									
Korean-Chinese	279	62.4	61.1	262	56.5	54.0	541	59.5	57.6
Han-Chinese	98	46.9	48.4	163	49.1	51.7	261	48.3	49.2
Korean	146	62.3	69.1	230	55.7	58.6	376	58.2	62.5
HBV infection rate[a,b,c]									
Korean	140	81.4	85.3	226	70.4	74.0	366	74.6	78.6
Korean-Chinese	279	81.4	79.7	262	76.3	74.1	541	78.9	77.0
Han-Chinese	98	63.3	63.4	163	59.5	59.8	261	60.9	60.7

[a]$P<0.05$ among 3 ethnic groups in male, by χ^2 test; [b]$P<0.05$ among 3 ethnic groups in female, by χ^2 test; [c]$P<0.05$ among 3 ethnic groups in total, by χ^2 test; [d]Age-standardized rates (standard population; Helong in 1997 and Yangpyung in 1995); [e]Korean-Chinese: Koreans in Yanbian in China, Han-Chinese: Chinese in Yanbian in China, Koreans: Koreans in Yangpyung in Korea.

Table 4 Serological profiles of hepatitis B virus markers by sex and ethnic groups[a]

Gender ethnic groups	Serological profiles* (%)								Total
	I	II	III	IV	V	VI	VII	VIII	
HBsAg	-	-	-	-	+	+	+	+	
Anti-HBc	-	-	+	+	-	-	+	+	
Anti-HBs	-	+	-	+	-	+	-	+	
Male Korean-Chinese	52(18.6)	16(5.8)	27(9.7)	145(52.0)	1(0.4)	2(0.7)	25(9.0)	11(3.9)	279(100.0)
Han-Chinese	36(36.7)	3(3.1)	8(8.2)	41(41.8)	2(2.0)	0(0.0)	6(6.1)	2(2.0)	98(100.0)
Koreans	26(18.6)	17(12.1)	22(15.7)	71(50.7)			4(2.9)		140(100.0)
Female Korean-Chinese	62(23.7)	17(6.5)	35(13.4)	125(47.7)	3(1.1)	1(0.4)	14(5.3)	5(1.9)	262(100.0)
Han-Chinese	66(40.5)	9(5.5)	8(4.9)	68(41.7)	4(2.5)	1(0.6)	5(3.1)	2(1.2)	163(100.0)
Koreans	67(29.6)	28(12.4)	21(9.3)	99(43.8)			11(4.9)		226(100.0)

[a]Korean-Chinese: Koreans in Yanbian in China, Han-Chinese: Chinese in Yanbian in China, Koreans: Koreans in Yangpung in Korea.

DISCUSSION

Since 1980, China has produced hepatitis B vaccines and by regulations, children must be vaccinated. However, vaccination against HBV was not mandatory in adults. Therefore, none of study subjects in China were vaccinated, while in Korea, 32.4% were vaccinated. It implies that the circumstances of HBV infection and transmission were different between China and Korea. Age-adjusted HBsAg positive rate of Korean-Chinese was 12.0%, higher than the 10% previously reported in China as a whole[14]. Moreover, the rate was higher than the 8.0% for the Korean-Chinese in Yanbian area during the 1980s[12]. The rate for Han-Chinese (7.2%) was less than the national level (10%) in China, and the same or less than that of other reports[2-5]. However, no other reports were found from Yanbian area, the differences did not reflect the chronological change. In Koreans, the rate for non-vaccinees was 4.1%, which was less than other reports (6.5%-13.3%)[6-10,15,16].

Korean and Korean-Chinese are the same race, but HBsAg positive rates were different and increased with time for Korean-Chinese and decreased with time for Koreans. The difference between Korean and Korean-Chinese seems to be caused mostly by vaccination. Other factors such as socioeconomic status, sanitary status and medical support appear to influence HBV infection and transmission[6,7,11,17]. The difference between Korean-Chinese and Han-Chinese resulted from cultural difference such as life style, food habits and susceptibility[6,7,11,17]. The rate for Korean-Chinese was more similar to the Han-Chinese than to Koreans, which suggests that environmental factors are more important than genetic factors on HBV.

Positive anti-HBc is difficult to determine definitely. Type IV (anti-HBs+, anti-HBc+, and HBsAg-) and VIII (anti-HBs+ anti-HBc+, and HBsAg+) are in recovery phases caused by the positive anti-HBs. But type III (anti-HBs-, anti-HBc+, and HBsAg-) and VII (anti-HBs- anti-HBc+, and HBsAg+) mean acute or chronic infection. The order of high anti-HBc positive rates among the three ethnic groups was Korean-Chinese, Korean and Han-Chinese. This order was too

difficult to interpret like the anti-HBc.

Positive anti-HBs means having immunity against HBV. Age-adjusted anti-HBs positive rate for Chinese (57.6% for Korean-Chinese and 49.2% for Han-Chinese) was higher than that of other reports[2-5], however, in Korean (the 62.5%) it was higher[8,18] or lower than that of other reports[7,19]. Even though the difference among the three ethnic groups was not statistically significant, the reason why the anti-HBs positive rate for Korean (62.5%) was the highest, can be explained by the different serological profiles. Among the anti-HBs positive Koreans, 20.9% was type II (anti-HBs+, anti-HBc-, and HBsAg-), which indicates remote past infection, but, 10.3% and 9.5% among Korean-Chinese and Han-Chinese. Other types like IV (anti-HBs+, anti-HBc+, and HBsAg-), VI (anti-HBs+, anti-HBc-, and HBsAg+) and VIII (anti-HBs+, anti-HBc+, and HBsAg+) indicate the recovery phase of acute infection as a whole (IV, recovery phase of HBV infection; VI, unknown; and VIII, circulating immune complex of HBsAg or reinfection with different HBsAg subtype or process of seroconversion from HBsAg to anti-HBs). Therefore, positive anti-HBs Koreans had more remote infections than Korean-Chinese and Han-Chinese, which could be also applied to the exploration of HBV infection rates. HBV infection was determined by having had any one of the positive HBV markers among HBsAg, anti-HBs and anti-HBc. The order of high HBV infection rates among the three ethnic groups was the same as anti-HBs, Korean, Korean-Chinese and Han-Chinese. The difference was statistically significant ($P<0.05$).

HBV infection rates in Korean-Chinese were 81.4% in males and 76.3% in females, which were 80.8% in males of Hunan area and 75.5% in female of Guangxi of China[5]. The rates for Koreans were 81.4% in males and 70.4% in females. Therefore, even if the HBsAg rates have been decreasing as compared with that of the 1980s, HBV infection rate did not drop. According to Maynard *et al*[20]. 70%-90% of the population were infected with HBV in the highly endemic areas. Hence, Korea and Yanbian were included in the endemic area.

The fact that HBV infection rates for Korean-Chinese and Koreans were higher than those of the Han-Chinese seems to be caused by susceptibility and cultural factors such as life style and dining habits. Ahn *et al*[19] reported the association between HBV infection and behavioral characteristics such as life style, dining habit and sanitary status. Therefore, to determine the reason for the higher rate of HBV in Koreans and Korean-Chinese, more studies dealing with genetic factors and behavioral factors are needed.

In regard to positive HBsAg rate, the results showed difference by ethnic groups in the same area. Consequently, for each of the areas and the ethnic groups, the HBV infection and transmission must be differentiated[21]. For the clarification of the natural course on HBV, more detailed immigration studies and follow-up efforts should also be made.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Establishment of transgenic mouse harboring hepatitis B virus (adr subtype) genomes

Yi Ping Hu[1], Wei Jiang Hu[1], Wen Chao Zheng[2], Jian Xiu Li[1], De Shun Dai[1], Xin Min Wang[1], Shu Zhong Zhang[1], Hong Yu Yu[3], Wei Sun[4] and Guang Rong Hao[4]

Subject headings hepatitis B virus; mice transgenic; genome; polymerase chain reaction; immunohistochemistry; microscopy, electron; danes particles

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INTRODUCTION

Hepatitis B virus (HBV) belongs to the group of hepatovirus, a major pathogen of human acute and chronic hepatitis B[1-4], which has a very close association with human hepatocellular carcinoma (HCC)[5-8]. For example, a statistical data from a hospital in Shanghai showed that 80% of HCC patients were positive for HBsAg (personal communication). The published data reported that people infected with HBV have a 200-fold greater risk of developing HCC than age-matched population not infected with HBV[9-11]. So HBV infection, including its consequences, is one of the key problems for the human health in the world. Since HBV only infects human beings and a few kinds of higher primates[12-14], the lack of culture cell and animal model impede the study of biological characteristics of HBV and human hepatitis B in the laboratory[15,16]. In 1985, the first transgenic mouse carrying HBV genes was generated[17], and thereafter the transgenic mouse system was applied in HBV studies[18-26].

HBV prevailing in the world has at least 4 subtypes[27-30], ayw, adr, ayr and adw, among which subtype adr is the most prevailing in China[31,32]. The complete genome of subtype adr has been cloned and analyzed from HBV infected population in China. It was identified that subtype adr was different from other 3 subtypes in DNA and protein sequence[33]. In order to produce the animal model for exploring adr subtype of HBV associated biomedical issues, we generated transgenic mice harboring complete genome from subtype adr of HBV by microinjection method, in which the HBsAg gene and HBeAg gene could be expressed, and the genomic DNA of HBV could be replicated and packed into complete virus particles. This model will be very useful for examination of many aspects of HBV and its associated biomedical issues *in vivo*.

MATERIALS AND METHODS

HBV genome of *adr* subtype was obtained from plasmid -padr-1-dimer. C57 mouse strain was bought from Animal Center of Shanghai Birth Control Research Institute. PCR primers for identification were designed by ourselves according to S region of HBV genome, primer 1: 5'CCCAACCTCCAATCACTCACC3', primer 2: 5'ACGAACCACTGAACAAATGGC3', synthesized by GIBCO BRL company (USA). The themostable DNA polymerase was bought from Sangon Co. (Canada), antigen-antibody ELISA kit of HBV was the product of Sino-American Bio-Engineering Co. (China). Primera gene Labeling System kit was bought from Promega Co. (USA). Antibodies for immunohistochemistry assay were products of Dako Co. (USA). Reagents for transmission electron microscopy were standard ones (China).

Preparation of HBV DNA and microinjection

padr-1-dimer has two copies of intact HBV (adr subtype) genome head to tail, cloned in BamHI site of plasmid pBR322 (Figure 1). Restriction enzymes *Sal* I and *Cla* I were used to digest *Padr*-1-dimer, followed by 7 $g \cdot L^{-1}$ agarose gel electrophoresis of the digested products, the 7.0 kb band was retrieved, purified and dissolved in TE buffer for microinjection. Microinjection and embryo manipulation were performed according to the methods described in References[34-36].

PCR and Southern-blotting of integrated transgenes

Genomic DNA from tail tissue of transgenic mice and normal mouse were prepared, and amplified with primers in S region according to the cycling program: 94℃, 30s; 60℃, 40s; 72℃, 45s; run 35

[1]Department of Cell Biology, Second Military Medical University, Shanghai 200433, China
[2]University of Wisconsin, Madison, WI 53705, USA
[3]Department of Pathology, Second Military Medical University, Shanghai 200433, China
[4]Center of Laboratory Animals, Second Military Medical University, Shanghai 200433, China
Yi Ping Hu, Professor, and now is working at Second Military Medical University , having more then 40 papers published.
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Correspondence to: Professor Yi Ping Hu, Department of Cell Biology, Second Military Medical University Shanghai 200433, China
Tel. 0086-21-25070240, Fax. 0086-21-25070240
Email. yphu@ smmu.edu.cn
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cycles in 50 μL reaction system. Fifteen μL of PCR products were loaded on 15 $g \cdot L^{-1}$ agarose gel in the presence of 0.5 μg ethidium bromide per mL. 380 bp band was visualized by UV fluorescence. Southern blot was performed on genomic DNA by agarose electrophoresis of 30 μg restricted DNA with *Bam* HI. Nylon filtrates were hybridized with the probe including HBV genome labeled with α-P^{32}-dATP for further characterization of the transgenic mice.

HBV gene expression in transgenic mice

HBV gene expression in serum Venous blood in eye sockets of mice was collected, serum was isolated by centrifugation, and then used for HBsAg and HBeAg ELISA or RIA.

HBV gene expression in tissues The distribution of HBsAg and HBcAg was assessed by the labeled-avidin-biotin detection procedure. Briefly paraffin-embedded sections were treated for 10 min at 37℃ with 30 $mL \cdot L^{-1}$ hydrogen peroxide and washed with PBS. Rabbit anti-HBsAg primary antiserum at 1:100 dilution, and goat anti-HBcAg primary antiserum at 1:200 dilution were applied over night at room temperature. After washing with PBS, a secondary antiserum consisting of biotin-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG was applied at 1:200 dilution for 40 min at 37℃, and then washed with PBS. Then the preparation was treated with 1:100 dilution ABC complex stained with DAB, and finally counterstained with hematoxylin.

Assay of serum antibody of transgenic mice

Fifty μL of the serum from transgenic mouse was assayed for anti-HBsAg, anti-HBeAg and anti-HBcAg by ELISA according to the Kit protocol.

Pathological findings analysis

Serum ALT and AST Serum ALT and AST were tested with auto-biochemical analyzer.

Histopathological study The specimens of liver, kidney, heart, spleen from transgenic mice were fixed in formalin, paraffin embedded, sectioned and stained with HE.

Examination of HBV Dane's particles in liver tissue from transgenic mice under TEM Several immunohistochemically HBV-positive mouse liver specimens were fixed over night at 4 ℃ in 40 $mL \cdot L^{-1}$ paraformaldehyde and 1 $g \cdot L^{-1}$ glutaradehyde in PBS. They were then postfixed in 10 $g \cdot L^{-1}$ OsO_4 in cacodylate buffer for 1hr at room temperature, dehydrated in gradient ethanol, and embedded in epoxy resin. This sections were cut on an LKB Ultratome III, mounted on copper grids, stained in uranyl acetate and lead citrate, and examined with electron microscope.

RESULTS

Microinjection and embryo manipulation

Hundreds of molecules of target fragment were microinjected into male pronuclei of fertilized eggs. Of 355 fertilized eggs microinjected, 262 of manipulated eggs survived; the survival rate was 73.8%. Thirteen of female recipient mice were transplanted with microinjected eggs through one of the ovarioles, and 2 of the transplanted mice were pregnant; the pregnant rate was 15.4%. Six small mice were born and all of them survived; the survival rate was 100%.

Founder mice and the identification of HBV DNA integration in transgenic mice

PCR and Southern-blotting results indicated that 4 mice were positive. So four founders were obtained. The founder mice were named HB *adr* dimer-*x*. The positive rate was 66%. Then the founders were mated with the normal mice of the same line. 27 F_1 offspring of the founder mice were born. In 14 of them PCR showed positive genomic DNA PCR. The offspring of F_1 were 56 mice, 28 of them were positive, and the positive rate was 50%. The exogenous genes can be transmitted through the germ line (Figure 2).

HBV genes expression in transgenic mice

HBV gene expression in serum The HBsAg and HBeAg were detected in the serum with ELISA and RIA. And the expression level of HBsAg was higher than that of HBeAg, and the gene expression was different in different founders (Table 1). The concentration of HBsAg in the serum was about 3000 $IU \cdot L^{-1}$.

Table 1 The ELISA result of founder mice and normal mice (A450)

Antigen	Control		Transgenic mice			
	Negative	Positive	HB-2	HB-3	HB-4	HB-5
HBsAg	0.01	1.42	0.17	0.06	0.26	0.23
HBeAg	0.01	1.56	0.06	0.06	0.08	0.09

Distribution of HBsAg and HBcAg in transgenic mice Immunohistochemical study of liver and kidney tissues from transgenic mice indicated that the distribution of positive hepatocytes was uneven, HBsAg-positive particles were located mainly in cytoplasm of hepatocytes. HBsAg expression level was weaker in kidney than in liver; the products of immune reactions of HBcAg-positive were not very certain, and mainly emerged in a few nuclei of hepatocytes from the individuals that were invariably HBsAg-positive (Figure 3). No obvious product of immune reaction from HBsAg and HBcAg positive mice was found in spleen, skin, testis and other detected tissues.

Serum antibody in transgenic mice The ELISA test indicated that there was no significant positive result of HBV antibodies in HBV transgenic mouse serum.

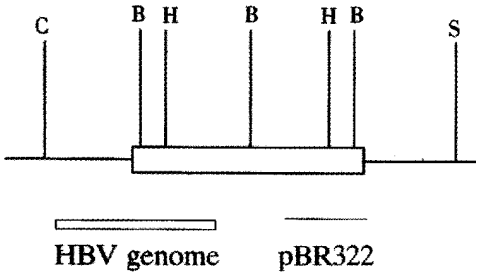

Figure 1 Restriction map and microinfection fragment of padr-1-dimer.
C: *Cla* I; B: *Bam* HI; H: *Hind* III; S: *Sal*I

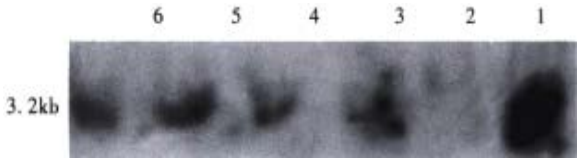

Figure 2 Southern-blot analysis of the HBV transgenic mice.
Lane 1: positive control; Lane 2: negative control;
Lane3-6: transgenic mouse

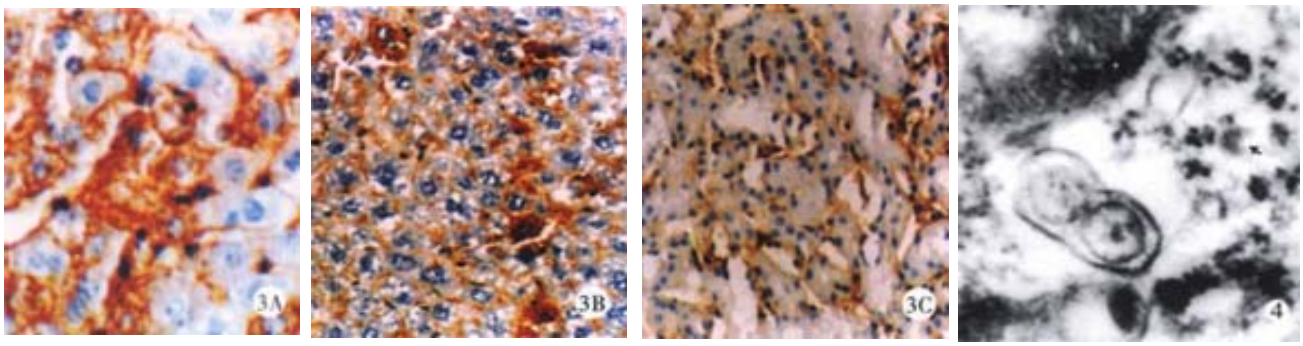

Figure 3 Immunohistochemical analysis of HBV antigens in transgenic mouse tissues.
A. HBsAg in the liver; B. HBcAg in the liver; C. HBsAg in the kidney
Figure 4 Dane's particles under electron microscope.

HBV Dane's particles in liver in transgenic mice under TEM HBsAg particles (spherial and filamental) were found in the endoplasmic reticulum of some hepatocytes, a few of in renal tubule-epithalia, and the cytoplasm of a few Kupffer cells (Figure 4).

Histopathological findings in transgenic mice

Histopathologic observation in transgenic mice tissues Mice have been monitored histologically for over 1 year without evidence of pathological changes in any organ, especially the liver and kidney. The serum ALT and AST assays indicated that there was no significant difference between the transgenic mice and the normal ones.(P>0.05) (Table 2).

Table 2 Aminotransferase in the transgenic mice (n = 12, $x \pm s$)

Mice	ALT(nkat·L^{-1})	AST(nkat·L^{-1})
Normal	698.5 ± 471.8	3597.4 ± 7669.9
Transgenic	708.5 ± 491.8	4349.2 ± 7248.1

DISCUSSION

The HBV transgenic mice show replication of the HB virus in the hepatocytes, but have no evidence of cytopathology of the liver and other organs or tissues.

In theory, HBV genes microinjected are complete in structure, and can be expressed and replicated in their bodies[37,38], because we have purposefully prepared the fragment which contains two copies of intact HBV (*adr* subtype) genomes head-to-tail to make it sure that all of four open reading frames of HBV genome are complete in structure. Four of the founders all showed serum HBsAg-positive, but the expression level is different. We think this is due to the different integrated site of HBV DNA. In general, the exogenous gene will be integrated into multi-sites in transgenic mice. This is similar with the other reports[39,40].

Immunohistochemical assays of several tissues from transgenic mice revealed that HBV gene tissue-dependent expressions were tissue-dependent, and the genes were mainly expressed in the liver and kidney, but the expression level was different; this is similar to HBV-infection in nature. Examination of liver and kidney tissue from transgenic mice under transmission electron microscope revealed the existence of Dane's particles and HBsAg particles in hepatocytes, which confirmed the ability of HBV genome replication in the transgenic mice.

Serum anti-HBs, anti-HBc and anti-HBe detected by ELISA were negative in all of the transgenic mice, and suggested that these mice were tolerant to HBsAg, HBcAg and HBeAg; this result was consistent with that of other author's reports[41]. Now, we obtain the F_3 generation positive mice. The HBV DNAs were able to be transmitted through the germ line of the mice from one generation to another.

The results of our experiments shows that HBVgenome introduced by microinjection is integrated into the mouse genome, and the HBV genes can be expressed, replicated and packaged. So these lines of HBV transgenic mice may be used as the animal model for the study on HBV and its associated biomedical issue. And it can also be applied in the selection of anti-HBV drugs and vaccine[42,43]. It is also useful for exploring the mechanism of HBV infection.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effects of glycyrrhetinic acid on collagen metabolism of hepatic stellate cells at different stages of liver fibrosis in rats

Ji Yao Wang, Qi Sheng Zhang, Ji Sheng Guo and Mei Yu Hu

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INTRODUCTION

Liver fibrosis is a dynamic course leading to cirrhosis from a various chronic liver diseases. The pathological basis of fibrosis is the disturbance of production and degradation of the extracellular matrix (ECM), which causes accumulation of ECM in the liver[1,2]. The deposition of collagen is derived primarily from collagen types I and III in liver fibrosis[3]. The main sources of ECM are the hepatic stellate cells (HSCs)[4,5], especially when HSCs are activated by hepatic injury[6,7]. One of the important methods for preventing liver fibrosis is the inhibition of proliferation and activation of HSCs so as to reduce production of collagen. Glycyrrhetinic acid (GA) has been clinically used in the treatment of liver diseases. It has anti-injury[8-10] and anti-viral[11-13] effects on hepatic diseases. The clinical trials have shown that GA could lower the serum aminotransferase level both in Asian[14,15] and European patients[16] with chronic hepatitis. Our previous studies also indicated that GA could down-regulate mRNA expression of types I and III procollagen in NIH3T3 cells[17] and in fibrotic livers of rats induced by alcohol and CCl_4[18]. However, the effect of GA on HSCs mRNA expression of types I and III procollagen is unclear. In this study, the effects of GA on HSCs mRNA expression of procollagen types I and III and collagenase were investigated and deposition of types I and III collagen in different stages of fibrotic livers in rats was also observed.

Department of Gastroenterology, Zhongshan Hospital, Medical Center, Fu Dan University (Shanghai Medical University), Shanghai 200032, China
Dr. Ji Yao Wang, graduated from Shanghai Medical University in 1967 and got the Master Degree of Medicine in 1981, Shanghai; Master Degree of Science in 1986, Canada. She is the Chairperson of Dept. Internal Medicine; Chief of the Division of Gastroenterology; Director of the Lab of Internal Medicine, professor of internal medicine and supervisor of postgraduate students in the Zhongshan Hospital. She has published more than 100 articles and is the chief editor of *Modern Treatment on Liver Disease* and *Textbook of Internal Medicine* (for 7-year curriculum medical students). She is also the associate editor-in-chief of *Clinical Economics.*
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Correspondence to: Prof. Ji Yao Wang, Department of Internal Medicine, Zhongshan Hospital, Medical Center, Fu Dan University (Former Shanghai?Medical University), Shanghai 200032, China
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MATERIALS AND METHODS

Animal model of liver fibrosis and drug treatment

Adult SD rats weighing 250 g-300 g were selected. They were distributed as the normal group, the model group, and the GA group. Each group contained the early (2 weeks), middle (6 weeks), and late stage (9 weeks) subgroups. There were 30 rats in each group and 10 rats in each subgroup. The liver fibrosis model was induced by the administration of CCl_4 and alcohol. Potenlini, an injectable compound whose active component is GA, was administered intraperitoneally in the GA group with 3 mL per rat three times a week, begining at 2 weeks prior to sacrifice. Rats were killed by the end of 2, 6 and 9 weeks respectively.

Isolation and culture of HSCs

HSCs were isolated from rat liver as described by Hu[19] with slight modifications. The HSCs showed a typical stellate-like shape containing fat droplet in cytoplasm. During the culture period, HSCs became larger and contained less amounts of fat droplet. By the end of 2 weeks, HSCs looked like myofibroblast. Cells were seeded in culture flask and maintained in DMEM media supplemented with 20% FCS and antibiotics. The media was changed every 48 h. After 2 weeks, when cell confluence was attained, they were harvested by the trypsinized method and applied to further studies or stored in liquid nitrogen.

Identification of HSCs

Freshly isolated HSCs could be distinguished by its autofluoresence characteristic of vitamin A in the lipid droplets at 328 NM. Immunohistochemistry showed that desmin and α-SMA were positive in 99% of cells after 2 weeks of culture. Besides, transmission electron microscopy confirmed the existence of lipid droplet in cytoplasm and revealed a purification of about 90% in freshly isolated cells.

^{3}H-TdR and ^{3}H-proline incorporating test

After two weeks of culture, the HSCs were collected and seeded on 96-well culture plates at a density of 1 × 10^5 cells/mL cultured media. Forty-eight hours later, GA (i.e.potenlini) was added into wells at a final concentration of 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/L, respectively, and incubated for 4 h, 24 h or 48 h, then ^{3}H-TdR or ^{3}H-Proline at a density of 18.5 KBq/per well were added and incubated for 24 h. The cells were then harvested with trypsin and the adhered cells were placed into glass fiber filter by multiple cell collector. The cells were baked at 80℃ for 2 h, scintillation fluid was added and the radioactivity in the cells was determined using scintillation counter.

Plasmid amplification and probe labeling

Plasmid pUCAU1U (containing procollagen type I cDNA fragment)[20], pHFS3 (containing procollagen type III Cdna fragment)[21], and pUC19A (containing collagenase cDNA fragment)[22], were amplificated in LB culture media. The plasmid DNA was extracted with a plasmid extracting kit (QIAGEN Incorporation, Germany). Plasmid was cleaved with restriction endonuclease and the target cDNA fragment was retrieved and phenolized. The cDNA fragment was labeled with DIG high primer technique (Boehringer Maannheim Incorparation,Germany). The probe was further purified by ethanol precipitation. Finally, the efficiency of probe labeling was determined using pseudo-hybridization, the optimal probe concentration of procollagen types I and III and collagenase was found to be 25, 35 and 25ìg/L respectively.

RNA extraction and Northern blot

HSCs RNA was extracted with a Rneasy mini-kit (Boehringer Maannheim Incorparation, Germany). Total RNA 5 μg was electrophorized on a 1% agarose/ 3% formadehyde gel. The RNA samples were stained with ethidium bromide and transferred overnight by capillary blotting in 20 × SSC to nylon membrane. The RNA was immobilized by baking for 30 min, at 120℃. Membranes were prehybridized (2 h) and hybridized (overnight) at 60℃ in high SDS solution. The membranes were washed at a stringency of 2 × SSC with 0.1% SDS at room temperature for 30 min and 0. 1 × SSC with 0.1% SDS at 68℃ for 30 min. The hybridization band was obtained by the chemoiluminescent method after film exposure for 5-10 min and then quantified by the scanning laser densitomitry.

Dot blots of types I and III collagen

The types I and III collagens were isolated by limited pepsin digestion. The livers were minced and homogenized thoroughly in cold distilled water and then centrifuged at 12 000rpm for 20 min. The precipitate (5 g) was suspended in 0.5M acetic acid and digested with pepsin for 24 h at 4℃ with stirring, and then centrifuged. The supernatant was incubated with NaCl (1.0M) overnight. The precipitate contained types I and III collagen which were purified by salt fractionation and their concentration was estimated by ultraviolet spectroscopy. Twenty μL of each sample was loaded on PVDF membrane and was blocked with 10% BSA for 60 min. The polyclonal antibodies (1:250 dilution) of types I and III collagen were added and incubated overnight. The membrane was then washed with PBST 3 times and blocked again with 10% BSA for 30 min. It was then incubated with the secondary antibody for 2 h at room temperature. After washing with 50mM Tris-HCl, the dot was obtained 5-10 min after incubation with DAB. The dot intensity was quantified by scanning laser densitomitry.

Statistical analysis

Data were expressed as mean ± SD. One way ANOVA and *t* test were applied for data analysis.

RESULTS

Histological examination and identification of HSCs

Histological examination (H&E and collagen specific staining) revealed the successful establishment of the rat liver fibrosis models at different stages. Two weeks after CCl_4 and ethanol treatment, denaturation and necrosis were the main microscopic changes in liver. By 6 weeks, except for denaturation, connective tissues began to enlarge and extend. By 9 weeks, pseudo-nodules formed and bands of connective tissues were found in the portal areas. The yield of HSCs was 1.6-1.8 × 10^7 cells per liver. The result of transmission electron microscopy, fluorescence microscopy (Figure 2) and immnohistochemical staining all showed that the purity of HSCs was high, which met the demand of further studies.

^{3}H-TdR and ^{3}H-proline incorporating test

Compared with the control group, GA had an inhibitory effect on cultured HSCs on incorporation of ^{3}H-TdR and ^{3}H-proline at 4 h, 24 h, and 48 h (Table 1), with a time dependent relationship. This inhibitory effect was significant in a dose-dependent manner when the concentration of GA was above 0.25 mg/L (^{3}H-TdR), and 0.125 mg/L (^{3}H-proline), respectively (Table 2).

Table 1 Effect of GA treatment with different duration on ^{3}H-TdR and ^{3}H-proline incorporation of HSCs

Drug	Duration (hrs)	^{3}H-TdR incorporation		^{3}H-proline incorporation	
		cpm	Inhibition rate(%)	cpm	Inhibition rate(%)
None		1540 ± 120		542 ± 102	
GA	4	1327 ± 198	15[a]	421 ± 16	22[a]
	24	1217 ± 254	21[a]	316 ± 18	42[b]
	48	1057 ± 121	31[b]	265 ± 84	51[b]

[a] Compared with control values, $P<0.05$; [b] Compared with control values, $P<0.01$.

Table 2 Effect of GA of different doses on ^{3}H-TdR and ^{3}H-proline incorporation of HSCs

Drug	Duration (hrs)	^{3}H-TdR incorporation		^{3}H-proline incorporation	
		cpm	Inhibition rate(%)	cpm	Inhibition rate(%)
None		1540 ± 120		542 ± 102	
GA	0.0625	1427 ± 175	7	441 ± 76	19[a]
	0.125	1321 ± 126	14	327 ± 71	40[b]
	0.25	1211 ± 137	21[a]	316 ± 57	42[b]
	0.5	1176 ± 134	24[a]	295 ± 81	46[b]
	1.0	1027 ± 121	33[b]	220 ± 63	59[b]

[a]Compared with control values, $P<0.05$; [b]Compared with control values, $P<0.01$.

Plasmid amplification and probe labeling

The purity of extracted plasmid DNA was high, and the ratio OD260/OD280 was over 1.6. The DNA yield of three kinds of plasmid all exceeded 130 μg. Electrophoresis showed that the yield of DNA fragment, which was cleaved with restriction enzyme, was satisfactory. On the other hand, the probe concentration of type I and III procollagen and collagenase was determined to be 15 mg/L, 35 mg/L and 100 mg/L, respectively.

The effect of GA on HSCs mRNA expression of types I and III procollagen and collagenase

At the end of 2, 6 and 9 weeks after the induction of rat liver fibrosis, HSCs mRNA expression of types I and III procollagen in the model group was higher than in normal group ($P<0.05$). However, HSCs mRNA expression of types I and III procollagen in GA group was lower than that in the model group, but was higher than the normal group (GA group vs model group and GA group vs normal group, $P<0.05$). HSCs mRNA expression of collagenase in the model group was higher than in normal group at each stage of liver fibrosis ($P<0.05$). And at the end of 9 weeks, the mRNA expression of HSCs showed a dropping tendency. HSCs mRNA expression of collagenase in GA group was also higher than in normal group, but there was no difference between the GA and the model group (Figure 1).

The effect of GA on liver deposition of types I and III collagen

At the end of the 2nd, 6th and 9th week, the liver deposition of types I and III collagen in the model group was higher than in normal group ($P<0.05$). The liver deposition of types I and III collagn in GA group was lower than in model group ($P<0.05$), but was still higher than in the normal group. In addition, the density of type I and III collagen in treatment groups were closer to the model group at the end of the 9th week than at the 2nd and 6th weeks (Figure 2).

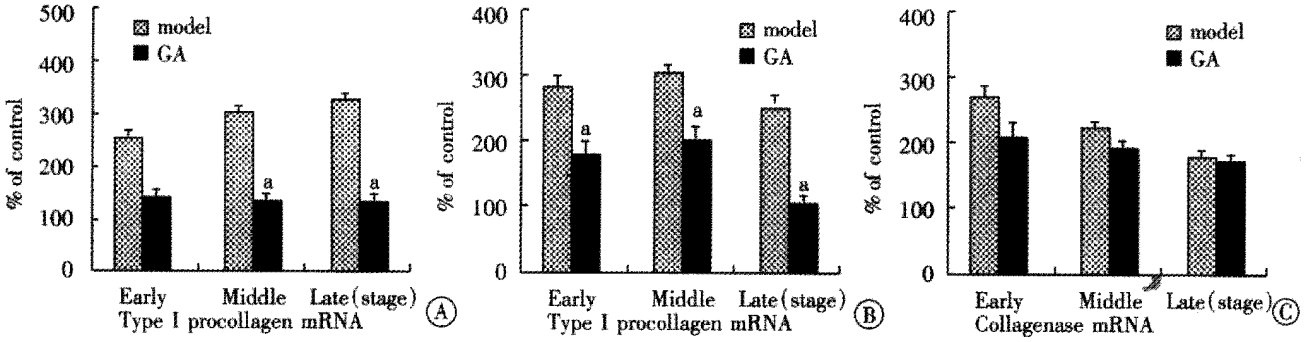

Figure 1 Densitometric analysis of collagenase, type III and I procollagen mRNA Northern blot. The result of Densitometric analysis after normalization against hybridization signals for normal groups was expressed as mean percentage ± SD of the control values ($n = 5$). The level of types I and III procollagen mRNA expression of HSCs in GA groups was lower than in model groups ([a]$P<0.05$). For the collagenase mRNA expression of HSCs, there was no significant difference between GA groups and model groups.

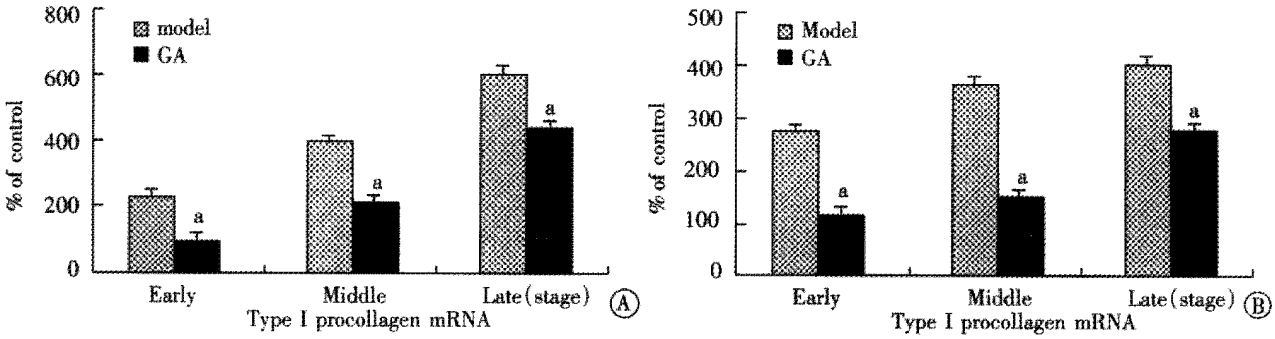

Figure 2 Dot blot densitometric analysis of types I and III collagen. The result of densitometric analysis after normalization against hybridization signals for normal groups was expressed as mean percentage ± SD of the control values ($n = 5$). The level of types I and III collagen densitometric values in GA groups was lower than in model groups ([a]$P<0.05$).

DISCUSSION

In normal livers, HSCs are situated in the Disse£§s spaces, separating hepatocytes from sinusoidal endothelium and being rich in fat drips. It has been well known that HSCs are responsible for the excessive production of ECM[23-25]. The central event in liver fibrosis is the activation of HSCs and subsequent transformation from quiescent vitamin A-rich cells to proliferative, fibrogenic and contractile myofibroblasts[26]. *In situ* hybridization demonstrated the specific transcript of procollagen[27,28]. It is rationale to choose HSCs as a target for pharmacological therapies for anti-fibrosis of liver. In this study, we observed that GA had an inhibitory effect on proliferation and collagen production of HSCs *in vitro*, which could be associated with the inhibition of activation of HSCs. The activation of HSCs is a pleiotrofic process. It involves a series of gene transcriptions. Finally, HSCs display a shape that is similar to myofibroblast-like cells while addition of GA delayed the transformation of HSCs into myofibroblast-like cells. It suggests that GA could serve as a proximal segment modulator to decrease the activation and collagen production of HSCs in culture.

It has been proved that the expression of type I and III procollagen is up-regulated in hepatic fibrosis [29,30]. In this study, we demonstrated that the expression of types I and III procollagen of HSCs increased in hepatic fibrosis of rats. At the end of the 2nd, 6th and 9th week, the expression of types I and III procollagen was down-regulated in GA treated group, but still higher than in normal group, which indicates that GA only decreases the expression of types I and III procollagen partially. We have previously reported that GA inhibits nuclear factor-κB (NF-κB) binding activity[31]. NF-κB is a pleiotropic transcription activator[33] that exists in many kinds of cells[32]. It binds NF-κB inhibitors (IκB) in the cytoplasm as an inactive form. A wide spectrum of cellular stimulating signals, including mitogen, cytokines, bacterial lipopolysaccharides, viruses and viral proteins, and oxidative injury, could induce the activity of NF-κB[34]. Inducers of NF-κB activity resulted in phosphorylation, ubiquitination and degradation of IκB proteins, thus releasing free NF-κB for its translocation into the nucleus to activate transcription. We presume that by this way, GA can down-regulate the expression of types I and III procollagen of HSCs. In normal liver, the production of collagen is relatively static, and only with moderate expression of mRNA of type III and IV procollagen and laminin. The mRNA expression of type I procollagen increased significantly in the formation of hepatic fibrosis. The ratio of type I:III was about 4:1[35] as observed in our study.

Recently, it has been found that the activity of interstitial collagenase is elevated in the early stage of hepatic fibrosis[23]. However, in the development of fibrosis, the activity of collagenase decreased[36-40]. In our study, the activity of collagenase increased at the early and middle stage of fibrosis, i.e. 2 or 6 weeks after CCl_4 treatment. At the end of the 9th week (late stage), it dropped to the level similar to that of normal groups. It could be due to the overexpression of tissue inhibitor of metalloproteinase in late stage of hepatic fibrosis[41,42]. GA reduced the mRNA expression of types I and III procollagen of HSCs, but not elevated the mRNA expression of collagenase of HSCs, which indicates that GA decreases the deposition of types I and III collagen by reducing the production of collagen, instead of dissolving the collagen. Therefore, at the end of the 9th week, GA was unable to obviously decrease the deposition of collagen. It suggested that the treatment for hepatic fibrosis with GA should begin at the early stage of fibrosis.

Based on the well-known mechanism of fibrosis, the treatment of fibrosis should include the following elements: removing the injurious stimuli; suppressing the hepatic inflammation; down-regulating the stellate cell activation and promoting the matrix degradation[26]. As we know, the major etiological factor of cirrhosis in patients in China is chronic hepatitis B. Histological improvement was found in the patients responding to antiviral therapy with lamivudine for HBV[43]. The result of a long-term follow-up study suggested that the proliferation of fibrous tissues was reversible[44]. Sun *et al*[45] indicated that antifibrotic therapy is important even in cirrhotic stage in which the fibrogenesis is still active. The results of our studies indicated that the effects on antifibrosis of GA might be exerted by down-regulating the binding activity of NF-κB and HSC activation, and by suppressing the hepatic inflammation. Our previous study also showed that the effect of GA on serum conversion of HBeAg[14]. Sato *et al*[46] reported that GA could inhibit the release of HBsAg from the infected hepatocytes. Therefore, GA appears to function at multiple phases of hepatic fibrogenesis. Furthermore, the more exciting report is that GA treatment could inhibit the occurrence of hepatocellular carcinoma[47]. It has been also reported that long term (2-16 years.) treatment by GA in chronic hepatitis C patients had no side-effect and was effective in preventing liver carcinogenesis[48].

Other Chinese herbal recipes have shown their features in antifibrosis. Varieties of recipes or herbal extracts, such as Xiao Chaihu Tang[49], Recipe 861[50], Yiganxian[51], Ganyanping[52], and Matrine[53] have been shown to be effective in prevention and treatment of liver injury and fibrosis with different mechanism and pathway. It implies that the clinical application using the combination of glycyrrhetinic acid with these medicines is an

interesting area for further investigations.

In conclusion, GA inhibits the proliferation and collagen production of HSCs in culture, down-regulates the mRNA expression of type III and I procollagen, and reduces the deposition of type III and I collagen in fibrotic liver. It can be a very useful drug for anti-fibrotic treatment in patients with chronic liver disease.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Expressions of ICAM-1 and its mRNA in sera and tissues of patients with hepatocellular carcinoma

Jing Xu[1], Ming Hui Mei[1], Si En Zeng[2], Qing Fen Shi[3], Yong Ming Liu[4] and Li Ling Qin[3]

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INTRODUCTION

The increased expression of ICAM-1 on a wide range of cells and in the sera of patients with malignancies, chronic liver diseases and inflammation diseases has been described since the late 1980s[1-22]. Recently rapid progress in studies on expression of ICAM-1 in patients with hepatocellular carcinoma (HCC) have been achieved, including clinical and experimental researches [23-31]. It is well known now that ICAM-1 expressed in two ways in HCC: ① membrane-bounded ICAM-1 on the surface of HCC cells (mbICAM-1), which did not express in normal liver cells, and ② soluble ICAM-1 (sICAM-1) in sera of the patients, the concentration of which is well correlated with the progress and prognosis of the disease[32]. However, little is known about whether sICAM-1 is a diagnostic marker for early detecting HCC and monitoring its postoperative recurrence. It has been demonstrated that measurement of sICAM-1 might be of clinical values for early diagnosis and monitoring recurrence of HCC, particularly in patients with normal or low serum level of α-fetoprotein (AFP)[33-37]. Another controversy in this field is what is the main source of high levels of circulating sICAM-1 in HCC patients. It was reported that there were two forms of sICAM-1: HCC-specific circulating form of ICAM-1 shedding from HCC cells, and inflammation-associated ICAM-1 upregulated by several cytokines, of which interferon-gamma (INF-gamma) was the main cytokine trigger for ICAM-1 expression[38]. To solve the two problems mentioned above has become the key to the study. For this purpose, we have further confirmed the clinical values of sICAM-1 detection in HCC compared with benign liver diseases and normal control, analyzed the serum levels of AFP and sICAM-1 in HCC; observed mbICAM-1 expression in different regions of HCC tissues with the immunohistochemistry, and measured the expression of ICAM-1 mRNA in tissue samples by reverse transcriptase polymerase chain reaction (RT-PCR).

[1]Department of Hepatobiliary Surgery, [2]Department of Pathology, [3]Institute of Hepatobiliary Surgery, [4]Department of Biochemistry, Guilin 541001, Guangxi Province, China
Jing Xu, graduated from Tongji Medical University as a postgraduate, now associate professor, specialized in hepatology, having 15 papers.
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Correspondence to: Dr. Jing Xu, 95 Leque Road, Department of Hepatobiliary Surgery, Guilin Medical College, Guilin 541001, Guangxi, China
Tel. 0086-773-2810411, Fax. 0086-773-2822194
Email. Jxu@gliet.edu.cn
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MATERIALS AND METHODS

Patients

Between January 1997 and July 2000, 151 patients with pathologically proven HCC either by surgical resection of liver tumors or by liver biopsies (131 men and 20 women; aged from 14 to 80 years, median 49) were treated in our department. Tumor was less than 5 cm in diameter in 21 patients,less than 10 cm in 43 and more than 10 cm or with extrahepatic metastasis in 87 cases. Liver cirrhosis was found in 137 patients. Hepatitis B surface antigen (HBsAg) was positive in 129 patients. Hepatitis C antibody was positive in 14 patients. The serum concentration of AFP was measured in all cases by radioimmunoassay (RIA) and its reference ranges were classified as follows: >200 μg/L as positive in 93; 20 μg/L-200 μg/L as questionable positive in 33 and <20 μg/L as negative in 25[35]. Treatment included surgical procedures (in 90 cases), interventioanl radiology (IR in 46) and percuteneous ethanol injection (PEI in 15). Among the surgically-treated patients, tumor resection was performed in 75, including radical resection in 42, nonradical in 33 due to intrahepatic vascular invasion on the resected margin pathologically or intrahepatic metastasis of tumor found intraoperatively. Laparotomy cathterization of the hepatic artery was undertaken in 15. The 46 patients underwent IR therapies including transcatheter arterial embolization (TAE) in 30 and transcatheter arterial infusion (TAI) in 16.

Follow-up of the patients undergoing hepatectomies

The patients undergoing hepatectomies were followed up monthly in the first year and once every two months in the second year postoperatively. During the follow-up, serum concentration of AFP and sICAM-1, liver function and image

examinations including BUS, CT or selective hepatic angiography were undertaken. Tumor recurrence was confirmed when intrahepatic lesion or portal vein tumor thrombi were found by liver image, and pathological diagnosis was established by reoperation or biopsy of the tumor.

ELISA for sICAM-1

Concentrations of sICAM-1 were measured with an enzyme-linked immunosorbent assay kit (Biosource Europe, Fleurus, Belgium) as described previously[31-37]. In all HCC patients, sICAM-1 levels were compared with their levels of serum AFP. As controls, serum levels of sICAM-1 were measured in 62 patients with chronic hepatitis B (CH), 60 with liver cirrhosis (LC) and 50 healthy blood donors.Immunohistochemistry of liver tissues -Tissues from tumor and adjacent region were obtained from 52 patients with surgical resection. Histologically normal liver tissues were obtained from 3 patients with liver hemangioma. To demonstrate the presence of ICAM-1 in the resected liver tissues, each fresh specimen was cut at -20℃ with the cryostat (4 μm- thick), mounted on sialinized adhesion microscope slides, dried overnight at room temperature, fixed with cold acetone (-20℃) for 10 min and chloroform for 20 min. Anti-human ICAM-1 mouse monoclonal antibody (Clone HA58, Pharmigen) was used. The slides were washed twice in TRIS/0.2% BSA and then incubated with sheep-anti-mouse-Ig (DAKO, Copenhagen, Denmark). And then the staining reaction was developed with a streptavidin-biotinylated-alkaline-phosphatase-complex (DAKO),slides were counterstained with hematoxylin, and nonreacting monoclonal antibody of IgG-1-isotype (DAKO) was used as negative control.

RNA extraction and DNA amplification

The total RNA was isolated from the fresh resected liver tissues by the acid guanidium thiocynate-phennol-chloroform method[39](Gstract, Maxim Biotech, Inc., San Francisco, USA). The concentration of RNA was determined from absorption at 260nm, and A 260: -80 ratios were > 1.7. RNA (10 μg)was subjected to electrophoresis in 1.5% agarose gels. The PCR primer for ICAM-1 and GAPDH were designed according to Vigano *et al*[40]. The following four primers presented by Dr. Shraven were used: ① (105) 5'-TGATGACATCAAGAAGGTGGTGAAG-3'; ② (1047)5'-TCCTTGAGGCCATGTGGGC-CAT-3'; ③ (825) 5'-GTCCCCTCAAAAGTCATCC-3'; ④ (1064) 5'-AACCCCATTCAGCGTCACCT-3'. The primer set was used to amplify an intron-spanning region of the human glyceraldeyde-3-phosphate dehydrogenase (GAPDH) gene. The latter gene provided a constitutively expressed internal control for complementary DNA contamination. The presence of ICAM-1 and GAPDH mRNAs was demonstrated by amplifying respective target sequences using PCR according to the instruction provided with the Technical Bulletin Kit (Promega). In brief, 3 μL (2.5 mM) RNA, and 50 pmol/L primers for ICAM-1 or GAPDH were added to each reaction mixture respectively, which included 0.4mM dNTPs 2 μL, 3 μL (1.5 mM) $MgSO_4$, AM V reverse transcriptase 1 μL (5U), TfI DNA polymerase 1 μL (5U), and AMV/TfI 5 × buffer 10 μL. The final reaction volume was 50 μL and was covered with 20 μL mineral oil. Then with PCR thermal cycle(Hema 480, China), RT-PCR reaction was run in the following procedures: ① 48℃ for 45 min, 1 circle; ② 94℃ for 2 min, 1 circle; ③ 94℃ for 30s, 60℃ for 1 min, 38℃ for 2 min, 30 circles; and ④ 68℃ for 7min, 1 circle. Five μL PCR product was placed on 1.5% agarose gel and observed by EB staining under UV light, the electrophoresis photo was transformed into computer, and ICAM-1 intensity was analyzed with MPIAS500 image system, while the GAPDH band intensity was subtracted as an internal standard.

Statistical analysis

Groups were compared by the Kruskal-Wallsi test and the *t* test.

RESULTS

sICAM-1 measurement

The concentrations of sICAM-1 measured in different groups are illustrated in Figure 1. The levels of sICAM-1 in the patients with CH (median -462 μg/L), LC (median-587 μg/L) and HCC (median -1120 μg/L) were significantly higher than that in the normal control (median -285 μg/L) (P<0.01). The levels of sICAM-1 in HCC group was also significantly higher than those in CH and LC groups (P<0.01).

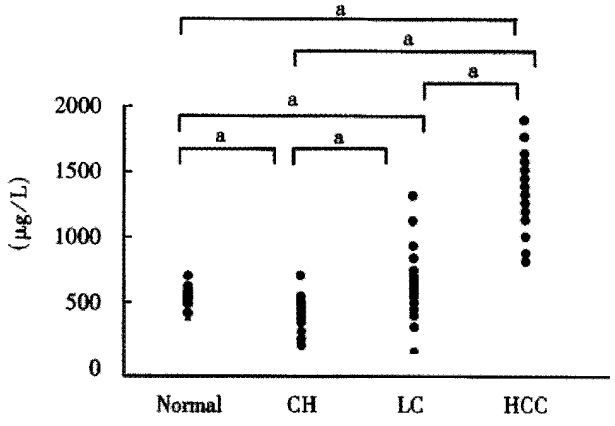


Figure 1 Serum concentration of sICAM-1 in normal controls (n = 50), in patients with chronic hepatitis (CH, n = 62), cirrhosis (LC, n=60), and hepatocellular carcinoma (HCC, n =151). ^aP<0.05, ^bP<0.01.

Comparative analysis of the serum values of sICAM-1 and AFP in HCC patients is listed in Table 1. There were 93 (62%) patients with positive serum AFP, and 58 (38%) with questionable and negative (33 and 25). However, the median values of sICAM-1 in these patients were 1597, 1456 and 1271 μg/L respectively. Statistical analysis showed no significant differences. In addition, analysis of ranges of sICAM-1 of HCC patients showed that 129 (85.4%) patients had a serum concentration exceeding 1000 μg/L, which was higher than the positive rate of serum AFP in the group of same patients.

Table 1 Comparative analysis of serum levels of sICAM-1 and AFP in HCC patients

Group	Number of patients	sICAM-1(μg/L)	AFP(μg/L)
AFP<20 μg/L	25	1271	18
AFP 20-200 μg/L	33	1456	121
AFP>200 μg/L	93	1597	26280

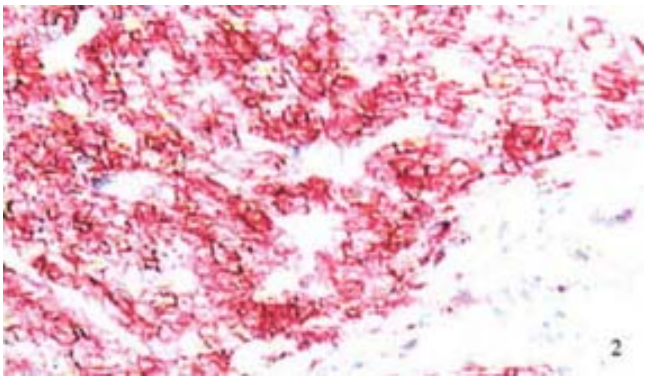
During the postoperative follow-up period of 6-61 months, tumor recurrence was confirmed in 41 patients. The median level of sICAM-1 of the patients was 1051 μg/L and 30 (73.2%) cases had a value higher than 1000 μg/L. On the other hand, there were 25 (60%) patients with serum AFP positive (>200 μg/L). In 6 patients with negative AFP, tumor recurrence was not detected by liver image until their serum levels of sICAM-1 had exceeded 2-4 times higher than before for 1-4 months.

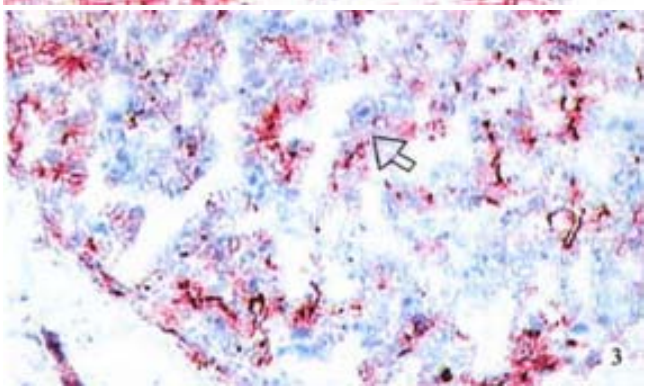
Immunohistochemical staining

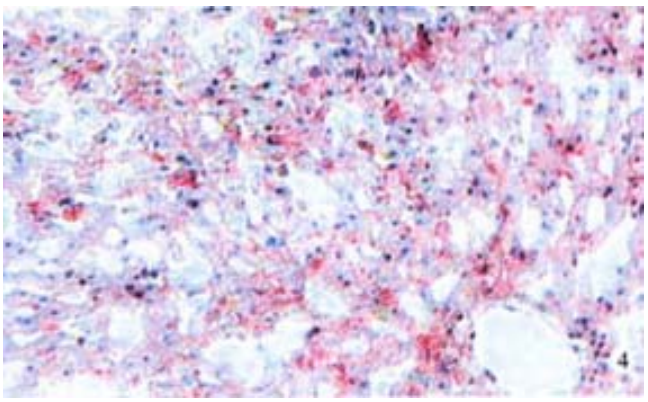
In all HCC samples, cancerous regions showed positive membrane staining for ICAM-1 with a honeycomb pattern (Figure 2). Parts of plasma of HCC cells showed strong positive reactions. In contrast to the cancerous areas, in noncancerous adjacent areas (Figure 3), the expression of ICAM-1 was negative, except those hepatocytes in severe cirrhotic areas. Normal liver tissue showed negative or very weak staining of the vascular endothelial cells and hepatocyts for ICAM-1 (Figure 4).

RT-PCR detection of ICAM-1

ICAM-1 mRNA in HCC tissues was detected by RT-PCR analysis (Figure 5). The result showed that freshly aspirated liver tissues expressed the gene coding for ICAM-1 because RT-PCR generated a DNA fragment corresponding to the predicted length, 943bp, of the ICAM-1 amplification product. In each tissue sample, all GAPDH amplification products were of 240bp length. The results revealed that the expression of ICAM-1 was stronger in the carcinomatous tissues of 28/43 cases (65.1%) than that in the paracacinomatous tissues of 13/43(30.2%) cases. Thirty-two of 43 cases had the levels of sICAM-1 >1000 μg/L.







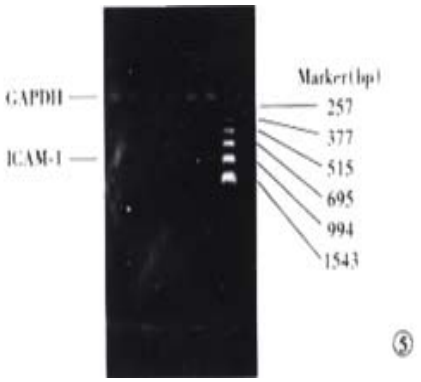


Figure 2 Immunohistochemical staining of ICAM-1 in hepatocellular carcinoma tissue is positive on the surface of tumor cells and in a honeycomb pattern.
Figure 3 Increased expression of ICAM-1 in paracancerous tissue was shown with severe cirrhosis by immunohistochemical staining.
Figure 4 ICAM-1 expression was negative in normal liver tissue by immunohistochemical staining (AEC method).
Figure 5 RT-PCR product gel electrophoresis. Three ìL RT-PCR products of ICAM-1 and GAPDH run on 1.5% agarose gel stained with EB. Lane 1 and Lane 4: the control for ICAM-1 and GAPDH. Lane 2 and 5: paracarcinomatous tissue for ICAM-1 and GAPDH. Lane and 6: HCC tissues for ICAM-1 and GAPDH.

The clinicopathological analysis of ICAM-1 mRNA and HCC is shown in Table 2. In the patients with serum levels of sICAM-1 above 1000 μg/L, the expression rates of ICAM-1 mRNA in HCC and adjacent tissues were 75.0% and 34.4%, respectively (P<0.01), however, in group with sICAM-1 lower than 1000 μg/L, the expression rate in those tissues were 36.4% and 18.2%, respectively (P>0.05). It is noticed that the expression rate of ICAM-1 mRNA was related to the severity of liver cirrhosis and intrahepatic metastasis of tumor. However, the expression rates of ICAM-1 mRNA were not correlated with the serum levels of AFP, differentiation of tumor and tumor sizes.

Table 2 Clinicopathological analysis of ICAM-1 mRNA and HCC %(n)

Clinical data	Cases	Positive tumor ICAM-1 mRNA	Positive paratumor ICAM-1 mRNA
Serum AFP>400 μg/L	25	68.0 (17/25)	32.0(8/25)
Serum AFP<400 μg/L	18	61.1(11/18)	27.8(5/18)
sICAM-1>1000 μg/L[a]	32	75.0(25/32)	34.4(11/32)
<1000 μg/L	11	36.4(3/11)	18.2(2/11)
Severe cirrhosis[a] Yes	30	80.0(24/30)	36.7(11/30)
No	13	30.8(4/13)	15.4(2/13)
Differentiation High	15	66.7(10/15)	33.3(4/15)
Low	28	64.3(18/28)	32.1(9/28)
Tumor size >5 cm	31	66.7(8/12)	25.0(3/12)
<5 cm	12	64.5(20/31)	32.3(10/31)
Intrahepatic metastasis[b] Yes	15	86.7(13/15)	40.0(6/15)
No	28	46.4 (13/28)	25.0(7/28)

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

DISCUSSION

HCC is one of the malignancies, which inflicts Chinese population severely. The prognosis of the disease is still dismal due to a delayed diagnosis, low resected rate and high recurrence rate of the tumor. Thus early diagnosis and effective postoperative monitoring are important to improve the surgical effectiveness for HCC[41]. The present study further confirmed our previous observations and other reports, in which elevated serum sICAM-1 was found in HCC, which was significantly higher than in benign liver diseases and the normal controls (Figure 1). As described by Shimizu *et al*[42], a sICAM-1 level above 1000 μg/L is a determinant for prognosis and progression of HCC. In our 151 patients with HCC, 129 (85.4%) cases had a high serum concentration of ICAM-1 above 1000 μg/L, which suggested that the positivity of sICAM-1 was higher than that of serum AFP. Although the serum levels of AFP in the HCC patients were different (Table 1), the sICAM-1 levels in the same group of patients showed no significant differences, particularly in cases with low levels of or negative AFP, indicating that measurement of sICAM-1 might be of diagnostic value for HCC.

Another interesting finding from our study is that in patients with AFP negative or questionable positive, sICAM-1 is a more sensitive serum diagnostic marker than AFP and other procedures, including BUS, CT, etc. In 41 patients with tumor recurrence detected during the postoperative follow-up, 73.2% patients had a high level of sICAM-1 above 1000 μg/L, however, the proportion of positive serum AFP in the same cases was only 60%. Moreover, in 6 of the 41 patients with negative AFP, tumor recurrence was diagnosed 1-4 months earlier by sICAM-1 than by liver image examination. We believe that sICAM-1 is not only a useful marker for predicting the progression and prognosis of the disease, but also a sensitive marker for diagnosing and monitoring HCC and its recurrence, especially for patients with low serum concentrations of AFP when the serum level of sICAM-1 was above 1000 μg/L[32-36].

As mentioned above, our clinical observations strongly supported that sICAM-1 might be of diagnostic value for HCC. However, it is unknown what is the main source of high level of sICAM-1 in HCC: HCC- specific ICAM-1 shedding from mbICAM-1 or inflammation-associated ICAM-1[38]. To distinguish the two forms of ICAM-1 is the ultimate goal of the study in this field[38]. Clinically, we have got the evidence that sICAM-1 is derived from HCC tumor cells, because we found in non-radical resected patients, the sICAM-1 concentrations maintained at a high level after the operation compared with those who underwent a radical resection of tumors, whose sICAM-1 levels would be decreased to the normal within 1-2 months postoperatively. This suggested that circulating sICAM-1 in HCC may originate mainly from tumor cell itself[36,37].

To verify our clinical findings that sICAM-1 is mainly derived from the HCC cells, we investigated the expression of ICAM-1 in HCC and its adjacent tissues with immunohistochemistry. The results showed that ICAM-1 expressed strongly in all specimens of HCC, but did not express in noncancerous regions, which reflected such a fact that high serum sICAM-1 levels of HCC patients might be attributable to tumor cells and a malignant transformation of hepatocytes. Our inference was supported by other authors[24,30,43,44]. Torri and Momosaky reported a high expression rate of ICAM-1 in HCC (80%-96%). Although three possible mechanisms for the expression of ICAM-1 were considered, Torri *et al* hold that malignant transformation of liver cells appeared to be the most important mechanism for the ICAM-1 expression in HCC. Thus they concluded that examination of ICAM-1 might yield significant information on the process of malignant transformation of hepatocytes[41,43]. In the nude mouse liver cancer metastasis model, Sun JJ *et al* demostrated that tissue ICAM-1 and sICAM-1 could indicate the stage

of HCC, potential of hepatoma cells for invasion and metastasis[45]. In addition, *in vitro* study by Momosaky *et al* showed that HCC cell itself markedly secreted soluble ICAM-1 into the culture supernantant in tumor cell lines[43]. On the other hand, no soluble ICAM-1 was shed from normal mouse hepatocytes regardless of the presence or absence of cytokine stimuli[43]. According to the observations from the present and other studies mentioned above, we think that high levels of sICAM-1 in HCC patients might be released mostly from the tumor cells.

After the clinical and immunohistochemical studies, we have recently investigated the correlation between serum levels of sICAM-1 and expression of its mRNA in HCC tissues. The results (Table 2) suggested that hepatocytes transformation may be the essential cause for strong expression of ICAM-1 mRNA, as in carcinomatous tissues of the 43 patients, the positive rate of expression was 65.1% (28/43). However, that in non-carcinomatous tissues was only 30% (13/43), which was quite different from the HCC tissues ($P<0.01$), and in normal liver tissues expression of ICAM-1 mRNA did not exist. Furthermore, the same correlation between the expression of ICAM-1 mRNA and the serum concentrations of sICAM-1 was observed. In patients with serum levels of sICAM-1 above 1000 μg/L, the expression rates of ICAM-1 mRNA in HCC and adjacent tissues were 75% and 34.4%, respectively, however, in group with sICAM-1 lower than 1000 μg/L, the expression rates were 36.4% and 18.2% respectively, which indicated that the expression of ICAM-1 protein was controlled by its correlative gene at the level of transcription regulation.

The expression rate of ICAM-1 mRNA was also related to the severity of liver cirrhosis and intrahepatic metastasis of tumor. Particularly in the later circumstances, the expression rate was much higher in metastatic patients (86.7%) than those without metastasis (53.6%), which suggested that the expression of ICAM-1 mRNA may play an important role in tumor intra and extrahepatic spread. These results were identical to our clinical observations, in which the proportion of sICAM-1 level >1000 μg/L was higher than that of AFP (73. 2% vs 60%) and sICAM-1 level was more sensitive than liver image examinations in indicating tumor recurrence.

Based on our studies on expressions of ICAM-1 and its mRNA in serum and tissues of HCC, we concluded that: ① Serum levels of sICAM-1 in HCC were significantly higher than that in benign liver diseases and normal controls, which indicated that it is useful to measure sICAM-1 to differentiate HCC from other benign lesions when the level of sICAM-1 is higher than 1000 μg/L. ② The high expression level of sICAM-1 might result from the strong expression of ICAM-1 in HCC tissue, which was controlled by its related gene. The source of sICAM-1 in HCC was originated mainly from HCC cells. ③ In HCC with intrahepatic metastasis, the expression of ICAM-1 mRNA was significantly high, which suggested that detecting sICAM-1 is of important value in predicting tumor recurrence after surgery. ④ With understanding of the mechanism of ICAM-1 expression and its origination, we believe that measurement of sICAM-1 may be of diagnostic value for HCC, particularly in patients with low levels of serum AFP.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Plasma endothelin in patients with endotoxemia and dynamic comparison between vasoconstrictor and vasodilator in cirrhotic patients

Feng Liu[1], Ji Xin Li[2], Chun Mei Li[2] and Xi Sheng Leng[2]

Subject headings hypertension, portal; liver cirrhosis; portosystemic shunt, surgical; endothelins; radioimmunoassay; epoprostenol; liver cirrhosis

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METHODS

We measured the concentration of endothelin in plasma using radioimmunoassay in 121 patients with cirrhosis and compared these values with 50 age- and sex-matched control subjects, and evaluated systemic endotoxemia. At the same time, perioperative plasma vasoconstrictor and vasodilator were clinically observed in 30 portohypertensive cirrhotic patients undergoing portosystemic shunt.

RESULTS

Plasma endothelin levels were higher in cirrhotic patients with ascites than in those without ascites. Femoral venous plasma endothelin levels averaged 90 ± 23 ng/L in cirrhotic patients versus 34 ± 8 ng/L in controls (P = 0.000), and that of cirrhotics with ascites was higher than those without 106 ± 17 ng/L *vs* 90 ± 23 ng/L(P = 0.002). Moreover, plasma endothelin levels increased in proportion to the severity of endotoxemia (r_s = 0.61, P = 0.034). Both the levels of plasma vasoconstrictors (ET, TX-) and of the vasodilator (PGI_2) were higher in portohypertensive cirrhotic patients (ET: 107.8 ± 25.9 ng/L*vs* 48.1 ± 9.4 (P = 0.000); TX-: 349.7 ± 198.4 ng/L *vs* 156.3 ± 54 (P = 0.000); PGI_2: 463.1 ± 108.3 ng/L *vs* 227.2 ± 46 (P = 0.000), and their concentrations decreased significantly in patients after portosystemic shunt (P = 0.002).

DISCUSSION

These results suggest that endothelin has significant influence on the portal vascular resistance of cirrhotic liver *in vivo* and may play an important role in the pathogenesis of portal hypertension[25-28]. Endotoxin may lead to the increased synthesis and release of endothelin. It could be that a dynamic balance between levels of vasoconstrictor and vasodilator in plasma exists in the pathophysiology of portohypertensive cirrhotic patients after portosystemic shunt.

INTRODUCTION

Portal hypertension is a common clinical syndrome characterized by an abnormal increase in portal blood to the systemic circulation, bypassing the liver. Recent studies have reported that humoral substances play an important role in the pathogenesis of portal hypertension, either by increasing vascular resistance at both the intrahepatic and porto-collateral sites or affecting splanchnic vasodilation with a concomitant increase in parto-collateral blood flow[1-6].

Endothelin (ET) released by endothelial cells is a 21-amino acid peptide with potent vasoconstrictor action. Endothelin comprises a family of four homologous isopeptides in human and animals (ET-1, ET-2, and ET-3, VIC)[7-14]. Most reported data are related to ET-1, which is the most powerful vasoconstrictor. Owing to a variety of reasons, reports concerning endothelin levels in cirrhotics are not consistent with each other. Endothelin concentrations in plasma have been reported to be increased in some studies and normal or reduced in others[15-20]. Present evidence suggests that endothelin may play an important role in modulating intrahepatic vascular resistance[21-24]. However, the relationship between vasoconstrictor (ET, TX-) and vasodilator (PGI_2) during portosystemic shunt has not been documented.

[1]Department of General Surgery, the Fifth Affiliated Hospital, Harbin Medical University, Harbin 150036, Heilongjiang Province, China
[2]Department of General Surgery, People's Hospital, Medical University, Beijing 100044, China
Dr. Feng Liu, graduated from Beijing Medical University as a postgraduate in 1995, now associate professor of general surgery, majoring hepatobilliary surgery, having 12 papers published.
Project supported by the National Natural Science Foundation and Ministry of Public Health of China, No.37600481
Correspondence to: Dr. Feng Liu, Department of General Surgery, the Fifth Affiliated Hospital, Harbin Medical University, Harbin 150036, Heilongjiang Province, China
Tel. 0086-51-5314098, Fax. 0098-51-5314088
Email.lfdlyy.163.net.
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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Adhesion molecule and proinflammatory cytokine gene expression in hepatic sinusoidal endothelial cells following cecal ligation and puncture

Rong Qian Wu, Ying Xin Xu, Xu Hua Song, Li Jun Chen and Xian Jun Meng

Subject headings cell adhesion molecules; sepsis; endothelium; cytokines; gene expression; polymerase chain reaction; RNA, messenger; liver/injuries; cecal diseases

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INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is thought to be a frequent consequence of sepsis[1-3]. Despite substantial advances in our knowledge and understanding of the basic pathophysiologic mechanisms[4-7], in critically ill patients infections and sepsis are still associated with a high mortality[8,9]. There is evidence that the development of tissue damage in sepsis and shock is closely associated with the release of an ever increasing number of mediators and accumulation of neutrophils at the sites of infection or injury[10,11].

The endothelium is an intimal layer of simple squamous cells which provides a continuous, fluent surface for circulating blood. It is not the passive, metabolically inert barrier that it was once thought to be, and it is now known to be a metabolically and physiologically dynamic tissue with multiple functions. On the basis of recent discoveries in the field of endothelial cell biology (such as endothelium-derived mediators and the expression of adhesion molecules), end othelial cells are now thought to be not only target cells of injury, but also actively involved in inflammatory reactions and subsequent organ damage[12,13].

Polymicrobial sepsis induced by cecal ligation and puncture (CLP) is a model of sepsis which reproduces many of the inflammatory and pathological sequelae that are observed clinically. Following CLP, animals develop bacteremia, hypothermia, hypotension, and damage to multiple organ systems[14]. The present study was designed to observe the gene expression of adhesion molecules and proinflammatory cytokines in hepatic sinusoidal endothelial cells with a CLP model, in order to investigate the role of endothelial cells in tissue damage during sepsis.

Institute of Surgical Research , General Hospital of PLA, Beijing 100853, China

Dr. Rong Qian Wu, graduated from General Hospital of PLA as a M.D. in 1999, now attending surgeon of general surgery, major field of research in sepsis and MODS, having 15 papers published.

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Correspondence to: Dr. Rong Qian Wu, Institute of surgical Research, General Hospital of PLA, Beijing 100853, China

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MATERIALS AND METHODS

Animal model and CLP

NIH mice were obtained from the animal center of the General Hospital of PLA. The mice were randomly divided into 2 groups: CLP group and sham group. Sepsis was induced in the CLP group by CLP. The mice were anesthetized, and the cecum was ligated below the ileocecal junction: intestinal continuity was maintained. The cecum was punctured twice with a 20-gauge needle and a small amount of cecal contents was expressed through the punctures. The incision was closed and 1 mL of normal saline was administered subcutaneously. Sham-operated mice underwent the same surgical procedure, but without CLP. The mice were sacrificed at 3 or 12 h after the procedure.

Isolation and purification of hepatic sinusoidal endothelial cells

Hepatic sinusoidal endothelial cells were isolated by collagenase perfusion of the liver, isopyknic sedimentation in a two-step percoll gradient, and selective adherence [15]. The purified sinusoidal endothelial cells were identified by staining with anti-von willebrand factor (vWF, factor VII I-related antigen). Flow cytometric analysis showed a purity greater than 85% in hepatic sinusoidal endothelial cells.

Analysis of adhesion molecules and proinflammatory cytokines mRNA by reverse transcription-PCR

Total RNA was extracted from endothelial cells. We used a phenol-chloroform extraction method reported by Chomczynski. The RNA was then quantitated spectrophotometrically. Total RNA from experimental samples was used to synthesize cDNA using AMV reverse transcriptase. β-actin and β_2-MG were used as internal control primers. The primers for the adhesion molecules and controls were as follows: β- actin (478bp), 5'AGG GAA ATC GTG CGT GAC ATC AAA 3', 5'ACT CAT CGT ACT CCT GCT TGC TGA 3'; β_2-MG (300 bp), 5'GGC TCG CTC GGT GAC CCT AGT CTT T 3',

5'TCT GCA GGC GTA TGT ATC AGT CTC A 3'; VCAM-1 (442bp), 5'CCT CAC TTG CAG CAC TAC GGG CT 3', 5' TTT TCC AAT ATC CTC AAT GAC GGG 3'; ICAM-1 (326bp), 5'TGC GTT TTG GAG CTA GCG GAC CA 3', 5' CGA GGA CCA TAC AGC ACG TGC AG 3'; E-selectin (435bp), 5'CCT GAA CTG CTC CCA CCC GTT CG 3', 5' GTG AAG TTA CAG GAT GAC TTA AAC GCA 3'; TNF-α (349 bp), 5'TTC TGT CCC TTT CAC TCA CTG G3', 5'TTG GTG GTT TGC TAC GAC GTG G 3' IL-1β (441bp), 5'ATT AGA CAG CTG CAC TAC AGG CTC 3', 5'AGA TTC CAT GGT GAA GTC AAT TAT 3' IL-6 (156bp), 5'TGG AGT CAC AGA AGG AGT GGC TAA G 3', 5'TCT GAC CAC AGT GAG GAA TGT CCA C 3'. Polymerase chain reactions were performed in a 25 μL reaction volume. A hot start was applied for 5min at 95℃. The amplification cycle (denaturation step at 94℃ for 30s, an annealing step at 55℃ for 30s and an extension step at 72℃ for 90s) was repeated 30 times and followed by a final extension for 10 min at 72℃. Amplified products were separated by electrophoresis in ethidium bromide-stained 1.5% agarose gel and visualized with UV illumination. The bands representing reaction product on the film were scanned by densitometry. A normalization quotient (Q) was calculated between the integrated optical density values (IOD) for the adhesion molecules and the β-actin or β_2-MG bands (Q = IOD, adhesion molecules band/internal control band). The level of adhesion molecules mRNA were expressed as the quotient of the integrated optical density values for the adhesion molecules and the β-actin or β_2-MG bands.

Statistical analysis

All data were reported as means ± SD. Data were analyzed by *t* test for comparisons between the two groups. A *P* value of less than 0.05 was deemed significant.

RESULTS

Adhesion molecules mRNA expression in hepatic sinusoidal endothelial cells

E-selectin mRNA levels markedly increased at 3 h after CLP in hepatic sinusoidal endothelial cells, and returned to baseline at 12 h after CLP. Increases in ICAM-1 mRNA level was found at 3 h after CLP, and this level became higher at 12 h after CLP. VCAM-1 mRNA expression in hepatic sinusoidal endothelial cells increased significantly 3h after CLP but declined at 12h after CLP (Table 1).

Table 1 Adhesion molecules mRNA expression in hepatic sinusoidal endothelial cells ($x \pm s$)

	E-selectin		ICAM-1		VCAM-1	
	3h	12h	3h	12h	3h	12h
Sham	0.22 ± 0.04	0.23 ± 0.04	0.26 ± 0.03	0.30 ± 0.05	0.37 ± 0.04	0.30 ± 0.05
CLP	0.85 ± 0.06[b]	0.24 ± 0.03	0.67 ± 0.04[b]	1.02 ± 0.10[b]	1.04 ± 0.14[b]	0.86 ± 0.05[b]

[b]$P<0.01$ *vs* sham group.

Proinflammatory cytokines mRNA expression in hepatic sinusoidal endothelial cells

A significant increase in TNF, IL-1 and IL-6 gene expression was observed at 3 and 12 hours after CLP. The level of TNFα and IL-1β at 3 hours was higher than 12 hours, and the level of IL-6 gene expression at 12 hours was higher than 3 hours (Table 2).

Table 2 Proinflammatory cytokines mRNA expression in hepatic sinusoidal endothelial cells ($x \pm s$)

	TNFα		IL-1β		IL-6	
	3h	12h	3h	12h	3h	12h
Sham	0.23 ± 0.04	0.22 ± 0.04	0.30 ± 0.04	0.39 ± 0.06	0.47 ± 0.05	0.49 ± 0.05
CLP	0.71 ± 0.03[b]	0.54 ± 0.07[b]	1.02 ± 0.12[b]	0.78 ± 0.08[b]	0.90 ± 0.05[b]	1.11 ± 0.14[b]

[b]$P<0.01$ *vs* sham group.

DISCUSSION

The liver, with its rich supply of blood and sinusoid, is directly exposed to bacteria and endotoxins drained from the GI tract[16-19]. Previously, researchers in our institute have reported that the liver is the most susceptible and vulnerable organ during sepsis and multiple organ failure [20].

Vascular endothelial cells form an interface between tissues and inflammatory cells. This unique location allows localization of the inflammatory reaction to the site of injury while protecting adjacent healthy tissue. Endothelial cells mediate the local inflammatory response through modulation of vascular tone, vascular permeability and stimulation of leukocyte extravasation[21,22]. The role of neutrophil extravasation and accumulation has been emphasized in recent years[23,24]. This is mediated by the induced expression of multiple cell adhesion molecules on the surface of neutrophils and endothelial cells[25,26]. These include the selectins which are a group of surface glycoproteins essential to leukocyte margination and rolling along the vascular endothelium. Specifically, endothelial E- and P- selectin and L-selectin are expressed on neutrophils[27]. Another group of cell adhesion molecules involved in endothelial cell-leukocyte interaction is the immunoglobulin supergene family. This group comprises intercellular adhesion molecules-1 and 2 (ICAM-1, ICAM-2), vascular cell adhesion molecules-1 (VCAM-1)[28]. The most well studied of these molecules is ICAM-1, which play a critical role in events subsequent to initial leukocyte margination. In this study, we found that the up-regulation of the expressions of adhesion molecules in liver sinusoidal endothelial cells is a crucial step for the migration of leukocytes to and accumulation at the site of inflammation. Although neutrophils are important for killing microorganisms, activation of recruited neutrophils coupled with excessive release of oxygen metabolites and proinflammatory mediators may induce tissue injury which can lead to organ dysfunction[29,30].

Cytokines are polypeptides or glycoproteins of low molecular weight. Most cytokines are not stored as preformed molecules, hence their production requires new gene transcription and translation. Unlike mediators derived from the classical endocrine system, cytokines are produced. The production of cytokines at various tissue sites depends, in part, on the proximity of the site to the injurious stimulus. In this study we assessed the gene expression of proinflammatory cytokines in hepatic sinusoid endothelial cells. Recent discoveries in the field of endothelial cell biology have shown its capability of production cytokines[31-33], but the role of endothelial cell-derived cytokines in sepsis induced tissue injury was largely ignored. Proinflammatory cytokines TNF and IL-1 are known to play predominant roles in the normal inflammatory response. Exaggerated endogenous production is likely responsible for the complications associated with sepsis such as tissue injury and ultimate organ failure[34-36]. We observed a significant increase in TNF and IL-1 gene expression shortly after the induction of sepsis. This indicates that endothelium is an important source of cytokines during sepsis, and may play a role in sepsis induced organ dysfunction. The consensus of the concept of systemic inflammatory responses has brought about a likely promising approach in the treatment of SIRS/MODS[37] -anti-inflammatory instead of anti-infection[38,39]. Various approaches aimed at interrupting the cascade of host inflammatory responses have been tested. These include interventions targeted at the inflammation effector cells as monoclonal antibodies or receptor antagonist to pro-inflammatory cytokines. However, many of these seemingly effective measures in experimental study failed when moved from the laboratory bench to clinical ward [40-44].

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Edited by Jason Carr

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effect of 5-2Aza-2'-deoxycytidine on the P16 tumor suppressor gene in hepatocellular carcinoma cell line HepG2

Li Hua Liu[1], Wen Hua Xiao[2] and Wei Wen Liu[3]

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies world wide[1,2], and is closely associated with infection of HBV and HCV and contamination of aflatoxin B1[3-6]. Although the molecular mechanisms of hepatocarcinogenesis remain poorly understood, an increasing number of genetic abnormalities have been recognized[7-10], for example, the *p*16 gene[11,12] the p53 gene[13-18], the E-cadherin gene[19], and the c-myc gene[20]. In relevance to HCC, inactivation of a tumor suppressor gene *p*16, which is important in the regulation of cell cycling, has recently been described in a significant proportion of patients with HCC[11]. An important mechanism of gene inactivation is aberrant methylation in the promoter region of the gene[11,21,22]. Evidence of p16 hypermethylation has also been documented in several human cancers[21-26]. However, to our knowledge, there were few studies on the *p*16 gene in HCC. 5-Aza-2'-deoxycytidine (5-Aza-cdR) is thought to act by inhibiting DNA methyltransferase that methylates cytosine residues in eukaryotic DNA[27]. Cedar *et al*[28] even took it as an experimental tool for demethylation. This drug appears to incorporate into the cellular DNA where it acts as a noncompetitive inhibitor of the maintenance methylase[29]. Methel moieties are thus passively removed when the DNA undergoes replication, and indeed, numerous inactive cellular and viral genes can be turned on following exposure to 5-Aza-cdR[30]. In order to determine whether *p*16 hypermethylation is involved in hepatocarcinogenesis, we determined the *p*16 gene protein, mRNA expression and methylation status in HCC cell line HepG2 before and after treatment with 5-Aza-cdR, and observed the growth change.

MATHERIALS AND METHODS

Cell line culture

Human hepatocellular carcinoma cell line HepG2 was obtained from the Laboratory of Gastroenterology, Southwest Hospital. It was cultured in RPMI 1640 supplemented with 100 mL/L fetal bovine serum, 100KU/L penicillin/ streptomycin and 5% sodium pyruvate at 37℃ in humidified atmosphere of 50 mL/L CO_2.

5-Aza-CdR treatment

Cells (approximately 1×10^5/mL) were plated in the vial, and treated with 5×10^{-7}M 5-Aza-cdR (Sigma Co.) for 24 h. The medium was changed after 24 h drug treatment and cultured for 9 days, to ensure complete recovery from the immediate toxic effects of 5-Aza-cdR[31]. Untreated cells were analyzed under similar conditions as a control.

Determination of cell cycle profile

Cells (2×10^6/100 mL vial) were plated and treated with 5×10^{-7} M 5-Aza-cdR. Cells were fixed after 7 days with 700 mL/L ethanol, and stained with propidium iodide (50 mg/L: Crze Co.). DNA content at each cell cycle stage was determined via flow cytometry.

Effects of 5-Aza-CdR on tumorigenicity

HepG2 cells (2×10^6) were injected into the right flank of 4-week-old Balb/c male nude mice 11 days after treatment with 5-Aza-cdR. Untreated cells were injected into the left flank under the same condition as a control. Tumor sizes were measured 4 weeks after injection.

[1]Department of Oncology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China (now working in Department of Gastroenterology, General Hospital of PLA, Lanzhou 730050, Gansu Province, China)
[2]Department of Oncology,[3]Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
Dr. Li Hua Liu, graduated from Third Military Medical University as a postgraduate in 2000, associate professor of gastroenterology,majoring in pathology of hepatocellular carcinoma, having 5 papers published.
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Correspondence to: Wen Hua Xiao, Department of Gastroenterology, 304th Hospital of PLA, Beijing 100037, China
Tel. 0086-10-66867321
Email. wenhua_x@mailcity.com
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Immunohistochemistry

Cells (1×10^6/3 cm dish) were cultured onto acid-washed 0.8 × 0.8 (mm) coverslips. The cells were fixed in 4% parafomaldehyde for 30 min at room temperature. The slides were washed in phosphate-buffered saline (PBS) three times for 3 minutes. The endogenous peroxidase activity was quenched by 10 minute incubation in methanol with 3% hydrogen peroxide (Zhongshan Chemical Co.). The fixation slides were then cleared with PBS. Nonspecific binding was blocked by applying normal goat serum in humidity chamber in a dilution of 1:10

for 30 minutes. Slides were blotted, and the anti-entire-human p16 antibody (Zhongshan Chemical Co.) (ready to use) as a primary antibody was applied for 1 hour at 37℃ in a humidity chamber. Secondary antibody (rabbit anti-mouse IgG) (Zhongshan Chemical Co.) diluted with PBS and normal human serum (40 μL rabbit anti-mouse antibody, 50 μL normal human serum, and 910 μL PBS) was applied for 1 hour at 37 ℃ in a humidity chamber. Finally, peroxidase-antiperoxidase conjugate diluted 1:100 in PBS was applied for 1 hour at 37℃ after washing with PBS. Slides were kept in diaminobenzidine tetrahydrochloride for 10 minutes (50 mg in 200 mL PBS with 25 μL of 30% H_2O_2) (DAB; Zhongshan Chemical Co.) . The manufacturer provides the presence (+) and absence (-) controls. The expression of p16 gene was observed under the light microscope and analyzed by the figure analysis method.

RT-PCR

RNA was isolated using Tripure isolation reagent (Boehringer Mannheim Co.). RNA was reverse transcribed according to the manufacturer's instructions of the access RT-PCR system (Promega Co.). cDNA was amplified using primers specific for both the p16 gene and GAPDH gene, the latter being used as a control. The primers used for *p*16 amplifications were located in exon 1 and exon 2 of the gene. Their sequences were 5'-AGCCTTCGGCTGACTGGCTGG-3'(sense) and 5'-CTGCCCATCATCATGACCTGGA-3'- (antisense). (Shanghai Sheng Gong Chemical Co., PAG pure). Standard PCR reaction was performed in total volume of 25 μL reaction mixture, including dNTP 0.2mM, 1 mM each primer 1 (sense) and Primer 2 (antisense), 1 mM $MgSO_4$, 0.1 U/mL of reverse transcriptase AMV, 0.1U/mL of Taq DNA polymerase and 100ng RNA. Conditions for *p*16 amplifications were 94℃ for 2 min, 40 cycles of 94 ℃ for 30s; 60℃ for 45s; and 68℃ for 90s , followed by incubation at 68℃ for 7 min. RT-PCR amplification product is 428 base pair[bp]. Primers for the GAPDH gene were 5'-CCACCCATGGCAAATTCCATGGCA-3' (sense), and 5'-TCTAGACGG CAGGTCAGGTCCAC-3'. (antisense) Conditions for GAPDH amplifications were 94℃ for 2 min, 40 cycles of 94℃ for 30s , 60℃ for 40s and 68℃ for 60s, followed by incubation at 68℃ for 7min. The amplification product is 598 bp. RT-PCR products were resolved on 2% agarose gel by figure analysis method.

PCR-based methylation assay

DNA was isolated with the Tripure isolation reagent. A PCR assay relying on the inability of some restriction enzymes, e.g. Hpa II or *Msp* I, to cut methylated sequences was used to analyze the methylation status of the second exon of the *p*16. DNA digests were performed according to the manufacturer's directions (Boehringer Mannheim Co.). DNA (1 μg) was digested for 2 h with 10 units of enzyme/μg of DNA. 200 ng of the digested DNA were amplified with primers flanking the restriction sites. The primers set used for methylation analysis of *p*16 exon 2 were 5'-CTGCTTGGCGGTGAGGGGG-3'(sense) and 5'-CCTCACCTGAGGGACCTTC-3'(antisense). The amplifiction products was 402 bp. Conditions were: 94 ℃ for 3 min, 35 cycles of 94℃ for 1 min, 57℃ 30s, and 72℃ for 40s, followed by incubation at 72℃ for 1 min. PCR products were resolved on 2% agarose gel. To rule out the possibility of incomplete restriction, all samples were digested twice with each of the enzymes in independent experiments. PCR ampifications from each of the duplicate digests were repeated at least twice to ensure reproducibility of the results.

Statistical analysis

t tests were used for statistical analysis. Significance was defined as $P<0.05$.

RESULTS

Cell cycle arrest after treatment with 5-Aza-cdR

HepG2 cells treated with 5-Aza-cdR were analyzed by flow cytometry to identify any change in its cell cycle profiles. The treated cells showed an increase in the proportion in G1,from 79.4% to 82.2% and a decrease in S, from 11.9% to 6.4%. Apoptotic rate increased from 6. 5% to 14.5%. These results indicated that 5-Aza-cdR may induce cancer cell division and apoptosis.

Effects of 5-Aza-cdR on tumorigenicity

Tumors induced via the injection of untreated HepG2 cells were approximately 1.32 cm^3 in size, whereas the tumors induced by HepG2 cells treated with 5-Aza-cdR averaged 0.98 cm^3 in size. These demonstrated that the growth-inhibitory effects of 5-Aza-cdR treatment *in vivo* were still recognizable.

Effect of 5-Aza-cdR on P16 protein expression

The positive 5-Aza-cdR-treated cells showed intense

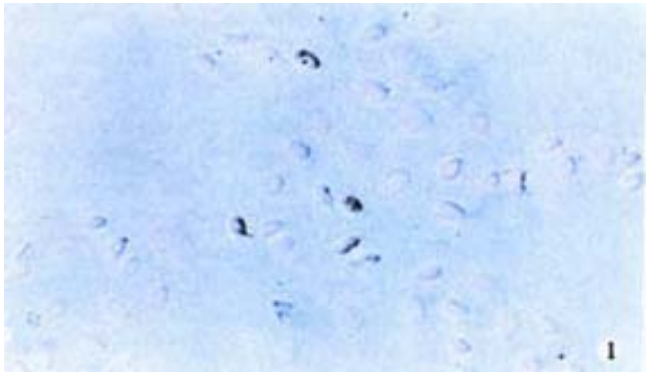
homogenous brown staining of their nucler. After treatment with 5×10^{-7} M 5-A2a-cdR, HepG2 showed a higher expression of P16 protein than untreated cells. We observed under light microscope 5 fields in every slide at random, measured the staining degree of every 124 positive cells by the figure analysis method, IOD was 212.81 ± 62.56 and 495.89 ± 52.12 respectively in the untreated group and the treated group. This showed a significant difference (P<0.05). Although *P*16 protein expression alternations are very complicated and still undefined, our data showed that *P*16 protein expression could be silenced by hyper methylation of *p*16 gene (Figures 1,2).

Effect of 5-Aza-CdR on p16 mRNA expression

Figure 3 shows that expression level of *p*16 mRNA in HepG2 cells was increased after treated with 5×10^{-7} M 5-aza-cdR. The *p*16 gene, was selected for this study, because it was known to frequently undergo *de novo* methylation during tumorigenesis and because 5-Aza-cdR might be highly effective in inducing the expression of the gene inappropriately silenced by *de novo* methylation[18]. These results demonstrated a strong correlation between hypermethylation of the *p*16 and transcriptional silencing of this gene.

Effect of 5-Aza-CdR on p16 gene methylation status

Before 5-aza-cdR treatment the *p*16 exon2 showed hypermethylation, and therefore could not be deaved by loth HpaII and *Msp* I. It has been known that only the DNA indigested with *Hpa* II or *Msp* I can be amplified. After treated with 5-aza-cdR, the *p*16 gene became demethylated, then could be deaved by loth of the enzymes, and this failed to be emplified. Our experiment demonstrated that after hypermethylation in the *p*16 exon2 and 5-aza-cdR could enhance demethylation (Figure 4).



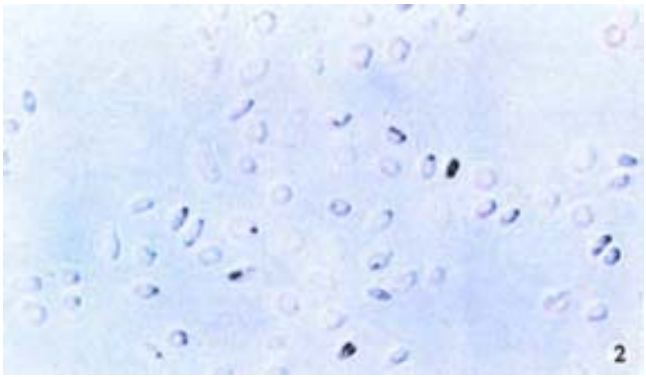


Figure 1 Immunohistochemical demonstration of p16 protein expression in HepG2 cell line before treatment with 5-Aza-cdR. SABC × 200
Figure 2 Immunohistochemical demonstration of p16 protein expression in HepG2 cell line after treatment with 5-Aza-cdR. SABC × 200

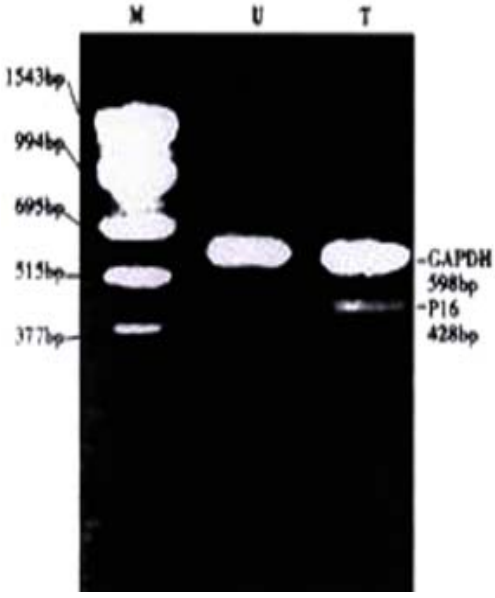


Figure 3 Effects of 5-Aza-cdR on the p16 mRNA expression in HepG2 cell line. Cells were treated with 5×10^{-7} M 5-Aza-cdR for 24 hours; after culturing for 9 days, p16 mRNA were determined by RT-PCR. U: untreated T: treated with 5-Aza-CdR

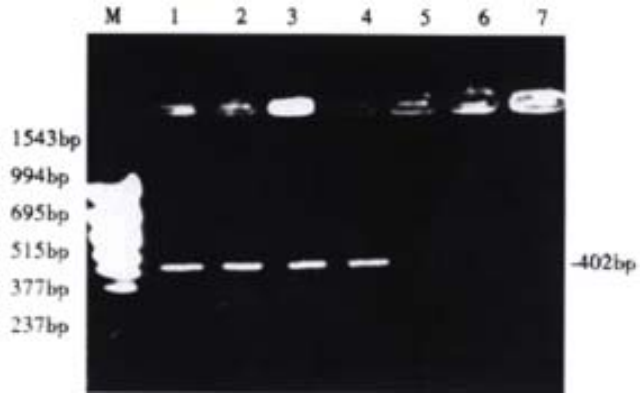


Figure 4 Detection of aberrant methylation of the p16 gene in HCC cell line HepG2. *Lane M*: molecular weight marker; *Lane 1*: Neither treated with 5-aza-cdR nor digested with *Hpa* II or *Msp* I; *Lane 2*: Untreated with 5-aza-cdR, but digested with *Hpa* II; *Lane 3*: Untreated with 5-Aza-cdR, but digested with *Msp* I; *Lane 4*: Treated with 5-aza-cdR, but not digested with *Hpa* II or *Msp* I; *Lane 5*: Treated with 5-aza-cdR, and digested with *Hpa* II; *Lane 6*: Treated with 5-aza-cdR, and digested with *Msp* I.

DISCUSSION

HCC is one of the most frequent human malignances worldwide. A number of tumor suppressor genic abnormalities are being recognized involving in the development of HCC[7-20]. The tumor-suppressor gene, p16,coding for an inhibitor of cyclin-dependant kinase (cdk) 4, was isolated and mapped to chromosome 9p21 [32,33], a locus frequently lost in diverse malignancies [34]. The *p*16 gene product p16 protein was originally isolated as a protein that was associated with cdk complexes[35] and thought to exert a negative type of control on cell proliferation through its binding to cdk4, thereby preventing cdk4 from forming an active complex with cyclin D protein[36]. As one of cancer-associated genes, the p16 gene abnormalities is arousing more and more attention. Germ-line mutations of the p16 gene have been identified in hereditary melanomas[37]. In addition,the gene is homozygously deleted or mutated in uncultured tumors of diverse origin[38], including pancreatic adenocarcinomas[39], melanomas[37], esophageal carcinomas[40,41], gliomas [42]lung cancer[43] and hepatocellular carcinoma[44]. Recently, it has been reported that inactivation of the *p*16 gene is frequently associated with aberrant DNA met hylation in all common human cancers[26]. However, there have been relatively few studies on expression of *p*16 in HCC.

Sun *et al*[45] found that DNA methyltransferase (MTase) mRNA levels were significantly higher in HCC and HCC cell lines (including HepG2 cell line), but they did not study the related genic methylation. Wong *et al*[46] demonstrated that aberrant methylation of the p16 gene occurred in 73% of HCC tissues. Hui *et al*[44] studied six HCC cell lines and found no p16 mRNA and protein in only one of them. After ruling out mutation and homozygous deletion of the p16 gene, they guessed that the silence of p16 gene might be associated with enhanced methylation of p16 gene, although the methylation status was not tested. However, p16 mRNA was detected in the other five HCC cell lines, and they thought that the lack of p16 gene transcription was not a frequent event in HCC cell line. The absence of p16 protein and presence of p16 mRNA expression strongly suggested that inactivation of p16 gene function could occur at the posttranscriptional level, but, the presence of expressions of both *p*16 mRNA and protein in HepG2, could not be explained. Boyes *et al*[47] discovered that the inhibition of gene transcription correlated with density of DNA methylation and the length of the promoter region. Hsieh[48] also found that the expression of gene transcription depended on CpG island methylation density; lower levels of methylation yielded a 67% to 90% inhibition of gene expression, higher levels of methylation extinguished gene expression completely. Numerous investigations described reactivation of genes by 5-Aza-cdR by acting on various loci of the inactive X chromosome[49], the VHL gene[50], the E-cadherin gene [51], the estrogen receptor gene[52] and the p16 gene[31]. In order to detect similar methylation changes in HepG2 cell line, we also used 5-Aza-cdR.

In this study, HepG2 cells treated with 5-Aza-cdR, showed demethylation of the p16 gene. The p16 mRNA and protein were all increased dramatically, cell cycle was arrested in G1, apoptototic rate increased and implanted tumor grew more slowly. According to our results, we thought that: p16 hypermethyla tion is associated with HCC cell line; MTase is increased in HepG2 cell line; 5'CpG island of p16 promoter was not methylated completely in this HCC cell line; p16 gene can be reactivated by inhibiting the activities of MTase; p16 methylation may be a common event during the establishment of the cell line *in vitro*; and p16 gene encodes a cell cycle regulatory protein that belongs to the cyclin-dependent kinase inhibitory protein family and regulates the G1/S phase cell cycle transition, and therefore, reactivated p16 may affect the growth of the HCC cell line. Xiao *et al*[53,54] found that 17p13.3 CpG island, PYN22.1, Rb gene all showed hypermethylation, we guess that 5-Aza-cdR may reactivate those genes.

The induction of p16 activation by 5-Aza-cdR *in vivo* and the decreased tumorigenicity in animal experiment suggest the chemotherapeutic potential of 5-Aza-cdR in the management of cancer.

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Edited by Lu HM
proofread by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Methylation status of c-fms oncogene in HCC and its relationship with clinical pathology

Jun Cui, Dong Hua Yang, Xiang Jun Bi and Zi Rong Fan

Subject headings carcinoma, hepatocellular/pathology; oncogenes; DNA methylation; receptors, colony-stimulating factor; genes, fms; gene expression

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INTRODUCTION

The mechanism that DNA hypomethylation leads to activation of oncogene and occurrence of malignant neoplasm is being increasingly recognized by researchers. Normal DNA methylation plays important role in stabilizing the phenotype of cell. DNA methylation status reduction and/or pattern alteration are related to activation and abnormally high expression of some oncogenes and cellular malignancy [1-6]. c-fms oncogene encodes for colony stimulating factor 1 receptor (CSF-1R)[7], c-fms/CSF-1R was highlyexpressed in hepatocellular carcinoma (HCC) tissue, but the mechanism remained obscure[8,9]. In this study, restrictive endonucleases *Hpa* II/*Msp* I digestion and Southern blot were used to study methylation status alteration of c-fms oncogene in HCC tissue and matching circum-cancer liver tissue, meanwhile the relationship between the alteration and clinical pathology of HCC was investigated. The gist of this study was to clarify the mechanism leading to c-fms oncogene high expression in hepatocellular carcinogenesis.

MATERIALS AND METHODS

Subjects

Thirty HCC patients were verified with pathological examination (25 males and 5 females, age range 32 -76 years, mean 55 years). Fresh hepatic tissue of HCC focus and circum-tissues 2 cm from HCC focus were cut off, normal control was from HBV negative hepatic tissue of renal transplantation donor. All the tissues were washed with physiological saline and put in freezing tube, and then they were put in liquid nitrogen for storage.

Main reagents

Restrictive endonucleases *Hpa* II/*Msp* I were purchased from Japan TaKaRa Bio-Company. DIG random labeling and detection kit were purchased from Boster Bio-Company. Nylon transfer membranes used for Southern blot were purchased from Shanghai Bio-Company. c-fms plasmid was kindly provided by academician Gu Jianren in the National Laboratory for Oncogene and Related Genes, Shanghai Cancer Institute.

METHODS

DNA extraction About 0.5g hepatic tissue was triturated in mortar, and a dissolvent (10 mmol/L Tris-Cl pH 8.0,10 mmol/L EDTA pH 8.0, 0.5% SDS) 500 μL was added. The preparation was digested with proteinase K and RNase, then it was extracted twice with saturated hydroxybenzene and once with chloroform isoamyl alcohol (chloroform:isoamyl alcohol = 24:1), and at last 2.5 volumes of absolute alcohol and 0.1 volume of 3 mol/L sodium acetate (pH 5.2) were added to precipitate the DNA. The extracted DNA was dissolved in TE buffer (pH 8.0), and DNA concentration was detersmined with an ultraviolet spectrophotometer.

Probe labeling

Plasmid extraction, restrictive endonucleases digestion, reclamation and purification were performed as the methods recorded in Molecular Cloning. The probe was labeled with DIG random labeling and detection kit.

Southern blot

Genome DNA 10 μg was digested with restrictive endonucleases Hpa II and *Msp* I 50U each for 12 hours respectively. Digested DNA was examined with 0.8% agarose electrophoresis. The buffer used for electrophoresis was 0.5 × TBE. Electrophoresis was ended when 2 kb marker shifted to the middle of the gel. Photo was taken under ultraviolet ray transmission. The gel was immersed in 0.25 mol/L

Department of Gastroenterology, Zhujiang Hospital, The First Military Medical University, Guangzhou 510282, Guangdong Province, China
Jun Cui, graduated from North China Coal Medical College in 1994 and got bachelor degree, graduated from China Medical University in 1997 with master degree, worked in Yantai Yu Huang-Ding Hospital, came to the First Military Medical University in 1998 to continue the study of doctor degree. Now physician-in-charge, lecturer, specialized in the genetic diagnosis and therapy for hepatocellular carcinoma, having 10 papers published.
Supported by the Natural Science Foundation of Guangdong Province, No 990422
Correspondence to: Jun Cui, Department of Gastroenterology, Zhujiang Hospital, The First Military Medical University, Guangzhou 510282, Guangdong Province, China
Tel. 0086-20-85143369
Email. Fanzr@fimmu.edu.cn
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HCl for 10 minutes, washed with distilled water for 20-30 minutes, and then immersed in denaturalizing liquid twice for 15 minutes each time. The gel was again washed twice with distilled water, and then it was immersed into neutralizing liquid twice for 15 minutes each time. A piece of nylon membrane of the same size as the gel was cut, it was immersed into 2 × SSC for 20 minutes. Transfer was performed for 18 hours at room temperature by the capillary transfer method. Then the nylon membrane was taken out and washed several times with 2 × SSC. After this, it was fixed for 20 minutes under long wave ultraviolet radiation. The transferred nylon membrane was sealed in a plastic bag and then pre-hybridizing reagent was perfused into the bag. The bag was put in a 42℃ water bath for 2 hours, and then pre-hybridizing reagent was decanted off. The bag was sealed, after the hybridizing reagent containing the DIG-labeled probe had been put in. The bag was put into a 42℃ water bath and hybridized for 20 hours. After hybridization the nylon membrane was taken out, and showed coloration according to the instruction of the DIG random labeling and detection kit.

Pathological examination and classification

The tumors were variously classified into unifocal or multifocal, and massive or nodular (a main tumor mass with satellites). The size and inner-condition of the tumors were recorded. The histological grading was based on Edmondson standard I-IV scale.

RESULTS

Southern blot of HCC tumor tissue and circum-cancer liver tissue

Hpa II and *Msp* I are isoschizomers. Both can digest CCGG sequence, but when the sequence is methylated into C-mCGG, only *Msp* I can digest while *Hpa* II can not. Methylation status of the gene may thus be determined by relying on such a difference. The data in this study showed that after digestion with *Hpa* II, hybridizing bands of less than 6.5kb in size were observed in 36.7%(11/30) of HCC tissue and 13.3%(4/30) of circum-cancer liver tissue, which resembled those of the hybridizing bands digested with *Msp* I. The results indicated that the methylation status of c-fms oncogene in the tissue was decreased (Figures 1,2).

Methylation status comparison of c-fms oncogene in HCC tissue and circum-cancer liver tissue

Hypomethylation rate of c-fms oncogene in HCC tissue and circum-cancer liver tissue was compared, the difference was determined, the result is shown in Table 1.

Table 1 Methylation status of c-fms oncogene (number of patients)

Methylation status	HCC tissue	Circum-cancer liver tissue
Normal methylation	19	26
Hypomethylation	11	4

Note: χ^2=4.36, P<0.05, hypomethylation rate of c-fms oncogene in HCC tissue was higher than that in circum-cancer liver tissue.

Relationship between hypomethylation of c-fms oncogene and clinical pathology

The relationship between hypomethylation of c-fms oncogene in hepatic tissue and the sex, age and pathological Edmondson scale of patients was analysed; the results are shown in Table 2.

Table 2 Relationship between hypomethylation of c-fms oncogene and clinical pathology

Item		Total number	HCC tissue		Circum-cancer liver tissue	
			Number	%	Number	%
Sex	Male	25	10	41.0	3	12.0
	Female	5	1	20.0	1	20.0
Age(yrs)	<60	22	7	31.8	2	9.1
	⩾60	8	4	50.0	2	25.0
Edmondson	I-II	14	2	14.3	1	7.1
scale	III-IV	16	9	56.3[b]	3	18.8

[b]P<0.05, *vs* Edmondson I-II.

Chi-square test showed that there was no significant relationship between hypomethylation of c-fms oncogene and sex and age of patients (P>0.05). There was significant relationship between Edmondson scale and hypomethylation of c-fms oncogene in HCC tissue (χ^2=5.66, P<0.05), while there was no significant relationship between Edmondson scale and hypomethylation of c-fms oncogene in circum-cancer liver tissue(P>0.05).

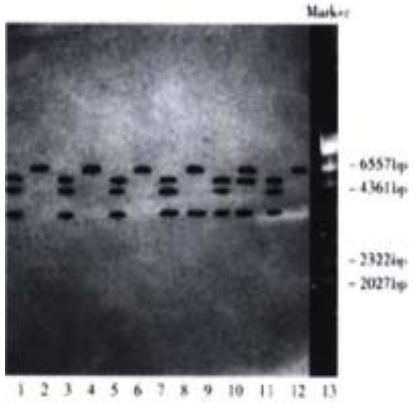

Figure 1 Southern blot of HCC tissue genome DNA digested with *Hpa* II / *Msp* I.
Lane 1, 3, 5, 7, 9, 11 *Msp* I digestion;
Lane 2, 4, 6, 8, 10, 12 *Hpa* II digestion;
Lane 13 λDNA / *Hind* III markers;
Lane 1, 2 normal control hepatic tissue;
Lane 3-12 HCC tissue;
Lane 8, 10 hybridizing bands of less than 6.5kb in size appeared after *Hpa* II digestion

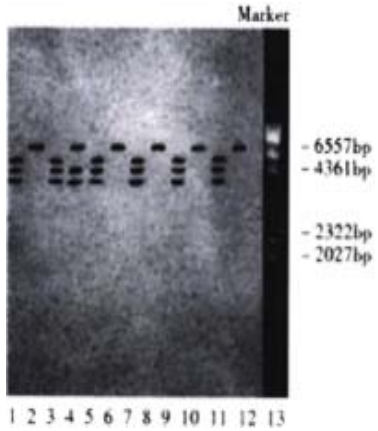

Figure 2 Southern blot of circum[a2]cancer liver tissue genome DNA digested with *Hpa* II / *Msp* I. Lane 1, 3, 5, 7, 9, 11 *Msp* I digestion; Lane 2, 4, 6, 8, 10, 12 *Hpa* II digestion; Lane 13 λDNA / *Hind* III markers; Lane 1, 2 normal control hepatic tissue; Lane 3-12 circum-cancer liver tissue; Lane 4 hybridizing bands of less than 6.5kb in size appeared after *Hpa* II digestion

DISCUSSION

In the process of cellular carcinogenesis, genetic and epigenetic mechanism contributes to abnormal expression of gene. The genetic mechanism involves the mutation and chromosome rearrangement, with which genetic products of abnormal molecular structures can be produced[10-19]. The epigenetic mechanism mainly refers to an altered methylation status of 5'-cytosine in the DNA sequence, which leads to an abnormal expression of gene in quantity, but no change in DNA sequence and genetic product [20-24]. In the process of cellular carcinogenesis, the methyl groups in CpG islands of DNA are lost gradually. Although the accurate mechanism between DNA hypomethylation and cellular carcinogenesis remains obscure, some data showed that DNA hypomethylation could affect chromatin condensation in the middle stage, which led to a change and/or rupture of chromosome[25-31]. DNA hypomethylation is a kind of molecular structure leading to abnormal expression of gene; it facilitates a high expression of gene[32]. Highly specific DNA methylation types exist in human genomes, and are reflected in genetic characteristics of DNA, such as transcription, duplication, recombination, transposition and mutation. One biological function of human genome DNA methylation is to keep promoter of gene in silent status for long. 5' methyl-cytosine in special DNA sequence can mediate the specific combination and interaction between DNA and some functional proteins. As the interactions locate in the core region which regulates biological function of gene, sequence-specific methylation of DNA can affect cellular function[33-39]. Furthermore, DNA methylation depends on combining special proteins, and it directly or indirectly induced conformational alterations of DNA. DNA methylation is a kind of modificatory mode of cellular DNA at transcription level. In human genome, CpG islands in house keeping genes including a great number of oncogenes are in the form of methylation. Transcription and expression of the methylated genes especially oncogenes are limited. Decline of DNA methylation status at special sites of oncogene induces abnormal expression of the gene[28,40-43].

c-fms oncogene locates at 5q 33.3 of human chromosome and encodes CSF 1R, which has tyrosine kinase activity[44]. c-fms/CSF-1R is highly expressed in hepatocellular carcinogenesis, and its expressing level in HCC tissue is higher than that in circum-cancer liver tissue. Through the mechanism of signal transduction, CSF-1R stimulates the growth and development of HCC [45-50]. The whole length of c-fms oncogene is 43kb, in which there are 49 CCGG sites. The data in this study indicated that the hypomethylation rate of c-fms oncogene in HCC tissue was higher than that in circum-cancer liver tissue, while no hypomethylation of c-fms oncogene in normal hepatic tissue was observed. So hypomethylation is an important activating mechanism of c-fms oncogene in hepatocellular carcinogenesis. The data also showed that the hypomethylation rate of c-fms oncogene in Edmondson III-IV HCC was higher than that in Edmondson I-II HCC. All the evidence indicated that hypomethylation of c-fms oncogene might be an important late genetic incident in hepatocellular carcinogenesis. HCC with hypomethylation of c-fms oncogene may show the characteristics of higher malignancy and rapid development, and provide a clinical guide in the selection of surgery, radiotherapy and chemotherapy, and also in judging the prognosis of patients.

c-fms/CSF-1R is abnormally high-expressed in hepatocellular carcinogenesis at a late stage, meanwhile hypomethylation of c-fms oncogene occurs, and the hypomethylation rate of c-fms oncogene in HCC tissue is higher than that in the circum-cancer liver tissue. These data indicate that hypomethylation of c-fms oncogene is a kind of molecular mechanism leading to abnormally high CSF-1R expression and promoting the occurrence and development of HCC. So blocking the process of hypomethylation of c-fms oncogene, consequently decreasing a high expression of CSF-1R may have great clinical significance in decreasing malignant phenotype of HCC and improving prognosis of patients.

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Edited by Lu HM
proofread by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Influencing factors of rat small intestinal epithelial cell cultivation and effects of radiation on cell proliferation

Xin Ze Ran, Yong Ping Su, Yong Jiang Wei, Guo Ping Ai, Tian Min Cheng and Yuan Lin

Subject headings intestine, small; intestinal epithelial cell; radiation; cells, cultured; radiation dosage; immunohistochemistry; microscopy, electron

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INTRODUCTION

Crypt epithelial cells in normal small intestine proliferate at a high speed. But they are very difficult to culture *in vitro* and passage stably. A lot of studies have been done [1-16]. Some domestic labs isolated and cultured crypt cells from embryonal intestines and aseptic animal intestine, but failed. We introduced normal rat epithelial cell line IEC-6 from the USA and its living condition for stable passage was successfully established after trials. The cell line was testified to be the small intestinal epithelial cell by electron microscopy, immunihistochemistry and enzymatic-histoch-emistry. It has been applied to some related research work[17-21]. It was found that many factors were involved in the culture system. Our present study focuses on the culture method and the influencing factors on IEC-6.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle Medium (DMEM), HEPES from Sigma Cooperation, ^{3}H-TdR with the radioactive concentration of 37 MBq/mL and activity ratio of 740GBp/mL is the product of the Chinese Nuclear Science Institute.

Apparatus

Carbon dioxide culture case, Model Queue 2721, USA; automatic liquid scintillation counter, Model 1217, Sweden; cell harvester, Model 2T-II, Zhejiang Province; and microplate, Japan.

Institute of Combined Injury, PLA, School of Preventive Medicine, Third Military Medical University, Chongqing 400038, China
Dr. Xin Ze Ran, graduated from Third Military Medical University in 1980, now engaged in the research of combined radiation injuries for 20 years and having 40 papers published.
Supported by the National Natural Science Foundation of China, No. 39100119
Correspondence to: Dr. Xin Ze Ran, Institute of Combined Injury, PLA School of Preventive Medicine, Third Military Medical University, Chongqing 400038, China
Tel. 0086-23-68752278, Fax 0086-23-68752279
Email. Rxz@mail.tmmu.com.cn
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Cell line

Intestinal epithelial cell line, No. 6, IEC-6 was provided by the General Hospital of Massachusetts, Boston, USA.

Culture liquid medium

Liquid DMEM/L was made up according to the protocol supplemented with HEPES 10 mmol, penicillin 10^5U, streptomycin 100 mg, sodium carbonate 3.7 g and fetal bovine serum 100 mmol. L-glutamine 200 mmol/L was added before use.

Establishment of cell passage and detection of activity

IEC-6 cell line was immediately put into 40℃ water bath to thaw after being taken from liquid nitrogen, centrifuged for 10 minutes at 1000r/min. Liquid medium was added per bottle after the supernatant was deserted. Then the bottle was put into the carbon dioxide case (10% CO_2, 18.6% O_2 100% relative humidity, 37℃). After the cells adhered to the wall, change the liquid once, then passage on the 5th day. When the cell was passaged or the activity was detected, the liquid medium was deserted and 0.02% EDTA 8 mL was added for digestion of 30 minutes at 37℃. The incompletely digested cells were scraped softly with curved tube, passed into centrifuge tube and centrifuged for 8 minutes at 1000r/min. Supernatant was deserted and the liquid medium was added to a certain concentration. The cells were then seeded onto 96-well plates and cultured for 72 hours. ^{3}H-TdR, 1.5uCi per well, was added at the 12th hour before the culture was stopped. At the end of the culture, the cells were digested with 0.02% EDTA, harvested on the glass fiber filter membrane, and heated at 80℃. When the membrane cooled down to the room temperature, 8 mL scintillation liquid was added, Cpm was measured with automatic liquid scintillation counter.

Scintillation liquid contained POPOP 0.4 g, PPo 4 g, xylene 1000 mL.

RESULTS AND DISCUSSION

Effect of IEC-6 density

IEC-6 cells at various densities in microplate wells were labeled with ^{3}H-TdR 18.5kBq and cultured for 72 hours to investigate its effect on proliferation. Table 1 shows that at a certain range of densities, ^{3}H-TdR incorporation increased with the IEC-6 amount, the peak was at 10×10^4/well. Positive

correlation was found between cell density (X) and ^{3}H-TdR incorporation (y) at the range of 1.25×10^4-10×10^4/well with the correlation coefficient $r = 0.956$ and regression equation $y = 2177X+3575$(min^{-1}). When the cell density was more than 11.25×10^4/well, there was negative correlation ($r = 0.986$, y) = 36782-1253 X), possibly due to the limit amount of nutrition, liquid evaporation and subject to changes of culture condition[22].

Table 1 Effect of density on ^{3}H-TdR incorporation in culture cell ($\bar{x} \pm s$)

Density ($\times10^4$/well)	n	Min^{-1}	Density ($\times10^4$/well)	n	Min^{-1}
1.25	18	3547 ± 681	11.25	20	23648 ± 1398
2.50	20	9941 ± 413	12.50	18	20593 ± 2245
5.00	18	17931 ± 2051	13.75	20	19812 ± 2310
7.50	18	19825 ± 2135	15.00	18	17638 ± 959
10.00	24	23789 ± 2536	17.50	22	14874 ± 881

Effect of culture time

Incorporation of ^{3}H-TdR was different after IEC-6 was cultured for 6, 12, 24, 48, 72 and 96 hours (Table 2). Within 72 hours, incorporation increased from 1846 ± 146 to 25727 ± 4006 (min^{-1}) along with the time prolongation and it reached its peak at 72 hour. But when the culture time extended to 96 hours, the incorporation decreased. This may be caused by the activity inhibition of some IEC-6 under non-physical conditions.

Table 2 Effect of culture time on IEC-6 cell proliferation ($\bar{x} \pm s$)

Culture time (h)	n	Min^{-1}	Culture time (h)	n	Min^{-1}
6	24	1846 ± 146	48	24	21258 ± 1240
12	26	4038 ± 363	72	24	25727 ± 2006
24	24	6367 ± 588	96	24	24355 ± 2079

Effect of different ^{3}H-TdR dosage

In this study, different dosage of ^{3}H-TdR was administered in the IEC-6 culture system. A linear correlation was found between the ^{3}H-TdR incorporation and dosage when the dose was below 55.5KBq/well. When larger dosage was used, the incorporations slightly increased or decreased (Table 3). The radioactive damage to cells and consequent inhibition of DNA synthesis by high concentration of ^{3}H-TdR contributed to the incorporation decrease. Generally, the dosage of 18.5 KBq/well ^{3}H-TdR to 10×10^4 cell yielded a satisfactory result of incorporation 2.5×10^4.

Table 3 Effect of ^{3}H-TdR dosage on IEC-6 cell proliferation ($\bar{x} \pm s$)

^{3}H-TdR dosage (kBq/well)	n	Min^{-1}	^{3}H-TdR dosage (kBq/well)	n	Min^{-1}
0.00	20	139 ± 29	27.75	21	27555 ± 1637
2.31	22	1333 ± 118	37.00	21	37235 ± 1485
4.62		10136 ± 1083	46.25	22	41874 ± 1213
9.25	24	16880 ± 1447	55.05	24	48072 ± 1676
18.50	24	24890 ± 1623	74.00	20	42430 ± 1735

Effect of pH in lipid medium

pH of culture medium is one of the most important factors in cell culture. To optimize the culture condition, the pH value was set at 6.0, 6.6, 7.26, 7.6, 8.0 and 8.8, and ^{3}H-TdR incorporation was measured respectively (Table 4). The incorporation was the highest at pH 7.26, lower pH at 6.6 and 7.6, and the lowest at pH 6.0 and 8.0. In the common sense, cells can survive when pH ranged from 6 to 8. Variant cells and animal species do not have the same optimal pH. It is believed that optimal pH has an effect on the survival of cells *in vitro* by adjusting the intracellular enzymes and proliferation factors. We therefore set the optimal pH 7.26 in IEC-6 culture medium.

Table 4 Effect of pH of culture medium on ^{3}H-TdR incorporation ($\bar{x} \pm s$)

pH	n	Min^{-1}	pH	n	Min^{-1}
6.0	20	4528 ± 660	7.6	28	12897 ± 1301
6.6	24	18771 ± 920	8.0	20	1305 ± 146
7.26	24	22510 ± 1448	8.8	20	636 ± 102

Effect of insulin and concentration of fetal bovine serum

Fetal bovine serum is one of the essential factors in cell culture *in vitro*. If the concentration of fetal bovine serum is too low, cells will die or have proliferation prohibited. When the concentration is too high, the osmotic pressure in culture medium will change and it will influence the survival of cells. In this study, we found that 10% of fetal bovine serum was optimal in culture medium. Content of glucose in DMEM was high (4500 mg/L) and insulin can speed up glucose oxygenolysis and transportation through cell membrane, so the use of glucose was accelerated in the cells. The results showed that incorporation of ^{3}H-TdR was higher in cells treated with insulin than in the cells (Table 5) without insulin treatment.

Table 5 Effect of insulin and concentration of fetal bovine serum on IEC-6 cell proliferation ($\bar{x} \pm s$)

Fetal bovine serum(%)	n	Min^{-1}	
		Insulin group	Control group
0.0	18	476 ± 22	510 ± 101
2.5	20	13111 ± 978	1901 ± 580
5.0	22	14756 ± 1094	9097 ± 1069
7.5	22	20262 ± 2012	14569 ± 1136
10.0	24	23666 ± 1114	18775 ± 1361
15.0	20	22743 ± 1728	17645 ± 1289
20.0	18	22590 ± 1603	16965 ± 1147

Repeatability measurement

To investigate the experimental method, stability and the researcher's error, repeatability was measured by dividing the same culture system of IEC-6 into 30 parts. The incorporation of ^{3}H-TdR was 24327 ± 808 (min^{-1}). The value ranged from 23921 to 24733 when P<0.01 and coefficient of variation was 3.32%.

Effect of ionizing radiation on IEC-6

Intestinal epithelial cells are sensitive to ionizing radiation. The changes of incorporation of ^{3}H-TdR showed the damage of ionizing radiation on cells which reflected the cell biological characteristics. When IEC-6 was not exposed to radiation, the incorporation was 24327 ± 808. Incorporation after 4Gy, 8Gy, 16Gy, 2Gy and 26y irradiation were 31.8%, 24.1%, 15.2%, 11.2% and 8. 3% of control. Significantly negative dose-effect relation was found with the relative coefficient r = -0.970 (Table 6).

Table 6 Effect of ionizing radiation dosage on ^{3}H-TdR incorporation in IEC-6 cell ($\bar{x} \pm s$)

Dosage(Gy)	Min^{-1}	(%)	Dosage(Gy)	Min^{-1}	(%)
0	13427 ± 803	100.0	16	3698 ± 371	15.2
4	7736 ± 765	31.8	18	3381 ± 235	13.9
6	7249 ± 472	29.8	20	3041 ± 327	12.5
8	5863 ± 594	24.1	22	2725 ± 348	11.2
10	4865 ± 586	20.0	24	2481 ± 263	10.2
14	4136 ± 424	17.0	26	2019 ± 154	8.3
			r = -0.970		

In summary, methods of IEC-6 culture, passage and activity detection established in this study have the advantage of easy handling, being reliable in results, using less amounts of cells and a good repeatability. Subjective error can be avoided in measurement of epithelial proliferation with radioactivity. These will provide an ideal method for the research[23-35] on intestinal epithelimm[36-42].

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Effects of Taxotere on invasive potential and multidrug resistance phenotype in pancreatic carcinoma cell line SUIT-2

Bin Liu[1], Edgar Staren[2], Takeshi Iwamura[3], Hubert Appert[2] and John Howard[2]

Subject headings pancreatic neoplasms; drug therapy, combination; drug resistance; glycoproteins; neoplasm invasiveness; polymerase chain reaction; taxotere; multidrug resistance

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INTRODUCTION

Development of drug-resistance to chemotherapy and subsequent metastasis of tumor are primarily responsible for treatment failure and the death from cancer. There have been many previous studies on the relationship between expression of multidrug resistance (MDR) phenotype P-glycoprotein (P-gp) and the malignant properties of tumors, but the results are often conflicting[1-8]. The difference in tumor types or MDR phenotype induced by specific agents might account for this discrepancy. Taxotere (TXT), a member of the family of taxanes, has antitumor activity through its effect of promoting the polymerization of tubulin[9,10]. Since microtubules are involved in many respects of cell functions, such as cell movement, intracellular protein translocation, an altered invasive ability has been confirmed in *in vitro* invasion assay[11-14] and *in vivo* metastasis assay[15] in tumor cells treated with taxane. Taxane has also been found to down-or up- regulate the expressions of metastasis-associated proteins or genes, such as metallo-poteinase[13,16-18], urokinase-type plasminogen activator[18], and E-cadherin[19]. Our previous work demonstrated that the intrinsic and acquired resistance to TXT in pancreatic adenocarcinoma (PAC) cell line SUIT-2 was mediated mainly by P-gp[20]. This study demonstrated that the invasive ability of TXT-resistant cells S2/TXT was not significantly greater than that of SUIT-2. However, S2/TXT cells have increased resistance to the anti-invasion effects of TXT as compared with their parental cells, SUIT-2.

[1]Department of General Surgery, the Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China
[2]Department of Surgery, Medical College of Ohio, Toledo, Ohio, USA
[3]Department of Surgery, Miyazaki Medical College, Miyazaki, Japan
Bin Liu, graduated from Xuzhou Medical College in 1983, having 14 papers published. He is an associate professor and Deputy Director of the Department of General Surgery.
Supported in part by Phône-Poulenc Rorer Pharmaceuticals INC.
Correspondence to: Dr. Bin Liu, Department of General Surgery, the Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu, China
Tel. 0086-516-5802003
Email. Liubinxy@pub.xz.jsinfo.net
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MATERIALS AND METHODS

Reagents

Taxotere, obtained from Rhone-Poulenc Rorer Pharmaceuticals Inc., was stored as 10mM stock solution in absolute ethanol at -20℃. This solution was further diluted in the medium and used in the cell culture immediately before each experiment. MTT (3-[4, 5dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) was dissolved in phosphate buffered saline (PBS) at a concentration of 5 g/L and filtered. The MTT solution was stored in the dark at 4℃ before use.

Cell cultures

Human pancreatic cancer cell line SUIT-2 was established by Iwamura *et al*[21]. This cell line was derived from a metastatic liver tumor of human moderately differentiated pancreatic carcinoma. Its sublines including S2-007, S2-013, S2-020 and S2-028 were cloned by soft agar culture and showed different metastatic potential[22]. Cells were cultured in plastic flasks with McCoy's modified medium supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1. 25% penicillin-streptomycin solution (Sigma) (designated as "culture medium" below) and maintained at 37℃ in humidified atmosphere containing 5% CO_2.

Development of TXT resistant SUIT-2 cell line (S2/TXT)

A SUIT-2 TXT resistance derivative was developed by growing the parental cell line SUIT-2 in increasing concentration of TXT. Initially, the cells were grown as monolayers in culture medium at 37 ℃ in 5% CO_2 humidity atmosphere and exposed to 0.1nM TXT with addition of fresh culture medium and drugs every 3 days. After 2 weeks, the cells were exposed sequentially to stepwise increasing concentration of the drug until a TXT concentration of 2 nM was achieved. These cells were maintained in a drug-free culture medium for at least 3 weeks before used in experiments.

MTT colorimetric assay

The MTT colorimetric assay was performed as described by Page *et al*[23]. Briefly, SUIT-2 and S2/TXT cells were grown within 96well microtitre plates (Costar) at 2×10^4 cells/100 μL of culture medium per well and acclimated for 6 hours. Various concentrations of drugs (100 μL) diluted in culture medium were added. Five duplicate wells were used for each determination. The plates were incubated at 37℃ in 5% CO_2 for 72 hours when the control cells reached 90% confluence, and 30 μL of MTT solution was then added to each well and the plates were incubated at 37℃ for another 4 h. The medium and MTT solutions were then aspirated and 150 μL of dimethyl sulfoxide (DMSO) (Sigma) was added. The plates were agitated on the shaker for 15 minutes and read on Bio-Tek Microplate reader EL 800 (Bio-Tek Instruments, Inc.) with DeltaSoft 3 software. Fraction of cell proliferation was defined as the ratio of optical density volume to that of controls. The IC_{50} was defined as the concentration of the drugs required to reduce the optical density by 50% in treated cells to that of the controls.

Reverse transcription-polymerase chain reaction (RT-PCR)

The isolation of total RNA was based on the method of Chomczynski and Sacchi[24]. After the SUIT-2 and S2/TXT cell lines grew to 90% confluence, the total RNAs were extracted from the cell lines using Trireagent (Biotechnique, Molecular Research Center, Inc.). The total RNA was also isolated from the SUIT-2 cells incubated with 0.4 nM of TXT for 24 h. The messenger RNA was quantitated by measuring its absorbance at 260 nm. Equal amounts of RNA were reversed transcribed using SuperScriptTM One-stepTM RT-PCR System (Life Technologies). The 25 μL PCR mixed in each tube containing 0.5 μL RT/Tag Mix, 3 μL of 5 mM $MgSO_4$, 5 μL diethy pyrocarbonate (DEPC, Sigma) treated distilled water, 3 μL mixed primer pairs, 12. 5 μL 2X reaction mix and 1 μg template RNA in DEPC water. After an initial denaturation in a programmable thermocycler at 94℃ for 2 minutes, PCR was carried out for 30 cycles with the thermal profile: denaturing at 94℃ for 30 seconds, annealing at 55℃ for 30 seconds and extension at 72℃ for 1 minute with an extra 10 minutes extension for the last cycle. After completion of the amplification cycles, 5 μL of each PCR product was electrophoresed at 60V for 1.5 h on a 1.2% agarose gel (GIBCOBRL) in Trizma base and glacial acetic acid EDTA buffer, together with a 100-bp DNA (GIBCOBRL). The specific primers for mdr1 used in this study were sense 5'-CCC ATC ATT GCA ATA GCA GG-3', antisense: 5'- GTT CAA ACT TCT GCT CCT GA-3'. The metastasis of the carcinoma involved many kinds of genes. The primers used to detect the metastasis-related genes in this study were MMP-2: sense 5'-GAG CTG AAG GAC ACA CTA AAG AAG A-3'; antisense 5'-TTG CCA TCC TTC TCA AAG TTG TAG G-3', MMP-9: Sense 5'-CAC TGT CCA CCC CTC AGA GC-3'; antisense 5'-GCC ACT TGT CGG CGA TAA GG-3', Intigrin α5: sense 5'-CAT TTC CAA GTC TGG GCC AA-3'; antisense 5'-TGG AGG CTT GAG CTG AGC TT-3', intigrin β1: sense 5'-TGT TCA GTG CAG AGC CTT CA-3'; antisense 5'-CCT CAT ACT TCG GAT TGA CC-3' and E-Cadherin: sense 5'-GTG ACT GAT GCT GAT GCC CCC AAT ACC-3'; antisense 5'-GAC GCA GAA TCA GAA TAA GAA AAG CAA G-3'. β-actin was used as controls. Its sense primer was: 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; antisense primer was: 5'- CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

Flow cytometry for Rhodamine-123 (Rho-123) accumulation assay

SUIT-2 and its subline S2/TXT cells were harvested in logarithmic growth phase with 0.25% trypsin and resuspended in phenol red-free DMEM medium at 1×10^6 cells/mL. For Rho-123 accumulation assay, aliquots of 1 mL cell suspension were preincubated with or without 5 μM Verapamil for 45 minutes at 37℃. Rho-123, 200 μg/L dissolved in DMEM, was added and incubated for 40 minutes at 37℃ in the dark. After incubation, cells were washed twice with and resuspended in ice-cold Rho-123 free DMEM with 5 μM Ver. The accumulation of Rho-123 in cells was analyzed with flow cytometry. Ten thousand cells per sample were analyzed. The fluorescence was measured on a logarithmic scale of 4 decades of log. These cell lines, which had not been exposed to Rho-123, were used to determine the background of autofluorescence under this condition.

Fibroblast conditioned medium (FCM)

The FCM was obtained by incubating NIH 3T3 cells (ATCC) in a serum free medium. Briefly, after NIH 3T3 cells grew to 70%-80% confluence in 10% FBS McCoy's medium, the medium was changed to DMEM containing ascorbic acid (50 mg/L), the cells were then incubated at 37℃ for 24 h. The medium was collected after spinning down the cells and stored at -80℃[25].

Cell invasion assay

Matrigel invasion ability of cells was assayed using a Transwell cell culture chamber[25] with an 8 μm pore size polyvinylpyrrolidone-free polycarbonate filter (Costar, Cambridge, MA). At first, the filter was coated with 200 μL Matrigel (Invitrogen, 0.25 μg/μL) and allowed to air-dry overnight. The Matrigel was reconstituted the following day with

200 μL DMEM at room temperature for 30 minutes. SUIT-2 and S2/TXT cells were harvested by trypsinization and resuspended in the culture medium at concentration of 2×10^5 cells/mL. Single cell suspension (400 μL) were placed in the upper chamber of the Transwell in the presence or absence of 0.4 nM of taxotere. The lower chamber contained 1 mL conditioned medium. After the cells were incubated at 37℃ for 24 h, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The filter was fixed with 3% glutaraldehyde, stained with Hematoxylin. Cells which had invaded to the lower surface of the filter in five microscopic fields of 150 × magnification, were counted in each filter. Triplicate samples were conducted. The data were expressed as the average cell number of 15 fields.

Statistics

The significance of different invasion ability between SUIT-2 and S2/TXT, before and after treatment with TXT, was analyzed with Student's *t* test. Values were expressed as mean ± SD.

RESULTS

The sensitivity of SUIT-2 and S2/TXT cells to TXT

The acquired TXT resistant cell line S2/TXT was established from SUIT-2 by culturing with stepwise increasing concentrations of TXT as described in the Materials and Methods. Its IC_{50} (8.1 nM) was 9.5 folds that of its parental cell line SUIT-2 (IC_{50}: 0.85 nM). No change of sensitivity to TXT was found in this cell line during 4 months of study. The doubling time and morphology were similar to that of its parental cell line SUIT-2.

Expressions of mdr1 and other metastasis related genes

The expressions of the major drug transporter pump gene *mdr1* were studied by RT-PCR. There were strong expressions of *mdr1* in TXT-resistant cell line S2/TXT and SUIT-2 cells treated with 0.4 nM TXT for 24 h, and no expressions in the parental cell line SUIT-2, which were sensitive to TXT. Expression of metastasis-related genes in S2/TXT, including MMP-2, MMP-9, Integrin α5, Integrin β and E-Cadherin, was different from the parental cell line SUIT-2. Incubated with 0.4 nM of TXT for 24 h, TXT did not up- or down-regulate these metastasis-related gene expressions (Figure 1).

Rho-123 accumulation assay

Transporter activity of P-gp in S2/TXT cells was assayed by accumulation and efflux of Rho-123 tested with flow cytometry. The accumulation of Rho-123 in SUIT-2 cells is much higher than that of S2/TXT cells. The addition of 5 μM Ver significantly elevated the intracellular Rho-123 level in the TXT-resistant SUIT-2/TXT cells but not in the TXT-sensitive SUIT-2 cells.

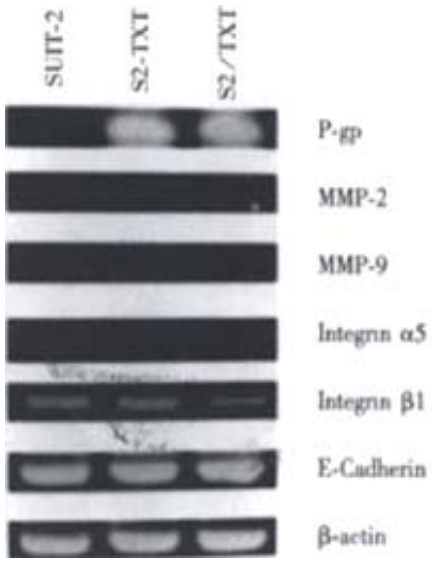

Figure 1 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of drug resistance gene messenger RNA *mdr1* in SUIT-2 and S2/TXT cell lines. RT-PCR was performed with 30 cycles of PCR amplification. The β-actin gene RT-PCR products confirms that intact mRNA is equally present in each of the cell lines. This figure shows that there were strong expressions of *mdr1* in TXT-resistant cell line S2/TXT and SUIT-2 cells treated with 0.4 nM TXT for 24 h, no expressions in the parental cell line SUIT-2. Metastasis-related gene expressions had no difference in these cells.

Invasion assay

The effects of TXT on the *in vitro* invasion ability of SUIT-2 and S2/TXT were examined using an invasion assay system with reconstituted Matrigel membrane. The invasion ability of S2/TXT, which expressed P-gp, was not significantly different from that of SUIT-2 (83 ± 28 *vs* 71 ± 22 cells per field, $P>0.05$). Treatment of the cells with 0.4 nM of TXT for 24 h significantly inhibited the cell invasion of SUIT-2 through the Matrigel basement membrane (71 ± 22 *vs* 17 ± 5 cells per field, $P<0.01$). However, the TXT, in the concentration tested, had no effect on invasion of drug resistant cell line S2/TXT (83 ± 28 vs 68 ± 24 cells per field before and after TXT treatment, $P>0.05$).

DISCUSSION

Pancreatic adenocarcinoma (PAC) currently remains one of the leading causes of cancer death throughout the world. Most patients are surgically unresectable at the time of diagnosis. For those who were resected, the risk of recurrence was extremely high[26]. Consequently, chemotherapy is an important approach for most patients with PAC. Taxane as a promising antitumor agent has been widely used in treatment of cancers. Unfortunately, the initial response to this agent may be hampered by the development of multidrug-resistant cells[27] and possible enhancement of malignant potential[28]. Taxane resistance of tumor cells may involve many mechanisms, but were most often

related to the expression of P-gp[20]. In this study, examination of the TXT effect on the *in vitro* proliferation capacity of SUIT-2 and S2/TXT cells revealed a higher sensitivity of SUIT-2 when compared to the S2/TXT variant. TXT challenge of the initially drug sensitive parental SUIT-2 cell lines resulted in the development of multidrug resistance together with simultaneous expression of P-gp. Active drug transporter pump P-gp in these TXT^{a2}resistant cells was confirmed by Rhodamine accumulation assay. However, in a study by Dumontet *et al*, only 44% of resistant clones were found to express the *mdr1* gene, and studies with labeled paclitaxel (Taxol, PTX) did not show altered accumulation in *mdr1* negative clones[29]. Other studies on the mechanisms of Taxane-resistance included the composition and the mutations in β-tubulin isotypes[29,30]. The different expression of P-gp or tubulin mutation might be related to the means by which the resistant cells were selected [31]. Multi-step selected cells often present a high level of Taxane-resistance mediated by P-gp, while single-step selection yields a low level Taxane-resistance cells with tubulin mutations[31]. Since severe tubulin mutations are very likely to affect cell survival, these cells will generally be lost during the selection.

As shown in this study, TXT had marked inhibitory effects on tumor cell invasion. These effects were also found in other tumor cells treated with taxol[11-13,32]. The basic effects of Taxane on tumor cell promoted the polymerization of tubulin and stabilizing microtubule assembly, thereby blocking cell replication in the late G2 mitotic phase of the cell cycle[10,33,34]. Since microtubules are also important components of cell motility and intracellular transport [19,35,36], it is possible that TXT inhibits the invasive and migratory ability through interference with the function of the fundamental part of the cytoskeleton, such as inducing rearrangements or changes of the microtubules, which might interfere with their functional ability to mediate cell movement and protease vesicle transports and secretions of the gelatinase[13]. Direct observation by microinterferometry demonstrated that taxol can suppress the mean area of protrusion and retraction of cells and reduced the spread of cell translocation [35]. The prevention of microtubule depolyme rization by taxol can freeze the cell in a spread conformation, thereby blocking motility[37]. In some cell types, microtubules are known to serve as tracks to transport vesicles and organelles[38]. Mark *et al* reported that relatively low levels of taxol can inhibit secretion of the M_r 72 000 and M_r 92 000 type IV collagenases plus an M_r 57 000 glatinase by blocking the cytoplasmic processing and packaging of the protease and completely inhibit cell attachment to matrigel, type IV collagen and plastic substrates *in vitro*. This has been shown to contribute to the reduced *in vitro* invasive ability and the establishment, growth and long-term survival of prostate tumor cells in SCID mice[13]. TXT did not, however, increase Integrin-mediated cell adhesion and cell spreading, which are attributable to microtubule depolymerization induced by microtubule disrupting agents[39].

Like other neoplasms, this pancreatic carcinoma cell line SUIT-2 is heterogeneous and consists of multiple subpopulations of cells with different invasive and metastatic properties[22]. These cells may be heterogeneous with regard to their sensitivity to chemotherapeutic agent and may contain different expressions of drug resistant phenotypes. Our previous studies have shown that the most TXT-resistant cell line in SUIT-2 and its subline (S2-007, S2-013, S2-020, S2-028) was S2-020 with strong expression of P-gp[21]. Although an *in vitro* study showed that S2-020 was also the most invasive toward Matrigel among these cell lines[22], there was no direct evidence that the expression of P-gp in this cell lines is responsible for its high invasive potential. On the contrary, it has been demonstrated that type I and IV collagenolytic activities are related to the malignancy of these cell lines[22,40].

The existence of different subpopulations in tumor increases the chance that cells with a high probability of survival will be selected when environmental conditions change. When SUIT-2 cell lines were treated with TXT, the highly invasive S2-020 with positive P-gp expression would be selected. However, the morphology of S2/TXT is totally different from that of S2-020 and *in vitro* invasive ability and morphology are similar to that of SUIT-2. Therefore, the expression of P-gp in S2/TXT was not due to the selection effect of TXT but to creation of TXT-resistant clone caused by potential of genetic mutation of TXT. In addition to the development of MDR, exposure tumor cells to some chemotherapeutic agents can cause activation or inactivation of genes with altered behavioral phenotype[41]. There was, however, no evidence that this occurred in this specific cell line treated with TXT, as shown by analysis of a number of metastasis-related genes. Thus, TXT appears capable of inducing the expression of P-gp without changing the intrinsic malignant characteristics of SUIT-2 cells.

In contrast to the study by Belotti *et al*, which show the PTX inhibits the motility of parental and PTX resistant cells equally[11], TXT did not inhibit the invasiveness of the TXT-resistant cell SUIT-2. This difference might be related to the mechanisms of taxane-resistance. Although the effect of PTX on cell motility and invasiveness is independent of

its effect on cell proliferation[11], these effects might still be based on the intracellular concentration of the drug. Studies have shown that the tumor cells can present TXT-resistance with normal intracellular concentration of TXT. This kind of resistance usually is mediated by changes of beta tubulin isotypes[31]. In such cases, the normal intracellular drug concentration in the TXT-resistance cell can be reached as in the TXT sensitive cells. In the S2/TXT cell line, which expresses active drug transporter pump P-gp, the intracellular concentration of TXT might not be high enough to act on microtubule and to inhibit cell invasion. It indicates that the resistance to the cytotoxic activity of TXT also confers resistance to the anti-invasion of the drug in the cell line SUIT-2. Therefore, the collateral anti-tumor effects of chemotherapeutic agents, as proposed by other studies[13], will disappear in this TXT-resistant cell line when treated with taxotere.

The relationship between P-gp expression and tumor malignancy is controversial. Although some studies have found that the expression of P-gp is casually related to a less aggressive pheno-type[4,42,43], in numerous cases, metastases exhibit a multidrug resistant pattern. Clinical and *in vitro* studies have also provided correlative results concerning the changes of metastatic potential following acquisition of the MDR phenotype[2,3,8,44]. To date, there has been no direct evidence showing that P-gp is involved intumor malignant potential. The invasive ability in the P-gp-positive S2/TXT cell line was not different from its P-gp-negative parental cell line SUIT-2, suggesting that TXT has no direct effect on increasing tumor malignant potential related to the induction of P-gp expression in this PAC cell line. The higher malignant potential associated with positive P-gp expression found in the other studies might be due to other invasive or metastatic related gene expressed simultaneously with P-gp.

From this study, we conclude that P-gp is primarily responsible for TXT resistance in PAC cell lines SUIT-2. However, expression of P-gp does not confer a more malignant invasive potential in this cell line with TXT resistance. Furthermore, TXT can inhibit the invasive ability of drug-sensitive cells but not drug-resistant cells.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Editorial

Helicobacter pylori: the primary cause of duodenal ulceration or a secondary infection?

M Hobsley and FI Tovey

Subject headings *Helicobacter pylori*/pathogenicity; Helicobacter infections/etiology; Helicobacter infections/complications; stomach ulcer/etiology

Hobsley M, Tovey FI. *Helicobacter pylori*: the primary cause of duodenal ulceration or a secondary infection? ***World J Gastroenterol***, 2001;7(2): 149-151

INTRODUCTION

It is generally accepted that *Helicobacter pylori (H. pylori)* infection has a role in duodenal ulceration. Eradication of *H. pylori* accelerates healing compared with placebo in the absence of control of gastric secretion and reduces ulcer recurrence. There is increasing evidence, however, that it may not be the primary cause of duodenal ulceration, but that it may be a secondary factor in a number of cases. This possibility is supported by four sets of observations:

1 *Geographical distribution*

In India and China there is a difference in prevalence of duodenal ulceration between the rice eating areas of the south and the drier wheat and millet eating areas of the north despite a similar high prevalence of *H. pylori* infection[1-3]. There is a similar situation along the West Coast of Africa. In the coastal area in the south where there is a diet of yams, sweet potato, manioc, plantains, rice and some white flour, the prevalence of duodenal ulceration is higher than in the northern savannah regions where much of the diet is millet. Both areas have a similar prevalence of *H. pylori* infection[4,5]. Again in Fiji the Indian population adhering to their traditional diet has twice the incidence of duodenal ulceration compared to the Fijian population despite similar *H. pylori* infection rates[6,7].

2 *H. pylori-negative duodenal ulceration*

There are many reports of *H. pylori*-negative duodenal ulceration unrelated to non-steroidal anti-inflammatory drugs (NSAIDs) varying in prevalence between 14% and 72% (Table 1)[8-26]. The figures from more developed countries suggest that *H. pylori*-negative duodenal ulceration occurs more frequently in areas where the overall prevalence of *H. pylori* infection in the community is low[20,27]. A paper from Greater Rochester, New York[11], reports a 48% prevalence of *H. pylori*-negative in white patients and 15% in non-white, with an overall prevalence of 39%. Parsonnet[28] has conducted a world-wide survey of reports and gives a figure of 40% *H. pylori* negative duodenal ulcers. Kurata *et al*[27] calculated that only between 48% and 64% of peptic ulcers are *H. pylori*-positive.

Table 1 Endoscopy reports of *Helicobacter pylori*-negative duodenal ulcer (DU) unrelated to NSAIDs

Authors	Places	Year	% *H.pylori*-negative DU
Oshowo[8]	UK, London	1999	30
Jones[9]	UK, Manchester	1986	41
Maher[10]	USA, Rochester	1987	43
Jyotheeswaran *et al*[11]	USA,Rochester	1998	39
Greenberg *et al*[12]	USA, Harvard	1997	40
Gislason *et al*[13]	USA, Baltimore	1997	30
Fenger *et al*[14]	Greenland (Inuit)	1997	50
Mirghani *et al*[15]	Sudan, Khartoum	1994	38
Kontou and Katelaris[16]	Australia, Sydney	1997	32.5
Uyub *et al*[17]	Malaysia, N. Peninsular	1994	
	Malayans		72
	Non Malayans		14
Petersen *et al*[18]	USA, N. Carolina	1996	26
Lanza *et al*[19]	USA, Houston	1996	30
Bruno *et al*[20]	USA, Military Hospital	1997	75
Sprung and Apter[21]	USA, Florida		
	Retrospective	1998	68
	Prospective	1998	69
Dres Pest *et al*[29]	Argentina	1996	33
	Early DU		59
	Chronic DU		22
Parsonnet[28]	All countries (meta-analysis)	1998	40
Sprung and Gano[22]	USA, Florida (retrospective)		52
Ciociola *et al*[23]	USA	1999	27
Henry and Batey[24]	Australia	1998	33
Pilotto *et al*[25]	Italy	2000	18.6
Lahaie *et al*[26]	Canada	2000	38

3 *Early cases of duodenal ulceration may be H. pylori-negative*

Dres Pest[29] from Argentina found a 78% incidence of *H. pylori* infection in patients with a history of chronic ulceration and only 41% in patients with a short history. He suggests that many patients with a short history may be free from *H. pylori* infection.

Department of Surgery, Royal Free and University College Medical School, London, 67-73 Riding House Street, London, WIP 7LD
Correspondence to: Professor M. Hobsley, Department of Surgery, Royal Free and University College Medical School, London, 67-73 Riding House Street, London, WIP 7LD
Tel. 0208 445 6507, Fax. 0208 492 0317
Email. hobsley@ucl.ac.uk
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4 Recurrence after eradication

Laine *et al*[30] from N. America have done a meta-analysis of seven trials subjected to strict criteria and report a recurrent duodenal ulcer rate of 20% within 6 months of *H. pylori* eradication in patients not on NSAIDs. Two other reports[31,32] excluding NSAIDs give figures of a 6% endoscopic recurrence within 3 years and of 18.9% clinical relapse within 7 years after eradication in patients remaining *H. pylori*negative. There are many other reports of recurrent ulceration in patients remaining *H. pylori* negative after eradication in which NSAID users have not been excluded.

DISCUSSION

The above findings strongly suggest that *H. pylori* infection is not a prerequisite for duodenal ulceration and that *H. pylori* infection when it occurs may be only a secondary factor.

It has been suggested that the differences mentioned in the geographical distribution of duodenal ulcer may be due to the higher prevalence of Cag A and Vac A virulent strains of *H. pylori* in these areas, but there is no evidence to support this[33-39].

The long held concept that duodenal ulceration is the result of a combination of increased acid output together with factors such as reduced bicarbonate in the duodenum reducing the ability of the duodenum to cope with the presenting acid level still remains valid. There may be other factors-smoking, genetic or dietary. Smoking has a chronic effect of increasing the ability to secrete acid[40]. There is convincing evidence that the differences in geographical or ethnic distribution of duodenal ulceration may be diet related[41,42]. Duodenal ulceration is less common in areas where unrefined wheat, certain pulses and millets form the staple diet and more common in areas where rice, refined maize or wheat flour, yams, manioc, plantains or sweet potatoes are the staple foods. Experiments in our laboratory on animal peptic ulcer models have confirmed the protective action against ulceration of the lipids present in certain food from areas of low duodenal ulcer prevalence and have shown that stored milled white rice and its oil are ulcerogenic[43-46]. There are several reports[47-62] showing the protective effect of certain dietary essential fatty acids, phospholipids and phytosterols against peptic ulceration in several experimental animal models.

In conclusion, the findings suggest that duodenal ulceration does occur independently of *H. pylori* infection and that *H. pylori* infection which may be coincidental or be acquired subsequently contributes to the chronicity of the ulceration. Subsequent infection is more likely to occur in areas where the prevalence of *H. pylori* infection is high. Treatment reducing acid secretion and raising the pH may contribute to *H. pylori* infection in ulcer patients.

This is presented as a paper for discussion and it is hoped that readers will respond with their points of view in the correspondence section of the journal.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Reviews

Hepatic encephalopathy as a complication of liver disease

Stephan vom Dahl, Gerald Kircheis and Dieter Haussinger

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INTRODUCTION

Hepatic encephalopathy (HE) is a frequent complication of chronic liver disease. It is defined as a characteristic functional and reversible alteration of the mental state, due to impaired liver function and/or increased portosystemic shunting.

In the brain of HE patients, neurons appear morphologically normal, but astrocytes show signs of Alzheimer type II degeneration, i.e. nuclear enlargement, peripheral margination of chromatin and prominent nucleoli. Ammonia is generally considered to play a central role in the pathophysiology of HE[1]. In HE, selective alterations of blood-brain barrier permeability, changes in cerebral energy metabolism, an increased GABA-ergic tone, changes in neurotransmitter systems and alterations of gene expression. e.g. of monoamine oxidase, peripheral-type benzodiazepine receptor (PTBR) and neuronal NO synthase are found (reviewed in[1-10]. The reversibility of HE symptoms and the reason for its precipitation by a variety of different factors has not been sufficiently explained yet (Table 1). Central insights into the etiology of HE have arisen from recent *in vitro* work with astrocytes. Astrocytes are important constituents of the blood-brain barrier, and uptake of substances from the blood into the brain is achieved by transastrocytic transport. Astrocytes communicate directly with neurons[11], regulate neurotransmitter processing and ionic milieu and provide substrates for neurons[12,13]. In brain, astrocytes are the only cells containing glutamine synthetase[14] and represent the major site of cerebral ammonia detoxification. Upon exposure to ammonia, cultured astrocytes develop Alzheimer type II changes. These findings prompted the idea that HE is a disorder of glial cells with a consecutive neuronal dysfunction[7,15,16].

Although the symptoms of HE in *acute or chronic* liver failure are different, there are good reasons to assume that the pathophysiology of both conditions is similar, but may involve different kinetics. In acute liver failure, astrocytes swell and brain edema develops[17]. HE in chronic liver disease is not accompanied by clinical signs of cerebral edema, but evidence for increased cell hydration has been given, as described later. A disturbance of astrocyte hydration is apparently a major pathophysiologic event in both forms.

MRS FINDINGS IN HE

MR-spectroscopy (H-MRS) studies in human brain initiated the idea that a disturbance of astrocyte cell volume homeostasis could be decisive for development of chronic HE[18-20]. ^{1}H-MRS can be used to study metabolic abnormalities in the human brain *in vivo* and allows a myo-inositol signal to be picked up, which represents an osmosensitive myo-inositol pool[18] of predominantly glial origin[21]. Myo-inositol is an organic osmolyte in astrocytes[22-24]. Such organic osmolytes play a decisive role in cell volume regulation: upon shrinkage, they accumulate inside the cells, in response to cell swelling they can be released from the cells via osmoregulated membrane channels[25,26]. Consistently, *in vivo* ^{1}H-MRS studies on the brain from cirrhotic patients with HE show a depletion of myo-inositol which is accompanied by an increase in the glutamine/glutamate signal[18,19,27-30], as shown in Figure 1. On the other hand, in cirrhotic patients, the implantation of transjugular intrahepatic-stent-shunt (TIPS) may lead to an aggravation of ^{1}H-MRS changes (Figure 2)[18] and a normalization of MRS findings after transplantation has been described[30]. *In vitro* studies from the rat have shown similar alterations after portocaval shunting[28]. There is a good correlation between the extent of these ^{1}H-MRS changes and the clinical severity of HE[18,19,29,30]. An increased glutamine/glutamate signal, together with adecrease of inositol signal, is observed in patients after TIPS implantation[18], as depicted in Figure 2. A high sensitivity and specificity of the myo-inositol signal for the diagnosis of HE cirrhotics has been reported[29,30], but these changes have also been reported in asymptomatic stages of hepatic encephalopathy[18,19,29,30].

Division of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University, Moorenstrasse 5, D-40225-Düsseldorf, Germany
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Correspondence to: Stephan vom Dahl, M.D., Division of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University, Moorenstr. 5, D-40225 Düsseldorf, Germany
Tel. +49-211-811-8764, Fax. +49-211-811-8838
Email. dahlv@uni-duesseldorf.de
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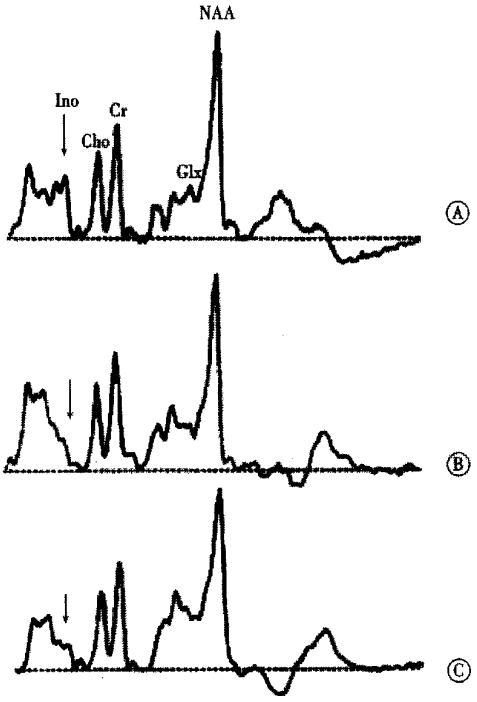

Figure 1 Parietal ^{1}H-MNR spectra from (A) a healthy person, patients with posthepatitic cirrhosis and latent (subclinical) HE (B) and manifest grade I-II HE (C). An increase in the glutamine/glutamate signal (Glx) and a decrease of the inositol signal (Ino) is observed. Further abbreviations: Cho, choline; Cr, creatine; N-acetylaspartate (NAA)[18].

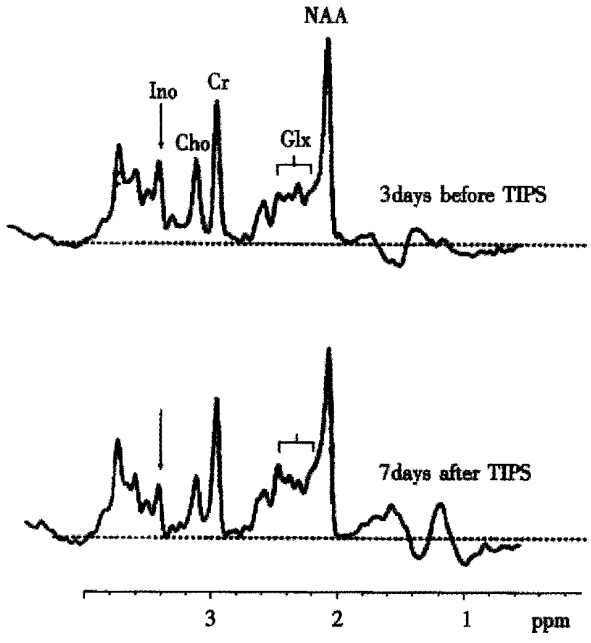

Figure 2 Parietal ^{1}H-MNR spectra from a 47-year-old patient with alcoholic cirrhosis 3 days before and 7 days after implantation of TIPS, showing an increased Glx signal and a decreased Ino signal. For abbreviations see Figure 1[18].

IMPAIRED CELL VOLUME HOMEOSTASIS IN HE AND FUNCTIONAL CONSEQUENCES FOR ASTROCYTES

The MRS findings in HE make an impaired cell volume homeostasis in brain likely and suggest that cellular non-cytotoxic edema[31] is present in hepatic encephalopathy. This edema is the result of an osmotically active intracellular accumulation of glutamine in response to hyperammonemia and a consecutive depletion of releasable myo-inositol and probably other osmolytes. Astrocytes swell in presence of ammonia[7,32]. Ammonia induces brain edema and intracranial hypertension in the portocaval shunted rat *in vivo* in a largely methionine sulfoximine-sensitive way[33]. PET studies with ^{15}N-ammonia on human brain from encephalopathic patients showed an increased cerebral metabolic rate for ammonia, suggestive of enhanced cerebral uptake of ammonia in HE and a stimulation of glutamine synthesis[34]. Astrocyte swelling also occurs *in vitro* under the influence of hyponatremia[35,36], some neurotransmitters[35,37], TNF-α[38] and benzodiazepines[7,37]. Apart from myo-inositol, recent data suggest that other organic osmolytes, such as taurine[21,22] and α-glycerophosphorylcholine[22,39], are depleted in order to counteract astrocyte swelling in HE. Small increases in astrocyte water content, as may occur in HE, could already have important functional consequences despite the absence of clinically overt increased intracranial pressure. Cell hydration is an independent signal which regulates cell function and gene expression, reviewed in[40-43] and a variety of different osmosignalling pathways linking cell hydration and cell function have been identified [44]. Extensive work from this laboratory has described the impact of cell hydration on cell function, cytoskeleton and gene expression[45], mainly in liver. In brain, cell hydration is also considered a key trigger of cell function. Swelling of astrocytes in culture activates via phosphatidylinositol-3-kinase extracellular regulated protein kinases (Erks)[36], i.e. members of the MAP kinase family with multiple functions, elevates intracellular calcium concentrations[46] and upregulates the peripheral type benzodiazepine receptor (PBR, reviewed in[47]) at the level of agonist binding[48] and mRNA (D. Häussinger and R. Fischer, unpublished results). Further, astrocyte swelling increases the pH in endocytotic vesicles[49] in an Erk-dependent osmosignalling pathway (R. Fischer and D. Häussinger). Several key findings in HE can thus partially be explained by an increase of astrocyte hydration. The endosomal alkalinization following astrocyte swelling could affect receptor densities and neurotransmitter processing and swelling-induced changes of the activity of plasma membrane transporters may underlie the selective changes in "blood-brain barrier" permeability of HE. Cell swelling stimulates glycogen synthesis and inhibits

glycogenolysis[45,50] and the increased deposition of glycogen in astrocytes in animal models of chronic HE[7] may reside on cell swelling. Astrocyte swelling leads to an increased expression of PBR and augments the synthesis of neurosteroids, which are potent modulators of neuronal $GABA_A$ activity [7]. Thus the interaction between astrocyte swelling, PBR expression and increased neurosteroid synthesis may explain the increased $GABA_A$-ergic tone in HE[9,51].

Table 1 Precipitating factors of HE

·Gastrointestinal or tissue bleeding
·Protein overload
·Sepsis
·Infection
·Catabolism
·Azotemia
·Acidosis
·Sedatives
·Diuretics
·Portocaval shunting (TIPS or surgical)
·Constipation

ASTROCYTE SWELLING AS AN INTEGRATIVE SIGNAL OF HE

An increase in astrocyte hydration, i.e. a low-grade cerebral edema, is a major pathogenetic event in the development of HE and induces a profound alteration of astrocyte function[20]. Altered astrocyte function may eventually lead to a disturbance of glioneuronal communication and present as the clinical syndrome of HE. Bleeding, infection, sedatives or electrolyte imbalance may precipitate HE in the cirrhotic patient (Table 1). Apart from ammonia, an increase of astrocyte hydration is also induced by hyponatremia, benzodiazepines and cytokines. Multiple factors could thus obviously result in a common pathogenetic endpath, i.e. glial swelling with its functional consequences. The osmolyte systems for counteraction of cell swelling are intact in non-cirrhotics and the precipitating conditions are well tolerated. In cirrhosis, however, organic osmolyte depletion is observed in order to compensate for glial glutamine accumulation and further challenges of cell volume can hardly be counteracted. The ^{1}H-MRS findings in nonencephalopathic cirrhotics could represent an early stage of a largely compensated disturbance of astrocyte volume homeostasis, where only few consequences yet for astrocyte hydration and function are observed. In response to HE-precipitating factors, a dysequilibrium of astrocyte volume results and hydration-dependent alterations of glial function will become clinically apparent. This unstable situation may explain the rapid kinetics of HE episodes and why severe brain edema with fatal outcome can occasionally develop in endstage cirrhotics[52].

DIAGNOSIS AND GRADING OF HE

Usually, HE is due to extensive porto-venous collateral shunting together with a decrease in hepatic function, resulting in increased cerebral ammonia load and diminished ammonia detoxification. Fulminant hepatic failure (FHF) means acute liver failure accompanied by hepatic encephalopathy. Sometimes, HE may be the result of metastatic liver disease[53], portal vein thrombosis[54], congestive heart failure[55] or constrictive pericarditis[56]. Even in the absence of overt liver disease, portosystemic shunting can induce HE[57], reviewed in[58]. Pre-TIPS encephalopathy is an important predictor of death during follow-up after placement of TIPS[59].

The symptoms of encephalopathy in all of these circumstances are characteristic, but unspecific. They range from subtle neuropsychologic derangements to coma. The diagnosis is made be the recognition of an appropriate hepatic disorder and the presence of encephalopathy in the absence of any other likely non-hepatic causes.

Foetor hepaticus and an increased blood ammonia concentration may contribute to the diagnosis.

For study purposes, an exact quantification of HE is required and defined by the West Haven Criteria [60,61]. The PSE index comprises the mental state, asterixis, number connection test results, electroencephalography and arterial blood ammonia concentrations. Subclinical hepatic encephalopathy (SHE) can only be diagnosed by subtle neuropsychological testing[62]. Preliminary results show that hepatic retinopathy, as detected by neurophysiological testing, very sensitively reflects the degree of HE (G. Kircheis and D. H-ussinger, unpublished observation) and responds to HE therapy. At the bedside, HE grade I is characterized by desorientation, whereas grade II HE shows spontaneous or inducible asterixis. In HE grade III, the patient is somnolent, grossly desoriented and precomatose, whereas grade IV represents coma.

THERAPY OF HE

Treatment of HE focusses on the pathogenetic events present in the individual patient. The most important therapeutic approach is to identify the precipating factors (Table 1) and to treat them vigorously. The required measures for precipitating factors are: therapy of GI bleeding together with bowel cleaning by lactulose, antibiotic treatment of concurrent infection, protein restriction, parenteral nutrition by an i.v. line and discontinuation of any diuretic therapy or sedatives. In patients with deterioration of HE after TIPS implantation, a

reduction stent may sometimes be necessary[63,64]. Apart from treatment of precipitating factors, additional therapeutic measures interfere with ammonia generation/disposal. In placebo-controlled studies, conflicting data exist on the efficacy of most therapeutic substances. Administration of lactulose is considered as gold standard in the treatment of HE, even for subclinical encephalopathy[65], but the beneficial effect of lactulose has not been precisely shown versus placebo, reviewed in[66]. In a recent randomized crossover trial, plasma ammonia and nitrogen balance were significantly better on vegetable protein diet as compared to an isonitrogenousanimal protein diet[67]. Neomycin is equally effective as lactulose, but a placebo-controlled trial on neomycin showed little effectivity[68]. Intravenous ornithine aspartate has proven its effectivity[69,70]and the benefit of orally administered ornithine aspartate is currently evaluated. Most studies on oral branched-chain amino acids (BCAA) showed clinical improvement of latent or low-grade HE and of protein tolerance[71], but studies on i.v. BCAA have not led yet to definite results. Benzodiazepine receptor antagonists have been reported to be of value in HE[72,73] but, to date with only modest success in some patients. In experimental cirrhosis, zinc supplementation reduces blood ammonia and increases liver ornithine transcarbamlyase activity[74] and a positive effect of zinc supplementation has also been shown in clinically overt HE[75].

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Hepatocellular transport proteins and their role in liver disease

Carmen Stanca[1], Diana Jung[2], Peter J. Meier[2], and Gerd A. Kullak-Ublick[1,2]

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MOLECULAR PHYSIOLOGY OF HEPATOCELLULAR TRANSPORT PROTEINS

Basolateral transport systems

Na^+-dependent bile salt uptake Uptake of bile salts into the liver was first characterized in experimental models such as the isolated perfused rat liver[1], isolated hepatocyte cultures and basolateral plasma membrane vesicles[2-4]. These studies indicated that more than 80% of taurocholate uptake but less than 50% of cholate uptake into hepatocytes is sodium-dependent[5-11]. Whereas unconjugated bile salts are uncharged molecules that can traverse membranes by passive nonionic diffusion, conjugation with glycine or taurine decreases their pKa values and necessitates the presence of a specific carrier protein for hepatocellular uptake[12].

The chief uptake system for conjugated bile salts in mammalian liver was isolated by expression and molecular cloning strategies and has been called the Na + - taurocholate cotransporting polypeptide (gene symbol: SLC10A1)[13-16]. Rat Ntcp consists of 362 amino acids with an apparent molecular mass of 51 kD[17,18] and is expressed exclusively at the basolateral membrane of hepatocytes (Figure 1)[17]. Ntcp mediates sodium-dependent uptake of taurocholate and other bile salts when expressed in stably transfected COS-7, Chinese hamster ovary (CHO) and hepatoblastoma (HepG2) cells or in cRNA injected *Xenopus laevis* oocytes, with apparent Km values between 17-42 μmol/L[10,13,17,19,20]. The only non-bile salt substrates that are transported by Ntcp are selected sulfated steroid conjugates such as estrone-3-sulfate[21] and dehydroepiandrosterone sulfate (DHEAS) [20]. In human liver, NTCP represents a 349-amino acid protein[14]. NTCP is structurally related to the intestinal bile salt transporter (IBAT), that also mediates the Na^+-dependent uptake of bile salts[22]and that is expressed not only in ileum, but also in the kidney[23] and in cholangiocytes[24].

Na^+-dependent taurocholate uptake is reduced in experimental models of cholestasis such as bile duct ligation[25], endotoxinemia[26,27] and partial hepatectomy[28], is reduced in primary hepatocyte cultures[29] and is absent in various hepatoma cell lines [30,31]. These changes in hepatic Na^+ dependent bile salt uptake correlate with expression levels of Ntcp. Thus, Ntcp mRNA and protein levels are decreased in bile duct ligation[25,32], endotoxinemia[26,33] and ethinyl estradiol induced cholestasis[34]. In patients with a diagnosis of extrahepatic biliary atresia and clinical evidence of cholestasis, NTCP mRNA levels are also decreased[35].

Na^+-independent hepatic uptake of amphipathic substrates: the organic anion transporting polypeptide family (OATP) Whereas uptake of conjugated bile salts into the liver is largely a Na^+-dependent process mediated by Ntcp, numerous other endogenous and xenobiotic compounds including non-bile salt organic anions and drugs are cleared from sinusoidal blood by carrier-mediated uptake into hepatocytes. Following hepatocellular uptake, many of these compounds are biotransformed in two phases. Phase I is mediated by cytochrome P450 enzymes and prepares the drug for conjugation by creating polar groups. Phase II conjugates drugs with a glucuronate, sulfate, glycine or methyl group and represents a detoxification step. The conjugates can then be excreted into bile or urine.

Na^+-independent hepatocellular uptake of bile salts and non-bile salt amphipathic compounds cannot be attributed to the function of a single transport protein, but is mediated by a family of transport proteins called the "organic anion transporting polypeptides" (Oatps) (Figure 1). In rat hepatocytes, at least three members of the Oatp

[1]Division of Gastroenterology and Hepatology and [2]Division of Clinical Pharmacology and Toxicology, Department of Internal Medicine, University Hospital, CH-8091 Zurich/Switzerland
Carmen Stanca, M.D., graduated from "Carol Davila" Medical and Pharmaceutical University in 1991, qualified in General Medicine in 1995 at the Emergency Hospital Bucharest. Current assignment: clinical research fellow at the Division of Gastroenterology and Hepatology, University Hospital Zurich/Switzerland, supported by "H+ Die Spitäler der Schweiz", by the Swiss Agency for Development and Cooperation (DEZA), and by the University Hospital Zurich/Switzerland.
Correspondence to: Gerd A. Kullak-Ublick, M.D., Division of Clinical Pharmacology and Toxicology, Department of Internal Medicine, University Hospital, CH-8091 Zurich/Switzerland
Tel. 0041 1 255 4097, Fax. 0041 1 255 4411
Email. kullak@kpt.unizh.ch
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family have been identified, called Oatp1 (Slc21a1) [36], Oatp2 (Slc21a5)[37] and Oatp4 (Slc21a10)[38]. Oatp1 is a 670 amino acid protein with an apparent molecular mass of 80 kDa that is localized at the basolateral membrane of hepatocytes[39-41] and at the apical membranes of kidney proximal tubular cells[39] and choroid plexus epithelial cells[42,43]. Many of the functional characteristics of Oatp1 indicate that it could represent the "multispecific bile acid transporter" identified in previous experimental models[3,44]. Thus, studies in numerous heterologous expression systems have shown that Oatp1 mediates the hepatocellular uptake of bile salts, bromosulphophthalein (BSP), conjugated steroids, thyroid hormones, leukotriene C_4, bilirubin monoglucuronide, ouabain, ochratoxin A, the anionic magnetic resonance imnaging agent gadoxetate (Gd-EOB-DTPA), the angiotensin-converting enzyme inhibitors enalapril and temocaprilat, the HMG-CoA reductase inhibitor pravastatin, and even oligopeptides including the thrombin inhibitor CRC-220, the endothelin antagonist BQ-123 and the opioid receptor agonists [D-penicillamine-2,5]enkephalin (DPDPE) and deltorphin II (for detailed review of the substrate specificities of Oatps/OATPs see reference[45]). The driving force for Oatp mediated substrate transport is not fully understood, although it has been shown that Oatp1 can mediate bidirectional transport of BSP[46] and anion exchange of taurocholate/HCO_3^-[47]. An important driving force for organic anion uptake via Oatp1 appears to be countertransport of reduced glutathione[48].

Oatp2 is a 661 amino acid protein with an apparent molecular mass of 92 kD at the basolateral plasma membrane of hepatocytes[41]. Oatp2 has also been detected in the retina[49], in endothelial cells of the blood brain barrier[43] and at the basolateral plasma membrane of choroid plexus epithelial cells[43]. Oatp2 is a close homologue of Oatp1 and transports bile salts, the cardiac glycosides ouabain and digoxin, and cyclic peptides[37,41]. An important difference between Oatp1 and Oatp2 is their acinar localization in the liver. Whereas Oatp1 is distributed homogeneously within the liver acinus [41,50], Oatp2 exhibits a heterogeneous lobular distribution with predominant expression in perivenous hepatocytes excluding the innermost 1-2 cell layers surrounding the central vein[41,51]. Interestingly, treatment of rats with phenobarbital, a known inducer of microsomal drug metabolizing P450 enzymes[52] and of hepatocellular ouabain, digoxin and thyroxine uptake[53-57], resulted in a significant increase in Oatp2 expression and in the appearance of positive immunofluorescence signals even in the innermost layer of perivenous hepatocytes[58].

Oatp4 (Slc21a10) can also mediate Na^+-independent uptake of bile salts in rat hepatocytes and represents a full-length isoform of the so-called "liver-specific transporter 1" or rlst-1[38,59]. Oatp4 transports numerous organic anions including taurocholate, BSP, conjugated steroids, prostaglandin E-2, leukotriene C_4, the thyroid hormones T_3 and T_4, and gadoxetate[38]. Oatp4 is 43% and 44% identical on the amino acid level with Oatp1 and Oatp2, respectively.

In human liver, at least four OATPs have been identified to date, called OATP-A (SLC21A3), OATP-B (SLC21A9), OATP-C (SLC21A6) and OATP8 (SLC21A8). OATP-C (also called OATP2 and LST-1)[60-62] and OATP8[63] are exclusively expressed at the basolateral membrane of hepatocytes and exhibit 80% mutual identity. The closest homologue expressed in rat liver is Oatp4, which is 64% and 66% identical with OATP-C and OATP8, respectively. Accordingly, the substrate specificities of human OATP-C and OATP8 and rat Oatp4 are very comparable[38,64]. Transport substrates of OATP-C include taurocholate (Km ~ 14-34 μM)[60,61], bilirubin monoglucuronide, DHEAS, estradiol-17β-glucuronide (Km~ 8 μM)[62], estrone-3-sulfate, prostaglandin E_2, thromboxane B_2, leukotriene C_4, leukotriene E_4, T_3 (Km~3 μM), T_4 (Km~3 μM)[60], pravastatin (Km~35 μM)[61] and BSP (Km~0.3 μM)[64]. OATP8 exhibits a closely overlapping substrate specificity compared with OATP-C but additionally transports the cardiac glycoside digoxin (similar to rat Oatp2) and is particularly efficient in transporting the oligopeptides BQ-123 (endothelin receptor antagonist), DPDPE (opioid receptor agonist) and cholecystokinin (Km~11 μM)[64,65].

OATP-B (SLC21A9) is also strongly expressed in human liver, with additional expression in spleen, placenta, lung, kidney, heart, ovary, small intestine and brain[64]. OATP-B is a 709 amino acid protein with an apparent molecular mass of 85 kDa that is localized at the basolateral plasma membrane of hepatocytes[64]. Compared to OATP-C and OATP8, OATP-B exhibits a limited substrate specificity for the organic anions BSP (Km~0.7 μM), estrone-3-sulfate (Km~6 μM) and DHEAS.

The fourth OATP known to be expressed in hepatocytes, albeit at relatively low levels, is OATP-A (SLC21A3)[66].Although OATP-A was originally isolated from human liver, it is predominantly expressed in human cerebral endothelial cells[67]. OATP-A is a 670 amino acid protein that transports bile salts, BSP (Km~20 μM), estrone-3-sulfate (Km~59 μM)[68], DHEAS (Km~6.6 μM)[69], the magnetic resonance imaging agent Gd-B 20790[70], the opioid receptor agonists DPDPE (Km~202 μM) and deltorphin II (Km~ 330 μM)[67], the antihistamine fexofenadine[71],

and the amphipathic organic cations APD-ajmalinium, rocuronium, N-methyl-quinine (Km~5 μM) and N-methyl-quinidine (Km~26 μM)[72]. Thus, in contrast to the preference of OATP-B, OATP-C and OATP8 for organic anions, OATP-A additionally transports amphipathic organic cations indicating that it can mediate substrate uptake into hepatocytes charge independently. Overall, the Oatp/OATP family of transporters plays a central role in hepatocellular organic anion and drug clearance.

Na^+-independent hepatic uptake of hydrophilic organic anions and organic cations: the organic ion transporter family (OAT/OCT) In addition to NTCP and OATPs, the basolateral hepatocyte membrane possesses a third family of transport proteins mediating substrate uptake, called the organic anion transporter (OAT) family[73]. This family comprises the OAT, the organic cation transporter (OCT)[74,75] and the organic cation transporter novel type (OCTN)/carnitine transporter families[73]. Whereas Oat1 is expressed only in rat kidney[76,77], Oat2 is expressed exclusively[78] and Oat3 predominantly[79] in rat liver. In human liver, only OAT2 (SLC22A7) has been isolated (Figure. 1). Oat2 mediates sodium-independent transport of α-ketoglutarate and salicylates, whereas Oat3 transports para-aminohippurate (PAH), estrone-3-sulfate, and the cationic compound cimetidine.

The first organic cation transporter, called OCT1, was cloned from rat kidney[80] and is expressed at the basolateral membrane of hepatocytes, small intestinal enterocytes and cells of the renal proximal tubule S1 segment[74]. In man, hOCT1 (SLC22A1) is expressed specifically in the liver (Figure 1) and mediates the hepatic clearance of small type I cations such as tetraethylammonium, N-methylnicotinamide, dopamine and choline[81,82]. No studies investigating the role of the OAT/OCT/OCTN transporter family in human liver disease have been performed to date.

Basolateral efflux pumps The basolateral membrane also possesses several members of the multidrug resistance protein family (MRPs) belonging to the superfamily of ATP-binding cassette (ABC) transporters (Figure 1). MRP1 (ABCC1) mediates the ATP-dependent efflux of glutathione S-conjugates[83], leukotriene C_4, steroid conjugates such as estradiol-17β-D-glucuronide and glucuronidated or sulfated bile salt conjugates[84]. MRP1 is normally expressed at very low levels in hepatocytes, but expression levels are increased in human hepatoblastoma HepG2 cells and SV40 large T antigen-immortalized human hepatocytes[85]. MRP3 (ABCC3) is expressed at the basolateral hepatocyte membrane[86] and mediates basolateral efflux of the organic anions estradiol-17β-D-glucuronide and S-(2,4-dinitrophenyl) glutathione, the anticancer drugs methotrexate and etoposide [87,88] and even of monovalent bile salts[89]. MRP5 (ABCC5) appears to be an anion transporter, however its expression level in the adult liver is very low[90]. MRP6 (ABCC6) is localized at the lateral membrane of hepatocytes and transports the cyclic pentapeptide and endothelin antagonist BQ-123[91-93]. Interestingly, mutations in the *MRP6* gene have been shown to be the cause of pseudoxanthoma elasticum[94].

Canalicular transport systems

Bile salt excretion Canalicular excretion represents the rate-limiting step in the overall secretion of bile salts from blood into bile. Whereas bile salt concentrations within the hepatocyte are in the micromolar range, canalicular bile salt concentrations are more than 1000fold higher, necessitating active transport across the canalicular hepatocyte membrane. Characterization of ATP-dependent taurocholate transport in canalicular membrane vesicles indicated the presence of a specific carrier system for monovalent bile salts[95,96], with an apparent Km for ATP-dependent taurocholate transport of ~ 2-20 μM[95-98].

The chief transport system that mediates the canalicular excretion of monovalent bile salts is the so-called "bile salt export pump" or Bsep (ABCB11), first cloned from pig[99] and subsequently from rat [100] and mouse liver[101,102]. Rat Bsep is a 1321 amino acid protein with 12 putative membrane-spanning domains, four potential -N-linked glycosylation sites, a molecular mass of ~ 160 kDa and with the structural features of the ABC-transporter superfamily[100]. The amino acid sequence is more homologous with the MDR family of transporters (~50%) than with MRPs. In membrane vesicles from transfected Sf9 insect cells, rat Bsep transports taurocholate with a Km of ~5 μM which is comparable to ATP-dependent transport in canalicular rat liver plasma membrane vesicles[100]. Bsep is expressed on the surface of canalicular microvilli as indicated by electron microscopic studies. In addition to taurocholate, rat Bsep also mediates ATP-dependent transport of glycocholate, taurochenodeoxycholate (Km ~ 2 μM) and tauroursodeoxycholate (Km ~ 4 μM).

The locus of the mouse Bsep gene on chromosome 2, band 2C1.3[101], corresponds to the locus of human BSEP on chromosome 2q24[103]. This region has been linked to the *Lith1* gene near D2Mit56 that confers genetic gallstone-susceptibility in the C57L/J mouse strain[104-106]. These mice overexpress Bsep[107] and exhibit relative hypersecretion of cholesterol into bile with subsequent cholesterol supersaturation[108]. The exact significance of overexpression of Bsep in these

mice is unclear, since it has been shown that functional ATP-dependent taurocholate transport activity in canalicular membrane vesicles is approximately 3fold lower compared to AKR/J gallstone-resistant mice, despite 3fold higher protein levels[109]. The functional decrease in canalicular bile salt excretion could be the cause of increased gallstone susceptibility in C57L/J mice.

The human *BSEP* gene locus has been identified as the positional candidate for progressive familial intrahepatic cholestasis type 2 (PFIC2), a progressive liver disease characterized by low biliary bile salt concentrations[103]. In PFIC2, BSEP is absent from the canalicular membrane and biliary bile salt concentrations are less than 1% of normal[110].

Excretion of non-bile salt organic anions The excretion of non-bile salt organic anions into bile is mediated by the canalicular multidrug resistance protein 2, MRP2[111]. MRP2 (ABCC2) has a molecular mass of 190 kD and the human protein exhibits 46% amino acid identity to human MRP1. Both rat and human MRP2 are expressed predominantly in the liver with exclusive localization in the canalicular membrane (Figure 1)[112-115]. The spectrum of organic anions transported by MRP2 is qualitatively similar to that of MRP1[84] and includes glutathione conjugates, glucuronides, leukotriene C_4 and divalent bile salts, but not monovalent bile salts[114,116]. A role for MRP2 in the canalicular excretion of reduced glutathione (GSH), a major driving force for the maintenance of bile salt-independent bile flow, has also been demonstrated[117]. Various structurally and functionally unrelated xenobiotics such as probenecid, glibenclamide, rifampicin, vinblastine, indomethacin and cyclosporin A were shown to inhibit excretion of the anionic fluorescent dye carboxy-2',7'-dichlorofluorescein (CF) by primary human hepatocytes, thus suggesting that organic anion excretion by human liver may be impaired by various drugs[118]. Mutations in the *MRP2* gene that lead to the synthesis of a truncated, non-functional protein, have been identified as the pathogenetic basis of hereditary chronic conjugated hyperbilirubinemia, discussed further below.

Phospholipid excretion The major lipid that is cosecreted into bile with cholesterol is phosphatidylcholine (PC). The constant replenishment of PC molecules from the inner to the outer hemileaflet of the canalicular membrane is mediated by the concerted action of ATP-dependent[119] and ATP-independent[120-122] PC "flippases". The ATP-dependent flippase has been identified as a class III multidrug resistance (MDR) P-glycoprotein, Mdr2 in mice and MDR3 in humans (ABCB4) (Figure 1)[123-125], a 170 kD canalicular protein. Mouse Mdr2 and human MDR3 are present in high concentrations in the canalicular membrane of hepatocytes. Mice lacking this protein are unable to secrete phosphatidylcholine (PC) into bile[123]. Conversely, in fibroblasts from transgenic mice expressing the human *MDR3* gene under a vimentin promoter, the transfer of radiolabeled PC from the inner to the outer leaflet of the plasma membrane is stimulated [124]. In addition, expression of mouse Mdr2 in secretory vesicles from the yeast mutant sec6-4, results in a time- and temperature-dependent enhancement of PC translocation to the inner leaflet of the membrane[126]. These data indicate that both mouse Mdr2 and human MDR3 function as physiological phospholipid translocators.

Copper excretion The liver is the central organ of copper homeostasis with a great capacity to store and excrete this metal. The degree of biliary copper excretion is directly proportional to the size of the hepatic copper pool, indicating that hepatocytes can sense the copper status in the cytoplasm and regulate copper excretion into bile accordingly[127]. The biliary excretion of heavy metals such as copper is an important detoxifying mechanism of the liver. Copper excretion is mediated by a copper transporting P-type ATPase called ATP7B that is expressed predominantly in the liver[128-130] (Figure 1). This 160 kD protein is localized to the trans-Golgi network[131] where it mediates the incorporation of copper into cuproenzymes such as ceruloplasmin. A truncated 140 kD isoform of ATP7B is localized to mitochondria[132], possibly explaining the abnormalities of mitochondrial morphology in Wilson's disease. Immunohistochemical studies in human liver indicate additional weak staining of ATP7B at the canalicular membrane[133]. A green fluorescent GFP-ATP7B fusion construct transfected into human hepatoma Huh7 cells localizes neither to the trans-Golgi network nor to the canalicular membrane, but to so-called late endosomes [134]. Copper incorporated into late endosomes is probably transported to lysosomes and subsequently excreted into bile by a process known as biliary lysosomal excretion[134].

Copper is presumably taken up into human hepatocytes via the copper transporters hCTR1 and hCTR2[135]. As the copper concentration of the hepatocyte increases, ATP7B redistributes from the trans-Golgi network to a cytoplasmic vesicular compartment[131] and to pericanalicular vacuoles[136] (Figure 1). After copper depletion, ATP7B returns to the trans-Golgi network. Thus copper can induce trafficking of its own transporter from the trans-Golgi network to the apical membrane, where it may mediate biliary copper excretion. Copper-induced redistribution of ATP7B may provide a mechanism to preserve copper when it is scarce and to prevent copper toxicity when levels become too high.

ROLE OF HEPATOCELLULAR TRANSPORTERS IN LIVER DISEASE

Hereditary defects of hepatocellular transporters

Progressive familial intrahepatic cholestasis Progressive familial intrahepatic cholestasis (PFIC) describes a group of autosomal-recessive disorders. The onset is usually during the first months of life, with severe and progressive intrahepatic cholestasis, proceeding to cirrhosis by the second decade. Diagnosis is based on the following criteria: ① progressive intrahepatic cholestasis and liver cell failure, after the exclusion of other causes of liver disease; ② lack of bile duct pathology (intrahepatic and extrahepatic); ③ a normal number of interlobular bile ducts[137]. Other signs and symptoms include pruritus, jaundice, hepatomegaly, wheezing and nosebleeds, cough, fat-soluble vitamin deficiency, cholelithiasis, short stature and delayed sexual development[138].

Three types of PFIC have been described[139] (Table 1). PFIC type 1 (PFIC1, Byler's disease) is caused by a mutation in the coding sequence of the *FIC*1 gene (*ATP*8*B*1, chromosome 18q 21-22)[140], that is expressed predominantly in liver and small intestine. The FIC1 gene product is a P-type ATP-ase putatively involved in the transport of phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of plasma cellular membranes[141]. Patients with a defective FIC1 protein encounter bouts of jaundice that later become permanent, severe pruritus, chronic watery diarrhea, high serum bile salts, but normal γ-GT and cholesterol levels in serum. Histologically, cholestasis is found with progression to cirrhosis, but without ductular proliferation.

PFIC type 2 (PFIC2, Byler syndrome) is caused by mutations in the *BSEP* gene (located on chromosome 2q24)[142] that lead to an absence of the bile salt export pump from the canalicular hepatocyte membrane[110]. Defective canalicular bile salt excretion results in an accumulation of bile salts within the hepatocyte and toxic damage. PFIC2 resembles PFIC1 clinically, biochemically and histologically, although the initial presentation is more severe with permanent jaundice from onset, and liver failure occurs more rapidly.

PFIC type 3 (PFIC3) is caused by homozygous mutations in the *MDR3* gene[143], that lead to an absence of the phospholipid export pump MDR3 from the canalicular membrane and to an absence of the major biliary phospholipid, *phosphat*-idylcholine, from bile. This results in toxic bile salt induced injury of the biliary epithelium. PFIC3 is characterized by elevated serum γ-GT levels, ductular proliferation, and an inflammatory infiltrate in the early stages which progresses to biliary cirrhosis[143,144]. Mice with a homozygous disruption of the *Mdr2* gene (which corresponds to *MDR3* in man) represent an animal model of PFIC3[123]. Overall, the analogy between the murine knockout model and human cholestatic liver disease indicates that the nonsuppurative cholangitis observed in Mdr2/MDR3 deficiency is caused by the high luminal concentration of free bile salts that are not sequestered in mixed micelles in the absence of phospholipids.

The appearance of lipoprotein X in the plasma of cholestatic mice has been attributed to the function of Mdr2. Bile duct ligation in control mice induced a dramatic increase in plasma cholesterol and phospholipid concentrations, mainly as lipoprotein X[145]. In bile duct ligated Mdr2 - / - mice, cholesterol and phospholipid concentrations were also increased but plasma fractionation revealed a complete absence of lipoprotein X. Plasma levels of cholesterol and phospholipid during cholestasis correlated very closely with the expression level of Mdr2, indicating first that the shift of hepatocellular lipid secretion from bile to plasma during cholestasis depends upon the formation of lipoprotein X, and second that the concentration of lipoprotein X is modulated by the activity of Mdr2. Thus, the elevation of serum cholesterol that is a common feature of cholestasis in man, could also be dependent upon the function of MDR3.

Benign recurrent intrahepatic cholestasis (*BRIC*) is also caused by a mutation in the *FIC*1 gene (ATP8B1) (Table 1). It is characterized by recurrent bouts of cholestasis in the adult, with symptom-free intervals lasting from months to several years. Unlike PFIC1, BRIC is not associated with progressive liver damage. Serum bile salt concentrations are elevated as the earliest markers of cholestasis. FIC1 is also expressed in the small intestine, where it appears to play a role in intestinal bile salt absorption. It is of interest that in non-symptomatic BRIC patients, fecal loss of bile salts due to intestinal malabsorption is increased[146].

Dubin-Johnson syndrome The Dubin-Johnson syndrome is an autosomal recessive disorder that is caused by impaired biliary excretion of certain cholephilic organic anions such conjugated bilirubin (Table 1). It is characterized by conjugated hyperbilirubinemia, increased urinary excretion of coproporphyrin I, deposits of a black pigment in centrolobular hepatocytes, and prolonged BSP retention[147]. In contrast to PFIC, hepatic function is preserved. The syndrome is produced by the absence of MRP2 protein from the canalicular hepatocyte membrane[148] due to mutations of the *MRP2* gene (ABCC2)[111,147,149]. Recently, the MRP2Delta(R,M) mutation, which describes the deletion of Arg1392 and Met1393, was shown to cause disturbed maturation and trafficking of the protein from the ER to the Golgi complex and impaired sorting of this glycoprotein to the apical membrane[150]. Absent MRP2 function may be compensated for by increased expression of MRP3

at the basolateral hepatocyte membrane, as suggested by immunofluorescence studies on liver sections from a Dubin-Johnson patient[86].

Wilson's disease Wilson's disease is an autosomal recessive disorder characterized by copper accumulation in the liver, brain, kidney and cornea secondary to inadequate biliary copper excretion. Under physiologic circumstances, biliary excretion represents the sole mechanism for copper excretion, and thus affected individuals have progressive copper accumulation in the liver. When the capacity for hepatic storage is exceeded, cell death ensues with copper release into the plasma, hemolysis, and tissue deposition [127]. The age at onset ranges from 3 to 40 years, with highly variable clinical manifestations. Hepatic dysfunction is the most common initial presentation in childhood, progressing from mild elevation of serum transaminases in asymptomatic individuals to chronic active hepatitis and cirrhosis. In some cases, severe chronic liver disease or fulminant hepatic failure may be the initial manifestations. The laboratory diagnosis of Wilson's disease is confirmed by decreased serum ceruloplasmin, increased urinary copper content, and elevated hepatic copper concentration.

Wilson's disease results from the absence or dysfunction of the ATP7B gene product, a copper transporting P-type ATPase encoded on chromosome 13. Molecular genetic analysis is complex, as more than 100 unique mutations have been identified and most individuals are compound heterozygotes. A database of Wilson's disease mutations can be retrieved online at *http://www.medgen.med.ualberta.ca*. Of these mutations, the H1069Q mutation accounts for more than 40% of the alleles in affected Northern European patients, whereas the *A778L* mutation is observed in 30% of alleles of Oriental patients[127]. Expression of the *H1069Q* mutant in a copper transporter-deficient cell line reveals that this mutation causes a defect in protein folding that results in mislocalization to the ER and rapid degradation[151]. The histidine residue at amino acid position 1069 appears to be essential for trafficking from the *trans* Golgi network in response to copper[151] (Figure 1).

Acquired defects of hepatocellular transporters

Extrahepatic cholestasis Extrahepatic cholestasis is produced by an obstruction of the hepatic or common bile duct secondary to cholelithiasis, neoplasms, or sclerosing cholangitis. A major risk factor for hepatocellular injury during bile duct obstruction is the increased intracellular concentration of potentially toxic bile salts[152]. This can be partly counteracted by the decrease in Ntcp expression that occurs in bile duct ligated rats[25, 32]. The human NTCP mRNA is also downregulated in cholestasis, as evidenced in 23 patients with a diagnosis of extrahepatic biliary atresia[35]. At the canalicular pole, the expression of the bile salt export pump, Bsep, is reduced to 50% of controls on the protein and to 32% on the RNA level[153]. Bsep expression is thus preserved relatively well compared to the marked decrease in expression of the canalicular multispecific organic anion transporter Mrp2[154]. The relative preservation of Bsep expression during bile duct ligation serves to maintain the canalicular efflux of bile salts that has been demonstrated experimentally[155].

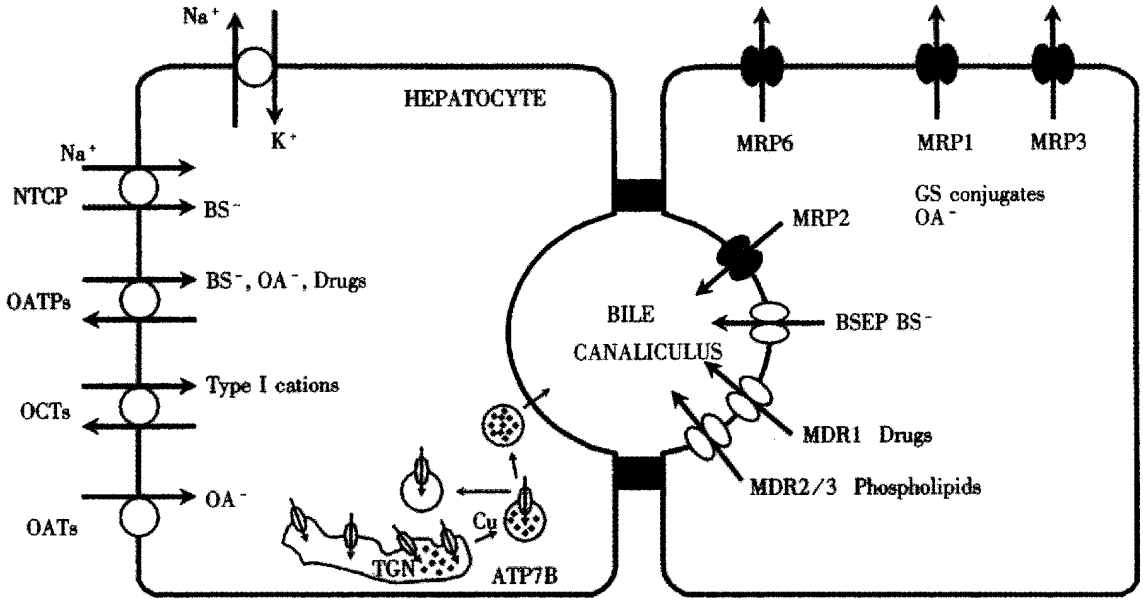

Figure 1 Hepatocellular transport proteins involved in bile salt, drug and organic substrate clearance by human liver.
Abbreviations: NTCP, Na^+-taurocholate cotransporting polypeptide; OATPs, organic anion transporting polypeptides; OCTs, organic cation transporters; OATs, organic anion transporters; ATP7B, Wilson ATPase; TGN, *trans*-Golgi network; MRP, multidrug resistance protein; MDR, multidrug resistance gene product; BSEP, bile salt export pump; BS, bile salts; OA, organic anions; GS, glutathione.

Table 1 Role of hepatocellular transport proteins in the pathogenesis of liver disease

Species	Transport protein	Gene symbol	Physiologic function	Alteration in liver disease
Basolateral transport proteins				
Rat / human	Ntcp / NTCP	SLC10A1	Na^+ dependent bile salt uptake	Decreased Ntcp expression in rat models of cholestasis[25,26,34] Decreased NTCP expression in human cholestatic liver disease[35]
Rat	Oatp1	Slc21a1	Multispecific uptake of organic anions and amphipathic compounds	Decreased Oatp1 expression in bile duct ligation[32] and in ethinyl estradiol induced cholestasis[34]
	Oatp2	Slc21a5	Multispecific uptake of organic anions and of cardiac glycosides (digoxin)	Not yet investigated
	Oatp4	Slc21a10	Multispecific uptake of organic anions and amphipathic compounds	Decreased Oatp4 expression in bile duct ligation and sepsis[59]
Human	OATP-A	SLC21A3	Multispecific uptake of organic anions and amphipathic compounds	Increased mRNA levels in primary sclerosing cholangitis (PSC)[180]
	OATP-B	SLC21A9	Multispecific uptake of organic anions and amphipathic compounds	Not yet investigated
	OATP-C	SLC21A6	Multispecific uptake of organic anions and amphipathic compounds	Decreased mRNA levels in primary sclerosing cholangitis[181] Not yet investigated
	OATP8	SLC21A8	Multispecific uptake of organic anions and amphipathic compounds	
Rat / human	rOCT1 / hOCT1	SLC22A1	Uptake of small hydrophilic organic cations (TEA, MPP, choline, dopamine)	Not yet investigated
Rat	OAT2	SLC22A7	Uptake of glutarate, salicylates, methotrexate, PGE_2 and PAH	Not yet investigated
Rat	OAT3	SLC22A8	Uptake of PAH, estrone-3-sulfate, ochratoxin A, cimetidine	Not yet investigated
Rat / human	Mrp1 / MRP1	ABCC1	Efflux of cytotoxic cations and non-bile salt organic anions	Increased expression in hepatoma cells[85] and sepsis[182]
Rat / human	Mrp3 / MRP3	ABCC3	Efflux of organic anions, bile salts and anticancer agents	Increased Mrp3 expression in Eisai Hyperbilirubinemic Rats and in bile duct ligation[91] Increased MRP3 expression in Dubin-Johnson syndrome and primary biliary cirrhosis[86]
Rat / human	Mrp6 / MRP6	ABCC6	Efflux of BQ-123	Not yet investigated
Canalicular Transport Proteins				
Mouse / rat / mBsep / Bsep / BSEP Human		ABCB11	Canalicular efflux of bile salts	Mutations in the BSEP gene and absence of the protein in patients with PFIC2, characterized by low γ-GT levels and reduced biliary bile acid excretion[103,110] *Cis*-inhibition by cholestatic drugs such as cyclosporine A[172] *Trans*-inhibition by the cholestatic estrogen metabolite estradiol-17β–D-glucuronide[172,175] Increased mBsep expression in C57L / J gallstone-susceptible mice, despite reduced bile salt excretory capacity[107,109]
Mouse / rat /	Mdr2 / Mdr2 / MDR3	ABCB4	Biliary excretion of phospholipids	Mdr2 -/- knockout mice exhibit an absence of phospholipids in bile and develop progressive liver disease with portal inflammation, bile duct proliferation and fibrosis[123] PFIC3, characterized by high γ-GT levels and absent lipoprotein X in serum, is caused by mutations in the *MDR3* gene (chromosome 7q21)[143] MDR3 mutations in PFIC3 are associated with intrahepatic cholestasis of pregnancy[171]
Rat / human	Mrp2 / MRP2	ABCC2	Canalicular excretion of organic anions	Decreased Mrp2 mRNA and protein levels in bile duct ligation and endotoxinemia[154,183] Decreased canalicular density of Mrp2 transporter molecules in endotoxinemia[183], taurolithocholate cholestasis[184] and bile duct ligation[154] Mutations in the rat *Mrp*2 gene cause hereditary conjugated hyperbilirubinemia[112] Mutations in the human *MRP*2 gene cause the Dubin-Johnson syndrome with absent protein expression[147,149] MRP2 function is inhibited by anabolic 17á-alkylated steroids[185,186] Decreased MRP2 mRNA but unchanged protein levels in PBC[187] Decreased MRP2 mRNA levels in PSC[181]
Human	FIC1	ATP8B1	Putative aminophospholipid translocator	P-type ATPase, positional candidate in genetic linkage analysis of PFIC1 (Byler's disease) and BRIC[141]
Human	AE2	SLC4A2	Canalicular Cl- / HCO_3^- exchange	Decreased AE2 expression on the luminal surface of cholangiocytes in PBC (increased expression secondary to UDCA treatment)[188]

Abbreviations: Ntcp / NTCP, rat / human Na^+-taurocholate cotransporting polypeptide; Oatp / OATP, rat / human organic anion transporting polypeptide; rOCT1 / hOCT1, rat / human organic cation transporter 1; OAT, organic anion transporter; Mrp / MRP, rat / human multidrug resistance protein; mBsep / Bsep / BSEP, mouse / rat / human bile salt export pump; Mdr / MDR, rodent / human multidrug resistance gene product; FIC1, familial intrahepatic cholestasis protein; AE2, anion exchanger 2; PSC, primary sclerosing cholangitis; PFIC, progressive familial intrahepatic cholestasis; PBC, primary biliary cirrhosis; BRIC, benign recurrent intrahepatic cholestasis; UDCA, ursodeoxycholic acid.

The molecular basis of reduced Ntcp expression in cholestasis has not been resolved. The *Ntcp* gene promoter appears to contain a response element for the farnesoid X receptor (FXR), a nuclear receptor that is responsive to bile salts[156-159]. This is suggested by recent studies in FXR knockout mice, which are unable to decrease Ntcp mRNA levels in response to bile acid feeding[160]. Since intracellular bile salt levels are elevated in bile duct ligation, decreased Ntcp expression is probably caused by suppression of Ntcp transcription via a cascade involving FXR. In the case of cholesterol 7á-hydroxylase (CYP7A1), the rate-limiting enzyme in cholesterol catabolism to bile salts, repression of gene transcription by bile salts has been extensively studied (for review, see reference[161]). Elevated intracellular bile salts activate FXR. This decreases levels of Ntcp and increases those of Bsep[160]. FXR also induces expression of the "short heterodimer partner" SHP, a nuclear receptor that suppresses bile acid synthesis by antagonizing the function of "liver receptor homolog-1" or LRH-1, an orphan receptor required for expression of CYP7A1[161]. Decreased expression of rat Oatp1 in bile duct ligation may also be mediated by FXR, since in FXR knockout mice bile acid feeding induces expression of (mouse) Oatp1[160]. These elaborate autoregulatory cascades ultimately serve to maintain hepatic cholesterol catabolism, and coordinate regulation of bile acid transporters and synthesizing enzymes is likely.

Sepsis-associated cholestasis Septic patients frequently exhibit cholestasis, the primary clinical manifestation of which is hyperbilirubinemia. In animal models of sepsis, reduced hepatic clearance of bile acids and organic anions is found[27,33,162]. The key mediators of sepsis induced cholestasis are inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β. These are liberated in response to endotoxemic stimuli, which can be induced experimentally by application of bacterial lipopolysaccharide (LPS). Both Na^+ dependent basolateral and ATP-dependent canalicular bile salt transport is reduced in hepatocyte plasma membrane vesicles isolated from LPS treated rats[27,33]. Direct administration of either LPS, TNF-α or interleukin-1β causes a decrease in Ntcp mRNA levels[26]. The decrease in Ntcp expression can be explained by decreased binding activity of ① the nuclear transcription factor hepatocyte nuclear factor 1 (HNF1) and ② a heterodimeric complex consisting of the retinoic acid receptor (RARα) and the retinoid x receptor (RXRα), to the *Ntcp* gene promoter[163, 164]. In the case of the human NTCP, dependence of gene transcriptional activity upon the CCAAT/ enhancer binding protein, the α form of which is reduced in sepsis[165], has been shown[166].

The reduction in bile flow that follows LPS administration is caused primarily by an 86% decrease in GSH secretion and a 25% decrease in HCO_3^- secretion [167], two major driving forces of bile salt independent bile flow. GSH is a substrate of Mrp2[117], the mRNA and protein levels of which are also reduced following treatment of rats with LPS[154]. The mechanism of decreased Mrp2 expression appears to be similar to Ntcp, since reduced binding of the RXRα:RARα complex to the rat *Mrp2* promoter secondary to IL-1β has been shown[164].

Cholestasis of pregnancy Intrahepatic cholestasis of pregnancy (ICP) has a high prevalence in Sweden and Chile and is characterized by pruritus and biochemical cholestasis. It is the clinical correlate of estrogen induced cholestasis. The familial clustering, the higher prevalence among relatives of patients with ICP and the susceptibility to oral contraceptive-induced cholestasis in families with a history of ICP implicates genetic factors in the pathogenesis[168-170]. Mutations of the *MDR3* gene in women with PFIC type 3 seem to predispose to ICP, although not all women with the mutation develop cholestasis[143,171].

The susceptibility to ethinyl estradiol in patients with a history of ICP suggests a role for estrogen metabolites in the pathogenesis. The cholestatic estrogen metabolite estradiol-17β-D-glucuronide (E-217G) has been shown to inhibit Bsep transport function[172]. E-217G, which is an Mrp2 substrate[173], probably *trans*-inhibits Bsep function from within the canalicular lumen, since Mrp2-deficient rat strains that are unable to secrete E-217G into the bile canaliculus do not develop cholestasis[174]. A recent study has confirmed that intact Mrp2 function is a prerequisite for the development of $Es_2$17G induced cholestasis [175]. The possible role of as yet unidentified genetic polymorphisms of the *BSEP* gene in the development of estrogen-induced cholestasis is currently under investigation.

Drug-induced cholestasis The liver is the major site of drug metabolism and elimination from the human body. The importance of drugs as hepatotoxins lies not in the overall number of cases, which is relatively small, but in the severity of some reactions and in their potential reversibility provided the drug etiology is promptly recognized. The most common causative agents include NSAIDs, antibiotics, newer antihypertensive agents, H_2-receptor blockers and psychotropic drugs. Drug induced hepatotoxicity can be divided into the three categories cholestatic, hepatocellular or mixed type injury, depending upon serum biochemistry. Cholestasis with hepatitis is seen with many drugs, notably chlorpromazine, psychotropic agents, erythromycins, clavulanic acid and NSAIDs. Pure cholestasis without hepatitis is observed most

frequently with estrogens, oral contraceptive steroids and 17α-alkylated androgenic steroids and less frequently with cyclosporine A, tamoxifen, griseofulvin, glibenclamide and others. Steroid jaundice caused by methyltestosterone and other C17-alkylated anabolic steroids is dose-related but is also dependent upon the individual susceptibility of the recipient. Whereas hepatic dysfunction is seen in most recipients of steroids, jaundice in seen in only few. A minor degree of hepatic dysfunction in women taking oral contraceptives which contain C-17 ethinyl estrogen and progesterone derivatives is relatively frequent. As mentioned above, women with a personal or family history of cholestatic jaundice of pregnancy are particularly prone to develop jaundice when taking oral contraceptives.

The following alterations of hepatocellular transporter function can be held responsible for the development of drug induced cholestasis. Selective interference of a drug or its metabolite with bile secretory mechanisms has been shown for C17-alkylated ethinylated steroids, the cholestatic bile acid lithocholic acid, and experimentally for icterogenin. *Cis*-inhibition of Bsep mediated [^{3}H]-taurocholate transport by cyclosporine A, rifamycin SV, rifampicin and glibenclamide is the likely mechanism for itrahepatic cholestasis caused by these agents[172]. Parenteral administration of cyclosporin A in rats inhibits both bile salt excretion and bile salt-independent bile flow, resulting in cholestasis[176]. In addition, bile salt synthesis decreases by about 50% and the total bile acid pool is reduced in rats following orthotopic liver transplantation. Selective interference with the sinusoidal -uptake of substances such as bilirubin and bromosulphophthalein has been shown for the tuberculostatic agents rifamycin SV and rifampicin. Both are mainly eliminated by hepatic uptake, metabolism and excretion into bile. Rifampicin increases serum bile salt concentrations in 72% of patients after the first dose[177], suggesting acute interference with sinusoidal uptake of bile salts. In the Xenopus laevis-oocyte expression system, rifampicin was shown to inhibit Oatp2 but not Oatp1 mediated taurocholate uptake. Both Oatp1 and Oatp2 were inhibited by 10 μmol/L rifamycin SV, whereas significantly higher concentrations of rifamycin SV and rifampicin were required to inhibit Ntcp[178].

The nonsteroidal anti-inflammatory agent sulindac, an established hepatotoxin, may also cause cholestasis by interference with the canalicular excretion of bile salts. Sulindac has been shown to follow the "cholehepatic shunt" pathway and induce choleresis[179]. However, when coinfused with taurocholate in the isolated perfused rat liver, sulindac causes cholestasis by reducing taurocholate secretion. Sulindac appears to be secreted into the bile canaliculus in unconjugated form via a canalicular bile salt export system and is passively absorbed by the bile duct epithelium, thereby inducing a bicarbonate-rich choleresis. Due to continuous cycling within the cholehepatic shunt pathway, high local concentrations of sulindac could be reached within the hepatocyte that cause cholestasis by inhibition of canalicular bile salt efflux[180-188].

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Transjugular portosystemic stent shunt in treatment of liver diseases

Michael Schepke and Tilman Sauerbruch

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INTRODUCTION

More than 10 years ago, an interventional technique for the creation of an intrahepatic decompressive shunt between a branch of the portal vein and a main hepatic vein using expandable metallic stents has been introduced for the treatment of portal hypertension[1,2]. This transjugular portosystemic intrahepatic stent shunt (TIPS) functions as a side to side shunt, similarly to surgical shunts. During the last decade, TIPS has become one of the major therapeutic options for patients suffering from the most common complications of liver cirrhosis, namely recurrent intestinal bleeding and refractory ascites. At present it is probably the most frequently performed portosystemic shunt procedure. TIPS insertion does not require abdominal surgery and has a lower procedure related mortality than conventional surgical shunts. In specialized centers, the rate of procedure related deaths is not higher than 1% as compared to 3%-15% reported for surgical shunts [3,4]. Thus, critically diseased or elderly patients who are not can didates for surgical shunts may be considered for TIPS insertion. However, every portosystemic shunt procedure has the potential disadvantage that the portal liver perfusion is reduced or completely stopped which may deteriorate liver function. Therefore, TIPS has to be compared with both surgical shunts and the non-shunt treatment alternatives, namely endoscopic treatment for bleeding varices and paracentesis for the treatment of refractory ascites.

In addition to the technical expertise required to perform intrahepatic stent placement, the proper selection of patients and indications remains a crucial issue. Numerous controlled clinical trials published within the last years help us weigh the role of TIPS among the different treatment options and to select the patients, who are most likely to benefit from this procedure.

Department of Internal Medicine I, University of Bonn, Bonn, Germany
Correspondence to: Prof. Dr. Tilman Sauerbruch, Department of Internal Medicine I, University of Bonn, Sigmund-Freud-Str.25, D-53127 Bonn, Germany
Tel. +49-228-2875216, Fax. +49-228-2874322
Email. sauerbruch@uni-bonn.de
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INTESTINAL BLEEDING

Bleeding from esophageal or gastric varices in patients with portal hypertension is still associated with a high mortality of about 30%-50%[5]. Prior to the first bleeding episode, medical treatment with unselective betablockers is the therapeutic standard in patients with a high bleeding risk, shunt procedures are not indicated for the primary prevention of bleeding[5]. Once a patient has survived the first episode of intestinal bleeding, the rebleeding rate without treatment is about 50%-70% within two years[6]. Thus, the application of therapeutic strategies for the prevention of rebleeding is mandatory.TIPS versus endoscopic treatment for the prevention of rebleeding from varices

The first line treatment for bleeding varices at most centers is endoscopic injection sclerotherapy or banding ligation. These procedures have proved effective in both treating the acute bleeding episode and preventing rebleeding[5]. Within the past years, numerous studies demonstrated that banding ligation is superior to sclerotherapy with respect to local side effects (treatment induced ulcers and bleeding), efficacy of variceal eradication, and rebleeding rate. Thus, endoscopic variceal ligation is considered to be the best established endoscopic treatment for patients with bleeding varices[7].

However, endoscopic procedures do not reduce portal pressure and thus, even after effective variceal eradication, recurrent varices may occur or patients may develop bleeding from extraesophageal varices. Since TIPS does reduce the portal pressure by about 50%, it was assumed to be more effective for the prevention of rebleeding. To date, eight controlled trials comparing TIPS with endoscopic therapy and two meta-analyses have been published[8-17].

Overall, according to the pooled data in the most recent meta-analysis[15], the results of the other meta-analysis published earlier are not substantially different[17]. TIPS is superior to endoscopic treatment with respect to rebleeding. The overall rebleeding rate within 13 and 33 months

was 21% in the TIPS-group as compared with 52% for endoscopic treatment. The number of patients needed to be treated by TIPS instead of endoscopic treatment to prevent one rebleeding episode was 3.3 (95% CI 2.6, 4.4). It was argued that the rebleeding rate in the endoscopically treated patients was rather high in these trials, probably because the centers that performed these trials are specialized in TIPS rather than in endoscopic treatment. Indeed, in trials comparing different endoscopic techniques for the prevention of rebleeding, the rebleeding rates for both sclerotherapy and banding ligation were lower (approximately 40%)[7]. However, since the pooled rebleeding rate for TIPS was only 21% in the published trials, and it may even be better today because of improved patients' surveillance, it can be concluded that TIPS is more effective than endoscopic treatment for the prevention of rebleeding. On the other hand, TIPS significantly increased the encephalopathy rate (31% versus 19% after endoscopic treatment). When 4-10 (95% CI) patients are treated by TIPS instead of endoscopic treatment, one more encephalopathy rate occurs. Only one trial that had been included in the meta-analysis in abstract form but has recently been published in full text demonstrated a significant reduction of mortality after TIPS[16]. In the meta-analysis, mortality was not significantly different between the TIPS and endoscopic groups. The most common cause of death among those patients treated with TIPS was liver failure, whereas variceal rebleeding was the most common cause of death in endoscopically treated patients[15]. Thus, with respect to bleeding related mortality, there was a trend in favor of TIPS, which, however, was not statistically significant[15].

In conclusion, TIPS is more effective for the prevention of rebleeding as compared to endoscopic treatment. This does, however, not translate into a reduction of mortality, most likely because TIPS may deteriorate liver function in some patients as indicated by a higher encephalopathy rate. Most centers prefer endoscopic banding ligation as first line treatment of bleeding esophageal varices and insert a TIPS when the endoscopic treatment fails (rebleeding despite endoscopic treatment). The clinical decision to change the therapeutic strategy from endoscopy to TIPS requires an exact evaluation of the patient's bleeding history and liver function. The severity of bleeding episodes, the prior endoscopic treatment and the site of bleeding have to be taken into account, e.g., one might change earlier from endoscopic treatment to TIPS in patients bleeding from gastric varices since they are more difficult to treat endoscopically and often bleed more severely than esophageal varices. Furthermore, TIPS is also effective in patients with ascites. In patients with recurrent variceal bleeding and concomitant severe ascites, it is reasonable to insert a TIPS earlier since these patients may also benefit with respect to ascites.

However, patients with a decompensated liver disease (bilirubin >85.5 μmol/L, Child's class C, a history of hepatic encephalopathy unrelated to bleeding) are likely to further deteriorate after TIPS insertion as compared to patients with a preserved liver function. These patients are not candidates for elective *TIPS*-insertion.

TIPS versus propranolol for the prevention of rebleeding

Propranolol effectively reduces portal pressure and related risk of rebleeding. Studies comparing unselective beta-blockers with TIPS for the prevention of rebleeding are lacking. Thirty to 40 percent of patients do not respond to propranolol with an adequate decrease of portal pressure. In a meta-analysis of nine randomized trials endoscopic sclerotherapy (which is inferior to ligation) has been shown to be more effective than propranolol to prevent variceal rebleeding[18] and TIPS is more effective than endoscopic treatment for this indication[15]. Thus, although there are no controlled trials on this topic, one may indirectly conclude that TIPS must be more effective for the prevention of variceal rebleeding than unselective beta-blockers.

Emergency TIPS for the treatment of uncontrolled bleeding

Uncontrolled acute variceal bleeding despite adequate first-line endoscopic treatment with or without vasoactive drugs continues to be a major clinical problem. The prognosis of these patients is poor and only the early diagnosis and treatment of an uncontrolled bleeding or an early rebleeding may improve the outcome.

Once the diagnosis of an uncontrolled bleeding despite adequate first-line treatment is made (which should be based on the Baveno criteria[19], emergency TIPS is recommended as second line treatment[20] since it is effective in both reducing portal pressure and arresting bleeding in >90% of patients[21]. However, since these critically ill patients frequently develop severe complications (e.g., sepsis, pneumonia, respiratory failure, hepatorenal syndrome) although the bleeding is controlled after TIPS, only about 50% of patients survive for more than two months after emergency TIPS insertion according to most series[21].

TIPS for bleeding extraesophageal collaterals

Some patients with portal hypertension bleed from

ectopic collaterals like duodenal or rectal varices[22]. These patients can also be effectively treated by TIPS insertion. However, atypically located varices occur more often in patients with portal hypertension due to a prehepatic obstruction like portal vein thrombosis. These patients are not candidates for TIPS, especially in patients with ectopic varices, the patency of the portal vein has to be proved prior to TIPS insertion.

TIPS for bleeding from hypertensive gastropathy

Since hypertensive gastropathy is associated with an elevated portal pressure and chronic or-*rarely*-acute bleeding from the gastric mucosa in these patients is difficult to treat with drugs or endoscopic procedures, TIPS has been evaluated for this indication. The largest recently published study found a beneficial effect of TIPS insertion with respect to rebleeding and endoscopic findings both in mild and severe hypertensive gastropathy and concerning transfusion requirements in patients chronically bleeding from severe hypertensive gastropathy[23]. This study emphasizes the importance to differentiate between patients with a real severe hypertensive gastropathy and patients with a gastric (antral) vascular ectasia (G(A)VE syndrome) because the former responds to TIPS insertion whereas the latter does not. Prior to TIPS-insertion, G(A)VE should be ruled out in these patients by endoscopy or-if necessary-by biopsy.

REFRACTORY ASCITES

The elevated portal pressure plays an important role in the pathogenesis of refractory or recurrent ascites. This clinical situation indicates a severe impairment of liver function. Thus, these patients should be evaluated for liver transplantation. If liver transplantation is not available or applicable, repeated large-volume paracentesis, implantation of a peritoneovenous shunt or TIPS insertion are the remaining treatment options.

Peritnoneovenous shunting is no longer routinely performed in most specialized centers since the occlusion and infection rate is high and some studies demonstrated an even increased death rate as compared to paracentesis[24]. Repeated paracentesis is effective and safe[24,25] but is associated with some disadvantages, e.g., the risk of bacterial peritonitis or local bleeding due to frequent punctures, adverse effects of high-dose diuretic treatment, intermittent tense ascites and risk of hepatorenal syndrome.

The majority of preliminary, uncontrolled studies that applied TIPS for the treatment of refractory ascites are promising[26-30]. A recently published, larger-scale controlled randomized trial comparing TIPS with repeated paracentesis for the treatment of refractory ascites demonstrated that TIPS is very effective for this indication[31]. Following TIPS insertion, 61% of patients had no ascites after three months as compared with 18% in the paracentesis group. In this study treatment with TIPS was independently associated with a better transplant-free survival in the mulitvariate analysis (one/two year transplant-free survival: 69%/58% in the TIPS group versus 52%/32% in the paracentesis group). In contrast, the only controlled randomized small study investigating the role of TIPS for the treatment of refractory ascites reported a significantly worse survival in the Child's class C patients treated with TIPS as compared to those treated with paracentesis[32]. However, since the mean baseline serum bilirubin was 30.8 μmol/L in the patients included in the first study[31], this discrepancy might be due to the fact that the patients in the latter study[32] had a more severe hepatic impairment. It must be stressed, that the number of patients included was relatively small in both studies[25,32] and 60[31] patients, respectively. Thus, larger studies with a higher statistical power are needed, in particular to determine the role of TIPS with respect to survival in those patients. However, it can be concluded from these two trials that TIPS is effective for the treatment of refractory ascites in patients with moderately impaired liver function (Child's class B or "good" C, serum bilirubin <51. 3 μmol/L). In patients with a more severe liver impairment TIPS may even accelerate the progression of liver failure and worsen the prognosis.

Hepatorenal syndrome

Patients with refractory ascites are at risk to develop a hepatorenal syndrome. This syndrome is characterized by renal insufficiency in patients with decompensated liver cirrhosis without preexistent kidney disease[33]. Especially the rapidly progressive form, in the literature commonly referred to as hepatorenal syndrome type I, has a very poor prognosis: 90% of patients died within a few weeks after diagnosis, the median survival was only two weeks[34]. Liver transplantation is the only definitive treatment for patients with hepatorenal syndrome, but many patients are not elegible for transplantation. Furthermore, due to the rapid course of this disease, even candidates for transplantation may die while waiting for a donor organ. A recent uncontrolled study evaluated TIPS in 41 non-transplant cirrhotic patients with hepatorenal syndrome (21 type I, 20 type II)[35]. The results were very promising. Even in the group of patients with type I hepatorenal syndrome, 50% of patients treated with TIPS were still alive

after six months as compared with 14% in the non-TIPS control group. TIPS cannot be recommended as an established treatment for hepatorenal syndrome before controlled randomized trials are published, but preliminary results indicate that TIPS is probably effective as a bridge to transplant for this serious condition.

SELECTION OF PATIENTS FOR TIPS

As it has been outlined, TIPS is an effective treartment for some severe complications of liver cirrhosis. However, like every portosystemic shunt procedure, TIPS may deteriorate liver function by reducing portal liver perfusion. Thus, most studies failed to demonstrate a survival benefit in patients treated with TIPS. Especially in patients with refractory ascites, a symptom of an at least moderate to severe hepatic impairment, selection criteria have to be evaluated very carefully. For clinical practice, the serum bilirubin has proved to be an important tool for this decision. In patients with a bilirubin >51.3 μmol/L TIPS should be inserted only in life threatening conditions like uncontrolled variceal bleeding. In the elective situation, patients with such a severe hepatic impairment may rather benefit from non-shunt treatment alternatives. In patients with a cholestatic liver disease, higher baseline bilirubin levels may be tolerable prior to TIPS insertion. Recently, two different scoring systems for the prediction of survival after TIPS based on pre-TIPS parameters have been published[36,37]. Both scoring systems contain the serum bilirubin. In addition to serum bilirubin levels, these studies identified the following risk indicators for poor prognosis after TIPS insertion. Pre-TIPS hepatic encephalopathy unrelated to bleeding, TIPS as emergency treatment for uncontrolled hemorrhage, alanin aminotransferase levels of >100U/L[36], impaired renal function, prolonged prothrombine time and viral or other non-alcoholic, non-cholestatic etiology of liver cirrhosis[37].

SUMMARY

During the last years, TIPS has definitely gained an important role within the different therapeutic options for patients with complicated liver cirrhosis. TIPS can be recommended as second-line treatment for gastroesophageal varices that bleed despite adequate endoscopic treatment and is also beneficial in many patients with refractory ascites. TIPS insertion is recommended as emergency treatment of otherwise uncontrolled variceal bleeding. Furthermore, although controlled trials are lacking, TIPS is probably also effective in more rare conditions like hepatorenal syndrome, hepatic hydrothorax and Budd-Chiari syndrome. However, TIPS insertion may also deteriorate liver function by reducing the portal perfusion and thus, patients have to be selected carefully.

As compared to surgical shunts, TIPS has the clear advantages of being less invasive and probably having a lower procedure related mortality. However, as indicated by the only randomized controlled trial directly comparing TIPS with a surgical shunt[38] (portocaval H-graft shunt), the relatively high TIPS-dysfunction rate due to thrombosis or endothelial hyperplasia remains a clinical problem requiring a careful follow-up of patients and should be addressed in the future development of this technique.

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Edited by Ma JY

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Historical origins of current IBD concepts

Joseph B. Kirsner

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INTRODUCTION

The "nonspecific" inflammatory bowel diseases, ulcerative colitis and Crohn's disease, represent a group of heterogeneous inflammatory and ulcerative diseases of the small and large intestines of unknown etiology, associated with many gastrointestinal and systemic complications. Appearing initially as isolated cases in Great Britain and northern Europe during the 19th and early 20th centuries, they have steadily increased numerically and geographically and today are recognized worldwide.

ULCERATIVE COLITIS

Matthew Baillie's 1793 *Morbid Anatomy of Some of the Most Important Parts of the Human Body* strongly suggests that patients were dying from ulcerative colitis during the latter part of the 18th century[1]. The first "impact" description of "ulcerative colitis" by Samuel wilks[2] of London in 1859 concerned a 42 year old woman who died after several months of diarrhea and fever. Autopsy demonstrated a transmural ulcerative inflammation of the colon and terminal ileum, originally designated as "simple ulcerative colitis", but a century later identified as Crohn's disease[3]. The 1875 case report of Wilks and Moxon[4] describing ulceration and inflammation of the entire colon in a young woman who had succumbed to severe bloody diarrhea was an early instance of ulcerative colitis.

In 1902 R.F. Weir[5] performed an appendicostomy in a patient with ulcerative colitis to facilitate colonic irrigation with potassium permanganate for a presumed infection. J. P. Lockhart-Mummery[6] of London in 1907, aided by the then new electrically illuminated proctosigmoidoscope, discovered carcinoma of the colon in seven of 36 patients with ulcerative colitis. By 1909, 317 patients had been admitted to seven London hospitals with an inflammatory and ulcerative disease of the colon[7]. Many had died from perforation of the colon, peritonitis, hemorrhage, sepsis and pulmonary embolism. Into the 20th century similar instances of "ulcerative colitis" were being reported in Europe and in the United States. Etiologic speculation included food and pollen allergy and a psychogenic disorder. Treatment later with sulfonamides (1938) and then antibiotics, beginning with penicillin (1946), re-emphasized the possibility of a bacterial infection. The favorable responses to ACTH and adrenal steroids during the 1950s[8] stimulated interest in immunological mechanisms as discussed later.

Pathology Initial pathologic descriptions of ulcerative colitis recognized the diffuse mucosal/submucosal involvement, beginning in the rectum and rectosigmoid, and advancing proximally to involve the entire colon in a diffuse inflammation of the mucous membrane with chronic inflammatory cells, lymphocytes, plasma cells, and eosinophiles, vascular congestion, goblet cell depletion, and crypt abscesses [9]. In 1933 Buie and Bargen[10] implicated vascular "thrombotic phenomena" as the patholgical basis for ulcerative colitis and in 1954 S. Warren and S. Sommers[11] described an inflammatory necrosis of arteries, veins, or both, leading to vascular occlusions and infarction of the colon in some patients with ulcerative colitis. A 1949 review implicated an etiologic agent in the fecal stream[12], as had been proposed by P. Manson-Bahr in 1943 and earlier by B.Dawson[13] in 1909.

"Natural" and experimental colitis Veterinarians long had been aware of inflammatory diseases of the small intestine and colon in animals (dogs, cat, horse, cattle, sheep, swine, rodents), attributable to bacteria, parasites, or viruses. However, despite morphologic similarities, none duplicated human IBD. Only the colitis in cotton top tamarins (saguinus oedipus) from colombia, housed in the United States, resembled human ulcerative colitis in its clinical and histologic features and response to sulfasalazine.

Many attempts to reproduce ulcerative colitis in animals (rabbit, guinea pig, hamster, dogs, mice, rats) during the 1920s-1960s[14] included nutritional depletion (vitamin A, pantothenic acid, pyridoxine), the local application of Shiga and staphylococcal toxins to colonic explants, the vasoconstriction induced by adrenalin

The Louis Block Distinguished Service Professor of Medicine, Department of Medicine, University of Chicago
Correspondence to: Joseph B. Kirsner, The Louis Block Distinguished Service Professor of Medicine, Department of Medicine, University of Chicago
Tel. 773-702-6101, Fax. 773-702-4028
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intraperitoneally in dogs, the intravenous injection of staphylococcus toxin in rabbits, enzymes (collagenase, lysozyme) intrarectally and intraarterially and carrageenan orally[15]. Topically (colonic) applied compounds (4%-10%) acetic acid, trinitrobenzene sulfonic acid in 50% alcohol), orally administered drugs (indomethacin, mitomycin-c), and inhibition of fatty acid oxidation[16] caused temporary colonic injury.

CROHN'S DISEASE

In 1612 Gullielmus Fabricius Hildenus (Wilhelm Fabry)[17] (1560-1634) noted at autopsy in a boy who had died after persistent "abdominal pain" and diarrhea that "the ulcerated cecum (was) contracted and invaginated into the ileum". G.B. Morgagni[18] (1682-1771) in his 1769 "De Sedibus et Causis Morborum" described ulceration and perforation of an inflamed distal ileum and enlarged mesenteric lymph nodes in a young man of 20 with a history of diarrhea and fever culminating in death after 14 days. Similar cases were reported by Combe and Saunders[19] and by Abercrombie[20]. Abraham Colles[21] of Dublin in 1830 described Crohn's disease among children and the complicating perianal, rectovaginal and rectovesical fistulas. In 1889 Samuel Fenwick[22], in a 27 year old woman with a history of diarrhea and weight loss, at autopsy observed "adherent loops of intestine with a communication between the cecum and adherent small intestine…" The lower end of the ileum was dilated and hypertrophied and the ileocecal valve was contracted to the size of a swan's quill. Early in the 20th century, case reports from Europe documented the occurrence of a similar condition associated with lower abdominal (inflammatory) masses, assumed to be "malignant" and, at a time of limited abdominal surgery, arbitrarily dismissed as "untreatable"[23].

The classic 1913 paper by T.Kennedy Dalziel[24], including 13 patients, antedated Crohn's contribution by nearly 20 years. The first patient had experienced bouts of cramping abdominal pain and diarrhea since 1901, progressing to intestinal obstruction and death. At autopsy, the entire small intestine was chronically inflamed and the mesenteric lymph nodes were enlarged. Dalziel attributed his "chronic interstitial ileitis" to Johne's mycobacterial intestinal disease of cattle.

By 1920 American physicians were reporting instances of hyperplastic, granulomatous lesions of the intestinal tract, originally identified as "hyperplastic intestinal tuberculosis." The clinical features were similar: young patients (children, teenagers, and young adults) often operated upon for "appendicitis", symptoms of fever, abdominal cramps, diarrhea, and weight loss. The disease usually involved the terminal ileum or ileocecal area. In a 20 year old man, three bowel resections were required within 18 months for recurrent intestinal obstruction[25]. In some countries (United States, England, Sweden) but not in others (Denmark, Norway), Crohn's disease was more commonly reported among Jewish people (Ashkenazi rather than Sephardic) regardless of native birth, immigrant history or orthodoxy.

Immediately preceding the paper by Crohn *et al*[26] in 1932, F.J. Nuboer[27] of Holland and M. Golob [28] of New York (1932) and in 1934 A. D.Bissell[29] of the University of Chicago reported instances of a similar disease. In 1936 Crohn *et al*[30] described 9 patients with combined ileitis and right-sided colitis. Fone[31] of Australia, noted that 40 of 41 patients had had at least one abdominal operation. Despite early European and American descriptions of colonic involvement by Crohn's-like inflammatory lesions[32, 33], the concept was not completely accepted in America until the 1959 and 1960 reports of Lockhart-Mummery *et al*[34,35].

Etiologic speculation included bacteria, viruses, abdominal trauma and impaired vascular and lymphatic circulation. In 1943, Tallroth[36], noting many eosinophils in histologic sections, termed the disease "ileitis allergica". The concept of an endolymphangitis provided the rationale for the 1936 experiments of Reichert and Mathes[37] who injected fine sand and the sclerosing solution of 26% bismuth oxychloride with Esch. Coli into the cannulated mesenteric lymphatics of dogs, producing an edema of the ileocecal area. Chess [38] in 1950 fed dogs silica and talc; and kalima *et al*[39] (1976) injected formalin solution into the mesenteric lymphatics, producing an endolymphangitis but not regional enteritis. Van Patter *et al*[40] in 1954 suggested that "the causative agent may be found in the fecal stream" entering the lymphatic system and causing lymphatic obstruction, dilatation and lymphoid hyperplasia but this possibility again went unnoticed.

Pathology of Crohn's disease In 1938 Coffey[41] emphasized the subacute or chronic, granulomatous inflammatory process, the tendency to intestinal stenosis and the fistula formation. In 1939 G. Hadfield[42] of England noted thickening of the ileum, fistulas from bowel to abdominal wall and to the urinary bladder, the giant-cell systems in the submucosa and in regional lymph nodes and the lymphedema of the submucosa. Warren *et al*[43] described the process as: "A progressive sclerosing granulomatous lymphangitis, probably a reaction to an irritative lipid substance in the bowel content." Rappaport's[44] 1951 study of 100 cases included 85

bowel resections and 15 autopsies; in 72 instances, sections from mesenteric lymph nodes, and in 35 appendices, documenting the gross features of Crohn's disease: adherent mesentery, thickened distal small bowel, enteric fistulas, intestinal narrowing, aphthous and linear serpiginous ulcers, a cobblestone appearing mucosa, and an asymmetrical distribution of disease. The tiny slit-like ulcer, located precisely over the M cell in the epithelium overlying lymphoid follicles in Peyer's patches[45], the granulomas, the focal distribution and the lymphoid prominence conveyed as "pathogenetic" histologic features of Crohn's disease.

EPIDEMIOLOGY

An epidemiological approach to inflammatory bowel disease was not feasible until the 1950s. Melrose [46] in 1955 collected information on 1425 patients with chronic idiopathic ulcerative colitis for the years 1946 to 1950 and proposed an incidence of 10.9% per 10 000 general admissions. The rate of 6.9% for the five Scottish towns in contrast to 15. 5% for the London hospitals was early recognition of the urban: rural IBD incidence differential. Houghton *et al*[47] in 1958, on the basis of 170 patients with ulcerative colitis and 32 with ileitis in Bristol, England for 1953, 1954, and 1955, estimated annual incidence rates of 0.85 per 1000 for ulcerative colitis and 0.14 per 1000 for regional ileitis. Ustvedt[48] of Norway in 1958, for the ten year period 1945-55, noted a mean annual rate of 1.2 per 100 000 population. Acheson[49] in 1960 analyzing data for 2320 male veterans discharged from U.S. Veterans Administration hospitals with diagnoses of regional ileitis, ulcerative colitis, or nonspecific enteritis, observed a fourfold increase of Jewish patients, over a sample of all discharges. Acheson[50] also noted a twentyfold increase in the incidence of ankylosing spond ylitis among U.S. veterans with IBD.

In the first population study of 231 patients with ulcerative colitis (excluding proctitis), Iversen *et al*[51], in Copenhagen county (Denmark) for the period 1961-1966, reported a disease incidence averaging 7.3 per 100 000 per year. A population study of Crohn's disease in two counties in central Sweden for the period 1956-1967[52] revealed a mean incidence of 2. 5/100 000 for the first six years of the 12 year span and 5.0 during the second six year period, a rising trend observed subsequently in other geographic areas.

Epidemiologic studies by Mendeloff *et al*[53-55] in the Baltimore area during the 1960s documented the rising incidence of ulcerative colitis during the first half of the 20th century, exceeding Crohn's disease in a proportion of 4 to 5:1. Mendeloff characterized the IBD population as follows: ① Males and females nearly equally affected; ② patients more commonly western than oriental, much more often of northern European origin; ③ more often urban than rural dwellers; ④ more often caucasian than colored; ⑤ more common among Jews (Originating often in northern Europe and North America) than among non-Jews, but not common among Israelis; and ⑥ more common in families than expected. For the period 1960 to 1979 Calkins and Mendeloff[55], comparing their first and second analyses, noted an increase in the age adjusted rate for Crohn's disease over ulcerative colitis, for whites of both sexes and for non-white females. Subsequent epidemiologic surveys[56] documented the worldwide distribution of IBD, the initially increased and now stabilizing incidence of ulcerative colitis, the rising incidence of Crohn's disease, appearing also in formerly "lagging" countries (Brazil, South Korea) and the unexpectedly high incidence of inflammatory bowel disease (especially Crohn's disease) in such areas as the North Tees Health District of England.

The implication of foods in the etiology of Crohn's disease during the 1960s-1970s, especially concentrated sugars, margarine, and fats, never attained scientific credibility.

Smoking and IBD The relationship between ulcerative colitis and non-smoking, especially the occurrence of ulcerative colitis among former smokers, was first reported by S.M. Samuelsson[57] in a 1976 thesis (Uni versity of Upsala). Rhodes *et al*. of Cardiff, Wales[58] in a 1982 mail questionnaire confirmed the hitherto recognized infrequency of cigarette smoking in patients with ulcerative colitis and the excess of cigarette smoking in Crohn's disease: eight percent of the ulcerative colitis series were current cigarette smokers compared with 42% of the group with Crohn's disease and 44% of controls. Forty eight percent of the ulcerative colitis group had never smoked compared with 30% for Crohn's disease and 36% for controls. The negative association between ulcerative colitis and cigarette smoking, especially among ex-smokers and the reverse relationship between smoking and Crohn's disease, subsequently was reaffirmed in studies from other geographic areas. The biologically complex tobacco-ulcerative colitis relationship is not exclusive to inflammatory bowel disease and is present also in patients with Parkinson's disease[59], and Alzheimer's disease.

PSYCHOGENIC RELATIONSHIP

Scientific recognition of the physiologic responses of the body to emotional stress originated with the classic obser vations of Cabanis (1796)[60],

Pavlov[61], and Cannon[62] (early 1900s). Psychogenic factors were "formally" implicated in ulcerative colitis in the reports of Murray[63] (1930) and Sullivan[64] (1935), who had been impressed with a chronological relationship between emotional disturbances and the onset of bowel symptoms in men and women with significant emotional disturbances involving their marriage, home life and interpersonal relationships.

Psychiatric precepts during the 1930s, 1940s, and 1950s emphasized an "ulcerative colitis personality", described as "immaturity of the patient, indecisiveness, over-dependence, and inhibited interpersonal relationships," together with critical emotional events including the loss of a loved one, feelings of social rejection, and "maternal dominance". The 1947 experiments of Almy *et al*[65], demonstrating the physiological effects of emotional stress upon the normal colonic mucosa (hyperemia, vascular engorgement , increased secretion of mucus, and augmented colonic motor activity) and, more pronounced in the ulcerative colitis colon, appeared consistent with the psychogenic hypothesis.

Psychotherapy (conventional and *psycho*-analytical) was an important part of medical treatment during the 1930s-1950s. In 1954 Grace, Pinsky, and Wolff[66] reported lower operability rates, fewer serious complications, and lower mortality rates in 34 patients with ulcerative colitis treated by stress-control therapy. However, in a series of 70 patients with severe ulcerative colitis treated by psychoanalytically oriented psychotherapy for three months, no specific value was observed in preventing surgical intervention on severe recurrences. Feldman *et al*[67] found no evidence of a psychogenic causation in a controlled study of 34 patients with ulcerative colitis.

Early clinical reports implicating emotional difficulties in ulcerative colitis had originated in retrospective reviews of often incomplete hospital records and in uncontrolled clinical observations. Later controlled clinical and critical studies did not support the concept[68,69]. A. Karush *et al*[70] in 1977 summarized the prevailing psychiatric view: "We do not claim that ulcerative colitis is'caused' by unusual reactions of the mind alone, we claim only that these reactions almost always play a vital role in the interaction of the four etiological determinants, genetic endowment, constitutional vulnerability, intrapsychic processes, and the external environment." Today, the role of emotions and stress in human disease has extended to the realm of the neurosciences[71], perhaps involving neuroimmune interactions as the basis of the emotional contributions to IBD. Emotional disturbances were less emphasized in Crohn's disease. Blackburn in 1939 considered a majority of 24 patients "abnormally introspective". Grace [72] and others were impressed with the relationship between stress and the onset or relapse of Crohn's disease. On the other hand, kraft and Ardali[73] and Crockett[74] regarded the psychological difficulties as consequences of chronic, recurrent, and frustrating illness and this view predominates today.

MICROBIAL ASPECTS—ULCERATIVE COLITIS

Bacterial causes of ulcerative colitis attracted attention during the early 20th century when bacterial origins of intestinal disease were first being identified, including bacillus coli (1909), streptococci (1911), and B. Coli communis (1913). None fulfilled Koch's postulates, yet, bacterial possibilities influenced the treatment of ulcerative colitis for many years. Hurst[75] administered a "polyvalent anti-dysenteric serum" intravenously, Leusden[76] an autologous vaccine of fecal bacteria and later sulfonamides and antibiotics were used extensively.

Focal infection (e.g. dental infection) was a popular cause of disease in the United States during the 1920s and encouraged the extensive removal of teeth, gallbladders and appendices. The occurrence of ulcerative colitis in a patient following removal of an abscessed tooth encouraged J.A. Bargen[77] to pursue the problem, experimentally and clinically. In 1925, Bargen *et al*[78] reported positive cultures from the rectal ulcerations in 80% of 68% ulcerative colitis patients and the occurrence of colonic lesions in rabbits injected intravenously with broth containing diplostreptococci. Cook[79] and Mayo microbiologist Edward Rosenow, in 1931, injected rabbits with diplostr eptococci cultured from abscessed teeth of patients with active ulcerative colit is and described a "diffuse hemorrhagic infiltration" of the colon. Cook also inoculated artificial cavities created in the teeth of dogs with a diplostreptoc occus isolated from the teeth of patients with ulcerative colitis. Diarrhea developed in seven of 15 animals and colonic ulcerations were observed proctoscopically for months. Bargen then treated patients with an autologous vaccine of diplostreptococci, with limited success. Studies by M. Paulson[80] and by Mones *et al*[81] had failed to confirm the experiments of Bargen and the diplostreptococcus concept soon lost scientific credibility.

Other bacteria implicated and similarly discarded for lack of decisive evidence included: the anaerobe spherophorus necrophorus[82], bacillus Morgagni, pseudomonas aeruginosa, hemolytic and non-hemolytic Esch. Coli, and viruses (e.g. lymphopathia venereum). Serological evidence of unusual response to known viruses (influenza,

mumps, measles, herpes, Cocksackie A, B, Echo, E-B, Adenovir us) in ulcerative colitis has been negative. The occasional increased titers of cytomegalovirus (CMV) have been in malnourished, secondarily immunodeficient patients. In the 1940s, studies of a possible etiologic relationship with lymph opathia venereum[83] proved negative[84].

Bacterial viral causes—Crohn's disease The many bacteria implicated in Crohn's disease included Boeck's sarcoid , mycobacteria (Kansasii [1978], paratuberculosis), anaerobic organisms (including Eubacteria strains Me_{46}, Me_{47}, B. Vulgatus, peptostreptococcus, aerobacter aerogenes, coprococcus, bifidobacteria), Campylobacter fetus ssp. Je juni, Yersinia enterocolitica, Chlamydia trachomatis), mycobacterial variant (Mycobact-erium Linda)[85], bacterial components[86] (lipopolysaccharides, peptidoglycans, *oligo*-peptides), metabolic products (toxins, necrosins) and viral protein elements (virions, prions); none achieved etiologic status. Serological studies of Epstein Barr, Echo A, B adenovirus, rotavirus, and Norwalk virus, as in ulcerative colitis, also was negative. Today, the possible role of an antecedent exposure to measles is under investigation.

Specific infections of the terminal ileum and colon in animals have been associated with tissue changes resembling Crohn's disease, including an enterocolitis in cocker spaniels (1954), mycobacterial paratuberculosis infection of the terminal ileum in cattle (Johne's disease) (1913), a terminal ileitis in swine, and a granulomatous colitis of Boxer dogs[87]. However, none of the animal diseases duplicated Crohn's disease.

IMMUNE MECHANISMS

Edward Jenner[88] in 1801 wrote that infection can alter the body in a manner that will cause its tissues to react with increased intensity to subsequent contact with the infective agent." More than 100 years elapsed before the important role of the gastrointestinal tract in the immune homeostasis of the body was demonstrated[89]. In 1919, Besredka[90] showed that oral "immunization of rabbits protected against otherwise fatal Shiga bacillus infection." In 1922 Davies[91] documented the presence of fecal antibody in the stools of patients with bacillary dysentery before serum antibody appeared. Subsequent observations by Heremans[92] (1960), Tomasi *et al*[93] (1965), and Bienenstock, among others, identified the IgA class of immunoglobulins and their role in the emerging field of mucosal immunity of the gastrointestinal tract. In 1938 I. Gray *et al* [94] induced an allergic reaction to a specific protein in the passive ly sensitized rectal mucosa of human subjects and the rhesus monkey and in the mucosa of the ileum and the colon in man (1940)[95,96]. The concept of an altered gut mucosal immune system in the pathogenesis of inflammatory bowel disease[97] developed in the context of a temporary interest in hypersensitivity (allergy) of mucous membranes of the gastrointestinal tract to foods, pollens, and other allergens[98,99].

Immune mechanisms in the late 1940s were implicated in various diseases of unknown etiology (e.g. rheumatoid arthritis). Several clinical events during the 1930 s and 1940s suggested to me the potential involvement of immune mechanisms in ulcerative colitis[100]. These included the abrupt onset of severe ulce rative colitis in a young woman who, with many others, had developed acute food poisoning at a family picnic in New York state; everyone recovered within 24 to 48 hours except for the patient, who developed ulcerative colitis from which she died several years later; the association of ulcerative colitis with other immune diseases (e.g. autoimmune hemolytic anemia); the ulcerative colitis developing years later in individuals who had experienced an acute amebic dysentery (1933-1934), the familial occurrences of inflammatory bowel disease, and the beneficial therapeutic effects of ACTH and the adrenal corticosteroids.

The immunologic resources and responses of the gastrointestinal tract, despite earlier observations, had not been fully appreciated. Kirsner and Palmer[101] wrote in 1954: "…Perhaps future studies should include the concept of vulnerability of the host, a person more susceptible to ulcerative colitis because of tissue hyper-reactivity." In 1956, utilizing the 1920 Auer [102] principle of local autosensitization to foreign protein, Kirsner and Elchlepp[103] produced immune complexes to crystalline egg albumin in rabbits and localized the complexes to the distal bowel via the rectal instillation of a non-inflammatory solution of very dilute formalin. An ulcerative colitis promptly developed in the same areas of the left colon demonstrated immunologically to contain the immune complexes and nowhere else. The Auer-Kirsner phenomenon was reproduced in 1963 by Callahan *et al*[104]. In colon-sensitized inbred mice. Kirsner and Goldgraber, inducing the cla ssic Arthus and the Shwartzman reactions in the rabbit colon, in 1958-1959 reconfirmed the immunologic responsiveness of the bowel.

Studies by Kirsner*et al*[105], O. Broberger *et al*[106] and by Bernier *et al*[107] had demonstrated heterogeneous hemaggluti nating and precipitating "antibodies" reacting with antigens of human colon mucosa in the sera of children and adult patients

with ulcerative colitis. Shorter[108] (1972), in recognition of the infant's more permeable in testine and immature intestinal defenses permitting the entry of bacteria and other antigens into the bowel, suggested an early "priming" of the gut mucosal immune system as "preparing" the bowel for the later development of an inflammatory bowel disease; a sequence of events similar to the earlier instances of food poisoning. Immunological interest in IBD increased and by the 1960s focused upon "autoimmunity", intestinal antigens, anti-colon anti bodies, abnormal serum immunoglobulins and an experimental immune colitis. The methodology was crude; the "antigens" and "antibodies" were inadequately characterized and a relationship to IBD was never established.

Though immune mechanisms are involved in IBD, immunologic studies, after approxi mately fifty years, have not yet demonstrated an antecedent vulnerability in patients or in healthy members of IBD families. Most of the immunologic phenomena described in IBD thus far, appearing and disappearing with the activity and quiescence of ulcerative colitis or Crohn's disease, represent secondary even ts, reflections of an over-active malfunctioning gut mucosal immune system. Nevertheless immunologic interest continues in the gut-associated mucosalimmune system, antigen-access M and dendritic cells of the intestinal epithelium, T cell antigen receptors and transgenic animal models[109]. Interest also is developing in the identification of antigen(s) (probably components of the intestinal flora) recognized by the serum anti-neutrophil cytoplasmic antibodies found in ulcerative colitis. The present view for ulcerative colitis emphasizes increased responsiveness of the gut muscosal immune system, involving Th1 T cells in Crohn's disease and Th2 T cells in ulcerativ e colitis in genetically vulnerable individuals. For Crohn's disease, immunological mechanisms also are involved in association with the intestinal inflammatory reaction probably involving a component of the intestinal flora.

M cell Two additionally important elements of the immune response in IBD are the intestinal (antigen access) M cell and the role of lymphokines / cytokines. The M (membranous) cell is a specialized epithelial cell characte rized by lumenal surface microfolds rather than microvilli overlying the gut- associated lymphoid tissues (also present in the colon and the appendix), which facilitates the selective uptake and transport of bacterial, viral, or food antigens from the intestinal lumen to the gut mucosal immune system. The membran ous (M) cell of the intestinal epithelium was identified in 1923 when Kumagai[110] demonstrated the uptake of ink, carmine dye, powdered erythro cytes, and living mycobacteria from the intestinal lumen into the rabbit appendi x and/or Peyer's patches, via specialized cells in the intestinal epithelium. In 1965 Schmedtje[111], studying the epithelium of the rabbit appendix, designated such cells overlying lymphoid follicles as "lympho-epithelial cells". Owen *et al*[112] (1974) coined the term M cells.

Inflammation, lymphokines, cytokines Cytokines are small to medium-sized proteins elaborated by "producer" cells responding to disease- inducing stimuli (injury or antigenic stimulation), influencing the behavior of particular target cells via specific surface receptors. Lymphokines is the arbitrary term applied to cytokines produced by cells involved in the immune system. Cytokines participate in the regulation of the immune response and help orchestrate the complex process of inflammation. The interrelationship of the immune response in IBD with the inflammatory process and the regulatory role of lymphocytes and cytokines are extremely important in understanding the nature of IBD.

Interest in the biology of inflammation and its involvement in immune reactions dates back nearly 100 years to the observations on cellular immunity (i. e. phago cytosis) by Elie Metchnikoff[113] in 1883, on humoral immunity by Paul Ehrlich[114] (1908), and in the 1930s and 1940s to the biochemical studies of inflammation by Valy Menkin[115]. McCord *et al*[116] in 1969 were the first to discover the enzyme superoxide dismutase (SOD) and proposed that the free radical is produced in mammalian systems. Babior[117] first demonstrated that activated polymorphonuclear cells produce large quantities of the superoxide anion radical. The possible role of reactive oxygen metabolites in intestinal injury or inflammation was first reported by Neil Granger *et al*[118] who demonstrated that post-ischemic microvascular injury in the small bowel could be attenuated by the intravenous administration of superoxide dismutase. M.B. Grisham *et al*[119] also suggested the possibility that immunologically-activated phagocytic leukocytes (e.g. PMNs, eosinophils, and macrophages) could be important contributors to the mucosal injury characterizing intestinal inflammation. In 1975, Gould[120] of England found increased levels of the cyclooxygenase derived prostaglandins (PGE_2) in the stools of patients with ulcerative colitis. Sharon *et al*[121] also noted elevated levels of prostagland ins in the colonic mucosa and the serum of patients with ulcerative colitis. The prostaglandins subsequently were identified as cytoprotective agents.

Interest in lymphokines/cytokines dates to the

1972 discovery of a factor produc ed by macrophages stimulating T cell responses to antigens, later designated as interleukin-1 (IL-1)[122] (perhaps known in the 1940s as endogenous pyrogen)[123] and to the discovery of interleukin-2 (IL-2) by Paetkau *et al*[124] and by Chem *et al*[125] in 1976. Sharon and Stenson demonstrated a 50-fold increase in the leukotriene LTB4 in the colonic mucosa of ulcerative colitis and postulated a pro-inflammatory role for LTB4 in both ulcerative colitis and Crohn's disease. Investigation of the important role of cytokines in the tissue reaction of ulcerative colitis and of Crohn's disease today is one of the most active research areas in IBD.

GENETIC ASPECTS OF INFLAMMATORY BOWEL DISEASE-EARLY OBSERVATIONS

The first published instances of familial IBD from the 1909 London symposium: (a) brother and sister, (b) father and sibling, and (c) father and siste r of a third patient, were considered "coincidences", and this view prevailed for more than 50 years. Reports of "familial" inflammatory bowel disease appeared in the 1960s and subsequently increased, indicating a genetic relations hip in IBD[126-129].

Ulcerative colitis In 1936 Moltke[130] described 5 fam ilies with ulcerative colitis. Sloan *et al*[131] (1950) noted 26 positive family histories among 2000 patients, kirsner and Palmer (1954) reported 6 family occurrences, and Banks, Korelitz, and Zetzel (1957), 9 families among 244 patients. Schlesinger and Platt (1958) obtained a family history of ulcerative colitis in 17% of 60 children with ulcerative colitis. An unusual sequence involved two brothers, who developed ulcerative colitis and succumbed to carcinoma of the colon within 15 years after onset of the disease[132]

Crohn's disease Crohn[133] in 1934 described regional ileitis in a brother and sister. Familial instances of regional enteritis subsequently were reported by other observers[134,135]. In the family described by Kuspira *et al*[136], six members were affected spanning three generations.

Familial patterns Familial distributions of IBD involved first-degree relatives (parent, child, or siblings) more often than second-degree or third-degree relatives (aunts, uncles, nieces, and nephews) in accord with a polygenic inheritance. In the 1963 Chicago study for ulcerative colitis, 50 of the 89 family members were brothers, sisters, and cousins, approximately the same generation as that of the probands and 11 were grandparents. For Crohn's disease, 15 of 22 family members involved brothers, sisters, and first- cousins. De Matteis[137] (1963) summarized 5 reports on ulcerative colitis comprising 20 parent-child combinations; mother and child were involved in 16 and father and child in 4. Among 32 reports on Crohn's disease involving 72 familial instances, mother and child were affected in 7 instances and father and child in 3.

The occurrence of IBD in three or more members of the same family, very strong support of a genetic relationship, included Spriggs (1934): ulcerative colitis in 2 brothers and a sister; Moltke (1936): brother, sister, and maternal aunt; B rown and Schieffley (1939): 2 sisters and 1 brother; Jackman *et al*[138] (1942): (a) mother, son, and mother's brother; (b) mother and 2 daughters with ulcerative colitis and nephew with regional enteritis; and Bacon (1958): tw in brothers and a sister.

Thayer's[139] (1972) family included a 21-year-old male with ulcerati ve colitis since the age of 8 who developed a carcinoma of the descending colon. A maternal aunt developed ulcerative colitis at the same time. One year after the death of the index patient, his brother, 2 years younger, developed ulcerative colitis and required colectomy and ileostomy. Within a year after this operation the boy's father developed ulcerative colitis and after 5 years of medical treatment, he also underwent a colectomy and ileostomy. The 8 members of the Morris family (1965) represented 3 generations, all with ulcerative colitis, 4 males and 4 females. The 7 affected members of the Ashkenazi Jewish f amily studied by Sherlock *et al* (1963) included 5 with Crohn's disease and 2 with ulcerative colitis. Seven IBD uninvolved relatives of the same family had varying degrees of deafness.

Intermingling of diseases-twins-genetic associations Ulcerative colitis was more likely to occur than Crohn's disease among the fami lies of probands with ulcerative colitis and a similar relationship held for probands with Crohn's disease. However, in approximately 25% of families, the dise ase incidence was mixed, suggesting a similar genetic susceptibility profile. The association of ulcerative colitis and Crohn's disease with genetically- mediated conditions, such as for ulcerative colitis: ankylosing spondylitis and Turner's syndrome; and for Crohn's disease: psoriasis and the Hermansky-Pudla k syndrome, added to the evidence. The survey of monozygotic twins demonstrated moderate concordance for ulcerative colitis and strong concordance for Crohn's disease; discordance was more common for ulcerative colitis than for Crohn's disease.

Early genetic surveys revealed an association between HLA-DR2 phenotype and ulcerative

colitis, between DR1, DWQW5 or B44C-W5 phenotypes with Crohn's disease, and HLA-DQB-1 genotype with Crohn's disease in children. Recent genetic linkage studies have identified gene loci in chromosomes 6 (possibly for ulcerative colitis), chromosome 16 (definitely for Crohn's disease), loci for chromosome 1 in the Chaldean patient population relocated near Detroit and a trend toward common genes for Crohn's disease and ulcerative colitis.

CONCLUDING COMMENT

The chronological events described for ulcer ative colitis and for Crohn's disease reveal diseases at least several centuri es old. The changing epidemiological patterns; the increases during the 19th century, especially in northern Europe and England, extending to the United States in the early 20th century; the prominence of ulcerative colitis during the first half and of Crohn's disease during the second half of this century; their frequency in the industrialized countries contrasting with under-developed cou ntries; their appearance in previously lagging, increasingly industrialized are as (e.g. Japan, Brazil), all are consistent with widespread environmental etiolo gic contributions (bacteria, viruses, and parasites, cytotoxic food additives, industrial, atmospheric, and water pollutants, chemicals, "stress", etc.) not exclusive to any particular geographic area or to any ethnic group, affecting genetically-vulnerable individuals in immune and genetically mediated complex tissue reactions.

The study of ulcerative colitis and Crohn's disease today involves many expandi ng scientific disciplines, including the biology of the intestinal epithelium, the molecular basis of inflammation, genetic, geographic epidemiology, molecular microbiology, intestinal immunology, molecular genetics and gastrointestinal neuro-endocrinology. The challenge for the next century will be to utilize these scientific advances in coordinated interdisciplinary research towards the ultima te understanding and control of two of the most intriguing diseases in medicine[140].

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Edited by Zhu LH

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Enteral nutrition and acute pancreatitis

Qiang Pu Chen

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INTRODUCTION

Acute pancreatitis (AP) is a common severe illness of the digestive tract with variable involvement of other regional tissues and/or remote organ systems[1-3]. Mild disease is associated with minimal organ dysfunction and rapid recovery, while severe disease is associated with multiple organ system failure and local complications such as necrosis, abscess, fistulas and pseudocyst formation [4-6]. Mild attacks account for 80% of hospital admissions for this condition and usually resolve in 5 to 7 days. Twenty percent of patients with severe acute pancreatitis (SAP) tend to have a more protracted hospital course with higher mortality, and are more likely to require a multidisciplinary treatment that includes Nutritional support[7-9]. Nutritional support in the patients with AP is both very critical and more complex. In the past years, parenteral Nutrition (PN) was recommended for patients with AP[10,11], but recently, significant progress has been achieved in the field of enteral Nutrition (EN)[12,13]. Clinical research has shown that early delivery of Nutrition via the gastrointestinal tract after severe injury can reduce septic morbidity in critically injured patients [14-18]. Enteral diets have been reported to decrease Gut permeability and maintain mucosal immunity and Gut-associated lymphatic tissue (GALT)[19-21]. These observations provide new insights into the use of EN. Therefore, more and more clinicians have begun to use this technique for patients with AP. This review examines metabolic alterations of AP and effects on pancreatic secreration of EN, evaluates the indications, feeding access and formulas of EN, and assesses the clinical role of EN in AP.

Department of Heptobiliary Surgery, The Affiliated Hospital of Binzhou Medical College, Binzhou 256603, Shandong Province, China
Dr. Qiang Pu Chen, graduated from Binzhou Medical College in 1983 and received his MS degree from Nantong Medical College in 1989. He is director of the Department of Heptobiliary Surgery, the Affiliated Hospital of Binzhou Medical College, and a member of the editorial board of the World Chinese Journal of Digestology. He is majoring in hepatobiliary surgery and clinical nutrition and has published 60 papers and 8 books.
Correspondence to: Dr Qiang Pu Chen, Department of Hepatobiliary Surgery, The Affiliated Hospital of Binzhou Medical College, 661, 2nd Huanghe Rd, Binzhou 256603, Shandong Province, China
Tel. 0086-543-3324131 Ext.6731, Fax. 0086-543-3264214
Email. drcqp@263.net
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METABOLIC ALTERATIONS AND AP

The metabolism of AP is very similar to sepsis, and is characterized by hyperdynamic changes, hypermetabolism, and catabolism. The hyperdynamic changes include increased cardiac output, decreased systemic vascular resistance, and an increase in oxygen consumption[22]. Hypermetabolism is seen in the majority of the patients[23] and is associated with increases in measured resting energy expenditure (REE) as high as 139% of that value predicted by the Harris-Bennedict equation (HB). Bouffard *et al* demonstrated that total energy expenditure was 1.49 times of the predicted resting energy expenditure using the Harris-Benedict equation in patients with SAP[24]. A variety of factors affect the REE from one patient to the next, or even the same patient during his or her hospital course. Sepsis N complicating pancreatitis may independently raise energy expenditure further. In one study, development of sepsis led to an increase in mean measured REE from 105% to 120% of the HBREE[23]. However, this is not a uniform finding of the hypermetabolism across the entire patient population. Dickerson *et al*[23] found that 38% of a group of pancreatitis patients were normometabolic (measured REE = HBREE±10%), and 10% were actually hypometabolic (measured REE<90% of the HBREE). With the variation of REE between patients with AP and with so many factors that can affect ultimate cumulative energy expenditure, indirect calorimetry may be useful to the clinician to measure caloric requirement accurately[23,24]. Catabolism is another important metabolic alteration in AP. Isotope techniques have been used to demonstrate that patients with SAP have a significantly higher urea production compared with controls, indicating both increased protein catabolism and diminished muscle protein synthesis. The resultant negative nitrogen balance is, therefore, a net effect of both of these changes[25]. Catabolism and proteolysis of skeletal muscle protein raises concentrations of aromatic amino acids, decreases levels of branched-chain amino acids, and accelerates ureagenesis. Nitrogen levels (from urea) in urine may increase up to 20-40 $g \cdot d^{-1}$[26]. Overall, the circulating pool of amino acids decreases to as low as 40% of normal levels. Circulating glutamine levels in the serum may drop to as low as 55% of normal levels, while levels in skeletal muscle may drop to as low as 15% of normal levels[26]

Errors in carbohydrate and fat metabolism also occur with AP, and may or may not be associated with underlying chronic pancreatitis. This may result from increased cortisol and catecholamine

secretion in the stressed state (which leads to an increase in the glucogen/insulin ratio), impaired beta-cell function, and insulin resistance. Gluconeogenesis is increased, while glucose clearance and oxidation are diminished. In AP, glucose intolerance occurs in 40%-90% of cases, and insulin is required in as many as 81% of patients. Isotope techniques have been used to demonstrate that patients with severe disease have an impaired ability to oxidize glucose compared with controls. Exogenuos glucose infusion causes almost complete suppression of gluconeogenesis from protein in normal subjects, but not in patients with AP. These changes are consistent with a state of hepatic insulin resistance, and are similar to those observed in patients with sepsis[27,28]. Micronutrient and vitamin deficiencies (such as hypocalcemia, hypomagnesmia,lower zinc levels, and thiamine and folate deficiencies), may also be present on admission or develop during hospitalization. Hypocalcemia occurs most often in as many as 25% of patients, presumably related to decreased parathyroid hormone secretion, increased stimulation of calcitonin, hypomagnesemia, and hypoalbuminemia[22-31]. The effects of increased metabolic demands are compounded by an inability or reluctance to maintain an adequate oral intake, so that patients become malnourished during the course of their illness. MalNutrition may be especially apparent in those who have an acute outbreak of chronic pancreatitis, because some patients with chronic alcoholic pancreatitis may also have suffered nutrient deficiencies.

In recent studies, the disease and its complications and metabolic changes have been associated with the release of cytokines and other mediators of inflammation, such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor α (TNF-α) and platelet-activating factor[32-36]. In addition, researchers have reported activation of the complement cascade, release of oxygen-derived free radicals and nitric oxide, and generation of prostaglandin E_2 and thromboxane A_2 from the metabolism of arachidonic acid[37,38]. These cytokines and mediators may cause systemic inflammatory response syndrome (SIRS), which in turn promotes a series of metabolic alterations and multiple organ failure[39]. If prolonged and combined with starvation, these changes can lead to a rapid loss of lean body mass, associated morbidity, and death[40]. Increased intestinal permeability in animals[41-44] and humans[45], along with anassociated impaired Gut barrier function, may lead to translocation of bacteria and endotoxins from the Gut lumen into systemic circulation[46], which contributes to the release of cytokines and systemic inflammatory responses. Experimental animal work has indicated that EN may prevent bacterial translocation in AP[47].

EFFECTS OF EN ON PANCREATIC SECRETION

The key pathological mechanism of AP is autodigestion of the pancreas and peripancreatic tissues by pancreatic enzymes. Oral and nasogastric feeding increases pancreatic secretion by stimulation of the cephalic and gastric phase, and it is suggested that early oral feeding may lead to recurrence of symptoms, elevations of serum amylase and lipase, and delayed complications[48,49]. In a study of 8 patients with AP, it was found that the interdigestive secretions of the exocrine pancreas were not different, within 72 h of the onset of mild to moderate disease, from those in 26 normal controls. Based on this evidence, the authors propose early inhibition of pancreatic secretion with somatostatin in the acute phase of the illness[50]. Animal studies[51,52] show that "pancreatic rest" reduces pancreatic synthetic activity and basal proteolytic and bicarbonate secretions, but evidence from human studies is less certain. A retrospective study suggests that early oral feeding predisposes patients to major peripancreatic infections, while prolonged nasogastric suction reduces the incidence of these infections[53]. However, randomized controlled studies[54] in patients with mild to moderate AP have, failed to establish the value and efficacy of putting the pancreas to rest by avoiding exocrine secretion. The definition of the pancreatic rest is variable. There are three main fluid volumes of pancreas juice: protein enzymes, and bicarbonate, which have different functions and reactions to stimulations. Of them, protein enzyme output is thought to be responsible for the autodigestion of the gland and perpetuation of the inflammatory process. One study showed that reducing the protein enzyme output from pancreas while sitimulating fluid volume and bicarbonate output put the pancreas to rest. From the available studies, in spite of some controversies, the reduction of pancreatic secretion or "putting the pancreas to rest" is necessary when dealing with the patients with AP in clinical practice.

The issue of whether or not the EN can successfully put the pancreas to rest is considerably more complex and controversial. In an early study in dogs, Ragins *et al*[55] demonstrated that intragastric delivery of nutrients (Vivonex) caused an increase in the volume, and protein content, and bicarbonate content of pancreatic secretions compared with those in saline-infused controls. Intraduodenal feeding only increases the volume of pancreatic secretions, but does not affect protein or bicarbonate secretion. In contrast, jejunal infusion showed no increase in any of the three components of pancreatic secretion. Other studies of dogs[56,57] also show that the intraduodenal delivery of elemental diets or pure amino acid solutions significantly increases pancreatic secretions, suggesting that the amino acid content of elemental diets is responsible for the stimulatory effects. In contrast, intrajejunal administration of nutrients is not associated with a significant change in the volume, protein content, or bicarbonate content of pancreatic secretions compared with controls.

Keith[58], in a study of two patients with chronic pancreatitis, reported changes in volume and amylase output in response to the intrajejunal infusion of an elemental formula. Keith concluded that bypassing the stomach, thereby minimizing acid secretion, played an important role in keeping the pancreas at rest. In direct contrast, other studies showed stimulation of pancreatic enzyme secretion in response to jejunal feeds[59,60].

Another factor affecting pancreatic secretion is the kind of feeding formulas. A study[59] on volunteers fed either an elemental diet or a food homogenate (via a nasoenteral tube placed at the duodenojejunal flexure) indicates that the latter has a greater stimulatory effect on the secretion of pancreatic lipase and chymotrypsin than the former. The authors suggest that this difference might be related to the greater nitrogen content of the food homogenate. Grant *et al*[61] demonstrated the effect of jejunal infusion of a formula with long-chain fat in a postoperative patient with an isolated duodenal fistula. Infusion of Osmolite into the jejunum resulted in a significant increase in lipase output, but no changes in amylase, bicarbonate, or fluid volume. Infusion of either Vinonex or Criticare, which are nearly fat-free, showed no significant increase in any component of pancreatic secretion. Similarly, Cassim and Allardyce[62], using a dog model, showed that an intact protein blended diet infused into the jejunum increased volume enzyme output, while an elemental formula increased only volume and bicarbonate with no change in protein enzyme output. Bodoky *et al*[63] randomized 12 patients undergoing pylorus-preserving pancreaticoduodenectomy for chronic pancreatitis. The patients received either EN via a needle catheter feeding jejunostomy (7 patients) or PN (5 patients). A catheter placed during operation in the pancreatic duct was used to collect pancreatic secretions. The authors found that evidence of the disease was mainly apparent in the pancreatic head and that the function of the pancreatic remnant was near normal. They did not find any difference in the volume of pancreatic secretions or the content of bicarbonate, protein, chymotrypsin or protein amylase between the two groups. Recently, a study[64] compared the effects of an elemental diet with an immune-enhancing formula administered by a jejunal route on pancreatic secretions after Whipple pancreaticoduodenectomy. The authors found a small but significant increase in pancreatic enzyme and bicarbonate secretion after jejunal feeding compared with the fasting state, but there was no significant difference in pancreatic enzyme output when the effects of the elemental and immune-enhancing feeds were compared.

Summarizing available evidence from both human and animal studies, one may conclude that oral, intragastric and intraduodenal feeding produce a significant stimulation of pancreatic secretions. In contrast, intrajejunal feeding has a smaller stimulatory effect. Elemental formulas (with individual amino acids and nearly fat-free) clearly cause less stimulation than standard formula with intact protein and long-chain fat. Therefore, intrajejunal feeding is the rational route of EN for patients with AP.

EN NECESSITY AND AP

Eighty percent of patients admitted for AP exhibit mild signs of symptoms of the disease, which usually has a self-limiting hospital course and is managed by intravenous fluid resuscitation and analgesia. These patients are likely to return to an oral diet within 7 days. The remaining 20% of patients admitted with SAP tend to have a more protrated hospital course, these patients have a more prolonged gastroduodenal atony, an increased risk for complications, and require surgical operations. This latter group is more likely to require Nutritional support by the enteral and/or parenteral route. Thus, it is necessary to identify the severity of AP before recommending enteral feeding. A variety of scoring systems has been devised to determine severity of AP and may actually be more accurate than clinical assessment. The Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system[65] and Ranson criteria[66,67] are two of the more useful scoring systems. Patients with a Ranson score of 2 or less or an APACHE IIscore of 9 or less are identified as having mild pancreatitis. In these cases, Nutritional support is considered unnecessary unless complications develop or the score begins to increase. In contrast, patients who score 3 or greater on the Ranson criteria scale or 10 or greater on the APACHE II scale, may be identified as having SAP and should be considered for Nutritional support particularly if the score increase in the first 48 h. The intrajejunal route of enteral feeding is the best, unless patients present with shock, massive bleeding of the gastrointestinal tract, intestinal obstruction, jejunum fistula or severe enteroparalysis.

ENTERAL ACCESS AND AP

There are three main categories of enteral access for patients with AP: ① nasojejunal tube, ② percutaneous gastrostomy/jejunostomy tube and ③ surgical jejunostomy with gastrostomy[68,69]. The choice of the route depends upon the phase of the disease and available expertise. During the early phase, resuscitation is the priority, and obtaining the access at this time must be weighed against the risk. Once the patient is believed to be stable, initial attempts at enteral feeding are probably reasonable. The first choice should be placement of a nasojejunal tube, because this technique is not invasive and easy to perform. The tube can be placed by using blind, pH, fluoroscopic and endoscopic-assisted techniques[70]. However, the endoscopic technique is a more popular method in clinical practice. One distinct advantage of endoscopic placement is that it can be performed at the bedside. An endoscopic placement method for

long nasojejunal tubes was described by Berger and Papapietro[71]. In 24 patients with AP, 28 tubes were placed using this method. In 15 patients with brain damage, traditional nasojejunal feeding tubes were placed without endoscopy. The position of both types of tubes was determined by fluoroscopy with the aid of contrast media. The results showed that the endoscopic placement method was simple and the tube was placed beyond the ligament of Treitz in all patients. No traditional tube was placed in the jejunum and contrast media filled the duodenum in all cases. The authors suggest that nasojejunal tubes can be easily placed beyond the ligament of Treitz with endoscopic aid and can be used for enteral feeding in patients with AP. Recently, Salasidis *et al*[72] introduced an air insufflation technique for placing the nasojejunal tube, reporting successful placement in 32 patients. This technique can also be used at patients' bedside[73].

Patients with gastroparesis and high nasogastric tube outputs may be good candidates for a combined gastric decompression / nasojejunal feeding tube. However, this kind of tube is more difficult to place and can be easily dislodged.

Patients who develop complications (pneumonia, ARDS) with protracted needs for enteral support may be candidates for percutaneuos enteral access. For most patients with AP, a combination of percutaneuos endoscopic gastrostomy and jejunostomy should be used for simultaneous gastric decompression and jejunal feeding[74]. The percutaneuos gastrojejunostomy can also be performed by radiologically-guided method, which has a higher success rate and fewer complications[75]. In some patients, a direct percutaneuos endoscopic jejunostomy technique[76], which allows placement of tubes directly in the jejunum with a success rate of around 85% and a minimal complications, is also a choice for enteral feeding.

Surgical jejunostomy is indicated for the patients requiring operations. There are many techniques used for jejunostomy: Witzel, Stamm, Marwedel, open gastrojejunostomy, needle catheter technique, and laparoscopy[12,77]. Among them, needle catheter jejunostomy is attractive for short-term (<4-6 wk) use. Myers JG *et al*[78] studied this technique in 2022 cases and reported a rate of 1. 55% for technique complications. For long-term use, the Witzel or Marwedel jejunostomy with 14, 16 or 18F catheters is the most (it more than 3) popular technique. One advantage of the large-bore tube is the easy administration of both enteral feedings and medications. Also, once the tube has been in place for approximately a week, it can be easily replaced should it become occluded or inadvertently dislodged[21]. No matter which kind of jejunostomy technique is used, it is necessary to perform a gastrostomy, allowing simultaneous gastric decompression and jejual feedings.

ENTERAL FORMULAS AND AP

Numerous enteral formulas are available today to meet various needs. They are generally classified as elemental (monomeric), semi-elemental (oligomeric), polymeric or specialized formulas[12]. All of these formulas contain varying concentrations of proteins, carbohydrates, and fats, depending on the patient's disease state. A number of factors such as paralytic ileus, glucose intolerance, fat intolerance with hypertriglyceridemia, and pancreatic enzyme deficiency, should be considered when selecting the enteral products for the patients with AP. In view of the metabolic features of AP, elemental diets (so-called chemically defined diets) should be considered as the first option. Although there is some variation among products, most elemental diets are lactose-free, are nearly fat-free (only 2%-3% of calories are derived from long-chain fat), and contain protein almost entirely in the form of individual amino acids. Examples include Precision HN (Sandoz, 1.3% calories as fat), Criticare HN (Mead Johnson, 3% calories as fat), and Vivonex High Nitrogen (Norwich Eaton, 0.87% calories as fat). These diets cause less stimulation of pancreatic exocrine secretion than standard formulas, and can lower pancreaticactivity, which is beneficial for treatment of the AP.

There is rational for use of a second category of formulas for patients with AP, that of seminelemental diets. One advantage of these diets is that the nutrients are more easily absorbed in the absence of digestive enzymes. An animal study on a ligated model of the pancreatic duct[79] and a human study with cystic fibrosis patients[80] have shown that protein in the form of small peptide chains may be absorbed more efficiently than individual amino acids. Although seminelemental formulas usually contain a higher percentage of fat calories than the elemental diets, only a small percentage is composed of long-chain fat. Most of the fat is in the form of medium-chain triglycerides (MCT), which can be directly absorbed across the small intestinal mucosa into the portal vein in the absence of lipase or bile salts. The enteral products such as Criticare HN (Mead Johnson, 5% of calories as fat, MCT), Pepti-2000 (Nutricia, 10% of calories as fat, MCT), and Vital HN (Ross, 11% of calories as fat, MCT) are all seminelemental formulas, and can be used effectively in clinical practice[12].

Another category of formulas is polymeric diets, which contain 50% to 55% carbohydrates, 15% to 20% intact proteins, and 30% fats. These diets are frequently used in patients with functional gastrointestinal tracts. Many polymeric diets have recently been added to the novel nutrient substrates, such as glutamine, arginine, ω-3 fatty acids, nucleotides, and fiber, which play important roles in some critically ill patients in the maintenance of mucosal integrity and immune status[12,81-83]. A recent study reported that, an average critically ill

patients who received a glutamine-enhanced enteral feed required a shorter stay in the hospital than patients who were fed a standard isocaloric isonitrogenous enteral feed[84]. The authors also documented a significant reduction in postintervention costs; the cost per survivor was 30% less in the glutamine fed group. The beneficial effects of immune-enhancing formulas have also been observed in critically ill patients[12,21,85-89]. Randomized, controlled studies reported that patients who received immune-enhancing enteral feeds containing arginine, nucleotides and ω-3 fatty acids (fish oil) after operation and trauma had a lower rate of postoperative infections and wound complications compared with patients receiving isocaloric, isonitrogenous control feeds[88,89]. Two recent meta-analyses of randomized controlled trials[86,87] comparing patients receiving standard EN with those receiving commercially available immune-enhancing feeds reported that, although immuno Nutrition has no effect on mortality rate, there is a significant reduction in infection rates, ventilator duration and length of hospital stay in these patients. At present, there are no available reports on the clinical effects of immune-enhancing diets on patients with AP. But recently, there was a case report[90] comparing the pancreatic output with respect to different feeding regimens in a patient who underwent a partial pancreatectomy for carcinoma. There was no difference in pancreatic exocrine secretion when the patient was fed jejunally with a polymeric immune-enhancing formula or supported with two different formulations of TPN. The authors suggest that jejunal feeding of polymeric immune-enhancing diet may be safe to administer patients with AP. Therefore, considering the above advandages, polymeric diets, particularly those containing glutamine, arginine, ω-3 fatty acids, nucleotides, and fiber, may be used in AP patients, but the beneficial effects require further study.

CLINICAL EXPERIENCES WITH EN

The use EN as therapy for patients with AP goes back to the 1970s when Voitk *et al*[91] demonstrated the beneficial effects of an elemental diet in 6 patients with complicated pancreatitis, reporting both positive nitrogen balance and weight gain. Since then a number of reports in the literature described successful use of enteral feeding in patients with pancreatitis without exacerbating the disease process. Most of the studies describe patients within whom the inflammatory process had peaked and begun to resolve, and the enteral feeding were used as transitional feedings.

Since the end of the 1980s, there is now renewed interest in early enteral feeding for AP. One large series[92] described the early use of EN within 48 hours of onset of AP. Among 83 patients with no evidence of ileus on admission for AP, 92% tolerated their enteral infusion well. Kudsk *et al*[93] reviewed an experience of feeding jejunostomies in 11 patients who underwent exploratory laparotomy for complications of pancreatitis. Two died, but the remaining 9 patients gradually improved on enteral feeding with none showing exacerbation of their diseases. No catheters were lost and mild diarrhoea was encountered only during the first week of therapy. This study indicates that prolonged jejunal feeding may be provided safety in patients with SAP without aggravating the disease. Parekh *et al*[94] showed similar beneficial effects of EN in 9 patients with AP in whom enteral feeding was commenced at a mean of 11 days after admission and continued for a mean of 16 days. This not only improved Nutritional status but was also accompanied by successful resolution of complications in 7 of the 9 patients. The authors suggest that stable patients who are unable to benefit from EN. Simpson *et al*[95] retrospectively reviewed nasoenteral feeding in 5 patients with acute alcoholic pancreatitis with a mean Ranson score of 1.8. None needed PN and the disease and its complications resolved in all 5. Nakad *et al*[96] described a study of early EN with SAP patients using double-lumen nasogastrojejunal tube within 60 h after admission. Severity was established by a mean Ranson score of 3.57. No patient developed a relapse, hypertriglyceridaemia or abnormalities of liver function, indicating that jejunal feeding can be used safely in SAP patients without reactivation of the inflammatory process.

Pupelis *et al*[97] reported 29 patients who had been operated on for SAP. They were randomized to receive either EN and conventional intravenous fluids postoperatively (n = 11) or conventional intravenous fluid alone (n = 18). Seventeen additional patients who had major abdominal operations for other conditions were also given EN and intravenous fluids and comprised the control group. Nutritional intake, duration of stay in the intensive care unit (ICU), hospital morbidity, mortality, and outcome were observed. Ten of the 11 patients given EN combined with conventional intravenous fluids survived, whereas 5 of the 18 given fluids alone died. The pattern of bowel transit in the fed group did not differ from that in the control group. The authors suggest that postoperative EN seems to be safe and effective in patients with SAP and may improve survival. However, another randomized, controlled study of EN *vs* conventional therapy (i.e. no Nutritional support) in patients with SAP provided no evidence of improved outcome in patients receiving Nutritional support in terms of organ dysfunction score or inflammatory markers such as antiendotoxin core antibody, IL-6, TNF receptor 1 and CRP[98]. Patients receiving enteral feeding had significantly worse abnormal intestinal permeability on the 4th day of therapy. However, this trial involved a total of only 27 patients and a median of 1.8 MJ/day was delivered over the first 4 d by EN,

which constituted 21% of daily caloric requirements. So, the results may be deliberated. Chen and Zhu[99] compared the effect of early EN and late EN. Thirty-eight patients were divided into an early group (start EN 3 to 4 days after operation) and a late group (start EN 7 days after operation). All patients received PN at first, and then were transferred to EN. The results indicated that patients tolerated the therapy well in both groups. In addition, early correction of hypoalbuminemia with more quickly improved serum albumin was observed in the group of early enteral feeding.

EN VS PN AND AP

Although there have been a number of studies comparing the clinical effects of EN and PN in postoperative, injured, burned, cancer, or critically ill patients[16-18,100-106], only three articles have been published that compare EN with PN in AP patients[107-109]. McClave *et al*[107] performed the first prospective, randomized trial comparing early EN with PN in 30 patients with mild, or acute chronic pancreatitis. EN was via nasojejunal tube and PN was via a central or peripheral line, both within 48h of admission. Efficacy was measured by the percentage of goal energy intake ($25kcal \cdot kg^{-1} \cdot d^{-1}$) achieved, days to oral diet and length of hospital stay. Although enterally fed patients lagged 1 day behind the other group in achieving energy goals, this difference disappeared by the fourth day. Mean Ranson, APACHE III and multiple organ failure scores decreased in the EN group and increased in the PN group, but these differences were not statistically significant. Patients in the latter group had significantly higher stress-induced hyperglycaemia over the first 5 days. There was a statistically insignificant trend towards earlier normalization of serum amylase, progression to oral diet, and decrease in hospital and intensive therapy unit stay in the EN group compared with the PN group. The mean cost of parenteral feeding was 4 times higher than that of enteral feeding. The authors suggest that EN for AP is not only safe and effective, but also significantly less costly than PN. Compared with PN, EN may promote more rapid resolution of the toxicity and stress response to pancreatitis. In another prospective randomized trial[108], comparing parenteral with enteral feeding in 38 consecutive patients with severe necrotizing pancreatitis, there was no statistically significant difference between the two groups with regard to intensive therapy unit support, use of antibiotics, hospital stay and number of days of Nutritional support. Nitrogen balance improved equally in both groups, but severe hyperglycaemia requiring insulin therapy was twice as frequent in the PN group compared with the EN group. Total complications and septic complications were significantly more common in the PN group, suggesting that EN should be the preferred method for Nutritional support in patients with SAP.

Windsor *et al*[109] recently published a randomized controlled trial of EN versus PN in 34 consecutive patients with AP who had a mean Glasgow score of 2 and APACHE II score of 8. After 7 d of Nutritional support, the EN group became better than the PN group with respect to CRP concentrations and APACHE II scores. Furthermore, the serum level of immunoglobulin M and endotoxin core antibodies increased in the PN group whereas it remained unchanged in the EN group. The total antioxidant capacity also fell in the former group and increased in the latter. There was a reduction in the requirement for intensive care, incidence of intra-abdominal sepsis, multiple organ failure, need for operative intervention, and mortality rate in the enterally fed group compared with the parenterally fed group. There was, however, no difference in hospital stay. The authors conclude that EN is not only feasible, but may modulate the inflammatory and sepsis response, reduce disease severity and improve clinical outcome and physiological parameters compared with PN. Erstad[110] reviewed the literature of both PN and EN in patients with AP from 1966 to 1999. The results show that the duration of AP and time to oral feedings is similar whether patients receive EN (i.e., jejunal tube feedings) or PN. Additionally, complications, length of stay, and costs are either similar or decreased with EN versus PN, suggesting that the EN rather than PN should be used to provide Nutrition to patients with AP. PN should be reserved for patients in whom nasojejunal feeding is not possible.

CONCLUSIONS

Patients with AP have a hypermetabolic and hypercatabolic state, resulting in mal Nutrition. Nutritional support for patients with AP is needed, particularly in SAP patients. Jejunal feeding is well tolerated and, unlike gastric and duodenal feeding, does not stimulate pancreatic secretions. EN by the jejunal route is feasible and safe, even in the early stage of AP. The elemental or semi-elemental formulas should usually be used. Although there is no definite evidence that EN support alters clinical outcome or the natural history in most patients with AP, at present, the beneficial effects of EN towards improving the Nutritional condition, protecting Gut barrier function, reducing translocation of bacteria and endotoxins, modulating the inflammatory and septic response, and decreasing the cost have been observed. Therefore, the EN rather than PN should be used to provide nutrional support for patients with AP. PN should be reserved for the patients in whom jejunal feeding is not possible. Furthermore, larger sample multi-center trials are needed to identifly the effects of EN on clinical outcome and the natural history in patients with AP, and the beneficial effects of formulas containing the novel nutrient substrates also require further study in the patients with AP.

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Edited by Jason Carr

P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Original Articles

Transepithelial transport of putrescine across monolayers of the human intestinal epithelial cell line, Caco- 2

Vladan Milovic, Lyudmila Turchanowa, Jürgen Stein and Wolfgang F. Caspary

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Abstract

AIM To study the transepithelial transport characteristics of the polyamine putrescine in human intestinal Caco-2 cell monolayers to elucidate the mechanisms of the putrescine intestinal absorption.

METHODS The transepithelial transport and the cellular accumulation of putrescine was measured using Caco-2 cell monolayers grown on permeable filters.

RESULTS Transepithelial transport of putrescine in physiological concentrations (>0.5 mM) from the apical to basolateral side was linear. Intracellular accumulation of putrescine was higher in confluent than in fully differentiated Caco-2 cells, but still negligible (less than 0.5%) of the overall transport across the monolayers in apical to basolateral direction.EGF enhanced putrescine accumulation in Caco-2 cells by four fold, as well as putrescine conversion to spermidine and spermine by enhancing the activity of S adenosylmethionine decarboxylase. However, EGF did not have any significant influence on putrescine flux across the Caco- 2 cell monolayers. Excretion of putrescine from Caco-2 cells into the basolateral medium did not exceed 50 picomoles, while putrescine passive flux from the apical to the basolateral chamber, contributed hundreds of micromoles polyamines to the basolateral chamber.

CONCLUSION Transepithelial transport of putrescine across Caco-2 cell monolayers occurs in passive diffusion, and is not influenced when epithelial cells are stimulated to proliferate by a potent mitogen such as EGF.

2nd Department of Medicine, Johann Wolfgang Goethe University, Frankfurt, Germany
Dr. Vladan Milovic, graduate of the Belgrade University School of Medicine (Yugoslavia), is a former recipient of Alexander von Humboldt and Knut & Alice Wallenberg Postdoctoral Research Fellowships, and is at present approaching his Senior Lectureship in Experimental Medicine at the Johann Wolfgang University in Frankfurt.
Correspondence to: Dr. med. Vladan Milovic, Medizinische Klinik II, J.W.Goethe Universität, Theodor Stern Kai 7, D-60590 Frankfurt, Germany
Tel. +49-69 6301-5326, Fax. +49-69 6301-6246
Email. Milovic@em.uni-frankfurt.de
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INTRODUCTION

Polyamines putrescine, spermidine and spermine are small aliphatic cations necessary for cell growth. If the cells are deprived from their polyamines they will stop dividing, resuming their proliferation rates after restoration of normal polyamine levels[1,2]. Polyamine content in the cell is tightly regulated: in addition to synthesis (via ornithine decarboxylase, synthesising putrescine from ornithine; and S-adenosylmethionine decarboxylase, an aminopropyl donor, synthesising spermidine and spermine), all mammalian cells are equipped with an efficient polyamine uptake system, regulated by mitogens[3-6] and capable of supplying the rapidly proliferating cells with increasing amounts of polyamines from the extracellular space.

Intestinal lumen is the main exogenous source of polyamines for the body. The prevailing polyamine in the intestinal lumen, putrescine, is present in human diet in high amounts[7,8]. In particular, food of plant origin contain excessive amounts of putrescine, while meat and meat products are rich in other two polyamines, spermidine and spermine. Postprandial putrescine concentrations in the duodenal and jejunal lumen can reach millimolar levels[9]. However, not longer than 2 h after meal, luminal polyamine content returns to baseline. Luminal putrescine is rapidly converted to metabolically active spermidine already in the intestinal wall[10,11]. However, surprisingly little is known about absorption mechanisms of luminal putrescine and its final metabolic fate, in spite of the findings that outlined the importance of luminal putrescine (and other polyamines) in both normal and neoplastic growth throughout the body. Luminal polyamines were found to support tumor

growth[12], and are found to accumulate in tissues of high demand, i.e., high proliferation rate, such as neoplasia[13,14] or skeletal muscle stimulated to grow by clenbuterol[15].

To elucidate the mechanisms of putrescine absorption from the intestinal lumen, we used Caco-2 cells grown on permeable filters as an experimental model. Caco-2 cells originate from human colonic adenocarcinoma, but after two weeks in culture, spontaneously differentiate into an enterocyte-like phenotype[16]. These cells form monolayers with well-developed tight junctions[17], and have been evaluated in detail as a model to study both transcellular and paracellular transport of nutrients and drugs in the gut[17-20].

MATERIALS AND METHODS

Materials

EGF (human recombinant, expressed in S. cerevisiae), unlabelled putrescine, spermidine and spermine were purchased from Sigma Chemie (Deisenhofen, Germany). [^{3}H]-putrescine was obtained from Amersham Buchler (Braunschweig, Germany). Cell culture media and supplements were purchased from Gibco BRL (Eggenstein, Germany).

Methods

Cell culture Caco-2 cells were obtained from the German Cancer Research Centre, Heidelberg. Cells of passage number 49-56 were used. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 25 mM Hepes, supplemented with 10% foetal calf serum, 100 U/mL benzylpenicillin and 10 mg/L streptomycin. The medium was changed every second day. The cells were routinely checked for Mycoplasma in monthly intervals.

For the experiments, the cells were expanded in tissue culture flasks (225 cm^2 growth area), detached by treatment with 0.5 g/L trypsin and 0.2 g/L EDTA in PBS, and, to allow monolayers to be formed and uptake to be studied in conditions closely resembling this in the human intestinal epithelium, reseeded to 10 cm^2 cell culture filters (ANOPORE; Nunc, Roskilde, Denmark); 2×10^6 cells were seeded to each plate. After 24 h, 1.5 mL of the medium was replaced with fresh medium and further maintenance was done as described above.

Putrescine uptake studies Cells were monitored for confluence by phase contrast microscopy. Confluence was reached at average after 6 or 7 days of culture. Confluent (day 7) or differentiated (day 14) Caco-2 cells were washed twice with PBS and synchronised in serum-free DMEM overnight. The studies on polyamine uptake into Caco-2 cells were done by adding 50 μM of each polyamine (0.1 μCi/mL) to the cell culture wells. After the desired period uptake was terminated by washing the cell monolayers twice with cold PBS to remove non-absorbed radioactivity from the cell surface. The filters were then removed by cutting using a sterile scalpel, and the cells were permeabilised with 1N NaOH, neutralised with 1 N HCl and the radioactivity was measured by standard liquid scintillation counting. In the experiments where a known enhancer of polyamine uptake into the cells, EGF was used, the procedure was carried out by the same manner except that the cells were incubated with 100 μg/L EGF for 12 h prior to the uptake experiments. Because EGF receptors in both confluent and differentiated Caco-2 cells are localised predominantly at the basolateral side [21], EGF was routinely added only to the basolateral chamber.

Permeability of putrescine across Caco-2 cell monolayers Transport studies using radiolabelled putrescine were performed on Caco-2 cells cultured on permeable filters. All experiments were carried out at 37℃ in serum-free DMEM under 'sink' conditions, as described by Artursson[18-20], using Caco-2 monolayers which were 14 days old. Transepithelial resistance was routinely measured using Millicell TER chamber (Millipore, Eschborn, Germany), and the value of 250 Ohm × cm^2 was considered sufficient to allow permeability studies.

In brief, all solutions were preheated to 37℃ and the experiments were performed at 37℃ ± 1℃ on a custom-built heating plate. The cell monolayers were washed with preheated DMEM and equilibrated in the same medium for 20-25 minutes prior to the transport experiments. The filters were then transferred to wells containing 1.2 mL fresh preheated DMEM. One millilitre DMEM containing radiolabelled test substance was added to the apical ("donor") chamber. The [^{14}C]-mannitol (MW 182, comparable to molecular weight of putrescine - 161.1) was used as a marker molecule to assess the integrity of the monolayers during the experiment. Radioactivity was used at concentrations of 10 000 or 20 000 Bq/mL for Caco-2 monolayers. Samples were taken from the apical solutions in order to measure the initial donor concentration. At four regular time intervals, the inserts were moved to new wells and samples were taken from the basolateral solution. All samples were analysed directly as described below.

The possible effect of putrescine adhesion to permeable supports was investigated by comparing the diffusion of [^{14}C]-putrescine across cell-free supports. No retarded diffusion of [^{14}C]-putrescine was observed, which showed that possible adhesion of putrescine to extracellular material could be neglected.

Analytical methods S-adenosylmethionine -decarb oxylase activity in Caco-2 cells was assayed as described

before[22], by measuring the amount of $^{14}CO_2$ liberated from S-adenosyl (carboxy ^{14}C)-L-methionine. The final volume of incubation medium consisted of 50 mM Tris-HCl pH 7.2, 0.2 mmol/L S-adenosyl (carboxyl ^{14}C)-L-methionine, 0.05 mmol/L pyridoxal-5-phosphate, 2.5 mmol/L DTT, and 0.1 mmol/L EDTA. The incubation period at 37℃ was 60 minutes. Radioactive samples were analysed using a liquid scintillation counter (Packard Instruments 1900CA TRI-CARB®; Canberra Pacard Instruments, Downers Grove, IL). Protein was measured by Coomassie blue assay, by kits obtained from Biorad Laboratories GmbH (Munich, Germany).

Calculations The apparent permeability coefficient (P_{app}, cm/s) was determined according to the following equation[18-20]:

$$P_{app} = \frac{K \cdot Vr}{A}$$

where K is the steady state rate of change in concentration in the receiver chamber (C_t/C_0) versus time (s), C_t is the concentration in the receiver compartment at the end of each time interval, C_0 is the initial concentration in the apical chamber at each time interval (mole/mL), Vr is the volume of the receiver chamber (mL) and A is the surface area of the filter membrane (cm^2).

Statistics The results were expressed as mean values ± SEM of four to six experiments. One-way ANOVA was used to compare means. A 95% probability was considered significant.

RESULTS

Basal and EGF-stimulated putrescine uptake in Caco-2 cells

Caco-2 cell monolayers were incubated with and without 100 μg/L EGF for 12 hours and then with 50 μM radiolabelled putrescine for different time intervals. Uptake rate of putrescine increased rapidly and was significantly higher in confluent than in differentiated Caco-2 cells (Figure 1). In confluent, 7 days old cells, EGF-induced putrescine uptake was significantly higher than in differentiated 14 days old cells, basolateral uptake being markedly higher than apical uptake when the cells were stimulated with EGF.

Transepithelial permeability of putrescine

The transepithelial resistance in Caco-2 cell monolayers reached a plateau of 234 ± 12 Ohm × cm^2 after approximately one week in culture and maintained this value for at least 40 days in culture, which was in agreement with previous findings[18-20]. Such monolayers of Caco-2 cells transported [^{14}C]-mannitol with P_{app} of 22 ± 4 × 10^6 cm/s, which was another proof of their well developed tight junctions and full functional integrity. When physiological amounts of putrescine (100 μM; 0.1 μCi/mL) was added to the apical chamber and flux into the basolateral chamber measured over time, transport was linear. Almost the entire applied concentration diffused to the basolateral chamber within a 60 min experimental period (Figure 2).

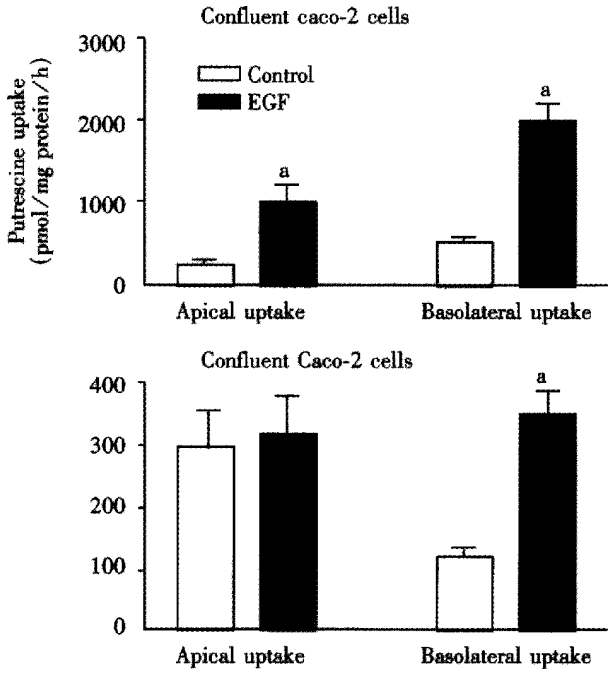

Figure 1 Apical *vs*. basolateral uptake of putrescine in confluent (day 7) and differentiated (day 14) Caco-2 cells in culture. The experiments were carried out as indicated in the Methods section. EGF (100 μg/L) was added to the basolateral chamber, and the cells were incubated for 12 h. Mean ± SEM, N = 4. $^aP<0.001$ *vs* control.

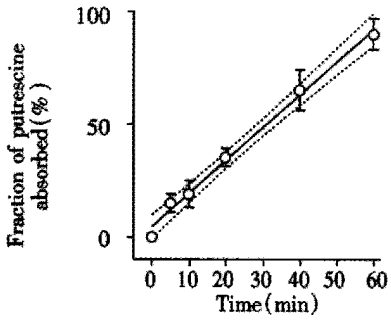

Figure 2 Transepithelial permeability of putrescine across Caco-2 cell monolayers. The experiments were done as described in Methods section, with 100 μM (0.1 μCi/mL) putrescine added to the apical chamber and incubation time 60 min. Mean ± SEM, N = 4.

Apparent permeability constants (P_{app}) for putrescine was in the range of 18-35 × 10^6 cm/s, which was similar to Papp of the standard marker for paracellular permeability, mannitol (Figure 3). There was no change in P_{app} values when the cells were incubated in a balanced salt solution without glucose (data not shown).

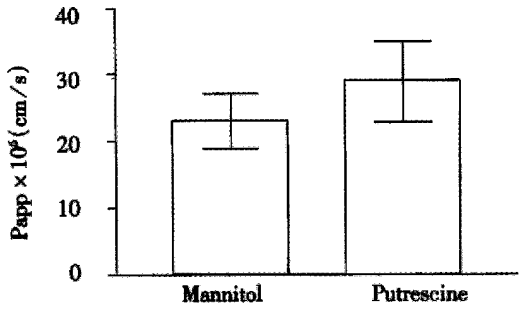

Figure 3 Apparent permeability coefficients for mannitol and putrescine. Each bar represents mean ± SEM of six experiments. Differences between the values are not statistically significant.

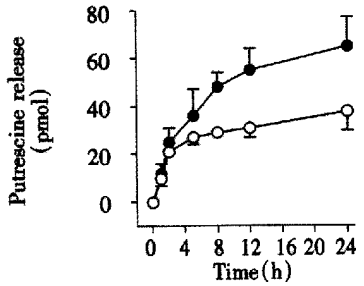

Figure 4 Putrescine release from Caco-2 cells pre-loaded with physiological concentrations (100 μmol / sample) putrescine ± EGF. Each data point represents mean ± SEM of six experiments.

Transcellular component of putrescine transport across the intestinal epithelium: putrescine uptake and release in Caco-2 cell monolayers

A 12 h incubation with 100 μg/L EGF stimulated not only putrescine uptake in CaCo-2 cells (Figure 1), but also its conversion to spermidine and spermine. This conclusion was based on the finding that EGF stimulated the activity of S-adenosylmethionine decarboxylase, the enzyme responsible for conversion of putrescine to spermidine and spermine by approx imately 90% (control: 114 ± 9 pmol CO_2/mg protein, *vs* EGF: 201 ± 8 pmol CO_2/mg protein, $P<0.01$). To investigate whether an increase of putrescine uptake in the intestinal epithelium can be more profoundly increased by a potent growth stimulus such as EGF (and therefore its general absorption influenced), we preloaded control and EGF-treated Caco-2 cell monolayers with physiological concentrations of putrescine (100 μmol), allowed to incubate for 2 h (time period previously shown to be sufficient for maximal uptake)[5], then washed the monolayers with PBS to remove non-absorbed radioactivity, and transferred to radioactivity-free cell culture wells. Release of the radioactivity originating from exogenous putrescine to the basolateral chamber in both control and EGF-treated cells was initially rapid, reaching plateau not earlier than after 6 h incubation (Figure 4). Up to 24 h only minute amounts of the radioactivity was released across the basolateral membrane, the values not exceeding 45 pmol; EGF did not have major influence on putrescine release.

DISCUSSION

Our results indicate that putrescine transport through the intestinal epithelial barrier is linear, the levels taken up by the epithelial cells not exceeding 0.5% of those passively transported across the intestinal epithelium. Treatment with EGF, known to up-regulate putrescine uptake in Caco-2 cells, did enhance putrescine accumulation in Caco-2 cells, but this had no overall impact on net transport of putrescine across the intestinal epithelium, in concentrations shown to exist postprandially in the intestinal lumen[9]. In spite of all limitations of the model used (i.e., absence of subepithelial tissue, inability to measure possible rapid metabolisation of absorbed putrescine in the intestinal wall), our data lead to the conclusion that, in the gut, putrescine is absorbed exclusively by passive diffusion.

A number of studies done in different experimental models show that polyamines rapidly disappear from the intestinal lumen after meal and are rapidly distributed in the body, being directed to tissues with highest demand, i.e. with the highest proliferation rate[23]. Our data may provide an explanation about the possible mechanism for the rapid disappearance of dietary putrescine from the gut lumen. At physiological pH putrescine is fully charged. Small, charged and hydrophilic molecules are usually absorbed via the paracellular pathway, and their transport is additionally facilitated by solvent drug[24]. Indeed, in our experimental model, permeability constant for putrescine did not significantly differ from this of a distinct paracellular marker sized similar to putrescine, mannitol (Figure 2).

The presence of several hundreds of micromoles putrescine in the postprandial duodenal and jejunal lumen, while its concentration in blood and tissues hardly exceeds tens of micromoles, clearly rises a need to offer an explanation for such a high supply and low utilisation of this polyamine in the body. Excess luminal putrescine has recently been attributed as an instant energy source in the gut[25]. Furthermore, endothelial cells in the gut are rich in diamine oxidase, the enzyme able to degrade excess putrescine to succinate and GABA[26]. In the only study of this kind done in humans, disappearance of putrescine from the human intestinal lumen was found to be linear; however, the amounts of putrescine itself, shortly after the putrescine-rich test meal, showed that no free putrescine was detectable in blood. Instead, its acetylated derivative was rapidly and markedly increased, while the amounts of spermidine and spermine increased less rapidly but progressively[27]. All these data may fit well into the hypothesis that putrescine, as well as other two polyamines, cross the intestinal epithelial barrier passively, and that they are then both rapidly metabolised in situ and

used as an instant energy source, or, also rapidly, metabolised into spermidine and spermine and then transported throughout the body.

Apart from transport across the intestinal epithelium, small amounts of putrescine in our study were also taken up by enterocyte-like Caco-2 cells. Putrescine uptake was also stimulated by EGF. Perhaps surprisingly, this uptake was higher across the basolateral then the apical membrane, and the difference was further increased after stimulation with EGF. The role of basolateral polyamine uptake when the gut is stimulated to grow has been described before[28-30], and putative polyamine transporters at the basolateral membrane of the enterocyte has been well characterised at the biochemical level[31,32]. Our data might also additionally confirm an interesting hypothesis that polyamines, via local circulation in the intestinal wall, do reach the enterocyte via their basolateral membranes rather than directly, via the brush border membrane of the enterocyte[33].

In conclusion, our data show that putrescine crosses the intestinal epithelial barrier passively, while only minor amounts are taken up by intestinal epithelial cells. Our data provide an explanation for a known rapid disappearance of dietary polyamines from the intestinal lumen, and their rapid distribution in the body, where they then exert their known growth-related actions.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Cholesterol crystal binding of biliary immunoglobulin A: visualization by fluorescence light microscopy

Frank Lammert, Stefan Südfeld, Norbert Busch and Siegfried Matern

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Abstract

AIM To assess potential contributions of biliary IgA for crystal agglomeration into gallstones, we visualized cholesterol crystal binding of biliary IgA.
METHODS Crystal binding biliary proteins were extracted from human gallbladder bile using lectin affinity chromatography. Biliary IgA was isolated from the bound protein fraction by immunoaffinity chromatography. Pure cholesterol monohydrate crystals were incubated with biliary IgA and fluoresceine isothiocyanate (FITC)conjugated anti IgA at 37 ℃. Samples were examined under polarizing and fluorescence light microscopy with digital image processing.
RESULTS Binding of biliary IgA to cholesterol monohydrate crystals could be visualized with FITC conjugated anti IgA antibodies. Peak fluorescence occurred at crystal edges and dislocations. Controls without biliary IgA or with biliary IgG showed no significant fluorescence.
CONCLUSION Fluorescence light microscopy provided evidence for cholesterol crystal binding of biliary IgA. Cholesterol crystal binding proteins like IgA might be important mediators of crystal agglomeration and growth of cholesterol gallstones by modifying the evolving crystal structures *in vivo*.

Department of Medicine III, University of Technology (RWTH) Aachen, Germany
Dr. Frank Lammert, graduated from RWTH Aachen as M.D. in 1993, 1995-1997 Research Fellow at Harvard Medical School (Boston), since 1998 Staff Physician at RWTH Aachen, since 1999 Junior Research Group on Genetics of Complex Hepatobiliary Diseases.
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Correspondence to: Dr. S. Matern, Department of Medicine III, RWTH Aachen, Pauwelsstraβe 30, 52057 Aachen, Germany
Tel. +49-241-8088590, Fax: +49-241-8888455
Email. smatern@post.klinikum.rwth-aachen.de.
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INTRODUCTION

Cholesterol gallstone formation involves multiple steps including hepatic secretion of lithogenic bile[1,2] and nucleation and growth of cholesterol crystals within the gallbladder[3]. Besides cholesterol supersaturation[4] and gallbladder hypomotility[2,5], kinetic protein factors that promote or inhibit cholesterol crystallization have been suggested to play key roles in cholesterol gallstone pathogenesis[6,7]. During the past decade, model bile studies indicated that several promoter or inhibitor proteins influence cholesterol crystallization *in vitro*[8-14]. Immunoglobulins were also identified as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones[15,16]. However, the significance of individual protein factors for the pathogenesis of the human disease remains controversial[17,18].

By preparing crystal adsorbed proteins via sucrose density gradient centrifugation and gel electrophoresis, we identified a subgroup of human biliary proteins that bind to cholesterol crystals, modify crystal morphology and inhibit cholesterol crystallization[19]. We demonstrated that secretory immunoglobulin A (IgA) is a major constituent of this group of cholesterol crystal-binding proteins[20]. To assess the importance of biliary IgA for cholesterol crystal agglomeration into gallstones *in vivo*, we now visualized and characterized the binding of biliary IgA to the cholesterol crystal surface.

MATERIALS AND METHODS

Chemicals and analytical procedures

Cholesterol was obtained from Eastman Kodak Co. (Rochester, NY, USA) and its purity was verified by HPLC to be >99%[21]. Sodium taurocholate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). *Concanavalin A* and *Helix pomatia*-lectin-sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were reagents or HPLC grade quality (Sigma Chemical Co.; Merck AG, Darmstadt, Germany).

Isolation of biliary IgA by immunoaffinity chromatography (Figure 1A)

Gallbladder bile from patients with cholesterol

gallstones was obtained by needle aspiration during cholecystectomy. The protocol was approved by the ethical committee of RWTH Aachen. To remove insoluble constituents, bile samples were ultracentrifuged at 100 000 × *g* for 1h. Biliary protein fractions were isolated by affinity chromatography using -Concanavalin A and Helix pomatia- lectin, as described[19,20].

Biliary IgA was isolated from the bound protein fraction by immunoaffinity chromatography[20]. Briefly, affinity columns were prepared by coupling rabbit polyclonal antibodies against the human immunoglobulin á-chain (Sigma Chemical Co.) to Aminolink coupling gel (Immunopure- Immoblization Kit, Pierce Chemical Co., Rockford, IL, USA). After washing the column with 1 M NaCl and equilibration with Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.4, 200 mM NaCl, 3 mM NaN_3), biliary proteins (2 - 3mg) were applied to the affinity column overnight. The column was washed extensively with TBS containing 10 mM sodium taurocholate to remove any unbound material and residual lipids. Afterwards, the bound IgA was eluted from the column with 10 Lm 0.1 M glycine/HCl (pH 2.5) into a tube containing 500 µL 1 M Tris/HCl (pH 9.5), and dialyzed against 25 mM ammonium bicarbonate. All steps were carried out at 4℃.

Immunofluorescence studies (Figure 1B)

Cholesterol monohydrate crystals were prepared by recrystallization from 95% (v/v) ethanol according to Igimi and Carey[22]. Five grams cholesterol was dissolved in 400 mL ethanol at 60℃ and cooled slowly to room temperature. To obtain predominantly small, intact crystals, the solution was stirred at 30 rpm. The cholesterol crystals were harvested by filtration through a 0.45 µm micropore filter (Millipore Corp., Bedford, MA) and checked under light microscopy.

Cholesterol monohydrate crystals were resuspended in phosphate buffered saline (PBS; 9.6 mM phosphate, pH 7.2, 2.7 mM KCl, 137 mM NaCl, 3 mM NaN_3) containing 1% (w/v) bovine serum albumin (BSA) to reduce non-specific crystal binding. Samples were incubated at 37℃ for 1 h and shaken at 100 rpm. After washing the crystals three times with PBS containing 0.1% (w/v) BSA (PBS/BSA), IgA was added in PBS/BSA to a final concentration of 200 µg/L. The sample was incubated for 2 h at 37℃ with rapid agitation (100 rpm). After another four washes with PBS/BSA, fluoresceine isothiocyanate (FITC)-conjugated anti-human IgA (Sigma Chemical Co.) was added in PBS/BSA (dilution 1 : 10). The sample was incubated for 1 h at 37℃. After two final rinses, the suspension was taken up in 1mL PBS/BSA. Samples without IgA or with human IgG (Sigma Chemical Co.) served as controls.

Small specimen (25 µL) were examined under polarizing and fluorescence light microscopy (Leica DM RB, fluorescence filter I3). Microphotographs (Minolta 9000) were processed with Corel Photopaint™ (Version 7.0; Corel Corporation, Dublin, IL).

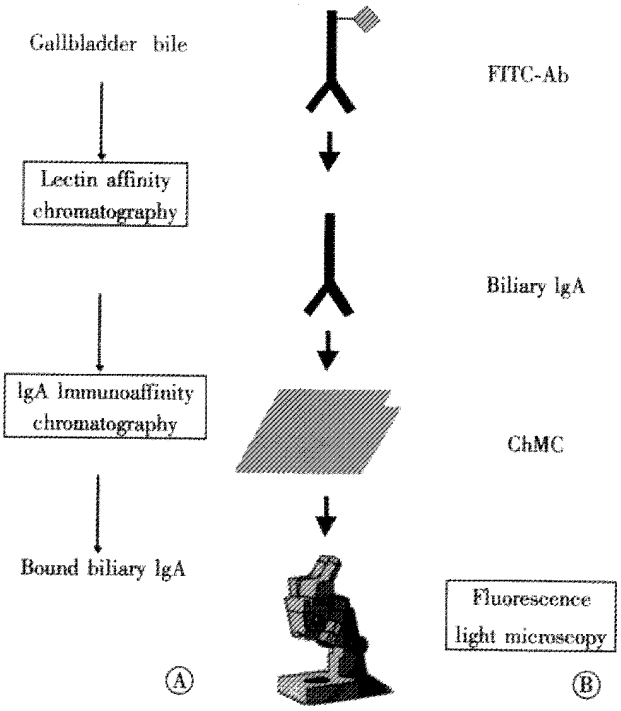

Figure 1 (A) Biliary IgA was isolated from human gallbladder bile by lectin affinity in sequence with immunoaffinity chromatography. (B) Cholesterol crystal binding of biliary IgA to cholesterol monohydrate crystals (ChMC) was visualized microscopically with fluoresceine isothiocyanate-conjugated anti-human IgA antibodies (FITC-Ab).

RESULTS

Utilizing the information on the identity of cholesterol crystal-binding proteins[20], we isolated biliary IgA by lectin affinity chromatography and immunoaffinity chromatography. The isolation procedure yielded electrophoretically homogeneous preparations of biliary IgA, as judged by silver-stained sodium dodecyl-sulfate polyacrylamide gel electrophoresis and immunoblotting (not shown), thus confirming the results of our previous study[20].

The immunofluorescence studies clearly demonstrate significant binding of biliary IgA to cholesterol monohydrate crystals. After incubation of the cholesterol crystal suspension with biliary IgA, followed by the secondary antibody conjugated to FITC, fluorescence is observed dispersed on the surface of the crystals. The left, middle and right panels of Figure 2A and Figure 2B display two representative cholesterol crystals by light, polarizing and fluorescence light microscopy,

respectively. The basic unit is the plate-like cholesterol monohydrate crystal, which shows an euhedral triclinic form with straight edges and a smooth surface (Figure 2A). In addition, the crystals tend to aggregate and to form more irregular structures (Figure 2B). The two right microphotographs of Figure 2A and Figure 2B show how the specific yellow FITC fluorescence is distributed on the crystal surface: Peak fluorescence signals occur at crystal edges as well as screwed and stepwise dislocations, consistent with crystal binding sites with a high affinity to IgA.

Figure 3 displays control samples with IgG (A) and without IgA (B). The crystals exhibit no or only little spotted fluorescence, with blank crystals set off against the green background activity.

Separate experiments were carried out to reduce residual non-specific binding by pre-incubation with BSA (data not shown), and 0.1% (w/v) BSA was used throughout the experiments. This is consistent with recent immunofluorescence studies demonstrating that albumin binds to randomly located little spots on the crystal surface[23], although albumin is not a crystal-binding protein *sui generis* and does not modulate cholesterol crystallization in model bile[24].

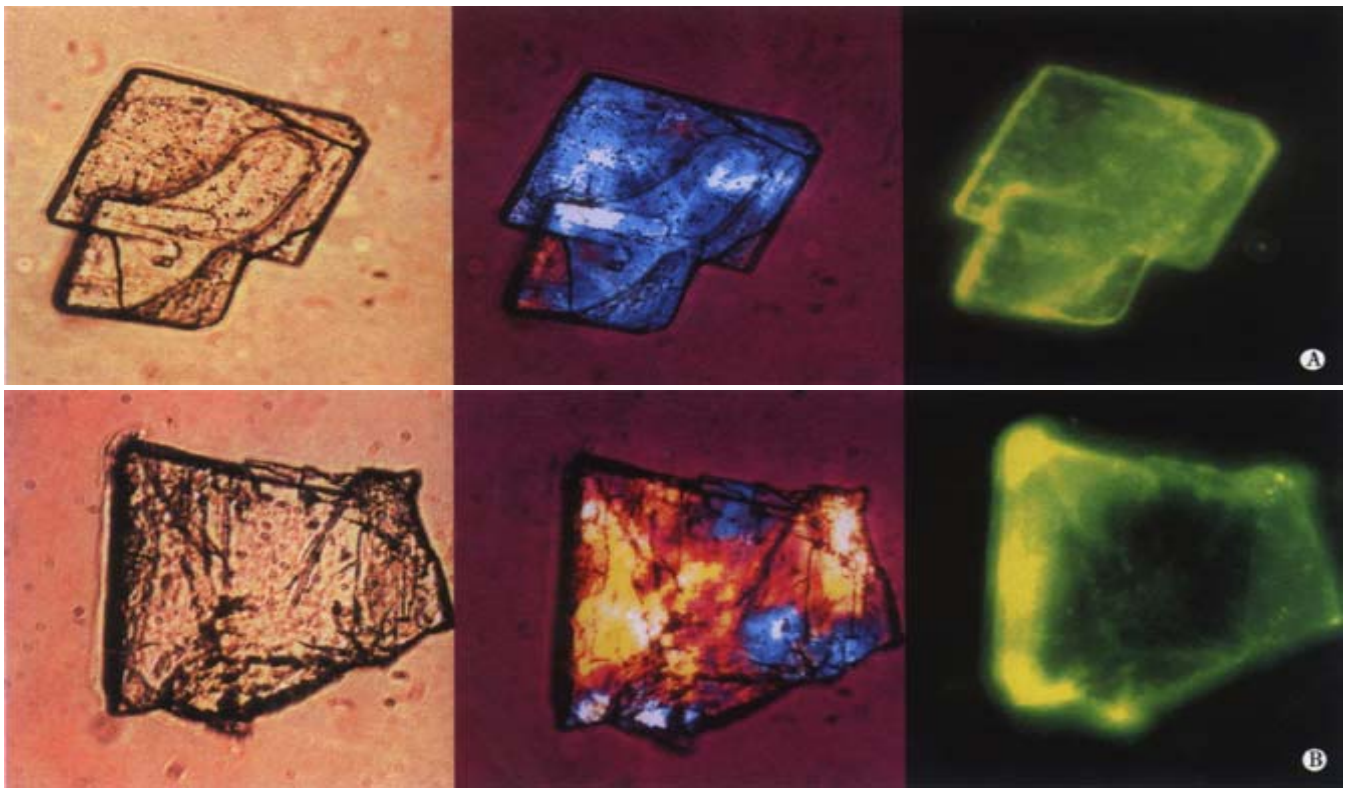

Figure 2 Binding of biliary IgA to cholesterol monohydrate crystals. Crystals were treated with biliary IgA, followed by FITC-Ab (see Figure 1). Left, middle and right panels of show light, polarizing and fluorescence light microphotographs, respectively.

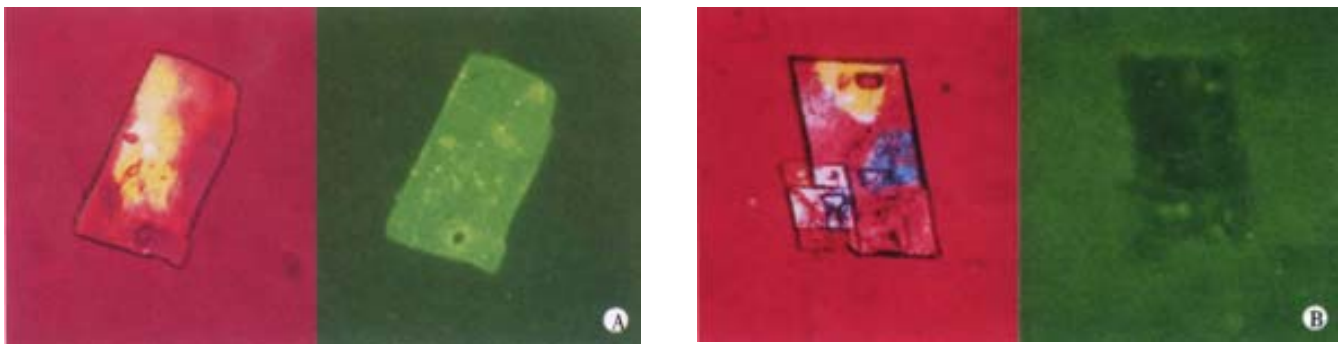

Figure 3 Cholesterol monohydrate crystals with IgG and FITC-Ab incubation (see Figure 1). Cholesterol monohydrate crystals without IgA incubation (FITC-Ab only). Left and right panels show polarizing and fluorescence light microphotographs, respectively.

DISCUSSION

Gallbladder bile contains several proteins that exhibit promoting or inhibiting effects on cholesterol crystallization *in vitro*. Besides physical-chemical determinants[4] and hypomotility of the gallbladder, these kinetic protein factors are assumed to modulate cholesterol gallstone formation[7,25]. Because systematic studies in reconstituted supersaturated human bile (cholesterol saturation index >1.6) did not affect cholesterol crystallization sequences or crystal detection times[17], biliary proteins might predominantly affect subsequent cholesterol crystal agglomeration and growth into mature stones[26].

Bile formation is a means of delivering immunoglobulin into the biliary system and the intestine. The major biliary immunoglobulin is IgA, which enhances the immune protection of mucus membranes and epithelial cells by binding injurious agents or toxins[27]. In humans, polymeric IgA is the predominant class of biliary immunoglob ulins[28,29]. It is secreted by the liver and the gallbladder[30] as complex (secretory IgA), consisting of IgA monomers and the secretory component that is necessary for the endocytotic path way of IgA across the biliary epithelial cells. Our previous studies demonstrated that secretory IgA inhibits cholesterol crystallization in supersaturated model bile in a dose-dependent manner; maximum crystal growth rate and final crystal concentration were significantly reduced even below physiological concentrations[20]. Furthermore, the presence of IgA and other crystal-binding proteins changed crystal morphology, favoring compact and regular microcrystals. However, direct visualization of IgA binding to the crystal surface was not demonstrated.

The interaction between biliary IgA and cholesterol monohydrate crystals could not be visualized by immunoelectron microscopy using anti-human IgA labeled with colloidal gold (mean particle size 4 nm), because the crystal structures were unstable under the conditions tested. We therefore employed immunofluorescence, which was successfully applied in parallel studies that investigated the protein contents of cholesterol gallstones[31] and biliary sludge[23]. Our fluorescence light microscopy studies confirmed cholesterol crystal binding properties of biliary IgA. These findings are in line with localization of IgA in biliary sludge where it was clearly associated with aggregations of cholesterol crystals, which resemble the concentric cholesterol layers at the periphery of stones[23]. These findings support the hypothesis that crystal-binding IgA might be an important modulator of cholesterol crystal agglomeration into stones and stone growth *in vivo*.

The interaction between IgA and cholesterol crystals could not only depend on the distinct immunoglobulin class but might predominantly rely on the variable ends of the light chains. This hypothesis is supported by the synthesis of specific antibodies against cholesterol upon injection of cholesterol-rich liposomes in rabbits and mice[32,33]. These antibodies recognize non-oxidized crystalline cholesterol[32], and analyses by solid-phase ELISA revealed that human sera contain varying levels of naturally occurring autoantibodies to cholesterol[34]. Similarly, humans might synthesize and secrete specific IgA antibodies to cholesterol into bile. This would explain why biliary IgA inhibits cholesterol crystal growth, in contrast to IgA from colostrum[20] and other immunoglobulins, which promote cholesterol crystallization in various experimental settings[15,35]. Using a modified ELISA technique for rapid, precise measurement of protein binding to cholesterol crystals[33], we have recently shown that binding of purified biliary IgA is more than 10-fold increased compared to colostrum IgA (Südfeld S, Lammert F, Busch N, Matern S; unpublished observations).

We speculate that crystal growth inhibitors like biliary IgA attach to the most rapidly growing sites like spiral and edge dislocations on the thermodynamically stable monohydrate crystals, thus changing crystal morphology, retarding crystal growth and decreasing final crystal concentration[19]. This concept applies to many crystal growth inhibitors in biomineralization. Concerning formation of other stones in humans, Nakagawa *et al*. reported a glycoprotein that prevents formation of urinary calcium oxalate stones in healthy persons[36]. From human pancreatic calculi, a small acidic glycoprotein could be isolated that inhibits calcium carbonate precipitation[37]. Other glycoproteins regulate the precipitation of calcium salts in bone and dentin[38,39]. Additional examples of crystal growth inhibiting glycoproteins are found in sera of polar fish and insects, whose 'antifreeze' proteins prevent freezing by adsorbing to ice crystal surfaces[40,41]. These examples illustrate that the concept of crystal growth inhibition by crystal binding probably applies to all inhibitor proteins in biomineralization.

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P.O.Box 2345 Beijing 100023, China
Fax. 0086·10·65891893 Tel. 0086·10·65897901
E-mail.wcjd@public.bta.net.cn www.wd.org.cn

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Mucin and phospholipids determine viscosity of gallbladder bile in patients with gallstones

Dieter Jüngst[1], Anna Niemeyer[1], Iris Müller[1], Benedikta Zündt[1], Günther Meyer[1], Martin Wilhelmi[2] and Reginald del Pozo[2]

Subject headings phospholipids/physiology; phospholipids/analysis; mucins/physiology; mucins/analysis; cholelithiasis/etiology; viscosity; bile/chemistry

Jüngst D, Niemeyer A, Müller I, Zündt B, Meyer G, Wilhelmi M, del Pozo R. Mucin and phospholipids determine viscosity of gallbladder bile in patients with gallstones. ***World J Gastroenterol***, 2001;7(2):203-207

Abstract

AIM An increased viscosity of gallbladder bile has been considered an important factor in the pathogenesis of gallstone disease. Besides lipids and proteins, mucin has been suggested to affect the viscosity of bile. To further clarify these issues we compared mucin, protein and the lipid componEnts of hepatic and gallbladder bile and its viscosity in patients with gallstones.

METHODS Viscosity of bile (mPa.s) was measured using rotation viscosimetry in regard to the non Newtonian property of bile at low shear rates.

RESULTS Biliary viscosity was markedly higher in gallbladder bile of patients with cholesterol (5.00 ± 0.60 mPa.s, mean ± SEM, *n* = 28) and mixed stones (3.50 ± 0.68 mPa.s; *n* = 8) compared to hepatic bile (0.92 ± 0.06 mPa.s, *n* = 6). A positive correlation between mucin and viscosity was found in gallbladder biles (*r* = 0.65; *P*<0.001) but not in hepatic biles. The addition of physiologic and supraphysiologic amounts of mucin to gallbladder bile resulted in a dose dependent non linear increase of its viscosity. A positive correlation was determined between phospholipid concentration and viscosity (*r* = 0.34, *P*<0.005) in gallbladder biles. However, no correlation was found between total protein or the other lipid concentrations and viscosity in both gallbladder and hepatic biles.

CONCLUSION The viscosity of gallbladder bile is markedly higher than that of hepatic bile in patients with gallstones. The concentration of mucin is the major determinant of biliary viscosity and may contribute by this mechanism to the role of mucin in the pathogenesis of gallstones.

Departments of Medicine II and Surgery[1], Klinikum Grosshadern, Ludwig Maximilians-University Munich, Munich, Germany and Facultad de Medicina, Universidad Católica de la Santìsima Concepción, Concepción, Chile

Dieter Jüngst, graduated from the University of Munich as M.D. and is an associate professor of medicine with special interest in biliary diseases.

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Correspondence to: Dieter Jüngst, M.D., Department of Medicine II, Klinikum Grosshadern, Marchioninistr. 15, 81377 Munich, Germany
Tel. 01149-89-7095-2376, Fax. 01149-89-7095-5374

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INTRODUCTION

Gallbladder mucin, a high molecular weight and densely glycosylated protein secreted by gallbladder epithelium, is the principal organic constituent of gallbladder mucus and biliary sludge[1-3]. It appears to play an important role in different stages of cholesterol or mixed gallstone formation[4]. Gallbladder mucin hypersecretion and accumulation in the gallbladder as a viscoelastic gel precedes the formation of gallstones in animals[5,6] and in humans[7,8]. Furthermore, the highly viscous gel on the luminal side of the gallbladder epithelium and the soluble mucin in bile are believed to enhance the residence time of lithogenic bile in the gallbladder, allowing the growth of cholesterol crystals and, thereby, serving as a nidus for gallstone formation[2,9]. In fact, an association between hexosamine concentrations in bile which derive mostly from soluble mucin and the viscosity of bile has been already described by Bouchier *et al*[10] many years ago. In a more recent study, capillary viscosimetry like Bouchier biliary viscosity at 37℃ was correlated closely with total protein concentration and hexosamine concentration in bile[11]. Few further studies dealing with bile viscosity are available, and in these studies capillary viscosimeters were also used[12-14]. According to these studies, bile behaves essentially as a Newtonian fluid at high shear rates. A Newtonian fluid has a constant viscosity, whereas a non-Newtonian fluid shows a disproportionate increase in viscosity at a low flow velocity. However, capillary viscosimeters lack standardization and are unable to measure the viscosity at low shear rates, at which bile shows a non-Newtonian behaviour. We used the Contraves LS-30 coaxial rotation viscosimeter to eliminate this problem. This

viscosimeter is very sensitive and has a wide measurement range, from 0.01 s^{-1} to more than 100 s^{-1} covering the range of both Newtonian and non-Newtonian flow.

Our study included bile samples from both hepatic and gallbladder biles from patients with mixed or cholesterol stones. The viscosity of bile was compared with the quantitatively most important constituents to investigate which component of bile affects its viscosity.

METHODS

Patients and collection of bile

Thirty-six patients, 25 women (mean age 40 ± 10.5 years) and 11 men (mean age 48.5 ± 11.4 years), who underwent laparoscopic surgery because of symptomatic gallstone disease and six patients with T-drainage after cholecystectomy were also included in the study. Gallstones were visualized by ultrasonography and reasonable gallbladder function was confirmed by determining that at least 30% of the fasting volume was emptied after the administration of a liquid test meal. Patients shown to have severely impaired gallbladder function or a loss of the gallbladder reservoir or cystic duct obstruction were excluded from the study. All patients gave informed consent after a detailed explanation of the procedure required for intraoperative bile collection. During laparoscopic surgery, the gallbladder was punctured with a 16-G needle and the bile was aspirated as completely as possible because of the known stratification of human gallbladder bile[15]. Stones were removed, washed with distilled water, dried, weighed and ground to a powder. The cholesterol content of the stones was measured chemically after extraction with organic solvent and expressed as percentage of dry weight[16]. T-tube hepatic bile was collected from 6 patients (3 women and 3 men) by gravity drainage within 24 hours after cholecystectomy.

Microscopy of bile and crystal observation time

After the collection, bile samples were mixed thoroughly and one drop was immediately examined under polarized light microscopy for cholesterol crystals. Four mL of gallbladder bile was centrifuged in polycarbonate tubes with a 50 Ti rotor at 37℃ for 1 hour at 100 000 × *g* in a Beckman L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA) to precipitate biliary "sludge". The supernatant was microscopically free of cholesterol crystals. Aliquots were incubated at 37℃ and examined daily under polarized light microscopy for up to 21 days[17].The time taken for cholesterol microcrystals to appear was defined as the crystal observation time and measured in days.

Analysis of bile composition

For the analysis of bile composition, duplicate aliquots were stored at -30℃ prior to determination. Cholesterol was determined colorimetrically with the Liebermann-Burchard reaction after double extraction of 1 mL methanolic bile sample with petroleum ether[18]. Phospholipids were measured as total biliary phosphate after hydrolysis at 150℃ with sulfuric acid, using the colorimetric assay of Fiske-Subbarow, and total bile salts were determined by a modified 3-α-hydroxysteroid dehydrogenase method [19,20]. The saturation index of each sample was calculated in native bile by dividing the cholesterol concentration with the maximum cholesterol solubility according to Carey and was corrected for the total lipid content of each individual bile[21]. Total protein was analysed using the Lowry assay after purification of biliary proteins[22]. This method has been validated extensively by adding known amounts of different serum proteins (albumin or γ-globulin) to samples of gallbladder bile with unknown protein content. In contrast to the fluorimetric assay which underestimates the amount of added protein, protein recovery was determined quantitatively by the Lowry assay.

Biliary mucin concentration was determined according to an assay recently described[23]. The assay is a modification of the classical method of Pearson *et al*[24] and Harvey *et al*[25]. Briefly, fresh gallbladder bile was diluted 1:1 (v/v) with 0.1 mol/L Tris-HCl buffer (pH 7.5) containing 0.44mol/L potassium thiocyanate and 0.02% NaN-3. The formation of lipid aggregates in vesicular form was avoided by addition of sodium cholate to this buffer (final concentration 12.5 mmol/L). The mixture was shaken overnight and centrifuged at 12 000 × g for 10 min. One mL of the supernatant was fractioned on a Sepharose 2B column (40 cm × 1.0 cm, Pharmacia AB, Uppsala, Sweden) at a constant flow of 0.3 mL/min. As for elution buffer, we used 20 mmol/L Tris, 140 mmol/L NaCl, 3 mmol/L NaN-3, pH 8.0 buffer containing 25 mmol/L sodium cholate. Column fractions of 1mL were collected and analyzed for glycoprotein by periodic acid/Schiff (PAS) assay[26]. The excluded PAS-positive glycoprotein fractions were further pooled and dialyzed for 12 h against distilled water to remove bile acids. Mucin concentration was measured in this fraction by the periodic PAS-assay using purified human gallbladder mucin standard.

Determination of viscosity

The rheologic measurements were carried out on a calibrated Contraves Low Shear-30 rotation viscosimeter, using a coaxial-cylinder system with a gap width of 0.5 mm (Contraves AG, Zürich, Switzerland). The rotation viscosimeter allows accurate measurements of viscosity of both Newtonian and non-Newtonian fluids. Programming

of measurements and the processing of the measured data were done utilizing the Contraves Rheoscan 30. To obtain a rapid standardized measurement within the entire range of shear rates (0.1 to 118s^{-1}), a computer program was used to enable measurements in one sample within 5 min[27]. These were repeated twice after an interval of 60s each. Thus, the final result of biliary viscosity represented the mean value of a total of 30 determinations. The viscosity was measured within 3 hours after the collection of the bile sample at laparoscopic cholecystectomy. One mL of the bile sample was taken for any viscosity assay and all measurements were performed at 37℃. All samples were centrifuged for 2 min at 12 000 × *g*, to eliminate sediment that could interfere with the determinations.

To study the effect of mucin on the rheological properties of human bile, purified mucin from porcine stomach (0.24-2.24 g/L) was added to gallbladder bile and the viscosity measured as shown above.

Statistical analysis

Values of each group of parametric data are expressed as means ± SEM. Non-parametric data (crystal observation time) are expressed as median and range. Group comparison was performed for parametric data using unpaired Student's *t* test and for non-parametric data using the Mann-Whitney U test. Spearman's correlation coefficient were calculated between variables and expressed as significant at the $P<0.05$ level.

RESULTS

Individual and total lipids, CSI, crystal observation time, protein, mucin and viscosity in gallbladder bile of patients with cholesterol and mixed stones and in hepatic bile are shown in Table 1. As expected, protein, mucin and lipid concentrations and viscosities were markedly higher in gallbladder bile compared to hepatic bile. A positive correlation between mucin and viscosity was found in gallbladder biles (Figure 1) but not in hepatic biles ($r = 0.01$;n.s.). Figure 2 shows the effect of adding physiological and supraphysiological amounts of mucin to gallbladder bile in relation to the viscosity. There was a dose dependent non linear increase of its viscosity by adding external mucin to native gallbladder bile at three different shear rates including the prestatic region (shear rate 0.1s^{-1}) in this experiment.

Furthermore, a positive correlation was determined between phospholipid concentration and viscosity ($r = 0.34$, $P<0.05$) in gallbladder biles (Figure 3). However, no significant correlation was found between total protein (Figure 4) or the other lipid concentrations and viscosity in both gallbladder (Table 2) and hepatic biles.

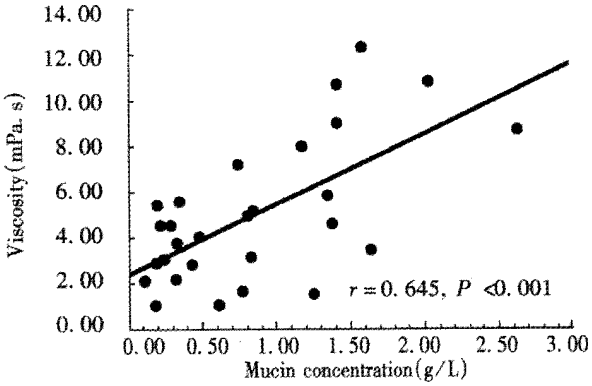

Figure 1 Correlation of viscosity and mucin concentration in 36 gallbladder biles from patients with gallstones.

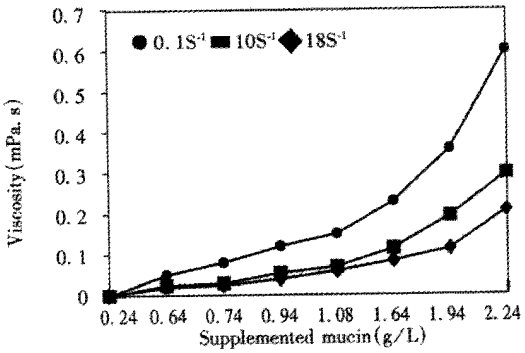

Figure 2 Increase of viscosity at 3 different shear rates including the prestatic region after supplementation of gallbladder bile with purified porcine mucin.

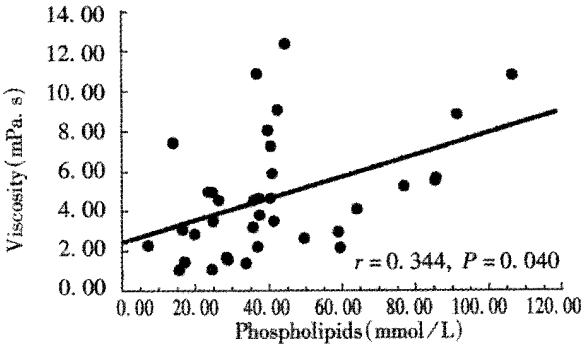

Figure 3 Correlation of viscosity and phospholipid concentration in 36 gallbladder biles from patients with gallstones.

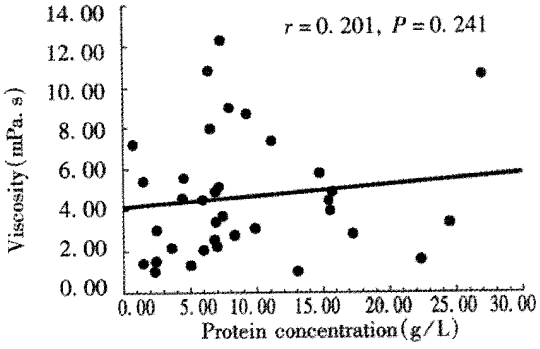

Figure 4 Correlation of viscosity and total protein concentration in 36 gallbladder biles from patients with gallstones.

Table 1 Cholesterol, phospholipids, bile acids, total lipids, CSI, protein, mucin, viscosity (mean ± SEM) and crystal observation time (median and range) in gallbladder bile of patients with gallstones and in hepatic bile

	Cholesterol stones (n = 28)	Mixed stones (n = 8)	Hepatic bile (n = 6)
Cholesterol (mmol / L)	21.1 ± 2.2	12.8 ± 1.7	1.9 ± 1.1
Phospholipids (mmol / L)	46.0 ± 4.7	26.6 ± 4.7	3.4 ± 1.3
Bile acids (mmol / L)	141.2 ± 15.1	65.9 ± 7.7	13.7 ± 7.4
Total lipids (g / dL)	11.3 ± 1.1	5.7 ± 0.7	1.0 ± 0.5
CSI	1.43 ± 0.09	1.79 ± 0.34	2.61 ± 1.68
Total protein (g / L)	10.4 ± 1.8	6.2 ± 0.9	2.8 ± 1.6
Mucin (g / L)	0.85 ± 0.12	0.39 ± 0.12	0.08 ± 0.04
Viscosity (mPa·s)	5.00 ± 0.60	3.50 ± 0.68	0.92 ± 0.06
Crystal observation time (days)	2.0 (1-13)	2.5 (1-14)	>21 (>21)

Table 2 Correlation coefficients between viscosity and mucin, protein, lipids and CSI in gallbladder bile of 36 patients with cholesterol or mixed gallstones

Viscosity (mPa·s)	Correlation coefficient (r)	Significance level
Mucin (g / L)	0.645	P<0.001
Total protein (g / L)	0.201	P = 0.24
Total lipids (g / dL)	0.238	P = 0.16
Cholesterol (mmol / L)	0.223	P = 0.19
Phospholipids (mmol / L)	0.344	P = 0.04
Bile acids (mmol / L)	0.161	P = 0.34
CSI	-0.141	P = 0.41

DISCUSSION

The aim of this study is to elucidate the relationship between viscosity and the main constituents of gallbladder and hepatic bile of patients with gallstones.

A major finding of our study is that mucin concentration is positively correlated to the viscosity of gallbladder bile. Similar results were obtained by Shoda *et al*[11], using a capillary viscosimeter, who determined a positive correlation between biliary viscosity and hexosamine concentration of gallbladder bile. In contrast to this report, we did not find a significant correlation between viscosity and the total protein concentration in gallbladder bile of patients with gallstones. These discrepancies might be explained by the different methods of protein determination in both studies. As stated above, the fluorescamine method used in the study of Shoda *et al*. might underestimate the total amount of protein in bile. The use of capillary viscosimeter instead of rotation viscosimetry in the study of Shoda is unlikely to explain the different findings, since bile behaves over a wide range of shear rates like a Newtonian fluid.

A further finding of our study was the positive correlation between phospholipids and viscosity in gallbladder bile. This correlation might be affected by the degree of concentration of gallbladder bile, although the other lipid components bile acids and cholesterol were not positively correlated to the viscosity. Furthermore, higher ratios of phospholipid to bile acids would favor the development of higher molecular weight micelles or vesicles which could favor a higher viscosity of bile. Although further studies have measured bile viscosity in patients with biliary drainage[28,29], the number of studies concerning gallbladder bile is very limited. This is astonishing, since an increased viscosity may play an important role in the formation of cholesterol crystals in gallbladder bile. According to Poiseuille's law bile flux through the cystic duct is inversely correlated to bile viscosity. Thus, increases in bile viscosity may lower the emptying of the gallbladder, thereby allowing more time for cholesterol crystal growth. Mucin has been shown to hydrophobically bind cholesterol in vesicles, promoting nucleation of solid cholesterol monohydrate crystals[30-32] and may also contribute thereby to an increase of the viscosity of gallbladder bile.

We investigated further the relation between viscosity and mucin concentration in human bile by adding purified porcine stomach mucin (range between 0.24 and 2.24 g/L) to native gallbladder bile. A non-linear increase of viscosity with the addition of mucin at 3 different shear rates including the prestatic region was observed. Particularly, after reaching a mucin concentration above 2 g/L, a greater increase in viscosity was found. Cholesterol crystal growth was promoted by already physiological concentrations of bovine gallbladder mucin and appeared to be maximal at 4 g/L [33]. Very high mucin concentrations (up to 20 g/L) have been found in mucin gels and sludge[34] and it has been proposed that crystal growth occurs within the mucin gel that is freqently seen before gallstone formation[2]. The concomitant increase in viscosity may contribute further to the retention of crystals in the gel phase of biliary mucin.

In summary, the most relevant finding of this study suggests that the concentration of mucin is the major determinant of biliary viscosity. Thus, an increased secretion of mucin by the gallbladder epithelium might contribute to a vicious cycle by inhibiting the emptying of the gallbladder and by this mechanism favoring the formation of gallstones.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Clinical characteristics and outcome of a cohort of 101 patients with hepatocellular carcinoma

Christian Rabe[1], Tillmann Pilz[1], Christoph Klostermann[1], Marc Berna[1], Hans H. Schild[2], Tilman Sauerbruch[1] and Wolfgang H. Caselmann[1]

Subject headings carcinoma, hepatocellular/etiology; carcinoma, hepatocellular/drug therapy; liver neoplasms/etiology; liver neoplasms/drug therapy; hepatitis B/complications; hepatitis C/complications; prognosis; cohort analysis; survival rate

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Abstract

AIM To conduct a cohort study of 101 patients with hepatocellular carcinoma (HCC) presenting to a tertiary care medical referral center in Germany between 1997 and 1999.

METHODS AND RESULTS Data were retrospectively analyzed by chart review. In 95 cases (72 males and 23 females) sufficient data were available for analysis. Twenty five (29%) of 85 patients were HBsAg or anti HBc positive, 21/85 (25%) were anti HCV positive, and 6/85 (7%) were positive for both HBV and HCV-markers. Age was significantly lower in HBV positive patients than in the other two groups. Thirty one (34%) of 90 patients had histories of alcohol abuse. In 79/94 (84%) patients, cirrhosis was diagnosed. Of these cirrhotic patients, 29/79 (37%) belonged to Child Pugh's group (CHILD) A, 32/79 (40%) to CHILD B, and 18/79 (23%) to CHILD C. AFP was elevated in 61/91 (67%) patients. A single tumor nodule was found in 38/94 (40%), more than one nodule in 31/94 (34%), and 25/94 (26%) had a diffusely infiltrating tumor, i.e. the tumor margins could not be seen on imaging procedures. Portal vein thrombosis was present in 19/94 (20%). Imaging data consistent with lymph node metastases were found in 10/92 (11%), while distant metastases were found in 8/93 (9%). According to Okuda 28/94 (30%) were grouped to stage I, 53/94 (56%) were grouped to stage II, and 13/94 (14%) were grouped to stage III. Survival data were available for 83 patients. The Kaplan-Meier estimate for median survival was 84 months. Factors influencing survival were the Okuda score, the presence of portal vein thrombosis, and the presence of ascites. The presence of non complicated liver cirrhosis by itself, distant metastases, or infection with hepatitis viruses did not influence survival. AFP positivity by itself did not influence survival, though patients with an AFP value greater than 100 μg/L did experience shortened survival. Treatment besides tamoxifen or supportive care was associated with prolonged survival. The influence of therapy on survival was most pronounced in Okuda stage II patients. There was longer survival in those Okuda stage II patients who were treated with percutaneous ethanol injection.

CONCLUSION Even in a low incidence area such as Germany, the majority of HCC is caused by viral hepatitis and therefore potentially preventable. Reflecting the high proportion of advanced stage tumors in our patients, the median survival was poor. Patients who received active therapy had a longer survival.

Department of Medicine I1, Department of Radiology2, University of Bonn, Germany

Christian Rabe, MD, graduated in 1992 from Hannover Medical School and has since trained at the Universities of Munich, Regensburg, and Bonn. He spent two years at the M. D. Anderson Cancer Center, Houston, as a DFG-research fellow and during medical school spent one year on a DAAD-scholarship in the Department of Pediatric Gastroenterology and Hepatology at the University of Chicago. Research interests include hepatocellular carcinoma and gene therapy approaches to hepatocellular carcinoma. He currently serves as editorial manager of the German center of the World Digestology Network.

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Correspondence to: Prof. Wolfgang H. Caselmann, Sigmund-Freud Str. 25, D-53105 Bonn, Germany
Tel. +49-228-287 5511, Fax. +49-228-287 4698
Email Caselmann@uni-bonn.de
Website www.bio-interactive.de/MolTher/index.html

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INTRODUCTION

Hepatocellular carcinoma (HCC) was responsible for the death of an estimated 609 000 individuals 1998 representing 1.1% of total mortality in WHO member states[1]. The incidence varies with geographic[1] area since factors predisposing to HCC development are unevenly distributed across continents and sometimes even within countries or within populations[2,3].

The incidence of HCC is clearly linked to the rate of chronic HBV infection[4,5]. A large percentage of HCC patients show serological or histological markers of past contact with HBV[3]. Prospective studies have demonstrated that men

chronically infected with HBV and expressing hepatitis B surface antigen (HBsAg) are about 70-fold more likely to develop HCC than their seronegative counterparts. Furthermore, even HBsAg-negative patients often exhibit serologic evidence, such as anti-HBc positivity, of past HBV-infection. Molecular mechanisms that account for that association are interaction of HBVproteins with signal transduction pathways, apoptosis, transcriptional machinery, and DNA repair[6]. Likewise, HCV infection predisposes to HCC[7]. While the molecular mechanisms of this association are not well understood at present, HCV proteins seem to interact with functions of the host cell in ways partially comparable to HBV proteins[8-12]. Examples include the capacity of HCV proteins for transactivation and their influence on apoptosis. The main difference between HCV and HBV related hepatocarcinogenesis is the lack of genomic integration of HCV sequences. It is interesting, however, that HCV frequently produces HCC only after liver cirrhosis has developed. This and the acquisition of HCV at a more advanced age may partially explain the older age at diagnosis and worse liver function compared to HBV-related liver cancer[13,14]. Therefore, HCC might be prevented by early antiviral therapy[15-17]. Besides hepatitis virus infection is associated with a large number of cases of HCC[18-23], liver cirrhosis itself regardless of etiology is a predisposing factor for HCC development[24,25]. In fact, most cirrhotics in Japan die from HCC[26]. Certain industrial[27], behavioral [28], or familial factors[29,30] also contribute to HCC development.

HCC can be cured by liver transplantation or liver resection[31-33]. However, due to the multifocal nature of the disease as well as coexisting conditions, only a small minority of patients can be treated surgically. This proportion of surgically treatable patients is likely to increase after institution of screening programs [34,35] that are based on the recognition of high risk groups[36] for the development of HCC. This is very important as the outcome of non-treatable patients remains poor[37,38].

While there is much data on the clinical characteristics and the outcome of patients with HCC in high risk countries in which the majority of HCC are viral hepatitis and/or aflatoxin-related[20-22,39], we know little about predisposing factors, clinical characteristics, and factors influencing outcome in low incidence areas such as northern Europe. Some of the data are contradictory with regard to factors predisposing to HCC development[18,40-43]. As incidence of HCC in many low-risk areas is rising albeit with local differences[44], presumably due to an increase in hepatitis virus-related liver cancer[45,46] and possibly rising rates of HCC development in women[47,48], an analysis of the present situation is needed to serve as a baseline for future refinements. The present study summarizes the data on a cohort of patients admitted to a hepatology unit at a tertiary medical referral center in Germany.

PATIENTS AND METHODS

Our institution is an academic tertiary care center located in the western part of Germany that receives the majority of patients as referrals from other hospitals. Using computer based searching for patients that presented for the first time with a diagnosis of hepatocellular carcinoma between 1997-1999, we identified 101 patients. Their charts were retrospectively reviewed. In 95 patients enough data were available for comprehensive analysis. These cases were further analyzed.

Diagnosis of HCC was made either by biopsy, postmortem examination or by diagnosing a new liver lesion in the proper clinical context in the absence of a demonstrable extrahepatic tumor and observing the clinical course. Liver cirrhosis was diagnosed by histology or by clinical evidence (signs of portal hypertension on endoscopic examination or characteristic imaging features on ultrasound examination).

A diagnosis of alcohol abuse was made using the criteria of Kubicka *et al*[40]. In brief, alcohol abuse was defined as a chronic alcohol consumption of more than 60 g/day for both sexes or when a history of alcohol abuse was noted in the patients' records.

Anti-HCV, HBsAg, Anti-HBc were measured using commercially available 2nd/3rd generation ELISA-based assays (Abbott Laboratories, Wiesbaden, Germany). Ultrasound examinations, CT scans, and chest X-rays were analyzed for the number of lesions, the size of the largest lesion, the approximate amount of liver volume taken up by the tumor, and the presence of extrahepatic lesions. The Okuda-score was calculated from imaging results and laboratory parameters. Patient survival was recorded by contacting the patients' primary care physician by mail or telephone.

For statistical analysis data were described by median and range. The number of patients with a specific trait was listed along with the number of patients for whom enough data was available to asses the absence or presence of this trait. Comparisons of continuous data between groups were performed using the Mann-Whitney-U-test (two groups) or the Kruskal-Wallis-test (more than two groups). Relationship between groups of nominal/ordinal data were explored using the chi-squared test, if necessary in its modification as the Fisher's exact test. To analyze survival data Kaplan-Meier-analysis was performed and survival differences between groups tested using the Log-rank-test. The Kaplan-Meier estimate for median or mean survival was reported depending on patient number.

RESULTS

Patient characteristics

Patient characteristics are summarized in Tables 1 and 2. Median patient age was 63 years (Range: 31-85 years) for men and 67 years (Range 42-83 years) for women. There was no statistically significant difference in age between men and women (U-test). The three most frequent concomitant diseases were diabetes mellitus in 32/95 (34%), arterial hypertension in 16/95 (17%) and nephrolithiasis in 6/95 (6%) patients. Apart from nephrolithiasis that was only diagnosed in men there was no predominance of one sex in these associated conditions. There was no difference in body mass index between those patients who suffered from diabetes and those who did not have diabetes. 24/32 (75%) Patients with diabetes suffered from liver cirrhosis.

Table 1 Patient characteristics: risk factors for HCC

Variable	Number/Total	Percentage
Sex		
Male	72/95	76%
Female	23/95	24%
Markers of past or present HBV infection		
Yes	25/85	29%
No	60/85	71%
Markers of HCV infection		
Yes	21/85	25%
No	64/85	75%
Markers of both HBV and HCV-infection		
Yes	6/85	7%
No	79/85	93%
History of alcoholism		
Yes	31/90	34%
No	59/90	66%
Cirrhosis		
Yes	79/94	84%
No	15/94	16%
Child score (only cirrhotics)		
A	29/79	37%
B	32/79	40%
C	18/79	23%
AFP elevated		
Yes	61/91	67%
No	30/91	33%

Table 2 Patient characteristics: age and clinical chemistry results

Variable	Median (Range)
Age	64 (31-85) years
GPT	32 (10-244) U/L
AP	214 (2-1490) U/L
Bilirubin	1.5 (0.4-14.4) mg/dL
Prothrombin time acc. to Quick	83 (25-122)%
Albumen	3.4 (1.9-4.6) g/dL
AFP	38 (0.9-831000) μg/L

Specific predisposing factors to HCC development

Hepatitis viruses Twenty-five/85 (29%) of HCC patients had markers of past or present HBV infection. Another 21/85 (25%) were infected by the hepatitis C virus (HCV), while 6/85 (7%) of patients had markers of infection with both viruses. There was a significant difference in age in patients with HBV, HCV, HBV/HCV coinfection, and those without hepatitis ($P<0.01$, Kruskal-Wallis-test). The median age (55 years, range 31-76 years) of patients with markers of HBV-infection was more than ten years lower than the median ages of patients without hepatitis (66, range 42-82 years), with HCV-infection (66, range 38-83 years), or with HBV/HCV-coinfection (69, 57-75 years). This is depicted in Figure 1. Interestingly, only 1/21 patients with HCV infection and none of the patients with HCV/HBV coinfection did not suffer from liver cirrhosis, while 2/24 patients with HBV-infection and 7/33 patients without hepatitis did not have cirrhosis.

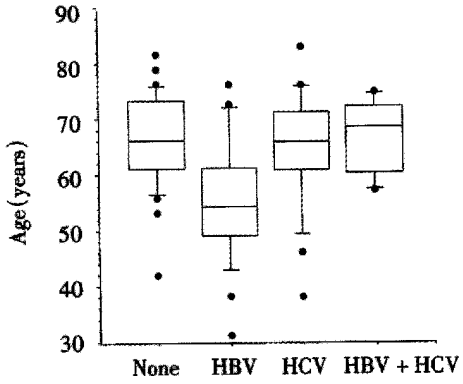

Figure 1 Box plots showing the patients' age according to hepatitis status. Patients with HBV-infections have a lower median age compared to patients with no hepatitis (None, n = 33), HBV-infection (HBV, *n* = 25), HCV Infection (HCV, *n* = 21), or HBV/HCV co-infection (HBV+HCV, *n* = 6).

Alcoholism Thirty-one/90 (34%) of HCC patients in our cohort were alcoholics. Age of alcoholics was not statistically different than that of patients with HCC from other causes ($P>0.05$, U test). Comparing the presence of alcoholism with the presence of viral markers, there was no apparent positive correlation between the presence of alcoholism and presence of markers indicating past or present HBV or HCV-infection. Indeed, only 9/81 patients were both alcoholics and had markers of hepatitis B or C, while 21/81 patients were alcoholics without markers of hepatitis and 39/81 patients had hepatitis but no alcoholism. 12/81 patients suffered neither from hepatitis nor alcoholism. Therefore it seems likely that a negative association between hepatitis and alcoholism exists in our cohort. This is confirmed by a borderline significant Fisher's exact test ($P<0.05$) for this inverse relationship.

Cirrhosis Seventy-nine/94 (84%) of HCC patients suffered from liver cirrhosis. The biggest percentage (40%) of these patients could be classified as CHILD-Pugh B, while 37% and 23% could be classified as CHILD A and C respectively. Age of cirrhotics was identical to that of non-cirrhotic patients ($P>0.05$, U test).

Tumor characteristics Tumor characteristics are listed in Table 3. 61/91 (67%) of patients had a positive AFP-test. The diameter of the largest tumor nodule in AFP positive tumors was significantly larger ($P<0.05$, *U*-test) than that of AFP negative tumors. A single tumor nodule was observed in 38/94 patients, while the remaining tumors were multinodular, with a diffuse growth pattern in 25/94. 20/89 tumors were smaller than 3 cm, 31/89 were between 3 and 5 cm, and 38/89 were larger than 5 cm. An involvement of both liver lobes was noted in 49/93 cases. In 19/94 cases an associated portal vein thrombosis was diagnosed. Ascites was seen during ultrasound in 43/95 patients. It was not possible to further classify ascites as malignant or due to liver cirrhosis retrospectively. Lymph node metastases were seen in 10/92 patients, while distant metastases (mainly lung metastases) were diagnosed in 8/93 patients.

Table 3 Tumor characteristics during initial presentation

Variable	Number / Total	Percentage
Type of tumor growth		
One nodule	38 / 94	40%
>1 Nodule	31 / 94	34%
Diffuse	25 / 94	26%
Size of largest nodule		
<3 cm	20 / 89	22%
3 cm-5 cm	31 / 89	35%
>5 cm	38 / 89	43%
Involvement of both liver lobes		
Yes	49 / 93	53%
No	44 / 93	47%
Portal vein thrombosis		
Yes	19 / 94	20%
No	75 / 94	80%
Lymph node metastases		
Yes	10 / 92	11%
No	82 / 92	89%
Distant metastases		
Yes	8 / 93	9%
No	85 / 93	91%
Presence of ascites		
Yes	43 / 95	45%
No	52 / 95	55%
Tumor stage according to Okuda		
I	28 / 94	30%
II	53 / 94	56%
III	13 / 94	14%

Outcome and factors influencing outcome

Survival data were available for 83 patients. The Kaplan-Meier estimate for median survival for all patients was 8.4 months. Survival curves according to Okuda stage are shown in Figure 2. There is a highly significant ($P<0.001$, Log-rank-test) relationship between tumor stage according to Okuda and patient survival. Kaplan-Meier-estimates for mean survival were 13.8 months for Okuda stage I, 9 months for Okuda stage II, and 1 month for Okuda stage III. By survival analysis other single factors influencing survival could be demonstrated: A significant ($P<0.02$, Log-rank test) influence on survival could be shown for the presence of portal vein thrombosis. The Kaplan-Meier estimate for mean survival in the absence of portal vein thrombosis was 10 months while the estimate for mean survival in the presence of portal vein thrombosis was 2.4 months. Likewise, patient survival was significantly shorter ($P<0.004$, Log-rank test) if ascites was present. The Kaplan-Meier estimates for median survival were 13 months in the absence and 3.8 months in the presence of ascites (Figure 3). The CHILD score was significantly correlated with survival of cirrhotic patients: CHILD A patients ($n = 27$) survived a median time of 14 months, CHILD B patients ($n = 29$) survived a median time of 6.6 months, while CHILD C patients ($n = 13$) survived a median time of 0.7 months. This influence of CHILD stage on survival was significant ($P<0.01$, Log-rank-test). CHILD stage was significantly correlated to Okuda score ($P<0.01$, Chi-squared test).

No difference in survival could be found for patients with or without hepatitis ($P = 0.8$, Log-rank test), AFP-positive versus AFP-negative patients ($P = 0.11$, Log-rank test), the presence of distant metastases ($P = 0.22$, Log-rank test), and for the presence or absence of liver cirrhosis ($P = 0.95$, Log-rank test).

While AFP positivity by itself was not associated with lower survival, AFP-levels greater than 100 ug/L ($n = 33$) were associated with a median survival of 3 months, while lower AFP-levels ($n = 46$) were associated with a survival of 12.5 months ($P<0.01$, Log-rank test).

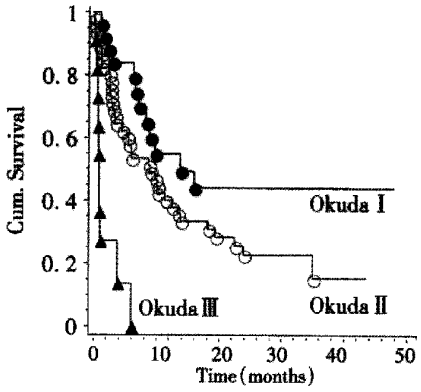

Figure 2 Kaplan-Meier-analysis of patient survival according to Okuda stage. Significant differences between groups ($P<0.001$, Log-rank test) can be seen.

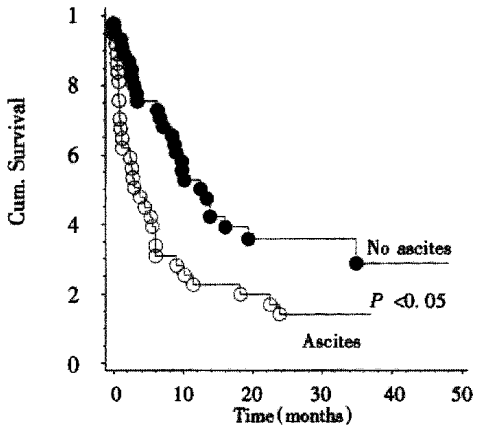

Figure 3 Kaplan-Meier-analysis of survival in relation to the presence or absence of ascites. Treated patients (open circles) lived significantly longer ($P<0.05$, Log-rank test) than their untreated counterparts (filled circles).

To investigate the influence of therapy on survival, the patients were divided in a group that received active therapy (17 percutaneous ethanol injection, 8 transarterial chemoembolisation, 11-I-131-Lipiodol administration, 2 resection, 2 octreotide for more than 4 weeks) and a group that received either supportive care (n = 36) or tamoxifen treatment (n = 13), that has recently been shown to yield no survival benefit compared to placebo. These patients were then stratified according to Okuda score. Only Okuda stage I or II patients were included. When survival was compared between both stratified groups overall there were significant differences (P<0.04, log-rank test) in survival between treated and untreated patients. For Okuda stage I the Kaplan Meier estimate for mean survival in the treatment group (n = 13) was 9 months, while the estimate for mean survival in the untreated group (n = 12) was 12 months. For Okuda stage II the Kaplan-Meier estimate for mean survival for the treatment group (n = 19) was 19 months while the estimate for mean survival for the untreated group (n = 27) was 7 months (Figure 4). While the use of percutaneous ethanol injection was not significantly related to increased survival overall(P = 0.18, Log-rank test); in Okuda stage II tumors there was better survival for patients receiving this treatment modality (P<0.02, Log-rank test). When only patients were analyzed who received either supportive therapy (n = 36) or tamoxifen treatment (n = 13), no difference for survival times could be found between tamoxifen treated patients and those who received supportive therapy.

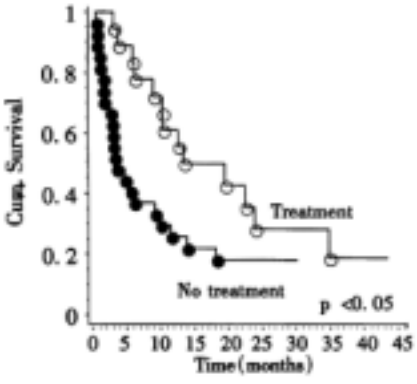

Figure 4 Kaplan-Meier-analysis of Okuda stage II patients in relationship to treatment. Patients with ascites (open circles) lived for a significantly shorter (P<0.05, Log-rank test) time than their counterparts without ascites (filled circles).

DISCUSSION

Patient characteristics

The age and sex distribution of our patient cohort closely reflected published data from other series. Interestingly, there was a high prevalence of diabetes mellitus in our patients. Other studies on HCC also reported a high prevalence of diabetes mellitus[49-51]. The body mass index of diabetic patients was similar to that of their non-diabetic counterparts as was the number of subjects with liver cirrhosis. The high prevalence of diabetes mellitus in our series can be interpreted as a consequence of the high prevalence of liver cirrhosis in HCC patients overall. Cirrhosis is associated with impaired glucose tolerance and diabetes in 20%-30% of cases[52,53].

Predisposing factors to HCC development

The presence of liver cirrhosis in 84% of our patients is well within the range of cirrhosis rates reported in the literature (Table 4). All but three of the patients with viral hepatitis were cirrhotics. The other HCCs can most likely be explained by the high prevalence of alcoholism in our patients. As alcoholism was not associated with viral hepatitis in our patients, alcohol abuse may have been an independent risk factor for HCC development. Liver cirrhosis was fairly advanced in most of our HCC patients as evidenced by the high proportion of CHILD B and CHILD C liver cirrhosis.In our study 29% of HCC had evidence of past or present HBV-infection, 25% had evidence of HCV-infection, and 7% had evidence of both HBV and HCV-infections. This is comparable to infection rates in northern Europe and some more recent studies from southern Europe (Table 4) that show that a rising proportion of hepatocellular carcinoma is HCV related. This is similar to Asian countries where one can also observe a clear relationship of HCC and hepatitis B and C (Table 4). The lower age of HCC-patients with HBV infection compared to that of HCC-patients with HCV-infection and HCC-patients without HBV/HCV-infection or with HBV/HCV-coinfection may be explained by two hypotheses: Firstly, HBV may have been acquired earlier in life than HCV. Secondly, HBV might be a more potent oncogenic stimulus than HCV. There is some evidence to support this latter hypothesis: HBV has been identified as a risk factor for HCC in individuals that are not cirrhotic. This may mean that the many interactions of HBV proteins with the host cell[6] and especially the transactivating function of HBV proteins coded by integrated viral sequences[54-56] can cause cancer regardless of cirrhosis development. Indeed, two of our 24 patients with HBV infection did not have cirrhosis. The older age of HBV/HCV coinfected patients compared to patients with HBV monoinfection might also be related to specific modes of transmission that occur only at an older age. It is a very interesting observation that the majority of HCC even in a low-risk area for viral hepatitis such as Germany are related to viral hepatitis and, therefore, potentially preventable. An important

factor in preventing liver cancer in Germany will be an effective reduction or eradication of HBV infection through general vaccination programs. This approach has already proved successful in Asia. Another possibility of preventing HCC could be interferon treatment of HCV infection. Although there is still some controversy if this may prevent HCC development, it seems very likely that successful interferon therapy can prevent progression to liver cirrhosis and will therefore reduce HCC incidence. HCV-related HCC occurs following the development of liver cirrhosis in the majority of cases. In our patients there was only one patient with HCV infection but without liver cirrhosis.

Tumor characteristics

Two thirds of our patients had a positive AFP-test. This is similar to the results of other German studies[41,43] and illustrates that other tumor markers are needed to detect early HCC in AFP-negative cases. This is emphasized by significantly larger tumor nodules in AFP positive cases. Patients with large tumor nodules may not be good candidates for liver transplantation or resection, the only curative therapies available for HCC.

As detailed in Table 3, most of the tumors were in an advanced stage at presentation. 70% of tumors were Okuda stage II or III. Involvement of both liver lobes was predominant in this study. there was also a high rate of portal vein thrombosis. The tumor stages in our patients were more advanced than the tumor stages in other German series, in which the percentage of Okuda stage II and III tumors ranged from 43% to 61%[40,41,57].

It is surprising, however, that despite locally advanced tumors, we detected only a relatively small rate of nodal or extranodal metastases. This may indicate that extrahepatic tumor spread in HCC does not generally present a major problem although it may become highly relevant in individual cases especially after liver transplantation.

Table 4 Comparison of viral parameters with studies reported in the literature

Author	Number	Cirrhosis	HBV	HCV	HBV+HCV (%)	Country
Northern/Central Europe						
Van Roey	154	60	52	55	NA	Belgium
Widell	95	NA	29	17	NA	Sweden
Kubicka	268	74.6	35.1	26.9	10	Germany
Petry	100	89.5	22	37	0	Germany
Peters	86	90	49	37	NA	Germany
This study	95	84	29	25	7	Germany
Southern Europe						
Kuper	333	NA	58	12	3	Greece
Stroffolini	1148	NA	12	71	5	Italy
Asia						
Kubo	330	NA	17	26	53	Japan
Shiratori	205	NA	11	71	13	Japan
Zhang	113	NA	63	11	NA	China
Chuang	128	NA	77	20	NA	China

Outcome and factors influencing outcome

Overall outcome was poor. Reflecting the large number of advanced tumors and the minuscule number of surgically treated patients in our study, the Kaplan-Meier estimate for median survival was only 8.4 months. This is similar to German results reported by Petry *et al*[41] and within the scope of survival results for medically treated patients reported by other authors[38,58]. Other authors had also observed the clear-cut relationship of survival to the Okuda stage of the tumor.

AFP positivity was not related to survival. However, when only tumors with AFP levels greater than 100 ug/L were compared to tumors with lower or negative AFP levels, survival was shortened for the patients with the higher AFP levels. This may reflect a larger tumor burden in the patients with higher AFP levels as evidenced by significantly larger tumor nodules in AFP positive patients. This relationship of decreased survival in patients with an AFP level greater than 100 ug/L had also been described by others[57]. The relation of CHILD stage to survival was expected since the CHILD stage reflects some of the same variables that are measured by the Okuda score. The disappointing survival of CHILD C cirrhotics with HCC (median survival of less than one month) might suggest that these patients are not treated but offered symptom-targeted supportive care. However, this has not been tested prospectively.

Comparing survival of tamoxifen treated patients to patients receiving supportive therapy, there was no survival benefit for tamoxifen treated patients. This matches results from prospective studies[59,60] that proved tamoxifen to be ineffective in HCC. Therapy other than tamoxifen was related to increased survival. As small tumors in cirrhosis have a relatively good prognosis[61], it was therefore important to assess the influence of therapy on survival without the confounding effect of Okuda stage. The analysis of treatment groups stratified by Okuda stage confirmed that treated Okuda stage II patients survived longer. Ethanol injection (PEI) was associated with improved survival in Okuda stage II patients. A favorable impact of PEI on survival was previously reported in the literature[62].

One has to be cautious in interpreting retrospective data such as ours. It may be possible that patients were offered therapy because they were in a better clinical state, even though their Okuda stages were identical. Prospective studies must address the effectiveness of therapy in HCC.

As HCC is mostly a problem confined to the liver, with most patients dying from liver related complications such as liver failure or variceal bleeding, an effective approach to HCC treatment may consequently be any therapy aimed at local tumor control such as PEI, high-frequency

thermocoagulation, or laser induced thermocoagulation[63,64] to prevent portal vein thrombosis and parenchymal displacement.

CONCLUSIONS

Our data show the majority of HCC seen at our institution to be related to viral hepatitis. Therefore, to reduce the incidence of HCC, the prevalence of viral hepatitis must be controlled through vaccination programs and possibly through antiviral treatment. As only about two thirds of HCC were AFP positive, additional tumor markers must be sought to identify patients with HCC early in their disease when they might still benefit from curative surgical interventions. Survival was poor, but therapy (in our series mostly TACE, ethanol injection, and ^{131}I-Lipiodol treatment) was associated with improved survival.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Preparation and activity of conjugate of monoclonal antibody HAb18 against hepatoma F (ab')$_2$ fragment and staphylococcal enterotoxin A Lian

Jun Yang, Yan Fang Sui and Zhi Nan Chen

Subject headings carcinoma, hepatocellular/immunology; liver neoplasms/immunology;superantigens; enterotoxins; antibodies, monoclonal

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Abstract

AIM To prepare the conjugate of staphylococcal enterotoxin A (SEA) protein which is a bacterial SAg and the F(ab')2 fragment of mAb HAb18 against human hepatocellular carcinoma (HCC), and identify its activity in order to use SAg in the targeting therapy of HCC.

METHODS MAb HAb18 was extracted from the abdominal dropsy of Balb/c mice, and was purified through chromatography column SP 40HR with Fast protein liquid chromatography (FPLC) system. The F(ab')$_2$ fragment of mAb HAb18 was prepared by papainic digestion method. The conjugate of mAb HAb18 F(ab')$_2$ fragment and SEA was prepared with chemical conjugating reagent N succinimidyl 3 (2pyridyldithio) propionate (SPDP) and purified through chromatography column Superose 12 with FPLC system. The molecular mass and purity of each collected peak were identified with SDS-PAGE assay. The protein content was assayed by Lowry's method. The antibody activity of HAb18 F(ab')$_2$ against HCC in the conjugate was identified by indirect immunocytochemical ABC method, and the activity of SEA in the conjugate to activate peripheral blood mononuclear cells (PBMC) was identified with MTT assay.

RESULTS The IgG mAb HAb18 was extracted, and purified successfully. Immunocytochemical staining demonstrated that it reacted with most of HHCC cells of human HCC cell line. There were two peaks in the process of purification of the prepared HAb18 F(ab')$_2$ SEA conjugate. SDS-PAGE assay demonstrated that the molecular mass of the first peak was about 130 ku, and the second peak was the mixture of about 45 ku and a little 100 ku proteins. The immunocytochemical staining was similar in HAb18 F(ab')$_2$ SEA conjugate and HAb18 F(ab')$_2$, i.e.the cytoplasm and/or cell membranes of most HHCC cells were positively stained. The MTT assay showed that the optical absorbance (A) value at 490 nm of HAb18 F(ab')$_2$ SEA conjugate was 0.182 ± 0.012, that of negative control was 0.033 ± 0.009, and there was significant difference between them ($P<0.05$).

CONCLUSION SPDP is a good protein conjugating reagent and can be used in preparing protein conjugate. The conjugate of mAb HAb18 F(ab')$_2$ fragment and SEA protein was prepared successfully in present study and can be used in the experimental study of HCC targeting therapy with the conjugate of SAg and anti HCC mAbs or their fragments.

INTRODUCTION

Superantigen (SAg) is a group of proteins which can conjugate with some Vβ fragments of the α or β region of T lymphocytic receptor (TcR) when submitted by major histocompatibility complex (MHC) II molecules. SAg can activate T lymphocytes efficiently and make them release a lot of cytokines[1-5]. SAg has great potential anti-tumor effect, but single SAg has some severe side effects to normal cells expressing MHCII molecules[6]. Using the conjugates of SAg and anti-tumor monoclonal antibody (mAb) or their fusion proteins to treat tumor is a focus in tumor targeting therapy research currently[7-21]. There has been no report about anti-hepatoma with mAb-SAg conjugate until now. The conjugate of SAg - Staphylococcal enterotoxin- A (SEA) and the F (ab')$_2$ fragment of mAb HAb18 against human hepatocellular carcinoma (HCC) was prepared with heterotype bifunctional conjugating reagent N-succinimidyl-3-(2-pyridyldithio) propionate

Department of Pathology, Fourth Military Medical University, Xi£§an 710032, Shaanxi Province, China
Dr. Lian Jun Yang, graduated from the Fourth Military Medical University as a Bachelor of Medicine in 1993, as a Master of Medicine in 1998, and as a M.D. in 2001. Now he is a lecturer in the Fourth Military Medical University, majoring the molecular pathology of tumors.
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Correspondence to: Dr. Lian Jun Yang, Department of Pathology, Fourth Military Medical University, Xi£§an 710032, Shaanxi Province, China
Tel. 0086-29-3374541 Ext.115
Email. yangsi@sina.com
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(SPDP), and the activities of the F(ab')$_2$ fragment of mAb HAb18 against HCC and SEA's activating peripheral blood mononuclear cells (PBMC) in the conjugate of HAb18 F(ab')$_2$ and SEA was identified in this study, laying an experimental base for the research of HCC targeting therapy with mAb-SAg conjugate.

MATERIALS AND METHODS

Cells, animals and main reagents

The hybridoma cells which secrete mAb HAb18 against HCC-associated antigens was established by our department[22]. This hybridoma was prepared by taking the cell suspension of some fresh human HCC tissue as antigen, and the cells were fused according to normal procedures for mAb preparation. Human HCC cell line HHCC was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Balb/c mice (body weight of 20 g-25 g) that were used to prepare the abdominal dropsy of mAb HAb18 were provided by the Experimental Animals Center of our university. Papain, mitosis promoter PHA, Methabenzthiazuron (MTT) and dithiothreitol (DTT) were the products of Serva Co. (USA). SPDP was synthesized and kindly provided by Prof. Wen Xue Wang, Department of Toxicology of our university. SEA protein and rabbit mAb against the F(ab')$_2$ fragment of mouse IgG were purchased from the Academy of Military Medical Sciences (Beijing, China). Immunohistochemical ABC kit was the product of Vector Laboratories Inc. (USA). Dimethyl benzidine (DAB) and dimethyl sulfoxide (DMSO) were the products of Sigma Chemical Co. (USA).

Extract of mAb HAb18 and preparation of its F (ab')$_2$ fragment

Extract and purification of mAb HAb18 The hybridoma cells which secrete mAb HAb18 (its immunoglobulin type is IgG), was cloned and selected again. The abdominal dropsy of the antibody was prepared by injecting 1×10^6 hybridoma cells into the cavum abdominis of every Balb/c mouse and collected at the aseptic environment. After pretreated with 0.01 mol·L^{-1} phosphate-buffered saline (PBS) (pH 7.5), the collected abdominal dropsy was purified through chromatography column SP-40HR (column volume was 2 cm × 18 cm, and velocity of flow was 10 mL·min^{-1}) with Fast protein liquid chromatography (FPLC) system (Waters 650E, Waters Co., USA), and the IgG part was collected. Then 227 g·L^{-1} solid ammonia sulfate was added at 4℃, agitated for 15-20 min, stood at 4℃ for more than 1 h, and centrifuged at 1 500 rpm for 5 min. The supernatant was aborted, while the deposit was dissolved in 0.01 mol·L-1 PBS (pH 7.4) and dialyzed with the same buffer for 12 h-20 h.

Identification of mAb HAb18 The antibody specialty of mAb HAb18 was identified by normal immunocytochemical ABC method. The procedures were as follows: ① HHCC cells were cultured in 6-well culture plates which contained some coverslips in RPMI1640 medium containing 150 mL·L^{-1} new born bovine serum at 37℃ until the cells grew into log phase; ② the cells were fixed in 950 mL·L^{-1} ethanol for 15 min; ③ 7.5mL·L^{-1} H_2O_2 was added and incubated at 37 ℃ for 10 min; ④ normal sheep serum was added and incubated at 37℃ for 30 min; ⑤ MAb HAb18 was added, while PBS was used as negative control, and incubated at 37℃ for 1h; ⑥ biotin-labeled sheep anti-mouse IgG mAb was added and incubated at 37℃ for 30 min; ⑦ ABC complex was added and incubated at 37℃ for 30 min; ⑧ the color was developed with DAB for 10-20 min; ⑨ the slides were counterstained with hematoxylin, dehydrated, cleared and mounted.

Preparation of the F(ab')$_2$ fragment of mAb HAb18

The F(ab')$_2$ fragment of mAb HAb18 was prepared by papainic digestion method. The procedures were: ① MAb HAb18 was dialyzed with 1 mol·L-1 NaAc (pH 5.5) for 12 h; ② papain was dissolved in NaAc buffer containing 3 mol·L-1 EDTA and 2 mol·L-1 DTT; ③ the dissolved papain whose mass was about 5% of the pre-digested mAb, was added into the mAb solution and incubated at 37℃ for 10 h; ④ the same concentration of papain was added again and incubated at 37℃ for 10 h-12 h; ⑤ 2 mmol·L-1 iodinate acetamide was added to terminate the reaction; ⑥ at last, the proteins were dialyzed with 0.01 mol·L-1 phosphate buffer (PB) (pH 7.5).

Preparation and purification of HAb18 F(ab')$_2$-SEA conjugate

SEA protein and mAb HAb18 F(ab')$_2$ purified through chromatography column with FPLC were respectively dissolved and dialyzed in 0.1 mol·L-1 PB (pH 7.5, containing 0.1 mol·L-1 NaCl). Then they reacted with SPDP, which was dissolved in DMSO and whose molecular value was twice that of HAb18 F(ab')$_2$ or SEA protein, for 30 min. SEA was dialyzed again in 0.1 mol·L-1 PB (pH 7.5, containing 0.1 mol·L-1 NaCl), and HAb18 F(ab')$_2$ was dialyzed again in 0.1 mol·L-1 PB (pH 4.6, containing 0.1 mol·L-1 NaCl). DTT whose final concentration was 50 m mol·L-1 was added into HAb18 F(ab')$_2$. Thirty min later, they were dialyzed in 0.1 mol·L^{-1} PBS (pH 7.5) in nitrogenous environment and at room temperature. Finally, the complex of SPDP and SEA was mixed with the reductive complex of HAb18 F(ab')$_2$ and SPDP, and they were stirred for 24 h. The conjugate was purified through chromatography Superose 12 column (column volume was 1 cm × 65 cm, and velocity of flow was 0.2 mL·min^{-1}) with FPLC system. The molecular

mass and purity of the protein of each collected peak were identified with SDS-PAGE assay (the concentration of separation gel was 120 $g \cdot L^{-1}$). The protein content was assayed by Lowry's method.

Identification of the activity of HAb18 F(ab')$_2$ in the conjugate

The antibody specialty of HAb18 F(ab')$_2$-SEA conjugate against HCC was identified by indirect immunocytochemical ABC method. The procedures were: ① HHCC cells were cultured in 6-well culture plates which contained some coverslips in RPMI1640 medium containing 150 $mL \cdot L^{-1}$ new born bovine serum at 37℃ until the cells grew into log phase; ② the cells were fixed in 950 $mL \cdot L^{-1}$ ethanol for 15 min; ③ 7.5 $mL \cdot L^{-1}$ H_2O_2 was added and incubated at 37℃ for 10 min; ④ normal sheep serum was added and incubated at 37℃ for 30 min; ⑤ HAb18 F(ab')$_2$-SEA conjugate was added, while HAb18 F(ab')$_2$ and PBS was respectively used as positive and negative controls, and incubated at 37℃ for 1 h; ⑥ rabbit anti-mouse IgG F(ab')$_2$ mAb was added and incubated at 37℃ for 30 min; ⑦ biotin-labeled sheep anti-rabbit IgG mAb was added and incubated at 37℃ for 30 min; ⑧ ABC complex was added and incubated at 37℃ for 30 min; ⑨ the color was developed with DAB for 10-20 min; ⑩ the slides were counterstained with hematoxylin, dehydrated, cleared and mounted.

Identification of the activity of SEA in the conjugate to activate PBMC

PBMC was extracted from fresh human peripheral blood that had been treated with anticoagulant reagent heparin by Ficoll's density gradient centrifugation method, and $2 \times 10^5 \cdot well^{-1}$ PBMC were added into 96-well flat-bottom culture plates. Then 0.1 $mg \cdot L^{-1}$ HAb18 F(ab')$_2$-SEA conjugate was added, while RPMI1640 medium containing 150 $mL \cdot L^{-1}$ new born bovine serum was used as negative control, 0.1 $g \cdot L^{-1}$ PHA and 0.02 $g \cdot L^{-1}$ SEA as positive controls. Six wells were used for each group of cells. They were cultured in CO_2 incubator at 37℃ for 60 h, then MTT (2 $g \cdot L^{-1}$, 50 $\mu L \cdot well^{-1}$) was added and incubated continually at 37℃ for 4 h. The medium was centrifuged and discarded, and 100 $\mu L \cdot well^{-1}$ DMSO was added. The culture plate was shaken for 10 min and the optical absorbance (*A*) value at 490 nm was detected with Bio-Rad ELISA instrument. The obtained data was statistically analyzed with Student's *t* test.

RESULTS

Extract and identification of mAb HAb18

After dialyzed, the abdominal dropsy of IgG mAb HAb18 was purified successfully with chromatography column SP-40HR (Figure 1). Immunocytochemical staining showed that the positive signal was brown, and located mainly within the cytoplasm and/or on the cell membranes. Most of the HHCC cells were positive. There was no detectable positive signal in negative control.

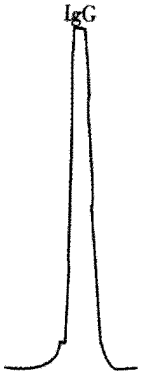

Figure 1 Chromatography for the purification of mAb HAb18.

Purification of HAb18 F(ab')$_2$-SEA conjugate

There were two peaks in the process of purification and elution of the prepared HAb18 F(ab')$_2$-SEA conjugate (Figure 2). SDS-PAGE assay demonstrated that the relative molecular mass of the first peak was about Mr. 130 and it was HAb18 F(ab')$_2$-SEA conjugate. The second peak was the complex of Fab whose relative molecular mass was about 45 and a little F(ab')$_2$ whose relative molecular mass was about 100 (Figure 3).

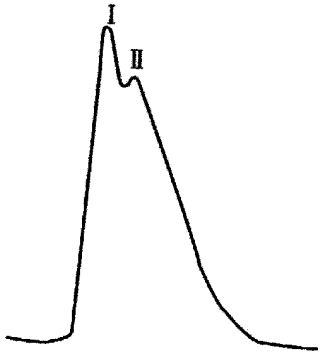

Figure 2 Chromatography for the purification of HAb18 F(ab')$_2$ SEA conjugate.

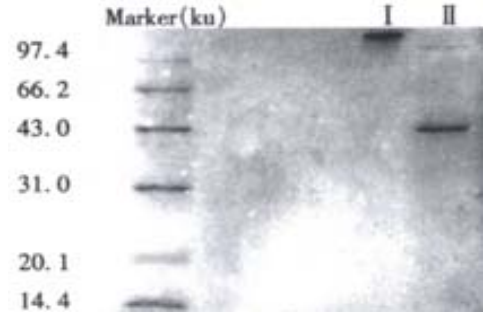

Figure 3 SDS-PAGE assay of the relative molecular mass of purified HAb18 F(ab')$_2$-SEA conjugate.

Identification of antibody activity of HAb18 F(ab')$_2$-SEA conjugate

The result of immunocytochemical staining was similar in HAb18 F(ab')$_2$-SEA conjugate and HAb18 F(ab')$_2$, i.e., the cytoplasm and/or cell membranes of most HHCC cells were positively stained, and no detectable positive signal was found in negative control (Figure 4).

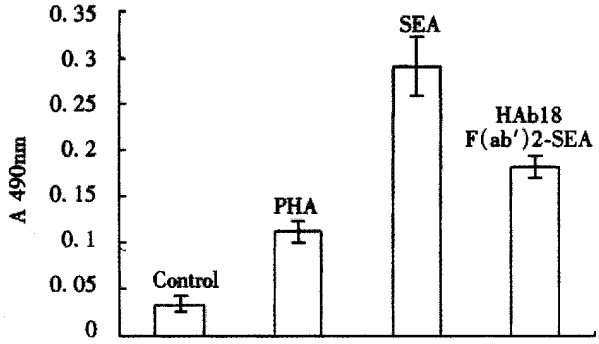

Figure 4 Distribution of HAb18 F(ab')$_2$ SEA conjugate in human hepatoma HHCC cells. ABC, ×400

Experimental observation on HAb18 F(ab')$_2$-SEA conjugate activating PBMC

The result of MTT assay showed that the *A* value at 490 nm of HAb18 F(ab')$_2$-SEA conjugate was 0.182 ± 0.012, those of PHA and SEA were respectively 0.112 ± 0.012 and 0.291 ± 0.032, that of negative control was 0.033 ± 0.009. The data of HAb18 F(ab')$_2$ SEA conjugate, PHA and SEA were all significantly higher than that of negative control (P<0.05, Figure 5).

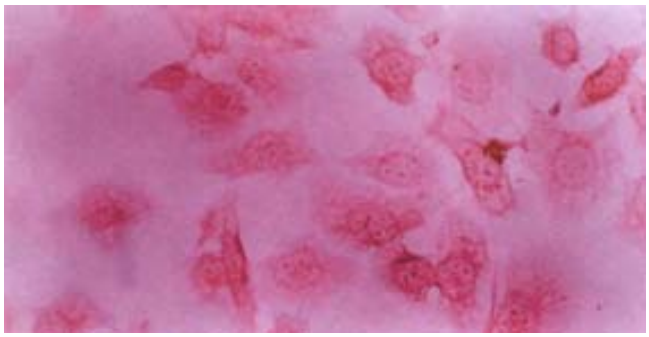

Figure 5 The MTT assay result of HAb18 F(ab')$_2$-SEA conjugate stimulating PBMC to proliferate.

DISCUSSION

HCC is a common malignant tumor, and there has been no effective treatment up to date[23,24]. Besides the 3 conventional therapeutics, i.e., surgical operation, chemotherapy and radiotherapy, targeting diagnosis and therapy of HCC with anti-HCC mAb have been studied extensively, giving a hopeful prospect to HCC treatment[25-36]. Targeting therapy is a common means of tumor immunotherapy, and is called "biological missile"[37-47]. The "warheads" of" "biological missiles" are usually radioactive nuclides, chemotherapeutants or toxins. Because of the radioactive pollution, "warheads" falling off and other side effects to normal tissue *in vivo*, the conjugate of mAb and radioactive nuclides, chemotherapeutants or toxins have limited effects and can not be used widely. SAg is a new "warhead" of "biological missiles". It can kill tumor cells through activating esoteric T lymphocytes, and make the activated T lymphocytes release much useful cytokines. The anti-tumor effect of mAb-SAg conjugate is significantly greater than that of bispecial mAb-mediated cytokines or mononuclear-microphage[8]. Dohlsten *et al*[7-9] have respectively prepared the chemical conjugates of SEA and anti-colon carcinoma mAb C215, C242, and the fusion proteins of SEA and the Fab fragment of mAb C215, C242 are prepared by gene engineering method. The cytotoxic experiment indicates that these conjugates or fusion proteins do not lose the ability of activating T lymphocytes. RT-PCR assay shows that SEA-activated monocytes express the mRNAs of many cytokines such as IL-1α, Il-1β, IL-2, IL-6, TNF-α, TNF-β and TNF-γ. Ihle *et al*[10] have prepared the fusion protein of SEA and staphylococcal protein A (SPA) which has a special affinity to IgG, and the conjugates of SEA and anti-CD7 mAb (IgG2a), anti-CD38 mAb (IgG1) respectively, and the cytotoxic experiment to MHCII negative acute T lymphocytic leukemia cells was performed. The results are excellent. The targeting treatment to severe combined immune deficiency (SCID) mice bearing intraperitoneally growing colon carcinoma colo205 cells using fusion protein SEA-C242 Fab shows that human T lymphocytes must be planted into SCID mice before treatment, indicating that SEA-C242 Fab needs to activate T lymphocytes to develop its anti-tumor effect[11]. Litton *et al*[12] have observed the reaction of tumors and their spherical tissues to fusion protein SEA-C242 with immunohistochemical staining and computer-assisted image analysis, and the results demonstrate that the T lymphocytes and monocytes concentrate in all the tumor tissues just several hours after the SEA-C242 Fab was injected. The main cytokines produced are TNF-α, IL-2, IL-4, IL-5, IL-10, IL-12, IFN-γ, GMSF and TGF-β. The authors consider that apoptosis in tumor cells happens because of the production of cytokines, T lymphocytes infiltration and CD95 (Fas) receptor expression. That is followed by obvious reduction of tumor mass, which is seen within 24 h after the SEA-C242 Fab infusion. In the previous studies of SAg treatment for tumors mAb-SAg conjugates or their fusion proteins were used. mAb-targeted SAg

was mainly used in the studies of anti-tumors including colon carcinoma, melanoma, lymphoma, neuroblastoma, etc. There have been some clinical reports about treating tumors with mAb-SEA conjugate[48,49]. Compared with complete mAb, the main advantages of mAb F(ab')$_2$ fragment are: ① Its volume is smaller, and easy to penetrate through tumor tissues; ② the human anti-mouse antibodies reaction is milder, and suitable for repeated treatments; ③ It does not conjugate with the Fc receptors on non-tumor cells, and can increase T/NT ratio *in vivo*. Therefore, in the present study, we used the F(ab')$_2$ fragment of mAb HAb18, but not intact mAb, and prepared the conjugate of the F(ab')$_2$ fragment of anti-HCC mAb HAb18 and SEA protein so as to use it in the experimental study of SAg anti-HCC in the future.

In the previous reports, the F(ab')$_2$ fragment of mAb is usually prepared by pepsic digestion method, and the papainic digestion method is mostly used to prepare the Fab fragment of mAb. The productive rate of preparing IgG fragments with papainic digestion is higher than those of other traditional methods and it is helpful in preserving the antibody activity. When an mAb is digested with papain, the F(ab')$_2$ fragment can be obtained at pH 5.5, and the Fab fragment can be obtained at pH 8.0[50]. The HAb18 F(ab')$_2$ prepared in this experiment contained a small amount of Fab, which can not be removed because of the limited conditions of purification. The second peak produced during purification of HAb18 F(ab')$_2$-SEA conjugate was the complex of HAb18 F(ab')$_2$ and Fab, but the amount of F(ab')$_2$ was significantly less than that of Fab. These results indicated that most F(ab')$_2$ had conjugated with SEA protein, whereas Fab seemed not prone to conjugate.

During the preparation of protein conjugate by SPDP method, the reagents with moderate reacting activity should be used, and the reacting conditions should be mild, such as at room temperature, in neutral pH value and water bath, so as to avoid denaturalization of proteins and loss of biological activity. SPDP is a commonly-used protein conjugating reagent. It can introduce sulfhydryl into protein molecules, and then make the protein molecules conjugate with other protein molecules with sulfhydryl exchange or sulfhydryl addition reactions. There are few side effects of this conjugating reaction, but SPDP also has some shortcomings, such as being prone to inactivate because of deliquescence, and there are many other influencing factors during the operating process. The results in present study suggested that the protein conjugate of mAb HAb18 F(ab')$_2$ and SEA can be prepared by SPDP method successfully, while the activity of proteins can be kept well.

Because the F(ab')$_2$ fragment of mAb HAb18 used in this study did not contain Fc fragment, mouse anti-human intact IgG antibodies can not react with it effectively, so the antibody activity of HAb18 F(ab')$_2$-SEA conjugate can not be identified by normal immunocytochemical method. In our experiment, the activity of mAb F(ab')$_2$ fragment in HAb18 F(ab')$_2$-SEA conjugate can be identified effectively by indirect immunocytochemical ABC method, in which rabbit anti-mouse IgG F(ab')$_2$ mAb was used as the second antibody.

SAg has a strong mitogenetic effect on PBMC because it can activate T lymphocytes effectively[51]. The A value of HAb18 F(ab')$_2$ SEA was significantly higher than that of negative control in this study ($P<0.05$), which indicated that the prepared HAb18 F(ab')$_2$ SEA conjugate had a significant effect on stimulating the proliferation of PBMC and can be used in the experimental study of HCC targeting therapy with mAb-SAg conjugate.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Combination of "low-dose" ribavirin and interferon alfa-2a therapy followed by interferon alfa-2a monotherapy in chronic HCV-infected non-responders and relapsers after interferon alfa-2a monotherapy

Perdita Wietzke-Braun, Volker Meier, Felix Braun and Giuliano Ramadori

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Abstract

AIM **To report on the efficacy, safety and tolerability of interferon alfa-2a combined with a "low dose" of ribavirin for relapsers and non-responders to alpha interferon monotherapy.**

METHODS **Thirty four chronic hepatitis C virus infected non responders to interferon alfa-2a monotherapy (a course of at least 3 months treatment) and 13 relapsers to interferon alfa-2a monotherapy (a dose of 3 to 6 million units three times per week for at least 20 weeks but not more than 18 months) were treated with the same dose of interferon alfa-2a used before (3 to 6 million units three times per week) and ribavirin (10 mg/kg daily) for 6 months. In complete responders, interferon alfa-2a was administered for further 6 months at the same dose used before as monotherapy.**

RESULTS **Seven (20.6%) of 34 non responders stopped the combined therapy due to adverse events, including two patients with histological and clinical Child A cirrhosis. In 17/27 (63%) non responders, the combined therapy was stopped after three months because of non response. Ten of the 27 non responders completed the 12 month treatment course. At a mean follow up of 28 months (16-37 months) after the treatment, 4/10 (15%) previous non responders still remained complete responders. All 13 previous relapsers completed the 12-month treatment course. At a mean follow up of 22 months (9-36 months) after treatment, 6/13 (46%) the previous relapsers were still sustained complete responders.**

CONCLUSION **Our treatment schedule of the combined therapy for 6 months of interferon alfa-2a with a low dose of ribavirin (10 mg/kg/day) followed by 6 months of interferon alfa-2a monotherapy is able to induce a sustained complete response rate in 15% of non responders and 46% of relapsers with chronic hepatitis C virus related liver diseases comparable to those obtained with the standard doses of ribavirin 1000 - 1200 mg/day. Randomized prospective controlled trials using lower total amounts of ribavirin in combination with interferon should be performed.**

Abteilung für Gastroenterologie und Endokrinologie, Georg-August-Universität, Göttingen, Germany
Correspondence to: Professor Dr. Dr. G. Ramadori, Abteilung für Gastroenterologie und Endokrinologie, Georg-August-Universität, Robert-Koch-Strasse 40, 37075 Göttingen, Germany
Tel. +49-551-39-6301, Fax. +49-551-39-8596
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INTRODUCTION

The hepatitis C virus (HCV) is the most common infectious agent associated with post-transfusion and community-acquired non-A-non-B hepatitis and cryptogenic cirrhosis[1]. Up to 85% of patients with acute HCV infection will develop chronic liver disease and spontaneous viral elimination is rare[2]. In studies with a follow up of 10-20 years, cirrhosis secondary to chronic HCV infection develops in 20%-30%[2,3] and is the most common indication for liver transplantation worldwide[4]. Patients with cirrhosis secondary to chronic HCV infection also have an increased risk for development of hepatocellular carcinoma, estimated to be between 1%-4% per year[5]. The introduction of alpha interferon (IFNα) for the treatment of HCV infection is an outstanding revolution in antiviral therapy[6]. However, alpha interferon monotherapy strategies can only induce a sustained response in 8%-21% of the patients with chronic HCV-related liver disease[7]. Thus, new therapeutic strategies were needed for chronic HCV infection in order to increase the sustained response rate in naive patients and patients in whom the

response to standard alpha interferon monotherapy was ineffective, in particular the relapsers and non-responders.

Systemic ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide), a broad-spectrum oral purine nucleoside analogue, has been used for the treatment of a variety of viral diseases[8]. In children with viral diseases, ribavirin is usually given orally at a dosage of 10 mg/kg daily[8]. In adult human immunodeficiency virus (HIV)-infected patients, ribavirin plasma levels between 6 and 12 μmol/L are required to achieve viral inhibition with acceptable toxicity[9]. Daily oral doses of 600 (1200) mg ribavirin resulted in a mean peak plasma concentration of 5.0 (11.1) μmol/L at the end of the first week. Clinical and hematologic toxicities were not noted at a ribavirin dosage of 600 mg/d for 2 weeks, but a dosage of 1200 mg/d for 2 weeks resulted in moderate to severe clinical and hematological adverse events (hematocrit decrease) [9]. Furthermore Glue *et al*. reported similar ribavirin pharmacokinetics comparing pediatric and adult patients[10].

In patients with chronic HCV infection, ribavirin monotherapy has been found to improve serum levels of hepatic transaminases and liver histology by decreasing hepatic inflammation and necrosis[11-13]. In 1991, oral ribavirin treatment in a dosage of 1000 mg/d for chronic HCV-infected patients weighing less than 75 kg and 1200 mg/d weighing more than 75 kg for 12 weeks was evaluated in a pilot study [14]. In 1994 and 1995, the first promising reports[15-18] on the combined therapy with alpha interferon and ribavirin in a dosage of 1000-1200 mg/d orally according to Reichard *et al*[14] were obtained in relapsers and non-responders as well in naive patients with chronic HCV infection. Therefore, we started a combination treatment with interferon alfa-2a (IFN) in non-responders and relapsers to IFN monotherapy. However, we used a lower dosage of oral ribavirin (10 mg/kg body weight daily) according to that used in child[8] and adult[9] HIV-infected patients and to pharmacokinetic studies performed in healthy volunteer and patient populations[10]. Duration of the combined therapy was 6 months, followed by a 6-month treatment with IFN monotherapy for responders to combined therapy in chronic HCV-infected patients who were previous non-responders or non-sustained responders (relapsers) to IFN alone. The results of safety, tolerability and virological efficacy during and after treatment are reported.

PATIENTS AND METHODS

Patients

Fourty-seven chronic HCV-infected patients (15 female, 32 male, mean age 48 years) with histological mild chronic hepatitis (n = 17), moderate chronic hepatitis (n = 18), severe chronic hepatitis (n = 5), cirrhosis (n = 5) and unknown histology (n = 2) received antiviral therapy. HCV genotypes were classified in 10 patients as I (1a), in 30 as II (1b), in 2 as IV (2b), in 4 as V (3a), and one was unclassifiable according to Okamoto[19] and Simmonds[20]. The cause for HCV transmission could be identified as lood-transfusions (n = 13), injection drug use (n = 3), occupational (n = 2), sexual/household (n = 1) and unknown (n = 28). All patients had been previously treated with IFN. Thirty-four patients had not responded. Non-response to the first IFN therapy was defined as the persistence of at least 3 abnormal aminotransferase activities during a course of at least 3 months of IFN treatment with monthly control and after IFN discontinuation, and by PCR which remained positive [21]. Thirteen patients had an initial response to a dose of 3-6 million units three times per week for at least 20 weeks but not more than 18 months[22], followed by relapse. The IFN treatment was discontinued for at least 3 months before the combination therapy was started. All patients had persistent elevations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), a positive third-generation anti-HCV test using an immunoblot procedure (CHIRON RIBA HCV 3.0 SIA, Ortho Diagnostic Systems Inc., Raritan, USA) and positive serum HCV RNA by reverse transcriptase/polymerase chain reaction (RT/PCR). Patients with decompensated liver disease, autoimmune disorders, thyroid gland alterations, active alcohol or injection drug abuse, history of major psychiatric disease, pregnancy, significant anemia (hemoglobin <12 g/dL), leukocytopenia (<3000 μL) or thrombocytopenia (<50000 μL) were not included. Active hepatitis A virus, HIV, hepatitis B virus, cytomegalovirus or Ebstein-Barr virus infections were excluded by convential laboratory tests. Informed consent was obtained in all treated patients.

Treatment

Combination therapy was given for 6 months with IFN and ribavirin. The IFN dose was adjusted to the previous IFN treatment. Twenty-two of 34 previous non-responders were treated with 6 MU subcutaneous (s.c.) t.i.w. and 12/34 with 3 MU t.i. w.. Eleven of 13 previous relapsers were treated with 6 MU t.i.w. and 2/13 with 3 MU t.i.w.. To improve virological efficacy and tolerability instead of using ribavirin at a dose of 1000-1200 mg/day, we administered ribavirin at 10 mg/kg body weight daily in three divided doses orally. This dose was chosen in accordance to the tolerability data obtained in HIV-infected children[8] whose pharmacokinetic data are similar to those obtained in adults[10] and in HIV-infected adults[9]. Patients who responded to the combination therapy were

treated for another 6 months with IFN monotherapy with the same preceeded dose (3 or 6 MU s.c. t.i.w.). After 12 months of treatment, the patients were followed up for at least 9 months.

Monitoring

Clinical examination, total blood cell counts, routine biochemical tests and detection of serum HCV RNA by RT/PCR were performed before and at monthly intervals during and after 6 months treatment, thereafter every 3 months.

Detection of serum HCV-specific RNA by RT/PCR

RNA was extracted from serum samples (140 μL) using the QIAamp viral RNA KIT (Qiagen, Hilden, Germany) according to the manufacturer's protocol. One-fifth of the extracted material from serum was subjected to a nested RT/PCR procedure essentially as described[23].

Determination of HCV genotypes

HCV genotypes were determined according to Okamoto *et al*[19] using RT/PCR with a type-specific primer of the core region. Genotypes determination was then confirmed by restriction enzyme digestion analysis as described previously[24].

RESULTS

Response to previous IFN monotherapy, combination therapy with IFN and ribavirin, subsequent IFN monotherapy for responders to combination therapy and outcome during follow-up for previous non-responders is depicted in Figure 1 and for previous relapsers in Figure 2.

Drop-outs

Seven of 34 non-responders to previous IFN monotherapy (20.6%) stopped the combination therapy because of insulin dependent diabetes mellitus ($n = 1$), sarcoidosis ($n = 1$), lichen ruber oris mucosae ($n = 1$), severe fatigue ($n = 1$) or depression and fatigue ($n = 3$). Two of seven drop-out patients had a histological and clinical Child A cirrhosis. During combination therapy and after withdrawal, transaminases remained unchanged at elevated levels and serum HCV RNA was still positive in all patients. There were no drop-outs in the 13 relapsers to the previous IFN monotherapy.

Outcome of combination treatment (6 months)

Twenty-seven non-responders to previous IFN monotherapy continued on the 6-month course of combination therapy. At the end of the 3 months, 17/27 (63%) patients did not respond to the combination therapy with unchanged elevated transaminases and positive serum HCV-RNA, so therapy was stopped. Ten of 27 (37%) had a complete response with transaminases in the reference ranges and negative serum HCV RNA.

All 13 relapsers to previous IFN monotherapy completed the 6-month course of combination therapy. At the end of 6 months, 13/13 (100%) patients had a complete response with transaminases in the reference ranges and negative serum HCV RNA.

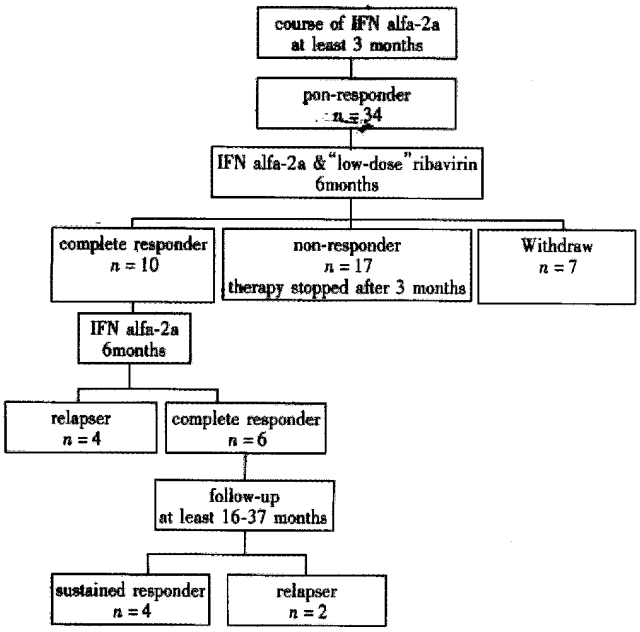

Figure 1 Outcome of 34 HCV infected non-responders to IFN monotherapy, including response to combined therapy with IFN and "low-dose" ribavirin for 6 months, response to followed IFN monotherapy for 6 months for complete responders to combined therapy and outcome during follow up after 12 months treatment.

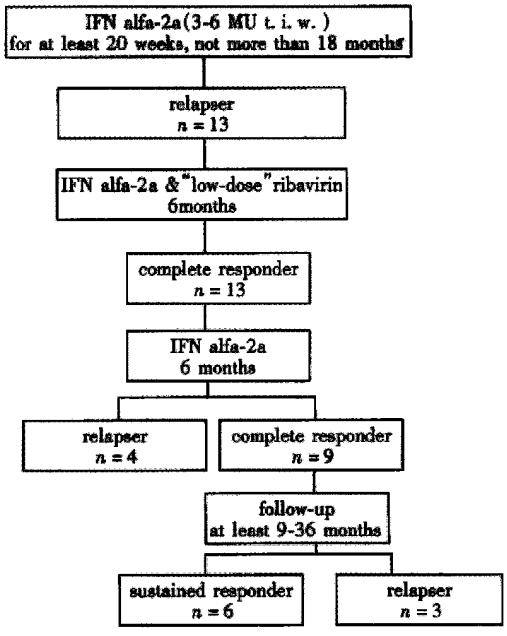

Figure 2 Outcome of 13 HCV-infected relapsers to IFN monotherapy, including response to combined therapy with IFN and "low-dose" ribivirin for 6 months, response to followed IFN monotherapy for 6 months for complete responders to combined therapy and outcome during follow-up after end of 12 months treatment.

Outcome of complete treatment (12 months)

The 10 non-responders to previous IFN monotherapy, who had a complete response at the end of combination therapy were treated with the same dose of IFN used before as monotherapy for a further 6 months. During the 6-month course of IFN monotherapy, 4/10 (40%) patients had a relapse within 6-months after the end of ribavirin treatment. Six of ten (60%) patients still had a complete response at the end of 12-month treatment course with transaminases in the reference ranges and undetectable serum HCV RNA.

All 13 relapsers to previous IFN monotherapy had a complete response at the end of combination therapy and were treated with the same dose of IFN used before as monotherapy for a further 6 months. During the 6-month course of IFN monotherapy, 4/13 (31%) patients had a relapse within 6 months after ribavirin treatment. Nine of 13 (69%) patients still had a complete response at the end of 12-month treatment with transaminases in the reference ranges and undetectable serum HCV RNA.

Follow-up (at least 9 months)

At the end of the 12-month treatment, 6 non-responders to previous IFN monotherapy became complete responders. At a mean follow-up of 28 months (16-37 months), 2/6 (33%) patients relapsed, 4/6 (67%) were sustained responders, including one patient with genotype II (1b) and cirrhosis.

At the end of 12-month treatment, 9 relapsers to previous IFN monotherapy became complete responders. At a mean follow-up of 22 months (9-36 months), 3/9 (33%) patients relapsed, 6/9 (67%) were sustained responders with transaminases in the normal range and undetectable serum HCV-RNA.

In summery, 4/27 previous non-responders (15%) and 6/13 previous relapsers (46%) are sustained responders.

Side effects

Combination therapy with IFN (3-6 MU s.c. t.i.w.) and "low-dose" ribavirin (10 mg/kg body weight) was well tolerated. Monitoring of side effects by questioning and clinical examination revealed arthralgia in 77%, fatigue in 27% and loss of weight in 19% of the patients. The most prominent side effects of combination therapy were alterations in total blood cell counts. Mild leukocytopenia (minimal 2000/μL in a patient without cirrhosis and 1400/μL in a patient with cirrhosis) and thrombocytopenia (minimal 79 000 μL in a patient without cirrhosis and 35 000 μL in a patient with cirrhosis) were generally seen. Decreases in hemoglobin is shown in Figure 3. Of 40 patients who continued on combination therapy 7 patients were withdrawn. There was no need of transfusion or drug dose reduction. All side effects were completely reversible within one month after treatment.

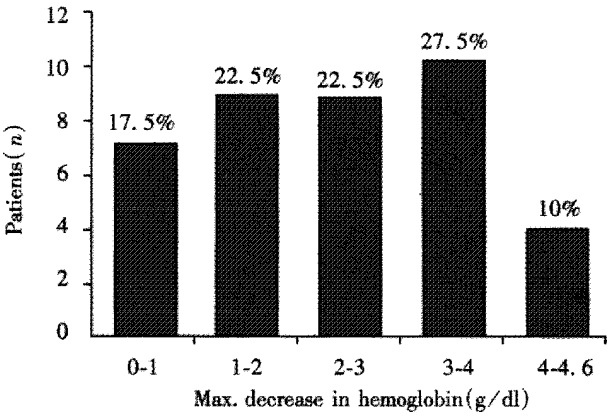

Figure 3 Decrease of hemoglobin of 40 patients (34 non-responders and 13 relapsers to IFN monotherapy) who continued on combination therapy with IFN and "low-dose" ribavirin.

DISCUSSION

In this retrospective uncontrolled analysis, we report on our experience of 47 chronic HCV-infected patients who received a combination treatment of IFN and "low-dose" ribavirin after previous failure to IFN monotherapy. Of the 47 patients, 34 were non-responders and 13 relapsers to the initial IFN monotherapy. Seven of 34 (20.6%) non-responders to the previous IFN monotherapy (two with histological cirrhosis), did not complete the 6 months course of combination therapy due to various side effects, but none of the 13 relapsers to previous IFN monotherapy. In comparison to our results, a meta-analysis of individual patient data from European centers revealed that about 10% of patients withdrawn from treatment[25]. Although our results have the drawback of being obtained under uncontrolled conditions, they have the advantage of being more realistic as every day experience is often different from that obtained under controlled study conditions.

In our patients, we found that a 6-month course of combined therapy with "low-dose" ribavirin induced a complete response at the end of treatment in 10 (37%)of 27 initial non-responders. In a similar retrospective analysis, 2 (12.5%) of 16 non-responders to IFN monotherapy were complete responders at the end of combination therapy and both patients relapsed during the follow-up period[26]. From our initial 13 relapsers to IFN monotherapy, 13 (100%) were complete responders at the end of 6-month combination treatment with "low-dose" ribavirin, in contrast to the multicenter study from Davis *et al*, who found 77% complete responders at the end of 6-month combination treatment with ribivarin in a dose of 1000-1200 mg per day depending on body weight[22].

Previous publications had suggested that duration of therapy longer than 6 months may reduce the number of relapsers[16,27]. Therefore, the 23 complete responders (10 initial non-responders and 13 relapsers to IFN monotherapy) at the end of 6-month combined therapy continued with IFN alone for another 6 months. Eight of 23 (35%) complete responders at the end of combined therapy with "low-dose" ribavirin (4 initial non-responders and 4 relapsers to IFN monotherapy) relapsed under IFN monotherapy within 3 months, suggesting that in a subgroup of patients a longer course of combined therapy could leed to sustained response.

During the follow-up period, 5 of 15 (33%) complete responders at the end of 12 months treatment (2 initial non-responders and 3 relapsers to IFN monotherapy) relapsed within 3 months. Also, these patients represent a subgroup who may benefit of a longer period of combined therapy. However, 4/27 (15%) initial non-responders (including one with histological cirrhosis and genotype I (1b) and 6/13 (46%) relapsers to IFN monotherapy were still complete sustained responders (16-37 months follow-up for initial non-responders and 9-36 months for initial relapsers to IFN monotherapy) after 12 months treatment.

A combined therapy with IFN and "low-dose" ribavirin for 6 months followed by 6 months of IFN monotherapy in those patients who responded to the combination therapy can induce sustained response not only in relapsers to IFN monotherapy, but also in some non-responders, among whom, one patient had histological sign of cirrhosis and genotype I (1b). This result is noteworthy as the monotherapy course was performed with a total amount of IFN which was previously shown to be effective in a large number of patients[27]. This suggests that retreatment with a higher dose of IFN for a longer period[28-30] is less promising than the combination therapy. Our experience also shows that a further 6 months of IFN monotherapy after combination therapy may help reduce the number of relapsers and that on the other hand the duration of the combination therapy of 6 months may not be sufficient for some patients. The results of this retrospective analysis are difficult to compare with those recently published by Sostegni *et al*[31], Davis *et al*[22], Pol *et al*[21] and by Milella *et al*[32] obtained treating patients who were non-responders to[21,31,32] or relapsed after[22,32] interferon alfa monotherapy with a combination therapy for 6 months, because the number of non-responders and relapsers we treated is small. However, it is also noteworthy that the lower dose of ribavirin we used seems to allow response rate may similar to those recently published in controlled studies[21,22,31,32]. This strategy received support in a recently presented abstract[33] where a ribavirin dose of 600 mg/day was as effective as 1000 mg/day as for as week 12 HCV RNA levels concerns during alpha interferon and ribavirin therapy in chronic HCV-infected patients. This is also of economic relevance as the ribavirin released into the market recently increases the costs of about 1000 Dollars per month. Our results may stimulate a prospective study where complete responders under combination therapy are treated for further 6 months with alpha interferon alone as has been recently suggested by Perasso *et al*[34].

In conclusion, combination therapy with "low-dose" ribavirin may represent a therapeutic alternative for at least some non-responders and relapsers.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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HCV-RNA positivity in peripheral blood mononuclear cells of patients with chronic HCV infection: does it really mean viral replication?

Volker Meier, Sabine Mihm, Perdita Wietzke Braun and Guliano Ramadori

Subject headings hepatitis C like viruses; hepatitis C, chronic; RNA, viral/blood; virus replication; monocytes; interferon alpha/therapeutic use; polymerase chain reaction

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Abstract

AIM To analyze the association of HCV-RNA with peripheral blood mononuclear cells (PBMC) and to answer the question whether HCV-RNA positivity in PBMC is due to viral replication.

METHODS HCV-RNA was monitored in serum and PBMC preparations from 15 patients with chronic HCV infection before, during and after an IFN-α therapy using a nested RT/PCR technique. In a second approach, PBMC from healthy donors were incubated in HCV positive plasma.

RESULTS In the IFN-α responding patients, HCV-RNA disappeared first from total RNA preparations of PBMC and then from serum. In contrast, in relapsing patients, HCV-RNA reappeared first in serum and then in PBMC. A quantitative analysis of the HCV-RNA concentration in serum was performed before and after transition from detectable to non detectable HCV-RNA in PBMC-RNA and vice versa. When HCV-RNA was detectable in PBMC preparations, the HCV concentration in serum was significantly higher than the serum HCV-RNA concentration when HCV-RNA in PBMC was not detectable. Furthermore, at no time during the observation period was HCV specific RNA observed in PBMC, if HCV-RNA in serum was under the detection limit. Incubation of PBMC from healthy donors with several dilutions of HCV positive plasma for two hours showed a concentration dependent PCR positivity for HCV-RNA in reisolated PBMC.

CONCLUSION The detectability of HCV-RNA in total RNA from PBMC seems to depend on the HCV concentration in serum. Contamination or passive adsorption by circulating virus could be the reason for detection of HCV-RNA in PBMC preparations of chronically infected patients.

Georg-August-Universität göttingen, Zentrum innere medizin, Abteilung FÜr Gastroenterologie Und Endokrinologie, Robert-Koch-Straβe 40, 37075 Göttingen, Germany

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Correspondence to: G. Ramadori, M.D., Georg-August-Universität Göttingen, Zentrum innere medizin, Abteilung FÜr Gastroenterologie Und Endokrinologie, Robert-Koch-Straβe 40, 37075 Göttingen, Germany
Tel. 0049-0551-396301, Fax. 0049-0551-398596
Email. gramado@med.uni-goettingen.de

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped singlestranded positive-strand RNA virus of the Flaviviridae family[1]. It is the major agent responsible for parenterally transmitted non-A, non-B hepatitis[2]. Acute infection with HCV is often clinically asymptomatic and the majority of patients develop a chronic hepatitis[3]. Treatment with interferon α (IFN-α) in combination with ribavirin, a synthetic guanosine analogue, is now the regimen of choice for patients chronically infected with HCV[4,5]. During drug administration, HCV-RNA may disappear both in serum and in peripheral blood mononuclear cells (PBMC)[6], but the proportion of patients responding to this therapy in terms of a sustained virological response varied around 40%. About 15%-20% of HCV infected patients progress to end-stage liver disease with cirrhosis and also hepatocellular carcinoma[7-10]. After orthotopic liver transplantation, reinfection of the graft with HCV is the rule[11,12]. It has been hypothesized that virus replication takes place at extrahepatic sites. Possible sites are the different cell fractions (PBMC, granulocytes or red blood cells/pellets) of the peripheral blood[13,14] or tissues like lymph nodes, pancreas, adrenal gland thyroid, bone marrow or spleen[15]. In particular, PBMCs have been suggested to function as an important extrahepatic reservoir or as a possible site for extrahepatic HCV replication[16-19]. This assumption was based on the demonstration of negative-strand HCV-RNA, the replicative intermediate of HCV, in association with PBMC from HCV infected patients in whose sera negative-strand HCV-RNA was not detected. The presence

of negative-strand HCV-RNA in extrahepatic compartments based on PCR detection assays has been suggested in many reports with very significantly different detection rates (from 0% to 100%). An extensive artefactual detection of negative-strand HCV-RNA due to self-priming and mispriming events is possibly responsible for these differences. This is especially true when a 5'-noncoding-region primer pair is used[20,21].

Other authors have also shown that the presence of HCV sequences in PBMC is compatible with passive virus adsorption via specific receptors or with contamination by circulating virus[22,23]. HCV has been found to bind to low density lipoproteins (LDL) and therefore, enter the PBMC via LDL-receptor[24-26]. Pileri and colleagues demonstrated the binding of HCV to CD 81, a 25-kD molecule of the tetraspanin superfamily expressed in various cell types including hepatocytes and B-lymphocytes[27].

The present study aims to analyse the association of HCV-RNA with PBMC and to answer the question whether HCV-RNA positivity in PBMC is really due to viral replication. Therefore, HCV-RNA detectability was serially monitored in both the serum and PBMC preparations from 15 patients with chronic HCV infection before, during and after IFN-α therapy. In a second approach, PBMC from healthy donors were incubated for a short time in HCV-RNA positive plasma to investigate the possibility of an attachment of HCV to PBMC.

MATERIALS AND METHODS

Patients

A total of 15 patients (8 women and 7 men; mean age 57.0 years; age range 41-79 years) (Table 1) infected with HCV as diagnosed by the presence of anti-HCV antibodies and HCV-RNA in serum were studied consecutively. All were anti-HCV and HCV-RNA positive for at least six months. The degree of the liver injury was estimated histopathologically according to established criteria [28,29]. Patients with active hepatitis B virus or human immunodeficiency virus infection and those with continued alcohol or drug abuse were excluded. The patients received an IFN-á therapy (3×10^6 to 6×10^6 IU IFN-α_{2a} three times weekly to 6×10^6 IU IFN-α_{2a} daily, Roferon A, Hoffmann La Roche, Basel, Switzerland; doses were adapted individually based on well-being and response parameters) over a period of 6 to 17 months. Blood samples were taken twice before the therapy, monthly during therapy and after therapy. Serum samples and PBMC preparations were stored at -80℃. Additionally, blood samples were collected from three healthy donors (VM, NN and BS) for isolation of HCV negative PBMC and from three untreated patients (EZ, SG and UP) with chronic HCV infection for isolation of HCV positive plasma. The study was approved by the local ethics committee of the Georg-August-University, G-ttingen, Germany.

Table 1 Clinical, virological and therapeutic parameters of 12 patients with chronic HCV infection and treated with IFN-α_{2a}

Patient No.	Age (yrs)	Sex	Genotype (U/L)	ALT	Therapy regiment (IFN-α)	Duration of therap (month)	Response
1	60	F	1b	30	$3 \times 3 \times 10^6$ IU / week	18	Respond
2	79	M	n.d.	n.d.	$2 \times 3 \times 10^6$ IU / week	19	Respond
3	51	F	1a	34	$3 \times 3 \times 10^6$ IU / week 800 mg Ribavirin / day	6	Relaps
4	62	M	1b	36	$7 \times 6 \times 10^6$ IU / week	6	Relaps
5	41	M	1b	40	$3 \times 3 \times 10^6$ IU / week	17	Respond
6	63	F	1b	46	$3 \times 6 \times 10^6$ IU / week	14	Relaps
7	61	M	1b	45	$7 \times 6 \times 10^6$ IU / week	6	Respond
8	48	F	1a / 1b	36	$3 \times 3 \times 10^6$ IU / week 800 mg Ribavirin / day	7	Relaps
9	63	F	1b	20	$3 \times 4.5 \times 10^6$ IU / week	12	Relaps
10	60	M	1b	36	$3 \times 6 \times 10^6$ IU / week	12	Relaps
11	53	M	n.d.	92	$3 \times 9 \times 10^6$ IU / week	12	Respond
12	43	F	1a	160	$3 \times 6 \times 10^6$ IU / week	12	Respond
13	53	F	1b	9	$3 \times 6 \times 10^6$ IU / week 800 mg Ribavirin / day	12	Respond
14	69	F	1b	22	$3 \times 6 \times 10^6$ IU / week	12	Relaps
15	63	M	1a	69	$3 \times 6 \times 10^6$ IU / week	9	Relaps

ALT: alanine transaminase; M: male; F: female; n.d.: not determined; respond: responder; relaps: relapser.

Preparation of PBMC

Human PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation[30]. Residual red blood cells were hypotonically lysed and cells were washed three times with phosphate buffered saline (PBS) pH 7.3. Cell preparations were routinely assessed for viability (>95%) by trypan blue dye exclusion. Typically, a PBMC preparation consisted of >98% lymphocytes and monocytes and <2% granulocytes as determined by morphology of cells stained according to Pappenheim.

Isolation of total cellular RNA

PBMCs obtained from approximately 30 mL peripheral blood were taken up in 3 mL guanidinium isothiocyanate (GTC) buffer[31]. The material was subjected to shearing forces by drawing it rigorously through a capillary needle. Subsequently, total cellular RNA was isolated by cesium chloride (CsCl) density gradient centrifugation[32] and the RNA concentration was determined photometrically.

Detection and quantification of serum HCV RNA

HCV specific RNA was extracted from serum samples (140 μL) using QIAamp viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For qualitative determination of HCV specific RNA one fifth of the extracted material was subjected to a nested RT/PCR procedure essentially as described[33]. For the quantitative measurement of HCV viral RNA the Amplicor HCV Monitor Test Kit (Hoffmann-La

Roche AG, Grenzach-Wyhlen, Germany) was used according to the manufacturer's protocol. Test results less than 600 copies/mL are below the lower limit of quantification of the test and should be reported as "HCV detected, less than 600 copies/mL".

Detection of HCV RNA in PBMC by nested RT/PCR-procedure

For the qualitative detection of HCV specific RNA in PBMC 100 ng of total cellular RNA were subjected to a HCV specific nested RT/PCR procedure essentially as described[33].

Incubation of HCV-RNA negative PBMC in HCV-RNA positive plasma

Blood samples were obtained from the three healthy donors (VM, NN and BS) for preparation of HCV-RNA negative PBMC and from the three patients (SG, EZ and UP) chronically infected with HCV for isolation of HCV-RNA positive plasma samples. The PBMCs were isolated as described before. The plasma samples were obtained by centrifugation (10 minutes, 14 000 rpm and 4℃) of the HCV-RNA positive blood samples. The HCV concentration was then measured in these samples using the Amplicor Monitor Test Kit. Afterwards each of the three different plasma samples were diluted 1:4, 1:16 and 1:64 with HCV-RNA negative plasma from a healthy donor to reduce the virus concentration; one undiluted sample was also used.

PBMC isolated from donor VM were then incubated with the plasma samples from patient UP, from donor NN in the plasma samples of patient EZ and from donor BS in the plasma samples of patient SG. Incubation was performed for 2 hours at 37℃ in an air incubator. After incubation the PBMCs were washed three times in PBS as described above. The total cellular RNA was then isolated by CsCl density gradient centrifugation and the HCV-RNA was detected with HCV specific nested RT/PCR technique as described.

RESULTS

HCV-RNA detectability in sera and in total RNA preparations of PBMC from patients with chronic HCV infection before, during and after an IFN-α therapy

The detectability of HCV specific RNA was monitored regularly before, during and after an IFN-α therapy in sera and in total RNA preparation of PBMC in 15 patients with chronic HCV infection by a nested RT/PCR procedure. HCV-RNA was below the detection limit in 102 serum samples taken during the IFN-α therapy. In none of these cases could HCV-RNA be observed in PBMC. In contrast, HCV specific RNA was only detectable in PBMC when HCV-RNA in serum was above the detection limit.

In patients who completely responded to the IFN-α therapy, the number of HCV-RNA copies decreased progressively until they became undetectable in serum samples. HCV-RNA in PBMC fell below the detection limit before positivity disappeared from the serum. In the patients relapsing after cessation of IFN-α therapy, HCV-RNA reappeared first in serum and later in PBMC. Serum HCV-RNA concentrations were measured quantitatively before and after transition from detectable to non-detectable HCV-RNA in PBMC preparation and vice versa. The transition from detectable to non-detectable HCV-RNA in PBMC during drug therapy was associated with a strong and significant decrease of serum HCV-RNA concentration (Table 2 and Figure 1). Vice versa, the transition from non-detectable to detectable HCV-RNA in PBMC, in the case of a breakthrough under therapy, or when the therapy was stopped and a relapse was observed, was associated with a marked and significant increase of serum HCV-RNA concentration (Table 2 and Figure 1). HCV-RNA was undetectable in PBMC if HCV-RNA concentration in serum fell below 2263 copies/mL and became detectable when the HCV-RNA level in serum was higher than 4708 copies/mL (Table 2).

In one patient (pt #15) after therapy and in four patients (pt #3, pt #4, pt #9 and pt #14) relapsing after cessation of therapy, the blood samples taken during these period did not include the moment, when HCV-RNA was detectable in serum and undetectable in PBMC.

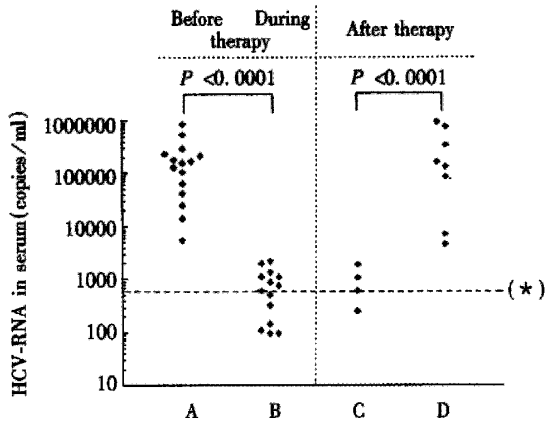

Figure 1 HCV-RNA concentration (copies / mL) in sera from patients with chronic HCV infection with regard to HCV-RNA detectability in PBMC before, during and after an IFN-α therapy. The transition from detectable to non-detectable HCV-RNA in PBMC during drug therapy was associated with a strong and significantly decrease of serum HCV-RNA concentration. Vice versa, the transition from non-detectable to detectable HCV-RNA in PBMC, in the case of a breakthrough under therapy, or when therapy was stopped and a relapse was observed was associated with a marked and significantly increase of serum HCV-RNA concentration. Columns A and D: HCV-RNA concentration, if HCV-RNA in serum and in PBMC was detectable. Columns B and C: HCV-RNA concentration, if HCV-RNA in serum was detectable and in PBMC undetectable. (*) Test results less than 600 copies / mL are below the lower limit of quantification of the test and should be reported as "HCV detected, less than 600 copies / mL".

Table 2 Detectability of HCV-RNA in serum and PBMC during an IFN-α_{2a} therapy

Patient No.	Before therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *pos.*)	During therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *n.d.*)	After therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *n.d.*)	After therapy HCV-RNA concentration (copy/mL) (HCV-RNA: serum pos./ PBMC *pos.*)
1	64717	<600	1)	1)
2	14300	<600	1)	1)
3	132224	<600	2)	167 898
4	107684	624	2)	135 242
5	161718	1404	1)	1)
6	5620	903	1108	938 861
7	183091	2041	1)	1)
8	172345	<600	1960	86 679
9	29542	775	2)	770 810
10	219018	1138	<600	7240
11	42601	<600	1)	1)
12	237335	<600	1)	1)
13	25097	2263	1)	1)
14	553901	1144	2)	4706
15	868996	2)	627	349 918

Abbreviations: 1) responder; 2) no sample available.

Incubation of HCV-RNA negative PBMC from healthy donors in HCV-RNA positive plasma from patients infected chronically with HCV

The results of the measurement of the HCV concentrations in the undiluted positive plasma samples of each patient with chronic HCV infection using the Amplicor HCV Monitor Test Kit were as follows: patient SG 119271 copies/mL, patient UP 337539 copies/mL and patient EZ 77518 copies/mL. The virus concentration of the dilutions steps in each patient is shown in Table 3. The results of incubation of PBMC from healthy donors in HCV-RNA positive plasma with different virus concentration are summarized in Table 3. After a two-hour incubation at 37℃, all PBMC samples incubated in the undiluted plasma samples were HCV-RNA positive. When PBMCs from donor BS were incubated in plasma samples from patient SG, the HCV-RNA was detectable only in the undiluted sample (119271 copies/mL, Table 3). After incubation of PBMC from donor VM in plasma samples of patient UP, the HCV-RNA was detectable in dilution step 1:16 (21096 copies/mL, Table 3). On incubation of PBMC from donor NN in plasma samples of patient EZ, the HCV-RNA was detectable in dilution step 1:4 (19290 copies/mL, Table 3). These results indicate a possible correlation between the detectability of HCV-RNA in PBMC and the HCV concentration in the plasma, since no HCV-RNA could be detected in PBMC when the HCV titer was below 19290 copies/mL.

Table 3 Detectability of HCV-RNA in PBMC of healthy donors after incubation with different dilutions of HCV-RNA positive plasma

Patients	HCV concentration (copies/mL)				PMBC of healthy donor	Detectability of HCV-RNA in PBMC of healthy donors	
	Undiluted sample	1:4	1:16	1:64		Before incubation	After incubation dilution step (copies/mL)
SG	119 271	29 818	7454	1863	BS	n.d.	Undiluted (119271)
UP	337 539	84 385	21 096	5274	VM	n.d.	1:16 (21096)
EZ	77 518	19 290	4822	1206	NN	n.d.	1:4 (19290)

DISCUSSION

In all cases of orthotopic liver transplantation, an HCV reinfection of the graft occurs[11,12]. Persistence of HCV at extrahepatic sites is considered to be responsible for the reinfection. Therefore, any extrahepatic association of virus, e.g., by productive replication or simply by adhesion to outer membrane structures, appears to be relevant. One important extrahepatic reservoir is possibly the whole blood, which consists of a liquid component (plasma) and different cell fractions (PBMC, granulocytes and red blood cells/platelets). In the literature it has been suggested that PBMC can function as an important extrahepatic reservoir and a possible site for HCV replication. The evidence for this assumption was based on the demonstration of negative-strand HCV-RNA in association with PBMC from HCV infected patients by a RT/PCR technique[16-19]. Other possible extrahepatic reservoirs were described by Laskus and colleagues in chronically HCV infected patients, additionally infected with the acqured immunodeficiency syndrome. HCV-RNA negative-strand could be detected by a Tth-based reverse transcriptase polymerase chain reaction in lymph nodes, pancreas, adrenal gland, thyroid, bone marrow and spleen[15]. The value of these findings is however controversial, since the presence of negative-strand HCV-RNA in extrahepatic compartments based on RT/PCR techniques has been described in many reports with a very large range of detection rate (from 0% to 100%). In fact, by using synthetic as well as biological templates, an extensive artefactual detection of negative-strand HCV-RNA, due to self-priming and mispriming events could be documented. This is especially true when a 5' noncoding region primer pair is used[20,21]. The

mispriming artefacts were directly correlated to the titer of positive strand and depend on the RT/PCR technique used[21]. Therefore, in this study no experiment was performed for the detection of negative-strand HCV-RNA in PBMC preparations.

In this work, the presence of HCV-RNA was qualitatively analysed in serum and in total RNA preparations of 15 patients infected chronically with HCV, before during and after an IFN-α therapy. During the observation period after beginning IFN-α therapy, it was noted that HCV-RNA disappeared first from total RNA preparations of PBMC and then from serum. In the case of a breakthrough under therapy or of a relapse at the end of therapy, HCV-RNA reappeared at first in serum and then in the total RNA preparation of PBMC. Therefore, we performed a quantitative analysis of the HCV titer at these points of transition, and obtained the following results: (a) HCV specific RNA in total RNA preparations of PBMC was only detectable when HCV-RNA in serum was above the detection limit of the assay; (b) the transition from detectable to non-detectable HCV-RNA in PBMC during drug administration was associated with a marked decrease of serum HCV-RNA concentration; (c) the transition from non-detectable to detectable HCV-RNA in PBMC in the case of a breakthrough or a relapse was associated with a marked increase in serum HCV-RNA concentration; (d) when HCV-RNA in PBMC was detectable, the HCV concentration in serum was significantly higher than when HCV-RNA in PBMC was not detectable (Figure 1): and (e) it was possible to contaminate PBMC of healthy donors with HCV by incubation for a short time (2 hours) at body temperature (37℃) in HCV positive plasma samples. These results are in agreement with those published by Cribier and colleagues (1998). These authors could show that HCV-RNA become detectable in the PBMC of healthy donors after an incubation of the cells with HCV-RNA positive serum with high virus concentration[34].

HCV-RNA in PBMC become undetectable in the *in vivo* study when HCV-RNA concentration in serum falls below 2263 copies/mL (Table 2) and in the *in vitro* experiments when the HCV-RNA level falls below 19290 copies/mL (Table 3). One explanation for this discrepancy could be that for the *in vitro* experiments we choose a short period of incubation, which however, better shows the adherence of HCV to blood cells.

These findings support the assumption that the presence of HCV sequences in total RNA preparations of PBMC is probably compatible with passive virus adsorption, with endocytosis of the virus or with contamination by circulating virus[22,23]. In 1992 Thomssen and colleagues described a possible mechanism of how HCV could bind to the surface of PBMC. They found, that HCV could bind to β-lipoprotein (LDL) and therefore possibly adhere in this form to specific lipoprotein-receptors on PBMC[25,26]. Recently, Agnello and colleagues demonstrated that HCV can enter the cells via the LDL-receptor[24]. The endocytosis of HCV by the LDL receptor was mediated both by VLDL or LDL and directly by HCV binding to the cell surface. Three kinds of cell lines (Hep G2, G4 and Daudi cells) were used in this study. After an incubation in HCV positive HCV-RNA serum positive-strand was detectable in each cell line by *in situ* hybridiziation. HCV-RNA negative-strand, as evidence for replication, was detected in the Hep G2 and Daudi cells, but not in the G4 cells, a B-lymphocyte cell-line[24]. These findings are in agreement with our results, that no replication occurs in PBMC. Another possibility for adherence of HCV to PBMC has been shown by Pileri and colleagues, who demonstrated the binding of HCV to CD 81, a 25-kD molecule of the tetraspanin superfamily, expressed in various cell types including hepatocytes and B-lymphocytes[27]. Fluorescence *in situ* hybridization of HCV-RNA in PBMC showed signals on the cytoplasmatic membrane of the cells. However, this could also be an indication for passive viral adsorption via a specific receptor or for a contamination by circulating virus. Other fluorescent signals appeared in granules in distinct submembrane areas or diffuse in the cytoplasm[35,36]. A possible explanation for these findings may be the ingestion of virus particles by phagocytosis particularly in macrophages or by endocytosis via the LDL-receptor.

HCV-RNA was not only detected in PBMC but also in other cell fractions of the whole blood such as granulocytes and red blood cells or platelets. Schmidt and colleagues have investigated the distribution of HCV in whole blood and in the different cell fractions. Whole blood contained significantly more HCV-RNA than pasma, which contained more HCV-RNA than PBMC, the lowest level of HCV-RNA was found in granulocytes and in red blood cells/pellets[13,14]. In the case of granulocytes, the virus may simply be ingested by phagocytosis and in the red blood cells or pellets. The HCV may be present because virus or virus-protein complexes could also adhere to specific receptors on the cell surface. Bronowicki and colleagues described the SCID mouse model as a possible *in vivo* model to analyse the issue of HCV-RNA persistence in mononuclear cells[37]. In their study, they injected PBMC from patients infected chronically with HCV into SCID mice. To exclude the possibility that just virus contamination and not true infection of the cells occurred, they incubated as a control sheep PBMC and human fibroblasts in HCV-RNA positive serum and injected them into

SCID mice. After injection of human PBMC, HCV-RNA was detected in 30% and 23% of blood cells and serum samples respectively of SCID mice. On the other hand, in the control mice, HCV-RNA sequences were not detectable either in serum samples or in PBMC. Therefore, they postulated the possibility of a replication of HCV in PBMC. In fact, the detection of no HCV-RNA in the control mice would argue against an adsorption of virus particles. The hypothesis was strengthened by detection of HCV negative-strand in two cell fraction samples of two SCID mice. However, no data were available showing that the sheep PBMC or human fibroblasts become HCV-RNA positive after the incubation in HCV-RNA positive serum.

Taken together, our findings could be explained as follows. During the IFN-α treatment, the HCV level is lowered by the inhibition of the hepatic virus replication. When the virus concentration decreases below a not yet exactly known upper limit, contamination of the PBMC becomes undetectable. At this time point, HCV is only detectable in serum for a short period and then disappears. On the other hand, it is also possible that IFN-α inhibits virus replication in both PBMC and the liver, and consequently, the HCV concentration in serum decreases in parallel with that of PBMC, although in lower amounts, the HCV-RNA continues to be detectable in the serum even when PBMC are negative. This indicaties that liver is the major site of replication. This could also explain that in the case of a relapse, the virus replication goes on in the liver and that the virus become detectable in the serum and then in the PBMC. The only approach to prove a true virus replication in PBMC is the detection of non-structural proteins (proteases, helicase or RNA-dependent RNA polymerase), which are important for virus replication, but this topic has not yet been investigated.

Our *in vitro* study, however, suggests that HCV-RNA positivity in PBMC may be due to the binding of the virus to blood cells and not to the true virus production. This would mean that PBMCs do not function as an extrahepatic reservoir for HCV when the diseased liver is explanted. In these cases, small amounts of HCV-RNA may still be present in the serum or attached to blood cells and be responsible for reinfection of the graft. This could happen even in the patients who are complete responders under IFN-α therapy immediately before transplantation.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Peripheral blood lymphocytes DNA in patients with chronic liver diseases

Vasiliy I Reshetnyak, Tatyana I Sharafanova, Ludmila U Ilchenko,Elena V Golovanova and Gennadiy G Poroshenko

Subject headings hepatitis, chronic/immunology; liver cirrhosis/immunology; DNA damage; lymphocytes

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Abstract

AIM Of this investigation is to reveal the damage to peripheral blood lymphocytes (PBL) DNA in the patients with chronic liver diseases.

MATERIALS AND METHODS Sixteen-nine patients with chronic liver diseases (37 patients with chronic viral hepatitis, 2 patients with liver cirrhosis of mixed etiology (alcohol + virus G), 30 women with primary biliary cirrhosis-PBC) were examined. The condition of DNA structure of PBL was measured by the fluorescence analysis of DNA unwinding (FADU) technique with modification. Changes of fluorescence (in %) reflected the DNA distractions degree (the presence of DNA single-stranded breaks and alkalinelabile sights).

RESULTS AND CONCLUSION The quantity of DNA single-stranded breaks and alkalinelabile sights in DNA in all patients with chronic viral hepatitis didn't differ from the control group, excluding the patients with chronic hepatitis (CH) C + G. Patients with HGV and TTV monoinfection had demonstrated the increase of the DNA single-stranded breaks PBL quantity. This fact may be connected with hypothesis about the viruses replication in white blood cells discussed in the literature. Tendency to increase quantity of DNA PBL damages in the patients with primary biliary cirrhosis (PBC) accordingly to the alkaline phosphatase activity increase was revealed. Significant decrease of the DNA single-stranded breaks and alkalinelabile sights in the PBC patients that were treated with prednison was demonstrated. Probably, the tendency to increase the quantity of DNA single stranded breaks and alkalinelabile sights in lymphocytes of the PBC patients was depended on the surplus of the blood bile acid content.

Central Research Institute of Gastroenterology, Moscow, Russia
Dr. Vasiliy Ivanovich Reshetnyak, graduated from First Moscow Medicine Institute as a doctor in 1977; got master degree at A.N. Bach Institute of Biochemistry, Russia Academy Sciences, Moscow in 1982; got doctor degree at Central Research Institute of Gastroenterology, Moscow in 1996, is now deputy director of Central Research Institute of Gastroenterology, Moscow; had more than 70 papers published, Deputy Editor-in-Chief of Russian Gastroenterological Journal with 1995; Vice-President of the Gastroenterological Scientific Society of Russia with 1998.

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Correspondence to: Dr. V.I. Reshetnyak, the Central Research Institute of Gastroenterology, shosse Entuziastov 86, Moscow, Russia, 111123
Tel. +7 095 304 30 19; Fax. +7 095 305 07 58, 7 095 304 30 35
Email. entero@aha.ru

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INTRODUCTION

The last years investigations demonstrated that chronic viral hepatitis are the systemic infections[1,2]. The replication of viruses hepatitis B (HBV), C (HCV) was revealed in mononuclear blood cells, bone marrow, lien and other organs. This fact accounts for polymorphism clinical signs of these infections and sometimes-unsuccessful treatment with interferon's therapy.

The role of recently revealed hepatitis G virus (HGV) for the autoimmune processes development is being discussed[3]. Phenomenon of viral immune supervision "avoid" is associated with disturbances of infected lymphocytes and monocytes immune control functions. Viral replication in blood cells with nucleuses may lead to the damage of lymphocytes genetic apparatus and the beginning of immunopathological reactions.

The immunocompetent cells condition plays significant role in the development of autoimmune process in the patients with PBC.THE AIM of this investigation is to reveal the damage to peripheral blood lymphocytes (PBL) DNA in the patients with chronic liver diseases.

MATERIALS AND METHODS

We studied 69 patients with chronic liver diseases: 43 females and 26 male, mean age ± SD: 40.8 ± 17.7 years (range 16-77 years). The study group consisted of 37 patients with chronic viral hepatitis, 2 patients with liver cirrhosis of mixed etiology (alcohol + virus G), 30 woman with PBC. Control group was formed of 10 healthy volunteers (5 females and 5 males).

The liver functional state was estimated with cytolysis enzymes activity (alanine and asparagine aminotransferases), indexes of cholestasis syndrome (activity of alkaline phosphatase, gammaglutamiltranspeptidase, and contents of cholesterol, bilirubin).

Hepatitis B markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAbsum, HBcAb IgM, HBV DNA),

hepatitis C markers (HCVAb IgG, HCVAb IgM, HCV RNA) hepatitis G markers (HGV RNA) and hepatitis TTV markers (TTV DNA) were revealed with immunoferment analysis method (IFA) and with polymerase chain reaction (PCR). The immunoglobuline's levels (IgM, IgG, IgA) were determined with IFA.

The liver biopsy was done to all patients with PBC and the main part of the patients with chronic hepatitis.

Lymphocytes were obtained from 20 mL of peripheral blood (obtained by venepuncture from healthy donors and patients with chronic liver disease) by centrifuge with the presence of Fikoll-pak solution during 30 minutes with 400 × *g*.

The condition of DNA structure of PBL was measured by the fluorescence analysis of DNA unwinding (FADU) technique as indicated by Birnboim H.C. and Jevcak J.J. (1981) with modification[4,5]. Method was created for alkalinelable sights and DNA single-stranded breaks registration. Suspension with 2 × 10^6 cells/mL was lysised. Lysate was treated with ethidium bromide, which selectively binds to double-stranded (ds) DNA. Ethidium bromide with DNA formed fluorescence complexes.

Each lysate was divided equally into 3 sets of tubes: ① a sample (P) used for evaluating total fluorescence of native DNA; ② a blank sample (B) in which DNA was completely unwound by alkaline and mechanical destruction; ③ a sample (T) used to estimate alkalinelabile sights and DNA single-stranded breaks which was obtained by alkaline. The percentage of dsDNA (D) was determined from the fluorescence of tubes P, B and T by equation: D = (P-B)/(T-B) × 100%.

The fluorescence was read with excitation wavelength 520 nm and an emission wavelength of 590 nm, and slit width 8 nm by a Jasco FP-550 spectrofluorimeter.

Changes of fluorescence (in %) reflected the DNA distractions degree (the presence of DNA single-stranded breaks and alkalinelabile sights).

Statistical methods

Conventional methods were used for calculation of means and standard deviations (SD). Results are shown as means ± SD. For skewed variables, non-parametric tests were used for comparisons between the groups (Mann-Whitney *U*-test), whereas Student's *t*-test was used for normally distributed variables. In all cases, *P* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

There were 37 patients with chronic viral hepatitis in our study. Chronic hepatitis B was revealed in 5 patients, chronic hepatitis C in 11 patients, chronic hepatitis B + C-in 1 patients, chronic hepatitis G-in 8 patients, chronic hepatitis TTV-in 4 patients, chronic hepatitis B + G-in 2 patients, chronic hepatitis C + G-in 3 patients, and chronic hepatitis B + C + G-in 3 patients.

Cirrhosis of mixed etiology (alcohol+virus G) was revealed in 2 of 69 studied patients. Replication of viruses was revealed in all patients with chronic viral hepatitis and liver cirrhosis with mixed etiology.

PBC was determined in 30 of 69 studied patients. The 1st-2 nd stage of PBC determined in 10 patients, the 3 rd stage of PBC was revealed in 14 patients and the 4th stage of PBC was revealed in 6 patients (accordingly to morphological H.Popper's classification, 1970)[6].

The percentage of $_{ds}$DNA, D, in control group was 83.5 ± 3.2. PBL DNA damages in patients with CH and PBC did not significantly differ from control group (Figure 1).

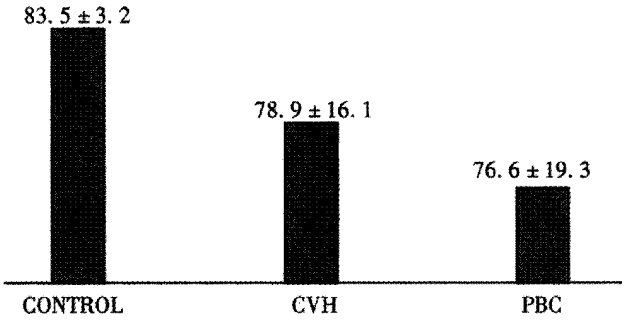

Figure 1 Percentage of double strained DNA in the blood lymphocytes (CVH: chronic viral hepatitis; PBC: primary biliary cirrosis).

The tendency to the increase of alkalinelabile sights and DNA single-stranded breaks in the patients with chronic liver diseases was determined.

Figure 2 shows diagram of DNA PBL content in the patients with viral CH. The quantity of DNA single-stranded breaks and alkalinelabile sights in all patients of this group didn't differ from the control group, excluding the patients with CH C+G ($\bar{x} \pm s$: D = 65.3% ± 12.9%, P<0.05).

Apparently the viruses C and B didn't possess a significant destructive effect on DNA PBL of the patients with chronic disease. But the patients with HGV and TTV monoinfection had demonstrated the tendency of increase of the DNA single-stranded breaks PBL quantity. This fact may be connected with hypothesis about the viruses replication in white blood cells discussed in the literature[3].

Increase of the HGV destructive effect on the DNA was revealed in the patients with chronic hepatitis of mixed (HCV + HGV) etiology (quantity of alkalinelabile sights and DNA single-stranded breaks is increased) (Figure 2).

Percentage of $_{ds}$ DNA in group of the patients with mixed HBV + HCV + HGV infections was decreased (D = 63.5 ± 20.7). Correlation between DNA structure change and hyperfermentemia degree was not revealed in the patients with different activity of chronic hepatitis.

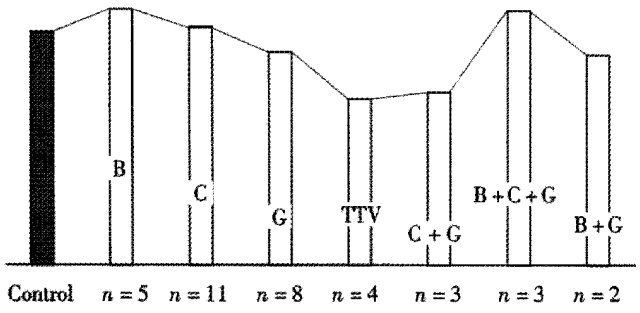

Figure 2 Percentage of double strained DNA in the blood lymphocytes of patients with chronic viral hepatitis (HBV: hepatitis B virus; HCV: hepatitis C virus; HGV: hepatitis G virus; TTV: TT virus).

So, monoinfection with HBV and HCV didn't significantly influence on PBL DNA structure in the patients with chronic disease. Monoinfection with HGV and TTV leads to increase of DNA single-stranded breaks in human PBL. Possibly, HCV increases virus G destructive effect on lymphocytes DNA structure.

Content of $_{ds}$ DNA in PBL in depending on cholestasis degree (activity of alkaline phosphatase, content of cholesterol and bilirubin), degree of histological process and used treatment was examined in the patients with PBC. Tendency to increase quantity of DNA PBL damages in the patients with PBC according to the alkaline phosphatase activity increase ($D_{aph<500un/}$ = 81.9% ± 15.2%, n = 11; $D_{aph>500un/L}$ = 74.8% ± 21.8%, n = 19) (Figure 3) was revealed.

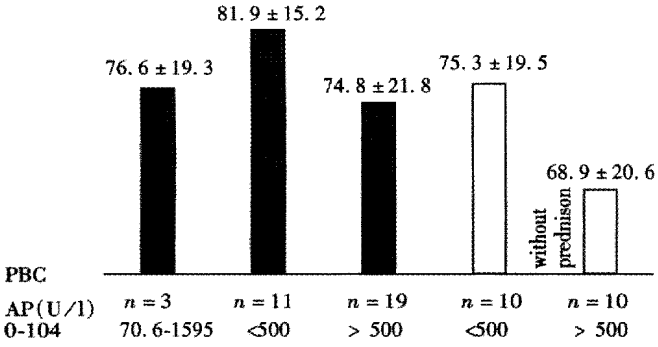

Figure 3 Percentage of double strained DNA in the blood lymphocytes of patients with PBC (PBC: primary biliary cirrosis; AP: alkaline phosphatase [normal range 0-104 u/l]).

The same data was demonstrated in 20 PBC patients without prednison treatment: percentage of $_{ds}$ DNA was 75.5% ± 19.5% in 10 PBC patients with alkaline phosphatase activity <500 u/L, and D = 68.9% ± 20.6% in 10 PBC patients with alkaline phosphatase activity >500 u/L (Figure 3). Dependence on $_{ds}$ DNA content according to other indexes of cholestasis and degree of histological changes was not demonstrated.

All PBC patients were divided into 4 groups according to the treatment methods: 6 patients were treated with prednison, 14 patients were treated with ursodeoxycholic acid (UDCA), 4 patients were treated with prednison and UDCA simultaneously, and 6 patients were treated with metabolic drug. The UDCA was not increased of D (Figure 4). Significant decrease of the DNA single-stranded breaks and alkalinelabile sights in the PBC patients, that were treated with prednison ($\bar{x} \pm s$: D = 94.5% ± 13.5%, n = 6, P<0.05, versus control D = 72.2% ± 19.9%, n = 20, P<0.05) was revealed (Figure 4).

The percentage of $_{ds}$ DNA, D, in lymphocytes in the PBC patients that were treated with prednison and UDCA simultaneously was 79.2% ± 5.3% (Figure 4).

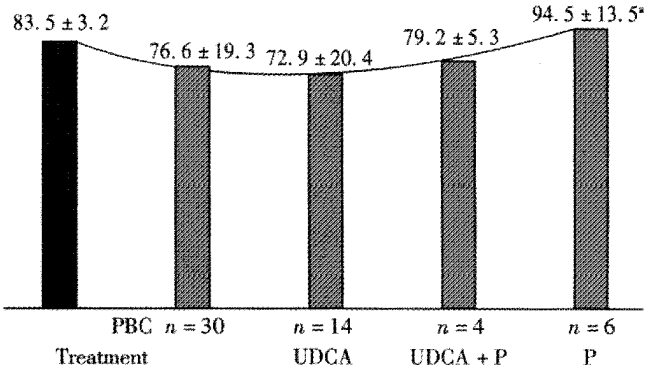

Figure 4 Percentage of double strained DNA in the blood lymphocytes of patients with PBC (PBC: primary biliary cirrosis; UDCA: urodesoxicholic acid; P: prednison; *P<0.05 *vs* control and all PBC patients).

Index D in the patients that were treated with UDCA, prednison and prednison with UDCA simultaneously had testified the suppression by bile acids the DNA reparation that was depended on prednison.

Probably, the tendency to increase the quantity of DNA single-stranded breaks and alkalinelabile sights in lymphocytes of the PBC patients was depended on the surplus of the blood bile acid content.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Immunohistochemical study of hepatic oval cells in human chronic viral hepatitis

Xiong Ma, De Kai Qiu and Yan Shen Peng

Subject headings hepatitis, viral, human; liver regeneration; oval cell; immunohistochemistry; colony stimulating factors

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Abstract

AIM To detect immunohistochemically the presence of oval cells in chronic viral hepatitis with antibody against c-kit.

METHODS We detected oval cells in paraffin embedded liver sections of 3 normal controls and 26 liver samples from patients with chronic viral hepatitis, using immunohistochemistry with antibodies against c-kit, π class glutathione S-transferase (π-GST) and cytokeratins 19 (CK19).

RESULTS Oval cells were not observed in normal livers. In chronic viral hepatitis, hepatic oval cells were located predominantly in the periportal region and fibrosis septa, characterized by an ovoid nucleus, small size, and scant cytoplasm. Antibody against stem cell factor receptor, c-kit, had higher sensitivity and specificity than π-GST and CK19. About 50%-70% of c-kit positive oval cells were stained positively for either π-GST or CK19.

CONCLUSION Oval cells are frequently detected in human livers with chronic viral hepatitis, suggesting that oval cell proliferation is asso ciated with the liver regeneration in this condition.

Shanghai Institute of Digestive Diseases, Renji Hospital, Shanghai Second Medical University, Shanghai 200001, China
Dr. Xiong Ma, graduated from Shanghai Second Medical University with master degree in 1998, now studying in Shanghai Institute of Digestive Disease for doctoral degree, majoring chronic liver diseases, having 10 papers published.
Corresponding to: Dr. Xiong Ma, Shanghai Institute of Digestive Diseases, Renji Hospital, 145 Shandong Zhong Road, Shanghai 200001, China
Tel. 0086-21-63200874, Fax. 0086-21-63266027
Email. xiongma@netease.com
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INTRODUCTION

Although essentially a quiescent organ, the normal adult liver can fully regenerate following surgical resection or injury. Liver regeneration is usually achieved by the entry of normally proliferatively quiescent, differentiated hepatocytes into the cell cycle, but, when hepatocyte regeneration is defective, oval cells can migrate outward from the portal tracts and then differentiate into hepatocytes[1-3]. The term oval cells described as small cells with oval nuclei that arise in the periphery of the portal tracts in rat models of hepatocarcin ogenesis and injury[4-7]. These cells are thought to have both clonogenic and bipotential capacity, i.e., the ability to proliferate and differentiate into cells of either hepatocyte or biliary epithelial cells[8]. There is also evidence that under certain conditions, oval cells can be induced to differentiate into non-hepatic lineages including intestinal and pancreatic epithelium[9]. The origin of oval cells and their precise location within the liver have remained enigmatic[10]. The aim of this study is to detect immunohistochemically the presence of oval cells in chronic viral hepatitis with antibody against c-kit.

MATERIALS AND METHODS

Tissue samples

Formalin-fixed, paraffin-embedded liver biopsy specimens from 26 patients with chronic liver diseases were obtained from the Department of Histopathology at Shanghai Institute of Digestive Diseases (Renji Hospital). Patient age ranged from 23 to 71 years, with mean age of 54 years. Twenty-six patients had been diagnosed having chronic viral hepatitis (twenty-one chronic hepatitis B; five chronic hepatitis C) with various degree fibrosis. There were mild fibrosis ($n = 5$), moderate fibrosis ($n = 8$), severe fibrosis ($n = 6$), and hepatic cirrhosis ($n = 7$). Three specimens of grossly normal liver tissues from the area surrounding benign angiomas were used as references. Specimens were fixed immediately in 10% neutral formalin and embedded in paraffin.

Primary antibodies

To highlight the presence of oval cells, three primary antibodies were used. The antibody against stem cell factor receptor, c-kit, was purchased from Oncogene Research Products. c-kit (Ab-1) is a purified rabbit polyclonal antibody raised against the

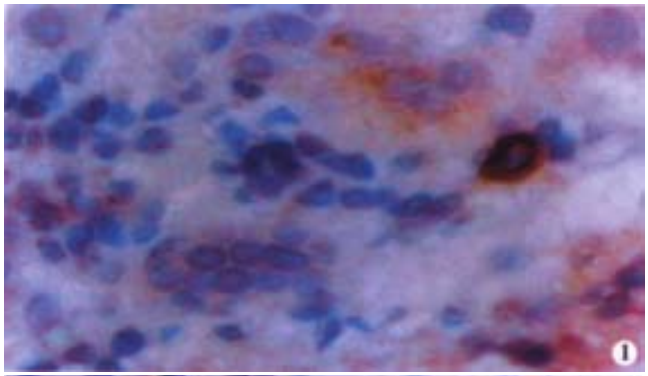
peptide (GSTASSSQPLLVHDDV), a sequence found at the Carboxyterminus corresponding to residues 961-976. Antibodies against π-class glutathione S-transferase (π-GST, clone 353-10) and cytokeratins 19 (CK19, clone BA17.1) were purchased from Dako Co, Denmark.

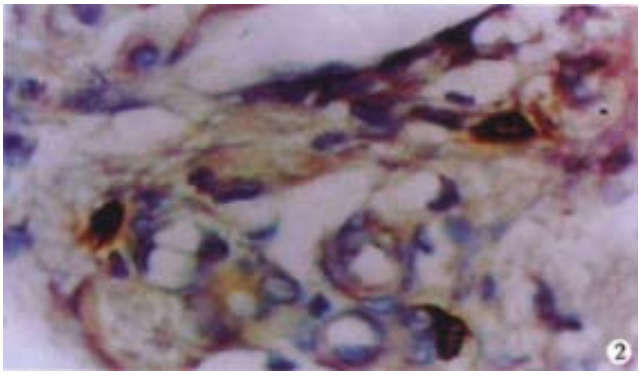
Immunohistochemistry

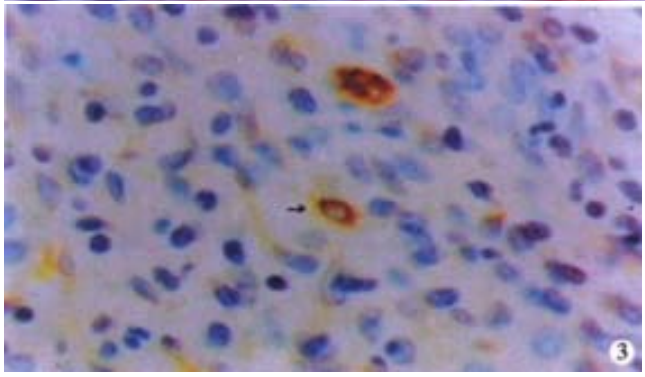
Immunohistochemical staining was performed on serial sections at room temperature, using the alkaline phosphatase method. The sections were deparaffinized in xylene and rehydrated through graded alcohol. The sections were boiled in 6M urea at 95℃ for 10 min for c-kit staining. Endogenous peroxidases were inactivated by immersing the sections in hydrogen peroxide for 10 minutes, then were incubated for 10 minutes with normal swine serum in Tris-buffered saline to block non-specific binding. The sections were subsequently incubated overnight at 4℃ with relevant antibodies (1:100 dilution respectively). The following day, the sections were incubated with biotinylated anti-mouse or anti-rabbit IgG (1:50 dilation, Maxim Biotech Inc., USA) for 45 minutes, followed by peroxidase-conjugated streptavidin (1:50 dilation, Maxim Biotech Inc.). The chromogenic reaction was developed with diaminobenzidine for 10 minutes, and all sections were counterstained with hematoxylin. Controls consisted of omission of the primary antibody.

RESULTS

Oval cells were not detected in normal liver tissue, but were detected in most liver tissues from patients with chronic viral hepatitis. Oval cells were characterized by ovoid nuclei from 7 μm × 9 μm to 12 μm×17 μm, small size, and scant cytoplasm (Figure 1). They were located predominantly in the periportal region (Figure 2) in hepatic cirrhosis, and were often found in close association with inflammatory cells in chronic active hepatitis (Figure 3). There were "transitional cells" in the parenchyma with size and structure between those of human oval cells and mature hepatocytes. They were moderately stained by CK19 antibody, had round nuclei, more cytoplasm, and were smaller in size than mature hepatocytes (Figure 4). c-kit antibody had higher sensitivity and specificity than π-GST and CK19. About 50%-70% of C-kit positive oval cells were stained positively for either π-GST or CK19. Some mature hepatocytes also expressed π-GST. Most mature bile ducts also expressed CK19.







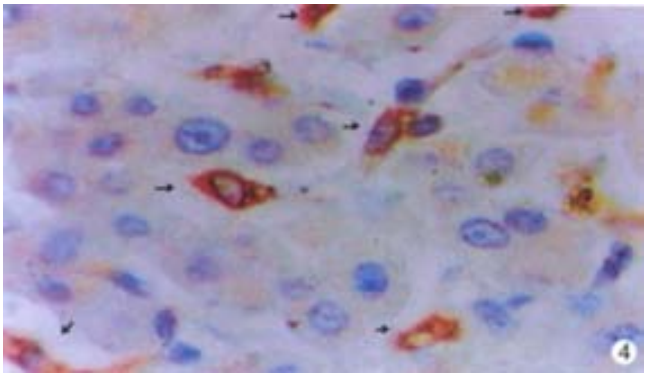


Figure 1 Oval cell identified by c-kit staining (Immunohistochemistry ABC method; original magnification: × 400)

Figure 2 Oval cells were located predominantly in the periportal region in hepatic cihrrosis (Immunohistochemistry ABC method; stained by c-kit; original magnification: × 400)

Figure 3 Oval cells were often found in close association with inflammatory cells in chronic active hepatitis (Figure 3). (Immunohistochemistry ABC method; stained by π-GST, original magnification: × 400)

Figure 4 "Transitional cells" from a patient with hepatic cirrhosis (Immunohistochemistry ABC method; stained by CK19; original magnification: × 400)

DISCUSSION

Hepatic oval cells proliferate under certain conditions, mainly when hepatocytes are prevented from proliferating in response to liver damage, and may be stem cells of hepatocytes and bile duct cells or the intermediate progeny of a hepatic stem cell[2]. Oval cells in animals are activated following administration of a variety of toxins and carcinogens alone or combined with other surgical or dietery regimens[11-19]. One of the models studied most is acetylaminofluorene treatment followed by partial hepatectomy, and an array of cytokines and growth factors have been shown to be an up-regulatory mechanism, is being delineated, for example interferon γ is implicated in orchestrating the process[20]. The oval cell itself however, probably represents the activated progeny of a dormant stem cell compartment although oval cells are readily identified in injury liver, one area of great controversy is the question of where these putative stem cells reside in the normal liver[3]. One suggestion is that they are present in the canals of Hering, that is the region where cells are transitional between the periportal hepatocytes and the biliary cells lining the smallest terminal bile duct[21]. Others suggest that there are cells which are located in the portal tracts, in the periductular region, or even that periportal hepatocytes have stem cell or metaplastic properties[2].

Oval cells are of great clinical interest since they may be the progenitor cells of both hepatocellular carcinomas and cholangio-carcinomas. Furthermore, they could be useful vehicles for *ex vivo* gene therapy for the correction of metabolic liver diseases[2]. However, identifying stem cells or their progeny in human liver has been a challenge. Oval cells, similar in morphology and antigenic profile to those seen in rodents, may be associated with many liver diseases in humans. These include submassive necrosis, focal nodular hyperplasia, primary biliary cirrhosis and primary sclerosing cholangitis, alcoholic hepatitis and cirrhosis, hepatoblastoma and HBV-associated hepatocellular carcinoma, genetic hemochro-matosis, and hepatitis C[22-30]. We detected the presence of hepatic oval cell in patients with chronic viral hepatitis. The human oval cell has a distinct morphology, with oval-shaped nuclei, small size and scant cytoplasm, and thus can be distinguished from the mature hepatocytes and inflammatory cells[1]. Oval cells express a wide variety of antigens that can be detected immunocytochemically. Although by no means specific for oval cells, many antibodies are very highly expressed, and can be used to highlight the presence of oval cells in histological sections[2].

Stem cell factor (SCF), a cytokine with structural similarity to the colony stimulating factors, was first isolated from conditioned medium of Buffalo rat liver cells[31]. Since then, it has become clear that SCF is critically important for early epithelial stem cell differentiation in hematopoiesis[32] or gamaetogenesis[33]. The corresponding receptor for SCF is encoded by the proto-oncogene c-kit, and exhibits a tyrosine kinase activity facilitated by its intracellular domain[34]. Target cells for SCF, which express c-kit, include mast cells[35], hematopoitic progenitor cells[32], and germ cells[33]. Recent studies suggest that SCF and c-kit may be involved in early growth and development of hepatic progenitor cells. Up-regulation of SCF and c-kit has been described in AAF-treated, partially hepatectomized rats, in which the carcinogen treatment inhibits the replication of hepatocytes and the induction of oval cells may be observed[19,36]. A similar increase of SCF and c-kit, accompanied by oval cell proliferation, has also been reported in bile duct-ligated rat[37]. Baumann U *et al* suggests that c-kit-positive cells may represent a hepatic progenitor cell population in normal and diseased pediatric liver[38]. In our study, we observed that some c-kit-positive cells with an oval-like morphology were present in human livers with chronic viral hepatitis, and that antibody against c-kit is useful in detecting the oval cells. We detected π-GST as a fetal GST in the majority of oval cells, supporting the view that oval cells display characteristics which resemble fetalhepatocytes or liver stem cells[29,39]. CK19n positive staining suggests that oval cells have characteristics of biliary epithelium[40]. The combined use of these antibodies and the knowledge of morphology allows us to reliably identify oval cells.

As hepatitis develops, hepatocyte necrosis is followed by an attempted secondary proliferation response of mature hepatocytes, but this proliferation response is often impaired in chronic liver diseases. Thus, oval cells may have a chance to proliferate and differentiate into hepatocytes or biliary epithelium cells[41]. We observed that oval cells were located predominantly in the periportal region, and were found in close association with fibrosis septa and inflammatory infiltrates. This location suggests that cytokines or other factors associated with the development of inflammation and fibrosis may be required to stimulate oval cell proliferation, differentiation and migration[42]. The matrix in fibrotic and cirrhotic livers is the binding site for both epidermal growth factor[43,44] and hepatocyte growth factor[45], whose receptors have been shown to be involved in proliferation of oval cells[46]. Additionally, the presence of “transitional

cells" may be evidence that oval cells are progenitors of hepatocytes.

While the debate on the source and location of hepatic stem cells is ongoing, two recent papers add a new dimension and offer a challenging alternative hypothesis to explain the origin of oval cells. By transplanting rat bone marrow into lethally irradiated recipients and following the fate of syngeneic cells using various markers, Petersen BE *et al*[47] reported striking changes in the livers of animals induced to regenerate following 2-AAF and CCl_4 treatment. Male donor marrow cells were visualized in female recipients. In a second model, marrow from dipeptidyl peptidase IV positive animals was transplanted into dipeptidyl peptidase IV deficients. In both cases, evidence was presented to suggest that the donor cells migrated into the livers of recipient animals and subsequently underwent differentiation to become hepatocytes, although it was less clear whether ductular cells of biliary phenotype developed[47]. A second recently published study describes a similar approach comprising a mouse marrow transplant model which, interestingly, did not include a liver injury step[30]. This new report provides confirmatory evidence that bone marrown derived haematopoietic stem cells can indeed give rise to hepatocytes[48]. The ability to identify and exploit a human hepatic clonal stem cell could have important clinical implications, since generating large numbers of differentiated and therefore fully functional human hepatocytes has enormous potential[49,50].

In conclusion, the presence of oval cells in human livers with chronic viral hepatitis indicates that oval cell proliferation may be one of the mechanisms in liver regeneration in this condition. The origin, growth, and differentiation of this cell is worth investigating further.

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Edited by Jason Carr

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Helicobacter pylori and gastric cancer: current status of the Austrain Czech German gastric cancer prevention trial (PRISMA-Study)

S. Miehlke[1], C. Kirsch[1], B. Dragosics[2], M. Gschwantler[3], G. Oberhuber[4], D. Antos[5], P. Dite[6], J. L uter[7], J. Labenz[8], A. Leodolter[9], P. Malfertheiner[9], A. Neubauer[10], G. Ehninger[1], M. Stolte[11] and E. Bayerdörffer[1]

Subject headings *Helicobacter pylori*; Helicobacter infections/complications; Helicobacter infections/drug therapy; stomach neoplasms/microbiology; stomach neoplasms/drug therapy; gastritis/microbiology; gastritis/drug therapy

Miehlke S, Kirsch C, Dragosics B, Gschwantler M, Oberhuber G, Antos D, Dite P, L uter J, Labenz J, Leodolter A, Malfertheiner P, Neubauer A, Ehninger G, Stolte M, Bayerdörffer E. *Helicobacter pylori* and gastric cancer: current status of the Austrian Czech German gastric cancer prevention trial (PRISMA Study). ***World J Gastroenterol***, 2001;7(2):243-247

Abstract

AIM To test the hypothesis that *Helicobacter pylori* eradication alone can reduce the incidence of gastric cancer in a subgroup of individuals with an increased risk for this fatal disease.

METHODS It is a prospective, randomized, double blind, placebo controlled multinational multicenter trial. Men between 55 and 65 years of age with a gastric cancer phenotype of *Helicobacter pylori* gastritis are randomized to receive a 7 day course of omeprazole 2 × 20 mg, clarithromycin 2 × 500 mg, and amoxicillin 2 × 1 g for 7 days, or omeprazole 2 × 20 mg plus placebo. Follow-up endoscopy is scheduled 3 months after therapy, and thereafter in one-year intervals. Predefined study endpoints are gastric cancer, precancerous lesions (dysplasia, adenoma), other cancers, and death.

RESULTS Since March 1998, 1524 target patients have been screened, 279 patients (18.3%) had a corpus dominant type of *H. pylori* gastritis, and 167 of those were randomized (58.8%). In the active treatment group (n = 86), *H. pylori* infection infection was cured in 88.9% of patients. Currently, the cumulative follow-up time is 3046 months (253. 38 patient years, median follow up 16 months). So far, none of the patients developed gastric cancer or any precancerous lesion. Three (1.8%) patients reached study endpoints other than gastric cancer.

CONCLUSION Among men between 55 and 65 years of age, the gastric cancer phenotype of *H. pylori* gastritis appears to be more common than expected. Further follow up and continuing recruitment are necessary to fulfil the main aim of the study.

[1]Medical Department I, Gastroenterology, Hematology, Oncology, Pulmonology & Infectious Diseases, Technical University Hospital Carl Gustav Carus, Dresden, Germany
[2]Gesundheitszentrum Süd, Vienna, Austria
[3]Medical Department 4, Krankenanstalt Rudolfstiftung, Vienna, Austria
[4]Institute for Pathology, General Hospital, Vienna, Austria
[5]Surgical Department, Ludwig-Maximilians niversity Hospital Groβ hadern, Munich, Germany
[6]Univerzitni nemocnice Jihlavska, Brno-Bohunice, Czech Republic
[7]Institute for Medical Statistics, Otto-von-Guericke-University, Magdeburg, Germany
[8]Department of Medicine , Evang. Jung-Stilling Hospital, Siegen, Germany
[9]Department of Gastroenterology, Otto-v.-Guericke University Hospital, Magdeburg, Germany
[10]Department for Hematology, Oncology & Immunology, University Hospital Marburg, Germany
[11]Institute of Pathology, Klinikum Bayreuth, Bayreuth, Germany
Correspondence to: Ekkehard Bayerdörffer, M.D., Medical Department I, Technical University Hospital Carl Gustav Carus, Fetscherstraβe 74, D-01307 Dresden
Tel. 0049-351-458-5645, Fax. 0049-351-458-4394
Email. bayerdoerffer@t-online.de
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INTRODUCTION

Gastric cancer is the second most common fatal malignancy in the world being responsible for at least 750 000 deaths annually[1]. Even though the incidence of gastric cancer is steadily decreasing in Western industrialized countries, the absolute number of diagnoses and deaths is likely to increase due to population growth and changing age structure of populations[2,3]. Following the first epidemiological reports of an association between *H. pylori* infection and gastric cancer in 1991[4-6], the International Agency for Research on Cancer, sponsored by the World Health Organization, categorized *H. pylori* infection as a definite human carcinogen in 1994[7]. Six years after that decision, the causal role of *H. pylori* in gastric carcinogenesis is still poorly understood, and the epidemiological data vary depending on the background prevalence of the infection and the design of the studies[8,9].

As suggested by an economical analysis based on the US data, a screen and treat strategy for *H. pylori* infection, even under conservative assumptions, may be a cost-effective strategy for gastric cancer prevention comparable to the costs of breast mammography screening programms[10]. This

strategy would become more cost-effective among populations at a higher background risk of gastric cancer [11]. However, valid prospective data on the benefit of *H. pylori* eradication for gastric cancer prevention are lacking. A non-randomized Japanese study on 132 *H. pylori* positive patients with early gastric cancer who had undergone endoscopic resection showed that after additional *H. pylori* eradication therapy there were no new occurrences of early gastric cancer during 2 years. However, among those who remained infected, there was a 9% recurrence rate of early gastric cancer[12]. Whether *H. pylori* eradication can lead to regression of gastric atrophy and intestinal metaplasia, histological risk markers for gastric cancer is still controversial[13]. The quality of endoscopic-bioptic follow-up studies of these parameters is often limited due to the confounding factor of sampling error, and due to a considerable lack of consensus among pathologists concerning the assessment of atrophy[14]. Decisive evidence that takes full account of all benefits and risks of preventive *H. pylori* eradication therapy could be derived only from adequately designed clinical trials with clearly defined study endpoints.

Recently, a corpus-dominant distribution of *H. pylori* gastritis has been recognized as a histological gastric cancer risk marker, which is frequently found not only in patients with gastric cancer at various clinical stages of disease[15,16], but also in healthy relatives of gastric cancer patients[17]. Based on histological studies, patients with a corpus-dominant *H. pylori* gastritis have an about 9-fold increased risk for gastric cancer[16]. This corpus-dominant gastritis has been termed as gastric cancer phenotype of *H. pylori* gastritis, and is the basis for the so-called "gastric cancer risk index" which has been suggested as a screening marker for *H. pylori* infected individuals in Germany being at an increasing risk for gastric cancer[18,19]. The major advantage of this screening marker is the homogenous distribution of the inflammatory response within antral or corpus mucosa which makes it less susceptible to sampling error.

In the present study, we are using this corpus-dominant phenotype of *H. pylori* gastritis as histological inclusion criteria to identify individuals with a clearly increased compared to the normal population. The aim of the present study is to investigate whether *H. pylori* eradication alone can reduce the incidence of gastric cancer in subjects with an individual high risk for gastric cancer.

PATIENTS AND METHODS

Recruitment and evaluation of patients

Gastroenterologists in hospitals and private practice in Austria, Czech Republic and Germany were asked to participate in the preparation of the study. Those who were interested were asked to obtain gastric biopsies for histological screening from all patients of the target age group who present for upper endoscopy, and to send these biopsies to one of the participating institutes of pathology.

At each endoscopic examination, two biopsies each from the antrum and the corpus were obtained according to the updated Sydney System[20]. In addition, one biopsy from the antrum and one from the corpus was taken for the rapid urease test (HUT-Test). At follow-up endoscopies, additional biopsies were obtained from macroscopically suspicious areas such as focal erythema, erosions or polyps.*H. pylori* colonization is detected by Warthin Starry. Hematoxilin & Eosin stain was used for assessment of gastritis according to the updated Sydney System[20], including the following parameters: grade and activity of gastritis, atrophy, intestinal metaplasia, lymphocytic aggregates, and degeneration of surface epithelium.

The first follow-up endoscopy was performed 3 months after treatment to determine successful eradication, and to increase the probability to detect preneoplastic changes or early gastric cancer which may have been overseen at the screening examination. Thereafter, follow-up endoscopies are scheduled in one-year intervals.

Inclusion criteria

Men between 55 and 65 years of age are eligible for inclusion in the study if they give informed consent to participate, if *H. pylori* is detected, and if the following histological criteria are present: ① grade of gastritis in the corpus $\geqslant$ grade of gastritis in the antrum; ② grade of activity of gastritis in the corpus $\geqslant$ grade of activity of gastritis in the antrum[18]. The grade and activity of gastritis in the corpus is required to be at least moderate or high according to the updated Sydney System[20].

Patients are not eligible for participation if one of the follwing criteria is present: *H. pylori*-associated diseases with strongly recommended indication for anti *H. pylori* therapy[21], type-A gastritis, a history of partial stomach resection, contraindications for biopsy sampling, present or history of malignant disease, expected residency in Germany, Austria or Czech Republic for the following five years, severe chronic disease with a survival expectancy of less than five years, benigne neoplastic lesions (adenoma, dysplasia) or early gastric cancer at the 3-month endoscopy, known allergy against the study medication.

Randomization and intervention

The randomization was carried out by the study secretariats at the University Hospitals in Dresden, Vienna and Brno. Patients receive either omeprazole 2 × 20 mg, clarithromycin 2 × 500 mg, and amoxicillin 2 × 1000 mg given for 7 days with meals[22], or omeprazole 2 × 20 mg and identically looking placebos. In case of allergy against penicilline, patients received either omeprazole 2 × 20 mg, clarithromycin 2 × 250 mg, and metronidazole 2 × 400 mg, or omeprazole plus

placebo. The trial was conducted in a double-blind fashion. Patients who did not agree to randomization received open anti *H. pylori* therapy, and were asked to return to regular follow-up endoscopies.

Statistics and ethics

The main study end points are benign neoplastic lesions (dysplasia, adenoma) in two subsequent examinations, gastric cancer, cancer of other origin, and death. The analysis is based on the intention-to-treat principle. *Chi*-square tests were used to compare categorial variables. The study was approved by the Ethics Committee of the University of Magdeburg, and by all Regional Ethics Committees in Germany, Austria and Czech Republic where patients were recruited.

RESULTS

In preparation of the trial, about 1300 gastroenterologists in Germany were informed by mail and invited to participate in the study. In addition, multiple information seminars were held in various regions of the country to explain organisatory matters, and to discuss critical questions. At initiation of the study in Germany, 500 gastroenterologists (232 in hospitals, 268 in private practice) and 201 pathologists stated interest and announced to participate in the study. However, during the first two years only 239 (47.8%) gastroenterologists became active by screening and recruiting at least one patient. Among those were 157 gastroenterologists in hospitals, and 82 doctors in private practice. Thus, between April 1998 and Dezember 2000, 1526 men at the age between 55 and 65 years were endoscopic-histologically screened, 749 in hospitals and 777 in private practice. The histological inclusion criteria was identified in 279 (18.3%) patients. Of these patients, 167 (58.8%) agreed to participate in the study, 86 were randomized to receive therapy to eradicate *H. pylori* (omeprazole plus antibiotics), while 81 were assigned to receive omeprazole plus placebo. One patient (1.2%) of the active treatment group prematurely discontinued treatment due to severe allergy. Otherwise no adverse side effects occurred leading to discontinuation of therapy. Of the 112 patients who declined randomisation and received open anti-*H. pylori* therapy, 109 stated to return to follow-up endoscopies in one to two-year intervals. Thus, 276 patients are currently under follow-up.

In the active treatment group, 54 (62.8%) patients have returned to control examinations so far. Among those, *H. pylori* infection has been cured in 48 (88.9%) patients. Those patients who did not become *H. pylori* negative did not receive a second course of anti-*H. pylori* therapy so far.

At present, 105, 50 and 24 patients have passed the 3-month, one-year, and two-year control endoscopy. Until January 2001, the cumulative follow-up time of all randomized patients was 3046 months (253.8 patient years). The median follow-up period of these patients was currently 16 months (range 3-35 months). So far, none of the patients developed gastric cancer or any preneoplastic lesion in the stomach. Three (1.8%) patients reached study endpoints other than gastric cancer. One patient of the active treatment group developed malignant melanoma 8 months after anti-*H. pylori* therapy. Two patients who were randomized to the placebo group died of myocardial infarction 6 and 7 months after therapy, respectively. Both patients had known coronary heart disease but were in stable condition at randomisation. Two patients of the placebo group received anti-*H. pylori* therapy, one of them due to active duodenal ulcer, the other patient due to persistent functional dyspepsia. Both patients, however, will continue the follow-up. Another 2 patients received cardiac surgery, one aortic valve replacement and one coronary bypass. Both patients will continue follow-up.

Table 1 Current status of the PRISMA study (initiation April 1998)

Endoscopy-histologically screened men between 55 and 65 years of age	Cases (n = 1526)	%
Presence of histological inclusion criteria	n = 279	18.3
Patients randomized	n = 167	58.8[a]
Non-randomized patients continuing follow-up	n = 109	39.1[a]
Patients with study endpoints	3	1.8[b]
Cumulative follow-up (months / years)	3046 / 253.8	

[a]Percentage of those who meet all inclusion criteria
[b]Percentage of all randomized patients.

Table 2 Currently conducted *H. pylori*-gastric cancer intervention trials with cancer endpoints

Name	Country	Target group	Age group	Sample size	Main endpoints
SCISC	China	Population	30-65	2400	Gastric cancer
NCI	China	Population	35-69	3400	Gastric cancer , dysplasia
JITHP	Japan	Population	20-59	5000	Gastric cancer
BUPA	U.K.	Population	35-69	56000	Gastric cancer
PRISMA	Germany Austria Tchech Republic	Male subjects with Corpus dominant gastritis	55-65	3000	Gastric cancer dysplasia,adenoma

DISCUSSION

Reliable epidemiological evidence on *H. pylori* and gastric cancer is still relatively sparse. Until today, only 10 seroepidemiological prospective have investigated the prevalence of *H. pylori* in a total of 800 gastric cancer cases. A combined analysis of these studies yielded a risk ratio of 2.5 for gastric cancer in people seropositive for *H. pylori* antibodies[9]. It may well be that the role of *H. pylori* in gastric cancer has been underestimated by these studies due to methodological reasons, such as poor sensitivity and specificity of serological tests available in the late 80s, or geographic variation of *H. pylori* antigens. In contrast, more recent studies indicate a much higher risk for gastric cancer in *H. pylori* infected individuals[23,24]. For example, a histology-based prevalence study in our patient

population has shown a *H. pylori* prevalence of more than 90% in patients with early gastric cancer, and a much higher relative risk for gastric cancer calculated after exclusion of other precancerous conditions such as type A gastritis[23]. Moreover, histological studies of our group have described a corpus-dominant pattern of mucosal inflammation, which is found in most *H.pylori* infected gastric cancer patients irrespective of the clinical stage[15,16], and also significantly higher in healthy relatives of gastric cancer patients compared to individuals without of family history of gastric cancer[17]: the so-called "gastric cancer risk index" which was developed on the basis of this gastric cancer phenotype of *H. pylori* gastritis[18] serves as histological inclusion criteria for the present study. A major advantage of this inclusion criteria is that it can be used to identify individuals with an increased gastric cancer risk independent of the presence of intestinal metaplasia which is always prone to sampling error. This gastric cancer risk index has recently been investigated in a Japanese patient population. In this study, a high prevalence of the gastric cancer phenotype of *H. pylori* gastritis was found in gastric cancer patients, but also with a similar frequency in control subjects[25]. In the study by Meining *et al*[18] duodenal ulcer patients were chosen as control group, because these patients rarely develop gastric carcinoma, and therefore probably would show histopathological features different from those of gastric cancer patients. In contrast, the Japanese study used gastritis patients without clinical relevant disease as control group, and were therefore unable to reproduce our previous findings.

To calculate the gastric cancer risk for target group in our study, we started from an average incidence of gastric cancer of about 62/100 000 per year in men aged 55 to 65 years[26]. Based on our data in patients with early gastric cancer[23], we calculated an incidence of 143/100 000 for *H. pylori*-positive men in this age group can be calculated. Under consideration of the histological inclusion criteria used in the present study which are found in 72% of early gastric cancer patients and in only 9% of *H. pylori* infected individuals without disease, we expect an incidence of gastric cancer as high as 1200/100 000 in our target patient group. In the present study, we observed that at least in men in the age group 55 to 65 years, the prevalence of a corpus-dominant pattern of *H. pylori* gastritis was higher than in the average population of infected individuals. By the selection of this high risk group for gastric cancer, we hope to be able to decrease the follow-up time of the study population and the number of recruited patients.

The key question is whether gastric cancer can be prevented by *H. pylori* eradication. It is worth mentioning that development of gastric cancer several years after *H. pylori* eradication has already been reported. For example, patients of the German MALT Lymphoma Trial which started in 1993 still remain under long-term endoscopic-histological follow-up[27,28]. Until today, 3 of these patients have developed early gastric cancer approximately 4 years after *H. pylori* eradication and complete remission of their lymphoma[29]. In addition, the extended follow-up of Japanese early gastric cancer patients who were treated with endoscopic mucosal resection and *H. pylori* eradication has shown that a metachronous gastric cancer may be inhibited to a great extent. However, one of these patients so far has developed a second gastric cancer four years after *H. pylori* eradication[30]. Although these reports suggest that gastric cancer may still occur after *H. pylori* eradication, they can not be transferred to the general population since those patients already had a gastric malignancy.

Relevant studies addressing the question of gastric cancer prevention by *H. pylori* eradication can be divided into those with precancerous lesions and those with gastric cancer as major study endpoint[13]. The latter studies vary considerably in their study design, but have in common that they require a much larger number of recruited patients and a longer follow-up compared with the studies using precancerous lesions as endpoints. Three of these trials are being conducted in Asian populations, while two studies, Including ours, are conducted in European populations (Table 2). The Asian studies are recruiting subjects from the general population in an area with a high population risk of gastric cancer following an endoscopy. One of the Chinese studies is also recruiting subjects to receive antioxidant micronutrients such as vitamins C and E, and therefore will be able to investigate the interaction between *H. pylori* eradication and dietary intervention in reducing the cancer risk. The British study aims to recruit the largest number of subjects with the longest follow-up, since the study is performed in a population with a relatively low gastric cancer risk. Men and women undergoing routine medical examination are randomized to testing for *H. pylori* and subsequent treatment if positive, or no testing. In contrast, the PRISMA study will assess the benefit of *H. pylori* eradication in patients with a high individual risk within a population of relatively low gastric cancer risk. By selection of a particular high-risk group it was expected to be able to reduce the necessary samplesize and the follow-up time. During the first two years of the study, however, we had to learn that at least in Germany the recruitment of subjects for this particular study-despite tremendous efforts by the principlal investigators[31-34] is extremely affected by the concerns of both patients and doctors regarding the possibility of being randomized to the control group. In our opinion, these concerns are mainly caused by the relatively broad knowledge about the association of *H. pylori* and gastric

cancer which has already been distributed by both professional and general media. Therefore, many patients expect and receive antimicrobial therapy if the infection has been diagnosed, despite the absence of strongly recommended indications for anti *H. pylori* therapy[21], and although the effect on cancer prevention is unproven. On the other hand, the certain benefit of regular control endoscopies which is the only measure to detect malignancy at a curable stage, and which would be provided by participation in the PRISMA study seems to be regarded as not acceptable or inconvenient for many patients and physicians. Due to these obvious difficulties in the recruitment of patients, it is currently planned to modify the study design and to continue without a placebo group. Although this measure will reduce the power of the study to a certain extent, it is believed that the key question can still be answered.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Development of early gastric cancer 4 and 5 years after complete remission of *Helicobacter pylori* associated gastric low grade marginal zone B cell lymphoma of MALT type

Andrea Morgner[1], Stephan Miehlke[1], Manfred Stolte[2], Andreas Neubauer[3], Birgit Alpen[3], Christian Thiede[1], Hermann Klann[4], Franz Xaver Hierlmeier[5], Christian Ell[6], Gerhard Ehninger[1] and Ekkehard Bayerd rffer[1]

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Abstract

AIM To report 3 of 120 patients on the German MALT lymphoma trial with *H. pylori* associated gastric MALT lymphoma who developed early gastric cancer 4 and 5 years, after complete lymphoma remission following cure of *H. pylori* infection.

PATIENTS AND RESULTS Three patients (two men, 74 and 70 years; one women, 77 years) with *H. pylori*-associated low-grade MALT lymphoma achieved complete lymphoma remission after being cured. Surveillance endoscopies were performed twice a year in accordance to the protocol. Four years after complete lymphoma remission in two patients, and after 5 years in the other, early gastric adenocarcinoma of the mucosa-type, type II a and type II c, respectively, was detetcted, which were completely removed by endoscopic mucosa resection. In one patient, the gastric cancer was diagnosed at the same location as the previous MALT lymphoma, in the other patients it was detected at different sites of the stomach distant from location of the previous MALT lymphoma. The patients were *H. pylori* negative during the whole follow-up time.

CONCLUSION These findings strengthen the importance of regular Long term follow-up endoscopies in patients with complete remission of gastric MALT lymphoma after cure of *H. pylori* infection. Furthermore, gastric adenocarcinoma may develop despite eradication of *H. pylori*.

[1]Medical Department I, Technical University Hospital, Fetscherstr. 74, Dresden
[2]Institute for Pathology, Klinikum Bayreuth, Preuschwitzer Str. 101, Bayreuth
[3]Centre for Internal Medicine, Universityhospital, Baldingerstr., Marburg
[4]Private Gastroenterologist, Exerzierplatz 12, Passau
[5]Med. Dept., Krankenhaus Kehlheim, Kehlheim
[6]Centre for Internal Medicine, HSK, Ludwig-Erhard-Str. 100, Wiesbaden, Germany
Correspondence to: Ekkehard Bayerd-rffer, M.D., Medical Department I, Technical University Hospital, Fetscherstr. 74, 01307 Dresden, Germany
Tel. +49-351-458-5645, Fax. +49-351-458-4394
Email. bayerdoerffer@t-online.de
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INTRODUCTION

Primary gastric low-grade marginal zone B-cell lymphoma of MALT type is a distinct disease entity with a characteristic histological presentation and clinical behaviour[1]. The role of chronic *Helicobacter pylori (H. pylori)* infection in the pathogenesis of gastric MALT lymphoma has become increasingly recognized. Thus MALT in the stomach is formed as an immunologic defense-system to control local infection caused by *H. pylori*. It is composed of antigen specific-reactive T-cells, plasma cells, some B-cells, and antigen-presenting, follicular, dendritic cells, and thus mimicing the lymphoid follicles known from other intestinal sites. Data were emerging to indicate that in fact low-grade MALT lymphomas in the stomach are a result of genetic changes probably affecting B-cells which clonally evolve from *H. pylori* related chronic gastritis[2]. There has been major progress in this area, including improvement of biopsy diagnosis, and especially, the start of a revolution in the treatment of low-grade gastric MALT lymphoma by eradicating *H. pylori* that lead to a complete remission in about 80% of cases[3-8].

Despite decreasing numbers of gastric cancer in western countries, it is still the second most common cause of death due to a malignant disease world wide[9]. Serological investigations in 1990 revealed that a high prevalence of anti *H. pylori* IgG antibodies is associated with a high incidence of gastric cancer in South America and China[10,11]. Thereafter, many case control studies reported on a 3-6 fold increased risk for gastric cancer in *H. pylori* positive individuals[12-15]. Since there are many other factors that are important for the development of gastric cancer, i.e. low vitamin C

level, gastric mucosal atrophy or hypergastrinaemia with an increase of nitroso compounds, the role of *H. pylori* is currently under investigation, thus the question if early eradication of the infection in high risk patients can prevent the development of gastric cancer[16,30].

Among the literature reviewed, only a few reports describe the development of synchronous gastric adenocarcinoma and primary gastric lymphoma[17-19]. However, only one report mentioned the probable co-factor *H. pylori* and the role of minimal invasive methods such as eradication therapy in the case of the lymphoma and endoscopic treatment for early gastric carcinomas[20]. The association of gastric lymphoma and the subsequent development of gastric adenocarcinoma is an exceptional finding, and has only been reported once with a gastric lymphoma of immunocytoma type in 1990 by a Spanish group[21].

In this paper, we reported 3 patients with *H. pylori* associated gastric MALT lymphoma who developed early gastric cancer 4 and 5 years, respectively, after complete lymphoma remission following cure of *H. pylori* infection.

PATIENTS AND METHODS

As reported earlier[5,7,22], 120 patients with primary gastric low-grade B cell MALT lymphoma in an early stage EI and *H. pylori* infection were included in the German MALT lymphoma trial starting from 1993. Cure of the infection leads to complete lymphoma remission in 79%, and partial remission in 10% of cases. Eleven percent of patients did not respond to antibiotic treatment.

Of the patients achieving complete lymphoma remission (n = 95; 79%) three developed an early gastric adenocarcinoma 4 and 5 years, respectively, after complete remission detected during routine follow-up endoscopy. Patient 1 (#1), a 74-year-old male, was included in the trial in March 1995 presenting with a gastric ulcer in the antrum and was treated in accordance to the protocol receiving antibiotic medication[5]. Four weeks later, the infection was cured, and after another 8 weeks, the patient achieved complete lymphoma remission. During the first year of follow-up, the patient was endoscoped every 3 months. Biopsy specimens were obtained each time and examined for continuous complete lymphoma remission and absent *H. pylori* colonization. Endoscopic controls were then performed twice a year. In June 1999, 4 years after complete lymphoma remission, routine control endoscopy showed a small flat elevation at the same location of the stomach where the MALT lymphoma ulcer was diagnosed 4 years earlier. Biopsies obtained from this area revealed an tubular adenoma partially transformed into a well-differentiated early gastric adenocarcinoma of the mucosa-type (m-type), 4mm in diameter, type IIa, intestinal type in accordance to the Laurén classification[23], UICC Ia (pT1G1pN × M × R0). *H. pylori* could not be detected either in the antrum or corpus mucosa. The early gastric adenocarcionoma was removed by endoscopic mucosa resection (EMR). Staging procedures revealed an adenocarcinoma of the left colon that was removed by surgery.

The second patient (#2), a 77-year-old woman, entered the MALT lymphoma trial in December 1995. Thirteen years earlier, the woman was diagnosed suffering from nodal Hodgkin's disease stage II b that was initially successfully treated with chemotherapy. In 1994, the Hodgkin's disease relapsed and the patient received another course of chemotherapy. In December 1995 she presented with a gastric ulcer at the angulus. Biopsy specimens revealed an early stage *H. pylori*-associated low-grade MALT lymphoma and she was enrolled in the study receiving antibiotic treatment. As described in patient #1, she underwent routine endoscopic control examinations after achieving complete lymphoma remission. Four years later, surveillance endoscopy revealed a complete erosion, 6 mm in diameter, opposite the ulcer scar where the MALT lymphoma was diagnosed with *H. pylori* negative. Biopsy specimens showed a well-differentiated tubular adenocarcinoma of the mucosa-type (m-type), 6 mm in diameter, type II c (according to the Japanese classification), intestinal type based on the Laurén classification[23], UICC I a (pT1G1pN × M × R0) that was removed by EMR (Figure 1).

The third patient (#3), a 70-year-old man, was diagnosed having an *H. pylori* associated low-grade MALT lymphoma in the upper corpus region in October 1994. He received antibiotic treatment and achieved complete lymphoma remission 12 months after cure of the infection. The patient was followed up in accordance to the protocol[5], and surveillance endoscopy 5 years after complete remission of the gastric MALT lymphoma revealed three flat elevations in the prepyloric antrum mucosa, approximately 5-6 mm in diameter. Endosonographic ultrasound revealed a mucosa-type tumor, and biopsies obtained during gastroscopy showed infiltrates of an intestinal type (Laurén) early gastric adenocarcinoma type II a and II c (Japanese classification), UICC Ia (pT1G1pN × M × R0). EMR was performed as well as additional argon plasma coagulation.

All histological evaluations were done by one central pathologist (M.S.). Sections were stained with H&E to grade gastritis, and Warthin-Starry stain to detect and grade colonization with *H. pylori*. Grading of the varibles of gastritis was done using the Sydney system[25], with slight modifications characterized by using a scoring system ranging from 0 = none to 4 = severe, and including the degree of replacement of foveolae by regenerative epithelium as an additional variable[26].

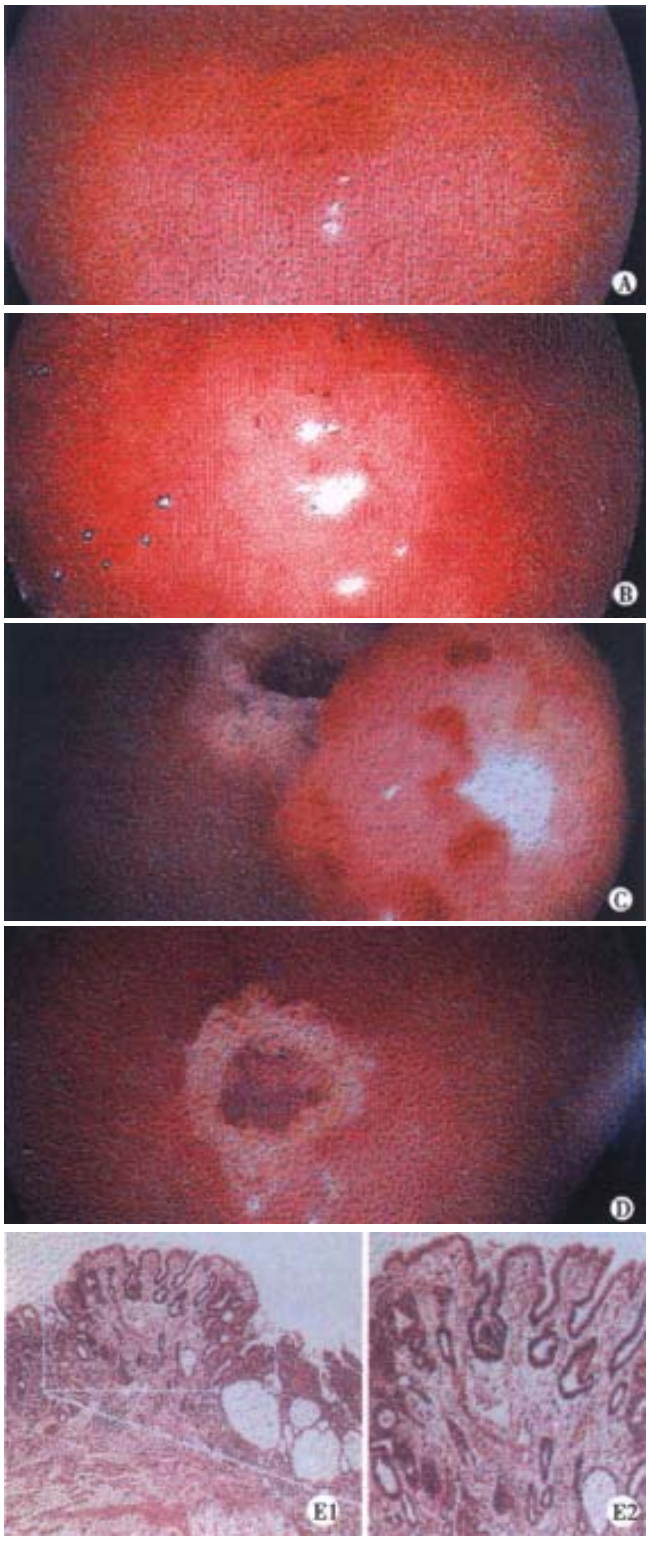

Figure 1 Endoscopic mucosa resection (EMR) in patient #2. A. Early gastric adenocarcinoma (EGC) in the antrum; ϕ 0.4cm, m-type, B. EGC in the antrum; ϕ 0.4 cm, m-type after injection of suprarenine, C. Mucosa after EMR, D. Ulcer after EMR, treatment with argon beamerplasma coagulation, E. H&E stain of the endoscopically resected mucosa showing a gastric intestinal type adenocarcinoma, restricted to the mucosa (m-type)

RESULTS

Briefly, 120 patients with primary gastric low-grade B cell MALT lymphoma in an early stage EI and *H. pylori* infection were included in the German MALT lymphoma trial starting in 1993. Cure of the infection leads to complete lymphoma remission in 79% (n = 95), and partial remission in 10% (n = 12) of cases. Eleven percent of patients (n = 13) did not respond to antibiotic treatment probably because of more advanced stages that were initially undergraded, or high-grade tumor components that were undetected[22]. Nine patients showed a lymphoma relapse, 8 local and 1 distant, 128 to 481 days after complete remission all but 1 *H. pylori* negative. The median follow-up time was 32 months.

Within the group of patients achieving complete lymphoma remission (n = 95; 79%) three patients aged 70-77 years, two men and one man, developed an early gastric adenocarcinoma 4 and 5 years, respectively, after complete remission detected during routine follow-up endoscopy, comprising an incidence rate of 3.15%. Based on the median follow-up time of 48 months, the annual risk for the development of a gastric adenocarcinoma following complete remission of gastric MALT lymphoma is 0.78%. In all patients, minimal invasive methodssuch as eradication therapy in lymphoma, diagnosed early and endoscopic treatment for early gastric carcinoma was employed (Figure 1). Endoscopic treatment of the carcinoma with EMR resulted in R0 resection of the cancer, and a follow-up examination 6 months after EMR in patients #1 and #2 revealed no remnant carcinoma infiltrates.

At the time of diagnosis of gastric adenocarcinoma, none of the patients showed *H. pylori* infection. In two patiens, the carcinoma was diagnosed near an area with intestinal metaplasia and signs of focal atrophy (Figure 2).

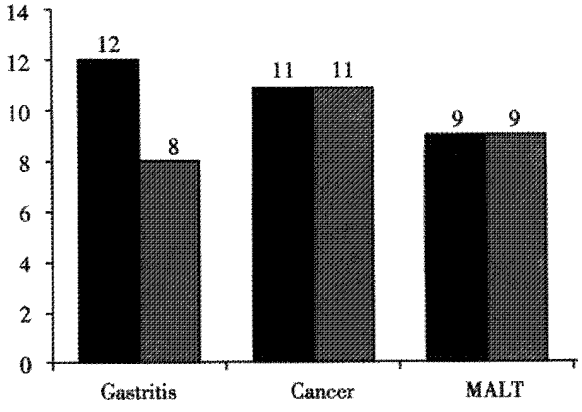

Figure 2 Medians of the summed gastritis score in antrum and corpus, formed by adding together the respective values of grade and activity of gastritis, replacement of foveolae by regenerative epithelium, and density of *H. pylori* colonization as presented in Reference 24.

DISCUSSION

The association of primary gastric MALT lymphoma and the subsequent development of gastric adenocarcinoma is an exceptional finding. In the review of the literature, we have found only 1 case of this subsequent association with an immunocytoma type gastric lymphoma[21]. The patient was treated with subtotal gastrectomy and adjuvant radiotherapy and developed an adenocarcinoma of the gastrojejunal anastomosis. As no one looked for *H. pylori* infection at that time the implicated factors in the development of this association are gastric resection and radiotherapy, and as it was also speculated, the non-Hodgkin's lymphoma itself. Reports on simultaneous MALT-type lymphoma and early adenocarcinoma of the stomach are more frequent[17-20]. It was speculated for a long time that patients with Hodgkin's disease and nodal non-Hodgkin's lymphoma seem to have an excess risk for other cancers, and a high incidence of other cancers (20%) has also been found in some series of patients with gastric MALT lymphomas[27]. Montalbán and co-workers[28]were able to show that in patients with gastric MALT lymphoma, other cancers do occur, but that there is no increase in risk above the background population. Among 136 patients with gastric MALT lymphoma, other cancers were detected in 11.7%, either prior to the diagnosis of MALT lymphoma, concomitantly or after diagnosis. Of all 136 patients investigated, only 53 had not received chemotherapy, radiotherapy or surgery as implicated factors, and of those, 2 (3.7%) patients developed a gastric adenocarcinoma. This cumulative incidence is in line with the cumulative incidence we report in this paper, that is 3.15% (3/95 patients).

The observation of concurrent or subsequent gastric adenocarcinoma and MALT lymphoma is of interest as both are etiologically related to *H. pylori*. For gastric MALT lymphoma, the causative association with this chronic stimulus is well established, and cure of the infection can lead to complete remission of early lymphoma in about 80% of cases[5,7,8]. As indicated earlier, serological investigations demonstrated a high prevalence of anti *H. pylori* IgG antibodies in an area with a high incidence of gastric cancer[10,11], and many case control studies reported on a 3-6 fold increased risk for gastric cancer in *H. pylori* positive individuals[12-15]. In one report analysing synchronous adenocarcinoma and low-grade B-cell MALT lymphoma, *H. pylori* was seen in 7 (78%) of 9 cases which is consistent with an etiological role for this organism in both tumors in the stomach[18].

Primarily on the basis of this epidemiological evidence, *H. pylori* has been classified as a definite human carcinogen in 1994[29]. Although several pathophysiological mechanisms have been identified which may contribute to the development of gastric carcinoma, the role of *H. pylori* eradication for disease prevention is currently under investigation[16, 30]. The aim of these studies is to test the hypothesis that *H. pylori* eradication alone can reduce the incidence of gastric cancer in a subgroup of individuals with an increased risk for developing gastric adenocarcinoma. A non-randomized Japanese study on 132 *H. pylori* positive patients with early gastric cancer who had undergone endoscopic mucosa resection showed that after additional *H. pylori* eradication therapy, no new early gastric cancer occurred during 2 years. Another 2 years later, i.e. 4 years after eradication therapy, one of these patients so far has developed a second gastric cancer. However, among those who remained infected, there was a 9% recurrence rate of early gastric cancer[33, 34]. Whether *H. pylori* eradication can lead to regression of gastric atrophy and intestinal metaplasia, histological risk markers for gastric cancer, is still controversial[16]. Based on all these evidences and, hence, the idea of a possible prevention of the development of gastric adenocarcinoma by curing chronic *H. pylori* infection, it is surprising that 3 patients within our MALT lymphoma trial developed gastric carcinoma despite eradication of the bacterium 4 and 5 years earlier in the scope of treating the gastric MALT lymphoma. These data may indicate that *H. pylori* eradication alone might not be sufficient to prevent cancer. However, it might have been successful, if cure of the infection probably occurred earlier in life. The timing of treatment might be crucial for the determination if the development of gastric adenocarcinoma can be prevented by *H. pylori* eradication alone. Although our report suggests that gastric cancer may still occur after *H. pylori* eradication, these data can not be transferred to the general population since those patients already had a gastric malignancy.

Based on the findings that MALT lymphoma patients, in fact, do not have an increased risk of additional neoplasms, there is no need to invoke or be concerned about general genetic instability of the host as a possible underlying mechanism for lymphoma-and/or carcinogenesis. Differences in the characteristics of *H. pylori* strains as well as different environmental factors in the infected hosts seem also being involved in the variable outcome of the infection[31,32]. Recently, a corpus-dominant distribution of *H. pylori* gastritis has been recognized as a histological gastric cancer risk marker, and studies concerning the degree and distribution of *H. pylori* have shown significant differences among *H. pylori* associated diseases[24]. As it was demonstrated by Meining *et al*[24], in both malignant diseases investigated, gastric

adenocarcinoma and gastric MALT lymphoma, the extent of gastritis was equal in the corpus and in the antrum mucosa. Thus both malignant diseases differ from the benign gastritis only in the extent of the gastritis in the antrum and corpus part of the stomach. Furthermore, the overall extent of gastritis in MALT lymphoma patients was not as severe as in patients who had a carcinoma or gastritis only. Gastric atrophy was much more frequent in gastric cancer patients than in MALT lymphoma patients (42% vs 6% in the antrum mucosa, 18% *vs* 4% in the corpus mucosa)[24]. The three patients with gastric adenocarcinoma presented here also have a gastritis equally expressed in the corpus and antrum mucosa but of a milder degree. The carcinomas diagnosed were found near or within intestinal metaplasia in two cases of consecutive atrophy, the histological risk markers for early gastric cancer. The exact reasons for the differences in distribution and degree of gastritis in different *H. pylori* associated diseases have not yet been fully understood. It seems possible, that patients developing a minimal to mild gastritis, equally distributed in the antrum and corpus mucosa are likely to have a higher risk of developing a gastric MALT lymphoma.

Our study population included 56 men and 64 women with a median age of 65 years (a range of 29-88 years). The three patients developing a gastric adenocarcinoma were 70, 74, and 77 years old, respectively. The incidence of cancer in the general population increases continuously with age, affected by changing patterns of population screening and intensity of attempts at cancer detection, and is about 120 for men and 50 for women in this age group in Germany. The relatively high median age in our MALT lymphoma study group is therefore, independently of other risk factors, associated with a high cumulative incidence of cancers. Appreciating the apparent increase in cancer incidence brought about by better screening methods and earlier detection, and comparing our results to a defined general population with accurately known cancer incidences, there is still to state that the association of primary gastric MALT lymphoma and the subsequent development of gastric adenocarcinoma is an exceptional finding, and that *H. pylori* gastritis must be considered to be associated with the development of these two malignancies with an approximately 6-7 fold increase of the overall incidence.

All cancers of our three patients were detected during routine surveillance endoscopy. Endoscopy was performed in each patient twice yearly in accordance to the MALT lymphoma protocol since these patients have achieved complete lymphoma remission after cure of *H. pylori* infection. Early gastric adenocarcinoma was detected 4 and 5 years after complete remission of lymphoma. Due to the early stage diagnosis of the gastric cancer, endoscopic therapy (mucosal resection = EMR) was successfully performed in each case. In contrast to most cases of synchronous MALT lymphoma and gastric carcinoma, the definite diagnosis of gastric lymphoma and carcinoma was obtained preoperatively. As it has been also stated by Müller *et al*[20], this seems to be in future times an essential prerequisite for employing minimal invasive methods such as eradication therapy in the case of the diagnosed early lymphoma and endoscopic treatment for early gastric carcinomas. This management of the diseases seems to be benefical and effective especially with regard to the life quality of the patients. However, to apply this treatment strategy, it is important to perform regular long-term follow-up endoscopies in patients with complete remission of gastric MALT lymphoma after cure of *H. pylori* infection.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Virulence and potential pathogenicity of coccoid *Helicobacter pylori* induced by antibiotics

Fei Fei She[1], Dong Hui Su[1], Jian Yin Lin[2] and Lin Ying Zhou[3]

Subject headings *Helicobacter pylori*/drug; *Helicobacter pylori*/pathogenicity; antibiotics/pharmacology; polymerase chain reaction

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Abstract

AIM To explore the virulence and the potential pathogenicity of coccoid *Helicobacter pylori* (*H. pylori*) transformed from spiral form by exposure to antibiotic.

METHODS Three strains of *H. pylori*, isolated from gastric biopsy specimens of confirmed peptic ulcer, were converted from spiral into coccoid from by exposure to metronidazole. Both spiral and coccoid form of *H. pylori* were tested for the urease activity, the adherence to Hep 2 cells and the vacuolating cytotoxicity to Hela cells, and the differences of the protein were analysed by SDS-PAGE and Western blot. The mutation of the genes including ureA, ureB, hpaA, vacA and cagA, related with virulence, was detected by means of PCR and PCR-SSCP.

RESULTS In the coccoid *H. pylori*, the urease activity, the adherence to Hep 2 cells and the vacuolating cytotoxicity to Hela cells alldecreased. In strain F44, the rate and index of adherence reduced from 70.0% ± 5.3% to 33% ± 5.1% and from 2.6 ± 0.4 to 0.96 ± 0.3 ($P<0.01$), respectively. The invasion of coccoid *H. pylori* into Hep 2 cell could be seen under electronmicroscope. SDS-PAGE showed that the content of the protein with the molecular weight over Mr 74 000 decreased, and the hybridiotional signal in band Mr 125 000 weakened, while the band *M*, 110 000 and Mr63 000 strengthened in coccoid *H.pylori* as shown in Western blot. The results of PCR were all positive, and PCR-SSCP indicated that there may exist the point mutation in gene hpaA or vacA.

CONCLUSION The virulence and the proteins with molecular weight over Mr74 000 in coccoid *H.pylori* decrease, but no deletion exists in amplification fragments from ureA, ureB, hpaA, vacA and cagA genes, suggesting that coccoid *H.pylori* may have potential pathogenicity.

[1]Department of Microbiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China
[2]Department of Molecular Medicine, Fujian Medical University, Fuzhou 350004, Fujian Province, China
[3]Laboratory of Electron Microscope, Fujian Medical University, Fuzhou 350004, Fujian Province, China
Fei Fei She, graduated from Fujian Medical University as a postgraduate in 1991, now associate professor of microbiology and immunology, specialized in molecular biology of pathogen, having 15 papers published.
Supported by the Natural Science Foundation of Fujian Province, China, No. 95A003
Correspondence to: Fei Fei She, Department of Microbiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China
Tel. 0086-591-3569309
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INTRODUCTION

Helicobacter pylori is an important pathogen that causes chronic gastritis and peptic ulcer and may be a risk factor for gastric carcinoma[1-9]. The organism exists in two forms, a spiral form and a coccoid form. The coccoid *H.pylori*, which can be induced by increased oxygen tension, extended incubation and exposured to antibiotics[10-14], is nonculturable but alive [12,15-17], and could be induced to revert to a virulent spiral form *in vivo*[11]. Therefore, the coccoid *H. pylori* has been suspected to play a role in the transmission of the bacteria and to be partly responsible for recrudescence of infection after antimicrobiol treatment. However, the pathogenesis of coccoid *H.pylori* remains unclear. In this study, three strains of coccoid *H.pylori* transformed from spiral form by exposure to metronidazole, were tested for the urease activity, the adherence to Hep-2 cells and the vacuolating cytotoxin activities to Hela cells, the differences of the protein were analysed by SDS-PAGE and Western blot, and mutation of the genes involving ureA, ureB, hpaA, vacA and cagA was detected by means of PCR and PCR-SSCP, in order to explore the virulence and the potential pathogenicity of coccoid *H.pylori*.

MATERIALS AND METHODS

Bacterial strains

Three strains (F_{44}, F_{45} and F_{49}) of *H.pylori* were isolated from gastric biopsy specimens of confirmed peptic ulcer patients. The isolates were spiral shape, positive for catalase, oxidase, urease, and cagA and vacA gene. Stock cultures were maintained in defatted milk at -80℃.

Cells

The Hep-2 cells and Hela cells were maintained in

1640 medium supplemented with 10% fetal calf serum, 200 IU/mL penicillin and 50 μg/mL streptomycin at 37℃ in 5% CO_2-95% air, and recultivated once or twice a week.

Cultivation of H.pylori and induction of coccoid forms

The stored strains of *H.pylori* were cultivated on Brucella agar with 5% sheep blood at 37℃ for 2-3 days under microaerophilic conditions (5% O_2; 10% CO_2; 85% N_2). After subculturing, the bacteria were harvested and suspended in Brucella broth with 10% fetal calf serum, and the suspension was divided into two parts, one as spiral *H.pylori*, the other added with metronidazole at a concentration of 1/2 of MIC values (MIC = 32 mg/L-64 mg/L) at 37℃ under the microaerophilic conditions for a few days until coccoid forms reaching 100%(about 2-3 days).

Examination of urease activity

According to the manufacturer's instructions of the kit detecting fastly urease activity (Sanqiang Company), the suspension of *H.pylori* (10^{10} cfu/mL) was added into the testing well in volume of 5 uL, and on the basis of reacting colors, the urease activity was divided into five grades ("+++ +" "+++", "++","+"and "-").

Assay for adherence to Hep-2 cells

To assay bacterial adherence, Hep-2 cells were grown to confluence on coverslips in culture flask, and the suspension of *H.pylori* (10^8 cfu/mL) were added in a total volume of 0.5mL for Hep-2 cultures and allowed to adhere for 3.5 h at 37℃ in 5% CO_2 95% air. Cultures were washed and stained with Wright-Giemsa stain, and both the amount of cells adhereed by bacteria and bacteria adhereing to cells were counted among one hundred cells under the light microscope. In addition, the culture with strain F_{44} was scraped using a glass club, centrifuged, and the pellet was embedded in Epoxy 618, then the ultrathin sections were cut and examined under a Hu-12A transmission electron microscope.

Assay for cytotoxicity to Hela cells

To assay vacuolating cytotoxicity of *H.pylori*, Hela cells were grown to confluence in 96-well plates (2×10^4 cells/well at the time of infection), the suspension of bacteria were swung, centrifuged, and the cell-free supernatants were concentrated 20-fold using polyethylene glycol M_r 20 000, then sterilized by passage through a 0.25 μm pore-size filter. The concentrated supernatants at the consistency shown in Table 4 were added in a total volume of 0.1 mL to Hela cells, and Brucella broth served as a negative control. After incubation for 48 h, cells were stained and observed under phase microscope. Wells, in which 50% or more cells were vacuolated, were defined as showing a cytotoxic effect[18].

SDS-PAGE of whole cell proteins

The suspension of *H.pylori* (6×10^{10} cfu/mL) were centrifuged, washed once with 0.5 mol/L Tris (pH 7.4), and the pellets were suspended in extraction buffer consisting of 0.75% Tris, 2% sodium dodecyl sulfate, 5% dithiothreitol, 10% glycerol and 0.1% bromophenol blue. The homogenate was heated for 5 min in a boiling water bath and frozen at -20℃ until use. According to Sambrook J *et al*[19], the protein solutions (10 μL) were electrophoresed on SDS-polyacrylamide gels with the stacking and the separating gels containing 5% and 10% acrylamide, respectively, and the gels were stained with Coomassie blue.

Western blot

According to Sambrook J *et al*[19], after SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane under a constant current of 100 mA for 7 h, and the antigenic profiles were studied by Western blotting using serum from the patients infected with strain F_{44} (diluted at 1/50).

PCR and PCR-single-strand conformational polymorphism (PCR-SSCP)

PCR primers and the size of the corresponding PCR products are shown in Table 1[20-22]. The suspension of bacteria were centrifuged, and the pellets were resuspended in distilled water(10^5 cfu/mL), heated in a boiling water bath for 1min to obtain the DNA template, which was added into PCR reaction system in volume of 1/5. PCR was performed as follows: denaturation at 95 for 5 min, followed by 30 cycles of denaturation (94℃ for 0.5 min);annealing (52℃ for 1 min); and extension (72℃ for 1 min), and final extension at 72℃ for 7 min. The PCR products were electrophoretically separated on 0.2% agarose gel, and stained with ethidium bromide.

Table 1 Oligonucleotide primers used for PCR reactions

Gene amplified	Primer sequence	Size of PCR product
ureA	HPU_1:5'-GCCAATGGTAAATTAGTT-3'	411bp
	HPU2:5'-CTCCTTAATTGTTTTTAC-3'	
ureB	HPU_{55}:5'-AATTGCAGAAATATCAC-3'	115bp
	HPU_{17}:5'-ACTTTATTGGCTGGTTT-3'	
hpaA	$HPYLO_1$:5'-GAATTACCATCCAGCTAGCG-3'	375bp
	$HPYLO_2$:5'-GTAACCTTGACAAAACCGGC-3'	
vacA	VA_1F:5'-ATGGAAATACAACAAACACAC-3'	259bp
	VA_1R:5'-CTGCTTGAATGCGCCAAAC-3'	
cagA	F_1:5'-GATAACAGGCAAGCTTTTGAGG-3	349bp
	B_1:5'-TCTGCCAAACAATCTTTTGCAG-3'	

ureA: urease gene A; ureB: urease gene B; hpaA: *H.pylori* adhesin gene A; vacA: vacuolating cytotoxin geneA; cagA: cytotoxin-associated gene A.

SSCP was performed as follows: the mixture consisting of 5 μL of PCR product and 3 μL of loading buffer (95% formamide, 200 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was heated at 100℃ for 5 min to denature double-stranded DNA and then plunged into ice for 5 min, and electrophoresed on 50% polyacrylamide gel under a constant voltage of 300v for 7 h, the gel was stained with 0.2% silve nitrate.

Statistical analysis

Analysis of data was performed using the Student *t* test. A value of $P < 0.05$ was regarded as statistically significant.

RESULTS

Urease activity

As shown in Table 2, the urease activity of coccoid *H. pylori*, which was transformed from spiral form by exposure to antibiotic in subinhibitory concentrations, decreased.

Table 2 Urease activity of*H.pylori*

Strain	Urease activity	
	Spiral form	Coccoid form
F_{44}	++++	+
F_{45}	++++	++
F_{49}	++++	+

Adherence to Hep-2 cells

According to the following formula, the rate and the index of adherence were calculated:

The rate of adherence = the amount of cell adherenced by bacteria/100×100%

The index of adherence = the amount of bactria adhereing to cells/100

For each coverslip five-fields (one field containing of one hundred cells) were counted, and the mean of all fields is shown in Table 3. As compared with the spiral forms ($P < 0.01$), the adherence of coccoid forms to Hep-2 cells decreased. In addition, invasion of coccoid *H. pylori* into cell could be seen under electron microscope (Figure 1).

Vacuolating activity to Hela cells

As shown in Table 4, vacuolating activity of coccoid *H. pylori* to Hela cell impaired after Hela cells were incubated with different concentrations of supernatants of coccoid *H.pylori* for 48 h.

Table 3 Adherence test of *H.pylori* to Hep-2 cells

	Rate of adherence			Index of adherence		
	F_{44}	F_{45}	F_{49}	F_{44}	F_{45}	F_{49}
Spiral form	70.0 ± 5.3	73.0 ± 5.1	72.6 ± 4.5	2.60 ± 0.4	3.1 ± 0.5	2.90 ± 0.4
Coccoid form	33.0 ± 4.3	40.1 ± 3.7	35.5 ± 4.1	0.96 ± 0.3	1.0 ± 0.3	0.98 ± 0.4
t	12.1	11.7	13.6	7.5	8.1	7.6
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 4 Vacuolating activity of *H.pylori* to Hela cells

Dilution of concentrated supernatants	Spiral form			Coccoid form		
	F_{44}	F_{45}	F_{49}	F_{44}	F_{45}	F_{49}
No dilution	+	+	+	-	-	-
1:10	+	+	+	-	-	-
1:20	+	+	+	-	-	-
1:40	+	-	+	-	-	
1:80	-	-	-	-		-

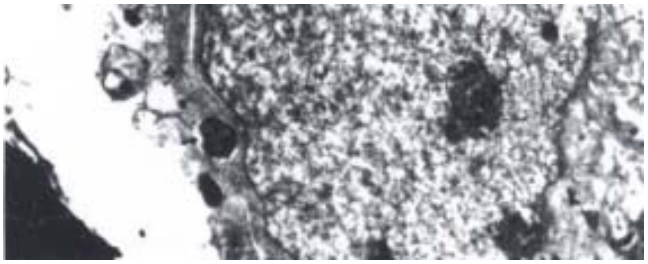

Figure 1 Invasion of coccoid *H.pylori* into Hep-2 cell. Transmission electron microscope, × 15 000

SDS-PAGE and Western blot

SDS-PAGE patterns are illustrated in Figure 2A. The content of the proteins with molecular weight over Mr 74 000 decreased, especially the band at Mr 125 000 was presented with deletion in coccoid *H. pylori*. The protein patterns of the three strains of coccoid *H. pylori* were similar. Western blot patterns showed that the hybridiotional signal in band M_r 125 000 weakens, meanwhile, strengthens in band M_r 110 000 and M_r 63 000 in all coccoid *H. pylori* as illustrated in Figure 2B.

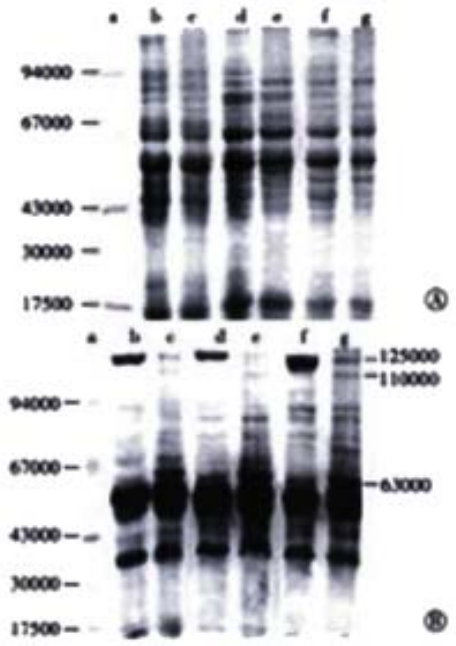

Figure 2 A. SDS-PAGE pattern of whole cell proteins of *H. pylori*, B. Western blot pattern of the proteins of *H. pylori*.
a. marker; b.d.f. spiral forms of *H.pylori*F_{44}, F_{45} and F_{49}, respectively; c.e.g. coccoid forms of *H.pylori* F_{44}, F_{45} and F_{49}, respectively.

PCR and PCR-SSCP

The result of PCR for strain F_{44} is illustrated in Figure 3. The genes detected by PCR, which included ureA, ureB, hpaA, vacA and cagA, were all positive in both spiral and coccoid *H.pylori*. The patterns of SSCP showed that there could exist point mutation in vacA gene of strain F_{44} and F_{45}, as well as in hpaA gene of strain F_{49} in coccoid forms. The pattern of SSCP for strain F_{44} is illustrated in Figure 4.

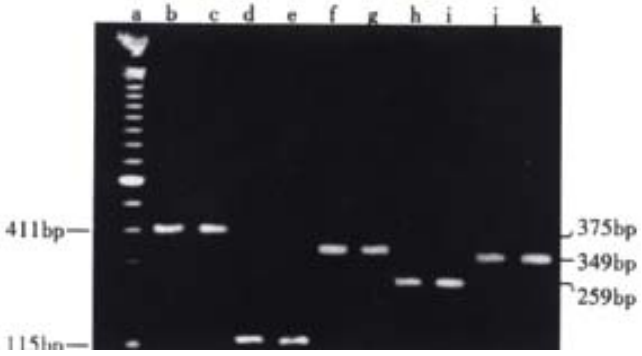

Figure 3 The results of PCR of *H.pylori* F_{44}.
a. PCR marker 100bp lader; b.c. ureA gene; d.e. ureB gene; f.g. hpaA gene; h.i. vacA gene; j.k. cagA gene; b.d.f.h.j. spiral forms of *H.pylori*; c.e.g.i.k. coccoid forms of *H.pylori*.

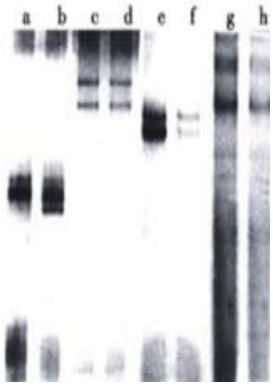

Figure 4 The pattern of PCR-SSCP of *H.pylori* F_{44}.
a.b. vacA gene; c.d. ureA gene; e.f. cagA gene; g.h. hpaA gene; a.c.e.g. spiral forms of *H. pylori*; b.d.f.h. coccoid forms of *H.pylori*.

DISCUSSION

H.pylori can convert into coccoid forms after induced by antibiotics *in vitro* or *in viro*[12,23,24]. It has been reported that coccoid *H.pylori* is able to colonize and to produce gastric alterations in the suitable animal model[11]. Costas M *et al*[25]compared the pre-with post-treatment isolates of *H.pylori* from the same patients by using 1-D SDS PAGE of proteins and considered that recurrent patients were not reinfected with a different strain but that there was recrudescence of the pre-treatment strain. Thus, it is reasonable to suppose that the viability of the coccoid form may account for the wide number of relapses in patients. However, as yet, the pathogenesis of the coccoid form remains unclear.

The putative pathogenic determinants of *H.pylori* can be divided into two major groups[26]: maintenance factors, which allow the bacterium to colonize and remain within the host, and virulence factors, which contribute to the pathogenetic effects of the bacterium. Both urease activity and adherence to epithelia cells of *H.pylori* are important maintenance factors[27-30]. In this study, It is shown that both urease activity and adherence to Hep-2 cell of coccoid *H.pylori* decreased, suggesting that virulence related to colonization in coccoid *H.pylori* reduced. Vacuolating cytotoxin produced by about 50%-60% of *H.pylori* strains[18,31-33] is one of the important virulence factors[34]. Infection with cytotoxin-producing *H.pylori* strains was more prevalent among patients with peptic ulcer disease[35-37]and gastric carcinoma[18,38-42] than among patients with gastritis alone. In our study, the decrease of vacuolating cytotoxicity in coccoid *H. pylori* was found, which may be related with the reduction of the volume of M_r 87 000 VacA and M_r 125 000 CagA proteins determining vacuolating cytotoxicity [34,43] by SDS-PAGE. These findings indicate that the coccoid *H.pylori* is less efficient in the colonizational virulence and vacuolating cytotoxicity,which may make it unlikely to induce an inflammatory response. Thus the alleviation of clinical symptom of the patients after antimicrobiol treatment does not necessarily mean eradication of *H.pylori*, it may also result from the convertion to coccoid form. As shown in the assay for adherence, a few coccoid *H.pylori* still adhered to Hep-2 cells, even invated into them, which indicates that coccoid *H.pylori* is likely to sustain in the host, thus making the recrudescence of infection possible.

In order to display the hereditary background of coccoid *H.pylori*, the genes related with virulence mentioned above (involving ureA, ureB, hpaA, vacA and cagA) were detected by PCR and PCR-SSCP. No deletion was in these genes, but there only existed the point mutation in genes hpaA or vacA. These data demonstrate that the coccoid *H.pylori* may revert into an infectious spiral form under the appropriate conditions and result in recudescence of infection, suggesting that coccoid *H.pylori* may have potential pathogenicity.

According to the features of coccoid *H.pylori*, it may escape the techniques usually applied for their detection such as cultivation and assay for urease activity. Because of no deletion in genes mentioned above in coccoid *H.pylori*, we can use PCR to detect these genes instead of conventional methods to determine whether the bacteria have been completely eliminated after treatment.

Some studies showed that the synthesis of some proteins of coccoid *H.pylori* increased such as 62KDa and >94KDa proteins[16,44,45]. In this study, the results of Western blot showed that the antigenic fraction with molecular weight in M_r 110 000 and M_r 63 000 was detected more intensively in all three strains of coccoid *H.pylori*, as compared with

spiral *H.pylori*. The fractions may be the one degraded from a high-molecular-mass antigen. Further studies are required to determine whether these antigenic proteins have special functions.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Stress kinase inhibition modulates acute experimental pancreatitis

F. Fleischer, R. Dabew, B. Göke and ACC Wagner

Subject headings pancreatitis, acute necrotizing/ enzymology; cerulein/therapeutic use;Ca^{2+} calmodulin dependent protein kinase/metabolism; rats

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Abstract

AIM To examine the role of p38 during acute experimental cerulein pancreatitis.

METHODS Rats were treated with cerulein with or without a specific JNK inhibitor (CEP1347) and/or a specific p38 inhbitor (SB203580) and pancreatic stress kinase activity was determined. Parameters to assess pancreatitis included trypsin, amylase, lipase, pancreatic weight and histology.

RESULTS JNK inhibition with CEP1347 ameliorated pancreatitis, reducing pancreatic edema. In contrast, p38 inhibition with SB203580 aggravated pancreatitis with higher trypsin levels and, with induction of acinar necrosis not normally found after cerulein hyperstimulation. Simultaneous treatment with both CEP1347 and SB203580 mutually abolished the effects of either compound on cerulein pancreatitis.

CONCLUSION Stress kinases modulate pancreatitis differentially. JNK seems to promote pancreatitis development, possibly by supporting inflammatory reactions such as edema formation while its inhibition ameliorates pancreatitis. In contrast, p38 may help reduce organ destruction while inhibition of p38 during induction of cerulein pancreatitis leads to the occurrence of acinar necrosis.

Department of Gastroenterology, Inselspital, University of Bern, Switzerland
Correspondence to: Andreas C.C. Wagner, Department of Gastroenterology, Inselspital, University of Bern, CH-3010 Bern, Switzerland
Tel. 0041/(0)31/6328025, Fax. 0041/(0)31/6329765
Email. Awagner@dkf4.unibe.ch
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INTRODUCTION

Acute pancreatitis is a common gastrointestinal disorder but remains enigmatic in its unpredictable clinical course ranging from mild to very severe, and life threatening pancreatitis. Due to our limited understanding of its underlying pathophysiology, the treatment of acute pancreatitis is still confined to general supportive measures with no causal approach. In order to better understand the pathophysiology of pancreatitis, we characterized the molecular mechanisms of the pancreatic stress response.

Members of the mitogen-activated protein kinase (MAPK) cascade are considered to play key roles in signal transduction pathways activated by a wide range of stimuli[1]. The three best characterized members of this growing family of serine/threonine kinases are extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. While ERK responds vigorously to growth factors and certain hormones, JNK and p38 are rather activated by stress stimuli and are widely believed to be part of the cellular stress response machinery[1-5]. Activation of these kinases requires phosphorylation of both tyrosine and threonine residues by upstream dual specific kinases. Active MAPK are responsible for the phosphorylation of a variety of effector proteins including several transcription factors[1].

In the pancreas, it has been shown that hyperstimulation with the cholecystokinin (CCK) receptor agonist cerulein, which induces acute pancreatitis in rats, can activate JNK in rat pancreas[6,7]. We and others have recently found that p38 is also expressed in the pancreas and rapidly activated by cerulein[8-10]. Due to the pattern and time course of activation in response to a variety of different secretagogues, JNK has been proposed as an important mediator early during cerulein-induced pancreatitis[6,10]. In addition, we have also shown that treatment with CEP1347, a specific inhibitor of the JNK pathway[11,12], ameliorates cerulein pancreatitis[13].

However, no data on the effects of p38 inhibition on the course of experimental pancreatitis is available so far. There is evidence that p38 may actually promote the protection through influencing HSP27 phosphorylation and actin stabilization[8,14]. Therefore, we have studied the effects of p38 inhibition with SB 203580 on the course of cerulein

pancreatitis, and compared the effects of p38 inhibition to those of JNK inhibition as well as inhibition of both kinases simultaneously. Our data show that both kinases influence pancreatitis differentially in that JNK inhibition ameliorates pancreatitis while p38 inhibition aggravates cerulein pancreatitis, resulting in the development of necrosis.

MATERIAL AND METHODS

Chemicals and antibodies

Sodium dodecyl sulfate (SDS), polyacrylamide, molecular weight and isoelectric focusing standards were products of BioRad (Hercules, CA, USA); nitrocellulose membranes were obtained from Schleicher and Schuell (Keene, NH, USA). All other chemicals were from Sigma (St. Louis, MO, USA). CEP-1347 was kindly provided by Cephalon Inc (West-Chester, PA, USA).

Preparation and treatment of pancreatic acini

Acini were prepared as previously described[15]. Briefly, pancreata from white male Sprague Dawley rats were digested with purified collagenase and dispersed by pipetting through polypropylene pipettes of decreasing orifice, followed by filtration through a 150 μm nytex screen. Acini were purified by centrifugation through 4% bovine serum albumin (wt/vol) and then preincubated for 30 min at 37℃ in N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES)-buffered Ringer solution (HR), pH 7.4, supplemented with 11.1 mM glucose, minimal Eagle's medium amino acids, 5 g/L bovine serum albumin, and 0.1 g/L soybean trypsin inhibitor. Buffers were gassed with 100% O_2. Acini were then kept resting at room temperature for 2 hours with and without 20 μM CEP-1347 and/or 50 μM SB203580. Incubation at 37℃ was then continued and acini stimulated with the indicated concentrations of cerulein. After 30 min stimulation, acini were pelleted and homogenized for assessement of MAPK, JNK and p38 activity in kinase/IP buffer, containing 50 mM glycerophos-phate, 2 mM Na_3V0_4, 1 mM each of NaF, EGTA, EDTA, DTT, PMSF, benzamidine, 10 mg/L each of aprotinine and leupeptin, 0.1% β-mercaptoethanol (v/v), 0.03% Brij 35 (w/w), 5% glycerol in phosphate buffered saline (pH 7.4, 150 mM NaCl, 16 mM $Na_2HPO_4 \cdot 2H_2O$ and 4 mM $NaH_2PO_4 \cdot 2H_2O$). Supernatants were used for measurement of amylase release, expressed as % total amylase content.

In vivo experiments

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Following insertion of a tail vein catheter, rats were then treated with a hyperstimulatory dose of cerulein (10 μg/kg i.v.). Controls received isotonic saline only. To assess the effects of stress kinase inhibition on cerulein-induced pancreatic stress kinase activation, rats were treated with 30 mg/kg CEP-1347, SB203580 or both, dissolved in Solutol (Boehringer Mannheim), by subcutaneous (s.c.) injection 4 h prior to cerulein injections. Controls received solutol alone. The rats were then sacrificed 30 min after cerulein injection and pancreata homogenized in stress kinase/IP buffer. This time point was chosen since it is known that pancreatic ERK, JNK and p38 are all strongly activated 30 min after cerulein hyperstimulation *in vivo*[6-10]. Pancreatic wet weight and trypsin activation as well as serum lipase and amylase were also determined.

To assess effects of cerulein and SB203580 on p38 Map kinase *in vivo*, activation of pancreatic MAPKAP kinase 2 was assessed following immunoprecipitation in an *in vitro* assay using recombinant HSP27 as substrate as described[13,16].

SDS-PAGE

One-dimensional gel electrophoresis was performed according to Laemmli, as previously described[17]. Five μg of protein were loaded per lane.

Light microscopy and histological evaluation

For histological evaluation, freshly removed pancreata were formalin (4%) fixed, ethanol dehydrated and, embedded in paraffin. Six micrometer slices were then stained with H&E and subjected to conventional light microscopy.

Serum amylase and lipase measurements

Measurement of serum amylase and lipase activity was performed using commercially available kits (Boehringer Mannheim) following the manufacturers instructions.

Measurement of trypsin activity

After incubation with or without cerulein, acini were pelleted (10 000 × *g*, 1min). Pellets were resuspended in 1mL HEPES buffer without BSA and sonicated. After a second centrifugation step (20 000 × *g*, 2 min), the supernatant was then analysed. Trypsin activity was measured fluorometrically using BOC- Glu-Ala-Arg-MCA as the substrate, according to the method of Kawabata[18] To allow quantification and comparison between experiments, a standard curve with known amounts of trypsin was generated by plotting trypsin concentration (0-100pg) against flouresence intensity (380lEx/460lEm). Trypsin content in individual samples was then normalized to total protein content (BioRad) using BSA as a standard and expressed as pg trypsin per mg protein.

Statistical analysis

Analysis was done using standard software (Sigma Plot, Systat). Data were compared by Student's *t* test, $P<0.05$ was considered significant.

RESULTS

Effects of CEP1347 and SB203580 on acinar stimulus secretion coupling and trypsin activity

We have previously shown that JNK inhibition does not influence acinar stimulus secretion coupling[13]. We, therefore, investigated effects p38 and/or JNK inhibition on cerulein-induced acinar amylase release (Figure 1A). Cerulein induced a dose dependent secretory response including the typical biphasic inhibition with hyperstimulatory amounts (●). Neither JNK nor p38 inhibition apparently altered the secretory dose response to cerulein. Thus, treatment with either 20 μM CEP1347 (▽), 50 μM SB203580 (▼) or both agents given simultaneously (○) did not alter maximal amylase release not the biphasic dose response. We also measured the effects of stress kinase inhibition on cerulein-induced acinar trypsin activation (Figure 1B). Active trypsin could be found even in unstimulated acini, but treatment with cerulein led to a dose dependent increase of acinar trypsin activity (●). Interestingly, using CEP1347 (▽), we observed a tendency towards reduced trypsin activation while SB203580 (▼) appeared to increase trypsin activation. However, in isolated acini, this effect was not statistically significant.

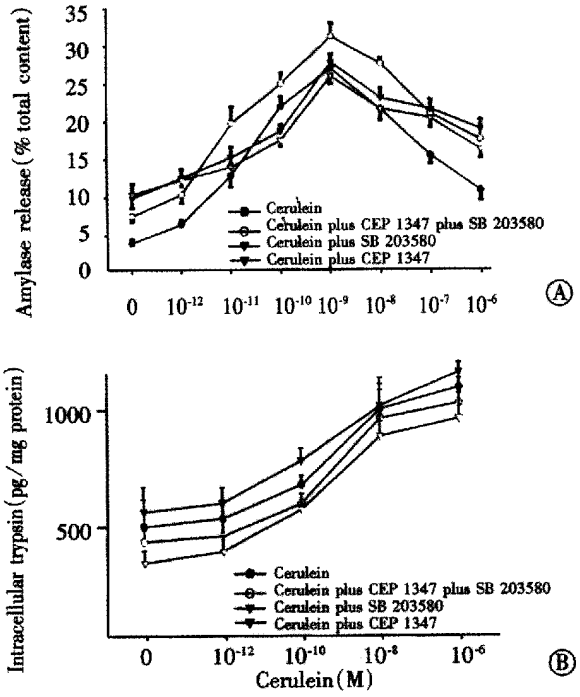

Figure 1 Effects of CEP1347 and SB203580 on acinar stimulus secretion coupling and trypsin activation. Acutely isolated acini were incubated with the indicated amounts of cerulein for 30 min (●). Acini were also treated with 20 μM CEP1347 (▽), 50 μM SB203580 (▼) or both agents simultaneously (○). A: Amylase release into the supernatant was determined and expressed as % of total content. Stimulus secretion coupling was not significantly altered by stress kinase inhibitors. B: Active trypsin was measured in acinar homogenates and expressed as pg / mg protein. Although SB203580 treated acini had a tendency towards higher amounts of active trypsin, this effect was not statistically significant.

SB203580 inhibits cerulein-induced p38 kinase activation in vivo

In order to compare the effects of JNK and p38 inhibition *in vivo*, we tested whether *in vivo* inhibition of pancreatic p38 activity could be accomplished. In contrast to JNK[9,10], p38 is constitutively active in the pancreas (Figure 2, lane 1). Treatment with 30 mg/kg cerulein resulted in strong p38 activation (lane 2). Treatment with 3 mg/kg SB203580 reduced cerulein-induced p38 activation by half while 30 mg/kg SB203580 suppressed cerulein-induced p38 activation almost completely (Figure 2, lanes 3 and 4). However, even the highest SB203580 dose could not reduce pancreatic p38 kinase activity below basal levels (Figure 2, lane 4). SB203580 had no apparent effect on cerulein-induced activation of JNK or ERK (not shown).

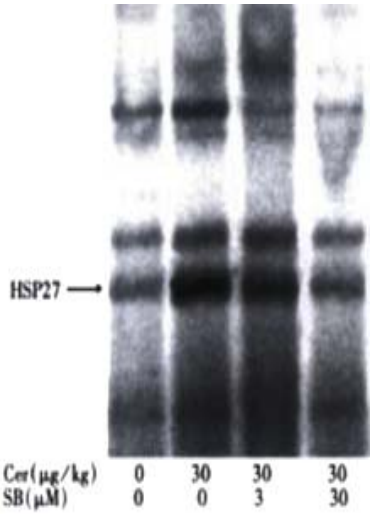

Figure 2 SB203580 inhibits cerulein-induced p38 kinase activation *in vivo*. Animals were treated with the indicated amounts of SB203580, sc, 2 h prior to cerulein hyperstimulation. *In vitro* phosphorylation of HSP27 following immunoprecipitation of MAPKAPK2 was used as read-out of p38 activity. 30 mg SB203580 almost completely abolished cerulein-induced pancreatic p38 activation.

Effects of CEP1347 and SB203580 on biochemical parameters of pancreatitis

According to our previous results, we used 30mg of either CEP1347[13] and/or SB203580 (Figure 2) given sc 4h prior to cerulein. In accordance to the results on acinar stimulus secretion coupling (Figure 1), both agents failed to alter the increase of serum amylase and lipase levels following pancreatitis induction (Figure 3A and B). As reported previously[13], treatment with CEP1347 to inhibit JNK activation significantly increased the dry to total pancreatic weight ratio after cerulein hyperstimulation, indicating reduced edema formation (Figure 3C). In contrast, SB203580 treatment to inhibit p38 kinase activation had no apparent effect on the cerulein-induced increase of pancreatic water content. However, when given simultaneously, SB203580 treatment abrogated the

effects of CEP1347 on the dry to total pancreatic weight ratio. Thus, animals pretreated with both CEP1347 and SB203580, apparently developed similar edema compared to rats treated with cerulein alone.

We also measured the pancreatic trypsin activity *in vivo* (Figure 3D). Compared to acini, pancreata from untreated rats contained very little active trypsin and trypsin activity was strongly induced following cerulein hyperstimulation. Thus, freshly isolated acini on average contained 500 pg active trypsin/mg protein while whole pancreata from untreated control animals on average contained only 15 pg/mg protein (Figure 1B and 3D). Accordingly, the fold increase of active trypsin after cerulein hyperstimulation was much higher *in vivo* (5 fold) rising to 72 pg/mg protein, compared with acini in which cerulein hyperstimulation induced only a 2-2.5 fold increase. The higher basal active trypsin content in acini may possibly be explained by the mechanical stress of the acinar preparation.

As in acini, CEP1347 given *in vivo* did not alter cerulein-induced pancreatic trypsin activation. In contrast, SB203580, interestingly, further increased pancreatic trypsin activation in response to cerulein. This effect was much more pronounced compared to isolated acini, possibly due to lower basal trypsin activity *in vivo*. Thus, SB203580 pretreated animals had significantly higher pancreatic trypsin levels following cerulein hyperstimulation when compared to animals pretreated with CEP1347 (Figure 3D). Again, when given simultaneously, CEP1347 treatment abrogated the effect of SB203580 on pancreatic trypsin activation.

SB203580 treatment leads to necrosis development during cerulein pancreatitis

In order to better compare the effects of CEP1347 and SB203580 on cerulein pancreatitis, histological assessment was also performed (Figure 4). Control pancreata showed regular acinar structure with polarized acinar cells grouped around a ductal lumen and lobules were separated by thin septi (Figure 4A). Cerulein induced the typical morphological changes of edematous pancreatitis including widened interstitial spaces and vacuole formation but without necrosis (Figure 4B). In accordance to previous results[13], JNK inhibition through CEP1347 pretreatment strongly reduced edema formation while vacuolisation was still visible (Figure 4C). Interestingly, inhibition of p38 with SB203580 without concomitant JNK inhibition aggravated cerulein pancreatitis with focal areas of necrosis (Figure 4E and F). Effects of either compound alone on cerulein pancreatitis were mutually abolished when both JNK and p38 were inhibited through simultaneous treatment with CEP1347 and SB203580 (Figure 4D) and no major difference in cerulein pancreatitis without any stress kinase inhibition was seen (compare panel B and D).

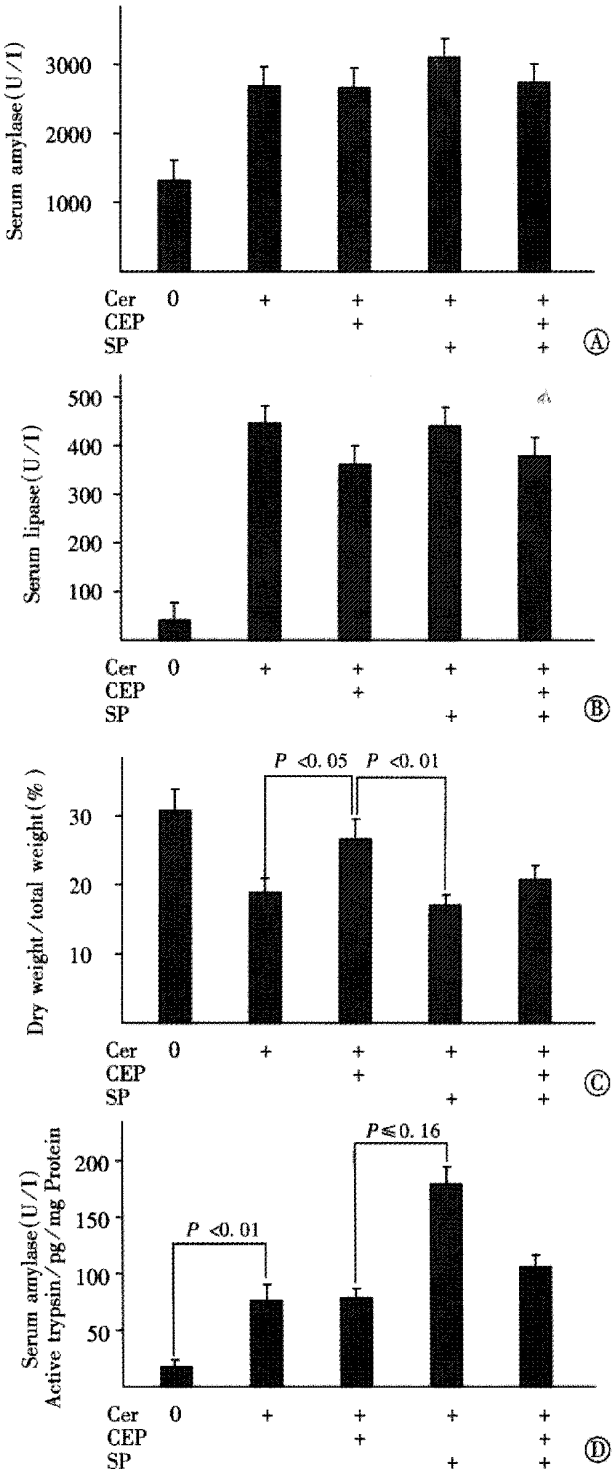

Figure 3 Effects of CEP1347 and SB203580 on biochemical parameters of pancreatitis. Animals were treated with 10 μg/kg cerulein iv and sacrificed 2 h later. As indicated rats were also pretreated with 30 mg/kg CEP1347 and/or SB203580 4 h prior to cerulein injections. Serum amylase levels (A), serum lipase levels (B), dry to pancreatic total weight ratio (C) and pancreatic active trypsin content (D) were determined after sacrifice. Kinase inhibitors had no apparent effect on the cerulein-induced increase of serum digestive enzyme levels. However, CEP1347 reduced the cerulein-induced increase of pancreatic water content and this effect was blocked by simultaneous treatment with SB203580. SB203580 further increased cerulein induced pancreatic active trypsin content and this effect was blocked by simultaneous treatment with CEP1347.

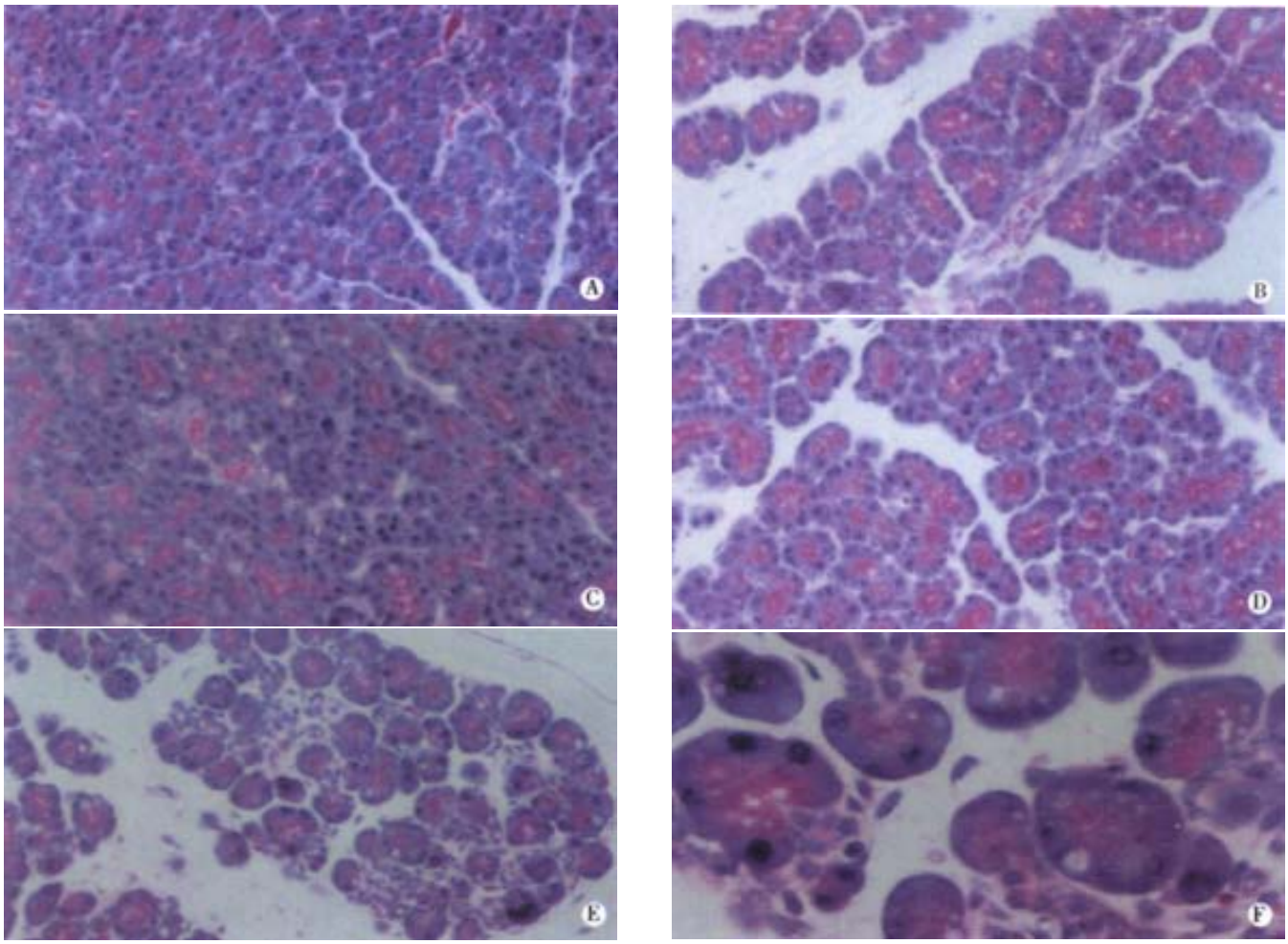

Figure 4 SB203580 treatment leads to necrosis development during cerulein pancreatitis. Animals were treated as in Figure 3. Pancreata were then removed and tissue sections HE stained for light microscopy. untreated control, cerulein alone, 30 mg/kg CEP1347 4 h prior to cerulein, 30 mg/kg CEP1347 and SB203580 prior to cerulein, and 30 mg/kg SB203580 prior to cerulein. Original magnification 50 fold (A-E). Panel F shows a higher (150-fold) magnification of panel E.

DISCUSSION

In our study, we investigated the effects of p38 kinase inhibition on cerulein pancreatitis in comparison to JNK inhibition. We used CEP1347 and SB203580 as tools to specifically inhibit JNK and p38 kinase, respectively. Previously we used CEP1347 for inhibition of pancreatic JNK[13]. CEP 30 mg/kg had no effect on other members of the ERK family. In addition, it has been shown that CEP1347 exhibits between 10-200 fold greater potency towards inhibition of the JNK pathway as compared with other kinases such as protein kinase C, protein kinase A, trk tyrosine kinase, myosin light chain kinase and phosphatidyl inositol 3 kinase[12,13]. We, therefore, believe that CEP1347 can safely be used as a tool to characterize the function of JNK during pancreatic stress. SB203580 has been extensively characterized and widely used as p38 inhibitor and is known to specifically inhibit p38 isoforms α and β but not γ and δ[19]. Nothing is known about pancreatic expression of p38 isoforms, but we (Figure 2) and others[8] have shown that the cerulein-induced p38 activation can be almost completely blocked through SB203580. This indicates that SB203580 is a useful tool to assess the role of p38 kinase in the pancreatic stress reaction early during pancreatitis induction. It is interesting to note that basal p38 kinase activity in the pancreas could not be suppressed by SB203580. This indicates that SB203580 insensitive p38 isoforms may be expressed in the pancreas while only those isoforms inhibitable by SB203580 may be responsive to hyperstimulation stress.

From our data it is clear that neither JNK nor p38 play any role in acinar stimulus secretion coupling, which is in agreement with previous reports by us and others[8,13]. However, our data provides further evidence that stress kinases play an important role early during pancreatitis. Although the exact roles of JNK or p38 for cellular stress responses are still not clear, it has been suggested that these pathways may at least in part be

redundant and may substitute each other[20]. Interestingly in our system, p38 and JNK pathways did not appear as redundant systems. JNK inhibition clearly ameliorated cerulein pancreatitis, reducing pancreatic edema formation following cerulein hyperstimulation. This indicates that JNK activation may promote pancreatic injury. Accordingly, JNK activation has been widely reported to induce cellular damage such as apoptotic or necrotic cell death[20].

In contrast, p38 inhibition aggravated cerulein pancreatitis, indicating that p38 activation may support protection of the pancreas against damage through hyperstimulation stress. Recent evidence from other systems indicates that p38 activation can indeed be protective. Thus, p38 is reportedly important for protection observed after ischemic precondition in myocardial cells and it is evidenced that p38 may exert protective effects via its substrate MAPKAPK2 (MAPK activated protein kinase 2)[14,21].

Our investigation does not allow definitive conclusions about the mechanism of SB203580 actions on the course of cerulein pancreatitis. Based on theoretical considerations, some speculations about mechanisms are possible, however. The deletorious effect of p38 inhibition was apparent biochemically with higher pancreatic levels of active trypsin following cerulein hyperstimulation *in vivo* and, most importantly, histologically with the appearance of necrosis. Premature intracellular trypsin activation has long been thought to play a key role in pancreatitis development[22-24]. Recent evidence to support the importance of active trypsin for the development of pancreatitis came from two sources. First, the discovery of the underlying genetic defect of hereditary pancreatitis showed that, trypsin mutations are sufficient to induce pancreatitis[25,26] second, acinar cells respond to secretory hyperstimulation with rapid conversion of the inactive zymogen precursor trypsinogen to its active form, trypsin[27-29]. Our data could, therefore, be interpreted that p38 kinase inhibition increases pancreatic active trypsin content, allowing necrosis to occur. Our model does not allow us to distinguish whether the active trypsin was mainly present intracellularly or in the pancreatic interstitial space.

Interestingly, it has also been reported that large amounts of trypsinogen are released into the interstitial space during cerulein pancreatitis and that activation of interstitial trypsinogen through concomitant infusion of enterokinase induces necrosis in that model[30]. It is believed that digestive enzymes accumulate in the interstitial space during pancreatitis induction due to missorting of zymogen granules to the basolateral rather than the apical membrane and that missorting is at least in part related to disturbances of the acinar cytoskeletal organization. In the pancreas, it has been demonstrated that secretagogues can activate the p38-MAPKAPK2 pathway[8,10], which has further indicated that secretagogues induce HSP27 (heat shock protein 27) phosphorylation via the p38-MAPKAPK2 pathway[8]. HSP27 phosphorylation via the p38-MAPKAPK2 pathway has further been shown to stabilize the acinar actin cytoskeleton against the effects of secretagogue stimulation[8]. In addition, there is evidence that the p38-MAPKAPK2-HSP27 pathway may be important for organ protection in the pancreas.

Although speculative, it appears possible that during cerulein pancreatitis induction, p38 activation may help reduce acinar damage through its action on MAPKAPK2-HSP27 with increased cytoskeletal stability and less accumulation of active trypsin in the interstitial space. In this model, interruption of the p38-MAPKPAK2-HSP27 pathway through SB203580 could then allow necrosis to occur after cerulein hyperstimulation. The exact mechanism of SB203580 action will have to be determined through additional studies.

In conclusion, we have demonstrated that stress kinases play no role in acinar stimulus secretion coupling. We have also shown that inhibition of JNK and p38 kinase oppositely influences the course of cerulein pancreatitis. SB203580 increases pancreatic active trypsin content and also induces necrosis following cerulein hyperstimulation although necrosis is typically not a feature of cerulein pancreatitis. Our current working hypothesis is that p38 kinase activation may stabilize the actin cytoskeleton via the p38-MAPKAPK2-HSP27 pathway. Inhibition of p38 kinase then might increase missorting of secretory granules with higher amounts of trypsinogen/trypsin secretion into the interstitial space and concomitant development of necrosis.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Mycoplasma infections and different human carcinomas

Su Huang, Ji You Li, Jan Wu, Lin Meng and Cheng Chao Shou

Subject headings Gastrointestinal neoplasms/ microbiology; mycoplasma infections; antibodies, monoclonal; immunohistochemistry

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Abstract

AIM To explore relationships between human carcinomas and mycoplasma infection.

METHODS Monoclonal antibody PD4, which specifically recognizes a distinct protein from mycoplasma hyorhinis, was used to detect mycoplasma infection in different paraffin embedded carcinoma tissues with immunohistochemistry. PCR was applied to amplify the mycoplasma DNA from the positive samples for confirming immunohistochemistry.

RESULTS Fifty of 90 cases (56%) of gastric carcinoma were positive for mycoplasma hyorhinis. In other gastric diseases, the mycoplasma infection ratio was 28% (18/49) in chronic superficial gastritis, 30% (14/46) in gastric ulcer and 37% (18/49) in intestinal metaplasia. The difference is significant with gastric cancer (χ^2 = 12.06, P<0. 05). In colon carcinoma, the mycoplasma infection ratio was 55.1% (32/58),but it was 20.9% (10/ 49) in adenomarous polyp (χ^2 = 13.46, P<0.005). Gastric and colon cancers with high differentiation had a higher mycoplasma infection ratio than those with low differentiation (P<0.05). Mycoplasma infection in esophageal cancer, lung cancer, breast cancer and glioma was 50.9% (27/53), 52.6% (31/ 59), 39.7% (25/63)and 41% (38/91), respectively. The mycoplasma DNA was successfully amplified with the DNA extracted from the cancer tissues that were positive for mycoplasma infection (detected with antibody PD4).

CONCLUSION There was high correlation between mycoplasma infection and different cancers, which suggests the possibility of an association between the two. The mechanism involved in oncogenesis by mycoplasma remains unknown.

Beijing Institute for Cancer Research, Peking University School of Oncology, Beijing 100034, China
Su Huang, received B.A from Jiangxi Medical College of China in 1994. Now she is a graduate student pursuing a Ph.D degree at the Peking University School of Oncology.
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Correspondence to: Dr. Cheng Chao Shou, Department of Biochemistry and Molecular Biology, Beijing Institute for Cancer Research and Peking University School of Oncology. No.1 Da Hong Luo Chang Street, Western District, Beijing 100034, China
Tel. 0086-10-66160960, Fax. 0086-10-66175832
Email. 6201@us.sina.com.
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INTRODUCTION

Mycoplasma is one of the smallest living organisms isolated from nature, and can be cultured in a special medium. Mycoplasmas spread widely at the cell membrane of many types of mammaliane cells. Some can enter these cells[1-3]. As a conditional pathogenic organism, mycoplasmas have been associated with many diseases[4-11]. Experimental data indicates that some mycoplasmas cause chromosomal changes and cell transformations *in vitro* through progressive chromosomal loss and translocations[12-17], but the association between mycoplasmas and cancer remains unclear. Monoclonal antibody (MAb) PD4 was prepared with human gastric cancer cell line MGC803 as immunogen[18]. The antibody specifically reacted with an antigen associated with some tumor cell lines[19]. Western blot analyses indicated that molecular weight of this antigen (P40) was about 40 kilo-Daltons. Our previous study indicated that MAb PD4 could inhibit the growth of Rat 3-3 and GCM3T3 cells, as well as the tumorigenicity in nude mice[20]. Recently, the antigen P40 was identified with N-terminal sequence analysis. The 16 amino acids at the N-terminus of P40 are identical with the N-terminus of P37, which originated from Mycoplasma hyorhinis, and the PD4 reacted with mycoplasma hyorhinis strongly (unpublished). These results indicate that MAb PD4 is an antibody raised to mycoplasma, not to tumor cells. We then used PD4 detecting mycoplasma infections in paraffin-embedded gastrointestinal carcinoma tissues and other cancers. Here we report the detection results which show a strong association between mycoplasma infections and different human carcinomas.

MATERIALS AND METHODS

Specimen selection

The examined specimens were selected according to the results of hematoxylin-eosin (H&E) staining evaluated microscopically. There are 90 samples of

archived gastric carcinoma tissues and 44 samples of archived normal tissue around carcinoma for comparison, 67 samples are from men and 28 samples are from women. The pathologic grand I-II, II-III and III were 23, 18 and 49 cases, respectively, in the 90 tumor specimens. We selected 47 cases of superficial gastritis, 46 cases of gastric ulcer, and 49 cases of intestinal metaplasia to serve as controls. Fifty-eight cases of colon carcinoma and 49 cases of adenomarous polyp were chosen for detection. Besides the gastrointestinal samples, 53 cases of esophageal carcinoma, 59 cases of lung carcinoma, 63 cases of breast carcinoma, and 91 cases of glioma were also detected. Except the specimens of glioma, which were from 307th Hospital of the People's Liberation Army, all of the samples were kindly provided by the Department of Pathology of the First Affiliated Hospital of Jiangxi Medical College of China.

B-SA immunoperoxidase stain

Tissues embedded in paraffin were microtome-sectioned into 4 μm slices. The slices were floated onto a tissue flotation bath and mounted on 3-aminopropyltriethoxysilane (APES)-treated slides. Sections were heat-immobilized in an oven at 60℃ for 30 minutes, deparaffinized with three changes of xylene, sequentially dehydrated in different concentrations of ethanol, and rinsed in distilled water. Endogenous peroxidase activity was blocked by immersing sections in 0.3% H_2O_2 for 5-10 minutes, followed by sequential rinsing in distilled water and in phosphate buffered saline (PBS). The primary antibody, mouse anti-mycoplasma hyorhinis monoclonal antibody PD4 (described above prepared by our laboratory, diluted to 10 mg/L) was applied to the slices. Unspecialized mouse IgG was used as a negative control. After being incubated for 1 hr at 37℃ or overnight at 4℃, the slices were rinsed three times in PBS for 5 minute each. The slices were stained with B-SA kit (Biogenex) according to the manufacturer's instruction. The results were evaluated by two investigators independently. (-) means no yellow staining was observed in cells, (+) means some cells were observed to be lightly stained, but less than 50%, and (++) indicates that over 50% were observed to be stained, or the staining was strong.

Extraction of DNA from tissues embedded in paraffin

Human gastric cancer tissues embedded in paraffin were microtome-sectioned into 10 μm slices. Two or more slices from each sample were placed into individual sterile autoclaved microcentrifuge tubes. Sterility was maintained at all times and the microtome blade was cleaned completely with ethanol between sectioning to prevent cross-contamination. Digestion buffer, consisting of 0.5% Tween-20, 50mM Tris (pH 8.5), 1mM EDTA, and 200 mg/L proteinase K (Sigma Chemical Co), was added into each tube and incubated overnight. The DNA was successively extracted with equal volumes of Tris-saturated phenol (pH 8.0), 1:1 phenol/chloroform (vol/vol) and then chloroform. one/10 volume of 10M ammonium acetate was added to each sample and the DNA was precipitated by the addition of 2.5 volumes of 100% percent cold ethanol. The DNA was incubated at -20℃ overnight and centrifuged for 20 minutes at 4℃, 12 000 rpm. The DNA was washed with 70% ethanol, and suspended in distilled water.

Mycoplasma infection detected by PCR

Primers used to amplify conserved mycoplasma 16sr DNA were synthesized by Shengong Co. (Shanghai) and the sequences are as follows: forward primer: 5'-TACGGGAGGCAGCAGTA-3'; reverse primer: 5'-TCAAGATAAAGTCATT-3'. The PCR program consisted of 35 cycles at 94℃ for 30 seconds, 48℃ for 30 seconds, and 72℃ for 20 seconds, and 72℃ 10 minutes for final extension[21,22]. The PCR products were analyzed with agar gel electrophoresis.

Statistical analyses

The data were analyzed using the χ^2 test implemented in a commercially available computer program. A value of $P \leqslant 0.05$ was considered significant.

RESULTS

Mycoplasma infection in gastric carcinoma tissues and other gastric diseases

The results indicate that the gastric carcinoma tissues with high differentiation had a higher mycoplasma infection ratio than that of low differentiation gastric carcinoma tissues ($P<0.005$, Table 1). Seven samples exhibited different levels differentiation in the same specimen. The mycoplasma infection in the atypical hyperplasia was higher than that in the carcinoma tissue; the mycoplasma infection was positive in the gastric adenocarcinoma or in the papilloma, but was negative in the gastric mucoid carcinoma.

Table 1 Mycoplasma infection in different grades of gastric carcinoma

Grades of differentiation	Total number of cases	Infection of mycoplasma				
		Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
I-II	23	3	12	8	20	87
II-III	18	7	9	2	11	61
III	49	30	14	5	17	39
Total	90	40	35	14	50	56

In other gastric diseases, the total mycoplasma infection ratio was 31.5% (45/142). This ratio was significantly lower than that observed in the cancer tissue ($\chi^2 = 12.06$, $P<0.05$, Table 2).

Table 2 Comparison of mycoplasma infection in gastric carcinoma and in the other gastric diseases

	Infection of mycoplasma			
	Negative cases	Positive cases	Total number of cases	Ratio of positive (%)
Chronic superficial gastritis	34	13	47	28
Intestinal metaplasia	31	18	49	37
Gastric ulcer	32	14	46	30
Gastric carcinoma	40	50	56	56
Total number of cases	137	95	50(mean)	40(mean)

Mycoplasma infection in colon carcinoma and adenomarous polyp tissues

In the 58 cases of colon carcinoma, the total infection ratio was 55%. As in the gastric carcinoma described above, the colon carcinoma tissues with high differentiation had a higher mycoplasma infection ratio than that of low differentiation colon carcinoma tissues (Table 3, $P<0.05$).

In the 49 cases of adenomarous polyp, there were 10 cases with mycoplasma infection. The positive ratio was 20.4%. The difference between the infection ratio of colon carcinoma and that of adenomarous polyp was significant ($\chi^2 = 13.46$, $P<0.005$).

Table 3 Mycoplasma infection in different grades of colon carcinoma

	Infection of mycoplasma					
Grades of differentiation	Total number of cases	Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
I-II	42	15	15	12	27	64
II-III	8	5	2	1	3	37
III	8	6	2	0	2	30
Total	58	26	19	13	32	55 (mean)

Mycoplasma infection in other carcinoma tissues

Beside the gastriointestinal carcinomas, other cancer tissues from human esophagus, lung, breast and brain were also analyzed (Table 4).

Table 4 Mycoplasma infection in other carcinoma tissues

	Infection of mycoplasma					
Types of carcinoma	Total number of cases	Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
Esophagus	53	26	21	6	27	50.9
Lung	59	28	23	8	31	52.6
Breast	63	38	17	8	25	39.7
Glioma	91	53	27	11	38	41.0
Total	266	145	88	33	121	45.5

Some immunoperoxidase stainings of different carcinoma are shown in Figure 1. The low differential gastric cancer (ring cell cancer) was negative reacted with PD4 (A), but the gastric adenocarcinoma was positive (B).

Detection of mycoplasma DNA in positive specimen by PCR

We selected 3 positive and 3 negative specimens detected by immunochemistry for mycoplasma DNA amplification. The specimen DNA was extracted and PCR was carried out by using mycoplasma 16srDNA primers. The mycoplasma hyorhinis DNA was used as control. The analysis of agar gel electrophoresis showed that mycoplasma DNA was amplified from all three positive specimens, as well as from the positive control of mycoplasma DNA, but there was no mycoplasma DNA amplified from negative tissues, which should be 142bp in size (Figure 2). This result corresponded with that of immunohistochemical detection.

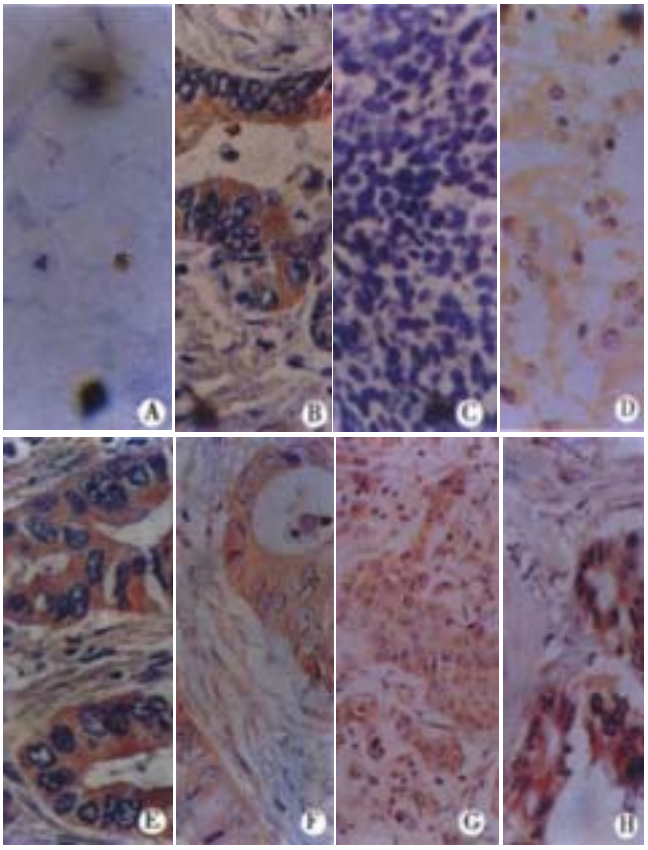

Figure 1 Immunoperoxidase stainings of different carcinoma tissues reacted with monoclonal antibody PD4 (× 400). Both A and B were gastric carcinomas. A (singnet-ring cell carcinoma) was negative, B (adenocarcinoma) was positive. C (glioma) indicated the negative reaction, D (glioma), E (lung cancer), F (esophagus cancer), G (breast cancer) and H (colon cancer) presented the positive reactions with antibody PD4.

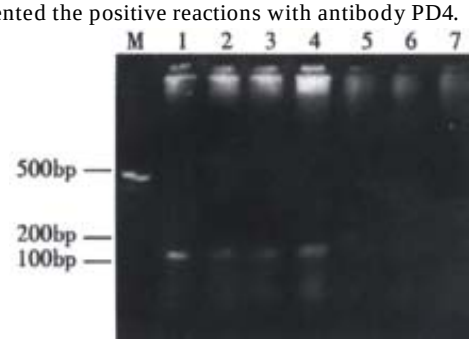

Figure 2 Amplification of mycoplasma 16srDNA from paraffin -N- embedded specimens. M: 100bp DNA Marker; Lane 1: Positive control; Lane 2-4: positive specimen; Lane 5-7: negative specimen. The arrow indicates the amplified mycoplasma DNA.

DISCUSSION

Mycoplasma exists widely in nature. Mycoplasma contamination in cultured cells is common and a major problem in bio-laboratory work. McAb, prepared with cultured tumor cells as immunogen and using these cells as selection targets, has been identified to be antibody against mycoplasma. Unfortunately, the molecules recognized by these antibodies are difficult to characterize and are usually researched as tumor-associated antigen. Meanwhile, these antibodies are regarded as tumor-specific[23,25]. McAb PD4 has been used as a tumor-specific antibody until its antigen was identified by protein sequencing. Because the characterization of tumor antigen is usually difficult, and mycoplasma contamination is common in cultured cells, researchers should be very careful in preparing antibody against tumor and take necessary steps to exclude the possibility of antibody binding to mycoplasma.

We observed that mycoplasma was present in 55% cases of gastrointestinal carcinomas ($P<0.05$) and 45.5% in otherdetected carcinomas. These results are similar to those presented by Philip[26] and Sasaki[27], where the rate of mycoplasma infection was 59.3% in ovarian cancer cases and 48% in gastric cancer, respectively. In gastrointestinal carcinomas, we found that cancer tissues with high differentiation had a higher mycoplasma infection ratio than that of low N differentiation cancers. The reason for this is unknown.

It may be logical to consider the correlation between the cancer and mycoplasma because of the high infection in the tumor tissues, but the role of mycoplasma as a causative or facilitative agent during tumor development has yet to be determined. Although the AIDS related *Mycoplasma fermentans* and *Mycoplasma penetrates* have been reported to induce cell transformation[15], and *Mycoplasma hyorhinis* was observed to increase invasiveness and inhibit cell contact inhibition *in vitro*[24], but direct evidence for a cause-and-effect relationship has not been discovered. In our recent work, we found that gastric cancer cell line MGC803 contaminated with *Mycoplasma hyorhinis* has a much higher capacity to form colonies on soft agar than that of MGC803 without mycoplasma contamination. Interestingly, we have observed McAb PD4 to inhibit colony formation of MGC803 with mycoplasma contamination, but no effect was observed on mycoplasma free MGC803 (unpublished). This result indicates that the protein recognized by McAb PD4 may play a critical role in colony formation increased by mycoplasma. Elucidating the mechanism by which PD4 inhibits colony formation will provide important information for understanding the relationship between mycoplasma infection and oncogenesis.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Inhibitory effect of voglibose and gymnemic acid on maltose absorption *in vivo*

Hong Luo[1,2], Toshiaki Imoto[2] and Yasutake Hiji[2]

Subject headings diabetes mellitus/therapy; maltose; gymnemic acid; alpha-glycosidases; intestinal mucosa; nutrition; voglibose

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Abstract

AIM To determine whether diabetic care can be improved by combination of voglibose and gymnemic acid (GA), we compared the combinative and individual effects of voglibose and GA on maltose absorption in small intestine.

METHODS The small intestine 30 cm long from 2 cm caudal ward Treitz's ligament of Wistar rat was used as an in situ loop, which was randomly perfused in recircular mode with maltose (10 mmol/L) with or without different dosages of voglibose and/or GA for an hour. To compare the time course, perfusion of 10 mmol/L maltose was repeated four times. Each time continued for 1 hour and separated by 30 minutes rinse. In the first time, lower dosages of GA (0.5g/L) and/or voglibose (2 μmol/L) were contained except control.

RESULTS Absorptive rate of maltose was the lowest in combinative group ($P<0.05$, ANOVA), for example, the inhibition rate was about 37% during the first hour when0.5 g/L-GA and 2 μmol/L voglibose with 10 mmol/L maltose were perfused in the loop. The onset time was shortened to 30 minutes and the effective duration was prolonged to 4 hours with the combination; therefore the total amount of maltose absorption during the effective duration was inhibited more significantly than that in the individual administration ($P<0.05$, U test of Mann Whitney). The effect of GA on absorptive barriers of the intestine played an important role in the combinative effects.

CONCLUSION There are augmented effects of voglibose and GA. The management of diabetes mellitus can be improved by employing the combination.

INTRODUCTION

It has been well known that there is an association between hyperglycemia and diabetic complication. Patients who develop non-insulin-dependent diabetes mellitus (NIDDM) even at age 65 years may live long enough to develop micro-vascular and neuropathic complication[1]. A cluster of risk factors including hyperglycemia, hyper-insulinaemia, hypertension, dyslipidemia and obesity is called metabolic *X*-syndrome due to the correlationship of them[2]. Similar to cardiac syndrome X, metabolic syndrome X often induces vascular dysfunction[3-5]. Diet regimen and the control of nutrient entry, with the aim of avoiding glucose and anabolic hormone peaks and reducing the rise of developing long-term complications, are broadly accepted as the basic treatment for diabetes mellitus[6,7].

In the ordinary diet, carbohydrates, which contain far more starch than the other carbohydrates, normally represent the quantitatively greatest part of human diet and the main energies supply even though in diabetes. Glucose represents more than 80 per cent of the final products of carbohydrate digestion. Maltose is a rather important product during starch hydrolysis. Therefore, the digestive process in which various glycohydrolases work successively to hydrolyze starch to the final product glucose and the absorption of glucose in the small intestine could be a target for the control of the nutrient entry. Voglibose, an N-substituted derivative of valiolamine isolated from the fermentation broth of Streptomyces hygroscopicus subsp. limoneus, is a potent and structurally novel inhibitor of the intestinal disaccharidases, which not only can be used for treatment of NIDDM but also for insulin-dependent diabetes mellitus (IDDM)[8-11]. It has a potently inhibitory effect on maltase but with a short inhibitory duration[12,13]. On the other hand, GA[14], a mixture of triterpene glucuronides, which

[1]Department of Physiology, Institute of Basic Medicine, Chinese Academy of Medical Sciences/School of Basic Medicine, Peking Union Medical College, Beijing 100005, China
[2]Departments of Physiology, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan
Supported by Japanese Government (Ministry of Education, Science and Culture of Japan, MONBUSHO) scholarship No. 933241 (1994-1999).
Correspondence to: Dr. Hong Luo, Department of Physiology, Institute of Basic Medicine, CAMS/PUMC, 5 Dongdansantiao Beijing 100005, China
Tel. 0086-10-65296463, Fax. 0086-10-65133604
Email. hongluo1@263.net
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was found in the leaves of the Indian plant, Gymnema sylvestre[15], inhibits glucose absorption of small intestine[16], but it needs a longer time and higher dosage to achieve its maximum effect.

In this experiment, the combinative effect of Voglibose with GA was examined on hydrolysis and absorption of maltose in rat small intestine.

MATERIALS AND METHODS

Animals

Male 8-9-week-old Wistar rats weighing 300 g ± 25 g (Shimizu, Kyoto), were housed in an air-conditioned room at 22℃ ± 2℃ with a nature lighting schedule for 1 to 3 weeks before experiment. They were fed with a standard pellet diet (Oriental Yeast Co., Kyoto) and tap water. Care and treatment of the animals conformed to Tottori University guidelines for the ethical treatment of laboratory animals.

Perfusion of small intestine in vivo

A modified technique of Barry *et al* was used[17]. Animals fasted overnight with free access to water, were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg bw, Dainabot). The abdominal cavity was opened by a mild line incision. The small intestine 30 cm long from 2 cm caudal ward Treitz's ligament was used as an *in situ* loop, which was emptied of its contents with Ringer's solution. L-shape cannulae were inserted into each end of the selected intestine and connected with a peristaltic pump (SJ-1211H, Atto, Tokyo). The abdominal cavity was closed and the loop was rinsed with Ringer's solution (145.4 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$ and 2.4 mmol/L $NaHCO_3$) by uncirculated perfusion for 1 hour. Then the intestine was perfused in the recircular mode with 10mmol/L maltose (Sigma) or maltose plus different dosages of voglibose (Takeda Chemical Industries Ltd., Osaka) and/or GA for one hour to determine IC_{50} (concentration of the drug achieving 50% inhibition of maltose absorption or hydrolysis) for each drug. To compare the combinative and individual effects, the animals were randomly separated into four groups in which each loop was perfused for four times (T, R1, R2 and R3). In the first time (T), 10 mmol/L maltose with or without GA (0.5 g/L) and voglibose (2 μmol/L) was perfused. After rinsing for 30 minutes with Ringer's solution, perfusion of maltose (10mmol/L) only was repeated 3 times (R1, R2 and R3) to examine the time course of recovery. The perfusates of 4 groups in the first perfusion (T) were as follows: ① control group: 10 mmol/L maltose, ② voglibose group: 2 μmol/L voglibose +10 mmol/L maltose, ③ GA group: 0.5 g/L GA +10 mmol/L maltose and ④ Combined group: 0.5 g/L GA + 2 μmol/L voglibose +10 mmol/L maltose. All perfusates were dissolved in Ringer's solution. All solutions were kept at 37℃ and pH was regulated at 7.5-7.8.

Measurement of maltose absorption and hydrolysis

Two samples each containing twenty microlitter of perfusion fluid were taken at an interval of 15 minutes during the perfusion period to measure the amount of glucose and maltose remained and kept at 0℃ to prevent further hydrolysis in the collected samples. One was used to measure the amount of glucose remaining at time t (Gt) after the beginning of perfusion. Maltose in the other sample was completely hydrolyzed by incubating it with enough alpha-glucosidase (Funakoshi) to determine total glucose remaining at time t (TGt). The amount of glucose was determined by glucose determining kit (Glucose Test B, Wako, Osaka). Thus, the extents of absorption and hydrolysis of maltose at time t were obtained as follows:

$$\text{Absorption (\%)} = (TGo-TGt)/TGo \times 100$$

$$\text{Hydrolysis (\%)} = (TGo-TGt+Gt)/TGo \times 100$$

where subscript 0 and t represent the perfused time when the sample was taken, and the Go almost equals 0.

GA extraction

Dry-Gymnema sylvestre leaves were obtained from Okinawa, from which GA was extracted with water, ethanol and diethyl carbonate according to slightly modulated Kurihara's method and freeze-dried to obtain GA powder[16].

Statistical analyses

Statistical analyses were performed with the U test of Mann-Whitney or ANOVA, which was indicated in the result when ANOVA was used. $P<0.05$ was considered as significant difference.

RESULTS

Under the present conditions of experiment, about 63% of maltose in the perfusion fluid disappeared during 60 min perfusion as a result of being hydrolyzed to glucose and successive absorption by the intestinal loop. For simplicity we express hereafter this phenomenon as 'absorption of maltose', as was defined in Methods.

When voglibose was present in the perfusion fluid, the absorption of maltose was inhibited dose dependently with apparent IC_{50} of about 6.06×10^{-6} mol/L. On the other hand, GA inhibited the maltose absorption with IC_{50} of 0.85 g/L, whereas the IC_{50} of voglibose on the hydrolysis of maltose in the loop was 1.8×10^{-6} mol/L. In order to

investigate the combined effect of the two inhibitors, we chose rather low doses of the drugs such as concentrations lower than IC_{50}'s. In Figure 1, time courses of the absorption of maltose are shown during 60 min perfusion with or without voglibose (2 μmol/L) and/or GA (0.5 g/L). At such a low dose as 2 μmol/L, voglibose showed slight inhibitory effect on the absorption of maltose and the significant inhibition was observed at 60 min after the beginning of the perfusion. GA (0.5 g/L) exhibited a significant inhibitory effect at 45 min after the beginning of the perfusion. When the two inhibitors co-existed in the perfusion fluid, inhibitory effect was more pronounced. The significant effect was attained at 30 min after the beginning of the perfusion. The inhibition rate of about 37% was achieved at the end of the perfusion and the percentage of maltose absorption was lowest when GA and voglibose were presented in the perfusate ($P<0.05$ *vs* control; ANOVA).

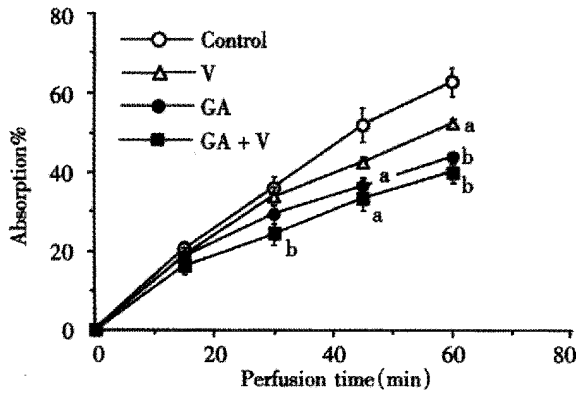

Figure 1 The inhibitory effects of GA (0.5 g/L), voglibose (V, 2 μmol/L) and the combination (GA+V) on 10 mmol/L maltose absorption. The maltose contained in the fluid at perfusion starting point was taken as 100%. Each bar is expressed as the Mean ± SE. ($^aP<0.05$, $^bP<0.01$)

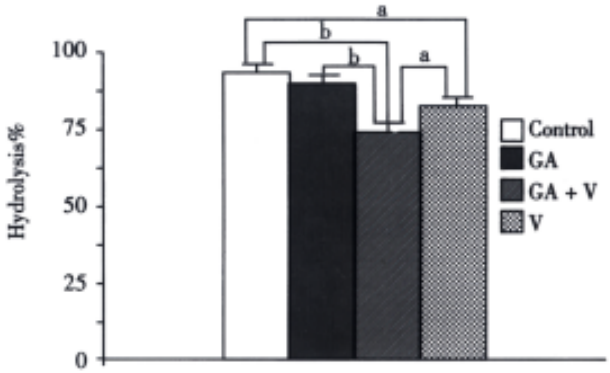

Figure 2 Alteration of maltose absorption following application with GA and voglibose. Each point is shown as the maltose absorption during 60 minutes perfusion of 10 mmol/L maltose following treatment with GA and/or voglibose, except the points of "CON" or "GA/V" which are shown as the absorption during first 1 hour perfusion with or without GA and voglibose. Others are the same as in Figure 1.

Time courses of the recovery from the inhibited states were compared with each other, where perfusion for 60 min with 10 mmol/L maltose only was repeated three times (R1, R2 and R3) inserting rinsing for 30 min between each perfusion, and the extent of recovery was assessed by the absorption of maltose at the end of each perfusion (Figure 2). As shown in the figure, the effect of voglibose was completely recovered during the first perfusion (R1). The effect of GA was still significant after R1 but completely recovered during R2. On the contrary, the suppressed absorption was still remained significant even after R2 and the complete recovery was attained during R3 in the combination group. The total inhibitory rates of maltose absorption in the 3 times perfusion (T, R1 and R2) were 5.39%, 22.21% and 35.55% in the 2 μmol/L voglibose, 0.5 g/L GA and combination groups respectively.

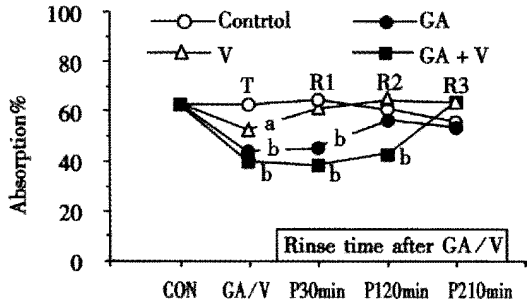

Figure 3 Hydrolytic rates of maltose in each group during R2 in Figure 2. The conditions are the same as in Figure 2.

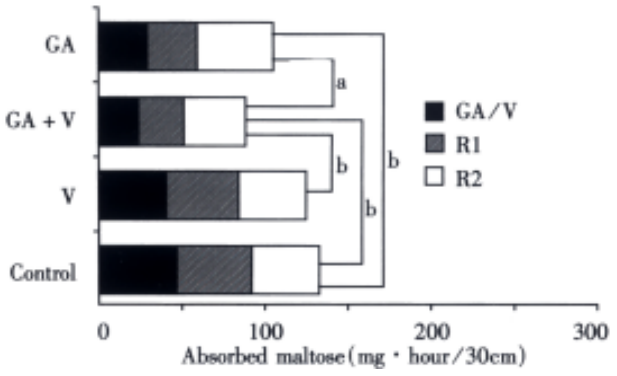

Figure 4 Total maltose absorption in 3 times perfusion during 4 hours. The absolute amounts of maltose absorbed during three perfusions (T, R1 and R2) are compared with each other.

To find the reason of the longer effective duration for the combined group of GA and voglibose, the hydrolytic rate of maltose during R2 in each group was measured (Figure 3). The hydrolysis in the combined group was the lowest among the four groups, although there was no significant difference between the GA and control

group.

Figure 4 shows the total amount of maltose absorbed during three perfusions (T, R1, and R2) in the rather low doses of voglibose (2 μmol/L) and GA (0.5 g/L). It was easily expected from the result shown in Figure 4, that the smallest amount of maltose was absorbed in case of mixing both inhibitors, reflecting the sustained inhibitory effect on the maltose absorption.

DISCUSSION

In the present study, the combinative effect of voglibose and gymnemic acid (GA) was investigated on the digestion and absorption in rat small intestine using maltose as a substrate. Voglibose is an inhibitor of the alpha-dissaccaridases and expected to suppress the absorption of maltose in small intestine as a result of inhibiting the hydrolysis of maltose. Apparent IC_{50} determined in the present experiment of perfusion was 6.6×10^{-6} mol/L for the absorption of maltose, which was about three times larger than that for the hydrolysis of maltose (1.8×10^{-6} mol/L). This difference of IC_{50} could be understood if the rate of hydrolysis of maltose in the intestinal loops was large enough compared to that of absorption of glucose. In fact, when 10mmol/L maltose only was perfused, free glucose appeared progressively in the perfusion fluid. Matsuo *et al*[18] reported the IC_{50} of voglibose on maltase originated from rat small intestine to be 6.4×10^{-9}mol/L *in vitro*. The difference could come from the methods employed in the two experiments.

It has been believed that the anti-diabetic effect of voglibose is due to the inhibition of the hydrolysis of disaccharidases. Recently Hirsh *et al*[19] reported that voglibose showed the inhibitory activity on the free glucose absorption *in vivo* with an IC_{50} near 3 mg/L (1.1×10^{-5} mol/L). The concentration of voglibose used in the present combinative treatment with GA and voglibose was 0.2×10^{-5} mol/L that was less than 1/5 concentration used in Hirshs' experiment. Therefore, the direct inhibitory effect of voglibose on the glucose absorption, if any, would be negligible in the present experiment.

The depressing effect of GA on the sweet taste sensation in human has been known for a long time [20-26]. The inhibitory effects of GA, a mixture of triterpene glucuronides, from the plant -Gymnema sylvestre, on the glucose absorption in the small intestine and the improvement of glucose tolerance have been noticed since the 1980s[15,16,27,28]. Recently we found that GA could inhibit the absorption of oleic acid and gluocose simultaneously[29]. Although the mechanism of the action of GA has not been fully understood, GA is thought to suppress the active transport of glucose suggesting the involvement of the interaction with Na^+ glucose cotransporter and/or ATPase[30-32] on the epithelial cells. The other mechanisms for GA's actions have been considered as participating in the glucose receptor[33] and insulin release[34].

By combining two inhibitors, faster, more effective and long-lasting inhibition of maltose absorption was achieved than those expected as the additive effect. Namely, under the present conditions, significant suppression of maltose absorption was attained at 30 min after the beginning of the perfusion and interestingly, the inhibitory effect was still significant even at 4 hr after the application, whereas almost complete recovery was attained at same time in the case of applying GA or voglibose alone. Therefore, the more effective reduction of postprandial hyperglycemia and hyperinsulinaemia could be expected with the combination.

In the present stage of our knowledge, the mechanism underlying this synergistic effect is not clear. However, as shown in Figure 3, GA enhanced significantly the inhibitory action of voglibose on maltose hydrolysis even at 4 hr after application, although no effect of GA alone on the hydrolysis of maltose can be observed simultaneously. It is well known that most of the disaccharidases produced by the enterocytes are binding with the membrane (under the unstirred layer) and a small amount of saliva and pancreatic amylase is in the glycocalyx[35,36]. The maltose is hydrolyzed during it passes through the glycocalyx and enterocytes[37]. Recently we have found that GA increased the function of unstirred layer by suppression of intestinal motility[38]. Is it possible that voglibose was kept longer time in/under the unstirred layer by GA.

The most common adverse effect of voglibose is hepatotoxicity and gastrointestinal disturbance[39-43] induced by fermentation of unabsorbed carbohydrate in the bowel and increments of gastrointestinal motility[44]. Unfortunately, the disturbance of digestive system also exists in diabetics[45,46]. Voglibose has only rarely been associated with systemic adverse effects, but in some cases acute ileus, pneumatosis cystoides intestinalis and acute dizziness have been reported[47-49]. These adverse effects tend to increase with higher doses of voglibose. GA may diminish the adverse effects not only by decreasing dosage of voglibose, but also suppressing the intestinal motility[38] and inhibiting the growth of anaerobia[50], because bacterial overgrowth plays a role in the development of gastrointestinal symptoms[51].

In summary, the combined effect of voglibose and GA is first reported here. With the combination, the onset time is shortened and the effective duration was prolonged each other, as a result the total amount of maltose absorption is inhibited significantly. Improvement in postprandial hyperglycemia, hyperinsulinaemia and insulin resistance, treatment of an overweight condition (syndrome[52]) and diminishing of the adverse effects of voglibose in diabetic control can be achieved by this combination.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Function and regulation of cholecystokinin octapeptide, β-endorp hin and gastrin in anorexic infantile rats treated with ErBao Granules

Yong Ping Du[1], Yue Ping Zhang[2], Shou Chuan Wang[3], Jian Shi[1] and Shao Hua Wu[1]

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Abstract

AIM To study the role of cholecystokinin octapeptide (CCK-8), β-endorphin (β-EP), and gastrin in an anorexic infantile rat model and no subsequent regulation of nose peptides by the Yunpi complex prescription ErBao Granule.

METHODS We fed infantile rats with special prepared forage. A liquid extract of ErBao Granule was administered to the rats daily for 3 weeks, CCK-8, β-EP, and gastrin concentrations in hypothalamus, gastric antrum, and plasma of the rats were measured by radioimmunoassay, and were compared with controls.

RESULTS Treatment of rats with ErBao Granule inhibited CCK-8 secretion and increased β-EP and gastrin secretion. CCK-8 concentration in hypothalamus and plasma of model control group increased significantly and correlated negatively with food intake of models, respectively. β-EP concentration in gastric antrum and plasma of model control group decreased significantly and showed a positive correlation with food intake of models, respectively. Hypothalamus concentration of β-EP was similar in models and controls. Gastrin concentration in gastric antrum of models was lower than in the blank control group, and correlated positively to food intake of models. Finally, CCK-8 concentrations in plasma of rats showed a positive correlation with plasma β-EP ($r = -0.68$, $P<0.05$).

CONCLUSION The increased plasma and hypothalamus concentration of CCK-8, decreased gastric antrum and plasma level of β-EP, and decreased gastric antrum concentration of gastrin are associated significantly with the anorexia of infantile anorexic rat models produced by special forage. ErBao Granule can reverse these changes, which may be the major mechanisms of ErBao Granule simulating feeding.

INTRODUCTION

In pediatrics it is well recognized that children may refuse to eat for long periods of time without any demonstrable organic disease, and that the origins of this anorexia are to a large extent unknown[1-3]. We investigated 300 cases of children with anorexia [4]. Among these cases (whose causes were clarified), 50.7% was caused by improper diet and feeding. The rest of them were correlated to improper diet and feeding too. Result of several studies supports this point[5-7]. In the view of traditional Chinese medicine, the principal pathogenesis of children anorexia is PiShiJianYun (dysfunction of spleen in transportation). Thus, the YunPi method (activating the spleen) is an important part of traditional Chinese medical treatment of anorexia in children and has been studied extensively by Chinese scholars[8-10]. Since 1989, ErBao Granule, a complex prescription constituted according to Yun Pi method, has been used to treat this disease and has been observed to be effective[11]. Many children who suffered from anorexia exhibited a normal appetite 2 weeks after ErBao Granule was administered. Further study for the mechanism of ErBao Granule stimulating feeding suggested that it may increase digestive enzyme secretion, and improve both intestinal peristalsis and intestinal absorption[11]. Other similar studies have reported the same result[12,13]. However, we did not think that the action of ErBao Granule on digestive function was the major mechanism of stimulating appetite, because children with anorexia showed no significant digestive

[1]Department of Traditional Chinese Medicine, Xijing Hospital, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
[2]Department of Pediatrics, Xijing Hospital, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
[3]Department of Pediatrics, Nanjing University of Traditional Chinese Medicine, Nanjing 210029, Jiangsu Province, China
Dr. Yong Ping Du, graduated from Shanxi Medical University as a undergraduate and from Nanjing University of Traditional Chinese Medicine as a postgraduate in 1993, attending physician, major in gastrointestinal diseases of children, having 20 papers published.
Project supported by the National Natural Science Foundation of China, No.39670896.
Correspondence to: Dr. Yong Ping Du, Department of Traditional Chinese Medicine, Xijing Hospital, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Tel. 0086-29-2534749
Email. dypzyp@pub.xaonline.com
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disturbances and malnutrition as compared with healthy children.

The regulation of appetite is a complex process that consists of both central and peripheral mechanisms and involves the integration of neurotransmitters, neuropeptides, hormones and metabolic signals[14-17]. Recently, a group of peptides, distributed in the brain and gut, have been discovered to be involved in appetite control mechanism[18]. Some brain-gut peptides stimulate eating, whereas others inhibit it. Inhibitors include cholecystokinin (CCK) and somatostatin, while enhancers include β-endorphin (β-EP) and neuropeptide Y. The secretion and release of some peptides are affected by food composition, and these peptides modulate appetite as a positive feedback or negative feedback factor at the same time[19]. We suggested the loss of appetite in children with anorexia might be related to an imbalance between the brain-gut peptides[20], because the main reason of anorexia for children is inadequate diet:more protein and fat, less mineral substance and vitamins[21,22].

To study the role of CCK, β-EP and gastrin, and their potential relationship with ErBao Granule, we used an anosexic infantile rat model[23]. ErBao Granule was used to treat the rats, and CCK-8, β-EP and gastrin concentration in hypothalamus and periphery of model rats and controls were measured.

MATERIALS AND METHODS

Animals

Forty-eight Sprague-Dawley rats (24 males, 24 females, aged 35 d-40 d,60 g ± 10 g, derived from Laboratory Animal Center of our university) were randomly divided into four groups of 12: a blank control group, a model control group, an ErBao low dose groups and an ErBao high dose group. Each rat was housed individually in a regulated environment (22℃ ± 2℃, 55% ± 10% humidity and 12 h light-dark cycle).

To establish the rat model with anorexia and treat models using ErBao Granule

The anorexic rat model was established by feeding infantile rats with forage we prepared ourselves. The model forage was a compound of ingredients including milk powder, dried minced fish , sugar corn powder, and soy bean powder. We mixed the ingredients together in 1: 1: 1: 1: 2: 2: 1.6, they were shaped into cakes, each weighing about 20 g. Dried by airing, the cakes were stored at 4℃. The major composition of model forage were measured and compared with common forage. Rats in blank control group were fed with common forage and in other three groups were fed with the model forage. All rats were allowed to drink and eat at will. Fresh food was provided each day at 09:00 and each rat's food intake was measured at the same time. ErBao Granule liquid extract (offered by Vegetable Drug Research Center of Nanjing University of Traditional Chinese Medicine) was administered (ig) for twenty days, beginning on the eighth day of the experiment. ErBao liquid extract 18.8 g/kg was given to the low dose group, and 37.6 g/kg to the high dose group. Equal amounts of saline was administered in blank control group and model control group. On the 29th day of the experiment, all animals were killed after an overnight fast.

Preparation of samples and RIA of three brain-gut peptides

Blood was obtained from the orbital artery and collected into chilled plastic tubes containing 1% heparin (10 μL/mL blood sample), miscing bene immediately and then placed on ice. Samples were centrifuged at 4℃ for 3 h. Supernatant fluid was obtained and frozen at -70℃ untilanalysis. The rats were decapitated. The brain and stomach were boiled in saline for 5 min. The hypothalamus was then separated from brain, and the gastric antrum mucosa was scraped. The hypothalamus was homogenized with glacial acetic acid (1 mol/L, 1 mL). The homogenate was incubated at room temperature for 100 min. We added NaOH (1 mol/L, 1 mL) and the suspensions were centrifuged at 4℃. Supernatant fluid was obtained and frozen at -70℃ until analysis. Levels of CCK-8, β-EP and gastrin in plasma and supernatant of animals were measured using commercial radioimmunoassay. The radioimmunoassay kits for CCK-8 and β-EP were provided by the Department of Neurobiology of the 2nd Military Medical University, and the gastrin immunoassay kits, were provided by the Institute of Atomic Energy Science of China. The standard curve for CCK-8 was r = 0.998, for β-EP, r = 0.999, for gastrin, r = 0.999, all fitted the quality control criteria.

Statistics

Data are presented as the means ± SE from 12 rats per group. Statistical analysis was performed using a two-tailed Student's t test, and correlations were examined using linear correlation. $P<0.05$ was considered significant.

RESULTS

Average daily food intake of rats in four groups

On day one, we observed no significant difference in daily food intake between rats in each group. On day seven, the daily food intake of animals in model control group, low dose group and higher dose group was significantly lower than that of the blank control group. The lower food intake of rats was maintained until the end of week 4 in model control group, whereas in low dose group and higher dose group, the daily food intake of rats increased significantly at the end of week 3 (Figure 1). The results indicate that the anorexia model was established successfully and ErBao Granule was effective to the model rats[23].

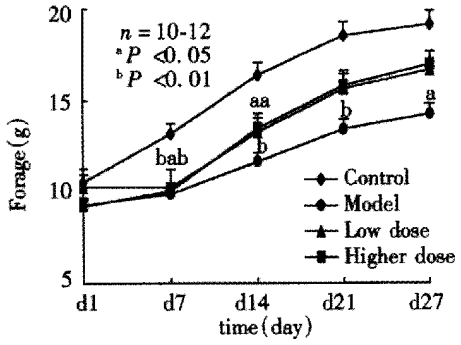

Figure 1 Daily food intake of rats in four groups. Rats of blank control group were fed with common forage. Rats of other three groups were fed with model forage. ErBao and saline were administered (ig) from the d 8 to d 28. Data are presented as the meanÀSE form 12 rats.
[a]Statistically significant difference from control, $P<0.05$, [b]$P<0.01$. At d 7, food intake of model control group, low dose group, and high dose group all was significantly lower than blank control group ($t = 3.76$, $P<0.01$; $t = 2.58$,$P<0.05$; $t = 2.83$, $P<0.01$, respectively). At d 14 food intake of the model control group, low dose group, and high dose group was still lower than blank control group ($t = 4.76$, $P<0.01$; $t = 2.53$, $P<0.05$; $t = 2.38$, $P<0.05$, respectively). At d 21, only model control group was lower than blank control group ($t = 4.71$, $P<0.01$). At d 27, still only model control group was lower than blank control group ($t = 2.33$, $P<0.05$).

Comparison of major composition between common forage and model forage

Each composition of forages was determined from parallel samples. The data were presented as the mean value. As compared with common forage, model forage showed higher concentration of coarse protein and fat (Table 1), lower concentrations of mineral elements (Table 2), and lower concentrations of vitamins (Table 3). Levels of Ca, Fe and Vitamin D were only 1/8, 1/10 and 1/7 of common forage, respectively. The results were similar to dietary survey from children with anorexia[22].

Table 1 Essential component of nutrient in two kinds of forage

Forage	Coarse protein (%)	Coarse fat (%)	Total carbohydrate (g/kg)
Model forage	18.03	39.74	389.43
Normal forage	16.12	14.99	501.05

Table 2 Content of mineral elements in two kinds of forage

Forage	Ca (%)	P (%)	Zn (mg/kg)	Cu (mg/kg)	Fe (mg/kg)
Model forage	0.17	0.08	22.7	7.24	42.39
Normal forage	1.36	0.21	40.46	9.64	428.20

Table 3 Content of vitamin in two kinds of forage (IU/g)

Forage	CaVit A	Vit D_3	Vit B_1	Vit B_2	Vit B_6
Model forage	11.1	2.3	5.3	3.3	4.7
Normal forage	21.5	17	7.3	14.3	19.9

CCK-8 level in four groups

CCK-8 concentration was significantly increased in hypothalamus (122.57 pmol/g ± 31.79 pmol/g) and in plasma (506.88 ng/L ± 113.32 ng/L) of model group compared with that of blank control group (89.15 pmol/g ± 17.94 pmol/g and 253.75 ng/L ± 95.09 ng/L). In high dose group, CCK-8 level dropped both in hypothalamus (95.55 pmol/g ± 20.68 pmol/g) and in plasma (322.14 ng/L ± 66.36 ng/L). In low dose group, it dropped to 100.00 pmol/g ± 18.83 pmol/g and 282.80 ng/L ± 75.63 ng/L, respectively. No significantly difference was observed between high or low dose group and blank control group (Figure 2). We found a negative correlation between food intake and CCK-8 concentrations of model control group in hypothalamus ($r = -0.67$, $P<0.05$) and in plasma ($r = -0.62$, $P<0.05$), and the hypothalamus level of CCK-8 was correlated positively to plasma level of CCK-8 in models ($r = 0.68$, $P<0.05$) (Figure 3).

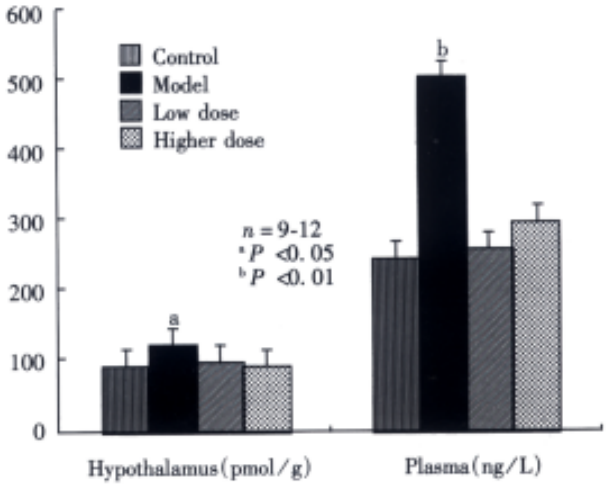

Figure 2 CCK-8 level in the four groups. Data are presented as the mean ± SE from 12 rats. [a]Statistically significant difference from control, $t = 2.55$, $P<0.05$; [b]$t = 5.81$, $P<0.01$.

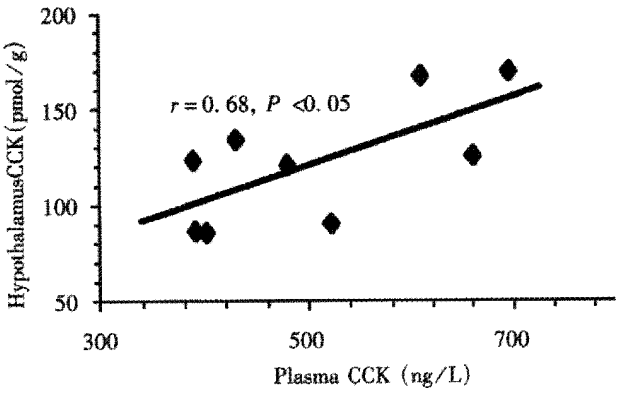

Figure 3 Correlation between hypothalamus CCK-8 and plasma CCK-8 level in model control group.

β-EP level in four groups

β-EP level in gastric antrum (1.45 μg/g ± 0.60 μg/g) and plasma (6.26 ng/L ± 1.73 ng/L) of models were significantly lower than those in the blank control group (6.28 μg/g ± 1.43 μg/g and 10.25 ng/L ± 4.86 ng/L), but hypothalamus concentration of β-EP showed no difference in rats of model control group (23.01 ng/g ± 8.01 ng/g) as compared to blank control group (24.83 ng/g ± 6.67 ng/g). In low dose group, β-EP levels in hypothalamus (28.05 ng/g ± 7.85 ng/g) and in gastric antrum (1.38 μg/g ± 0.32 μg/g) showed no obvious changes compared with model control group. Plasma level of β-EP (8.82 ng/L ± 2.86 ng/L) rose to common level. However, in high dose group, β-EP levels in hypothalamus (41.56 ng/g ± 8.74 ng/g) and gastric antrum (16.49 μg/g ± 4.07 μg/g) showed significant increases compared with those in other three groups, and plasma concentration of β-EP (8.43 ng/L ± 1.59 ng/L) raised to normal level (Figure 4). Food intake in model group correlated positively with β-EP in gastric antrum (r = 0.73, P<0.01) and in plasma (r = 0.58, P<0.05), but not in hypothalamus. We also found a negative correlation between CCK-8 and β-EP in plasma of model control group (Figure 5).

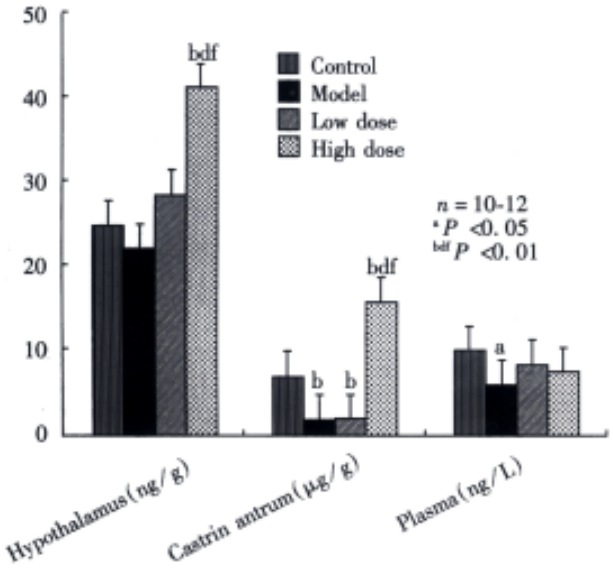

Figure 4 β-EP level between groups. Data are presented as the mean ± SE from 9-10 rats. [a]Statistically significant difference from blank control, [b]P<0.05, [d]P<0.01; from model control, [f]P<0.01; from low dose, P<0.01. In hypothalamus, β-EP level in the high dose group was higher than in the blank control group (t = 4.79, P<0.01), model control group (t = 4.82,P<0.01), and low dose group (t = 3.55, P<0.01). In gastric antrum, β-EP levels of model control group and low dose group were lower than in the blank control group (t = 9.05, P <0.01; t = 10.79, P<0.01), but β-EP level of high dose group increased more significantly than blank control group (t = 7.75, P<0.01), model control group (t = 11.34, P<0.01), and low dose group (t = 12.76, P<0.01). In plasma, β-EP level of model control group was lower than in the blank control group (t = 1.79, P<0.05).

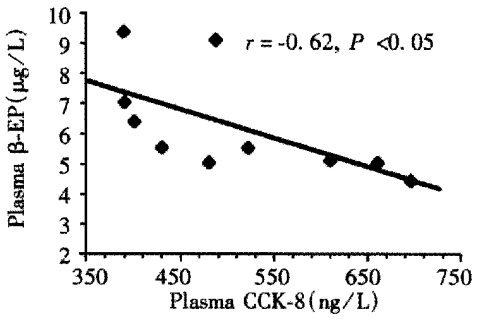

Figure 5 Correlation between plasma CCK-8 and plasma β-EP level in model control group.

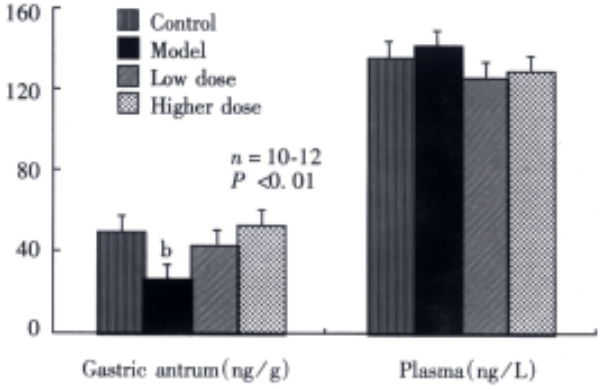

Figure 6 Gastrin level in four groups. Data are presented as the mean ± SE from 10-12 rats.[b]Statistically significant difference from blank control, t = 2.53, P<0.01.

Gastric level in four groups

Gastrin concentration in gastric antrum of model control group (27.04 ng/g ± 13.28 ng/g) was significantly lower than that of blank control group (42.78 ng/g ± 13.43 ng/g). In both the low and high dose groups, gastrin levels in gastric antrum increased to 42.78 ng/g ± 13.43 ng/g and 52.27 ng/g ± 12.37 ng/g, respectively. This increase was similar to that observed in the blank control group. Plasma concentration of gastrin remained constant in model control group, low dose group and higher dose group as compared to blank control group (Figure 6). Food intake of model control group showed a positive correlation with gastrin in gastric antrum of model group (r = 0.70, P<0.05), but not in gastrin level in plasma.

DISCUSSION

We observed an abnormal secretion status of CCK-8, β-EP and gastrin in hypothalamus and periphery of infantile rat model with anorexia produced by feeding our specially prepared forage. However, the secretion was regulated by ErBao Granule. CCK-8 is one of the most widely distributed peptides in the brain and gut. There is now strong evidence that endogenous CCK-8 is important in producing satiety in a variety of species[24,25], and it has been demonstrated to be a powerful suppressor of food

intake as compared with other peptides[26]. It has been reported that CCK-8 concentration increased in hypothalamus and gastric antrum of rat model with Spleen Deficiency[27]. No previous study has examined CCK-8 concentration in children with anorexia. However, some researchers have suggested that CCK-8 might play a role in the pathologic inhibition of food intake in idiopathic senile anorexia and anorexia nervosa [28,29]. In the present study, the model forage resulted in an increased endogenous CCK-8 secretion and release in brain and peripheral tissue of rats. Considering the negative correlation between food intake and elevated CCK-8 levels, we hypothesize that CCK-8 may be responsible, at least in part, for anorexia in children.

It remains unclear whether endogenous CCK is acting peripherally or centrally to produce satiety[30, 31]. Some studies have described that circulating CCK-8 suppressed food intake by inhibiting gastric emptying[32]. However, other investigations researches conclude that the satiety effect of endogenous CCK might not be mediated by circulating CCK[33,34]. Previous studies suggested that the satiety effect of CCK is primarily mediated by the type A receptor which is predominantly located in the periphery[35]. However, recent histochemical studies discovered that the A-type receptors are apparently present in the CNS to a greater extent than previously described[24], which indicated that CCK produces satiety by two modes:peripheral action and central action. Our study found food intake was correlated negatively with elevated CCK-8 concentration not only in plasma, but in hypothalamus of models as well, and that the hypothalamus concentration of CCK-8 was positively correlated with plasma CCK-8 level. These results agree with two modes by which CCK-8 act to inhibit food intake:one is peripheral endocrine effect, the other is a central neurocrine effect. ErBao Granule inhibited CCK-8 secretion and release both in the hypothalamus and intestines of models. We suggest that ErBao Granule might stimulate feeding by both of the two modes described above[36].

β-EP is an endogenous opiod peptide. Unlike CCK-8, β-EP can stimulate feeding significantly[37,38]. Although it has been reported that central opioids produce positive reinforcing effects, whereas peripheral opioids produce opposite effects acting as anorectic factors[39-41], a great deal of studies reported that β-EP levels in both CSF tissue and serum were reduced in anorexia of aging[42]. This suggests that β-EP concentrations in both of central and periphery play an important role in pathologic reduction of feeding. In our study, the food intake of models were positively correlated with decreased β-EP concentration in gastric antrum and plasma. We suggest that decreased peripheral concentration of β-EP might be one of the factors inhibiting feeding in models.

In the present study, ErBao Granule stimulated peripheral β-EP secretion and release. This inecreased stimulation was dose dependent, and inecreased from low dose group to the high dose group. This result indicates that the regulation of ErBao Granule on β-EP concentration in tissues was associated with dose. In addition, ErBao Granule significantly increased β-EP secretion in hypothalamus of high dose group, although there was no predominant change in hypothalamus of models, presumably because of a compensatory action in attempting to overcome anorexia. In addition, we also found a negative correlation between CCK-8 and β-EP in plasma of model group, which agree with the premise that there exists antagonism between CCK-8 and opioid system in the control of feeding behavior[43,44].

Gastrin is one of major hormones released by gastric mucosa after eating food. It has been demonstrated that gastrin is closely related to secretion and motility of the digestive tract[45,46]. Previous studies have reported abnormal high/low secretion of gastrin in patients with Spleen Deficiency syndrome and Liver-Qi Stagnation syndrome related to digestive functional disturbance[47-49]. Yet, the evidence for the role of gastrin in the control of food intake is lacking. The decreased gastrin level in serum of patients with anorexia nervosa suggested that the decrease of gastrin concentration might be one of the factors in pathologic inhibition of food intake[50]. In our study, gastrin concentration decreased in gastric antrum of models compared with controls, but not in plasma, and there was a positive correlation between higher/lower levels of gastrin and food intake in models. Therefore, we suggest that gastrin in gastric antrum might play a role in appetite control mechanisms. Furthmore, ErBao Granule may increase feeding of models by modulating gastrin secretion in gastric antrum,either directly or indirectly[51].

In summary, our results indicate that increased CCK-8 concentrations in plasma and hypothalamus, decreased β-EP level in gastric antrum and plasma, and decreased gastrin concentration in gastric antrum are associated significantly with the anorexia in rat models. The Yunpi complex prescription can regulate these changes which may be the major mechanisms of simulating feeding.

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Edited by Jason Carr
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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Clinicopathological analysis of patients with gastric cancer in 1200 cases

Wei Xin Niu, Xin Yu Qin, Han Liu and Cheng Pei Wang

Subject headings stomach neoplasms/pathology; stomach neoplasms/surgery; lymphatic metastasis; neoplasm invasiveness; gastrectomy

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INTRODUCTION

Gastric cancer is one of the most common fatal malignancies in the world. The prognosis is generally poor in advanced gastric cancer. The low survival is related to delayed diagnosis, metastasis and recurrence after operation. The aim of this paper was to find correlation between clinical factors and biologic behavior of gastric cancer in a series of 1200 patients undergoing surgical resection.

PATIENTS AND METHODS

Between November 1992 and December 1999, 1200 patients with stomach cancer confirmed by pathology underwent radical operations. The mean patient age at operation was 54.2 (range 22-89) years, 836 patients were male and 364 were female, with a mean postoperative hospitalization of 16.2 (range 6-127) days.

We analyzed the following clinicopathologic and surgical factors: age, sex, hemoglobin, operation manners, operation time, amount of transfusion during operation, postoperative hospital stay, postoperative complications, positive proximal margin, location of tumor, tumor size, differentiation, depth of tumor invasion, lymph nodes and lymphatic metastasis rate. Frequency of positive lymph nodes = numbers of metastatic lymph nodes / all lymph nodes excised × 100%.

Statistics

All data were analyzed by SPSS statistics program. The comparisons were made by Chi-square test, one-way ANOVA, linear and multivariate regression analysis, $P<0.05$ was considered as significant.

Surgical Department, Zhongshan Hospital, Fu Dan University Medical Center, Shanghai 200032, China
Dr. Wei Xin Niu, graduated from Shanghai Medical University as postgraduate in 1991, now associate professor of surgery, having 16 papers published.
Correspondence to: Dr. Wei Xin Niu, Surgical Department, Zhongshan Hospital, Fu Dan University Medical Center, 136 Yixueyuan Road,Shanghai 200032, China
Tel. 0086-21-64041990 Ext.2810 or 2276
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RESULTS

Of these 1200 patients, 768 (64%) underwent distal gastrectomy, 72 (6%) proximal gastrectomy via abdomen and 264 (22%) via thorax, and 96 (8%) underwent total gastrectomy. Distal and total gastrectomy had more numbers of clearances of lymph nodes than the other operational approaches. The postoperative complications occurred in 96 patients (96/1200, 8%), including gastric retention in 22 (22/96, 23%), anastomotic leakage in 18 (18/96, 18.7%), infection of incision in 16 (16/96, 14.6%), disruption of wound in 8 (8/96, 8.3%), and thoracic cavity effusion in 8(8/96, 8.3%). The complication was most common in proximal gastrectomy via abdomen (16/96, 17% patients) (Table 1). The overall mortality was 0.4% (5/1200).

The diameter of the neoplasm was positively correlated with the depth of infiltration and lymphatic metastasis rate while hemoglobin was the opposite. One hundred and seventy-nine (14.9%) of 1200 were early gastric carcinoma (EGC) with metastasis of lymph nodes in 21 patients (21/179, 11.7%). The frequency of positive lymph nodes in these patients was 3%-4% less than in advanced gastric cancer (Table 2). In linear regression analysis, age and diameter of the tumor were negatively correlated with the preoperative hemoglobin ($P<0.001$). The diameter of the tumor was positively correlated with age and the frequency of positive lymph nodes ($P<0.01$).

The patients with tumor of bad differentiation were younger than the other groups, who had larger tumor diameter and higher frequency of positive lymph nodes. The degree of differentiation was not related with the depth of tumor invasion on the gastric wall (Table 3). The tumor diameter on the corpus and fundus was larger than the others, which had higher frequency of positive lymph nodes (Table 4). The proximal gastric cancer, bad differentiation and frequency >30% positive lymph nodes were more common in female than in male (Table 5).

Multiple analysis demonstrated that sex, location of tumor, tumor diameter, depth of tumor invasion and differentiation play an important role in the metastasis of lymph nodes (Table 6).

Table 1 Comparison of operation manner with numbers of lymph nodes, time for operation, amount of blood transfusion during operation, hospitalization days and complications ($\bar{x} \pm s_{\bar{x}}$)

Manners of operation	N (1200)	Numbers lymph nodes	Time for operation (hours)	Amount of blood transfusion (mL)	Hospitalization stays (days)	Complication (%)	Positive resection margin (%)
Distal gastrectomy	768	11.7 ± 0.3*	3.3 ± 0.04	426.5 ± 17.1*	16.9 ± 0.7	9.1	3.8
Proximal gastrectomy via abdomen	72	9.6 ± 0.4	4.0 ± 0.1*	629.5 ± 43.3*	18.3 ± 1.5	17*	8.2*
Proximal gastrectomy via thorax	264	8.2 ± 0.2	3.3 ± 0.03	771.5 ± 19.5	15.5 ± 0.6	1.6	8.7*
Total gastrectomy	96	13.8 ± 0.7*	4.6 ± 0.1*	768.2 ± 47.6	19.8 ± 1.7	12.9	9.3*
P		<0.0001	<0.0001	<0.0001	>0.05	<0.001	= 0.01

*Compared with other operative approaches.

Table 2 Comparison of depth of infiltration with age, diameter, hemoglobin, and lymphatic metastasis rate ($\bar{x} \pm s_{\bar{x}}$)

Depth of invasion	N (1200)	Age (yrs)	Diameter (cm)	Hemoglobin(g/L)	Lymphatic metastasis rate (%)
pT1(m)	114	51.6 ± 1.2	2.3 ± 0.4	12.1 ± 0.3	3.2 ± 0.8
pT1(ms)	65	55.7 ± 1.5*	2.5 ± 0.7	11.3 ± 0.4*	4.1 ± 1.3
pT2	91	56.8 ± 1.4*	3.1 ± 0.3	11.2 ± 0.1*	9.8 ± 1.6*
pT3	95	57.1 ± 1.2*	4.2 ± 0.6*	11.4 ± 0.2*	20.4 ± 2.9*
pT4	835	56.9 ± 0.3*	5.6 ± 0.1*	11.2 ± 0.1*	37.1 ± 1.2*
P		<0.003	<0.0001	<0.001	<0.0001

*Compared with pT1(m).

Table 3 Comparison of differentiation with age, diameter, hemoglobin and lymphatic metastasis rate ($\bar{x} \pm s_{\bar{x}}$)

Differentiation	N (1200)	Age (yrs)	Diameter (cm)	Hemoglobin (g/L)	Lymphatic metastasis rate (%)
I	37	61.4 ± 1.4	3.5 ± 0.2	10.7 ± 0.4	10.3 ± 3.2*
II	161	57.9 ± 0.8	4.1 ± 0.3	11.1 ± 0.1	26.1 ± 2.5
III	329	58.6 ± 0.7	4.2 ± 0.2	11.2 ± 0.3	22.7 ± 1.4
IV	673	53.2 ± 0.3*	4.9 ± 0.3*	11.8 ± 0.1*	33.6 ± 1.2*
P		<0.0001	=0.004	=0.01	<0.0001

*Compared with other groups.

Table 4 Comparison of tumor site with age, diameter, hemoglobin and positive lymph node rate ($\bar{x} \pm s_{\bar{x}}$)

Location of tumor	N (1200)	Age (yrs)	Diameter (cm)	Hemoglobin (g/L)	Lymphatic metastasis rate (%)
Pylorus	27	54.3 ± 2.9	3.9 ± 0.7	12.6 ± 0.7	13.9 ± 3.2
Antrum	379	56.6 ± 0.5*	4.6 ± 0.2	11.5 ± 0.3	26.3 ± 1.4
Incisura	372	54.2 ± 0.4	3.3 ± 0.4	12.5 ± 0.1	22.5 ± 1.3
Corpus	91	55.2 ± 1.2	6.4 ± 0.5*	12.2 ± 0.4	38.3 ± 4.2*
Fundus	331	59.8 ± 0.6*	5.6 ± 0.2*	12.8 ± 0.2	35.1 ± 1.9*
P		<0.0001	<0.0001	>0.005	<0.001

*Compared with other locations.

Table 5 Comparison of sex with tumor location, differentiation, depth of invasion and positive lymph node rate ($\bar{x} \pm s_{\bar{x}}$)

Sex	Location (%)			Differentiation (%)			Depth of invasion (%)			Frequency of metastatic lymph node (%)	
	Proximal	Middle	Distal	Well	Middle	Bad	pT1	pT2	pT3	<30	>30
Male (836)	31	2	40	18	30	52	14	8	78	64	36
Female (364)	40	3	25	13	21	66	15	7	78	56	44
P		<0.001			<0.001			>0.05			=0.01

Table 6 Multi-factors analysis of lymphatic metastasis in gastric patients

Related factors	Regression coefficient	Standard error	Standard regression coefficient	*P*
Constant	-24.3	7.1		0.001
Age	-0.006144	0.079	-0.22	0.438
Sex	-6.489	2.027	-0.092	0.001
Tumor location	2.326	0.780	0.087	0.003
Diameter of tumor	2.368	0.459	0.165	0.0001
Depth of invasion	7.043	0.786	0.285	0.0001
Differentiation	3.687	1.146	0.094	0.001

DISCUSSION

Gastric cancer remains one of most common causes of death. Although the etiology of gastric cancer is still unclear, but studies have shown that many factors are associated with the development, metastasis of gastric cancer, and recurrence after operation[1-9]. Recent studies suggest that infection with *Helicobacter pylori* may play an important role in the development of gastric cancer[10-15]. It has been proposed that *Helicobacter pylori* infection may produce acute and chronic gastritis, intestinal metaplasia, dysplasia, and eventually resulting in gastric cancer. Some abnormal expression[16-18] in gene is involved in carcinogenesis of gastric cancer such as matrix metalloproteinases gene, *p*53 gene and dinucleotide repeat sequence gene. Abnormal contents of some trace elements may also be one of the risk factors in gastric cancer[19,20].

Early gastric cancer (EGC) has been considered to be a form of gastric malignancy with a relatively good long-term prognosis compared to that of advanced gastric cancer because of rare metastasis in lymph nodes[21-26]. In Japan, EGC is diagnosed in 30%-50%, due to partly at least the extensive use of endoscopy and mass screening programs[27-29]. In this study, the proportion of EGC diagnosed in all patients is 14.9%, similar to the proportion in the United States and Europe[30,31]. In recent years, endoscopic treatment has become increasingly popular as an alternative to surgical treatment of patients with EGA in hope of offering superior quality of life (QOL)[32]. However, because of presence of metastasis in 10%-20% and skip metastasis of lymph nodes, whether the rationale for a standard resection with systematic lymphadenectomy is necessary is still a controversial issue[33-37].

Different operative approaches were carried out according to the different locations of the tumor. In our study, the number of lymph nodes excised were the largest in total gastrectomy, followed by distal gastrectomy which may be related to the resection of all or most parts of omentum. The number of lymph nodes excised in proximal gastrectomy via a transabdomen was similar to via transthorax. There was shorter time for operation and lower frequency of complication in proximal gastrectomy via transthorax while lower blood transfusion in proximal gastrectomy via transabdomen. The postoperative hospitalization stay and the positive resection margin was same between them. The complications varied among different operations: gastric retention was common in distal gastrectomy while thorax effusion and infection of lung were mainly found in total gastrectomy.

Although the overall incidence of gastric cancer has remained stable in the West, there is well-documented shift from distal to proximal lesion. The clinical relevance of this shift is that the overall prognosis for patients with proximal gastric cancer is worse than for those with distal tumor. This difference in survival may be attributed to a variety of factors, ranging from an increased biologic aggressiveness of proximal tumors to an advanced stage of presentation[38,39]. In study, a higher frequency of positive lymph nodes was found in gastric cancer located on corpus and the fundus which may be associated with the larger diameter of the tumor in corpus and the fundus. In tumors with larger diameters there were worse differentiation, deeper infiltration, and higher frequency of positive lymph nodes. Apparently, the prognosis will be worse in these patients. The present results also show that the more proximal lesions, bad differentiation, and the higher >30% frequency of positive lymph nodes can be found in female than in male.

The numbers of metastatic lymph nodes play an important role in the long-term outcome after curative resection[40-43]. Thus it is suggested that extended lymphadenectomy should be performed in advanced gastric cancer[44-47]. Our multivariate analysis indicated that among six clinicopathologic variables (age, sex, location of tumor, tumor diameter, depth of invasion and differentiation), the depth of invasion was the most important factor influencing metastasis of lymph node.

In conclusion, this retrospective study has shown that clinicopathological characters in gastric cancer varied with sex, location, and diameter of the tumor. The depth of invasion plays a very important role in metastasis of lymph node. The prognosis in female with gastric cancer may be worse than in man. Because metastasis of lymph nodes may occur even in patients with EGC, radical gastrectomy witi lymphadenectomy may be necessary in all stages of gastric cancer.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Expression of insulin like growth factor II and its receptor in hepatocellular carcinogenesis

Zi Rong Fan, Dong Hua Yang, Jun Cui, Han Rong Qin and Chun Chi Huang

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INTRODUCTION

Insulin-like growth factor II (IGF-II) is a mitogenic peptide of 74 kD and is mostly synthesized in fetal liver tissue. IGF-II is believed to play an important role in fetal growth and development and is involved in cellular proliferation and differentiation[1-5]. Recently, several researchers have reported increased expression of the IGF-II gene in human hepatocellular carcinoma(HCC) and adjacent non-cancerous liver tissues[6-10]. It is suggested that the aberrant overexpression of insulin-like growth factor II might be related to the deregulation of growth control in hepatocytes during malignant transformation via an autocrine or paracrine mechanism [11-14]. We have designed a study using an *in situ* hybridization technique to assess the effect of IGF-II and its receptor mRNAs on the carcinogenesis and development of HCC.

MATERIALS AND METHODS

Tissue samples

Thirty surgically resected HCC specimens were collected in the Tumor Hospital affiliated to Sun-Yet Medical University during the period of 1996-1997. All the HCC specmens were classified according to Edmondson-Steiner criteria[15]. Eighteen chronic hepatitis and 25 liver cirrhosis specimens were taken by liver biopsy. The tissues were fixed in 10% paraform and embedded in paraffin. Continuous sections were cut for *in situ* hybridization staining of IGF-II and IGF-IIR, respectively.

Department of Gastroenterology, Zhujiang Hospital, The First Military Medical University, Guangzhou 510282, Guangdong Province, China
Zi Rong Fan, graduated from The First Military Medical University, physician-in-chief, lecturer, master degree of gastroenterology, he has ?published 20 papers.
Project supported by the National Nature Science Foundation of China, No. 39470774
Correspondence to: Zi Rong Fan, Department of Gastroenterology, Zhujiang Hospital, The First Military Medical University, Guangzhou 510282, Guangdong Province, China
Tel. 0086-20-85143361
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Hybridization probes

We designed the oligonucleotide probes of IGF-II and IGF-IIR. They were synthesized at the Shanghai Biochemistry Research Institute. The sequence of the oligonucleotides probes is: IGF-II, 5'-GTG-CTT-CTC-ACC-TTC-GCC-TTC-GCC-TCG-TGC-TGC-ATT-G-3'[16] IGF-IIR, 5'-ACA-ATG-CCT-GTC-TGT-GGG-ACC-ATC-CTG-GGA-AAA-CCT-GTC-T-3'[17].

In situ hybridization

The *in situ* hybridization protocols used are described by Tomita *et al*[18]. Briefly, the hybridization solution contained 50% formamide, 5 × SSC, 0.15 mol/L NaCl, 0.1% degraded herring sperm DNA, 0.1% bovine serum albumin, 0.1% SDS, 0.1% polyvinylpyrrolidone (PVP), and digoxin labeled the oligonucleotides probes of IGF-II or IGF-IIR and were kept at 40℃ overnight. The slides were digested prior to proteinase K (30 mg/L) treatment at 37℃ for 30 minutes. After the procedures, the slides were counter stained with nuclear fast red.

Controls

The fetal liver tissues with a high level of expression of insulin-like growth factor II served as positive controls. Negative control slides were treated with PBS and were subjected to all other steps of the staining procedure.

DNA extraction and Southern-blot hybridization

Genomic DNA was prepared using the proteinase K and Phenol-Chloroform extraction method. Southern-blot hybridization was used to detect HBV-DNA integration in various kinds of liver tissues[19].

Statistical analysis

Chi-square test was used for significance analysis. The difference was regarded as significant if P value was less than 0.05.

RESULTS

Expression of IGF-II and IGF-IIR mRNAs in chronic hepatitis, liver cirrhosis and HCC tissue

In normal adult human liver sections and negative control slides, IGF-II and IGF-IIR mRNAs were

not detected exclusively in the hepatocytes. However, in normal fetal liver tissue, very strong signals of IGF-II and IGF-IIR mRNAs were detected. The positive or ratios of IGF-II and IGF-IIR mRNAs increased stepwise starting with chronic hepatitis (33.3%), then HCC (66.7%) and finally liver cirrhosis (72.0%). There was a statistically significant difference in IGF-II and IGF-IIR mRNAs expression between chronic hepatitis and liver cirrhosis ($\chi^2 = 6.34$, $P<0.05$) or HCC ($\chi^2 = 5.04$, $P<0.05$). No significant difference of IGF-II and IGF-IIR mRNAs expression was observed between liver cirrhosis and HCC ($\chi^2 = 0.29$, $P>0.05$). The positive expression of IGF-II and IGF-IIR mRNAs was anchored locally in hepatocellular cytoplasm in a diffuse pattern. The signal intensity of IGF-II and IGF-IIR mRNAs was found to vary with cell type. The lower signal intensity was detected in chronic hepatitis sections, whereas the stronger signal intensity was detected in liver cirrhosis and HCC tissue. An intensive signal intensity was detected in liver regenerative nodules, liver cell dysplasia(LCD) and poorly differentiated HCC cells.

Expression of IGF-II and IGF-IIR mRNAs in differently differentiated HCC tissue

Expression of IGF-II and IGF-IIR mRNAs in HCC tissue of Edmondson grades I-IV were 0, 25.0%, 76.2% and 100.0% respectively. The poorly differentiated tissues (Edmondson grades III and IV) showed higher positive ratios than the well-differentiated tissues (Edmondson grades I and II). (comparison among all groups: $\chi^2 = 9.48$, $P<0.05$).

Relationship between expression of IGF-II/IGF-IIR mRNAs and HBV-DNA integration ratios

HBV-DNA integration ratios in chronic hepatitis, liver cirrhosis and HCC tissues were 61.1% (11/18), 84.0% (21/25) and 83.3% (25/30), respectively. The positive ratios of HBV-DNA integration in chronic hepatitis, liver cirrhosis and HCC tissues were 54.5% (6/11), 81.0% (17/21) and 76.0 (19/25), respectively. The negative ratios of HBV-DNA integration in chronic hepatitis, liver cirrhosis and HCC tissues were 0% (0/7), 25.0% (1/4) and 20.0 (1/5), respectively. These data showed that IGF-II and IGF-IIR mRNAs expression was closely related to HBV-DNA integration.

DISCUSSION

The occurrence of HCC is a process which involves multiple-steps[20,21]. Chronic hepatitis, liver cirrhosis and liver cell carcinogenesis are three important different steps in the occurrence of HCC. Our study demonstrated that IGF-II and IGF-IIR mRNAs were both abnormally expressed in chronic hepatitis, liver cirrhosis and HCC tissue. The expression ratio of IGF-II and IGF-IIR mRNAs in liver cirrhosis was the highest, while that in chronic hepatitis was the lowest. The expression of IGF-II and IGF-IIR mRNAs in liver cirrhosis and HCC was remarkably higher than that in chronic hepatitis. The results suggest that IGF-II and IGF-IIR participate in the process of HCC development at different stages[22,23].

IGF-II and IGF-IIR were expressed at low levels in chronic hepatitis. With the progression of chronic hepatitis to liver cirrhosis, the expression of IGF-II and IGF-IIR increased significantly (especially in regenerative nodules and of liver cell dysplasia). Not only the number of positive cells increased, but also the intensity of the positive signal, which suggests that their over-expression was related to the formation of liver cell dysplasia and liver cell regenerative nodules[24,25]. It is generally accepted that regenerative nodules and liver cell dysplasia are pre-cancerous pathological changes of HCC[26]. The over-expression of IGF-II and IGF-IIR in the pre-cancerous pathological changes of HCC suggests an early event in hepatocellular carcinogenesis. IGF-II and IGF-IIR possibly may play important roles at the early stage of hepatocellular carcinogenesis[27].

In the developmental process of chronic liver disease, repeated inflammation and necrosis of liver cells occurs, which leads to over-expression of IGF-II and IGF-IIR. All these stimulate the abnormal proliferation of liver cells, which creates conditions for the formation of regenerative nodules and liver cell dysplasia. The hepatocytes of liver cell dysplasia and regenerative nodules proliferate actively. IGF-II and IGF-IIR were highly expressed in the two kinds of pre-cancerous pathological changes of HCC, which further stimulates the abnormal proliferation of pre-cancerous liver cells and increases the mutation rate of pre-cancerous liver cells[28]. Their high expression could also induce the malignant transformation of pre-cancerous hepatocytes under certain conditions and eventually lead to hepatocellular carcinogenesis[29-31]. If the over-expressions of IGF-II and IGF-IIR are related to the abnormal proliferation of pre-cancerous hepatocytes, they could be regarded as markers for abnormal proliferation of pre-cancerous hepatocytes[32-34]. The pre-cancerous lesions with high expression of IGF-II and IGF-IIR may be high risk factors of carcinogenesis[35,36].

The expressions of IGF-II and IGF-IIR were related to the differentiation of HCC. Stronger positive signal were detected in poorly differentiated HCC cells. The expression of IGF-II and IGF-IIR in poorly differentiated HCC cells were significantly

higher than that in well differentiated HCC cells. All these magnify the signal transduction, stimulating cells to grow persistently what accelerates the growth of HCC cells and maintained the malignant phenotype of HCC cells[37-41]. If the expressions of IGF-II and IGF-IIR are related to the differentiation of HCC cells, it IGF-II and IGF-IIR may be regarded as a marker for HCC differentiation, which could contribute to better determination of prognosis for patients with HCC[42].

We observed that the expressions of IGF-II and IGF-IIR mRNA in liver cirrhosis were higher than that in HCC. This observation is similar to results reported by Yang Dong-Hua[43]. The over-expressions of IGF-II and IGF-IIR mRNAs were mainly detected in regenerative nodules and liver cell dysplasia. Their over-expressions provided necessary conditions for the malignant evolvement of pre-cancerous liver cells. Once the pre-cancerous liver cells became malignant, they got auto-growth ability and did not simply depend on the high expression of IGF-II and IGF-IIR to maintain their malignant proliferation. Although the expressions of IGF-II and IGF-IIR in HCC tissue were less than that in pre-cancerous liver cirrhosis tissue, when compared with normal more redundary hepatocytes and non-pre-cancerous liver tissue, the expressions of IGF-II and IGF-IIR in HCC tissue were higher. So the expressions of IGF-II and IGF-IIR in liver cirrhosis tissue were higher than that in HCC tissue.

Human HCC frequently occurs in patients with chronic hepatitis B virus (HBV) infection[44-46]. However, the exact molecular biological mechanism by which HBV leads to HCC is still unknown. HBV-DNA integration was reported in 80%-90% of HCC tissue derived from HBV infected patients, which would lead to the deletion, rearrangement, translocation or replication of cellular DNA at the integration site[47,48]. Therefore, the integration of HBV-DNA might play an important role in pathogenesis of some HCC cases. The 11th chromosome is the main site for HBV-DNA integration. The IGF-II gene is located on the short arm of 11th chromosome[49]. Hence, the integration of HBV-DNA on ^{11}P chromosome was probably related to the aberrance of IGF-II gene and therefore lead to abnormal over-expression of IGF-II[50]. Another possibility is that the instability of genome DNA caused by chronic liver disease interrupts the normal regulating sequence of IGF-II gene and therefore leads to the replacement, deletion and transition of IGF-II gene, leading to the persistent over-expression of IGF-II and eventually resulting in hepatocellular carcinogenesis. Our data indicates that the over-expressions of IGF-II and IGF-IIR were significantly higher with than without HBV-DNA integration in liver disease tissue. However, the accurate regulating mechanism and possible motivating or initiating relationship between over-expression of IGF-II/IGF-IIR and HBV-DNA integration remained obscure.

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Edited by Jason Carr

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Rapid detection of sepsis complicating acute necrotizing pancreatitis using polymerase chain reaction

Wei Zhong Zhang, Tian Quan Han, Yao Qing Tang, Sheng Dao Zhang

Subject headings pancreatitis/diagnosis; polymerase chain reaction/methods; multiple organ failure; septicemia; gram[a2]negative bacteria

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INTRODUCTION

Acute necrotizing pancreatitis usually takes a severe clinical course and is associated with multiple organ dysfunction. With the further understanding of pathophysiological events of acute pancreatitis and the therapeutic measuses taken by the clinicians, the patients can pass through the critical early stages, and then the septic complication caused by translocated bacteria, mostly gram-negative microbes from the intestines ensues[1]. During this stage, the clinical manifestation is not specific and is characterized by systemic inflammatory response, but bacterial cultures are often negative.

Identification of minute quantities of microbial-specific DNA has been made possible by using polymerase chain reaction techniques[2-19] and this method has been used to detect and identify specific pathogen in clinical specimens. It has been shown that PCR method is more sensitive than conventional blood cultures for detecting microbial products in blood[20-22].

The current study was performed to evaluate the technique of PCR with the universal primers targeting bacterial 16S rRNA genes in diagnosing the systemic infection secondary to acute necrotizing pancreatitis.

MATERIALS AND METHODS

Patients and sample collection

Between May 1998 and May 1999, 22 blood samples were obtained from 13 patients with CT or surgically confirmed acute necrotizing pancreatitis who were admitted consecutively to surgical ICU in Ruijin Hospital, Shanghai. There were 8 men and 5 women, the average age was 56.6 ± 8.9 years, and the average APACHE II scores were 10.5 ± 2.2 points.

The blood samples were drawn if the patients presented two or more of the following conditions: ① temperature more than 38℃ or less than 36℃, ② elevated heart rate more than 90 beats per minute, ③ respiratory rate more than 20 breaths per minute or $PaCO_2$ less than 32 mmHg, and ④ white blood cell count more than 12 000/cu mm, less than 4 000/cu mm, or more than 10% immature band forms. And the foci of infection were documented[23-26].

Twelve mL of blood was drawn from each patient, of which 2 milliliter was collected in sterile Na_2 EDTA anticoagulant Eppendorf tubes and stored at 4℃ until DNA extraction was performed, 10 milliliter was sent for conventional blood cultures.

At the same time, 10 blood samples were obtained from 10 healthy volunteers for controlled study.

Bacterial strains

The bacterial strains used were clinical isolates collected from Ruijin Hospital and identified by automated Vitek system. The strains were cultured at 37℃ on blood agar plates until DNA extraction was performed.

DNA extraction

Blood was transferred from Na_2EDTA tubes to sterile 1.5 mL Eppendorf tubes, red cells were lysed in 0.32M sugar-5 mmol $MgCl_2$-0.01M Tris-Cl -1% Triton-x for 10 minutes at room temperature. After centrifugation for 5 minutes at 5 000 rpm, the supernatant was discarded and sediment was preserved for DNA extraction.

The sediment was lysed in 10% Chelex-100 (Sigma) - 0.03% Sodium dodecyl sulfate - 1% Tween 20-1% Nonidetp-40 for 5 minutes at 95℃. After centrifugation (5 000 rpm) for 10 seconds, 5 μL of the supernatant was directly used for PCR amplification[2].

[1]Department of Surgery, Huangyan First Hospital, Huangyan 318020, Zhejiang Province, China
[2]Department of Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
Dr. Wei Zhong Zhang, graduated from Shanghai Second Medical University receiving master degree of surgery in 1999; he is devoted to basic and clinical investigation on severe acute pancreatitis and has one paper published.
Correspondence to: Dr. Tian Quan Han, Department of Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
Fax. 0086-21-64373909
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Oligonucleotide primers for PCR

One set of oligonucleotide primer pair was synthesized by the Promega Company, Shanghai Office. The target DNA sequence was the 16S rRNA gene. This set of primers was 5'-GGC GGA CGG GTG AGT AA-3' and 5'-ACT GCT GCC TCC CGT AG-3' to amplify a 255 bp region.

Positive and negative controls

DNA from clinical isolates of *E.coli* was extracted in the same manner as outlined previously. This DNA was used in PCR reactions to determine if the PCR reaction was successful. In addition to a positive control, each PCR experiment contained a reagent negative control that consisted of all PCR reagents but without DNA to determine whether the potential contamination was present.

Microbial DNA amplification

PCR assay was established according to the protocols described by Widjojoatmodjo *et al*[2]. The PCR mixture (50 μL) contained 50 mM Tris-HCl, 200 mM each deoxynucleoside triphosphate (dNTP), 0.4 μM each primer and 1.0 u of Super-Taq Polymerase (Promega Company, Shanghai Office) and 7 mM $MgCl_2$.

The PCR was performed in a DNA Thermal Controller (MJ, Research, INC, USA) as follows. The first step of 5 minutes at 94℃ was followed by 30 cycles of 30 seconds at 94℃, 10 seconds at 72℃ and 1 minutes at 55℃; and extension period of 2 minutes at 55℃ completed the cycling squence.

Identification of PCR products

After amplification, 5 μL of PCR products was run on a 1% agarose gel in 0.5 × TBE. DNA bands were detected by ethidium bromide staining and visualized by UV light photography.

Blood cultures

Blood obtained for culture was collected from patients in a sterile manner and inoculated directly into aerobic and anaerobic bottles. The procedure was performed in the department of clinical diagnosis, Ruijin Hospital.

Statistical analysis

Statistical analysis was done by using the Chi-square test. The difference was considered significant at $P<0.05$.

RESULTS

There was only 1 positive blood culture in the 22 blood samples of 13 patients (4.55%). The organism was *Escherichia coli* (Table 1). But PCR amplification was positive for 8 samples (36.36% $P<0.05$ *vs* culture) from patients and all clinical isolates, yielding the 255 bp band (Figure 1). No DNA amplification occurred in the blood samples from volunteers.

Table 1 Results of PCR and blood culture data for ANP patients

Sample No.	Age (yr)	Gender	Blood culture	PCR	T (℃)	HR (beats / min)	R (breaths / min)	WBC (10^9/L)
1	53	M	-	+	39	120	20	13.5
2*	36	M	+	+	39.1	116	22	14.3
			-	+	38.6	130	25	17.9
			-	-	38.1	114	22	9.8
3*	51	M	-	+	39.1	130	24	17.1
			-	-	39.1	140	24	18.3
4	55	M	-	+	38.3	100	22	17.5
5	67	F	-	-	38.2	110	24	12.8
6*	54	M	-	-	38.5	116	34	14.4
			-	-	37.9	116	22	12.2
			-	-	37.2	95	18	18.7
			-	-	37.9	118	23	18.3
7	57	M	-	-	39.5	120	26	18.6
8*	51	M	-	+	39.7	180	Mechanic	19.2
			-	-	39.2	130	Mechanic	13.4
9*	54	F	-	+	39.6	128	Mechanic	37.7
			-	-	41.3	130	Mechanic	18.5
10	60	F	-	-	39.3	170	Mechanic	9.3
11*	61	F	-	-	39.1	126	28	20.4
			-	+	38.5	116	22	8.9
12	60	M	-	-	38.5	108	22	16
13	74	F	-	-	38	116	Mechanic	18.6

The data are time-ordered in the same patient; *Mechanic*- means mechanical ventilation and ANP is the abbreviation of acute necrotizing pancreatitis.

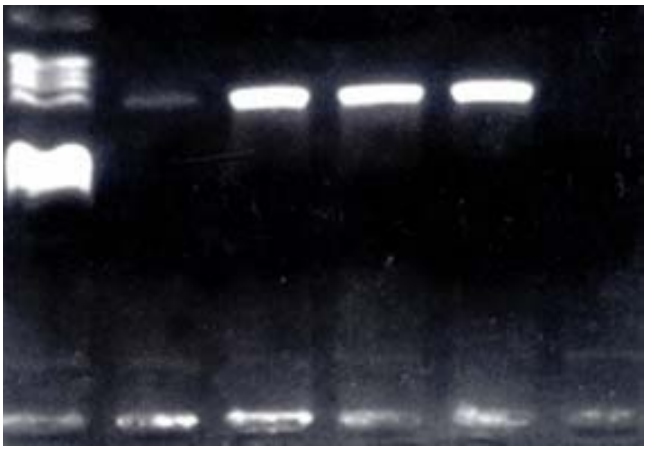

Figure 1 Agarose gel electrophoresis of amplified DNA from clinical samples and controls and volunteers.
Lane 1:DNA marker; 2: Case 2; 3: Case 8; 4: *Escherichia Coli*; 5: *Staphylococcus aureus*; 6:human leucocyte

DISCUSSION

During the late course of acute necrotizing pancreatitis, starting from the second week, local and systemic complication caused by translocated bacteria from intestines are dominant. The infection occurs in 30% to 40% of patients with acute necrotizing pancreatitis. Around 80% of deaths in patients with acute necrotizing pancreatitis are caused by septic complication[1]. But during this stage, the clinical manifestation is not specific and

characterized by systemic inflammatory reaction and the blood culture is usually negative; this will levy a heavy toll on the clinician for the prompt management of the patients.

Recent studies showed that blood culture techniques, such as volume of inoculated blood, culture media could significantly influence the recovery of bacteria in clinically suspected septic patients and culture is more time-consuming[27-30].

Molecular biology techniques, such as PCR have been used in making a specific and sensitive diagnosis of bacterial infection[2,8,9,12,13,15,16]. The 16S rRNA sequence is highly conserved through the phylogenetic tree. The conserved sequences of the 16S rRNA have led to the development of conserved primers for PCR for the detection of eubacteria. Recently the PCR with universal primers targeting 16S rRNA genes has been used widely to define bacteria[2,15,16,31-41].

With the protocol described by Widjojoatmodjo *et al*[2,31], we developed PCR assay by using the 16S rRNA genes as the amplification targets. In this assay, we found no DNA amplification in healthy blood cells, suggestive of high specificity of these primer pairs. The disadvantage of PCR technique is the contamination of DNA templates, and therefore we employed negative controls at each PCR experiment to safeguard against the potential contamination of stock PCR reagants with microbial DNA products in the environment, and this study showed no false-positive results (Figure 1).

The gold standard of identifying sepsis is blood culture; however, the clinical sepsis is observed in the absence of documented infection in more than 50% of patients with MOF[21] and the prevalence of positive blood culture is around 12%. The positive rate of blood cultures in our study was 4.55% (propably due to small sample), whereas the PCR-positive rate was 36.36% ($P<0.05$), which signifies that this detection method has higher sensitivity than blood culture.

Another advantage of this PCR assay is its ability to perform serial measurements in the same patient for detection of bacterial DNA in the blood, as shown in patients 2, 3, 6, 8, 9 and 11 (Table 1), because PCR is time-saving (less than 8 hours) and blood cultures usually take much longer time (at least 2 days).

However, this detection method cannot identify whether it represents living invading organisms or dead ones engulfed by phagocytes, so this approach cannot differentiate between controlled and invasive infections. Until methods that quantitate bacterial DNA are developed[36], we should combined the results of PCR assays with relevant clinical information to determine whether the sepsis is present. Furthermore, if we apply multiple oligonucleotide primers in the PCR assay[21], there would be a higher PCR-positive rate.

In conclusion, the PCR assay with universal primers targeting 16S rRNA genes is more sensitive in detecting the sepsis secondary to acute necrotizing pancreatitis and this may prompt us to take more aggressive approach to the disease.

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Edited by Lu HM
proofread by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Can ultrasound predict the severity of acute pancreatitis early by observing acute fluid collection?

Yan Luo[1], Chao Xin Yuan[1], Yu Lan Peng[1], Pei Lin Wei[1], Zhao Da Zhang[2], Jun Ming Jiang[3], Lin Dai[1] and Yun Kai Hu[1]

Subject headings pancreatitis/classification; acute fluid collection; pancreatitis/ultrasonography; C reactive protein; prognosis

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INTRODUCTION

The spectrum of acute pancreatitis (AP) ranges from a mild spontaneously resolved disorder to severe disease with a mortality up to 20%-48.4%[1-3]. sAP is defined as the AP with organ failure and/or local complications which developed from acute fluid collection (AFC) including necrosis, abscess, pseudocyst formation into or around the pancreas[4]. sAP is only about 15%-25% but almost all of mortality and morbidity of AP is concentrated in it[5]. Early diagnosis and assessment of severity in AP are still far from ideal. The role of clinical assessment is reliable but for its subjectivity the value in the early detection of severity is limited[1, 6]. C-reactive protein (CRP) is a promising laboratory marker of severity, even though it is not specific for AP[1]. Single or multiple biochemical criteria and different scoring systems have been used as prognostic indicators, but none has been proven satisfactory in clinical practice[1,6].

The aim of this study is to investigate the prognostic significance of evaluating the severity of AP by observing AFC using ultrasonography.

[1]Ultrasound Department of First Affiliated Hospital of West China University of Medical Science, Chengdu 610041, Sichuan Province, China
[2]Surgery Department of First Affiliated Hospital of West China University of Medical Science, Chengdu 610041, Sichuan Province, China
[3]Tranditional Chinese Medicine, Department of First Affiliated Hospital of West China University of Medical Science, Chengdu 610041, Sichuan Province, China
Dr. Yan Luo, graduated from West China University of Medical Sciences as a postgraduate in 1990, now chief and lectuer of ultrasound department, specialized in ultrasound diagnosis in abdominal and vascular diseases, having 10 papers published.
Supported by Clinical Key Subject Fund of the Health Administration, No.97050234.
Correspondence to: Dr Yan Luo, Ultrasound Department of First Affiliated Hospital of West China University of Medical Science, 37# Guxue Street, Chengdu 610041, China
Tel.0086-28-5422304, Fax. 0086-28-5422987
Email. luoyian@mail.sc.cninfo.net, luoluohei@sina.com.cn
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SUBJECTS AND METHODS

General information

A total of 627 patients who underwent ultrasound examination for AP between 1996-01/1998-12 in the 1st Affiliated Hospital of West China University of Medical Sciences were analyzed retrospectively. Among them 293 were male and 334 female, aged from 4 to 82 years, with an average of 43.02 years; 502 had mild acute pancreatitis (mAP), and 125 severe acute pancrititis (sAP). All examinations were performed by attending doctors in the ultrasound department using AI Performa or Diasonics 2D Gateway ultrasound machines with 3. 5-5MHz probes.

The primary or first ultrasound examination

Usually on the d1 or d 2 after patient admission to our hospital, routine upper abdominal scanning was performed, with particular observation of the size and the echogenicity of pancreas, the pancreatic and bile ducts. Additional attention was made to peripancreatic AFC, including the lesser sac, anterior pararenal spaces, posterior pararenal spaces, peritoneal cavity, and even thoracic cavity.

Follow-up ultrasound examinations

Continued observation of the pancreas with further attention of those spaces mentioned above, as well as the presence or absence of AFC, the quality and the nature of the fluid (i.e., necrosis, abscess or psydeocyst). If necessary, an ultrasound-guided needle aspiration was performed in order to determine the nature of the fluid and to relieve any pressure build-up. Typically repeated scans were performed 3-7 days following the initial scan. However, this interval was dependent upon the severity of AP. For analysis, the patients were classified into four groups according to the fluid collection: no AFC, 1 site AFC, 2 sites AFC, more than 2 sites AFC.

Treatment

All patients were treated by the methods, including traditional Chinese therapy, aggressive intravenous fluid resuscitation, intensive monitoring of vital functions, supportive therapy. Operative intervention was used only when local complication occurred despite maximal conservative therapy.

Clinical parameter

Age, sex, etiology, hospital stay, local

complication, number of operation, mortality. sAP and mAP were diagnosed according to the clinical features, symptoms, physical examination, laboratory tests and image findings.

Data collection

The size of the pancreas, the number of spaces where there were AFC, as well as any regression or resolution in AFC were recorded. If fluid had resolved, the amount of time necessary for complete resolution and any complications associated with AP as well as the amount of time necessary to develop such complications were monitored. Meanwhile, the response to therapy (either medical or surgical), mortality, and length of hospital stay were noted.

Statistical analysis

Continuous variables were analyzed by *t* test. Discrete variables were analyzed by the χ^2 test and rank sum test using SPSS. $P<0.05$ was considered significant.

RESULTS

The number and distribution of AFC and the clinical outcome are summarized in Table 1.

Table 1 AFC and clinical outcome

	0 site	1 site	2 site	>2 sites	Total
Mild AP	256(51)	91(18.1)	75(14.1)	80(15.9)	502(100)[a]*P*
Severe AP	10 (8)	13(10.4)	18(14.4)	84(67.2)	125(100)[a]*P*
n	263	101	91	144	599
Mean hospital stay	12.2±6.14 [b]P [c]P [d]P	15.8±9.89 [b]P [e]P [f]P	19.4±10.13 [c]P [e]P [g]P	28.4±19.68 [d]P [f]P [g]P	17.8 ±14.37 [h]P
Local complication	0	22	16	40	78
Operation	0	2	5	33	40
Death	2	2	1	15	20

n: the number of mean hospital stay, exclude dead patients and the patients who discharged themselves without complete cure. Value in parentheses are percentage.

[a]$P<0.00001$, $\chi^2 = 147.78$, DF = 3.

To analyze the mean hospital stay using Kruskal-wallis test, [h]$P<0.00001$, $\chi^2 = 161.47$, DF = 3; furthermore using Wilcoxon test (l test) to analyze the mean hospital stay between every two group: [b]$P<0.00001$, l = 4.09; [c]$P<0.00001$, l = 6.69; [d]$P<0.00001$; [e]$P = 0.0206$, l = 2.32; [f]$P<0.00001$, l = 8.02; [g]$P<0.00001$, l = 5.07

In the local complication, all 22 local complications in one site were necrosis or pseudocyst formation in sac with or without infection; 56 local complication in two and more than two sites were sac necrosis formation with another space necrosis.

The operation meant all kind drainage for local complication during AP, did not include cholecytectomy after AP.

In dead cases, 4 cases with 0 and 1 site AFC died within 48 h of admission for multisystem organ failure with an average of 28 h; 16 cases with two and more than two sites AFC died within 2-35 days with an average of 18.6 d. Three patients died in wk 1, 4 died in wk 2, and 9 died in wk 3.

The prognostic significance of the severity of AP using AFC number are shown in Tables 2 and 3.

Table 2 To prognosticate the severity of AP using 2 and more than 2 sites AFC

		Clinical sAP	Diagnosis mAP	Total
	2 & >2 sites	102	155	257
US	< 2 sites	23	347	370
	Total	125	502	627

Sensitivity 81.6%, speciality 69.2%, accuracy 70.2%.

Table 3 To prognosticate the severity of AP using more than 2 sites AFC

		Clinical sAP	Diagnosis mAP	Total
	>2 sites	84	80	164
US	2 & < 2 sites	41	422	463
	Total	125	502	627

Sensitivity 67.2%, speciality 84.1%, accuracy 80.7%.

DISCUSSION

Acute pancreatitis is an acute inflammatory process of pancreas, with varible involvement of other regional tissue or remote organ system, some inflammatory cytokine related to it and any high pressure of the pancreatic duct can lead to accumulation of pancreatic juice into pancreas or spill into the peripancreatic area[7-20]. As pancreas is situated in the anterior portion of the retroperitonium and behind the lesser sac, the fluid can accumulate in the lesser sac and the vast retroperitoneal space (especially in anterior pararenal space). The retroperitoneal space is bounded by the posterior parietal peritoneum in front and the transverse fascia behind , extending from the diaphragm to the upper brim of the pelvis. As soon as the pancreatic fluid and exudate enter the retroperitoneal space, they spread freely under tension, extending downward through the lateral part of the retroperitoneal space toward the iliac fossa and upward to the undersurface of the diaphragm and mensenteries involved[21-24].

AFC is quite common in acute pancreatitis[25], while more than 50% can resolve spontaneously, only part of them will develop into local complications, which include necrosis, abscess and pseudocyst formation into or around the pancreas[7]. Once local complications ensue, the onset of severe acute pancreatitis is established[4].

sAP is only about 15%-25% but almost all of mortality and morbidity of AP is concentrated in it[26-35]. The principle benefit of early correct assessment of severity in AP is that patients who require intensive monitoring and therapy are detected at once. The role of clinical assessment is reliable but for its subjectivity the value in the early detection of severity is limited[1,6]. C-reactive

protein (CRP) is a promising laboratory marker of severity, even though it is not specific for AP[1]. Multiple-factor biochemical criteria and different scoring system, such as Ranson's early prognostic signs and Acute Physiology and Chronic Health Evaluation (APACHE) have been used as prognostic indicators, but none has been proven ideal in clinical practice[1,6].

Contrast-enhanced CT is proven to be able to predict the severity of AP by scoring the degree of pancreatic tissue necrosis[36-39], yet the main and widely accepted indication for dynamic CT in AP is clinical suspicion of life threatening local complication and to plan invasive intervention 2-3 weeks after admission for its costliness and X-ray exposure. Therefore, the candidate with AP for contrast-enhanced CT was about 14%[1], and 11.5% patients receiving contrast-enhanced CT in our study was considered to be adequate. Preliminary experiences with magnetic resonance imaging have not identified advantages compared with contrast-enhanced CT[40].

Our data showed that the distribution of AFC is different in mAP and sAP, 96.4% (256/266) patients without AFC had mAP while 51.2% (84/164) patients in more than 2 sites AFC group had sAP. The number of AFC was related to the severity of AP, the hospital stay of patients, the emergence of local complication, and the operation possibility and mortality which is similar to other trials[37]. In other words, 69.1% patients in mAP had no AFC or 1 site AFC while 67. 2% patients in sAP had more than 2 sites AFC, and 2 sites AFC in both mAP & sAP were 14.1%, this seemed to imply that 2 sites AFC is the watershed for mAP and sAP in ultrasonography, when there are 2 sites AFC in AP patients, further examinations should be made and more close attention should be paid. The sensitivity, specificity and accuracy were 81.6%, 69.2% and 70.2% respectively if using 2 and more than 2 sites AFC to distinguish mAP and sAP. The sensitivity, specificity and accuracy were 67.2%, 84.2% and 80.1%, respectively if using more than 2 sites AFC.

In conclusion, the number of AFC is related to the severity of AP, the hospital stay of patients, the emergence of local complication, the operation possibility and mortality. Our preliminary experiences showed that ultrasound can be used as an early and elementary prognostic indicator for severity of AP by observing AFC.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Reviews

Detection of alcoholic liver disease

Harriet Gordon

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INTRODUCTION

Alcohol has been used in society over centuries and all over the world for its mood lifting properties and taste. It is probably, however, the commonest drug of abuse world wide and unfortunately causes considerable morbidity, mortality and social disruption. In 1990 the cost to the USA was more than $100 billion and 100 000 lives[1].

The relationship between alcohol and mankind is well documented from the earliest times. Wine-making equipment was found in the remains of an early neolithic village in Northern Iran dated about 5 000 BC[2]. In the Bible Noah 'planted a vineyard: and he drank of the wine, and was drunken'[3].

Beer was first produced in Ancient Mesopotamia, Egypt and Greece. There is evidence that alcohol was used for religious purposes and recreationally in Egypt around 3 000. The Ancient Greeks worshipped the God of wine, Dionysius, and seem to have been the first to develop large-scale wine fermentation and production, with export to other countries. The Romans in turn worshipped Bacchus, their God of wine, and were significant wine producers, planting vineyards across Europe. It is not just wine production that has survived through the ages. Barley provided both bread and beer from the first agricultural communities, but mead, made from fermented honey, was the preferred choice for most of Western Europe until Tudor times. Beer, brewed with hops, was introduced later from Germany.

Alcohol has long been an accepted part of human daily life, and throughout the centuries there has been evidence that, for both men and women, consumption gradually increased. This is due, at least in part, to the boiling of the water in the brewing process. An alcoholic beverage was wellknown as an adjunct in the treatment of cholera. For example, in the seventeenth century the water supply for Nottingham, Great Britain, came from the sewage laden river Leen, but there was a plentiful supply of ale houses (about one for every eighty people) thus ensuring some relatively clean fluid to drink[4].

However, there are long-term side effects and problems with excessive drinking. In 1726 the Royal College of Physicians in England issued a statement to the House of Commons asking for an increase in taxes on spirits to act as a disincentive to this 'great and growing evil'[5]. Concern for the effects of alcohol misuse continued and at the start of the twentieth century the British Prime Minister, Lloyd George, addressed factory workers during the first World War with 'we are fighting Germany, Austria, and drink; and, as far as I can see, the greatest of these three deadly foes is drink'[6]. As a result licensing laws were introduced in Britain limiting the hours during which alcohol could be served, and these are currently still in place[7].

Immoderate alcohol consumption may result in a broad spectrum of medical, psychiatric and social problems. These in turn are an expensive burden on any health service and society at large. However alcohol is also widely available and enjoyable so there have been attempts to identify levels of drinking at which alcohol related damage occurs.

LEVELS OF ALCOHOL RELATED DAMAGE

Harmful drinking is alcohol consumption that is causing actual physical or psychological harm[8]. Alcohol dependency refers to those individuals who 'have a compulsion to drink...the same amount each day...and suffer withdrawal symptoms on stopping' (Diagnostic and Statistical Manual of Mental Disorders, DSM III)[9]. This is the most obvious immediately attributable disorder directly related to alcohol misuse.

Regular excessive alcohol consumption is known to cause a wide range of diseases and disorders. Alcohol permeates every system in the body as it is water soluble. Every system in the body is therefore liable to alcohol induced damage, and the spectrum of deaths attributed to alcohol misuse reflects this. The commonest causes of death in the general population in the UK are cardiovascular causes (44.5%), cancer (28.6%) and then accidents (12.6%). These remain the three commonest causes for alcohol misusers, but accidents constitute a much larger proportion (44.1%) in this group[10]. The overall mortality in

Consultant Physician and Gastroenterologist, The Royal Hampshire County Hospital, Winchester, Hampshire, UK
Correspondence to: Dr. Harriet Gordon, Consultant Physician and Gastroenterologist, The University Department of Medicine, The Royal Free Hospital, London NW3 2QG
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individuals misusing alcohol is three and a half times that for the general population.

Alcoholic liver disease forms the largest component of gastrointestinal causes of alcohol related mortality. The first histologically identifiable change seen in alcoholic liver disease is fatty liver. It is usually asymptomatic and can develop within days of heavy drinking[1]. Histologically, droplets of triglyceride can be seen within the hepatocytes. This can progress to alcoholic hepatitis, severe fibrosis and finally cirrhosis.

The level of drinking that constitutes misuse and results in alcohol-related damage was first studied in France. The risk of developing significant liver injury increases with increasing alcohol intake above the threshold levels[11]. It has been suggested that the threshold level for developing liver injury, with or without cirrhosis, may be as low as 30 g per day of alcohol and that with increasing alcohol intake there is increasing risk[12]. Alternatively a study looking at a series of 400 male autopsies showed no significant features of alcoholic liver disease in those who it was estimated had drunk less than 40 g per day. Those drinking 40 g-80 g per day had an increased incidence of fatty liver and alcoholic hepatitis, while those drinking more than 80 g per day had an increased incidence of liver cirrhosis. The threshold for liver damage was seen to be 60 g per day, or 49 units per week in this study[13]. Cirrhosis may develop after only a minimal alcohol intake over a short period of time, or, despite drinking considerable amounts over a life time, never develop. Only 20% of chronic alcohol misusers progress to cirrhosis and the reasons for this have been postulated to be a combination of genetic and environmental factors, and are the subject of continued research.

One of the difficulties in establishing the level of alcohol intake required for liver damage to occur is that despite the common usage of alcohol, exact quantification of the amount drunk on an individual or population basis is difficult to estimate. In the UK, information on alcohol consumption comes from two main sources: Customs and Excise data and population surveys. Customs and Excise data have some confounding factors: they do not include 'home brew.

Population surveys consistently produce lower figures than the Excise data. The Office of Population Censuses and Surveys (OPCS) survey in 1987 estimated alcohol consumption to be 4.2 litres absolute alcohol per head per year, while Excise data estimated this at 7.4 litres per head per year[14]. Population surveys are difficult because, by their nature, they are based on several elements of subjectivity. There is a significant non-response rate which could be amongst the higher or problem drinkers. In general, individuals tend to underestimate their consumption to within, or close to, 'sensible limits'.

The UK Department of Health has suggested that 'sensible limits' for drinking are 21 units per week for men and 14 units per week for women. A unit contains 8g absolute alcohol and is approximately half a pint of beer, a glass of wine or a single measure of spirits. In December 1995 they changed this to 3-4 per day for men and 2-3 for women, in an attempt to reduce binge drinking.

The Royal Colleges of Physicians and Psychiatrists have collaborated to give indications of harmful levels of alcohol intake, and to demonstrate the relationship between alcohol intake and physical harm (Table 1). It was agreed that the threshold for definitely harmful drinking is 50 units per week for men and 35 units per week for women, while that for heavy or hazardous drinking is >35 units per week for men and >25 units per week for women[5].

Table 1 The royal college of physicians (UK) advice on 'safe' and at risk drinking[5]

	Men(units per week)	Women(units per week)
Low risk	21	14
Hazardous	22-49	15-35
Harmful	50^{+}	36^{+}

The problems related to alcohol misuse are preventable. There is a need for an effective screening method for the early detection of alcohol misuse so as to provide support services and then the monitoring of progress. To do this effectively there is a need for objective markers of alcohol misuse.

DETECTION OF ALCOHOL MISUSE

The early detection of alcohol misuse is vital, so that the physical and psychological damage can be limited and reversed where possible. Once those drinking at misuse levels have been identified they need to be monitored through treatment. The Royal College of Physicians (UK) recommend that 'Every person seen in general practice or in hospital should be asked about his or her alcohol intake as a matter of routine, along with questions about smoking and medication, and the answers recorded'[5].

HISTORY AND QUESTIONNAIRES

The history is the most important means for detecting alcohol misuse[15]. The history should cover current and past alcohol intake, and identify quantity and frequency of intake. Unfortunately, although self-report has been shown to be reliable and reproducible, it is subjective, and often is, intentionally or unintentionally, an underestimate.

Questionnaires have been used to try to improve

the identification of alcohol misuse. The CAGE questionnaire[16] consists of four questions; two or more positive answers warrant further investigation:

1. Have you ever felt that you should Cut down your drinking
2. Have people Annoyed you by criticising your drinking
3. Have you ever felt Guilty about your drinking
4. Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover (an Eye opener).

This tends to be too sensitive, having a high false positive rate but, if combined with self-report, can detect 90% of alcohol misusers[17]. The MAST (Michigan Alcohol Screening Test) is best at detecting alcohol misusers who have had complications and has been modified to the MmMAST (Malmo modified Michigan Alcoholism Screening Test)[18]. The AUDIT (Alcohol Use Disorders Identification Test) was developed by the WHO collaborative group and was designed to detect early heavy drinking. It consists of ten questions to be used in primary care[19] and has been shown to have a sensitivity of 92% and specificity of 94% in detecting harmful or hazardous drinking[20]. The CAGE, MmMAST and AUDIT were compared in Occupational Health and detoxification clinic settings and compared with self-report for alcohol intake[21]. Over all the sensitivities for the CAGE and MmMAST were 100% and for the AUDIT was 91% amongst the alcohol-misusers, but the AUDIT had the best performance in the Occupational Health setting.

CLINICAL SIGNS

There are a number of cutaneous signs of chronic alcohol misuse but these may be found in those with no significant liver disease. They include spider naevi, telangiectasiae, palmar erythema, gynaecomastia and Dupuytren's contracture. The mechanisms by which these develop are unknown, and with the exception of Dupuytren's contractures, they may all regress with abstinence. There may be truncal obesity mimicking Cushing's Syndrome and parotid enlargement.

In the withdrawal phase of alcohol misuse there may well be sweating, tremor and tachycardia, all of which are difficult to distinguish from thyrotoxicosis.

BIOLOGICAL MARKERS OF ALCOHOL MISUSE

These are particularly valuable to screen or confirm a suspicion of alcohol misuse as they are objective, are useful serially for monitoring, and may be helpful in motivating the patient. These markers can be divided into those indicating recent alcohol drinking, and those of chronic alcohol misuse.

Markers of recent alcohol consumption

Ethanol is the most obvious confirmation of recent drinking and can be detected in breath, serum or urine. It is useful to validate self-report, if positive. Alcohol is eliminated at the rate of $1g \cdot kg^{-1} \cdot hr^{-1}$, usually in 4-6 hours from breath and blood, and within 8 hours from urine, although the elimination rate is affected by the chronicity of alcohol misuse. Most patients know this and adapt their habits so that their clinical value is limited.

Methanol is present in the body and in small amounts in alcoholic beverages as a congener. Both ethanol and methanol are metabolised via alcohol dehydrogenase. However alcohol dehydrogenase has a much higher affinity for ethanol, so this is preferentially metabolised. The level of methanol therefore accumulates during ethanol metabolism and does not start to fall until ethanol has been removed. In practice this is at least 2-6 hours after ethanol has ceased to be detectable[22]. It can be detected in blood or urine.

Serotonin metabolites The urinary metabolites of serotonin (5-hydroxytryptamine) are 5-hydroxytryptaphol (5-HTOL) and 5 hydroxy indole acetic acid (5-HIAA). These are natural substrates. Normally 5-hydroxytryptamine (5-HT) is metabolised predominantly to 5-HIAA by aldehyde dehydrogenase, but a small amount is metabolised to 5-HTOL by alcohol dehydrogenase. However after alcohol ingestion, alcohol is metabolised to acetaldehyde, which than inhibits aldehyde dehydrogenase. Therefore there is a shift towards 5-HTOL and an increase in the 5-HTOL:5-HIAA ratio. The increase is dose-dependent and can be detected 5-15 hours after the ethanol has been eliminated. In urine, methanol and serotonin metabolites can be detected up to 18 hours after drinking, long after the ethanol is cleared[22]. The sensitivity and specificity of the 5HTOL:5HIAA ratio is proportional to the alcohol intake above 200 $\mu mol \cdot L^{-1}$. This is however affected by serotonin containing foods, for example bananas, and disulfiram which both increase the 5HTOL level but not the 5HIAA level. This can be resolved by using the 5HTOL/creatinine ratio in addition to the 5HTOL/5HIAA ratio.

In summary, both methanol and an increase in the 5-HTOL:5HIAA ratio can be detected after ethanol has been metabolised but neither test is routinely available. Ethanol remains the most frequently used test, whether in breath, urine or

serum.

Markers of chronic misuse

The markers most commonly evaluated are those readily available as part of routine screening: erythrocyte mean corpuscular volume (MCV), serum aspartate aminotransferase and alanine aminotransferase (AST, ALT), and gamma glutamyl transferase (GGT).

Erythrocyte mean cell volume (MCV) is thought to be elevated as a result of direct toxicity by ethanol[24]. It becomes elevated after six weeks of alcohol misuse but, in view of the half life of the erythrocyte it remains elevated for up to three months and so has a limited use in monitoring alcohol intake. The sensitivity is higher in women (86.3%) than in men (63.0%)[24]. False positives are found in hypothyroidism, vitamin B_{12} and folate deficiency, non-alcoholic liver disease and in some patients who smoke[25].

Serum aspartate amino transaminase (AST) and serum alanine transaminase (ALT) are markers of liver damage as opposed to alcohol misuse. Both transaminases are found in hepatocytes but AST is also found in skeletal and myocardial cells. In alcohol related liver damage, the AST is elevated more than the ALT, at least in part as a reflection of alcohol related skeletal damage. This is the reverse of the normal pattern in acute hepatocellular disease (for example acute viral hepatitis) where the ALT exceeds the AST.

False positive results are found in non alcoholic liver disease, muscle damage and myocardial damage. Despite these, the specificity is reasonably high at >90% (Table 2).

Table 2 Sensitivity and specificity of markers for detection of hazardous and dependent alcohol use

		Sensitivity(%)	Specificity(%)
MCV	Hazardous consumption	20-30	64-100
	Dependence/alcoholism	40-50	64-100
AST	Hazardous consumption	10-30	>90
	Dependence/alcoholism	35-50	>90
ALT	Hazardous consumption	10-20	>80
	Dependence/alcoholism	20-50	>80
GGT	Hazardous consumption	20-50	55-100
	Dependence/alcoholism	60-90	55-100

Adapted from Conigrave *et al*[23]

AST itself has a mitochondrial (mAST) and cytosolic component. It appears that alcohol selectively affects the mitochondrial component following damage to this organelle so that the serum increase in alcohol misusers is mAST. This has been proposed as a more sensitive marker of alcohol misuse. There is also a small increase in non alcoholic liver disease and it has therefore been suggested it should be used as a ratio of mAST to total AST[26].

Serum gamma glutamyl transferase (GGT) increases in alcohol misuse in a dose-dependent manner, and is often the first marker to be elevated[23]. It is less sensitive in women than men[27,28]. The exact mechanism of elevation of GGT in alcohol misuse is unclear. The enzyme may be released by hepatic cell injury or by induction following exposure to alcohol. In alcoholic liver disease a component of the increase is also from hepatocyte cholestasis and hepatocyte damage. It increases after five weeks of drinking more than 50 g per day. It usually increases to three times the upper reference limit, but will normalise within five weeks of abstinence, with a half-life of 26 days, although this is lengthened in chronic liver disease[29].

Some individuals misusing alcohol never have an elevated GGT; in some chronic alcohol misusers initially high levels fall despite continued drinking. False positives are seen in non-alcoholic liver disease, including fatty liver, biliary tract disease, obesity, diabetes, pancreatitis, hyperlipidaemia, trauma and heart failure, and with microsomal inducing drugs such as anti-epileptics[25].

The varying sensitivity and specificity makes it an unsuitable marker to be used alone for screening, but it is useful to confirm a clinical suspicion of alcohol misuse. Several isoforms of GGT exist and can be separated by electrophoresis. The pattern in alcohol abuse is distinctly different from not only that of healthy volunteers, but also from non alcoholic liver disease. It is, however, the same as that in those taking anti-epileptic drugs since both result in enzyme induction. It has been suggested that the analysis of GGT isoforms may improve the specificity of GGT for alcohol misuse[30].

Combinations of markers

As can be seen from Table 2 none of the routinely available markers has sufficient sensitivity or specificity to be used alone, and in practice a combination is usually used. AST and GGT both have higher sensitivity in men than women while MCV is higher in women. Each of MCV, AST and GGT are raised by a different mechanism and so used in combination will pick up varying parts of the alcohol misusing population. If two or more markers are positive then the number of false positives fall and the specificity is seen to increase[29,31].

The clinical case mix affects the test performance of any given marker. The sensitivity of the test is highest where there are a high number of

severe alcohol misusers, in for example the alcohol treatment centre. The sensitivity is lowest in the general community[23]. Chick *et al* found a sensitivity of 40% for in patient alcohol misusers and 23% for those in the setting of employment screening[31]. Sillanukee *et al* found a sensitivity of 4. 7% for MCV for detecting alcoholics in the context of a detoxification centre, but only 22% for detecting heavy drinkers voluntarily attending for health screening. Similarly using GGT there was a sensitivity of 65% in the detoxifica tion centre, but only 35% within the community[32].

Other markers

Serum urate is routinely available and may be elevated in 40% of male and 25% of female alcohol misusers. False positives results are seen in gout, renal disease and with some drugs.

Serum triglycerides are often measured for other reasons but increase after one - week of drinking in 40% of alcohol misusers, and normalise within one week of abstinence. False positives are seen in hyperlipidaemia, diabetes, obesity and with some drugs.

It can be seen that the currently available routine markers do not have sufficient sensitivity or specificity to be used alone to detect alcohol misuse. Considerable research has been undertaken to try to find any other potentially more useful markers. One such marker is carbohydrate deficient transferrin (CDT).

Transferrin is a large glycosylated protein which binds and transports iron in the plasma. It has terminal carbohydrate units containing sialic acid. Alcohol intake of greater than 60 g per day for two weeks results in loss of some or all of the sialic acid component of transferrin and hence the term - carbohyd rate deficient transferrin. Initial research gave promising results with sensitivities of 100% and specificities of 97%[33], confirmed by other workers[34-37] and prompted the development of commercial assays: CDTect (Pharmacia and Upjohn, Sweden) and AXIS %CDT (AXIS Biochemicals, Norway). There has been a considerable amount of research using these commercial assays and variation in the reported results[38,39]. It seems that in development of the assays there has been some loss of sensitivity and specificity, particularly in women and those with liver disease. This marker is increa singly being used in Europe, and often in combination with other markers.

SUMMARY AND CONCLUSIONS

Alcohol has been used in society over centuries and all the evidence we have ind icates that, to society as a whole, the risks are heavily outweighed by the benefits and it is particularly expensive in health terms. A means to identify those at risk is required so that these individuals can be targeted for help. This in turn requires a means for monitoring. Ideally detection should screen for alcohol misuse at a level at which damage occurs.

Histories and questionnaires are still the commonest initial means of detection of alcohol misuse. They are cheap, easily administered but are subjective. They still provide the 'gold-standard'. If the history remains uncertain and there is a suspicion of alcohol misuse biological markers provide objectivity, and a combination of markers remains essential in detection. The three commonest markers in current practice were GGT, AST and MCV. However these show problems with detection, particularly in the context of liver disease. Serum carbohydrate deficient transferrin initially showed promise as having a high sensitivity and specificity and could be ideally suited for both screening and monitoring. However following development of commercial assays, the sensitivity and specificity is not as promising as early work had suggested. Research continues in both investigating and refining markers of misuse.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Treatment of *Helicobacter pylori*

Adam Harris

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INTRODUCTION

Using an evidence-based approach this review discusses the current treatment of *Helicobacter pylori* infection in patients with peptic ulcer disease, functional (non ulcer) dyspepsia or gastro oesophageal reflux disease (GORD). It also briefly addresses the potential role of eradication of *H. pylori* in preventing gastric cancer.

PATIENTS WITH DUODENAL ULCER DISEASE (DU)

In a patient with a well documented DU, who is not taking non steroidal anti-inflammatory drugs (NSAIDs), *H. pylori* may either be assumed to be present (more than 90% prevalence) or may be confirmed at endoscopy by tissue biopsy (for rapid urease test, histology or microbiological culture). *H. pylori* eradication therapy (see below) may then be prescribed. *H. pylori* eradication therapy, if successful, will be effective in healing the ulcer regardless of whether the patient is seen at the initial presentation of the disease, or at a recurrence[1]. Patients on long-term treatment with histamine-2 receptor antagonists (H_2RAs) or proton pump inhibitors (PPIs) for DU should also be offered *H. pylori* eradication treatment, regardless of whether they are symptom-free, or still experiencing symptoms, because eradication of the bacterium will cure the disease[1].

DUs heal quickly and completely after eradication of *H. pylori*, so that a separate healing course of anti-secretory therapy is unnecessary in an uncomplicated ulcer[2,3]. After a course of eradication therapy it may be acceptable to await the clinical outcome, i.e. an improvement in symptoms, rather than to formally test for the presence or absence of *H. pylori*. Recurrence of symptoms signifies either failure of *H. pylori* eradication, or the "unmasking" of some other disease, for example, GORD[4]. In such cases, further management will not be clear unless the outcome of eradication therapy is known. The best way to determine this is either through the ^{13}C-urea breath test (^{13}C-UBT) or possibly, a faecal antigen test, performed no sooner than four weeks after eradication therapy[5].

COMPLICATED DU

Complications of DU such as bleeding or perforation are associated with appreciable morbidity and mortality, especially in the elderly. There is good evidence that eradication of *H. pylori* decreases the risk of re-bleeding and reperforation from DU[6]. In such patients *H. pylori* eradication therapy should be followed by treatment with an H_2RA (such as ranitidine 300 mg at night) for a further four weeks until either repeat upper g.i. endoscopy with biopsies to confirm re epithelialisation of the ulcer crater and to assess *H. pylori* status, or a ^{13}C-UBT. If *H. pylori* has been successfully eradicated (and the patient is not taking NSAIDs) anti-secretory therapy is no longer needed. The prevalence of *H. pylori* in patients with complicated DU may be lower than that in patients with simple DU and it is therefore important to confirm that the patient is colonised with the bacterium before prescribing eradication therapy and stopping anti-secretory medication[7].

GASTRIC ULCER

The main point of difference in the management of a patient with *H. pylori* associated GU is the need to exclude malignancy in an apparently benign GU. Patients with GU should therefore be re-endoscoped about 8 weeks after *H. pylori* eradication therapy to confirm healing, obtain further biopsies if re-epitheliasation is incomplete and to ascertain *H. pylori* status.

Eradication of *H. pylori* leads to healing of GU and markedly decreases the incidence of relapse[1,8]. The effect of eradication of *H. pylori* on GU complications is unknown at present. Anti secretory maintenance treatment should therefore be started after successful eradication of *H. pylori* in those patients with GU who have a history or haemorrhage or perforation, until complete healing of the ulcer is confirmed at follow up endoscopy.

H. PYLORI AND NSAID-ASSOCIATED ULCER

Despite several studies, no clearly defined guidelines about the relationship between NSAIDs, gastro-duodenal ulceration and *H. pylori* have emerged[9,10]. NSAIDs and *H. pylori* appear to be independent risk factors for gastro-duodenal ulceration and ulcer bleeding. There is some evidence that *H. pylori* eradication may prevent the development of ulcers in patients starting

Consultant Gastroenterologist, Kent and Sussex Hospital, Tunbridge Wells, Kent TN4 8AT, England
Correspondence to: Adam Harris, M.D. MRCP, Consultant Gastroenterologist, Kent and Sussex Hospital, Tunbridge Wells, Kent TN4 8AT, England
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NSAIDs[10]. However there is other evidence that *H. pylori* associated gastritis may even be beneficial, because of prostaglandin release protecting against mucosal injury by NSAIDs[9]. Indeed it appears that NSAID associated ulcers heal more rapidly after treatment with PPI in the presence of *H. pylori*[9]. In a *H. pylori* positive patient with a history of peptic ulcer disease, *H. pylori* should be eradicated before starting treatment with NSAIDs because the NSAIDs may increase the likelihood of ulcer relapse[9]. There is no evidence that eradication of *H. pylori* is of benefit in decreasing the dyspepsia associated with treatment with NSAIDs[9].

H. PYLORI AND GASTRIC CANCER

Infection with *H. pylori* is associated with a three to six fold increase in the risk of developing non-cardiac (body and antrum) stomach cancer[11]. Although prevention of gastric cancer through eradication of *H. pylori* is potentially extremely important in global terms (750 000 deaths attributable annually to the neoplasm), it must be emphasised that, at present, there is no evidence that eradication of *H. pylori* decreases that risk. Nor is it known at what stage *H. pylori* has to be eradicated to prevent the progression of chronic gastritis to atrophy, intestinal metaplasia, dysplasia and eventually to invasive cancer. Although infection with *H. pylori* is very common, the life-time risk of developing non-cardiac stomach cancer in infected individuals in the developed world is estimated to be less than 1%[11]. In subjects with other risk factors for stomach cancer, such as one or more first degree relatives with this condition, or the presence of gastric mucosal dysplasia or intestinal metaplasia found at gastroscopy, it seems reasonable to offer *H. pylori* eradication therapy. It is important however to discuss with the patient the possible side effects of the treatment, the lack of evidence to support this practice and the possibility of treatment failure.

PATIENTS WITH FUNCTIONAL DYSPEPSIA AND H. PYLORI INFECTION

Functional dyspepsia is defined as pain or discomfort in the central upper abdomen which originates in the upper gastrointestinal tract, which has been present for at least three months and in the absence of organic disease, such as peptic ulcer or GORD[12]. This diagnosis accounts for up to 60% of patients with non-NSAID associated dyspepsia[13]. The evidence for an association between *H. pylori* and functional dyspepsia is uncertain. Several well designed, randomised and controlled trials assessing the efficacy of *H. pylori* eradication treatment in patients with functional dyspepsia have been published in the past three years but have produced discordant results[13-16]. A recent systematic review has found that eradication of *H. pylori* was significantly superior to placebo in treating functional dyspepsia (relative risk reduction 9%, 95% confidence interval 4%-14%) suggesting that one case of dyspepsia would be cured for every 15 patients treated[17]. The mechanism by which eradication of *H. pylori* decreases dyspepsia in these patients is unclear. It is possible that the patients that benefited may have had an ulcer diathesis which was not active at the time of endoscopy.

PATIENTS WITH GORD

There is unequivocal evidence that infection with *H. pylori* is the principal cause of peptic ulcer disease and there appears to be a small, but definite association between the bacterium and functional dyspepsia[1,17]. The relationship between *H. pylori* and GORD is however still unclear[18,19].

The prevalence of *H. pylori* is not increased and may actually be decreased in patients with GORD[20]. Recent studies have reported that significantly fewer patients with GORD are infected with *H. pylori* than healthy, age and sex matched individuals[20,21]. More importantly, there appears to be a negative correlation between the prevalence of *H. pylori* infection and the severity of oesophagitis; patients with erosive (grade III) oesophagitis or Barrett's columnar lined oesophagus are significantly less likely to be infected with *H. pylori* than patients with either a normal oesophagus or milder degrees of oesophagitis[22,23]. This inverse relationship has been further assessed according to the subtypes or strains of *H. pylori*; patients with cagA positive strains appear to be significantly less likely to develop erosive oesophagitis or Barrett's oesophagus[24-27]. Furthermore the prevalence of cagA positive strains appear to be significantly less frequent in patients with oesophageal adenocarcinoma or dysplasia, conditions which may result from longstanding and severe GORD[25].

ERADICATION OF *H. PYLORI* AND GORD

There is no convincing evidence that GORD improves after eradication of *H. pylori*[18,19]. There is conflicting data regarding the development of GORD following eradication of *H. pylori*. Some studies have found that patients develop endoscopic oesophagitis after eradication of *H. pylori* either for duodenal ulcer disease or functional dyspepsia[28-30] but these findings were not confirmed in another study[31]. The prevalence of heartburn does not appear to increase between 6 months and 3 years after eradication of *H. pylori* in patients with either functional dyspepsia, GORD or DU disease[32-36].

Another reason to carefully evaluate the role of *H. pylori* eradication therapy in patients with GORD is the recent finding that the treatment of GORD may be more effective in the presence of the

bacterium: pantoprazole was significantly more effective at healing the oesophagus in the presence of *H. pylori*[37]. It was found that healing of oesophagitis (grades II and III) was significantly greater after 4 and 8 weeks treatment with pantoprazole in *H. pylori* positive patients compared with *H. pylori* negative patients. On that point, anti-secretory therapy also appears to more effective in the presence of *H. pylori* in patients with functional dyspepsia[38]. The exact mechanism for these most interesting findings is unclear at present.

Patients with GORD and *H. pylori* infection who need prolonged treatment with standard or high doses of PPIs, according to one uncontrolled study, may be at increased risk of developing atrophic gastritis[39]. During profound acid suppression with PPIs *H. pylori* may migrate from the antrum to the more proximal parts of the stomach leading to chronic active corpus gastritis which may progress to atrophic gastritis, which is associated with increased risk of carcinoma[40]. The latter changes do not occur with profound acid suppression in patients without *H. pylori* infection. It has therefore been recommended that patients with GORD who need prolonged treatment with a PPI should have their *H. pylori* status determined and if positive, eradication therapy should be given. This recommendation is based on hypothesis, rather than on scientific evidence, and it has to be borne in mind that *H. pylori* eradication may render PPIs to be less effective[37,38]. Further studies are needed before these contradictory considerations can be resolved[41].

H. PYLORI ERADICATION TREATMENT

The aim of treatment of *H. pylori* in any therapeutic context is eradication of the organism from the foregut. Eradication is defined as negative tests for the bacterium four weeks, or longer, after the end of anti-microbial therapy[42]. Failure to detect *H. pylori* on tests done less than four weeks after the end of therapy may give false negative results because clearance, or uppression of *H. pylori* may occur during treatment, followed by rapid recrudescence of the original infection.

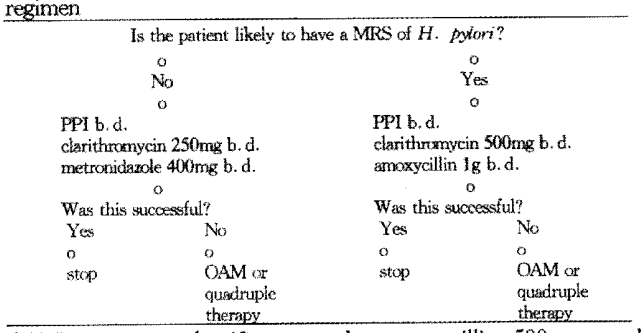
Antibacterial treatment of *H. pylori* is difficult because of the very rapid development of resistance to anti microbial agents, especially to nitroimidazoles, such as metronidazole and tinidazole, and clarithromycin[41]. The prevalence of resistance to these anti-microbial agents varies with gender, ethnic group and country of origin (Table 1)[41]. It was recently reported from Hong Kong that almost 50% of pre-treatment strains of *H. pylori* were resistant to metronidazole and over 10% to clarithromycin[42]. The efficacy of treatment for *H. pylori* is significantly decreased in the presence of pre-treatment anti-microbial resistance and the likelihood of this should influence the chosen regimen (Table 1, Figure 1)[5,41].

Monotherapy or dual therapy (PPI or ranitidine bismuth citrate (RBC) with an antibiotic) cannot be recommended and should not be used because of inconsistent and highly variable results[5,41].

Table 1 Risk factors for nitroimidazole resistance in *H. pylori*

- ☐ Previous use of nitroimidazoles, e.g. gynaecological infections, infective diarrhoeas
- ☐ Failed *H. pylori* eradication regimen containing a nitroimidazole
- ☐ Urban or inner city areas
- ☐ Patients born in emergent countries

Figure 1 How to choose a one week *H. pylori* eradication regimen



OAM – omeprazole 40 mg o. d., amoxycillin 500 mg and metronidazole 400 mg t. d. s.
MRS – metronidazole-resistant strain of *H. pylori*

LOW-DOSE TRIPLE THERAPY

The most overall effective *H. pylori* eradication regimens reported to date combine either a PPI (esomeprazole, lansoprazole, omeprazole, pantoprazole, rabeprazole) or RBC with two of the following: amoxycillin, clarithromycin, or a nitroimidazole (metronidazole or tinidazole)[5,42,43-49] (Table 2). These regimens are commonly prescribed for one week and the tablets are taken twice daily. There are few side effects (the most common are nausea, diarrhoea and taste disturbance). Results from large, randomised and controlled trials have consistently shown *H. pylori* eradication in about 90% of treated patients[44,46-49].

Table 2 Low dose triple therapy

Therapy	PPI or RBC Amoxycillin Clarithromycin	PPI or RBC Clarithromycin Metronidazole
Dosing	b.d.	b.d.
	1 g b.d.	250 mg b.d.
	500 mg b.d.	400 mg b.d.
Duration		One week
Side effects	Nausea, diarrhoea,	taste disturbances
H. pylori eradication	90%	90% in MSS
		75% in MRS

PPI- proton pump inhibitor; RBC- ranitidine bismuth citrate; b.d.- twice daily;
MSS- metronidazole-sensitive strain of *H. pylori*;
MRS- metronidazole-resistant strain of *H. pylori*

A one week course of omeprazole 40 mg once daily in combination with amoxycillin 500 mg and metronidazole 400 mg three times daily has been shown to be effective even in the presence of pre-treatment metronidazole resistant strains (MRS) of *H. pylori*, with 75% eradication[50]. Side effects are more common and compliance more complex than with the twice daily regimens, so that this regimen is best reserved for first line treatment failures (Figure 1).

CIASSICAL TRIPLE THERAPY

Bismuth based triple therapy was the first multi drug treatment to be widely investigated and used in clinical practice. Originally, it consisted of 14 days treatment with colloidal bismuth subcitrate 120 mg qds, together with metronidazole 400 mg tds and either amoxycillin, or tetracycline 500 mg qds. Unfortunately side effects are frequent and the regimen is complicated to follow with more than 11 tablets to be taken daily. Furthermore, the efficacy of the triple therapy is dependent on the susceptibility of *H. pylori* to metronidazole, with eradication in only 50% of those patients who are colonised by a metronidazole-resistant strain of *H. pylori*[5,41].

QUADRUPLE THERAPY

Classical bismuth-based triple therapy has been reported to be more effective when co-prescribed with a PPI. More than 90% *H. pylori* eradication is possible with a combination of omeprazole, colloidal bismuth subcitrate, tetracycline and metronidazole given for 7 days[5,41,51,52] (Table 3). Efficacy is highly dependent on patient's compliance with the complicated prescription. Furthermore, these quadruple regimens have been shown to work in 75%-80% of patients who failed first line eradication therapy with metronidazole and/or clarithromycin containing regimens[51,52].

Table 3 Quadruple therapy

Therapy	Omeprazole Colloidal bismuth subcitrate Tetracycline Metronidazole
Dosing	o.d. 120 mg q.d.s. 500 mg q.d.s. 400 mg q.d.s.
Duration	One week
Side effects	Common: nausea, diarrhoea, taste disturbances
H. pylori eradication	>75% in MRS >90% in MSS

o.d.- once daily; q.d.s.- four times daily;
MRS- metronidazole-resistant strains of *H. pylori*;
MSS- metronidazole-sensitive strains of *H. pylori*

FIRST-LINE TREATMENT (FIGURE 1, TABLE 2)

In areas with low (<30%) prevalence of pre treatment metronidazole-esistant strains of *H. pylori*, a one week triple therapy regimen consisting of a PPI, metronidazole and clarithromycin is recommended at present. Patients' compliance is likely to be good because of twice daily dosing and few side effects. If metronidazole resistance is likely (Table 1), a PPI in combination with amoxycillin and clarithromycin given for one week is preferable (Table 2).

SECOND-LINE TREATMENT (FIGURE 1, TABLES 2,3)

After a proven failure with a regimen containing metronidazole, the patient is likely to be colonised by a metronidazole-resistant strain of *H. pylori*. In this case, a PPI in combination with amoxycillin and clarithromycin given for one week should be used, with around 90% success. If *H. pylori* eradication is unsuccessful after a clarithromycin- and metronidazole-containing regimen or the patient is likely to harbour a pre-treatment metronidazole resistant strain of *H. pylori*, then either omeprazole in combination with amoxycillin and metronidazole[50] or quadruple therapy are the only logical options (Table 4), with approximately 75% success[51,52].

Table 4 Current indications for *H. pylori* eradication therapy

Diagnosis	Established evidence-based indications
Non-NSAID DU	+
Non-NSAID GU	+
NSAID DU or GU	-
Functional dyspepsia	+ or ?
GORD	-
Risk of gastric cancer	+ or ?
MALT lymphoma	+

Key: +, good / excellent evidence in favour of treatment;
-, unclear or negative outcome from treatment;
?, equivocal or mild benefit in favour of treatment

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Endoscopic difficulties in the diagnosis of upper gastrointestinal bleeding

M. N. Appleyard, C.P. Swain

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INTRODUCTION

Bleeding from the upper gastrointestinal (GI) tract remains common, with a reported annual incidence of up to 172 per 100 000[1], which has if anything increased from earlier series. Case fatality was recently reported as 14%[2], which has probably not changed over several decades. These figures may reflect a rising proportion of elderly patients and increasing non-steroidal anti-inflammatory use, but occur despite apparently better treatments and understanding of the underlying pathophysiology of peptic ulcer disease. Of patients in whom a diagnosis is confirmed, more than 90% suffer from Peptic Ulcers, oesophageal or gastric malignancy, varices, Mallory Weiss syndrome, erosive disease and oesophagitis[1,2]. This report will focus on the some of the less common aetiologies of upper GI bleeding which are sometimes difficult to identify at endoscopy.

Angiodysplasia

Gastrointestinal angiodysplasia are the most common cause of obscure chronic blood loss from the digestive tract with small bowel angiodysplasia accounting for up to 40% of obscure GI bleeding[3]. The pathophysiology is unknown, but has been suggested to result from low grade venous obstruction of submucosal veins as they cross muscle layers[4]. It is said to be more prevalent in chronic renal failure patients[5]and in patients with aortic stenosis, although, recent reports have failed to confirm this link[6]. Isolated gastric angiodysplasia commonly occurs on the greater curve of the body (Figure 1), whereas small bowel angiodysplasia are often multiple and widespread but may cluster in the proximal jejunum.

Department of Gastroenterology, Barts and The London NHS Trust, London E1 1BB, UK
Correspondence to: M. N. Appleyard MRCP, Department of Gastroenterology, Barts and The London NHS Trust, London E1 1BB, UK
Tel. 0207-377-7443, Fax. 0207-377-7441
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Osler-Weber-Rendu Syndrome is an autosomal dominant condition characterized by angiodysplastic lesions involving the skin, mucosal membranes and organs other than the GI tract (Figure 2). Patients present as children or adults with epistaxis and up to 40% develop chronic iron deficiency secondary to GI bleeding usually after the age of 50. Their endoscopic appearance is indistinguishable from other angiodysplastic lesions, but they tend to be more widespread.

Acquired angiodysplastic like lesions such as those due to radiation damage can also lead to upper GI bleeding.

Various thermal coagulation devices, including heater probes, bipolar probes, the Nd:YAG laser and the argon plasma coagulator appear to be successful in treating these lesions. Coagulation should begin at the central feeding arteriole and work peripherally. Bleeding is common during treatment and usually self limiting. The depth of injury should be minimized, especially in the small bowel and right colon,in order to avoid either frank perforation, or the post-coagulation syndrome, in which patients develop rebound tenderness without detectable intraperitoneal gas. Laser treatment can cause deep injury relatively easily and must be used carefully. Our primary treatment modality is the bipolar probe because it causes more superficial injury than other thermal methods. Complication rates are low for gastric lesions and although no data exists, are also thought to be low in the small bowel. Colonic complications after laser therapy have been reported in up to 10% cases and include partially treated lesions and perforation. Treatment is not required for incidental lesions. Treatment of isolated gastric lesions will often terminate bleeding, whereas treatment of small bowel lesions can more often only hope to reduce transfusion requirements since many lesions are not reached and treated and new lesions will develop with time. The frequency of further endoscopic treatment sessions depends on clinical assessment, rate of recurrence of anaemia and transfusion requirements. Some patients will maintain a stable haemoglobin on iron therapy alone. A placebo-controlled trial mainly in patients with Osler Weber Rendu disease demonstrated decreased transfusion requirements in patients taking 0.05 mg ethinyl oestradiol and

1.0 mg of norethindrone daily[7].

Dieulafoy's lesion

Dieulafoy's lesion is a cause of diagnostic difficulty in patients with repeated haematemesis; the exposed, eroded vessel in a very small ulcer is difficult to spot at endoscopy (Figure 3) and accounts for perhaps 2% of upper GI bleeds[8]. It was described in detail by Dieulafoy in 1896[9], who described "Exulceratio Simplex": bleeding from a simple acute submucosal ulcer of small size. Histology demonstrates an artery with a diameter of 1 mm-3 mm usually surrounded by a small ulcer, less than 5 mm in diameter. Often no inflammation, sclerosis or aneurysmal dilatation is seen. The aetiology is uncertain with most cases occurring in the elderly. NSAIDS and *Helicobacter pylori* have not been implicated. Patients usually present with significant upper GI haemorrhage. The lesions endoscopically are commonly located high on the posterior aspect of the lesser curve within 3 cm of the gastro-oesophageal junction, but similar lesions have been identified throughout the GI tract. They are often difficult to see when not actively bleeding. Adequate inflation to distend the folds in the upper stomach, a retroflexed endoscope and close examination of the mucosa posteriorly on the lesser curve may help to identify this entity. Multiple examinations are commonly required and the abnormality is sometimes diagnosed when pulsatile arterial bleeding is seen coming from apparently normal mucosa. Endoscopic therapy is successful in more than 90% of cases. Adrenaline is frequently injected into the base prior to definitive treatment with electrocoagulation or, more recently, band ligation.

Gastric antral vascular ectasia (Watermelon Stomach)

This syndrome is a rare cause of GI bleeding, although increasingly recognized. It was first described in 1952 by Rider *et al* and used to be called gastric antral vascular ectasia. Jabbari *et al* [10] coined the phrase "watermelon stomach" to describe the endoscopic features: visible columns of vessels traversing the antrum in longitudinal folds converging on the pylorus and resembling the stripes of a watermelon (Figure 4). There is often marked mucosal and submucosal thickening which has been demonstrated with endoscopic ultrasound. It is said to occur more commonly in women, 71% in the largest series[11], with the mean age of presentation being 73 in women and 68 in men. Approximately 90% of patients present for evaluation of occult bleeding and persistent iron deficiency anemia, which characteristically fails to respond to iron therapy. Up to 62% of patients have associated autoimmune or connective tissue diseases, the most common being the CREST syndrome and pernicious anemia, which can confusingly normalize the mean corpuscular volume. Atrophic gastritis seems almost invariable with the majority having achlorhydria and hypergastrinaemia. Cirrhosis and portal hypertension occurs in up to 60% in some series, but is not a feature in more recent series. This lesion can be confused with portal hypertensive gastropathy. The diagnosis is usually made endoscopically if the characteristic lesion is seen. However, histology can help demonstrate the vascular nature of the disorder, with dilated and thrombosed laminar propria capillaries with reactive fibromuscular hyperplasia. It has been suggested that watermelon stomach arises from traumatic gastric peristalsis in a similar fashion to that seen in other prolapse syndromes such as stoma sites, solitary rectal ulcer and haemorrhoids. Excellent long term results have been reported with the use of endoscopic therapy. Nd:Yag laser has been the most studied with an average of 3-4 sessions being required to ablate visible disease and terminate transfusion requirements in over 90% of cases in follow up of up to 6 years. Other treatments such as bipolar or heater probes and the argon plasma coagulator, have also been shown to be successful. However, Nd:Yag laser, which can be used to "paint" the stripes of the watermelon, may require fewer treatments due to its increased depth of injury. Surgical antrectomy has also been reported to be successful, but is rarely necessary since endoscopic therapy is highly effective. Pharmacological agents such as steroids and 5-hydroxytryptamine antagonists have been used in small numbers of patients in uncontrolled trials with perhaps some success. Recognition of this characteristic lesion is important since it is commonly dismissed by less experienced endoscopists as antral gastritis.

Cameron erosions

Erosive disease is an uncommon cause of severe upper GI bleeding; however, some lesions warrant mentioning as they are often overlooked or missed at endoscopy. Cameron erosions were described by Cameron and Higgins as chronic linear erosions positioned on the crests of folds at the diaphragmatic impression in one-third of 109 patients with a large hiatus hernia[12]. They suggested that the erosions were caused by the mechanical trauma of the folds rubbing together during diaphragmatic movement with breathing (Figure 5) and the erosions were more common in patients with anaemia, perhaps due to blood loss from these erosions.

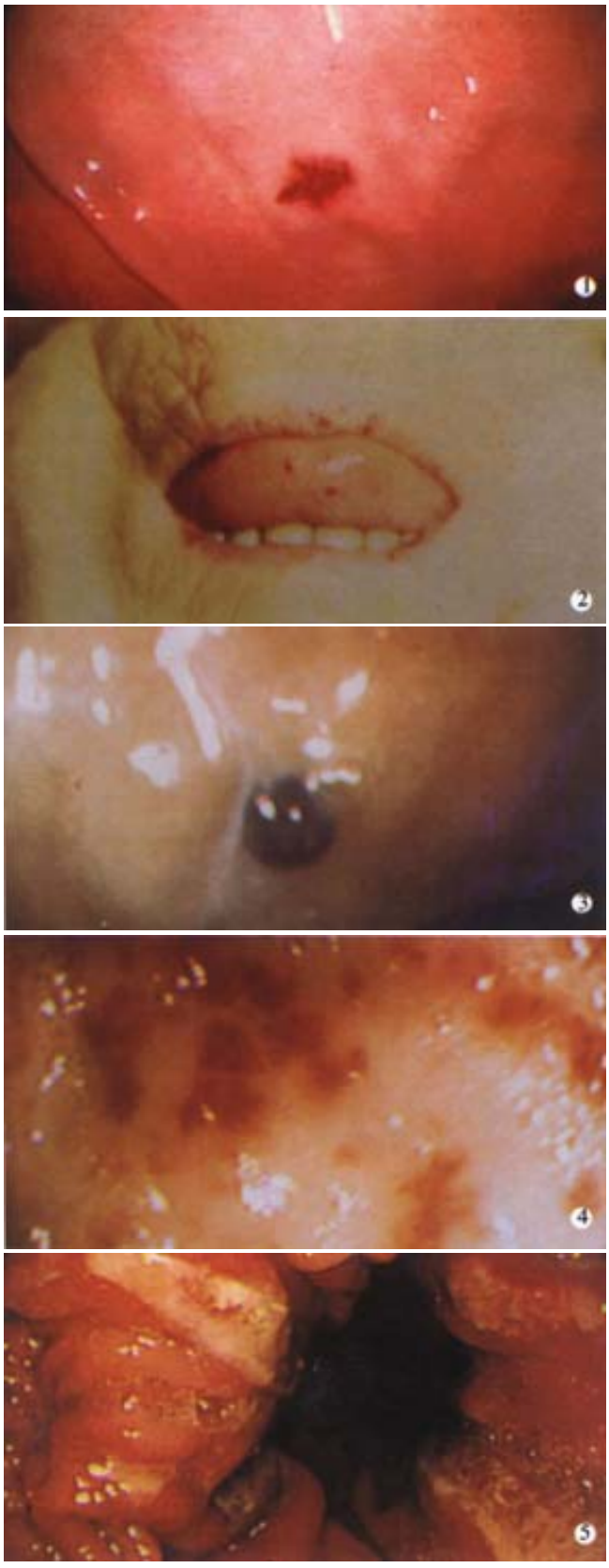

Figure 1 Cutaneous angiodysplasia in Osler-Weber-Rendu Syndrome.
Figure 2 Large gastric angiodyslasia.
Figure 3 Gastric Dieulafoy.
Figure 4 Watermelon stomach.
Figure 5 Cameron erosions.

Prolapse erosions

Prolapsing gastropathy is a syndrome characterized endoscopically by a focal area with subepithelial haemorrhage and, occasionally, erosions within a few centimeters of the cardioesophageal junction. This mucosal area may be seen to be the apex of a knuckle of gastric mucosa, most commonly coming from the 10 o'clock position which prolapses into the distal oesophagus during retching, often prior to haematemesis. Shepherd *et al* described the histological features at endoscopic biopsy of 21 cases of prolapsing gastropathy, describing inflammation in 85%, submucosal haemorrhage in 38% and superficial ulceration in 10%[13].

Unusual upper GI malignancies

Adenocarcinoma accounts for 90% of gastric tumours with lymphoma accounting for 5%, stromal tumours 2% and the rest including carcinoids, metastases and others. GI involvement occurs in 50% of non Hodgkin's lymphoma, with the stomach being the most common extranodal site. 95% of gastric lymphomas are non-Hodgkin's lymphoma. Lymphomas are often clinically silent early on, but progress to signs and symptoms of advanced gastric cancer including upper GI bleeding. Endoscopically lymphomas have a wide range of different appearances and may present as enlarged gastric folds, mucosal nodularities, multiple polypoid masses with or without ulceration, or with a diffuse infiltrative process (Figure 6). One unusual feature is that peristalsis is often preserved. Diagnosis can be difficult, sometimes requiring full thickness biopsy, but when combined with endoscopic ultrasound (EUS) diagnostic accuracy approaches 100%. Treatment is according to histology and includes helicobacter eradication for MALT lymphomas.

Stromal tumours such as leiomyomas tend to be indolent and slow growing, but can be aggressive. They most commonly present with GI blood loss, and are occasionally ulcerated but often appear entirely submucosal at endoscopy (Figure 7) and conventional biopsy usually fails to make the diagnosis. EUS and deep biopsy can assist in making the diagnosis. Surgical management should be considered for large gastric lesions of this type: small oesophageal stromal cell tumours seem to have a low incidence of malignant change. Carcinoid tumours only rarely cause GI bleeding. Like stromal cell tumours they are often submucosal requiring deep biopsies to make the diagnosis. Kaposi's sarcomas do not tend to bleed as much as their vascular appearance would suggest, but may bleed especially if the patient has a low platelet count. Multiple tumours metastasize to the stomach causing bleeding: these include secondary melanoma, and

cancer of the breast, lung, ovary, colon, liver and testes. Melanoma lesions can have a characteristic bullseye appearance or more rarely be amelanotic. Primary renal cancer on the right side can erode and bleed into the duodenum. A common problem with bleeding tumours is the diffuse, often friable area that needs to be treated. Endoscopic treatment of these lesions is often unrewarding, but includes the use of laser, argon plasma coagulator and injection of alcohol. Definitive treatment with resection is sometimes helpful in selected patients and some tumours are radiosensitive.

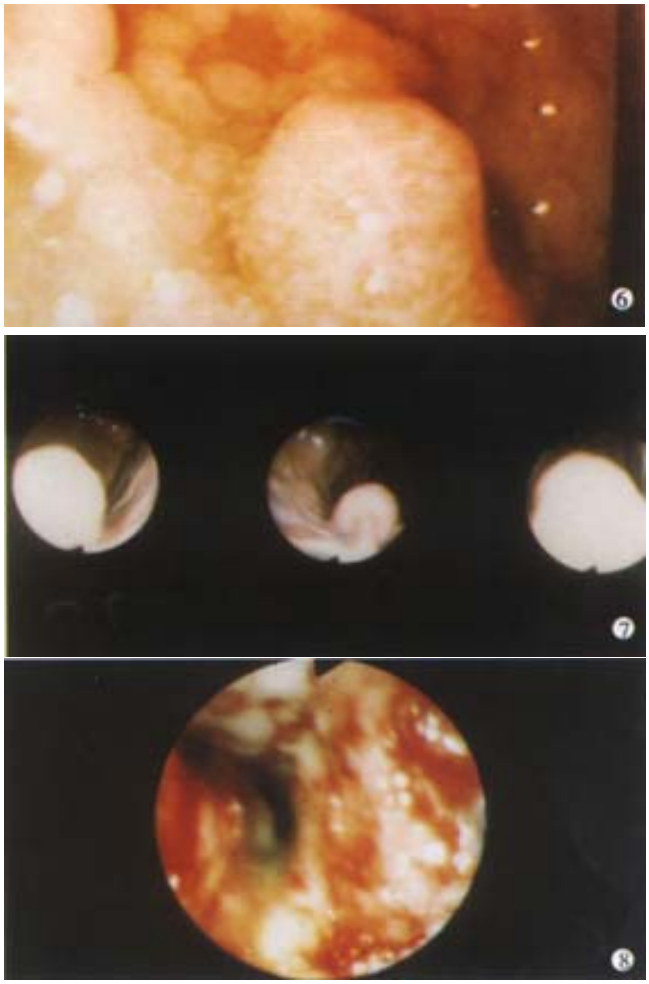

Figure 6 Nodular gastric lymphoma.
Figure 7 Gastric stromal tumour showing Schindler's sign: tenting and loss of gastric folds as mucosa is stretched over the submucosal tumour.
Figure 8 Intestinal amyloid.

Other vascular disorders

There are some other rare vascular lesions that can cause GI bleeding. The Blue Rubber Bleb Naevus Syndrome is an example of intestinal haemangioma which is an autosomal dominant condition causing GI bleeding in infants and children. These lesions are raised blue vascular lesions that can be multiple in the bowel associated with similar cutaneous lesions. The endoscopic lesions are usually redder in colour than the dark blue lesions seen on the skin. Endoscopic therapy has been successfully used, but care needs to be taken since the lesions can be transmural: some authors suggest that endoscopic therapy should be used only intraoperatively. Rarely, conditions involving the blood vessel itself can lead to bleeding. Connective tissue diseases such as pseudoxanthoma elasticum, vasculitis and infiltrating conditions such as amyloid all affect the integrity of the blood vessels resulting in bleeding (Figure 8).

Haemobilia

Bleeding from either the biliary tree (haemobilia) or from the pancreatic duct (Wirsungorrhagia) into the duodenum can be difficult to identify and may require the use of a side viewing endoscope to make the diagnosis. In earlier series, the most common cause of haemobilia was accidental trauma, accounting for 40%, with operative trauma, systemic infection, gallstone disease and aneurysms roughly contributing 15% each. Recent series indicate iatrogenic trauma accounting for 40% and accidental trauma 20%[14]. Classically, patients present with the triad of pain, jaundice and melaena, although only 40% of patients present in this way. A history of chronic pancreatitis or pseudocyst may be a pointer to a bleed from the pancreatic duct. Asymptomatic melaena and haematemesis with a normal endoscopic appearance are other recognized presentations. Endoscopically, blood is seen coming from the ampulla in less than 40% of patients and clot in the bile duct or pancreatic duct may be demonstrated during ERCP. It has been suggested that the endoscopic appearance at the ampulla of a filiform clot suggests biliary bleeding and of fresh bleeding a pancreatic origin. Angiographic or CT findings of an aneurysm, pseudoaneurysm or arterio portal venous fistula may be needed to make diagnosis. Management is often difficult. Some patients can be managed conservatively. However, if surgery is required 75% of patients can expect haemostasis. Embolization of aneurysms is occasionally effective.

Oesophageal apoplexy is a rare condition presenting with pain and haematemesis after swallowing and a large submucosal haematoma is seen, which can occlude the lumen and may be associated with a mucosal tear.

Conditions such as portal hypertensive gastropathy and aorto-enteric fistulae are only mentioned briefly here. A history of abdominal aortic graft surgery should prompt a careful

endoscopic examination of the second and third parts of the duodenum. If aorto-oesophageal fistula is suspected, usually in a patient with a massive bleed and a history of surgery, sepsis or trauma to the thoracic aorta, CT investigation should be undertaken prior to endoscopy in theatre since endoscopy can precipitate torrential bleeding.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Non-pharmacological treatments in the irritable bowel syndrome

A Leahy and O Epstein

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INTRODUCTION

The irritable bowel syndrome (IBS) is a gastrointestinal disorder characterised by chronic lower abdominal pain and disordered defaecation associated with bloating, tenesmus and extra intestinal symptoms including urinary frequency, dyspareunia, fibromyalgia and functional upper gastrointestinal symptoms. Currently there is no unifying hypothesis which adequately explains the pathogenesis of the disorder although a number of physiological and psychological abnormalities have been described. These include altered visceral sensitivity, abnormal intestinal motility and abnormalities of cortical processing of afferent stimuli from the gut. These observations are set against a background of abnormal psychological profiles and an over representation of negative early life experiences. Failure to identify the cause of IBS has led to the development of a range of therapies, some designed to influence the physical effects of the disorder and others to influence the psychological features of the syndrome.

Most IBS patients managed in primary care respond to dietary modification, conventional pharmacological interventions and reassurance. However, when considering any therapeutic efficacy in IBS, it is necessary to weigh the therapeutic effect against the placebo response rate which has been reported to range from 40%-70%.[1] Approximately 15% of IBS patients are resistant to medical therapy. Psychological treatments are usually reserved for these refractory patients and those who relapse despite an initial response to medical treatment. Tricyclic antidepressants have been widely used in IBS and are probably effective through anticholinergic and analgesic effects rather than antidepressant activity. Placebo-controlled trials of tricyclics indicate that even at low doses, they are helpful in the management of abdominal pain and diarrhoea in IBS[2].

Over the past two decades, there have been various attempts to treat IBS using non-pharmacological approaches. As evidence has accumulated to support a role for these interventions, both patients and gastroenterologists have shown increasing interest in exploring this approach to IBS treatment. In this review we consider the evidence for psychopathology in IBS and the efficacy of non-pharmacological interventions.

PSYCHOLOGICAL MORBIDITY IN IBS

From the very first reports of IBS in the medical literature, psychological factors have been recognised as an important component of the syndrome. In 1859, the cause for IBS was attributed to overeating, over-drinking and excess of "sexual or other emotional excitement, sedentary life, damp or hot atmosphere, and the abuse of purgatives, but above all aloes". A "highly excitable condition of nervous centres" was postulated as the cause of IBS, and in 1892, IBS patients were described as manifesting "hysterical, hyperchondriacal and neurasthenic personalities"[3].

With the development of scientific method in medical research, attempts have been made to provide both qualitative and quantitative measures of psychological morbidity in IBS. At least ten studies have assessed the prevalence of psychological disorders in IBS patients[4]. Between 42%-64% of IBS patients meet criteria for a psychiatric diagnosis compared to a median incidence of 19% in patients with organic gastrointestinal disease, and 16% in healthy controls. The most common diagnoses are a generalised anxiety disorder and depression. There is no unique psychological profile which characterises IBS. This higher prevalence of psychological dysfunction in IBS appears to relate only to those sufferers who seek medical advice. Indeed, the majority of individuals fulfilling the criteria for IBS never seek medical attention and these non-consulters have a similar psychological profile to asymptomatic controls[5-7]. This, in turn has provided evidence that psychological factors rather than severity of symptoms influences the decision to seek medical attention. Most IBS consulters have been shown to have experienced negative early life experiences and heightened anxiety about health status. These observations suggest that management in primary and secondary

Centre for Gastroenterology, Royal Free and University College Medical School, London, UK
Dr. A Leahy MRCP, Gastroenterology Specialist Registrar; Dr. O Epstein FRCP, Consultant Gastroenterologist.
Correspondence to: Dr. Owen Epstein, Centre for Gastroenterology, Royal Free Hospital, Pond Street, London NW3 2QG, UK
Received 2001-03-20 **Accepted** 2001-04-15

care should focus not only on symptoms, but also on those psychological factors which have caused the patient to seek medical advice.

There is strong evidence that stress is an important factor in the pathogenesis of IBS[8]. Stress of major "life events", rather than the stress of everyday living, has been implicated as a trigger factor in many patients. In addition, patients often recognise that periods of excessive stress exacerbate their symptoms. In patients with IBS there is good evidence that stressful life events are more frequent, and the effects on GI function more profound, than controls[9,10]. It is against this background of anxiety, depression, stress and consulting behaviour patterns that psychodynamic strategies have been developed to manage IBS.

PSYCHOTHERAPY IN IBS

Psychotherapy is a treatment that primarily seeks to relieve symptoms by exploring the patient's underlying psychological conflicts and emotional disturbances. The first reported study of psychotherapy in IBS included 101 patients who were randomized to medical therapy with or without psychotherapy[11]. The psychotherapy was delivered over ten sessions and focused on mechanisms for coping with stress and resolving emotional problems. Psychotherapy was found to produce a greater improvement in abdominal pain and bowel dysfunction than medical therapy alone. Although symptoms were positively influenced, this short term therapy did not significantly influence the underlying psychopathology. The authors suggested that this might be explained by the relatively mild psychological morbidity in their patients, as the presence of a serious psychological disorder was an exclusion criteria for the study. This study, although of interest, and often quoted, has methodological flaws including the decision to exclude patients with more severe psychological disturbance in whom this therapy might have been of greater value. In addition, the study did not include a placebo arm or select placebo non-responders. The study left it unclear whether the improvement of physical symptom was a result of the psychotherapy or the considerable attention invested in the psychotherapy group.

These pitfalls were addressed by a later study of psychotherapy in IBS performed by Guthrie *et al*[12]. One hundred and two patients refractory to previous medical treatment were randomized to receive psychotherapy or supportive listening. The psychotherapy concentrated on developing a healthy patient-therapist relationship, recognizing other inter-personal relationships and their possible role in the patient's presenting problems. Psychotherapy was found to be superior to supportive listening in terms of improving both physical and psychological symptoms and the improvement in gastrointestinal function correlated significantly with improved psychological well-being. Excellent study design and attention to detail has made this study a benchmark for both pharmacological and non-pharmacological studies in IBS.

BEHAVIORAL THERAPY IN IBS

Behavioral treatment seeks to address and deal with current issues, anxieties and behavioral patterns rather than indulging in deep analysis of past experiences. An uncontrolled trial of behavioral therapy has been reported with a 4 year follow up[13]. In this study the authors used a combination of progressive muscle relaxation, thermal biofeedback, cognitive therapy and IBS education. All but two of the nineteen patients who were available for long-term follow-up rated themselves as at least 50% improved. Symptom diaries were used to demonstrate that the therapy resulted in significant reductions in abdominal pain, diarrhoea, nausea and flatulence. However, like many studies in IBS, the study was inadequately controlled. A more carefully controlled trial of behavioral therapy has been reported in IBS[14]. Forty-two patients were randomly allocated to receive either conventional medical treatment or behavioural therapy with a nurse therapist. The therapy concentrated on behaviour modification, bowel retraining and pain management techniques. It was noted that there were improvements in a number of physical and psychological symptoms, but no significant differences was found when the treatment group was compared with controls. The authors concluded that their approach to behaviour modification was no more effective than conventional medical therapy and reassurance.

HYPNOTHERAPY IN IBS

Hypnotherapy is a state of unusual concentration on the suggestions of the therapist and a willingness to follow their instructions[15] Whorwell *et al* have reported well constructed controlled trials of hypnotherapy in IBS. The technique is focused around a specific "gut directed" hypnosis protocol where the patient is taught to assert control over gut function and imagery whilst in an hypnotic state[16]. Patients are given a simple account of intestinal smooth muscle physiology and hypnotised in a standard manner. The patient is then requested to place their hand on the abdomen and to sense both a positive feeling of abdominal warmth and increased control over gut function. During hypnosis, visualisation is also employed, using the analogy of a gently flowing river and a gently flowing bowel to reinforce a positive bowel image.

Whorwell's initial study randomised thirty refractory IBS patients to seven sessions of either hypnotherapy or psychotherapy. When compared to psychotherapy, hypnotherapy was found to have a greater impact on abdominal pain, bowel habit, abdominal distension and general well-being. It

should be noted, however, that the response rate in the psychotherapy group was much lower than those reported from other centres[11,12]. Further experience of gut directed hypnotherapy has been reported in 250 IBS patients indicating an overall response rate of approximately 80%. Factors predicting a less satisfactory response to hypnosis includes atypical symptoms, older age and more profound psychological disturbance[17].

Two other independent groups have obtained similar results using gut directed hypnotherapy[18,19]. Both these studies were uncontrolled but do appear to support the value of hypnotherapy in IBS. In addition, it has been reported that hypnotherapy in groups of up to eight patients is as effective as individual therapy[18]. Further support for the effectiveness of hypnotherapy in IBS derives from studies on the effect of hypnotherapy on gut function. In two separate studies, hypnotherapy has been reported to reduce rectal sensitivity and colonic motility[20,21].

RELAXATION THERAPY IN IBS

Recognition that stress is a major factor in IBS has provided a basis to explore stress management as a therapy in these patients. Stress management has been compared to conventional therapy in a trial of 35 patients[22]. A physiotherapist delivered a median of six sessions aimed at recognizing the relationship between symptoms and stress, and teaching relaxation exercises. IBS symptoms were relieved in two thirds of patients receiving stress therapy, and only a small number of patients receiving conventional therapy. A small pilot study also found that teaching progressive muscle relaxation was effective in improving gastrointestinal symptoms[23]. Whilst these studies are encouraging, larger controlled studies are required to make a firmer statement on the potential of relaxation therapy in IBS.

BIOFEEDBACK THERAPY IN IBS

Biofeedback is a behavioural technique that uses visual or auditory cues to teach patients to alter physiological responses. With biofeedback, physiological events which are not normally appreciated by the patient are sensed by a technological interface and amplified to give the subject visual or auditory feedback. Patients soon learn to influence the loop and manipulate these physiological events thereby modifying organ function. A new form of biofeedback therapy has been developed and tested in IBS patients[24]. The biofeedback loop is based on the polygraph ("lie detector") which monitors tiny changes in electodermal conductivity occurring in response to stress and relaxation. Changes in cutaneous electrical activity are electronically transformed into a computerised animation of the gut shown on the computer screen. This animation can be controlled by the patient who learns to manipulate the computerised representation of bowel movement using a combination of mental and physical relaxation. In a study of computer aided gut directed biofeeback, 40 IBS patients who were refractory to conventional treatment underwent 4 half hour biofeedback sessions. Eighty percent of the patients learned to achieve progressively deeper levels of relaxation, and in 50%, the technique was reported helpful in controlling bowel symptoms on almost every occasion they became troublesome. The relaxation technique also resulted in significant reductions in global and bowel symptom scores. A control group was not included as it is not possible to administer placebo biofeedback but the study was restricted to treatment refractory patients (ie presumed placebo non responders). An independent group has also recently reported that biofeedback approach is beneficial in managing IBS[25]. In this uncontrolled study, all comers were entered and there was no attempt to select patients. Sixty patients received biofeedback with improvements in abdominal pain, urgency of defaecation and global well-being.

COMPLEMENTARY THERAPY IN IBS

A small open pilot study of acupuncture produced a significant improvement in general well-being and abdominal bloating[26]. There has not been any large controlled trial assessing any form of complementary treatment in IBS.

SUMMARY

Over the last two decades evidence has mounted to suggest that non-pharmacological therapies may be helpful in IBS. Like IBS trials of pharmacological therapies, the studies are often small and poorly controlled. The trials designed to account for a high placebo response rate have either compared non-pharmacological strategies with conventional treatment, or selected only patients who were placebo non-responders. There is broad agreement from the few adequately controlled trials that psychotherapy offers a clear additional therapeutic benefit over and above medical treatments[12]. Hypnotherapy appears to be particularly potent, and, in expert hands, produces consistently impressive therapeutic results even in patients refractory to conventional IBS treatment[17].

Most IBS patients respond to standard medical treatments. Psychological strategies are time consuming, labour intensive and generally unavailable to the relatively large numbers of patients who might benefit. Consequently, these therapies are best reserved for selected patients who fail to respond to reassurance and education, dietary manipulation, antispasmodics and low dose amitriptyline. Increasingly, patients are expressing a preference for non-pharmacological treatment

strategies. Where resources allow, it is not unreasonable to offer these patients a psychodynamic approach as first-line therapy. Ideally, the gastroenterologist should have access to a range of treatment strategies including diet, drugs, psychotherapy, hypnotherapy, relaxation therapy and biofeedback. New approaches such as gut directed, computer-aided biofeedback are particularly attractive as, unlike the interpersonal therapies, this mode of biofeedback does not require highly trained therapists and can be self-administered[24]. Whatever the choice of non-pharmacological therapy, there is evidence that both the doctor and patient can expect symptom improvement, especially when conventional medical measures have failed.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Lasers in gastroenterology

Laurence B. Lovat and Stephen G. Bown

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INTRODUCTION

Endoscopy has revolutionised our management of many gastrointestinal disorders over the past 30 years. We are increasingly able to diagnose gastrointestinal (GI) tumors at an early stage, and endoscopic therapy has made a difference to the outcome of GI haemorrhage. We still rely on surgery for cure of cancer but as diagnostic techniques improve the goal of minimally invasive diagnosis and therapy appears ever more attainable. As populations get older, it is also increasingly desirable. Laser light can be used for both diagnosis and therapy in the gut. This article reviews the value of lasers in these areas.

HOW LASERS WORK

Biological effects

Lasers are sophisticated sources of monochromatic light in the visible and near infrared part of the optical spectrum. The ones of most interest to gastroenterologists are those where the beam penetrates living tissue well and which can be transmitted via thin, flexible fibers, so they can be used with flexible endoscopes. These can be used to deliver light as heat to cause thermal contraction of soft tissue. The most important laser in this group has been the Neodymium yttrium aluminium garnet (NdYAG) laser with a near infrared beam at 1064nm. Short, sharp shots from this laser cause thermal contraction in soft tissues, which provides good haemostasis. Longer shots at high power can vaporise tissue and coagulate the underlying layers, which is effective for debulking advanced cancers. At much lower powers, it is possible to coagulate a larger volume of tissue without vaporisation.

The other main group of effects is *photodynamic* where there is no increase in tissue temperature, but laser light is used to activate a previously administered photosensitising drug. This causes the release of highly reactive singlet oxygen which causes cell death by necrosis and apoptosis over a prolonged period. This can be used to completely eradicate small tumours.

A minor application is to use pulsed lasers endoscopically to fragment gall stones. These effects are summarised in Table 1.

Table 1 Laser effects used in gastroenterology

Laser effect	Clinical use
High power thermal:	Haemostasis Cutting or debulking of tissue by vaporisation and coagulation
Low power thermal: (Interstitial laser photocoagulation,ILP)	Gentle coagulation of lesions within solid organs
Photochemical: (Photodynamic therapy, PDT)	Non-thermal destruction of tissue by activation of a previously administered photosensitising drug
Pulsed shock wave	Fragmentation of gall stones

Non-biological effects

All living tissue display a number of interactions with light, which are altered in areas of dysplasia. Intrinsic fluorescence may be detected at endoscopy by exciting the tissue with blue laser light and using special detector cameras on the endoscope. Elastic scattering depends on the different way light is scattered depending on the density of cellular and nuclear packing. These approaches may allow us to detect premalignant lesions of the GI tract that would otherwise beinvisible at conventional endoscopy.

THERAPEUTIC USES FOR LASER

Palliation of advanced cancers

The main role of high power, thermal lasers like the NdYAG in current practice is for palliation of advanced, inoperable cancers of the upper and lower gastrointestinal tract. Under direct vision, nodules of exophytic tumour can be vaporised and underlying tumour coagulated either to relieve obstruction or to reduce blood loss (Figure 1). The incidence of complications is low, although it often takes several treatments to achieve optimum recanalisation. These laser beams are dangerous if viewed directly, so safety filters must be fitted to fibreoptic scopes. There is no risk to operators with video scopes, although filters are required to protect the chips in the camera.

Most patients with cancers of the oesophagus or gastric cardia present when the disease is too

National Medical Laser Centre, Institute of Surgical Studies, Royal Free and University College Medical School, London
Correspondence to: Dr Laurence Lovat BSc PhD MRCP, Senior Lecturer in Gastroenterology, National Medical Laser Centre, UCL, 67-73 Riding House Street, London W1W 7EJ.
Tel. 020-7679-9060, Fax. 020-7813-2828
Email. l.lovat@ucl.ac.uk
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advanced for there to be any prospect of cure and the main aim of treatment is to relieve dysphagia as simply and rapidly as possible[1]. The main endoscopic options are stent insertion and laser therapy. Tumor dilatation and insertion of a silicone rubber stent was standard practice in Europe until 5 years ago, although these stents were never popular in North America. Using this approach most patients were unable to eat any more than pureed food. Since their introduction in the early 1990s, expanding metal stents have become popular as they require less dilatation (hence less risk of perforation) and are easier to insert. It is becoming clear with experience that they are far from ideal, particularly as the relief of dysphagia has never been shown to be better than silicone stents. New designs are appearing at regular intervals and the current generation of metal stent are covered to prevent tumour ingrowth and conical in shape to prevent stent slippage. Nevertheless, intractable pain occurs in 10% of patients after stent insertion and up to 40% will need further endoscopic interventions[2,3].

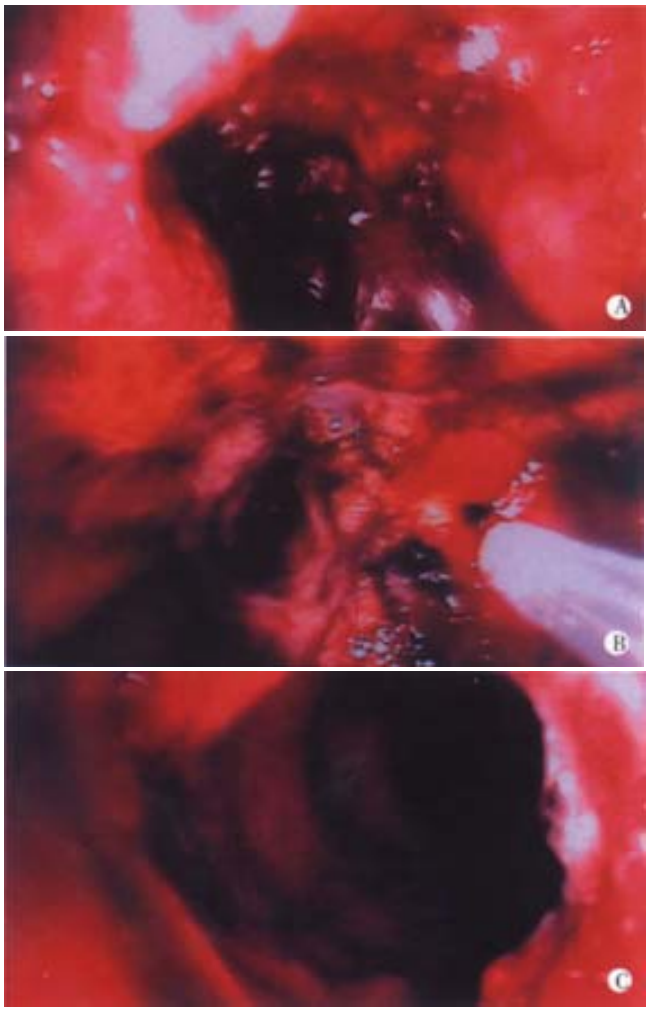

Figure 1 Advanced, obstructing carcinoma of the oesophagus: (a) at presentation; (b) during laser therapy; (c) after two endoscopic laser treatments. The oesophageal lumen has been re opened and the patient's dysphagia has been relieved.

Laser therapy has been shown to improve dysphagia to a similar degree as stents, and does not cause pain. The disadvantage is that laser therapy alone has to be repeated on average every 5-6 weeks. The addition of a palliative dose of external beam radiotherapy can increase this to 9 weeks and a single fraction of brachytherapy (intraluminal radiotherapy) will bring relief of dysphagia for a median of 5 months. Since the median survival time for advanced disease is less than this, a single course of laser and brachytherapy is an attractive option. The relative merits of lasers and stents are summarised in Table 2. Common sense dictates that the two are complimentary rather than competitive. An eccentric, exophytic tumour is best debulked with the laser whereas a circumferential tumour with little exophytic component is best stented. A fistula must be stented, whereas high cervical tumours can seldom be stented. What little data there is on comparative costs suggests that the lifetime treatment costs are similar for each of these approaches[4].

Table 2 Comparison of modalities for palliation of malignant dysphagia

	Laser	Conventional stent	Self expanding metal stent
Technique	Basically safe (risk of perforation if dilatation also needed)	10% risk of perforation on insertion	Usually safe & easy to insert
Cost	High setup cost Low patient costs	Low cost	High cost
Contra indications	Fistula No endoscopic target	High lesion Tracheal compression	High lesion Tracheal compression Care with lesions crossing cardia
Dysphagia post therapy Repeat Therapy	Variable, can be close to normal Possible. Usually required after 6-8 weeks	Semi-solids some solids Stent can be adjusted	Variable, can be close to normal Difficult to adjust once inserted. Second stent or laser debulking for tumour overgrowth
Enhancement of dysphagia relief with radiotherapy	Yes	No	No

A future direction may be the combination of laser palliation of dysphagia with radical chemoradiotherapy in inoperable patients. This approach is attractive as many patients with advanced disease present with severe malnutrition caused by their dysphagia. Radical treatment is not possible in a cachectic patient, but if dysphagia is overcome, patients regain weight and are able to tolerate intensive therapy. Long term data are lacking but early results suggest that this approach can lead to prolonged survival in at least some patients who have previously been thought to be terminally ill.

The situation with the small percentage of

rectal and recto-sigmoid cancers that are not suitable for surgery is very similar[5]. Endoscopic laser therapy can simply and effectively relieve symptoms due to exophytic tumour, usually as a day case procedure with minimal sedation. Bleeding, obstruction, mucus discharge and tenesmus can be helped, and the effect can be prolonged by the addition of palliative, external beam radiotherapy, although laser treatment cannot help pelvic pain or obstruction due to extraluminal tumour. Expanding metal stents are now available to relieve some distal colonic strictures, but they are unlikely to find an important role in palliating low rectal cancers.

Haemostasis for benign lesions

Lasers were first used with flexible endoscopes in the mid 1970s for the control of haemorrhage. Endoscopic treatment with the NdYAG laser significantly reduced the rebleeding rate after haemorrhage from peptic ulcers in a number of studies. Later trials showed that injection sclerotherapy worked just as well, and was simpler and cheaper. The only benign lesions for which endoscopic laser therapy retains an important role for control of blood loss are vascular lesions like hereditary telangiectasia, angiodysplasia and watermelon stomach. These can be managed by sclerotherapy and various thermocoagulatory modalities such as argon plasma coagulation, but laser treatment is effective, simpler to apply and needs less treatment sessions, especially when multiple lesions are present.

Interstitial laser photocoagulation

High power NdYAG laser is used during endoscopy, with short shots of a second or two, typically at 50-80W. The laser fibre is held away from the surface of the target to vaporize and coagulate tumour tissue under direct vision. An alternative approach is to insert the tip of the laser fibre directly into the target area and to use a much lower power (3-5W) to gently "cook" diseased tissue over a period of several minutes. This is known as interstitial laser photocoagulation (ILP). There is no selectivity of effect. Both normal and malignant tissue will be necrosed if heated to a high enough temperature for long enough. ILP can be used for the percutaneous treatment of small hepatic metastases (particularly from previously resected, primary colorectal cancers) in patients who are unsuitable or unfit for partial hepatectomy[6]. The key to using this technique succesfully is the imaging. Fibers can be inserted through needles positioned percutaneously under ultrasound, CT (computerised tomography) or MR (magnetic resonance) imaging. The best way to assess the effect is on contrast enhanced CT scans taken 24 hours after treatment, which show laser necrosed areas as new zones of non-enhancement. The size of the zone of necrosis depends on the number of laser fibres used, but it is possible to necrose lesions up to about 4 cm-5 cm in diameter. Treated areas heal mainly with regeneration of normal liver. ILP is only appropriate for hepatic metastases in patients with a small number of clearly identifiable lesions and no evidence of extrahepatic disease, but for these individuals, it may slow down the progression of their disease, and is likely to be complimentary to chemotherapy. Radiofrequency heating can achieve a similar thermal effect without the need for lasers.

Photodynamic therapy

Photodynamic therapy (PDT) is a technique for producing localised necrosis of tissue with light after prior administration of a photosensitising agent[7]. Drugs that are activated by red light are usually chosen as red light penetrates deeply into tissue, but mucosal disease (e.g. dysplasia in Barrett's oesophagus or squamous oesophagus) might be conveniently treated with green light, which penetrates only 1 mm-2 mm. On their own, neither the light nor the photosensitising drug produces any effect. The biological effect is photochemical with no increase in tissue temperature, so is quite different from the thermal techniques discussed above. ILP and PDT are compared in Table 3. In particular, PDT produces remarkably little effect on connective tissue like collagen, so it is possible to get full thickness necrosis in the wall of the gastrointestinal tract with minimal risk of perforation[8]. The effects produced vary with the photosensitiser used and may prove of value in a surprisingly wide range of conditions. Potential applications are summarised in Table 4.

Table 3 Comparison of interstitial laser photocaogulation (ILP) and photodynamic therapy (PDT)

	ILP	PDT
Nature of biological effect	Thermal	Photochemical
Wavelength of light used	Infrared (805-1064nm)	Green (510-530nm) Red (630-675nm)
Typical laser power per fibre	3-5 W	0.1-0.3 W (higher for illuminating hollow organs)
Effect on connective tissue	Destroyed	Largely unaffected
Healing	Resorption & scarring, some regeneration	Regeneration, sometimes with scarring
Selectivity of necrosis between tissue of origin of tumour and other adjacent tissues	None	Possible between mucosa and underlying muscle in hollow organs
Selectivity of necrosis between tumour and tissue of origin of tumour	None	Minimal
Cumulative toxicity	None	None

Table 4 Potential targets for photodynamic therapy in gastroenterology

Small, inoperable tumours in endoscopically accessible sites
Areas of dysplasia, especially in Barrett's oesophagus
Localised pancreatic cancer
Bile duct cancer
As an adjunct to surgery
Palliation of advanced cancers controversial
Eradication of *Helicobacter pylori* speculative

Tumors of the gastrointestinal tract

PDT is an attractive option for treating small tumours of the gastrointestinal tract in patients who are unsuitable for surgery. In a series of 123 patients with early oesophageal cancers treated with PDT using the photosensitiser porfimer sodium (Photofrin), a complete response (no evidence of tumour on endoscopy or biopsy) was seen in 87% at 6 months[9]. The disease specific survival was 75% although the overall 5 year survival was only 25%, This means that in the majority of the patients, the cancer was not the main cause of death. These patients were inoperable due to comorbidity and this minimally invasive therapy was well tolerated. Care must be taken not to treat too extensive a lesion as circumferential scarring in the muscle layer can cause a stricture. Strictures occured in 35% of the patients in this series, although they did all respond to dilatation. PDT can be applied at any endoscopically accessible site in the upper or lower gastrointestinal tract, but it cannot treat any lesion that has spread beyond the site of origin as, for example, to local lymph nodes.

Although the light for PDT is applied locally, the drug is given systemically, which means that the whole body is photosensitised, including the skin. This can be a problem, as there is a risk of skin damage due to drug activation by ambient light. With the photosensitiser porfimer sodium, patients must avoid bright sunlight for up to 3 months, although with the sensitiser mTHPC (meso-tetrahydroxyphenyl chlorin) it is 2-3 weeks and with ALA (5-amino laevulinic acid), it is only 1-2 days.

PDT has been proposed for the palliation of advanced malignant dysphagia. This was the first application for which PDT was licensed in the USA (using porfimer sodium). PDT does provide some relief in this situation, but there are very few cases that can be helped by PDT if NdYAG laser therapy or stent insertion fail, and it is certainly not desirable to make patients photosensitive for much of their remaining life[10]. PDT may be of value to treat tumour that has grown over or through a stent that cannot be adjusted and which cannot tolerate the heat from a NdYAG laser. In general terms, it seems more logical to licence PDT for treating early oesophageal cancers, as has been done by the Japanese authorities, although the UK seems more likely to follow the American pattern, at least initially.

Premalignant lesions of the gastrointestinal tract

ALA is a naturally occurring substance which is converted *in vivo* through a series of derivatives to haem via protoporphyrin IX (PPIX). PPIX is a photosensitiser, and after exogenous administration of ALA, enough PPIX accumulates to produce a PDT effect on activation with red or green light[7] (Figure 2). However, in contrast to porfimer sodium and mTHPC, PPIX accumulates primarily in the mucosa of hollow organs. This makes it possible to destroy mucosa (normal or abnormal) without damaging the underlying muscle layer. This is exactly what is required in the treatment of Barrett's oesophagus with dysplasia. In a recent report, using ALA PDT, high grade dysplasia was cleared in 10 of 10 and carcinoma in situ was cleared in 17 out of 22 patients with Barrett's oesophagus[11] and in a randomised placebo controlled trial, this modality had a significant impact on patient outcome[12]. There were no oesophageal strictures, in contrast to 35% of 100 patients with high grade dysplasia or intramucosal carcinoma arising in Barrett's oesophagus who were treated with PDT using porfimer sodium[13]. However, ALA PDT brings its own problems, as the depth of necrosis does not exceed 2 mm and this is not always sufficient. It is reassuring that the necrosed, neoplastic Barrett's mucosa healed with regeneration of normal squamous mucosa, but follow up biopsies showed that in some cases, there was still untreated columnar mucosa under the new squamous mucosa.

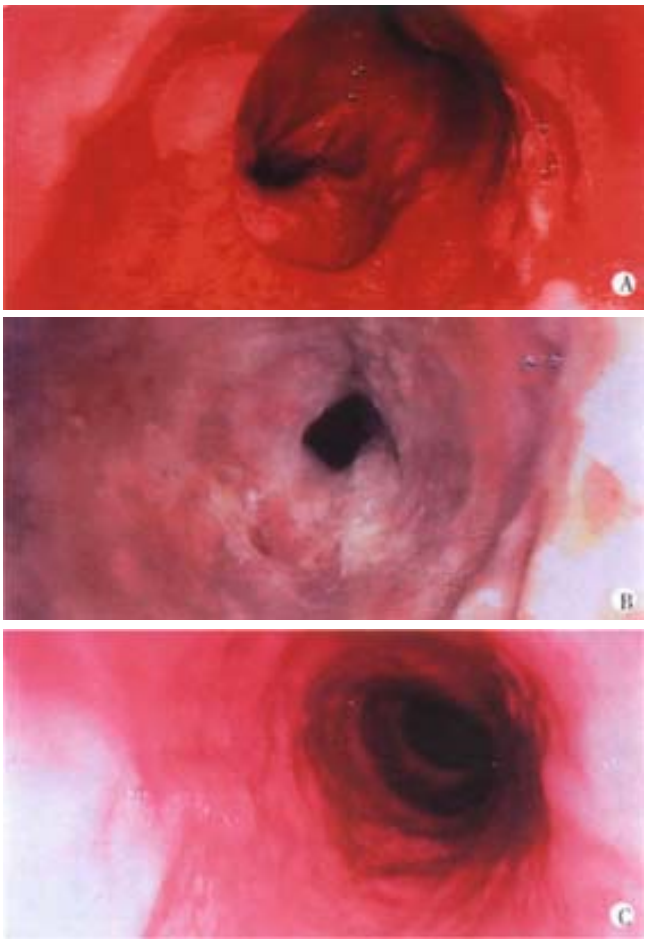

Figure 2 Photodynamic therapy for dysplasia in Barrett's oesophagus: (a) prior to therapy, columnar lined mucosa is seen at endoscopy. Dysplasia is not visible, but has been confirmed histologically; (b) one day after treatment, there is widespread sloughing of the oesophageal mucosa; (c) one month later, the oesophagus has completely healed with some regeneration of squamous mucosa.

Endoscopic management of Barrett's oesophagus is still a difficult problem. Thermal ablation with an argon plasma coagulation or KTP or NdYAG laser involves moving a small therapeutic beam across the area to be treated under direct endoscopic vision. It is easy to undertreat, and leave abnormal mucosa, or overtreat, with the risk of muscle scarring or even perforation. With PDT, balloon light delivery systems are available which illuminate all the relevant mucosa evenly, and there is very little risk of perforation, but using porfimer sodium, there is a high risk of a stricture as there is no selectivity of effect between the mucosa and underlying layers. PDT with ALA is the only technique at present that seems able to produce a selective effect, necrosing mucosa but not the underlying muscle, but to date, the results are no better than using other ablative techniques. More research is needed to find reliable ways of ensuring that the full thickness of the abnormal mucosa is ablated. In the present situation, surgery is still the treatment of choice for patients with high grade dysplasia or early carcinoma in Barrett's oesophagus. PDT should be reserved for those who are unfit for surgery. Endoscopic treatment of Barrett's oesophagus without evidence of dysplasia should be limited to the context of clinical trials. Since dysplasia and even cancer has been reported to occur in areas treated by PDT which have reverted to 'normal' squamous epithelium after treatment, long term surveillance endoscopy is still needed in patients treated this way.

TUMOURS OF THE PANCREAS AND BILE DUCT

Pancreatic cancer

Most of the applications of PDT in gastroenterology have been for lesions of the luminal gut. Interest is now growing in using this modality to treat localised tumours of the pancreas and bile ducts. Animal studies have shown that the pancreas and adjacent normal tissues can tolerate PDT and that necrosis can be produced in cancers transplanted into the hamster pancreas. These results justified a pilot clinical study[14]. The technique is shown in Figure 3. Sixteen patients with small, localised, pancreatic cancers, not involving major blood vessels, were sensitised intravenously with mTHPC. Three days later, up to 4 needles were inserted into the tumour percutaneously under computerised tomography (CT) guidance and laser fibres passed through the needles to activate the drug in the tumour. Contrast enhanced CT scans a few days later showed new areas of devascularisation in the pancreas consistent with PDT induced necrosis (Figure 4). There were no serious complications, and most patients were out of hospital within a week. The median survival from diagnosis was 12.5 months with the longest surviving subject living for 34 months. These preliminary data are encouraging but randomised trials are needed to establish just how successful this aproach will be. At present, it is far too early to judge what role PDT may develop in the management of this unpleasant cancer.

Cholangiocarcinoma

The median survival in patients with unresectable hilar cholangiocarcinoma is 4-6 months due to refractory cholestasis or sepsis. PDT may have a role in the treatment of bile duct cancers, particularly for relief of obstructive jaundice when biliary stents are ineffective or become occluded. Two small uncontrolled studies have shown prolongation of survival in this group of patients to more than one year and interstingly, areas of tumour not directly illuminated have also undergone regression, presumably due to conduction of light through the biliary tree[15,16]. Larger, controlled studies are needed in this area.

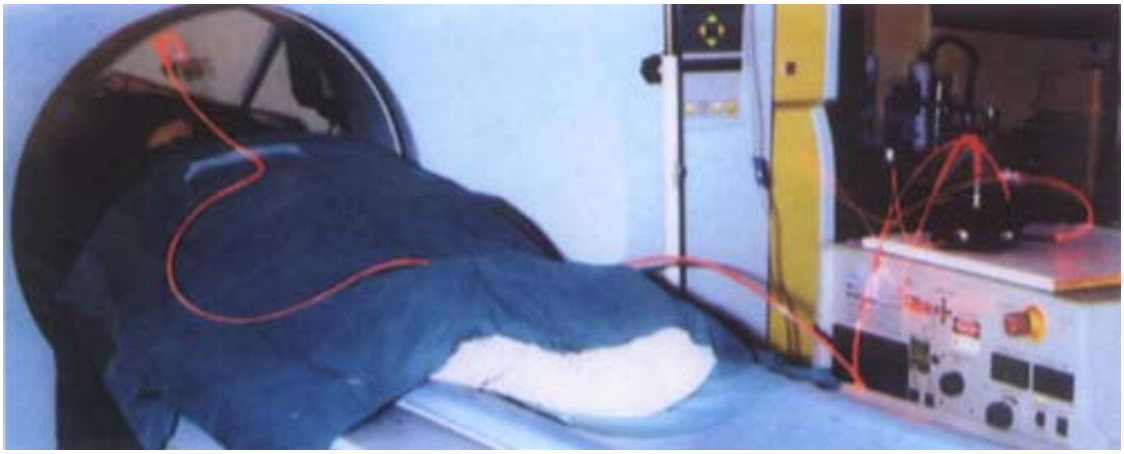

Figure 3 Photodynamic therapy for treatment of a small pancreatic cancer. Three days after photosensitisation with mTHPC, light is delivered to the tumour via fibres passed through needles positioned percutaneously under CT guidance. (Reproduced with permission from Bown SG, Br Med J, 1998;316:754-757)

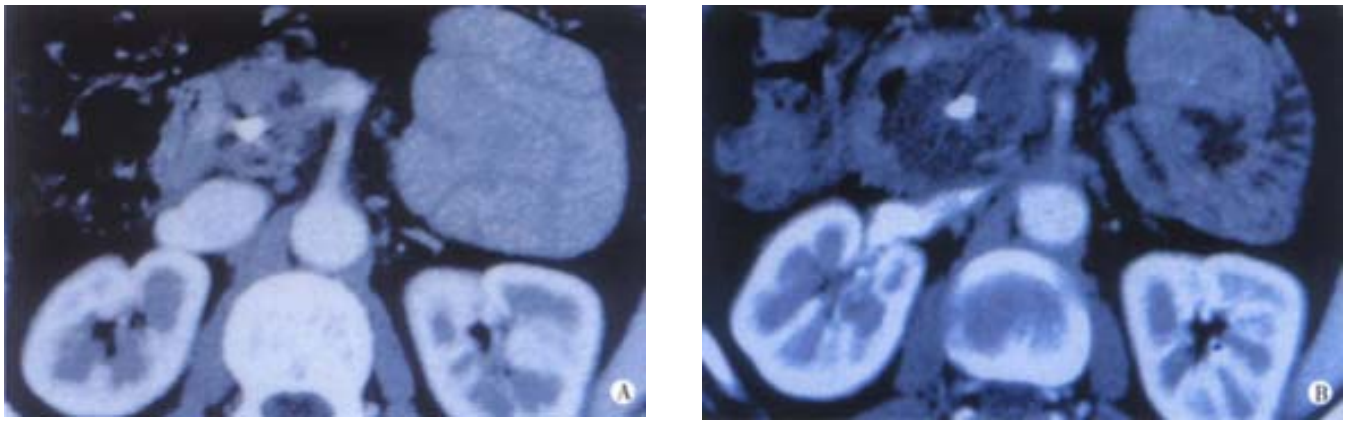

Figure 4 CT scans (a) before and (b) one week after PDT for pancreatic cancer. The tumour surrounding the biliary stent seen in the first scan has been replaced by a low density area of necrosis.

FUTURE APPLICATIONS OF PDT

A logical application of PDT is as an adjunct to surgery, to destroy small tumour deposits that are not visible to the naked eye or which involve areas that can not be resected. One randomised trial has been reported looking at PDT as an adjunct to resection of rectal cancers, but there was no difference between the two groups in the incidence of local recurrence[17].

A more speculative application of PDT is for the treatment of *Helicobacter pylori*. With the increasing incidence of antibiotic resistance, it would be attractive to have an alternative therapy and all sites colonised by *H. pylori* are easily accessible endoscopically for light delivery. The organism is certainly sensitive to PDT in culture, using methylene blue as the photosensitiser, and preliminary *ex vivo* experiments have also given encouraging results[18], but it would take considerable technical ingenuity to get adequate drug and light to all relevant sites to make this worth trying clinically.

TISSUE SPECTROSCOPY

Tissue spectroscopy is a way of interrogating tissue to obtain a characteristic 'tissue signature' that reveals its underlying histology. A number of spectroscopic techniques exist but all exploit the interaction between specific properties of light and the tissues under interrogation.

FLUORESCENCE ENDOSCOPY

All tissues exhibit a natural fluorescence. In luminal organs, dysplastic mucosa has a different fluorescence spectrum to normal tissue and this can be detected using specially modified endoscopes to emit blue light (either laser or a broader band source) and very sensitive cameras that detect green or red light. The technique is already proving useful for detecting early lesions in the bronchial tree. The accuracy of this technique is also under investigation for dysplasia in Barrett's oesophagus or in chronic ulcerative colitis but in both cases, the findings are confounded by the weak signals generated and the small differences between the fluorescence signal of normal, inflamed and dysplastic tissues. Studies are also underway to enhance the differences between normal and dysplasia by giving a drug exogenously that is selectively concentrated in the areas of dysplasia but this does not as yet appear to be beneficial. Although the idea is promising, real clinical application still seems a long way away.

ELASTIC SCATTERING: 'OPTICAL BIOPSY'

This technique exploits the difference in scattering of light of various wavelengths by different sized particles- the same principle that explains why the sky is blue and sunsets are red. It uses white light passed down a probe placed through the biopsy channel of an endoscope. This is a point measurement, like a biopsy but has the advantage of almost instantaneous diagnosis. Again, this is in early stages of development, but it is cheap, does not require complex equipment and initial reports suggest a high degree of accuracy[19,20].

CONCLUSIONS

Thermal laser is an established tool for endoscopic palliation of advanced gastrointestinal tract cancers. Interstitial laser photocoagulation may help in the management of patients with a small number of isolated hepatic metastases. However, the most important new applications of lasers being developed in gastroenterology are in photodynamic therapy, particularly for the endoscopic treatment of dysplasia in Barrett's oesophagus; for the treatment of small inoperable tumours in the gastrointestinal tract and in the pancreatobiliary

system. Finally, light based 'optical' methods are under investigation for instantaneous diagnosis in the gut and these may enable more accurate and faster diagnosis than have been available hitherto. The latter techniques are only at an early stage of clinical trials, but if these studies are successful, PDT and optical diagnosis could find an important role in gastroenterology practice.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Original Articles

Transplantation of human hepatocytes into tolerized genetically immunocompetent rats

Edwin C. Ouyang, Catherine H. Wu, Cherie Walton, Kittichai Promrat and George Y. Wu

Subject headings liver/cytology; immune tolerance; cell transplantation

Ouyang EC, Wu CH, Walton C, Promrat K, Wu GY. Transplantation of human hepatocytes into tolerized genetically immunocompetent rats.***World J Gastroenterol***, 2001;7(3):324-330

Abstract

AIM To determine whether normal genetically immunocompetent rodent hosts could be manipulated to accept human hepatocyte transplants with long term survival without immunosuppression.

METHODS Tolerance towards human hepatocytes was established by injection of primary human hepatocytes or Huh7 human hepatoma cells into the peritoneal cavities of fetal rats. Corresponding cells were subsequently transplanted into newborn rats via intrasplenic injection within 24h after birth.

RESULTS Mixed lymphocyte assays showed that spleen cells from non-tolerized rats were stimulated to proliferate when exposed to human hepatocytes, while cells from tolerized rats were not. Injections made between 15d and 17d of gestation produced optimal tolerizaton. Transplanted human hepatocytes in rat livers were visualized by immunohistochemical staining of human albumin. By dot blotting of genomic DNA in livers of tolerized rats 16 weeks after hepatocyte transplantation, it was found that approximately 2.5×10^5 human hepatocytes survived per rat liver. Human albumin mRNA was detected in rat livers by RT-PCR for 15wk, and human albumin protein was also detectable in rat serum.

CONCLUSION Tolerization of an immunocompetent rat can permit transplantation, and survival of functional human hepatocytes.

Department of Medicine, Division of GastroenterologyHepatology, University of Connecticut Health Center, Rm. AM045, 263 Farmington Avenue, Farmington, CT-06030
Correspondence to: George Y. Wu, M.D., Ph.D., Department of Medicine, Division of GastroenterologyHepatology, University of Connecticut Health Center, Rm. AM045, 263 Farmington Avenue, Farmington, CT 06030
Tel. (860) 679-3158, Fax. (860) 679-3159
Email. wu@nso.uchc.edu
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INTRODUCTION

Transplantation of allogeneic hepatocytes into immunocompetent rodents has been shown to result in rejection of donor cells by host immune system, with T cell activation, and a delayed type hypersensitivity reaction[1-3]. This can been overcome by generalized immunosuppression or through the use of genetically immunodeficient animals[4-8]. Another strategy to avoid rejection is by inducing immunological tolerance specifically to the transplanted cells. In this regard, it has been demonstrated that the ability of the immune system to distinguish between endogenous and exogenous antigens develops shortly before birth. Studies have shown that if foreign antigens are introduced during this formative period, when the animals mature, they will be tolerant to those antigens[9], permitting the survival of allogeneic (cardiac) transplants in rats[10,11]. We hypothesized that, if human liver cell antigens could be introduced into normal rodents at the appropriate time during fetal development, those animals could be rendered tolerant and serve as suitable hosts for human hepatocyte transplants after birth without either genetic or pharmacological generalized immunosuppression. If successful, these animals could serve as convenient animal models for studying mechanisms of human hepatitis viral infections, serve as models for testing new antiviral agents, and as means for testing hepatotoxicity of drugs.

MATERIALS AND METHODS

Animals

Pregnant Sprague Dawley rats, 250 g to 300 g of body mass (Charles River Co., Inc., Wilmington, MA) were maintained on 12 h lightdark cycles, and fed *ad lib* with standard rat chow in the Center for Laboratory Animal Care at the University of Connecticut Health Center. All animal procedures were approved by Institutional Animal Care and Use Committee and conformed to USDA and NIH animal usage guidelines.

Cells

Cryopreserved human primary hepatocytes were obtained from Clonetics Corp. (Walkersville, MD) and kept in liquid nitrogen until use. Frozen cells were thawed, washed with human hepatocyte medium (Clonetics Corp.) plus 5 $g\cdot L^{-1}$ insulin and 0.39 $mg\cdot L^{-1}$ dexamethasone, and then spun at 50 × g for 10 min at 4℃. Cell viability was measured by trypan blue exclusion staining (approximately 65% of the cells were viable, and 90% were parenchymal

hepatocytes). Human hepatoblastoma cell lines Huh7 and HepG2, human fibroblast IMR-90 and human kidney 293 cells were grown in Dulbecco Modified Eagle's medium (DMEM) with 100 mL·L^{-1} fetal bovine serum (FBS) and antibiotics.

Intrafetal intraperitoneal injections of human hepatocytes

At 15 d to 17 d of gestation, groups of pregnant rats were anesthetized by intramuscular injections of ketamine (40 mg·kg^{1} body mass) and xylazine (5 mg·kg^{1} body mass). Laparatomies were performed under sterile conditions; gravid uteri were exposed, and transilluminated by a high intensity lamp (Fiberlite MI150, DolanJenner Industries, Lawrence, MA). Human hepatocytes or Huh7 cells, 1×10^5 cells in 10 μL PBS, were injected through the uterine wall into the peritoneal cavities of rat fetuses using a sterile 200 μLHamilton syringe with a 28 gauge beveled point needle (Hamilton Inc., Reno, NV).

Cell transplantation

Within 24 h of birth, newborn rats were placed on ice for 2-5 min. Under sterile conditions, left paracostal incisions were made, and primary human hepatocytes or Huh7 cells, 1×10^{10} cells·L^{-1} in200 μL PBS were injected over 30 into the spleen by sterile Hamilton syringe.

Sample collection

Peripheral blood samples were drawn from tail veins, spun, and serum stored at -20℃. Liver samples were collected either by sacrificing animals or by performing partial hepatectomies. Samples were fresh frozen in liquid nitrogen, and stored at -80℃.

Mixed lymphocyte assays

The tolerance of host animals towards human hepatocytes was assessed by mixed lymphocyte assays in which the proliferation of host spleen cells was measured after exposure to exogenous antigens[12]. Briefly, spleens were removed from tolerized or control animals, 1 wk after cell transplantation or for non-transplanted controls one week after birth, and dispersed into RPMI1640 medium (Gibco-BRL) with 50 mL·L^{-1} FBS. Stimulator cells (primary human hepatocytes, Huh7, IMR-90 and 293 cells) were gamma irradiated with 20Gy to inhibit proliferation. Irradiated stimulator cells, 0.5 mL of 3×10^8·L^{-1}, were mixed with 0.5 mL of 1×10^9·L^{-1} rat spleen cells, pulselabeled with 37kBq of ^{3}H-thymidine (2960TBq·mol^{-1}, Amersham Life Science), and then incubated at 37℃ with 50 mL·L^{-1} CO_2 for 72 h. After trichloroacetic acid (TCA) precipitation, cells were harvested onto Whatman glass fiber filter papers (Whatman), washed successively with phosphate buffered saline (PBS), TCA and ethanol. Filter papers were counted in a scintillation counter (Tri-CARB 4530, Parkard). Spleen cells from untreated rats as well as stimulator cells incubated alone served as controls. All experiments were performed with triplicate animals, and the results expressed as $\bar{x} \pm S\bar{x}$ in units of nBq·cell^{1}.

Detection of human albumin gene sequences in rat liver

To detect human hepatocytes that survived transplantation in rat livers, human albumin gene sequences were sought as specific markers using a 5' primer (5'-CTGGTCTCACCAATCGGG-3') and a 3' primer (5'-CTGGTCTCACCAATCGGGGG-3'). Genomic DNA extracted from Huh7 cells served as a positive control. Genomic DNA from untreated rats, and rats tolerized without transplantation were used as negative controls.

Quantitation of human albumin DNA in rat liver

To quantify the number of human hepatocytes present in rat livers, dot blots using probes specific for the human albumin gene were performed by modifying the method of Kafatos[13] with a ^{32}P-labeled 1750bp *Bam*H I /*Bst*e II human albumin DNA fragment excised from palb-3, a plasmid containing the complete human albumin gene[14]. All assays were performed in triplicate, and the results were expressed as $\bar{x} \pm s_{\bar{x}}$. Genomic DNA from known numbers of Huh7 cells was measured in an identical fashion.

Detection of human albumin mRNA in rat livers

To determine whether transplanted human hepatocytes retained liver-specific transcription, the presence of human albumin mRNA was sought by RT-PCR after extraction according to the method of Chomczynski[15] using primers for human albumin (sense 5'-CCTTGGTGTTGATTGCCTTGCTC-3', antisense 5'-CATCACATCAACCTCTGGTCTCACC-3') and rat albumin (sense 5'-CGGTTTAGGGACTT-AGGAGAACAGC-3', antisense 5'-ATAGTGTCCCA-GAAAGCTGGTAGGG-3'). The expected size of PCR products for human and rat albumin mRNA were 315 bp and 388 bp, respectively.

Detection of human albumin in rat liver

Sixteen weeks posttransplantation, groups of rats were sacrificed, and livers sectioned into 5 μm slices in tissue freezing medium (Triangle biomedical Sciences, Durham, NC). Immunofluorescence staining was performed using a modification of the method of Osborn[16] using monoclonal mouse antihuman albumin antibody (Sigma, St. Louis, MO) and goat anti-mouse IgG second antibody conjugated with Texas Red (Amersham Pharmacia Biotech). Immunohistochemical staining for human albumin was done according to the method of Kieran[17]. Tissue samples were examined using confocal laser scanning microscopy (LSM-410, Zeiss, Germany).

Assays for human albumin in rat serum

To measure human albumin in rat serum, Western blotting was performed in a manner similar to the

method of Gershoni[18] using monoclonal mouse anti-human albumin antibody (Sigma, St. Louis, MO) and rabbit anti-mouse IgG second antibody conjugated with horseradish perioxidase (HRP) (Sigma, St. Louis, MO). The signal was detected by an enhanced chemiluminescence method (ECL kit, Amersham) and exposed to film.

RESULTS

Mixed lymphocyte assays were used to detect changes in immune response as a result of intrafetal injections. In these assays, spleen (responder) cells taken at wk 1 after birth were mixed with irradiated stimulator cells (primary human hepatocytes, or controls consisting of Huh7 human hepatoblastoma cells, IMR-90 human fibroblasts, or 293 human kidney cells). Figure 1, lane 1 shows that spleen cells from animals that were not injected with hepatocytes intrafetally, incubated alone (without any stimulator cells) had baseline uptake of (1446 ± 111) nBq·cell^{-1}. Irradiated hepatocytes incubated alone only took up (222 ± 100) nBq·cell^{-1}. Lane 2, in contrast, the spleen cells from lane 1, from rats with no intrafetal injection, but subsequently exposed to irradiated human hepatocytes, lane 3, were stimulated to take up (10842 ± 1585) nBq·cell^{-1}, a 7.5-fold increase. But, when spleen cells from rats that had intrafetal injection of primary human hepatocytes, were subsequently exposed to irradiated primary human hepatocytes, lane 4, they were not stimulated as uptake was only (1390 ± 139) nBq·cell^{-1}. To determine whether the lack of stimulatory effect was hepatocytespecific, spleen cells from animals injected intrafetally with primary hepatocytes were exposed to human IMR-90 fibroblasts. In contrast to hepatocyte stimulator cells, the spleen cells were stimulated by the fibroblasts, taking up (14373 ± 1473) nBq·cell^{-1}, lane 5, which was similar to the uptake of cells from rats not intrafetally injected with hepatocytes, (13956 ± 2085) nBq·cell^{-1}, lane 7. In another control, uptake by 293 human kidney cells was also stimulated in spleen cells from rats either intrafetally injected with hepatocytes, lane 6, or not, lane 8. Irradiated IMR-90, lane 9, and 293 cells, lane 10, incubated alone had negligible uptake, indicating that the contribution of these cells could not account for the observed increases in uptake results found in lanes 5 and 7.

To determine whether transformed human hepatocytes could be also used to induce immunological tolerance, Huh7, and HepG2 human hepatoblastoma cell lines, were compared to primary human hepatocytes in terms of induction of tolerance. Figure 2 shows that spleen cells from rats not injected intrafetally with hepatocytes, and subsequently exposed to primary hepatocytes, lane 1; Huh7 cells, lane 2; or HepG2 cells, lane 3 all had uptake ratios significantly and substantially greater than cells from rats intrafetally injected and subsequently exposed to the corresponding cells, lanes 4, 5 and 6, respectively.

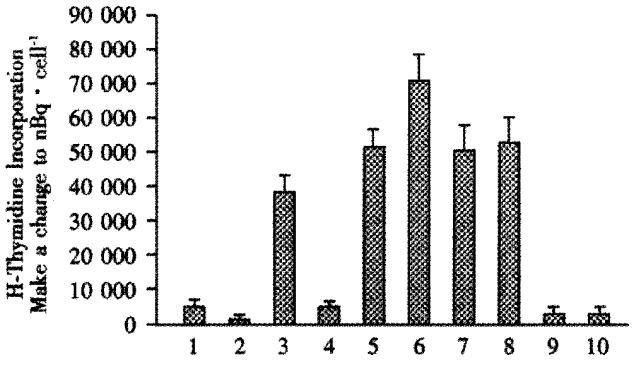

Figure 1 Mixed lymphocyte assays. Rat spleen (responder) cells, from 3 rats per group, were incubated either alone, or with (stimulator) irradiated primary human hepatocytes, IMR-90 human fibroblasts, or 293 human kidney cells in the presence of ^{3}H-thymidine. The incorporation of radioactivity was used as a measure of proliferation of rat spleen cells induced by exposure to foreign cells. When performed, rats were intrafetally injected with primary human hepatocytes on d16 of gestation. Mixed lymphocyte assays were performed at wk1 after birth. Spleen cells from rats neither injected intrafetally with hepatocytes, nor transplanted, lane 1; irradiated primary human hepatocytes incubated alone, lane 2; spleen cells from rats neither intrafetally injected nor transplanted, but which were incubated with irradiated hepatocytes, lane 3; spleen cells from rats intrafetally injected and transplanted and subsequently exposed to irradiated hepatocytes, lane 4; responder spleen cells from intrafetally injected and transplanted, exposed to irradiated IMR-90 fibroblasts, lane 5, and 293 kidney cells, lane 6; spleen cells from animals neither intrafetally injected nor transplanted, but exposed to irradiated IMR-90 cells, lane 7, or 293 cells, lane 8; irradiated IMR-90 and 293 cells incubated alone, lanes 9 and 10, respectively. Results were expressed as means ± S. E. *indicates statistical significance, P<0.05.

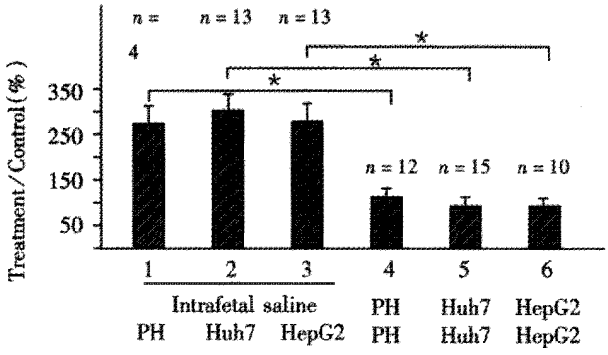

Figure 2 Mixed lymphocyte assays for measuring tolerance induced by different types of human hepatocytes. Rats were intrafetally tolerized with either primary human hepatocytes (PH), or Huh7 cell, or HepG2 cells. All assays were performed at wk1 after birth and show radioactive incorporation by spleen cells from rats that were injected intrafetally with only saline, and subsequently incubated with primary hepatocytes, hepatoblastoma cell lines Huh7, or HepG2, lanes 1, 2 and 3, respectively. Radioactive uptake of spleen cells from rats intrafetally injected with primary hepatocytes, Huh7 or HepG2 cells and incubated with their corresponding irradiated cells is shown in lanes 4, 5, and 6, respectively. The number of rats in each group is indicated on the top of each column. Results are expressed as percentage of controls (spleen cells from untreated rats incubated alone) as $\bar{x} \pm s_{\bar{x}}$. Duncan's test was used to analyze the significance between different treatment groups. *indicates significant differences between groups 1 and 4, between 2 and 5; and 3 and 6, P < 0.05.

Because of the difficulty in obtaining primary human hepatocytes, immunohistochemical methods for detection of transplanted cells were established using Huh7 hepatoblastoma cells. To visualize these cells in livers of rats that were previously tolerized intrafetally and transplanted with Huh7 cells, staining with monoclonal mouse antibody against human albumin was performed. Figure 3 shows that staining was detectable in rat livers on day 1 after transplantation mostly as single cells, but with occasional pairs, fairly evenly distributed throughout the parenchyma, panel A and with high magnification, panel B. Seven days after birth, clusters of 2 and 3 cells each were visible, and single cells less common, panel E and with higher magnification, panel F. No human albumin was detected in rats that were tolerized with Huh7, but were not transplanted, panel C and at higher magnification, panel D, confirming that the antibody was specific for human albumin and lacked cross reactivity with endogenous rat albumin.

Laser scanning confocal microscopy was used to visualize transplanted hepatocytes by staining with monoclonal antibody against human albumin. Figure 4 shows that in tolerized rats transplanted with human hepatocytes, human albumin was detected at week 16 post-transplantation, panel B. In tolerized rats without transplantation, no staining was detected, panel C. Rat liver sections stained with secondary antibody alone, panel D demonstrated that the staining was not an artifact due to nonspecific interaction with second antibody. As expected samples stained with monoclonal goat anti-rat albumin antibody, panel A, resulted in positive signals for rat albumin in virtually every parenchymal cell in the liver.

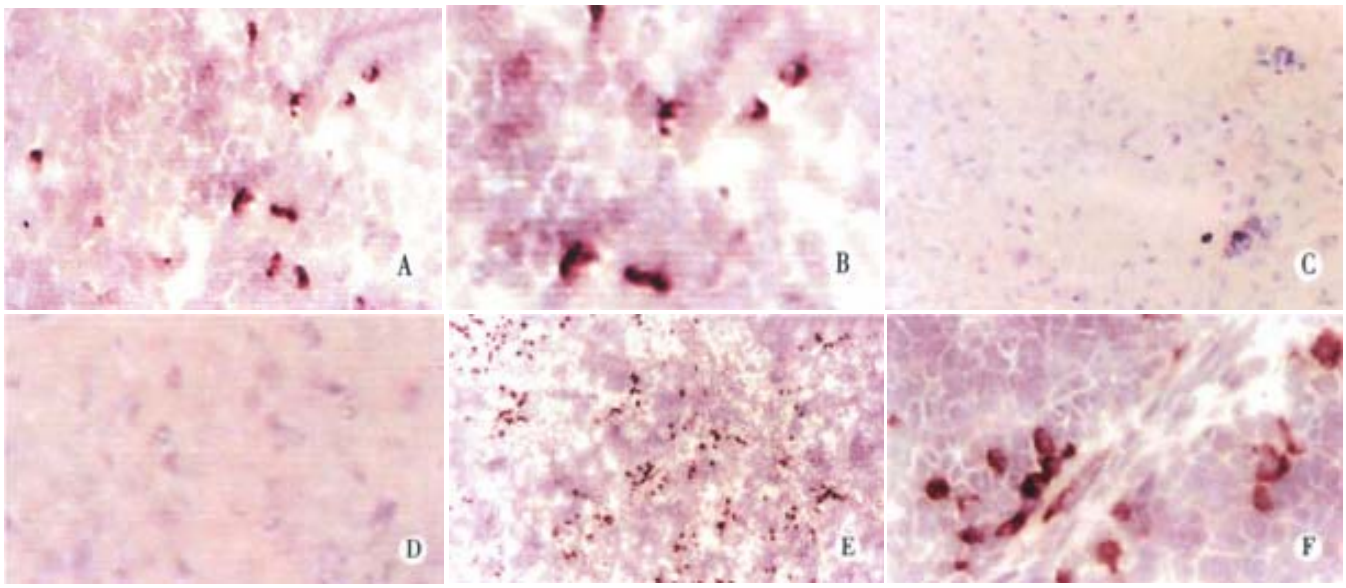

Figure 3 Immunohistochemistry for detecting human albumin in rat livers. Antibody against human albumin was visualized using a DAB method as described in Materials and Methods. Fifteen SD fetal rats were tolerized with Huh7 cells. Ten newborn rats were subsequently transplanted with Huh7 cells on d1 after birth, and the rest were not transplanted. Panel A, a representative rat that was tolerized and transplanted with Huh7 cells, sacrificed on d1 post-transplantation, magnification ×125; panel B, same as panel A, ×250; panel C, a representative rat that was tolerized with Huh7 cells, but without transplantation, ×125; panel D, same as panel C, ×250; panel E, a representative rat that was tolerized and transplanted with Huh7 cells, sacrificed on d7 after birth, ×125; panel F, same as panel B, ×250.

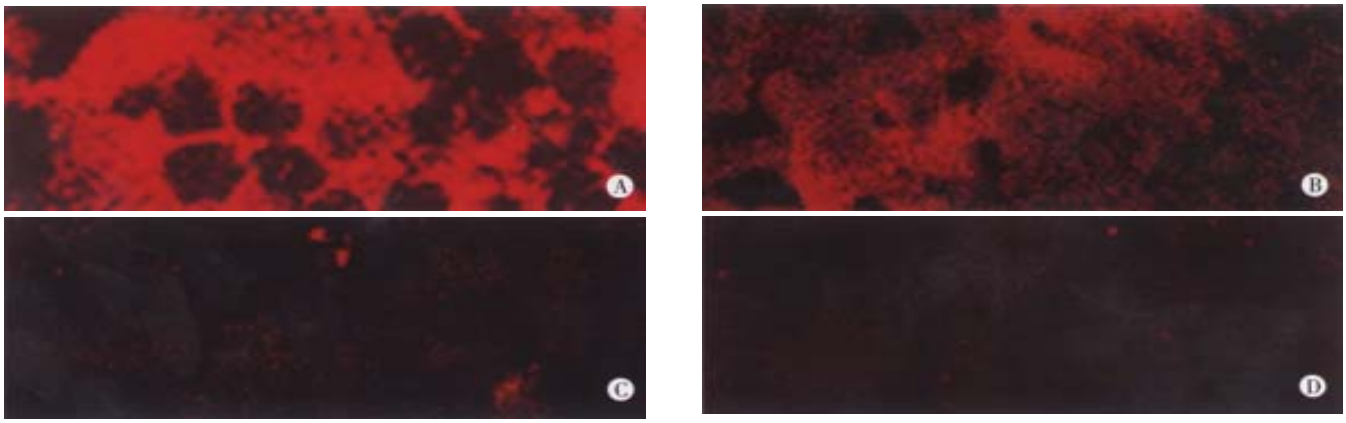

Figure 4 Confocal immunofluorescence microscopy for detection of human albumin in rat livers at wk16 post-transplantation. Panel A, a representative rat intrafetally injected with primary hepatocytes and subsequently transplanted with those same cells, stained with monoclonal goat anti-rat albumin. Panel B, a section from the same sample stained with monoclonal mouse anti-human albumin antibody. Panel C, a representative rat intrafetally injected with primary human hepatocytes, but not transplanted with hepatocytes, stained with anti-human albumin antibody. Panel D, the same sample as in Panel A stained with only second antibody. Magnification, ×250.

To estimate of the number of human hepatocytes present in rat liver transplanted with human hepatocytes, human albumin DNA sequences were detected by amplification of rat liver genomic DNA by PCR. Figure 5, lanes 3-5 show human albumin DNA extracted from 10^4, 10^3 and 10^2 Huh 7 cells, respectively. PCR produced expected 307 bp products with decreasing intensities of signals. DNA from a rat intrafetally injected with primary human hepatocytes and transplanted with those cells, lane 6 produced a band at the expected position. In contrast, a littermate intrafetally injected with human hepatocytes, but without hepatocyte transplantation, lane 7, produced no detectable human albumin DNA signal. Bands at the bottom of the gels are due to excess primers. Neonatal rats sustained intrasplenic transplantation of human hepatocytes well, with a mortality rate of about 5%.

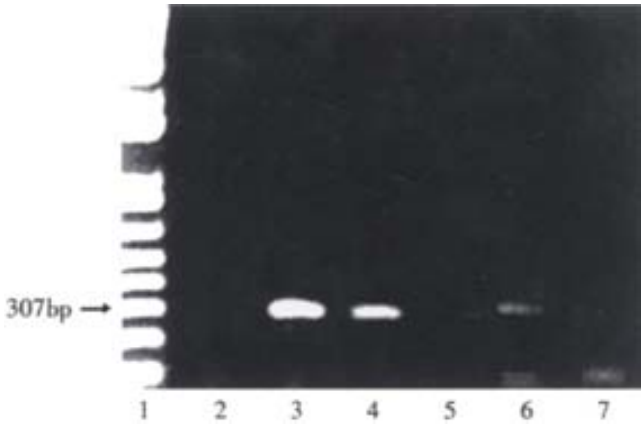

Figure 5 Detection of human albumin DNA in rat liver genomic DNA 16wk post-transplantation. From livers of animals treated as described in Figure 4, DNA was extracted, and assayed for the presence of human albumin sequences by PCR as described in Materials and Methods. Lane 1, molecular markers; lane 2, liver from untreated rats; lanes 3 to 5, 10^4, 10^3 and 10^2 Huh7 cells, respectively; lane 6, a representative rat intrafetally injected with primary human hepatocytes, and transplanted with those cells; lane 7, a rat intrafetally injected with primary hepatocytes, but not transplanted. The expected position of the amplified human albumin sequence is indicated by the arrow corresponding to 307 bp based on the DNA molecular markers in lane 1.

To quantitate the amount of human albumin gene present, dot blotting for human albumin DNA was performed. In Figure 6, the upper row of panel A, shows that liver samples from a representative rat intrafetally injected and transplanted with primary human hepatocytes had positive signals for human albumin DNA at 6 wk and 16 wk post-transplantation, with little obvious change in signal between the time points. In contrast, a littermate tolerized with human hepatocytes, but not transplanted, lower row, had no signals at the same time points indicating that the human albumin signal detected in the upper panel were not due to residual DNA from the tolerization procedure or cross reactivity with rat sequences. In panel B, the plasmid palb3, was loaded in serial dilutions as standards for human albumin DNA. Based on the amount of human albumin DNA in 10 (g rat liver DNA, the number of surviving human hepatocytes was calculated to be 2.5×10^5 cells per whole adult liver 16 wk posttransplantation. The ratio of human to rat hepatocytes that were present, 16 weeks posttransplantation, was calculated to be approximately 1 human cell per 6×10^3 rat hepatocytes.

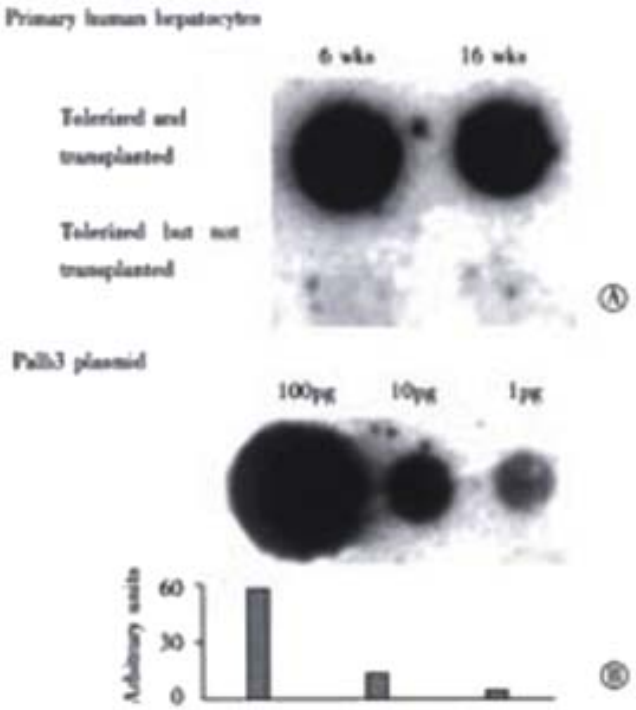

Figure 6 Quantitation of human albumin DNA in rat livers by dot blotting. Panel A, upper row, shows DNA extracted from liver samples from a intrafetally injected, and transplanted rat at wk 6 and 16 post-transplantation, respectively. Lower row shows results from a rat tolerized but without transplantation at the same time points. All dots were hybridized with a ^{32}P-labeled probe for human albumin DNA. Panel B shows a plasmid, palb-3, containing the complete human albumin gene, applied in decreasing amounts, 100 pg, 10 pg and 1 pg as standards.

To assess albumin gene transcription in rat livers, RTPCR of albumin mRNA was performed. In Figure 7, panel A, lane 3 shows that human albumin mRNA extracted from Huh7 cells was detected by RT-PCR by the presence of a product with the expected size of 315 bp. As expected, the same sample failed to generate a signal when rat albumin primers were used indicating that the human primers were specific for the detection human albumin mRNA, lane 7. In tolerized rats 16 wk after human hepatocyte transplantation, human albumin mRNA was also detected as a 315 bp band, lane 4. However, no human albumin mRNA was detected in a littermate intrafetally injected,

but without transplantation, lane 5, or from a nonfetally injected, and nontransplanted rat, lane 2. However, a band corresponding to the rat albumin amplification product of 388 bp was demonstrated in a representative rat neither intrafetally injected, nor transplanted, lane 6; a rat intrafetally injected, and transplanted, and sampled 16 weeks after transplantation, lane 8; and a rat intrafetally injected, but without transplantation, lane 9. As standards, RNA extracts from Huh7 cells were amplified with primers for human albumin, and as expected produced decreasing signals of 315 bp products, lanes 10-12.

The transcriptional activity of transplanted human hepatocytes as a function of time was measured over the entire experimental period of 16 wk. Figure 7, panel B, lane 3 shows that human albumin mRNA extracted from Huh7 cells in culture and detected by RTPCR produced a product with the expected size of 315 bp. Human albumin mRNA levels from rats were not obviously different at wk2, 6, and 16 after transplantation, lanes 4, 5 and 6, respectively, suggesting that, within the limits of the assay, the function of transplanted human hepatocytes, at least with regard to albumin production, remained unchanged for at least 16 weeks.

Figure 8 shows Western blots of serum from a representative rat tolerized and transplanted with primary human hepatocytes. A band with migration corresponding to 56 ku, the expected size of human serum albumin, as shown by standard human albumin in lane 1 was found in serum from a rat intrafetally injected and transplanted with primary human hepatocytes 1 wk posttransplantation, lane 3, and remained detectable at 2 and 3 wk posttransplantation, lanes 4 and 5, respectively. There was no cross reactivity of antibody with rat liver not transplanted with human cells as shown in lane 2. These data support the conclusion that human hepatocytes can be transplanted, and survive in the livers of intrafetally tolerized rats, and remain sufficiently active to secrete detectable amounts of human serum albumin into the circulation.

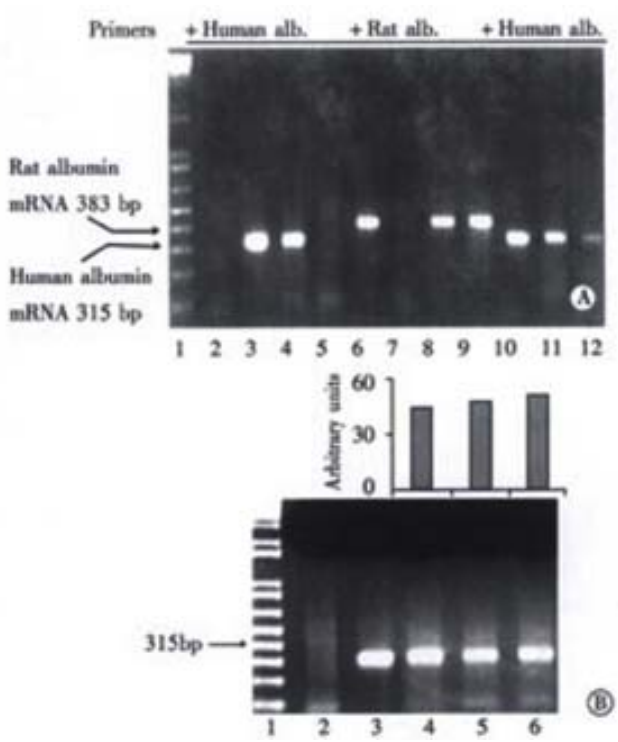

Figure 7 Detection of human albumin mRNA in rat livers by RT-PCR. Panel A, RT-PCR of RNA extracts from liver samples collected at wk16 post-transplantation. Lane 1, 1 kb plus molecular markers; lanes 2 and 6, non-intrafetally injected, non-transplanted rats; lanes 3 and 7, Huh7 cells as positive control; lanes 4 and 8, rats intrafetally injected, and transplanted with primary human hepatocytes; lanes 5 and 9, rats intrafetally injected, but not transplanted with human hepatocytes. For lanes 2 to 5, samples were amplified with primers for human albumin DNA, and for lanes 6 to 9, samples were amplified with primers for rat albumin DNA. In lanes 10 to 12, DNA from 10^4, 10^3 and 10^2 cultured Huh7 cells were amplified with primers for human albumin. The expected positions of human and rat albumin mRNA products at 315 bp and 388 bp, respectively, are indicated by arrows. Panel B, Time course of human albumin mRNA expression in the rat livers. Lane 1, 1 kb plus molecular markers; lane 2, samples from a nontolerized and non-transplanted rat; lane 3, Huh 7 cells as a positive control; lanes 4 to 6, samples from a representative rat tolerized and transplanted with primary human hepatocytes collected at wk2, 6 and 16 post-transplantation, respectively.

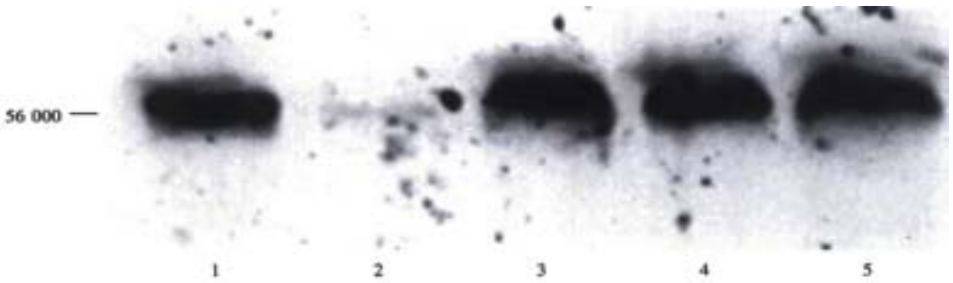

Figure 8 Detection of human albumin protein in rat serum by Western blots. Western blots of serum samples from a representative rat intrafetally injected and transplanted with primary human hepatocytes at post-transplantation wk1, lane 3; wk 2, lane 4; and wk 3, lane 5. Lane 1: standard human albumin and lane 2: standard rat albumin.

DISCUSSION

The developing immune system in the fetus allows a unique opportunity for the induction of tolerance to specific cells without generalized suppression of the immune system. *In utero* injection of donor cells directly into the peritoneal cavities of fetuses during gestation has been shown previously to result in a donor-specific tolerance[10,11,19]. With regard to the timing of cell injections for induction of tolerance, previous studies in normal mice demonstrated that *in utero* injections of splenocytes on days 14 to 16[20] were satisfactory for induction of donor-specific tolerance and prolongation of the survival of allogeneic skin grafts. In our experiments, intrafetal injections performed on any of three days, from day 15 to 17 of gestation, resulted in equal effectiveness in establishing tolerance to human hepatocytes.

Livers of tolerized rats transplanted with human hepatocytes was found to contain the human albumin gene up to 16 weeks post-transplantation, the duration of the experiment, Figures 5 and 6. The transplanted cells were functional as indicated by the presence of human albumin mRNA, Figure 7, and human albumin gene product, Figures 4 and 8. The presence of antibodies to human albumin was not detected (data not shown).

Chimeric liver models have been described previously, but in immunocompromised hosts. For example, normal adult woodchuck hepatocytes were transplanted into uPA/RAG2 knock out mice via intrasplenic injection. These cells eventually reconstituted 90% of those mouse livers[6]. Similarly, immortalized primary human hepatocytes have been transplanted into RAG-2 knock out mice[7]. Recently, primary human hepatocytes were transplanted under the kidney capsule of SCID/NOD mice[8]. The results demonstrate that when transplanted into immunocompromised hosts, human hepatocytes can not only survive, but also maintain differentiated cellular function in foreign hosts and even in unnatural locations. Our current data further indicate that general immune deficiency or suppression is not required, and intrafetal tolerization is sufficient to prevent rejection. Furthermore, the technique could be used to generate tolerance to other cell types, that might result in development of other useful animal models.

The mechanism of tolerance induction via intrauterine exposure to foreign antigen has been ascribed to T- cell clonal deletion[20]. Recently, Knolle and colleagues[21,22] described another method of inducing tolerance in the liver to foreign antigens: direct injection of foreign antigens into portal circulation resulted in the presentation of foreign antigens by liver endothelial cells to $CD8^+$ T cells. Such a model for tolerance has not been tested specifically for induction of tolerance to xenografted human hepatocyte transplantation.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Effects of aminoguanidine on nitric oxide production induced by inflammatory cytokines and endotoxin in cultured rat hepatocytes

Guo Liang Zhang, Ye Hong Wang, Hui Ling Teng and Zhi Bin Lin

Subject headings nitric-oxide synthase/antagonists & inhibitors; nitric oxide/biosynthesis; liver/cytology; cells, cultured/drug effects; endotoxins/pharmacology; immunologic and biological factors/pharmacology

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Abstract

AIM To study the effects of aminoguanidine (AG) and two L-arginine analogues N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) and N$^{\omega}$-nitro-L-arginine (L-NNA) on nitric oxide (NO) production induced by cytokines (TNF-α, IL-1β, and IFN-γ) and bacterial lipopolysaccharide (LPS) mixture (CM) in the cultured rat hepatocytes, and examine their mechanisms action.

METHODS Rat hepatocytes were incubated with AG, L-NAME, L-NNA, Actinomycin D (ActD) and dexamethasone in a medium containing CM (LPS plus TNF-α, IL-1β, and IFN-γ) for 24 h. NO production in the cultured supernatant was measured with the Griess reaction. Intracellular cGMP level was detected with radioimmunoassy.

RESULTS NO production was markedly blocked by AG and L-NAME in a dose-dependent manner under inflammatory stimuli condition triggered by CM *in vitro*. The rate of the maximum inhibitory effects of L-NAME (38.9%) was less potent than that obtained with AG (53.7%, $P<0.05$). There was no significant difference between the inhibitory effects of AG and two L-arginine analogues on intracellular cGMP accumulation in rat cultured hepatocytes. Non-specific NOS expression inhibitor dexamethasone (DEX) and iNOS mRNA transcriptional inhibitor ActD also significantly inhibited CM-induced NO production. AG (0.1 mmol·L^{-1}) and ActD (0.2 ng·L^{-1}) were equipotent in decreasing NO production induced by inflammatory stimuli *in vitro*, and both effects were more potent than that induced by non-selectivity NOS activity inhibitor L-NAME (0.1 mmol·L^{-1}) under similar stimuli conditions ($P<0.01$).

CONCLUSION AG is a potent selective inhibitor of inducible isoform of NOS, and the mechanism of action may be not only competitive inhibition in the substrate level, but also the gene expression level in rat hepatocytes.

INTRODUCTION

The pathogenesis of inflammatory sepsis and multiple autoimmune diseases (such as insulin-dependent diabetes mellitus, arthritis and viral hepatitis) is linked to the overproduction of nitric oxide (NO), a potentially toxic molecule, which is likely to be responsible in part for the cytotoxicity and mutagenicity of the inflammatory process. NO has been reported to cause DNA damage and mutations, and to induce apoptosis in several cell types for example macrophages, hepatocytes and pancreatic β-cells[1-7]. The analogues of L-arginine, N$^{\omega}$-nitro-L-arginine (L-NNA) or N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) may be useful in treating patients with the above diseases. However, the known inhibitors of the inducible enzyme can also exert their actions on the constitutive isoform NO synthase (cNOS), which resulted in an inappropriate vasodilatation or hypertension[8-10]. Thus, to inhibit selectively the inducible NOS (iNOS) may yield less side effects.

Aminoguanidine (AG) has effects on several enzyme systems, it interferes with non-enzymic glycosylation, leading to its potential use as a treatment for the complications of diabetes. Furthermore, recent work has showed that AG can also be used as a novel selective iNOS inhibitor in rat isolated vassels[11-13], although the mechanism

Department of Pharmacology, School of Basic Medical Sciences, Beijing University, Beijing 100083, China
Dr. Guo Liang Zhang, graduated from Xinxiang Medical College in 1982, got Ph.D. at Nagoya City University Medical School, Japan in 1994, finished postdoctoral research at Beijing Medical University in 1996, now an associate professor of pharmacology, specialized in hepatic pharmacology, having 15 papers published.
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Correspondence to: Dr. Guo Liang Zhang, Department of Pharmacology, School of Basic Medical Sciences, Beijing University, Beijing 100083, China
Tel. 0086-10-62091421, Fax. 0086-10-62015681
Email. yuankui@public.bta.net.cn
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underlying this is unclear. The aim of this study was to determine the effects of AG and two nonselective NOS inhibitors L-NNA and L-NAME, on the NO production and cGMP release induced by cytokines and LPS in rat cultured hepatocytes.

MATERIALS AND METHODS

Reagents

Collagenase (Type IV, 340kU·g^{-1}), bovine insulin, lipopolysaccharide (LPS, *E. coli* 0111:B4), N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), N$^{\omega}$-nitro-L-arginine (L-NNA), aminoguanidine (AG), dexamethasone were purchased from Sigma Chemical Co.; human recombinant (rh) tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ) were from Academy of Military Medical Sciences (Beijing), and Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL; Bacille Calmétte-Guérin vaccine (BCG) was obtained from the National Vaccine and Serum Institute (Beijing), [^{3}H]-radioimmunoassay kit for cyclic guanylate monophosphate (cGMP) was bought from Beijing Institute of Nuclear Research. All the reagents were diluted in medium and prepared freshly before use.

Isolation and culture of hepatocytes

Hepatocytes were harvested from male adult Wistar rats (weighing 180 g-220 g) using an *in situ* collagenase perfusion technique[14]. After inhalation anesthesia, the abdomens of the animals were opened and shaved, the portal vein was exposed and cannulated. Then the liver was perfused at 37 ℃ *in situ* first with a calcium-free phosphate-buffered saline solution (PBS). This perfusion was continued for 5 min, then it was switched to 0.5 g·L^{-1} collagenase and 10 g·L^{-1} bovine albumin in PBS buffer for 15 min. The liver was removed and the cells were combed gently in tissue culture medium. Hepatocytes were pelleted, washed, and separated from nonparenchymal cells by differential centrifugations at 50 × g. Viability of cells exceeded 90% as determined by trypan blue exclusion. Hapatocytes were plated onto 6-well plastic tissue-culture plates (1 × 10^9 cells·L^{-1} in each well). Medium in the control consisted of DMEM with L-arginine (0.5 mmol·L^{-1}), insulin(1 μmol·L^{-1}), Hepes (15 mmol·L^{-1}), L-glutamine, penicillin, streptomycin, and 100 mL·L^{-1} low-endotoxin newborn calf serum. After overnight incubation, the medium was changed with a cytokines mixture (CM) containing LPS (10 mg·L^{-1}), IL-1β (10KU·L^{-1}), TNF-α (500KU·L^{-1}) and IFN-γ (100KU·L^{-1}). Other experimental conditions included addition of NOS inhibitors (L-NAME, L-NNA or AG), actinomycin D (ActD) or dexamethasone (DEX) to the CM. After primary cultures were maintained for 24 h at 37℃ in 50 mL·L^{-1} CO_2, hepatocytes or cultured supernatants were collected for nitrite and cGMP assays[15].

Determination of NO production and cGMP levels

To determine the amount of NO produced by hepatocytes, the culture supernatants were assayed for the stable oxidative product, nitrite, by an automated procedure based on the Griess reaction, as previously described[7]. The intracellular levels of cGMP were determined using a [^{3}H]-labeled radioimmunoassay (RIA) kit, as described[14,15].

Statistical analysis

Data were expressed as $\bar{x} \pm s$. The significance of differences was determined with the Student's *t* test. Statistical significance was established at a *P* value <0.05.

RESULTS

Effect of aminoguanidine and N$^{\omega}$-nitro-L-arginine methyl ester on nitrite production

Being consistent with our previous results, the inflamatory factors induced a large amount of NO in a time- and dose-dependent manner in the primary culture of rat hepatocytes. Both L-NAME and AG inhibited LPS and cytokines-induced NO production (Table 1) in a dose-dependent fashion. On the other hand, AG completely blocked NO production stimulated by inflamatory factors in rat hepatocytes, however,the rate of the maximum inhibitory effects of L-NAME (38.9%) was less potent than that obtained with AG (53.7%, $P<0.05$).

Table 1 Inhibitory effect of aminoguanidine and L-NAME on CM stimulated *in vitro* nitrite production in primary cultured rat hepatocytes

Dose (μmol·L^{-1})	*n*	c (NO_2^-) (μmol·L^{-1})	Inhibition rate (%)	Dose (μmol·L^{-1})	*n*	c (NO_2^-) (μmol·L^{-1})	Inhibition rate (%)
Control	6	6.2±1.0		Control	6	6.5±1.3	
CM	16	12.3±4.1^b		CM	8	12.6±3.7^b	
CM+Aminoguanidine				CM+Aminoguanidine			
0.1	16	9.6±3.9	22.0	0.1	9	10.1±1.9	19.8
1	16	8.4±2.6^a	31.7	1	9	9.4±2.5	25.4
10	16	6.9±2.8^d	44.0	10	9	8.5±3.0^a	32.5
100	16	5.7±1.9^d	53.7	100	9	7.7±2.4^a	38.9
1000	16	5.5±2.1^d	55.3	1000	9	7.8±2.8^a	38.1

CM (Cytokines mixture): IL-1β 10KU·L^{-1}, TNFα 500KU·L^{-1}, and IFNγ 100KU·L^{-1} plus LPS 10 mg·L^{-1}; Cultured hepatocytes were stimulated *in vitro* with CM in the absence or presence of inhibitors (aminoguanidine or L-NAME) at various concentrations for 24 h. Amounts of nitrite in the supernatant were assayed 24h after start of stimulation *in vitro*. $\bar{x} \pm s$, *n* = 6-16 rats (3 well for each treatment in each experiment); ^bP <0.01, compared with control, ^aP<0.05, ^dP<0.01 compared with corresponding CM.

Effect of aminoguanidine, L-NNA and L-NAME on intracellular cGMP accumulation

To confirm that AG and two L-arginine analogues NOS inhibitor L-NAME and L-NNA, inhibit NO production induced by inflamatory stimuli, the effects of the three compounds on CM-induced accumulation of intracellular cGMP (a sensitive but non-specifical index of NO production) were investigated. Two L-arginine analogues L-NAME and L-NNA almost completely inhibited cytokines-stimulated intracellular cGMP accumulation from 3.0±1.5 (pmol·L^{-1}) to 1.4±0.4 (pmol·L^{-1}) with L-NAME, and to 1.3±1.1 (pmol·L^{-1}) with L-NNA (Table 2). AG on intracellular cGMP accumulation was also parallel with NO production, although the inhibitory effect of AG seemed weaker as compared with that of the two L-arginine analogues to 1.48±0.8 (pmol·L^{-1}).

Table 2 Effect of aminoguanidine, L-NAME and L-NNA on intracellular cGMP accumulation induced by CM *in vitro*

Groups	*n*	c (cGMP) (pmol·L^{-1})	Inhibition rate (%)
Control	7	1.29±0.6	
Cytokines mixture (CM)	12	3.01±1.5[a]	
CM+Aminoguanidine (0.1mmol·L^{-1})	7	1.48±0.8[c]	50.9
CM+L-NNA (0.1mmol·L^{-1})	7	1.33±1.1[c]	55.8
CM+L-NAME (0.1mmol·L^{-1})	7	1.36±0.4[c]	54.8

CM (Cytokines mixture): IL-1β 10KU·L^{-1}, TNFα 500KU·L^{-1}, and IFNγ 100KU·L^{-1} plus LPS 10 mg·L^{-1}; Cultured hepatocytes were stimulated *in vitro* with CM in the absence or presence of inhibitors (aminoguanidine, L-NAME, or L-NNA, respectively) for 24 h; Levels of intracellular cGMP were assayed 24 h after start of stimulation *in vitro* (CM or CM were co-incubated with inhibitors,respectively); $\bar{x} \pm s$, n = 7 rat or 12 rat (4 replicates for each treatment in each experiment); [a]P<0.05 compared with control, [c]P<0.05 compared with CM.

Comparison of inhibitory effects of aminoguanidine, L-NAME, DEX and ActD on nitrite production stimulated by CM in vitro

NO production was blocked by AG and L-NAME under inflammatory condition triggered by CM *in vitro*, AG (inhibition 52.2%) is a more potent inhibitor than L-NAME (35.5%, P<0.01, Table 3), the former completely blocked NO formation induced by CM *in vitro*. Similarly, both dexamethasone (DEX, 33.9%) and actinomycin D (ActD, 53.8%), inhibitors of non-specific NOS expression and iNOS mRNA transcription, also inhibited CM-induced NO production in rat hepatocytes. Furthermore, AG and ActD were equipotent in decreasing NO production triggered by inflammtory stimuli *in vitro*, and both effects more potent than that induced by L-NAME under similar stimuli condition (P<0.01).

Table 3 Effect of AG, L-NAME, DEX or ActD on rat hepatocytes NO production stimulated by CM only *in vitro*

Groups	*n*	c (NO^{2-}) (μmol·L^{-1})	Inhibition rate (%)
Control	12	6.0±2.4	
Cytokines mixture (CM)	12	12.1±4.5[b]	
CM+Dexamethasone (10 μmol·L^{-1})	12	8.1±2.3[c]	33.9
CM+L-NAME (0.1 mmol·L^{-1})	12	7.8±1.6[c]	35.5
CM+Aminoguanidine (0.1 mmol·L^{-1})	12	5.8±1.1[d]	52.2
CM+Actinomycin D (0.2 ng·L^{-1})	15	5.7±2.3[d]	53.8

CM (Cytokines mixture): IL-1β 10KU·L^{-1}, TNFα 500KU·L^{-1}, and IFNγ 100KU·L^{-1} plus LPS -10 mg·L^{-1}; cultured hepatocytes were harvested from rats that control group stimulated *in vitro* with CM in the absence or presence of inhibitors (AG, L-NAME, ActD or DEX, respectively) for 24 h; the amount of nitrite in the supernatant was assayed 24 h after start of stimulation *in vitro*. $\bar{x} \pm s$, n = 12 or 15 rats (3 wells for each treatment in each expriment); [b]P<0.01, compared with control. [c]P<0.05, [d]P<0.01, compared with CM.

DISCUSSION

Recent work showed that AG, a nucleophilic hydrazine compound, is structurally similar to L-arginine in that these compounds contain two chemically equivalent guanidino nitrogen groups and to L-arginine analogues that competitively inhibit NO synthase. The present study showed that AG completely prevents inflammatory stimuli induced formation of NO and confirmed that it is a potent inhibitor of the cytokine-inducible isoform NOS. On the other hand, administration of L-NAME failed to completely suppress the NO production induced by inflamatory stimuli in rat hepatocytes. This result was not associated with the previous observation[16,17] that AG and two L-arginine analogues, L-NNA and L-NAME, were equipotent in inhibiting the NO production induced by endotoxin only in rat aortic rings, suggesting that the role of AG may be due to incomplete inhibition of NOS, or that the development of immunological hepatic damage[18-21] may involve both NO-sensitive and NO-insensitive phases.

It has been recognized that the cNOS is Ca^{2+} dependent and produces small amounts of NO that activate soluble guanylate cyclase, resulting in the formation of cGMP, which mediates endothelium-dependent relaxation and neural transmission. NO is produced in much larger amounts by the iNOS, which is Ca^{2+} independent and appears to mediate the cytotoxic actions of macrophages on target cells, and which may implicate in the existence of other cGMP-independent mechanisms[22-25]. The present results showed that AG failed to inhibit completely the formation of cGMP by inflammatory stimuli, in contrast to NO production. Therefore, the effects of AG and two L-arginine analogs on the cGMP level were different, supporting the hypothesis that AG is a selective inhibitor for inducible isoform of NOS.

The specificity and possible mechanisms of

various L-arginine analogs as NOS inhibitors have been investigated. Recent work suggested that L-NAME and L-NNA may affect NOS activity by modifying electron transfer through iron centers, and then limits arginine transport or utilization[26,27]. However, it is unlikely that a difference in the transport of AG into the cells can explain the lack of effects of this inhibitor on cNOS since it effectively inhibits iNOS[28]. Furthermore, the difference between in affinity or in arginine binding sites for the constitutive and inducible isoforms of NOS may not be used to explain the differences between the effects of L-arginine analogs and AG, since iNOS is not only regulated in the enzyme activity but also in the gene expression level[29,30]. Moreover, in the present study the results showed that AG and transcriptional inhibitor ActD were equipotent in decreasing NO production triggered by inflammtory stimuli *in vitro*, and both effects more potent than that induced by dexamethasone, the latter have non-specifical and post-transcriptional inhibitory effect on NOS enzyme activity [31-33], suggesting that there is possibility of other mechanism in the AG inhibition, which in addition to competitive inhibition in the substrate level, also in gene transcription level. Further experiments will be required to determine whether AG directly affected the transcription of the iNOS gene or via the modulation of an intermediary protein(s) activity which indirectly influence on mRNA stability.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Alterations in metastatic properties of hepatocellular carcinoma cell following H-ras oncogene transfection

Qing Wang[1], Zhi Ying Lin[2] and Xiao Li Feng[3]

Subject headings liver neoplasms/pathology; carcinoma, hepatocellular/pathology; genes, ras; neoplasm metastasis

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Abstract

AIM To demonstrate the relationship between H-ras oncogene and hepatocellular carcinoma (HCC) metastasis.

METHODS Activated H-ras oncogene was transfected into SMMC 7721, a cell line derived from human HCC, by calcium phosphate transfection method. Some metastasis related parameters were detected in vitro, including adhesion assay, migration assay, expression of collagenase IV (c IV ase) and epidermal growth factor receptor (EGFR).

RESULTS The abilities of H-ras-transfected cell clones in adhesion to laminin (LN) or fibronectin (FN), migration, c IV ase secretion increased markedly, and the expression of EGFR elevated moderately. More importantly, these alterations were consistent positively with the expression of p21, the protein product of H-ras oncogene.

CONCLUSION H-ras oncogene could induce the metastatic phenotype of HCC cell in vitro to raise its metastatic potential.

[1]Department of Microbiology, Medical Center of Fudan University, the former Shanghai Medical University, Shanghai 200032, China
[2]Liver Cancer Institute, Zhongshan Hospital, Shanghai 200032, China
[3]Shanghai Institute of Biochemistry, Academy Sinica, Shanghai 200031, China
Qing Wang, earned master degree from Shanghai Medical University in 1996, now a senior lecturer of microbiology, specialized in the role of oncogenes on tumor metastasis, having 8 papers published.
Correspondence to: Qing Wang, Department of Microbiology, Medical Center of Fudan University, Shanghai 200032, China
Tel. 0086-21-64041900 Ext.2543
Email. zmswq@public4.sta.net.cn
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INTRODUCTION

Hepatocellular carcinoma (HCC) is common in China[1-6], and metastasis occurs early with poor prognosis[7-13]. Numerous studies on various human solid tumors have shown that H-ras oncogene is associated with tumor metastasis[14-21]. However, therelationship between H-ras and HCC metastasis remains an open question[22-25]. In the present study, we transferred activated H-ras genes into SMMC 7721, a cell line derived from human HCC, by the method of calcium phosphate transfection. The metastatic properties of ras-transfected clones were detected *in vitro*. This research was conducted to investigate the influence of H-ras oncogene on the metastatic characteristics of this liver cancer cell line from each link in the chain of tumor metastasis: adhesion-degration-migration, in order to reveal the relationship between H-ras oncogene and metastatic behavior of HCC cell.

METERIALS AND METHODS

Materials

Carrier plasmid pSV_2-neo and recombinant plasmid pSV_2-neo-*ras* (with activated H-*ras* DNA inserted at BamH I site) were gifts from Professor Luo, Director of Department of Biophysics, Fudan University. Human HCC cell line SMMC 7721 was provided by the Liver Cancer Institute, Zhongshan Hospital. Calcium phosphate transfection kit was purchased from Promega Company. DACO $p21^{ras}$ antibody, purchased from Sigma Company, could recognize specifically the 126-140 amino-acids of C-terminal. Antibodies of c IV ase and EFGR were products of Oncogene Company.

Methods

Transfer of recombinant plasmid into SMMC 7721 The method of calcium transfection, was used according to the protocol in the kit. Transfected clones were selected by G418.

Southern blotting The presence of the transfected ras oncogene in the DNA of the clones was assessed by Southern blot. Briefly, total DNA was digested with *Bam* H I, separated by electrophoresis in a 8 $g \cdot L^{-1}$ agarose gel, and transferred to nitrocellulose. The filter was then probed with H-*ras*-T_{24} DNA (6.6kb *Bam* H I fragment of plasmid pT_{24}) which had been radiolabeled with [^{32}P]dCTP.

Following hybridization, the filter was washed and X-rayed.

Immunochemistry The avidin-biotin-peroxidase complex (ABC) method was employed to detect the expression of c IV ase, EFGR and p21. The results were graded according to the percentage of positively stained cells: - less than 5%; + 5%-25%; ++ 25%-50%; +++ 50%-75%; and ++++ above 75%.

Cell adhesion assay The cell adhesion assay was performed as previously described by Busk *et al*. In brief, some wells of polystyrene 96-well flat-bottom microtiter plates were coated with increasing concentrations of laminin (LN) or fibronectin (FN), and additional wells with poly-lysine (positive control) or 10 g·L^{-1} bovin-serum albumin (BSA) (negative control) respectively. Cells were added after all the plates were blocked with 10 g·L^{-1} BSA. The plates were then incubated for 2 h at 37 ℃ in humidified CO_2. Non-adherent cells were removed and the attached cells were fixed and stained. The relative number of cells in each well was evaluated by measuring the absorbance (A) at 595 nm with a Microplate Reader. The percentage of cells attached to the experimental wells was calculated according to the formula as follows:

$$\frac{A\text{ (experimental well) - A(mean of BSA-coated wells)}}{A\text{ (mean of poly-lysine well) -A (mean of BSA-coated wells)}} \times 100\%$$

The data were expressed as $x \pm s_x$ of triplicate wells.

Cell migration assay The wound assay described by Birch *et al* was used to determine the random migration capacity of various clones. Cells were plated into the wells of 24-well plates and incubated until the cultures were subconfluent. A wound track (approximately 4 mm in size) was scored in each well. Replicate wells were terminated at 8, 16 and 24 h after wounding by fixing and staining the cell cultures with 10 g·L^{-1} crystal violet in methanol. The stained cells were then examined under an inverted microscope.

RESULTS

Identification of transfected cell clones

The four clones transfected with recombinant plasmid pSV_2-neo ras (named RC_1, RC_2, RC_3 and RC_4) and the two clones transfected with carrier plasmid pSV_2-neo (named NC_1 and NC_2), along with SMMC 7721, were tested for both Southern blot analysis and p21 expression. The presence of the transfected ras oncogene in RC_1-RC_4 was confirmed by Southern blotting, while it was absent in NC_1, NC_2 and SMMC 7721. The immunochemistry staining showed that the percentage of positive stained cells of RC_1-RC_4 was 71%, 76%, 55% and 49%, respectively. However, it was less than 5% in SMMC 7721, NC_1 and NC_2. The staining grade of these cell clones is presented in Table 1. The results showed that H-*ras* DNA had been transferred into SMMC 7721 successfully and it could express its protein product normally.

Table 1 Expression of p21, c IV ase and EGFR in different cell clones

	Staining grade						
	SMMC 7721	NC_1	NC_2	RC_1	RC_2	RC_3	RC_4
p21	-	-	-	+++	++++	+++	++
c IV ase	+	+	+	+++	++++	+++	+++
EGFR	+	+	+	+++	+++	++	++

Detection of metastasis-related parameters

Adhesive ability When ras transfected clones were assessed for their ability to bind LN or FN, it was found that there was a substantial difference in the adhesive capabilities of these variants (Figure 1). The attachment percentage of RC_1-RC_4 to LN increased by different degree as compared with SMMC 7721, the maximal was up to 69.7%, 74.4%, 38.5% and 55% respectively. Similar results were observed in adhesion assay to FN. The adhesive capabilities of NC_1 and NC_2 had no significant difference from that of SMMC 7721, suggesting that the carrier plasmid itself had no effect on cell metastatic properties.

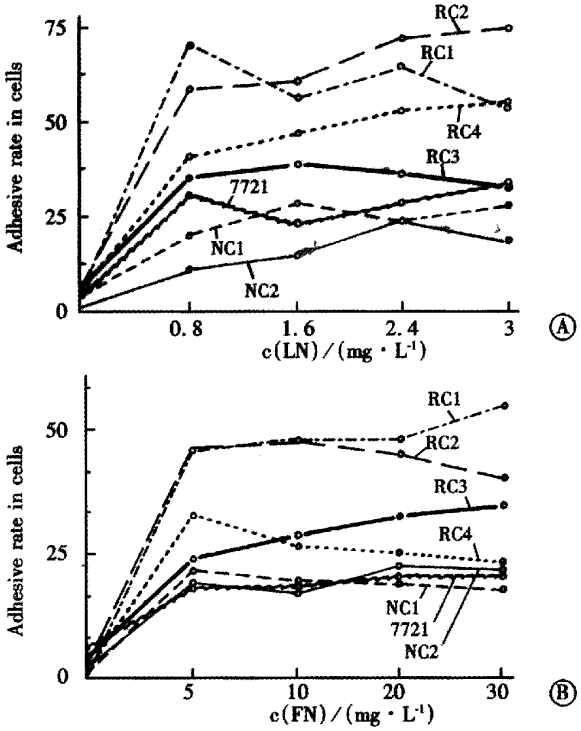

Figure 1 Attachment of different cell clones to LN or FN ($x \pm s_x$).
A: To increasing concentrations (0, 0.8, 1.6, 2.4 and 3mg·L^{-1}) of LN
B: To increasing concentrations (0, 5, 10, 20 and 30 mg·L^{-1}) of FN

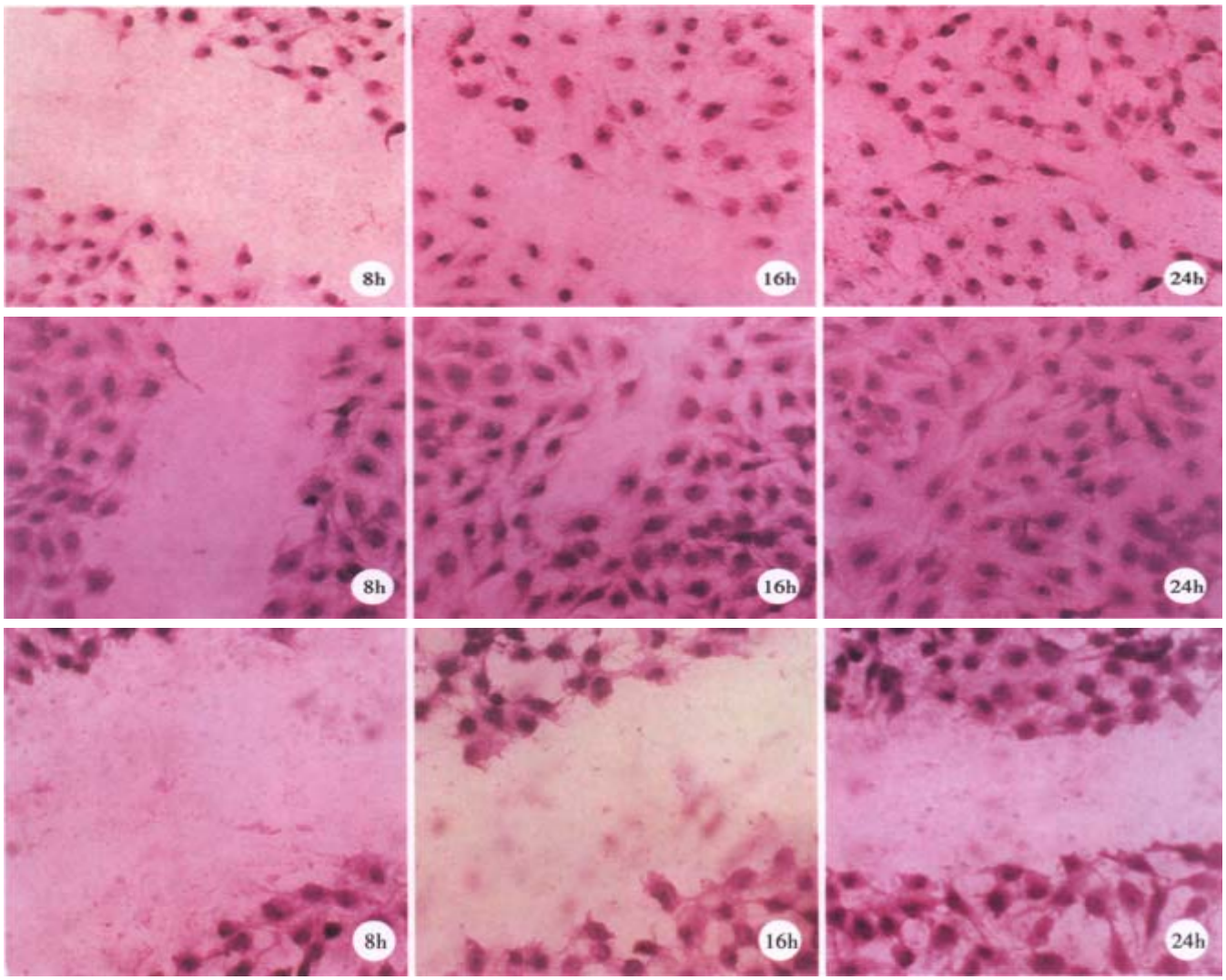

Figure 2 Migration ability of representative clones. Subconfluent monolayers of the clones were "wounded" at time 0. The cells were allowed to migration into the cell-free area for 24 h then fixed and stained with crystal violet.
A: RC_1; B: RC_2; C: SMMC 7721

Migration assay The migration of the different clonal lines was analyzed by using the "wound" system *in vitro*. Wounds of approximately4mm were made in subconfluent monolayers of the different clones and cells were allowed to migrate into the cell-free area over a 24 h period. Representative experiments using three clones are illustrated in Figure 2. The cell-free areas were filled up with cells within 24 h in the tests of RC_1-RC_4, but they still remained empty even after 24 h in the tests of SMMC 7721, NC_1 and NC_2.

Expression of c IV aes and EGFR The expressions of c IV aes and EGFR were significantly different before and after ras transfection (Table 1) and these alterations were consistent positively with the expression of p21.

DISCUSSION

The process of tumor invasion and metastasis can be divided into three steps at molecular level: adhesion, degration and migration. This consecutively complex process involves many kinds of cytokines, enzymes and cell surface receptors [26-29]. Ras gene has been implicated in these processes through the signal transduction pathway [30-42]. In malignant tumors, cell-matrix interactions are very important for tumor invasion and metastasis. LN and FN, major components of the basement membrane, are involved in several biologic activities. We investigated the adhesive abilities of H-*ras* transfected SMMC 7721 cells to LN and FN. The results showed that the adhesive abilities of different cell clones raised in different

degree. The reason that the adhesive ability of RC_3 to LN had no significant increase as against SMMC 7721, may be contributed to the heterogeneity of transfected clones. Some data have shown that the property of transfected clones is not expressed stably and that heterogeneity may develope during the growth of the clones. Experimental studies with several different tumors have suggested that the instability causing the heterogeneity of metastatic properties is due to a variety of genetic and epigenetic processes.

c IV ase is also associated with tumor metastasis[43]. It is considered that activated or overexpressed H-*ras* gene can induce the secretion and synthesis of c IV ase directly. Ura *et al* revealed that the transcription level of c IV ase gene was obviously higher in BEAS-2B cells transformed by H-*ras* gene than in their parent cells. The c IV ase secretion ability of these cells increased, and metastatic behavior *in vitro* was positively related to c IV ase secretion *in vivo*. Our results showed that the c IV ase expression level increased markedly following *ras*-transfection, the percentage of positively stained cells increased 2 to 5 times after transfection. It was indicated that oncogenic p21 ras may upregulate translational efficiency by activating the eukaryotic translation initiation factor 4E (eIF-4E), thereby enhancing the protein expression of ras-induciable genes.

EGFR is known to be interrelated with and interact on ras gene in cell signal transduction pathway[44-47]. It has some effects on tumor cell attachment, secretion of proteolytic enzymes, cytoskeleton structure and cell migration[48-52]. We found that the expression of EGFR in ras-transfected clones increased moderately, and the expressions of EGFR and c IV ase had definite relevance to p21 expression.

In summary, we have demonstrated that H-*ras* oncogene can induce the metastatic phenotype of human HCC cell *in vitro*, to raise its metastatic potential. The detections of some metastasis-related parameters, such as cell adhesion ability, migration ability, expressions of c IV ase and EGFR may have predictive value in the metastatic potential of HCC clinically.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Expression of nuclear factor-κB in hepatocellular carcinoma and its relation with the X protein of hepatitis B virus

Shuang Ping Guo[1], Wen Liang Wang[1], Yu Qiang Zhai[2] and Yi Ling Zhao[1]

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Abstract

AIM In this study we investigated the relationship of the X protein of HBV and nuclear factor-κB (NF-κB) and the expression of NF-κB in human hepatocellular carcinoma tissues.

METHODS Immunohistochemistry SP method was used to detect the expression of NF-κB and the X protein of HBV in human hepatocellular carcinoma tissues of 52 cases. Gene transfection mediated by lipofectamine was used to transfect the eukaryotic expression vector pCDNA3-1-HBX of HBV x gene into human hepatocellular carcinoma cell line HCC-9204 and NF-κB was detected.

RESULTS NF-κB was widely expressed in human hepatocellular carcinoma tissues in a total of 52 cases and its expression was related to the X protein of HBV. NF-κB was localized both in the cytoplasm and the nuclei of hepatocellular carcinoma cells in 11 cases which were positive for the X protein of HBV while in 41 cases negative for the X protein of HBV, NF-κB was only localized in the cytoplasm of hepatocellular carcinoma cells but translocated to the nuclei of hepatocellular carcinoma cells after the eukaryotic expression vector pCDNA3-1-HBX was transfected into HCC-9204 cells.

CONCLUSION This study strongly suggests that the nuclear factor NF-κB is widely expressed in hepatocellular carcinoma tissues in different styles according to the expression of the X protein of HBV. NF-κB is abnormally activated in hepatocellular carcinoma, which is probably related to the X protein of HBV. The X protein of HBV can activate NF-κB to translocate into nuclei of hepatocellular carcinoma cells.

[1]Department of Pathology, Xijing Hospital of the Fourth Military Medical University, Xi'an, China
[2]Department of Urology, the Central Hospital of Xi'an, China
Correspondence to: Shuang Ping Guo, Ph.D, Department of Pathology, the Fourth Military Medical University, Xi'an, China
Tel. 0086-29-3375497 or 3374541
Email. guoshuangping@sina.com
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INTRODUCTION

Hepatitis B virus is closely related to human hepatocellular carcinoma, but the mechanism of HBV in tumorigenesis of hepatocellular carcinoma is still unclear. HBV-associated hepatocellular carcinoma is a dominant type in China. It is reported that the X protein of HBV plays a key role in the tumorigenesis of HBV-associated hepatocellular carcinoma and it participates in many signal tranduction pathway of hepatocellular carcinoma cells[1,2].

NF-κB was extensively studied since it was first found in 1986. It plays an important role in physiologic and pathologic conditions as an inducible nuclear factor. More than 60 proinflammatory genes involved in controling. The differentiation, immuno stimulation, apoptosis, chemoattraction, cell adhension, extracellular matrix degradation have been shown to be regulated by NF-κB[3].However, concerning the relatetionship of the X protein of HBV to the activation of NF-κB and the role of NF-κB in the tumorigenesis of hepatocellular carcinoma is unclean. In this study we investigated the expression of NF-κB in hepatocellular carcinoma tissues and its relationship with the X protein of HBV.

MATERIALS AND METHODS

Material

Human hepatocellular carcinoma cell line HCC-9204 was established by the Department of Pathology, the Fourth Military Medical University. 52 cases of hepatocellular carcinoma tissues were collected from on Department. The main reagents such as rabbit NF-κB p65 antibody was purchased from Sant Cruz Company. DNA random primer labelled reagent kit and ALLN inhibitor were purchased from Bornglinman Company. The primer of HBV x gene was synthesized by Shengon Biology Company in Shanghai. G418 were purchased from Promega Company. Lipofectamin was purchased from GIBCORL Company. Other reagents were bought from Zhongshan Company, Beijing.

Method

Detection of NF-κB in hepatocellular carcinoma

Immunohistochemistry SP method was used to detect the expression of NF-κB in 52 cases of human hepatocellular carcinoma tissues. The main steps

were as follows: ① Tissues were treated with 3% H_2O_2 methanol at room temperature for 30 minutes and then incubated in 5% goat antiserum for 30 minutes at 37℃. ② Rabbit anti p65 and HBX antibody were added to adjacent tissue sections respectively and incubated overnight at 4℃. The dilution of p65 and HBX were 1: 80 and 1:50. ③ Biotin labelled goat anti-rabbit IgG was added to the sections and incubuated at 37℃ for 30 minutes. ④ SP complex was added and then DAB-H_2O_2 was used for the colour reaction. The tissues sections were washed with PBS (0.01M, pH 7.4)between each step.

Gene transfection and selection The eukaryotic expression vector pCDNA3.1-HBX of HBV x gene and the empty vector pCDNA3.1 were transfected into human hepatocellular carcinoma cell line HCC-9204 respectively by gene transfection mediated by lipofectamine. The main steps were as follows. ① HCC-9204 cells were seeded in culture plate. A liquid (100 μL 1640 serum free medium containing 2 μg plasmid) and B liquid (100 μL 1640 medium free of serum containg 10 μL lipofectamine) were mixed at room temperature for 30 minues to form DNA-lipofectamine complex. ② HCC-9204 cells were washed with 2 mL 1640 serum free medium twice. The DNA-lipofectamine complex were diluted with 0.8 mL 1640 medium and added to cells. The cells were cultured at 37℃ 5% CO_2 condition for 5 hours. ③ These the cells were cultured in 10% vitulary serum for 72 hours. (4) Cells were cultured in the selective medium containing G418 700g·L^{-1} for 1 week, and then cultured in the selective medium containing G418 500g·L^{-1}. Untransfected HCC-9204 cells were cultured in the same selective medium as control test.

Detection of HBV x mRNA in HCC-9204 cell *In situ* hybridization was used to detect HBV x mRNA in the fourth passage of HCC-9204 cells which were transfected with the eukaryotic expression vector pCDNA3.1-HBX of HBV x gene. DNA probe was labelled by using DNA random primer method. Control experiment: HCC-9204 cells which were not transfected served as negative control. Empty controls were those without adding any probes. The main steps were as follows: ① The cells were fixed in 4% paraformaldehyde for 30 minutes and were digested in 25 g·L^{-1} protease for 5 minutes at 37℃. ② The forehibridization liquids were added to the cells for 2 hours at 42℃, and then the hybridization liquids were added to the cells at 42℃ overnight (the constitutions of the hybridization liquid was 50% mathane amide, 1 x Denharts liquids, 10% sulfuric acid dextran,0.5 g·L^{-1} herring DNA, probes labled with digoxin). ③ The cells were washed with 2 × SSC, 1 × SSC, and buffer 1 sequencesly. ④ Anti-digoxin alkaline phosphatase complex (1:500) diluted with 1% goat serum -0.3% Triton X-100 was added to the cells for 2 hours at room temperature and then washed with buffer 1 and buffer 3 respectivily. ⑤ Chromogenic liquids (100 μL buffer III,4.5 μL NBT, 3.5 μL BCIP) was added to the cells in the darkness and observed under microscope. Buffer IV was used to terminate the chromogenic reaction.

Detection of the X protein of HBV in HCC-9204 cell Immunofluorimetry was used to detect the X protein of HBV in the fourth passage of HCC-9204 cells which were transfected with pCDNA3.1-HBX or the empty vector pCDNA3.1. HCC-9204 cells untransfected served as the negative control. The main steps were as conventional. The dilution of HBx antibody was 1:50.

Detection of NF-κB in HCC-9204 cells Immunohistochemistry SP method was used to detect NF-κB in cells which were transfected or not transfected by HBV x gene. The dilution of rabbit p65 NF-κB is 1:80. The detailed steps were as the above.

RESULTS

Expression of NF-κB in hepatocellular carcinoma tissues

Immunohistochemistry SP method was used to detect the expression of NF-κB in 52 cases of hepatocellular carcinoma tissues. It was found that NF-κB was widely expressed in hepatocellular carcinoma tissues. The cells which were positive for NF-κB distributed diffusely. The cells used were hepatocellular carcinoma cells and the expression of NF-κB was closely related to the expression of the X protein of HBV. In 11 cases of hepatocellular carcinoma tissues which were positive for X protein of HBV, NF-κB was localized both in the cytoplasm and the nuclei of hepatocellular carcinoma cells, while in 41 cases of hepatocellular carcinoma tissues which were negative for X protein of HBV, NF-κB was localized only in the cytoplasm of hepatocellular carcinoma cells (Figures 1,2).

Expression of NF-κB in HCC-9204 cells which were transfected with the eukaryotic expression vector pCDNA3.1-HBX of HBV x gene

Gene transfection and selection Gene transfection which was mediated by lipofectamine was used to transfect the eukaryotic expression vector pCDNA3. 1-HBX of HBV x gene and the blank vector pCDNA3.1 respectively into HCC-9204 cells. The cells were cultured in the selective medium containing G418. Most of the cells died after 2 weeks, only a few anchorage-dependent cells grew clonely. All of the control cells untransfected died 2 weeks after they were cultured in the same condition. It was suggested that gene transfection were successful primarily.

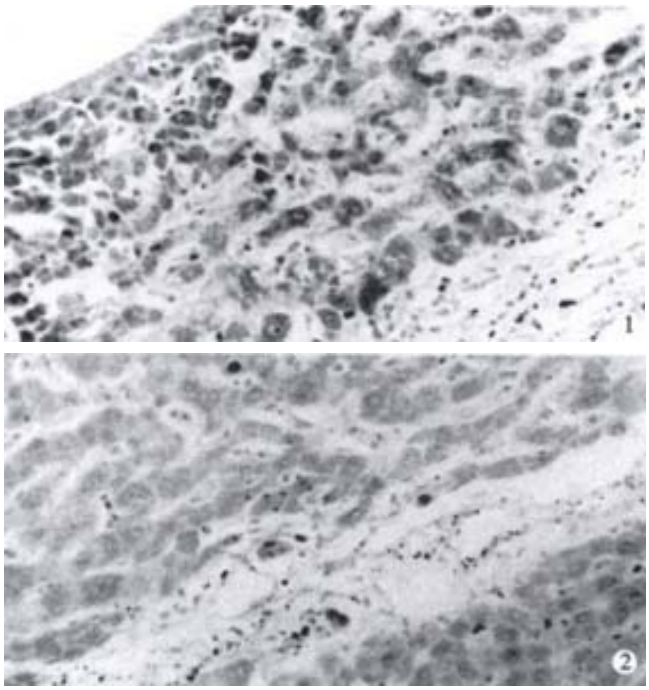

Figure 1 Expression of NF-κB in hepatocellular carcinoma which is positive of the X protein.
Figure 2 Expression of NF-κB in hepatocellular carcinoma which is negative of the X protein.

Detection of HBV x mRNA in HCC-9204 cells *In situ* hybridization was used to detect HBV x mRNA in cells which were transfected with the eukaryotic expression vector pCDNA3.1-HBX of HBV x gene. It was shown that positive siganls were loacted in the cytoplasm of HCC-9204 cells, while the nuclei were negative. There was no positive signal in both cytoplasm and nuclei of HCC-9204 cells untransfected served as the negative control (Figure 3).

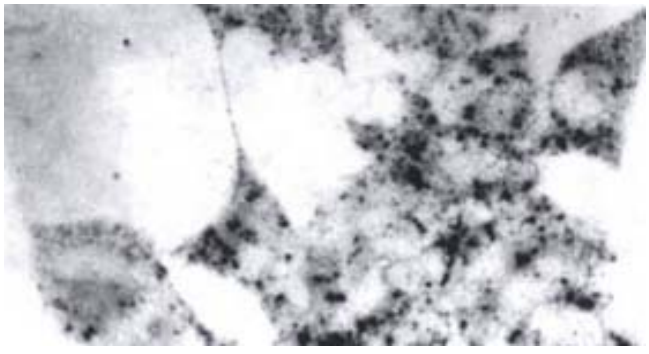

Figure 3 HBV x mRNA in HCC-9204 cells which were transfected with pCDNA3.1-HBX. (×400)

Detection of the X protein of HBV gene in HCC-9204 cells Expression of the X protein of HBV gene in the fourth passage of HCC-9204 cells which were transfected with pCDNA3.1-HBX or the blank vector pCDNA3.1 were detected by using immunofluorimetry. It was observed under fluorescent micrscope that the yellow-green fluorescence was localized in both cytoplasm and nuclei of hepatocellular carcinoma cells which were transfected with pCDNA3.1-HBX, but none in the cell not transfected (Figure 4).

Figure 4 X protein in HCC-9204 cells which were transfected with pCDNA3.1-HBX. (×400)

Expression of NF-κB in HCC-9204 cells which was transfected with eukaryotic expression vector of HBV x gene It was found NF-κB was localized in both cytoplasm and nuclei of hepatocellular carcinoma cells. NF-κB was localized only in cytoplasm of hepatocellular cells which were not transfected by the eukaryotic expression vector pCDNA3.1-HBX (Figures 5,6).

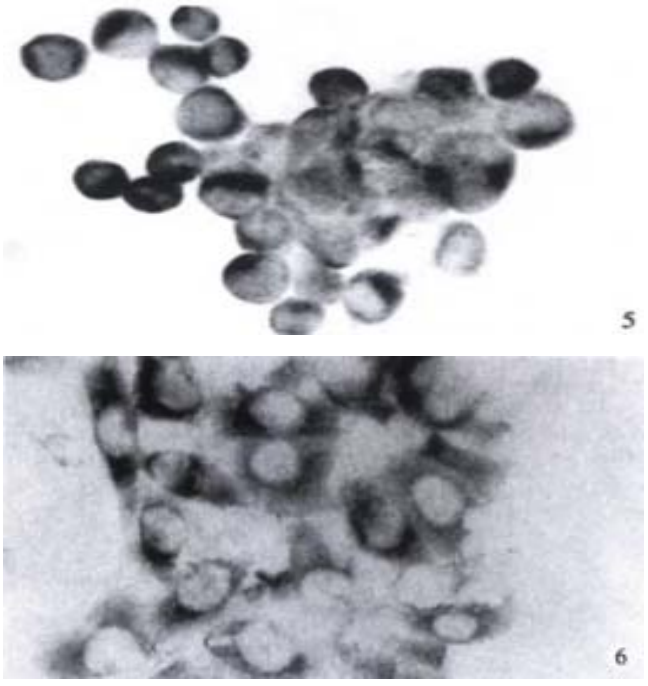

Figure 5 Expression of NF-κB in hepatocellular carcinoma cells which is positive for X protein of HBV. (×400)
Figure 6 Expression of NF-κB in hepatocellular carcinoma cells which is negative for X protein of HBV. (×400)

DISCUSSION

An estimated 300 million hepatitis B virus (HBV) carriers in the world are at an increased risk for the development of chronic active hepatitis (CAH), cirrhosis, and hepatocellular carcinoma[4]. HBV associated HCC is among the 10 most frequent cancers worldwide. At least 250 000 cases of HCC are diagnosed annually, less than 3% survive 5 years. The relative risk of HBV carrier developing HCC approaches 200:1, which is one of the highest relative risks known for human cancers[5,6]. Among the four proteins translated from the hepatitis B virus geneome, the x gene produt (the X protein) has drawn much attention for its role as a transacting factor for exploitation of the host cell machinery. Up to now, several significant discoveries had been made regarding the functions of the X protien, and from these studies, the X protein was established as essential in viral replication, HCC, and activation of certain signal transduction pathyway[7-11]. Although there is a cotroversy about the role of the X protein in viral replication, the x-gene product was shown to be required for the replicatiion of woodchuck hepatitis virus in animal studies, which were considered to reflect the *in vivo* phenomenon more precisely. Generally, it is believed that the X protein contributes to the generation of hepatocellular carcinoma. The X protein was reported to induce transformation of NIH3T3 cells. Forthermores, the development of HCC was observed in HBx transgenenic liver. The possible mechanism is based on the ability of the X protein to activate cellular signal transduction pathways. The X protein stimulates the ras /raf/mitogen-activated protein kinase cascade, leading to the activation of AP-1-dependent transcriptional activation[1,2]. In addition, c-Jun, N-terminal kinase were shown to be activated by the X protein[4,12-15]. Probably, through the combined actions of the above mechanisms, the X protein contributes to tumorigenesis[16-20]. In fact, the X protein has been shown to deregulate the cell cycle check point cotrols through the activation of ras.

Originally defined as the nuclear factor bound to the B site of the immunoglobin κ light chain gene enhancer in B lymphocytes[3], NF-κB is now known to be a family of dimeric transcription factors. NF-κB is ubiquitously expressed in non-B cells inactive form sequestered in the cytoplasm with specific inhibitory proteins termed IêBs[21-25]. NF-κB/Rel have been implicated in the inflammatory response and synthesis of adhesion molecule. When cells are stimulated by TNF-α and interleukin-1 (IL-1), I-κB proteins associated with NF-κB in the cytoplasm become phosphorylated, ubiquitinated, and degraded. Degradation of I-κB proteins frees NF-κB proteins, which then translocate into the nucleus, where they activate transcription[26-28]. Recently, it was demonstrated a role for the NF-κB/Rel family in survival of B lymphocytes. Downregulation of NF-κB/Rel activity induces apoptosis of normal and transformed murine B cells, whereas ectopic expression of c-Rel leads to survival of B lymphoma cells. Interestingly, mice lacking the p65 subunit of NF-κB displayed embryonic lethality, accompanied by massive liver apoptosis on the 15th and 16th days of gestation[29]. These findings suggest that expression of NF-κB/Rel activity in murine hepatocytes acts directly to promote survival of these cells and suggest that apoptosis observed in the hepatocytes of mice lacking relA is a direct effect of p65 deficiency[30,31]. In addition, many cells are resistant to stimuli that can induce apoptosis. The activation of the transcription factor nuclear NF-κB by tumour necrosis factor (TNF), ionizing radiation was found to protect from cell killing. Inhibition of NF-κB nuclear translocation enhanced apoptotic killing by these reagents but not by apoptotic stimuli that do not activate NF-κB[32-34]. Furthermore, NF-κB is also related to cell proliferation, transformation[35]. The abnorma activation of NF-κB was ralated to the tumorigensis of maglinant cancer, for example, in Hodgkin's lymphtoma, breast carcinoma and other cancers[36-50].

The X protein of HBV has been shown to be closely related to human hepatocellular carcinoma, but the relationship of the X protein of HBV and NF-κB and its role in the tumorigenesis of hepatocellular carcinoma, has not been elucidation. We firstly detected the expression of NF-κB in hepatocellular carcinoma tissues using immunohistochemistry SP method. It was shown that NF-κB was expressed diffusely in hepatocellular carcinoma tissues and its localization in cells was related to the expression of the X protein of HBV. In hepatocellular carcinoma tissues which was positive for X protein of HBV, the NF-κB was expressed in both cytoplasm and nuclei of hepatocellular carcinoma cells, whereas in hepatocellular carcinoma tissues which were negative for X protein of HBV, the NF-κB was expressed only in the cytoplasm of hepatocellular carcinoma cells. Gene transfection further indicated that the X protein of HBV could activate NF-κB translocate it to the nucleus.

This finding strongly indicated that there was abnormal activation of NF-κB in human hepatocellular carcinoma tissues which was probably related to the X protein of HBV. The X protein can activate NF-κB and translocate it to the nuclei from the cytoplasm of hepatocellular carcinoma cells. Gene transfection further demonstrated that the X protein of HBV can activate NF-κB. Activated NF-κB probably regulate the target gene transcription in the nuclei of hepatocellular carcinoma cells. The role of NF-κB in tumorigenesis of hepatocellular carcinoma was probably related to the cell cycle. Recently, it was reported that cyclinD1 is a target gene of NF-κB and activated NF-κB can initate the transcription of cyclinD1 and regulation of G_0/G_1 to-S phase transition. This led to loss of the normal regulation of cell cyccle and cell proliferation and

transformation.

In conclusion, NF-κB is abnormally activated in huamnn hepatocellular carcinoma, which is related to the role of the X protein of HBV, but the detailed mechanism of NF-κB in the tumorigensis of HBV-associated hepatocellular carcinoma worths further study. This study has important significance, because it sheds new light in revealing the role of the key nuclear factor NF-κB in the tumorigenesis of HBV-associated hepatocellular carcinoma from a new primt of view.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Antihepatoma effect of alpha-fetoprotein antisense phosphorothioate oligodeoxyribonucleotides *in vitro* and in mice

Xing Wang Wang[1], Jin Hui Yuan[1], Ru Gang Zhang[1], Li Xia Guo[1], Yong Xie[2] and Hong Xie[1]

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Abstract

AIM To evaluate antihepatoma effect of antisense phosphorothioate oligodeoxyribonucleotides (S-ODNs) targeted to alpha-fetoprotein (AFP) genes in vitro and in nude mice.

METHODS AFP gene expression was examined by immunocytochemical method or enzyme-linked immunosorbent assay. Effect of S-ODNs on SMMC-7721 human hepatoma cell growth in vitro was determined using microculture tetrazolium assay. *In vivo* antitumor activities of S-ODNs were monitored by measuring tumor weight differences in treated and control mice bearing SMMC-7721 xenografts. Induction of cell apoptosis was evaluated by fluorescence-activated cell sorter (FACS) analysis.

RESULTS Antisense S-ODN treatment led to reduced AFP gene expression. Specific antisense S-ODNs, but not control S-ODNs, inhibited the growth of heaptoma cells *in vitro*. *In vivo*, only antisense S-ODNs exhibited obvious antitumor activities. FACS analysis revealed that the growth inhibition by antisense S-ODNs was associated with their cell apoptosis induction.

CONCLUSION Antisense S-ODNs targeted to AFP genes inhibit the growth of human hepatoma cells and solid hepatoma, which is related to their cell apoptosis induction.

[1]Department of Biotherapy, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai 200031, China
[2]Department of Biology, Hong Kong University of Science and Technology, China
Dr. Xing Wang Wang, earned Ph.D. from Shanghai Institute of Materia Medical, Chinese Academy of Sciences in 1997. Now a professor at Shanghai Institute of Cell Biology, Chinese Academy of Sciences.
Supported by the National Postdoctoral Science Foundation of China, No.199711.
Correspondence to: Professor Hong Xie, Department of Biotherapy, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China
Tel. 0086-21-64315030 Ext.2139, Fax. 0086-21-64331090
Email. xiehong@sunm.shcnc.ac.cn
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INTRODUCTION

Primary hepatocellular carcinoma (PHCC), one of the most common malignancies in the world, is an aggressive cancer. The mean survival time from establishment of the diagnosis is only about 4 months (2 months if the diagnosis is made late). It causes approximately 250 000 deaths annually. Although much less common in western Europe, the Americas, and Australia than elsewhere, PHCC is responsible for approximately 10 000 deaths per year in the United States. A number of strategies such as surgery, radiation, chemotherapy, and biological response modifiers have been applied for the treatment of PHCC, there is still no satisfactory method that can obviously improve the overall survival rate. Most of the treatment protocols are related to significant side effects and, at best, result in an overall median survival of 7-8 months[1]. Therefore, PHCC with a high fatality rate and short disease period has been one of the major health problems in the world.

Alpha-fetoprotein (AFP) is a major serum protein produced by the liver or yolk sac in fetal life in mammals and other vertebrates, and it is hardly detectable in normal adult life. However, AFP is often elevated to a significant level in association with development of PHCC, and has been defined as an oncofetal antigen[2]. Currently, AFP has become one of the most important markers in the diagnosis of PHCC[3]. The biological roles of AFP have been widely investigated. Two functions of AFP have withstood the test of time, i.e., the carrier-transport of various ligands and the regulation of certain aspects of the immune responses. AFP binds to a large variety of ligands such as fatty acids, estrogens and phytosteroids. The nature of AFP ligand(s) can orient cells toward multiplication or differentiation. The immunoregulatory activity of AFP is another important character. AFP has been found to inhibit T- cell-dependent mitogen responses and allogenic one-way mixed lymphocyte reaction[4,5]. AFP also suppresses the natural killer cell activity and induces suppressor T cells. AFP causes selective and rapid down regulation of monocyte major histocompatibility complex class II molecules. The immunological activities of tumor necrosis factor, interleukin-1, transforming growth factor, and interferon are also decreased by the treatment with AFP[6,7]. The concept of AFP as an immuno-

regulatory agent provides an attractive explanation for the immunologic tolerance during pregnancy, since the fetus may be considered an allograft in the mother's body. Furthermore, it is also conceivable that AFP may some day be employed to treat certain autoimmune disorders such as rheumatoid arthritis[8]. The third function of AFP, only recently described, is the regulatory control of cell growth. AFP synergizes growth factors such as epidermal growth factor and insulin like growth factor-I to cause a marked increase in the proliferation of porcine granulosa cells. Placental cells undergo increased proliferation *in vitro* in response to AFP. AFP has also been found to regulate the proliferation of human mammary tumor cells[9]. AFP can inhibit apoptosis in HL-60 human leukemia cells and AFP receptors may play a role in anti-cellular senescence.

However, the relationship between AFP and PHC is still far from clear. Our recent investigations indicate that human AFP can enhance the proliferation of mouse hepatoma H-22 and human hepatoma SMMC-7721, BEL-7404 or QGY-7703 cells *in vitro*[10-12]. Similar growth stimulatory effect of low concentrations of AFP has also been obtained in human hepatoma Hep G_2 cells. These results have an important implication that AFP may function as a hepatoma growth stimulator, thus suppression of AFP gene expression and its biological activities may become a new strategy for the treatment of AFP-associated tumors such as PHCC[13]. Antisense oligodeoxynucleotides can inhibit gene expression by forming RNA-DNA duplexes, thereby preventing mRNA translation and are now commonly used to investigate their role in the treatment of human malignancies, both *ex vivo* and *in vivo*[14]. In this study, we investigated the antiproliferative action of AFP antisense phosphorothioate oligodeoxyribonucleotides (S-ODNs) on an established SMMC-7721 human hepatoma cell line *in vitro*. We also evaluated the efficacy of AFP antisense S-ODNs in the treatment of human hepatoma xenografts growing in nude mice.

MATERIALS AND METHODS

Tumor cells and tumor implants

A previously characterized human hepatoma cell line SMMC-7721 derived from a male patient with liver cancer was used for the present studies. The cell line as monolayer cultures was maintained in RPMI-1640 medium supplemented with 10% heat inactivated new born calf serum, 100 units/mL of penicillin and 100 mg/L of streptomycin, at 37℃ in a 5% carbon dioxide atmosphere in a humidified incubator[15]. The culture medium was replaced with fresh medium every two to three days. The SMMC-7721 hepatoma cells were also employed for *in vivo* studies. The solid tumor was obtained by subcutaneous injection of 2.5×10^6 viable cells into the right flank of male Balb-c nude (nu/nu) mice. Subcutaneous implantation resulted in 100% tumor obtained by days 6-7 after tumor cell injection.

Synthesis of S-ODNs

S-ODNs complementary to the translational initiation region of AFP mRNA (antisense: 5'-ACTTCATGGTTGCTA-3', 15 mer; sense: 5'-TAGCAACCATGAAGT-3', 15 mer) were used in the present study[16]. They were with phosphorothioate residues in the last two linkages at each end of the oligomers. Oligo-dT15 having the same modifications as the sense or antisense S-ODNs was used for controls. These oligomers were synthesized by β-cyanoethyl-phospho-ramidite chemistry using a model 381 A automated DNA synthesizer, as suggested by the manufacturer. Deprotection and purification were carried out according to the protocol on the user's manual (Applied Biosystems).

Analysis of AFP gene expression

AFP gene expression in cells was determined by avidin-biotin-peroxidase complex immunocyto-chemical method. Briefly, adhesive cells were fixed in 4% paraformaldehyde. Rabbit anti-human antibody (Dako) was used as the first antibody, while normal rabbit serum served as negative control. The second antibody was biotinylated sheep anti-rabbit immunoglobulins. Final incubation was in rabbit avidin-biotin-peroxidase complex. All incubations were made at 37℃. The positive intensities of AFP in cells were analyzed with a Leitz MPV-3 microspectrophotometer and expressed as absorbance at 460 nm.

AFP content in the serum of nude mice bearing SMMC-7721 xenografts was evaluated by two-site sandwich enzyme-linked immunosorbent assay. Briefly, the mice were killed by cervical dislocation the second day after the final injection of S-ODNs and the serum samples from the animals in each group were collected. AFP-containing serum was pipetted into the relevant wells of antiserum-precoated microtiter plate. The plate was incubated at 37℃ in a humid chamber for 1 h after which it was washed three times. Antibodies to AFP, labeled with horseradish peroxidase, were added to each well at a concentration of 1 mg antibody/L. After a 45-min incubation at 37℃ with the antibody conjugate and subsequent washing, 0.001% hydrogen peroxide and O-phenylenediamine 0.4 g/L were added. The color development was stopped 15 min later at 37℃ by the addition of sulfate 4 mol/L. The absorbance of each well was measured at 492 nm using an automatic microplate reader. Standards of defined concentrations were run in each assay allowing the construction of a calibration curve by plotting absorbance *versus*

concentration. The AFP concentration in the serum was then calculated from this calibration curve and expressed into μg/L.

In vitro cell growth assay of established cell line

SMMC-7721 cells were seeded in the wells of 96-well culture plates. After 24 h, AFP antisense S-ODNs were added. Equimolar amounts of AFP sense and Oligo-dT 15 were used in control experiments. In this procedure, the culture medium was removed and the S-ODNs added directly to the cells for 30-60 seconds to facilitate uptake, whereafter the culture medium was added. Cell growth was determined using the microculture tetrazolium assay. Briefly, after culture, the cells were incubated with 800 mg/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Fluka, Switzerland), which is used to assay the activity of mitochondrial dehydrogenases. Four hours later, 10% sodium dodecyl sulphate-5% isobutanol-0.12% hydrochloric acid solution was added to solubilize the formazan product. The plate was then incubated at 37℃ for another 12 h. The absorbance at 570 nm was measured with a model EL × 800 enzyme-linked immunosorbent assay automated microplate reader (Bio-Tek).

In vivo administration of S-ODNs

Blab/c male nude (nu/nu) mice, 6-8 weeks in age and 22 g-24 g in body weight, were purchased from the Tumor Biology Laboratories, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. Mice were housed under pathogen-free conditions and given acidified, autoclaved water and a γ-irradiated commercial diet ad libitum. All manipulations were performed under sterile conditions in a laminar air flow hood. All procedures involving animals and their care were in accord with institutional ethical guidelines in compliance with national and international laws and policies. Each experimental group of mice included six animals. S-ODNs were dissolved in 50 μL sterile distilled water and administered 8 days after tumor cell implantation by injecting them directly into the tumor every 24 h for 8 consecutive days. Tumor weights were calculated from caliper measurements according to the previous method with the formula: tumor weight (mg) = length (mm) × width 2 (mm)/2. Antitumor activity of S-ODNs was assessed by using percent tumor weight inhibition, calculated by dividing the mean tumor weight of the treated group by the mean tumor weight of the control group, subtracting the resulting value from 1, and multiplying that value by 100. Toxicity was evaluated based on the apparent drug-related deaths and net body weight loss. Death in a treated mouse was presumed to be treatment-related if the mouse died within 7 days after the last treatment. Net body weight loss was calculated as a percentage of the mean net body weight of untreated mice.

Detection of cell apoptosis

Cell suspensions from *in vitro* cultures and tumor xenografts were used for fluoroescence-activated cell sorter (FACS) analysis. Cell sample preparation and propidium iodide staining for FACS analysis were performed according to the method reported previously. Briefly, the cells were fixed with 80% ethanol at 4℃ for 60 min. The ethanol-treated cells were washed twice with phosphate-buffered saline, pelleted by centrifugation and resuspended in a solution containing 50 mg/L propidium iodide and 100 mg/L RNase A. The percentage of apoptotic cells was determined by the fluorescence of individual cells measured by FACS flow cytometry (Becton Dickinson, San Jose, CA).

Statistical analyses

The experimental results summarized in Table 1 and the apoptosis data in Table 3 were analyzed by Student's *t* test for statistical significance. The statistical significance of AFP protein expression differences in Table 3 was evaluated using one-way analysis of variance. The results in Table 4 were analyzed by Mann-Whitney U-test for statistical significance. Differences were considered significant at P value <0.05.

Table 1 Specific inhibition of SMMC-7721 human hepatoma cell growth by alpha-fetoprotein (AFP) antisense phosphorothioate oligodeoxyribonucleotides (S-ODNs) *in vitro*

Concentration (μmol/L)	Hepatoma cell growth (absorbance at 570 nm)		
	Sense	Antisense	Oligo dT15
0	0.83±0.10	0.83±0.10	0.83±0.10
6.3	0.84±0.08	0.50±0.08 (39.8)[a]	0.82±0.11
12.5	0.76±0.06	0.44±0.04 (46.9)[a]	0.87±0.07
25.0	0.72±0.13	0.42±0.03 (49.4)[a]	0.88±0.18

SMMC-7721 cells were incubated with various concentrations of S-ODNs for 48 h *in vitro*. A specific growth inhibition was observed by the treatment with antisense S-ODNs. Values in parentheses: percent inhibition. Each value represents mean ± SD of 3 separate experiments.[a]P<0.05 (Student's *t* test) compared with 0 μmol/L.

Table 2 Reversal of SMMC-7721 human hepatoma cell growth inhibition of AFP antisense S-ODNs by hybridization competition *in vitro*

Antisense (μmol/L)	Sense (μmol/L)	Hepatoma cell growth (absorbance at 570nm)	Suppression (%)
0	0	1.00±0.08	
0	10	0.95±0.07	5
10	0	0.46±0.02	54
10	10	0.51±0.05	49
10	100	0.95±0.05	5

SMMC-7721 cells were incubated with combined AFP sense and antisense S-ODNs for 48 h *in vitro*. The cell growth suppression induced by antisense S-ODNs was reversed by hybridization competition of sense S-ODNs. Each value represents mean ± SD of 3 separate experiments.

Table 3 Inhibition of AFP protein expression and apoptosis induction in SMMC-7721 human hepatoma cells by AFP antisense S-ODNs *in vitro*

Concentration (μmol/L)	AFP protein expression (absorbance at 460 nm)			Apoptosis (%)
	Antisense	Sense	Oligo-dT^{15}	
0	0.32±0.04	0.32±0.04	0.32±0.04	7.3±5.2
6.3	0.22±0.03 (31.5)[a]	ND	ND	ND
12.5	0.19±0.05 (40.6)[a]	ND	ND	26.7±7.8[a]
25.0	0.15±0.02 (53.1)[a]	0.31±0.06	0.31±0.02	47.9±7.5[a]

Cells were incubated with different S-ODNs for 48 h *in vitro*. AFP gene expression was determined by using the avidin-biotin-peroxidase complex immunocytochemical assay. Antisense S-ODNs specifically blocked AFP gene expression in the cells. The cells were also analyzed for apoptotic cell population with a fluorescence-activated cell sorter. AFP antisense S-ODNs induced apoptosis of the hepatoma cells. Values in parentheses: percent inhibition. Each value represents mean ± SD of 2 separate experiments. [a]$P<0.05$ (One-way analysis of variance for protein expression and Student's *t* test for apoptosis) compared with 0 μmol/L. ND: not done.

Table 4 Therapeutic efficacy of AFP S-ODNs in SMMC-7721 hepatoma bearing nude mice

Group (mg/day × day)	Mean tumor weight (mg)								Toxic deaths	AFP content (% inhibition)
	0*	2	4	6	8	10	12	14		
Control	96.7±	191.4±	336.9±	504.8±	636.3±	823.9±	1056.7±	1201.6±	0/6	—
(0)	51.1	48.3	67. 8	80.2	68.8	187.6	182.0	208.0		
Antisense	83.1±	145.2±	215.8±	286.5±	356.7±	520.0±	653.1±	776.6±	0/6	68.9[a]
(0.25 × 8)	33.7	43.8	49.6	49.2	52.5	56.1	128.8	61.3		
		(24.2)	(35.9)[a]	(43.2)[a]	(43.9)[a]	(36.9)[a]	(38.2)[a]	(35.4)[a]		
Antisense	84.8±	130.3±	202.3±	254.2±	310.8±	449.9±	593.9±	737.3±	0/6	73.8[a]
(0.50 × 8)	34.6	36.9	47.9	43.2	56.9	108.3	72.3	146.7		
		(31.9)[a]	(39.9)[a]	(49.6)[a]	(51.2)[a]	(45.4)[a]	(43.8)[a]	(38.6)[a]		
Sense	100.5±	201.4±	339.1±	510.4±	629.6±	829.5±	1034.9±	1191.9±	0/6	3.4
(0.25 × 8)	33.5	44.2	71.1	114.1	109.4	164.5	198.1	181.3		
Sense	86.9±	187.7±	359.7±	509.5±	659.7±	837.7±	1011.0±	1232.3±	0/6	0
(0.50 × 8)	25.6	42.3	59.6	111.8	101.7	147.3	142.9	149.0		

Mice were treated the eighth day after tumor cell implantation with S-ODNs following different schedules. Untreated mice received normal saline only. *Time after S-ODN administration (day). Values in parentheses: percent inhibition. Each value represents mean ± SD of 6 samples. [a]$P<0.05$ (Mann-Whitney *U* test) compared with untreated mice and mice treated with sense S-ODNs.

RESULTS

In vitro studies

As shown in Table 1, when SMMC-7721 cells were exposed to AFP antisense S-ODNs for 48 h, the growth of the cells was significantly suppressed as compared with untreated cells. Moreover, the antisense oligomers showed concentration-dependent effect although not following a linear relationship. However, the inhibitory effect of the sense S-ODNs was much weaker than that of the antisense S-ODNs. No obvious inhibitory influence of Oligo-dT15 on SMMC-7721 cell growth was observed. The growth of unhepatoma HL-60 leukemia cells was not affected by the three above-mentioned oligomers (data not shown). It was suggested that the antiproliferative effect of the antisense S-ODNs may be due to a hybridization-based mechanism. In order to confirm it, a 10-fold excess of complementary sense S-ODNs was added to the culture system for 48 h. The obvious reversal of the SMMC-7721 cell growth inhibition of 10 μmol/L antisense S-ODNs was obtained by the hybridization competition. The addition of equal amounts of antisense and sense S-ODNs produced intermediate degrees of growth suppression (Table 2). The unmodified phosphodiester oligomers were not testable *in vitro* because of their poor stability in culture medium.

We examined AFP gene expression in SMMC-7721 cells by immunocytochemical method. The positive grains were found to diffuse throughout the cytoplasm of SMMC-7721 cells. Reaction products were visible in almost all the cells although in various amounts. In contrast, we could not detect positive grains when rabbit anti-human AFP primary antibody was replaced by normal rabbit serum. It is indicated that SMMC-7721 cells could express AFP protein. Similar results were obtained by using BEL-7404 human hepatoma cell line [17,18]. Treatment of SMMC-7721 cells with AFP antisense S-ODNs for 48 h markedly downregulated AFP protein expression. Downregulation of AFP protein expression by the antisense oligomers was specific, since the sense S-ODNs and Oligo-dT15 having the

same modification did not downregulate AFP protein expression (Table 3). The results obtained from HuH-7 human hepatoma cell line were similar. These data argue strongly that the growth inhibition of AFP antisense oligomers is likely caused by the sequence-specific blocking of AFP protein expression in SMMC-7721 cells. To determine whether the decrease in AFP protein expression was due to a downregulation of AFP gene expression induced by antisense S-ODNs and not merely related to the apoptotic status of the cell population, AFP protein expression was analyzed 6 h after S-ODNs treatment The results indicated that treatment with 25 μmol/L antisense S-ODNs resulted in an obvious inhibition of AFP protein expression, whereas no evidence of an increase in apoptosis was observed at this time point. The same concentrations of sense S-ODNs and oligo dT-15 had no effect on the AFP protein expression and hepatoma cell apoptosis (data not shown).

After exposure of exponentially growing SMMC-7721 cells to AFP antisense S-ODNs for 48 h, cells showed a hypodiploid DNA content indicative of apoptosis as determined by FACS analysis (Table 3). The presense of apoptosis was also confirmed by morphologic observation. It is concluded that SMMC-7721 cells are rapidly induced to undergo apoptosis by AFP antisense S-ODNs.

In vivo studies

Nude mice bearing SMMC-7721 tumors were treated with 0.25 and 0.5 mgS-ODNs per day for 8 consecutive days. As shown in Table 4, a marked decrease in tumor weight was observed after antisense S-ODNs treatment. Moreover, treatment with 0.25 mg per day proved less effective than treatment with 0.5 mg per day. No obvious toxicity was observed in mice receiving either 0.25 or 0.5 mg per day of antisense S-ODNs for 8 days.

In order to verify whether the antitumor effect observed *in vivo* was due to a specific antisense effect, AFP protein content in the serum of untreated mice and mice treated with S-ODNs was measured by using avidin-biotin-peroxidase complex method. The results indicated that treatment with AFP antisense S-ODNs decreased the AFP protein in the serum (Table 4). FACS analysis of the DNA content histograms of hepatoma cells from tumors of untreated mice revealed an accumulation in the hypodiploid region representative of about 40% of the cell population, indicating apoptotic cell death This cell death was expected, since the FACS analysis was performed when the tumors were at a late stage of growth (day 16 of growth). Similar levels of apoptosis were found in tumor cells from sense S-ODN-treated mice. Treatment with AFP antisense S-ODNs produced a marked increase of the percentage of apoptotic tumor cells to about 80% (Figure 1).

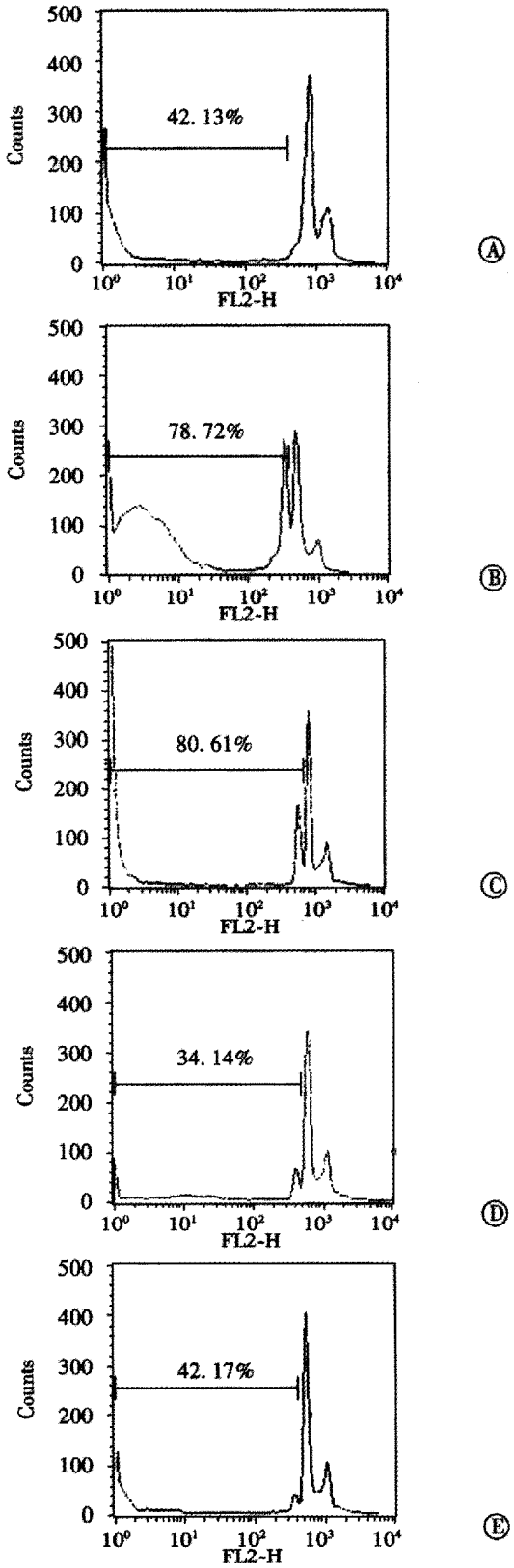

Figure 1 Effect of AFP antisense [S]ODNs on the *in vivo* induction of apoptosis. Representative fluorescence-activated cell sorter analysis of DNA content (propidium iodide fluorescence) in untreated (A), 2 mg antisense (B), 4 mg antisense (C), 2 mg sense (D) and 4 mg sense [S]ODN (E)-treated SMMC-7721 hepatoma cells. Apoptosis analysis of the cells was done by using tumor xenografts of nude mice treated with [S]ODNs for 8 days. Percentages indicate the proportion of cell population with a subdiploid DNA content.

DISCUSSION

Since AFP was found in the mid 1950s,this oncofetal protein has demonstrated clinical utility as a PHCC marker. But its biological activities in mammals have still remained an enigma. To date, only two functional roles of AFP (i.e., ligand / carrier transport and modulation of immune responses) have been ascertained. In the last decade, the growth regulatory properties of AFP have aroused interests as a result of studies involving ontogenetic and oncogenic growth in both cell cultures and animal models. Particularly, the effect of AFP administration on the development of experimental tumors in mammals has been investigated *in vivo* with the use of carcinogens. The influence of murine AFP on the threshold dose, mean tumor size, regression time, and number of progress or for moloney sarcoma virus-induced tumors in mice was studied. AFP-treated mice developed larger tumors, required a longer period for regression and had a significantly higher mortality. Furthermore, AFP, but not murine albumin or transferrin,allowed the growth of tumors when normally subthreshold doses of virus were injected. The effect of AFP could be abrogated by pretreatment with an anti-AFP antibody. AFP, but neither albumin nor transferrin, also accelerated the appearance of plasmacytomas. Chicken-AFP-treated quails developed tumors with shorter latent periods than those of the tumors that developed in untreated quails after inoculation with Rous sarcoma virus[19]. The *in vivo* tumor growth stimulation by AFP can be explained by its immunosuppression. Cell-mediated immunity is an important and central mechanism of host resistance to malignant neoplasms. Recently, we found that antihepatoma effects of AFP antisense S-ODNs were more significant in normal mice than in nude mice. Moreover, thymus weight of the normal mice was obviously increased after AFP antisense S-ODNs treatment (data not shown). It is suggested that there exists a relationship between AFP and the susceptibility of hepatoma cells to immunity-mediated cytotoxicity. If the effective immune system does not exist, however, can AFP still enhance the growth of tumor cells It was found that AFP stimulated, in a dose-dependent manner, human mammary tumor cell growth induced by platelet-derived growth factor *in vitro*; ablation of endogenous AFP by anti-AFP monoclonal antibody affinity chromatograph significantly reduced the proliferative activity of human mammary tumor cells. Recent studies indicated that AFP did directly stimulate the proliferation of hepatoma cells *in vitro*, independent of its immunosuppression. In the authors'view, AFP should be considered as a direct or indirect factor associated with tumor growth.

The precise relationship between AFP and PHC is not known. AFP was highly expressed in populations of hepatic oval cells during the early stages of carcinogenesis. These cells did not show the histological patterns that were diagnostic of trabecular hepatocellular carcinoma[20,21]. AFP-expressing hepatic oval cells in hepatocellular foci and nodules proliferating after carcinogenic treatment seem to be precursor cells of the hepatic carcinoma. On the other hand, high levels of AFP in the fully developed PHCC, or in the serum of the host, are associated with more aggressive behavior, and increased anaplasia[22]. The present study also demonstrated that AFP antisense S-ODNs are effective inhibitors of hepatoma cell growth *in vitro* and *in vivo*. Based on all the data, it is strongly inferred that AFP may play a role in the generation and development of PHCC.

The present results indicated that AFP antisense S-ODNs exhibited significant antihepatoma activities *in vitro* and *in vivo* by the sequence-specific blocking of AFP gene expression. AFP sense oligomers and Oligo-dT15 as controls had no or at least much weaker effects. Likewise, AFP antisense S-ODNs could also inhibit BEL-7404 human hepatoma cell proliferation *in vitro*[23,24]. We thus propose the possibility of suppressing human hepatoma proliferation by specifically decreasing AFP gene expression with antisense oligomers. The recent use of antisense oligomers as a therapeutic tool represents a newly established strategy for treating diseases[25]. Therefore, the successful designing of antisense S-ODNs for blocking the AFP gene expression in human hepatoma cells may not only further confirm the growth stimulatory effect of AFP, but also become an important therapeutic approach for PHCC[26]. However, the antitumor effects of AFP antisense S-ODNs were obtained at relatively high concentrations that might be difficult to achieve clinically for a number of reasons, among which are high cost and difficulties in the large-scale production of S-ODNs. Hopefully, the development of new generation ODN analogues or a more efficient delivery of S-ODNs, perhaps using liposomes as vehicles, will lead to therapeutic applications in humans. Thus this antisense therapy awaits further investigations[27,28]. And, antihepatoma therapeutic strategies relying on the use of AFP antisense S-ODNs may be enhanced by using these compounds in combination with other antitumor agents[24].

It is well-known that activation of an endonuclease in apoptotic cells results in extraction of the low molecular weight DNA following cell permeabilization, which, in turn, leads to the decreased stainability with DNA-specific fluorochromes. Thus, measurements of DNA content by FACS make it possible to identify

apoptotic cells. Using the FACS method, we have observed that AFP antisense S-ODNs induced apoptosis of SMMC-7721 hepatoma cells. Moreover, the presence of apoptotic cells has also been confirmed by morphologic observation. Therefore, although the mechanisms of antihepatoma action of AFP antisense S-ODNs are not fully understood, the induction of hepatoma cell apoptosis may be responsible for their growth-inhibitory effect.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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APC and K-ras gene mutation in aberrant crypt foci of human colon

Ping Yuan[1], Meng Hong Sun[2], Jin Sheng Zhang[1], Xiong Zeng Zhu[2] and Da Ren Shi[2]

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Abstract

AIM To study the genetic alteration in ACF and to define the possibility that ACF may be a very early morphological lesion with molecular changes, and to explore the relationship between ACF and colorectal adenoma even carcinoma.

METHODS DNA from 35 CRC, 15 adenomas, 34 ACF and 10 normal mucus was isolated by means of microdissection. Direct gene sequencing of K-ras gene including codon 12, 13 and 61 as well as the mutation cluster region (MCR) of APC gene was performed.

RESULTS K-ras gene mutation frequency in ACF, adenoma and carcinoma was 17.6% (6/34), 13.3% (2/15), and 14.3% (5/35) respectively, showing no difference ($P>0.05$) in K-ras gene mutation among three pathologic procedures. The K-ras gene mutation in adenoma, carcinoma and 4 ACF restricted in codon 12 (GGT→GAT), but the other 2 mutations from ACF located in codon 13 (GGC→GAC). K-ras gene mutation was found more frequently in older patients and patients with polypoid cancer. No mutation in codon 61 was found in the three tissue types. Mutation rate of APC gene in adenoma and carcinoma was 22.9% (8/35) and 26.7% (4/15), which was higher than ACF (2.9%) ($P<0.05$). APC gene mutation in carcinoma was not correlated with age of patients, location, size and differentiation of tumor.

CONCLUSION ACF might be a very early morphological lesion in the tumorogenesis of colorectal tumor. The morphological feature and gene mutation status was different in ACF and adenoma. ACF is possibly putative "microadenoma" that might be the precursor of adenoma. In addition, the development of a subgroup of colorectal carcinomas might undergo a way of "normal epithelium→ACF→carcinomas".

INTRODUCTION

Colorectal cancer (CRC) is a complex pathological procedure in which multiple genes are involved during multiple steps. It is widely accepted that the order of "normal epithelium→hyperplastic epithelium→adenoma→cancer→cancer with metastasis" exists in majority of coloretal carcinoma[1,2]. The corresponding molecular order is demonstrated as follows: APC→altered methylation[3]→K-ras→MCC/DCC→P53. The activation of K-ras gene and inactivation of APC gene are frequent early events in the carcinogenesis of colorectal carcinoma[4-8]. Recent studies showed that aberrant crypt foci (ACF) is the earliest morphological lesion detectable in colorectal epithelium[9,10]. We studied CRC, adenoma and ACF with the aim to understand the relationship between ACF and colorectal neoplasm.

MATERIALS AND METHODS

General data of patients

Thirty-five CRC were obtained from an unselected cohort in which the patients underwent initial curative resection in 1999 in the Cancer Hospital of Fudan University. Twenty CRC occurred in men and 15 in women. The age of the patients ranged from 28 to 85 years. The median age was 54.2. Fourteen of 35 patients had their tumor in proximal colon, and 21 in the distal part of large intestine (including rectum). Macroscopically, 22 were ulcerative type with more endophytic extension and 11 showed exophytic growth (including rape flower like polypoid and nodal). Histologically, 6 were highly, 22 were moderately and 7 were poorly differentiated. Fifteen adenomas during the same time period were collected from 9 men and 6 women. In histological type, 5 were tubular, 6 tubular villous, and 4 villous adenoma. The macroscopically normal epithelium 5 cm apart from

[1]Department of Pathology, Medical College of Fudan University,
[2]Department of Pathology, Cancer Hospital/Cancer Institute, Fudan University, Shanghai 200032, China
Dr. Ping Yuan, Studying Province, studying in Medical College of Fudan University, worked in Department of Pathology, Wannan Medical College, having eighteen papers published.
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Correspondence to: Dr. Meng Hong Sun, Department of Pathology, Cancer Hospital/Cancer Institute, Fudan University, Shanghai 200032, China
Tel. 0086-21-64175590 ext.3357, Fax. 0086-21-64174774
Email. menghongs@hotmail.com; pingyuan61@hotmail.com.
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the tumor site served as ACF study and that 10 cm at least apart from the primary tumor as normal control.

Identification of ACF and histological microdissection

Cryostat frozen sections (5 μm, stained with 0.1% methylene blue for 5 min) of epithelium 5 cm apart from the primary tumor were used for identifying ACF according to Shpitz and Gregorio *et al*[11-15]. ACF was determined by two qualified pathologists when the following features appeared: large crypts, tight arranging, dark and overlapping nuclei, dysplastic morphology, "saw tooth like" and elongated luminal surface, follicular distribution and no inflammation cells or lymph follicle (Figure 1). Ten slides of 8 μm from 10 normal epithelia, 34 identified ACF and 35 CRC were stained with methylene blue and subsequently microdissected under the dissect microscope (40 folds). Individual crypts from CRC, adenoma, ACF and normal epithelium were isolated with scalpels and transferred to the centrifuge tube for DNA extraction. One crypt or about 100 cells from each case were used for the study[16] (Figure 1).

DNA extraction of minimal amount tissue

Microdissected tissue samples were digested in 50 μL cell lysis buffer (0.5 M Tris-Cl, pH 8.9, 20mM EDTA, 10mM NaCl, 1% SDS), digested with proteinase K (500 mg/L) overnight at 37℃. Genomic DNA was purified using DNA extract kit (DX Biotech Co. Ltd., Shanghai). The precipitation was suspended in TE for using[17,18].

Primer design and PCR amplification

Computer PC gene analysis software was used and according to the study by Losi[19-23]. The primers for K-ras gene encompassing exon 1, 2, the primers for APC gene encompassing the mutation cluster region in exon 15 (codon 1263-1596) were designed. PCR products were checked in agarose gel for size confirmation. All primers and PCR protocols are listed in Table 1.

Table 1 Primers for K-ras and APC gene sequencing

Genes	Region	Codon	Size of PCR	AT	Sequence
K-ras	exon1	1-54	163	56℃	5-GACTGAATATAAACTTGTGG
					5-CTGTATCAAAGAAGTGTCCT
K-ras	exon2	31-84	161BP	54℃	5-GACTGTGTTTCTCCCTTCT
					5-GGCAAATACACAAAGAAAG
APC	15-A	1263-1393	390BP	54℃	5-GTGTAGAAGATACTCCAATA
					5-GTGAACTGACAGAAGTACAT
APC	15-B	1338-1436	295BP	56℃	5-CAGGGTTCTAGTTTATCTTC
					5-TTCTGCTTGGTGGCATGGTT
APC	15-C	1412-1515	310BP	56℃	5-GGAATGGTAAGTGGCATAAT
					5-AAATGGCTCATCGAGGCTCA
APC	15-D	1496-1596	300BP	56℃	5-ACTCCAGATGGATTTTCTTG
					5-GGCTGGCTTTTTTGCTTTAC

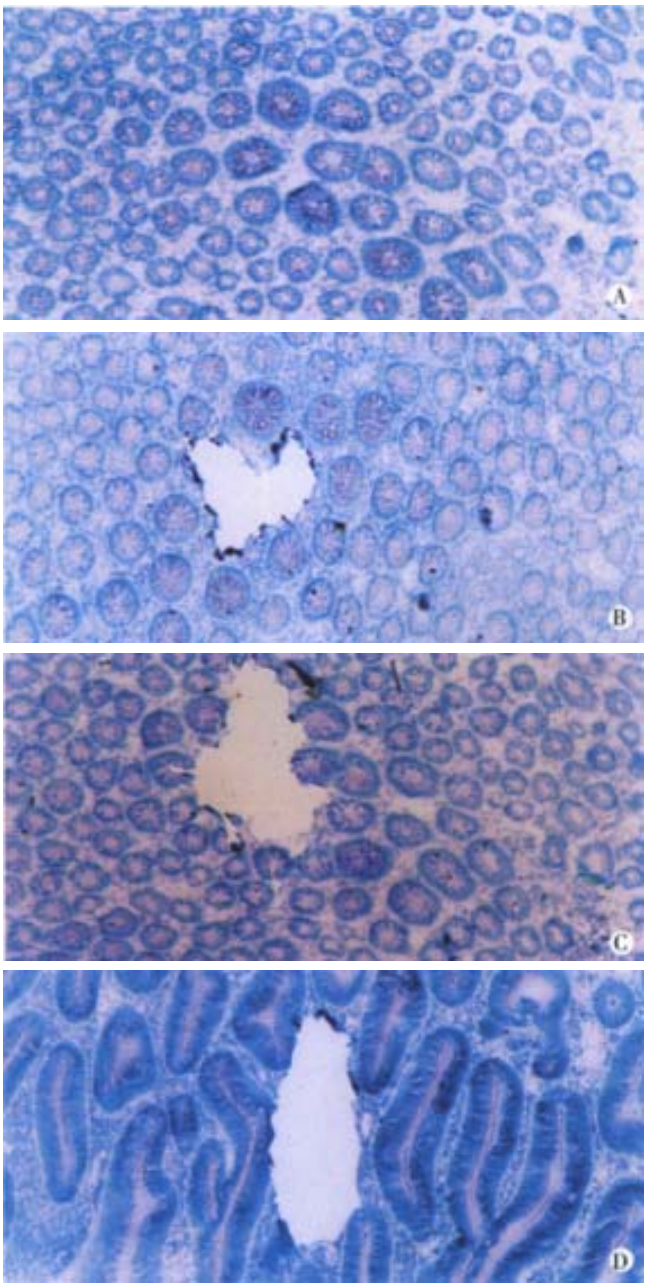

Figure 1 A. morphology of ACF; B-C. microdissection of ACF; D. microdissection of adenoma.

PCR products purification and sequencing

PCR products were purified with QIAquick PCR kit as described. Sequencing reaction was performed with Ready Reaction Kit from PE Company (96℃ 10"→55℃5"→60℃4', 25 cycles). The reaction product was precipitated in 100% ethanol (2.5 volume) and 3M NaAc (0.1 volume), and washed with 75% ethanol. The precipitation was resuspended in TSR (template suppression reagent), denatured at 94 ℃ and snap cooled at 4℃ before the automatic electrophoresis.

Statistical analysis

Chi-square study was used for the comparison between two groups.

RESULTS

K-ras gene mutation and its feature-Five of 35 (14.3%) carcinomas, 2 of 15 (13.3%) adenomas and 6 of 34 (17. 6%) ACF showed K-ras gene mutation. The mutation frequency was comparable among three types of tissues (P>0.05). No mutation was detected in normal epithelium. The mutation in carcinoma, adenoma and 4 ACF located at the second nucleotide of codon 12 (GGT →GAT). Two mutation in ACF located at the second nucleotide of codon 13, (GGC→GAC). The carcinoma and ACF patient No.30 shared the same mutation at codon 12. No mutation of codon 61 was found. K-ras mutation in carcinoma was related to the age and macroscopic type. The patients with mutation (median age of 70.8 years) were older than the patients without mutation (median age of 52.3 years) (P<0.01). Four out 5 carcinomas with mutation were polypoid carcinoma (P<0.05). All of 6 ACF with mutation were obtained from distal colon, most of their primary carcinomas showing polypoid (5 cases). The patients with mutation (median age of 68.4 years) in their ACF were older than that without mutation (median age of 43) in their ACF. This is coincident with the mutation in carcinomas. One of 2 adenomas with mutation was villous adenoma and another one was tubular adenoma (Figure 1).

APC gene mutation and its feature

Eight out of 35 carcinomas (22.9%) and 4 of 15 adenomas (26.7%) showed mutation in APC gene. The mutation frequency was close in two tissue types (P>0.05). Only 1 of 34 ACF showed mutation. APC gene mutation in carcinoma was not related to the age, gender, tumor site, macroscopic type and histological differentiation. All 8 mutations were scattered in region A (2 cases), region B (4 cases), and in region C and D (1 case), respectively. Among the 4 cases of adenomas with APC mutation, 2 were villous adenomas and other two were villous tubular adenomas; 2 mutations in region A, 1 in region C and 1 in region D. A mutation in region A was demonstrated in both ACF and primary carcinoma (Figure 2). APC gene mutations were displayed in Table 2. Altogether 13 mutations were detected in regions A →D of APC gene, 5 were stop codon, 1 was nonsense mutation, the other were point mutation.

Table 2 APC gene mutation

DNA	Region	Codon	Mutation	Amino acid exchange
N 3 CRC	A	1354	TTT→TTA	Phe→Leu
N17 CRC	A	1309	GAA→TAA	Stop cocon
N14 CRC	B	1389	TCT→TTT	Ser→Phe
N 5 CRC	B	1357	GGA→AGA	Gly→Arg
N21 CRC	B	1365	GGT→GGC	nonsense
N26 CRC	B	1398	AGT→ACT	Ser→Thr
N28 CRC	C	1465	GTG→GCG	Val→Ala
N33 CRC	D	1547	GAA→TAA	Stop codon
Tubular villous adenoma	A	1301	ins1 A	Stop codon
Villous adenoma	A	1309	GAA→TAA	Stop codon
Villous adenoma	C	1490	ins8 TTATTACA	Frame shift
Tubular villous adenoma	B	1367	CAG→CAC	Gln→His
ACF	A	1309	GAA→TAA	Stop codon

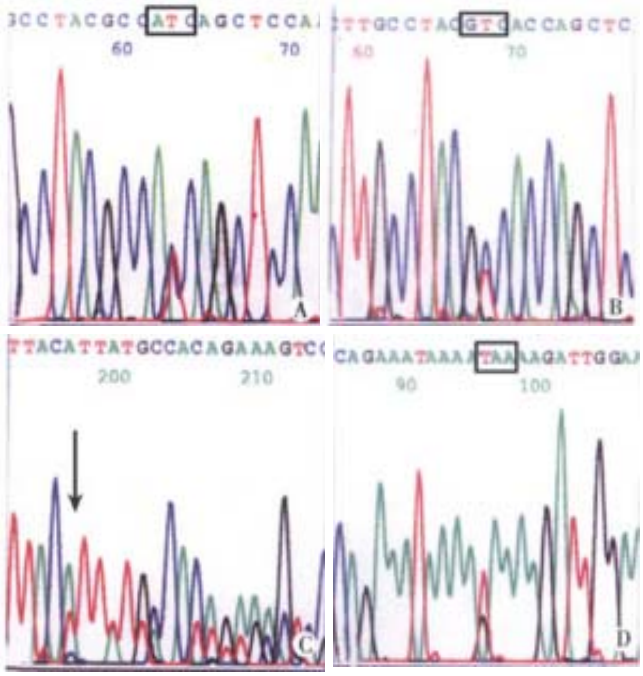

Figure 2 A. N30 CRC K-ras 1 12 GGT→GAT (CCA→CTA); B. Tubular-adenoma K-ras 1 12 GGT→GAT (CCA→CTA); C. ACF 6 K-*ras*-1 13 GGC→GAC (CCG→CTG); D. N14 CRC APC-B 1389 TCT→TTT.

DISCUSSION

In 1987, Bird[24] established a mouse model of colorectal carcinoma by injecting F334 mouse with carcinogen AOM. He observed the changes of methylene blue stained large intestine mucus in different stages and found for the first time the earliest morphological change in normal colorectal mucus. Several or dozen aberrant crypts, scattering in the crypt level, were termed as aberrant crypt foci. It is recently reported that similar lesions exist in the human colorectal epithelium apart from the carcinoma[25-27]. The order "normal epithelium→ metaplasia→adenoma→carcinoma→carcinoma with metastasis" is widely accepted by most authors. APC and K-ras gene mutation is the early event in the carcinogenesis[28-30]. Our results revealed that normal epithelium showed no mutation, but K-ras gene mutation appeared in ACF, adenoma and carcinoma with a close rate (P>0.05), suggesting that K-ras mutation initiates in ACF stage and maintains during the process of the carcinogenesis. This demonstrates the possible relationship of ACF to carcinoma and ACF as a preneoplastic lesion in the carcinogenesis of colorectal carcinoma[31, 32]. K-ras gene point mutation at codon 12 and 13 endows the epithelium with transformation ability. All mutations in carcinoma and adenoma located at codon 12 (4/6), while 1/3 mutation (2/6) of ACF was found at codon 13 (GGC→ GAC). In the previous reports by American and European scientists, the K-ras gene mutation located both at codon 12 and 13, more frequently at codon

12[16,33-37]. Japanese and Chinese scientists reported the similar results as ours[22,38,39], the mutation in carcinoma limited in codon 12. The reason might be the difference in the genetic predisposition, the food, the environment and the pathogeny. The cell clone with mutation at codon 13 might have weaker clone selection and such cells have, therefore, weaker ability to expand themselves to grow out. ACF is reported to be located more frequently in rectum than in colon, more in distal than in proximal colon[40]. All 6 ACF with K-ras gene mutation in the current study located in distal colon, implying the same site of predisposition of colorectal carcinoma. ACF might be the earliest morphological lesion with detectable molecular genetic alteration in it.

APC gene was cloned, isolated and defined as a tumor suppressor gene in 1990. Germline mutation of the gene is responsible for the pathogenesis of familial adenomatous polyposis (FAP). Thirty-five sporadic CRC and 15 adenomas had similar frequent APC gene mutation in the MCR (P>0.05). It is coincident with previous report[41-45]. APC gene mutation was not correlated with age, tumor site, macroscopic type and histologic differentiation. This is identical to the previous demonstration that APC gene is involved very early in the carcinogenesis of sporadic CRC. There have been many reports about the APC gene mutation in sporadic adenoma. APC gene mutation occurred even in the adenoma <0.3 cm. The mutation was more frequently found in villous and tubular villous adenoma than in tubular adenoma. Our results support this documentation. Two of 4 adenomas with APC gene mutation were villous adenoma and 2 were tubular villous adenoma. The same mutation in codon 1309 (GAA→TAA) was found simultaneously in ACF, adenoma and primary carcinoma. This mutation was also found in other 4 of 15 adenomas. The mutation at codon 1309 is also the hot spot in Chinese sporadic colon tumors. Exactly alike the other reports, the mutation at codon 1301, 1309 and 1547 lead to stop codon. It is reported that the mutations cause most commonly truncation of the protein. The truncated protein binds to the wild type protein, causing a negative effect and decreasing their function as a tumor suppressor[46-48]. Because APC gene is too large to be wholly sequenced and the mutations scatter throughout over the gene, it is difficult for us with so less cases to find more mutation characterization of this gene.

ACF differ with adenoma in morphological and molecular level on the following points: ① ACF is surrounded by normal crypts, ② cells in ACF are not so dysplastic as that in adenoma, ③ mitosis is rare, ④ ACF is much smaller than adenoma, ⑤ APC mutation is rare, ⑥ mutation in K-ras gene at codon 13 is detectable. The above fact suggests the hypothesis that ACF is a preneoplastic lesion. The concept "microadenoma" could be used to describe such morphological lesion[49]. Nucci and Kobayashi have suggested that ACF could be added to the order: "normal epithelium→ACF→adenoma→carcinoma"[50-52]. Many studies demonstrated that K-ras gene mutation is induced by the APC gene mutation. We found in our study in contrast that 6/34 ACF displayed K-ras gene mutation. Only 1 out of 6 showed APC gene mutation. The possible interpretations are: ① not all K-ras gene mutations are induced by APC gene mutation, ② adenoma formation could occur in the basis of APC and K-ras gene mutation, ③ ACF formation is related to K-ras gene mutation but not to APC gene, ④ there might be another path of CRC. In addition, 27 of 34 CRC in our study were not accompanied with adenoma, but the ACF is proved to possess the same molecular alteration as in the primary CRC. We speculate that except the classical path of CRC, there might be another path "normal epithelium→ACF→carcinoma", without the stage of adenoma. More detailed molecular approach to understanding the role of ACF in this path is necessary.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Modulation of postoperative immune and inflammatory response by immune-enhancing enteral diet in gastrointestinal cancer patients

Guo Hao Wu, Yan Wei Zhang and Zhao Han Wu

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Abstract

AIM To evaluate if the administration of an enteral diet supplemented with glutamine, arginine and ω-3-fatty acids modulates inflammatory and immune responses after surgery.

METHODS A prospective randomized double-blind, clinical trial was performed. Forty-eight patients with gastrointestinal cancer were randomized into two groups, one group was given an isocaloric and isonitrogenous standard diet and the other was fed with the supplemented diet with glutamine, arginine and ω-3-fatty acids. Feedings were started within 48 hours after operation, and continued until day 8. All variables were measured before operation and on postoperative day 1 and 8. Immune responses were determined by phagocytosis ability, respiratory burst of polymorphonuclear cells, total lymphocytes lymphocyte subsets, nitric oxide, cytokines concentration, and inflammatory responses by plasma levels of C-reactive protein, prostaglandin E2 level.

RESULTS Tolerance of both formula diets was excellent. There were significant differences in the immunological and inflammatory responses between the two groups. In supplemented group, phagocytosis and respiratory burst after surgery was higher and C-reactive protein level was lower ($P<0.01$) than in the standard group. The supplemented group had higher levels of nitric oxide, total lymphocytes, T lymphocytes, T-helper cells, and NK cells. Postoperative levels of IL-6 and TNF-α were lower in the supplemented group ($P<0.05$).

CONCLUSION It was clearly established in this trial that early postoperative enteral feeding is safe in patients who have undergone major operations for gastrointestinal cancer. Supplementation of enteral nutrition with glutamine, arginine, and ω-3 fatty acids positively modulated postsurgical immunosuppressive and inflammatory responses.

Department of General Surgery, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Correspondence to: Guo Hao Wu, Department of General Surgery, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Tel. 0086-21-64041990 Ext.2365, Fax. 0086-21-4038472
Email. wughmc@online.sh.cn
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INTRODUCTION

Patients with gastrointestinal cancer frequently suffer from protein calorie malnutrition. This is due, largely to a systemic anorexia in conjunction with varying degrees of local anorexia in response to obstructive symptoms. In addition, the metabolic response to injury that is seen in these patients postoperatively produces a redistribution of endogenous macronutrients, causing further depletion of body fat stores and lean body mass. If the injury response persists and nutritional support is not instituted, pre-existing malnutrition will be exacerbated and the risk of postoperative complications as well as morbidity and mortality will be increased. It is well known that major surgery and other types of trauma are associated with severe alterations of the host defense mechanisms, making the patients highly susceptible to septic and inflammatory complications. Nutritional support after injury may modulate the immune, inflammatory, and metabolic responses, and the clinical outcome of critically ill subjects. In different experimental and clinical settings, enteral *vs* parenteral route, early *vs* delayed enteral feeding, and immuneenhancing enteral *vs* standard diets are related to better results[1-3]. Supplementation of enteral diets with glutamine, arginine and ω-3 fatty acids has been reported to improve clinical outcome after major operations and to reduce the risk of septic complications above and beyond the role of standard enteral formula[4].

The purpose of this study is to compare the effect of early enteral nutrition with immune enhancing enteral diet with standard diets on postoperative immune, inflammatory and metabolic response in a group of cancer patients. The endpoints of the study were the evaluation of the

immune response by polymorphonucler (PMN) cell respiratory burst and phagocytosis ability, total lymphocytes, lymphocyte subsets, nitric oxide (NO), and cytokines concentrations, the inflammatory response by C-reactive protein (CRP), α_1-1antitrypsin, fibrinogen levels, and prostaglandin E_2 level.

MATERIALS AND METHODS

Study protocol

In this prospective, randomized, and blinded study, 48 adult patients, aged between 34 and 75 years, with gastrointestinal malignancies, who were scheduled for major abdominal surgery due to their cancer, were included in the study. Exclusion criteria were clinical relevant alterations of the pulmonary, cardiovascular, renal, intestinal or hepatic function; history of recent immunosuppressive therapy (including preoperative radiochemotherapy) or immunological disease; ongoing infection; intestinal obstruction; or emergency surgery. The comparability of the groups is shown in Table 1.

Table 1 Characteristics in 2 groups of GI cancer

Characteristics	Supplemented (n = 25)	Control (n = 23)
Age / y	55.2±12.1	52.6±9.8
M / F	16 / 9	15 / 8
Site of cancer		
Stomach	14	13
Colorectum	8	7
Pancreas	3	3
Type of operation		
Gastrectomy (subtotal or total)	14	13
Miles or Dixon procedure	8	7
Pancreatoduodenectomy	3	3
Time of surgery / min	220±45	245±60
Operative blood loss / mL	400±325	450±400
Transfused patients	22	20

Patients were randomized to receive either an immuneenhancing enteral diets, Stresson (Nutricia; n = 25) or a standard diets, Nutrison (Nutricia; n = 23) supplemented with protein power to make the diets isonitrogenous. Table 2 summarizes the nutrient content of these diets. Enteral feeding was started within 48 h of operation via a needle catheter jejunostomy, or a nasoenteric tube, and was delivered by continuous pump infusion. The diets were started at half strength 50 $mL \cdot h^{-1}$, and all patients reached their nutritional goals by 72 h after the initiation of enteral nutrition. The goals of nutritional delivery in the study included 146 $kJ \cdot kg^{-1} \cdot d^{-1}$ and protein 2.2 $g \cdot kg^{-1} \cdot d^{-1}$. Patients had to receive enteral nutrition for a minimum of 7 days to be included in the study. All patients received intravenous fluid (50 $g \cdot L^{-1}$ dextrose and normal saline solution) and other electrolytes as indicated.

Table 2 Composition of Diets

Variables	Composition of formula (per 100 mL)	
	Supplemented	Control
Calories (kcal)	100	125
Protein (g)	4.00	7.50
Glutamine	0.40	1.30
Arginine	0.16	0.89
Fat (g)	3.89	4.17
LCT	3.89	2.45
MCT	0.00	1.72
EPA	0.00	0.079
DHA	0.00	0.030
*n*6:*n*3	5:1	3.45:1
â carotene (mg)	—	0.40 (66.70 μg RE)
Vit. E (mg TE)	0.81	4.92
Vit.C (mg)	5.00	13.30
Osmolarity (mOsm / L)	250	380

During the postoperative period, patients were evaluated prospectively for a number of symptoms: nausea, vomiting, abdominal cramping, abdominal distention, and diarrhea. Advanced the tube feeds or adjust the rates as the patients tolerate.

On admission, the following baseline variables were determined in all patients: plasma levels of glutamine, arginine, CRP, α_1-1antitrypsin, and fibrinogen. polymorphyonuclear (PMN) cell function, nitric oxide (NO), lymphocyte subsets (CD3, CD4 and CD8), natural killer cells, circulating cytokine level (IL-1, IL-2, IL-6 and TNF-α) and prostaglandin E-2 (PGE-2) level were also determined. Baseline variables were reassessed 1 day and 8 days after surgery.

Immune response assays

PMNs were separated on a sodium metrizoate and methylecellulose column by the method of Boyum and suspended in pHadjusted (7.2-7.4) Eagle's medium at a density of 5×10^9 cells$\cdot L^{-1}$. The oxidative metabolism of PMNs was assayed by measuring the superoxide production with nitroblue tetrazolium (NBT) red uction test[5]. The PMNs ability to phagocytose zymosan particle was detected as described by Brain. The degree of phagocytosis was expressed as percentage of PMNs in which zymosan particles were detected over a hundred PMNs. The production of NO by macrophages was measured by the Greiss reaction. This is an indirect colorimetric assay of NO. Greiss reaction consists of measurement of stable end breakdown products of NO such as nitrite which are considered to be reliable markers for NO formation.

The cytokines (IL-1, IL-2, IL-6, TNF-α) concentration in the supernatant were measured using commercially available ELISA kits. Total lymphocyte and lymphocyte subset were assayed by flow cytometry.

Inflammatory response assays

Prostaglandin E_2 (PGE_2) level in the supernatants were measured using the PGE_2 ^{125}I-scintillation proximity assay system. Plasma levels of CRP, α_1-

antitrypsin, and fibrinogen were measured with standard techniques.

Statistical analysis

Data were presented as the $\bar{x} \pm s$ and analyzed by analysis of variance (ANOVA) using Sigmastat software (Jandel Scientific, San Rafael, CA, USA). Statistical significance was predetermined as $P<0.05$.

RESULTS

The preoperative diagnoses and operations were similar between the two groups. At the time of entry into the study, there were no significant differences in nutritional state or type of disease, the age ranges were similar in both groups. There were no significant differences between groups in mean age, usual body weight, mean duration of operation, or the number of patients who received transfusions during the procedure or postoperatively (Table 1). Tolerance of both formula diets was excellent and there were no differences between the groups. No patient was withdrawn because of intolerance. Three patients in immune enhancing enteral diet group and two patients in the standard diet group developed diarrhoea.

Plasma arginine and glutamine levels are shown in Table 3, at baseline, there were no significant differences between the groups, but decreased1d after operation in both groups. After 7d feeding, the study group had a higher plasma levels of arginine and glutamine, but, in the control group, no variation was observed. As expected, plasma levels of PGE_2, CRP, α_1-antitrypsin, and fibrinogen were increased after operation in both groups. In patients receiving immune enhancing enteral diet, the levels of CRP were significantly lower than in the standard diet group over the 7d feeding. However, the levels of PGE_2, α_1-antitrypsin and fibrinogen had no significant differences between the groups at the end of study (Table 3).

Table 3 Variations of glutamine, arginie, PGE_2, and acute phasere actents

Variables	Period	Supplemented	Control	*P*
GLN (nm/mL)	Baseline	558.7±62.5	584.1±51.5	NS
	D 1	386.0±74.6	412.4±65.3	NS
	D 8	722.4±116.5	555.0±72.2	<0.05
ARG (nm/mL)	Baseline	62.7±21.0	58.5±23.1	NS
	D 1	56.2±18.6	50.9±20.0	NS
	D 8	115.4±47.2	66.7±31.1	<0.001
PGE_2 (pg/mL)	Baseline	62.2±26.4	73.7±42.1	NS
	D 1	167.7±73.8	184.4±91.3	NS
	D 8	85.5±53.0	112.6±84.0	NS
CRP (g/L)	Baseline	0.4±0.1	0.3±0.1	NS
	D 1	4.8±2.3	4.6±1.8	NS
	D 8	2.0±1.1	3.9±2.3	<0.05
α_1-antitrypsin (g/L)	Baseline	15.5±0.2	13.2±0.2	NS
	D 1	42.0±3.4	40.9±4.7	NS
	D 8	28.2±2.8	30.0±3.1	NS
Fibrinogen	Baseline	32.4±3.3	30.6±3.1	NS
	D 1	36.8±4.2	35.5±4.4	NS
	D 8	34.5±3.5	35.0±3.9	NS

Total lymphocyte and lymphocyte subset analyses are depicted in Table 4. At baseline, the study and control groups did not have significant numbers of total lymphocytes, T lymphocytes (CD3), T helper (CD4), T suppressors (CD8) or natural killer cells. A significant postoperative decrease in total lymphocyte, CD3, CD4 and natural killer cells were observed in both groups, but they recovered after 7 days feeding in the supplemented group. No significant difference was noted in CD8 cell numbers.

Table 4 Variations of lymphocytes

Variables	Period	Supplemented	Control	*P*
Total lymphocytes	Baseline	1.62±0.41	1.73±0.38	NS
No. cells×10^9/cm^3	D 1	1.12±0.39	1.55±0.33	NS
	D 8	1.55±0.33	1.18±0.35	<0.05
CD3 (%)	Baseline	72.2±11.1	69.0±9.8	NS
	D 1	60.1±8.8	57.7±10.3	NS
	D 8	76.4±14.5	60.3±9.3	<0.05
CD4 (%)	Baseline	45.0±8.5	42.5±9.2	NS
	D 1	38.2±9.4	37.7±9.0	NS
	D 8	57.5±7.8	40.0±8.6	<0.05
CD8 (%)	Baseline	23.3±7.4	24.5±7.7	NS
	D 1	22.7±8.0	22.2±8.0	NS
	D 8	19.6±6.6	23.1±5.9	<0.05
CD4/CD8	Baseline	1.9±0.8	1.7±0.7	NS
	D 1	1.7±1.2	1.7±0.9	NS
	D 8	2.9±1.4	1.7±0.8	<0.01
NK cells (%)	Baseline	17.7±7.1	18.3±6.9	NS
	D 1	11.1±8.2	10.2±7.2	NS
	D 8	16.6±5.4	11.1±4.3	<0.05

Circulating cytokine levels of IL-1, IL-2, IL-6 and TNF-α are displayed in Table 5. At baseline, there were no significant differences between groups. There was a significant postoperative decrease in all cytokines . After 7d of feeding, the absolute levels of IL-1 and IL-2 as well as the changes from baseline were not different. However, IL-6 and TNF-α concentrations were significantly lower in the group given immune enhancing enteral diet on postoperative d8.

Table 5 Variations of cytokines (pg/mL)

Variables	Period	Supplemented	Control	*P*
IL-1	Baseline	2520±566	2480±502	NS
	D 1	1375±304	1528±312	NS
	D 8	2020±385	2155±450	NS
IL-2	Baseline	1255±226	1310±252	NS
	D 1	892±173	900±186	NS
	D 8	990±201	1156±192	NS
IL-6	Baseline	405±141	432±128	NS
	D 1	365±110	389±143	NS
	D 8	277±106	762±199	<0.001
TNF-α	Baseline	260±96	236±101	NS
	D 1	178±85	159±93	NS
	D 8	202±104	345±133	<0.05

The ability of PMNs to phagocytose zymosan particles is shown in Figure 1. Both groups had a similar baseline phagocytosis ability. A significant

decrease in phagocytosis was observed after surgery in the control group. Conversely, phagocytosis ability remained similar to the preoperative values in the study group. The PMN oxidative metabolism is shown in Figure 2. At baseline, there are no significant differences between the groups. A sharp postoperative increase of the oxidative metabolism compared with preoperative values was observed in both groups, the levels still remained higher in the study group than the control group (P<0.05). Figure 3 gives the variations of NO. The circulating levels of NO increased after operation in both groups, but, the study group had significantly higher NO levels than control group postoper atively (P<0.05).

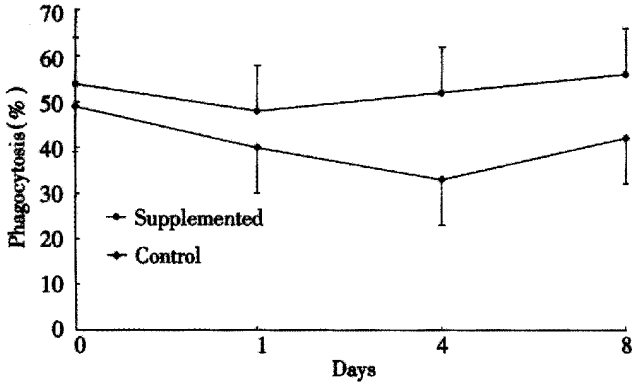

Figure 1 Variations of the phagocytosis ability.
[a]P<0.01 *vs* control, [b]P<0.01 *vs* preoperative day.

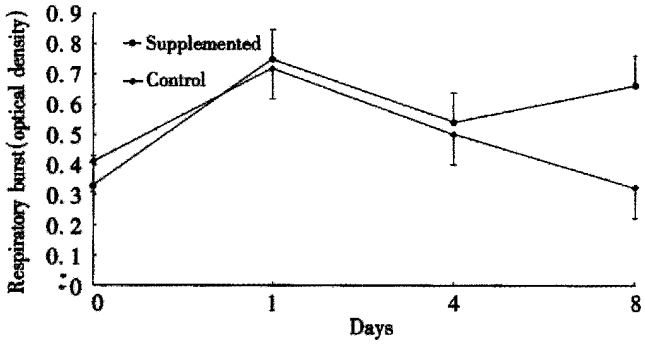

Figure 2 Variations of the respiratiry burst of PMN.
[a]P<0.05 *vs* control, [b]P<0.01 *vs* preoperative day.

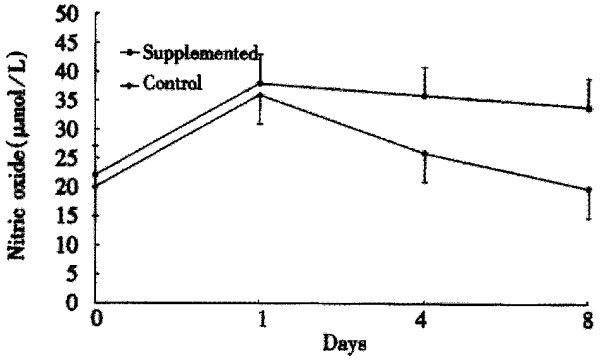

Figure 3 Variation of the nitric oxide production.
[a]P<0.05 *vs* control, [b]P<0.01 *vs* preoperative day.

DISCUSSION

Malnutrition in patients with cancer has been shown to have increased risk of postoperative complications. Effects to reduce the impact of malnutrition initially focused on the use of parenteral and enteral nutrition for perioperative nutritional support. Enteral feeding has gained popularity for the nutritional support of surgical patients. Biologically, there have been several reasons reported including better substrate utilization, prevention of mucosal atrophy, preservation of normal gut flora, gut integrity, and immune competence. Early enteral vs parenteral feeding in traumatized and surgical patients is gaining wide consensus after the promising results showing good tolerance and notable reduction of septic morbidity[1,6-9]. Our data confirm that early postoprative enteral feeding may be carried out safely in patients who have undergone major operations for cancer. Gastrointestinal side effects were minimal and were similar in both groups. Though diarrhoea, bloating, and abdominal cramps occurred, there were no major gastrointestinal complications in either group.

Current investigations in enteral nutrition have focused on the ability to modulate the metabolic and immune response to injury via specially formulated enteral diets. Specific nutrients shown to have the potential to modulate immune function include glutamine, arginine, and ω-3 fatty acids as well as nucleotides. These specialized diets have generally been termed “immuneenhancing diets” (IEDs). A number of recent studies have compa red IEDs with standard enteral diet in the management of patients with trauma, critically illness and cancer[3,10,11]. Although evidence appears to be accumulating in support of some clinical benefits for IEDs, the specialized diets used in most of these studies generally delivered more nitrogen and often more calories than the control “standard” diet, thus making it difficult to conclude that the beneficial effects noted were due to the special nutrient supplements. The current study was intentionally designed such that both groups of patients received isocaloric and isonitrogenous diets, and differed only in their glutamine and arginine content, fatty acid compositi on, and the levels of the antioxidant micronutrients vitamin C, E and carotene. Our purpose is to examine the influence exerted by the dietary supplements on postoperative immune, inflammatory and metabolic response in a group of cancer patients.

Both cellular and humoral immune function are compromised in patients undergoing operations for cancer. The immune suppression is a common phenomenon and seems to be related to both postoperative outcome and to diseasefree survival. There are many reports about the use of enteral nutrition during the early posto perative period to ameliorate immune dysfunction induced by the

tumor and operation[12]. Certain nutrients such as glutamine, arginine, and ω-3 fatty acids as well as nucleotides, may act pharmacologically on the immune system. It has been suggested that these nutrients may improve host immune defences[13-16]. Our data show that plasma glutamine and arginine levels were decreased 1d after operation in both groups. It is likely that glutamine uptake in certain tissues increase, and glutamine utilization may exceed endogenous glutamine production after injury. It is indicated that glutamine supplementation upregulates human immune cell number and function. Glutamine is critical for human lymphocyte proliferation in *in vitro* system. Our study demonstrated that enteral glutamine supplement ation could increase plasma glutamine concentrations, and might improve immune function.

Arginine is a semiessential amino acid which may become essential in catabolic states. Supplementation with arginine improved wound healing and enhanced immune function in animals by decreasing the T- cell dysfunction associated with injury. Additionally, arginine is a precursor for nitrates, nitrites, and nitric oxide . Nitric oxide is particularly important as a vasodilator but also participates in immunologic reactions, including the ability of macrophages to kill tumor cells and bacteria, and it may inhibit the development of precursor cytoloytic T- cells. In the present study, the increased NO levels observed 1 and 8 days after operation in the supplemented group may be a mechanism involved in the enhanced immune response early after surgery as shown by the improved phagocytosis ability and respiratory burst of PMNs.

Cytokines play a vital and integral role in both cellular and humoral immunity. The different mechanisms of action of the cytokines can be summarised as a coord inated immune response of the organism to exogenous or endogenous stimuli such as cancer, infection, injury, and operation. Measurement of cytokine concentrations may, therefore, give information about the immune response in patients after major operations. In this study, the mean IL-1 and IL-2 concentrations were only a slight but not significant difference between the two groups until the postoperative d8. The IL-6 and TNF-α concentrations were similar before operation and the first postoperative day in both groups, and they both showed a decline in IL-6 and TNF-α concentrations on the first postoperative day. Subsequently, IL-6 and TNF-α concentrations exceeded preoperative levels in the group that received the standard diet and kept rising as compared with the group given the supplemented diet, whose IL-6 and TNF-α concentrations remained below the preoperative values. As both IL-6 and TNF-α are important mediators of the acute phase reaction, it seems that systemic inflammatory responses were reduced in the group given the supplemented diet.

The acute phase reaction is a systemic inflammatory reaction to injury, including fever, tachycardia, leucocytosis, and changes in circulating protein concentrations. It has been proposed that injury promotes the switching of protein synthesis from constitutive to acute phase proteins through the release of proinflammatory cytokines such as IL-6 and TNF-α. In the present study, CRP levels significantly increased 1d after operation in both groups, but, this increase in patients receiving the supplemented diet was significantly lower than in the standard diet group after 7d feeding. A reduction in CRP concentrations may therefore be at least partly responsible for the lower postoperative levels of IL-6 and TNF-α in the group given the supplemented diet. ω-3 fatty acids, e.g., present in fish oils or canola oil, can replace arachidonic acid, which is derived from ω-6 fatty acids, in cell membranes and have modulating effects on immune function. Because the arachidonic acid metabol ite PGE_2 is known to inhibit the function of many immune cells, decreasing its generation by supplementing diets with ω-3 fatty acids would theoretically result in a relative enhancement of the immune response. Our data demonstrated that macrophage PGE_2 production increased after operation in both groups. However, there was no trend toward significance between the supplemented and standard diet groups on the d8 after operation, and it was an unexpected finding. Many of the immunosuppressive effects of the ω-6 fatty acids are found to be related to the immunosupp ressive prostaglandins, particularly PGE_2. It has been shown in previous studies that increasing the ratio ω-3 fatty acids in the diet effectively alters the monocyte/macrophage lipid membrane, decreasing the production of PGE_2. This decrease in immune inhibition thus should potentiate immune function. The normal to increased level of PGE_2 seen in the monocytes from supplemented diet group represents several alternatives. It is likely that the nutritional fat source used in the present study was not an effective source of ω-3 fatty acids. The duration of feeding might be insufficient to induce the changes in cell membrane fatty acids necessary to alter PGE_2 production. Alternatively, arginine or other contents of the supplemented diet may differentially affect PGE_2 production. Higher PGE_2 levels may be a counterregulatory phenomenon that results from an autocrine effect of the higher levels of cytokines produced by these monocytes.

The heterogeneity of T lymphocytes responsible for cell-mediated im munity is well known. There are two major types of T cells; CD4 cells recognize antigens associated with major histocompatibility complex (MHC) class II molecules, whereas CD8 suppressor cells recognize antigens associated with

MHC class I molecules. CD4 T cells play an important role in immunoregulation, and CD8 cells provide an inhibitory function, suppressing cellmediated immune responses. In the present study, supplemented diet improved immune response in surgical patients as demonstrated by increase in peripheral mature total lymphoc ytes, T lymphocytes, Thelper cells, and NK cells. Alterations in the number and ratio of the various Tcell subsets may subsequently influence the effectiveness of the overall immune response.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Inhibiting effect of antisense oligonucleotides phosphorthioate on gene expression of TIMP-1 in rat liver fibrosis

Qing He Nie, Yong Qian Cheng, Yu Mei Xie, Yong Xing Zhou and Yi Zhan Cao

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bstract

AIM To observe the inhibition of antisense oligonucleotides (asON) phosphorthioate to the tissue inhibitors metalloproteinase-1 (TIMP-1) gene and protein expression in the liver tissue of immunologically induced hepatic fibrosis rats. The possibility of reversing hepatic fibrosis through gene therapy was observed.

METHODS Human serum albumin (HSA) was used to attack rats, as hepatic fibrosis model, in which asONs were used to block the gene and protein expressing TIMP-1. According to the analysis of modulator, structure protein, coding series of TIMP-1 genome, we designed four different asONs. These asONs were injected into the hepatic fibrosis models through coccygeal vein. The results was observed by RT-PCR for measuring TIMP-1 mRNA expression, immunohistochemistry and in situ hybridization for collagen I, III, special staining of collagen fiber, and electron microscopic examination.

RESULTS Hepatic fibrosis could last within 363 days in our modified model. The expressing level of TIMP-1 was high during hepatic fibrosis process. It has been proved by the immunohistochemical and the electron microscopic examination that the asON phosphorthioate of TIMP-1 could exactly express *in vivo*. The effect of colchicine was demonstrated to inhibit the expressing level of mRNA and the content of collagen I, III in the liver of experimental hepatic fibrosis rats. However, the electron microscopy research and the pathologic grading of hepatic fibrosis showed that there was no significant difference between the treatment group and the model group ($P>0.05$).

CONCLUSION The experimental rat model of hepatic fibrosis is one of the preferable models to estimate the curative effect of anti-hepatic fibrosis drugs. The asON phosphorthioate of TIMP-1 could block the gene and protein expression of TIMP-1 in the liver of experimental hepatic fibrosis rats at the mRNA level. It is possible to reverse hepatic fibrosis, and it is expected to study a new drug of anti-hepatic fibrosis on the genetic level. Colchicine has very limited therapeutic effect on hepatic fibrosis, furthermore, its toxicity and side effects are obvious.

The Center of Infectious Disease Diagnosis and Treatment of PLA, Tangdu Hospital, Forth Military Medical University, Xi'an 710038, Shaanxi Province, China

Dr. Qing He Nie, graduated from Qinghai Medical College as a doctor in 1983, got master degree at Beijing 302 Army Hospital in 1993, got doctor degree at the Third Military Medical University in 1998, engaged in postdoctoral research at the Fourth Military Medical University from 1998 to 2000, now an associate professor, specialized in clinical and experimental research of infectious diseases, had more than 90 papers published, coauthor of ten books, first author of one book.

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Correspondence to: Dr. Qing He Nie, The Center of Infectious Disease Diagnosis and Treatment of PLA, Tangdu Hospital, Forth Military Medical University, Xi'an 710038, Shaanxi Province, China
Tel. 0086-29-3577595
Email. nieqinghe@hotmail.com huangj@fmmu.edu.cn

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INTRODUCTION

In China, the incidence of liver cirrhosis is still high for quite a long period in the future[1-5]. The pathological basis of hepatic cirrhosis is fibrosis[6-14]. Many factors inducing liver injury and inflammation will lead to chronic liver disease, and hepatic fibrosis is inevitable[15-25]. Researchers have paid more attention to reversing the hepatic fibrosis[26,27].

Recently, studies on the role of tissue inhibitors of metalloproteinase-1 (TIMP-1) and tissue inhibitors of metalloproteinase-2 (TIMP-2) in the process of hepatic fibrosis have attracted more attentions[28-33]. The main aim of our study is to seek for an approach of reversing hepatic fibrosis, to investigate the role of TIMPs in the pathogenesis of hepatic fibrosis and to observe the fluctuation of TIMPs in patients with various liver diseases in order to set up a new laboratory diagnotic index of hepatic fibrosis. It had been considered as the target gene and the antisense oligonucleotides (asON) phosphorothioate was used to inhibit the gene and

protein expressing in experimental rat hepatic fibrosis.

In 1973, Miller *et al*[34] reported that non-ionic oligonucleotide and its derivant could specifically inhibit cellular DNA/RNA replication, transcription, translation. In 1978, Stepherson *et al*[35] obtained favorable anti-virus effect in infected fibroblast by Rous sarrcoma virus using 13-polyoligonucleotide. Since then attention has been made to the studies of the application of antisense oligonucleotide technology in antineoplastic and antiviral therapy[36,37]. This technology has been carried out under clinical trial stage[38-40]. In present study, we attacked the rats with HSA in order to establish experimental immune hepatic fibrosis model, and tried to block the gene and protein expression of TIMP-1 in rats with asON, so as to investigate the possible mechanism of reversing hepatic fibrosis.

MATERIALS AND METHODS

Design and synthesis of antisense oligonucleotide

According to the whole TIMP-1 cDNA sequence in rats[41], we analysed the sequences of modulator, structural protein and coding region and then designed four different antisence oligonucleotide as follows:

DNA Seq1 5'-GGCGCCATCGTGGTATCTGC-3'
Seq2 5'-GCTCTAGCGTGTCTCTAGGA-3'
Seq3 5'-GATAAACAGTGTTCAGGCTTC-3'
Seq4 5'-GTTCAGGCTTCAGCTTTTGC-3'

Twenty-polyligodeoxyribonucleotide phosphorthioate was automatically synthesized by the 391A PCRMATE EP DNA synthesis machine (ABI Company, USA). AsON was modified by phosphorthioate using TETD/acetonitrile method and purified by high-performance thin layer chromatography (purity>99%), which was accomplished by Wang SQ and Wang XH (Chinese Academy of Military Medical Sciences).

Animal experiment

Forty healthy adult female Wistar rats, weighing 120 g-150 g (provided by Experimental Animal Center of FMMU), were employed in the study. Immune hepatic fibrosis rat model was produced by immunological attacking with HSA, a method introduced by Wang *et al*[42]. Anti-mouse monoclonal antibody IgG was bought from Coulter Company (France).

The animals survived from the experimental attack were randomly allocated as follows. Model group: animals with hepatic fibrosis did not receive any interventional factors. Treatment group: animal models in this group received colchicine (0.28 $mg \cdot kg^{-1}$) treatment, six times a week, lasting 3 months. Experimental group: animals in this group received asON phosphorthioate through coccygeal vein injection (20 $\mu g \cdot g^{-1}$), every other day, 15 times in all. Control group (normal group): animals in this group were injected with same quantity of N.S. Three months after the beginning of injection, all animals were killed under narcosis, and their liver samples were kept in N_2, 100 $mL \cdot L^{-1}$ formalin or glutaraldehyde for designed investigations.

Detection parameters

Pathologic observation Some hepatic sections were stained with hematoxylin and eosin, while other sections for *Von Gieson* and *Masson* special staining.

Transmission electron microscope The liver samples were fixed with glutaraldehyde, and examined with electron microscope.

Immunohistochemical staining of TIMP-1 The liver samples were embedded with paraffin, and serial sections at 4 μm thickness were prepared. SP immunostaining was performed as described by streptomycin avidin-peroxidase immunochemistry kit (purchased from Maxim Biological Technology Company). Paraffin was removed from the sections with xylene and rehydrated with graded ethanol. After repairing the antigens, nonspecific binding sites were blocked by a 20 min preincubation with 100 $mL \cdot L^{-1}$ normal human serum. The sections were incubated with monoclonal antibody against TIMP-1 at 4℃ overnight, and then secondary antibody at 37℃ for 30-40 min, avidin-peroxidase at 37℃ for 20 min, finally added DAB to be colorated. After several washings, the sections were counterstained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and the sections mounted with gum for microscopic examination and photography.

Immunohistochemical staining of collagen I, III Immunohistochemical kits of collagen I, III were purchased from Bo-Shi-De Biological Technology Limited Company (Wuhan, China). The numbers of antibodies of collagen I, III were BA0325, BA0326 respectively. The immunostaining was performed as described by the kit.

Procollagenase I, III *in situ* hybridization We used digoxin-labeled probes to detect the mRNA expression of procollagen I, III. The *in situ* hybridization kit was purchased from Bo-Shi-De Biological Technology Limited Company (Wuhan, China, No. MK1171). *In situ* hybidization was performed according to the manufacturer's directions. Briefly, the paraffin embedded serial sections(thickness 4 μm), were dried at 80℃, paraffin was then removed by xylene and

rehydrated in graded ethanol. The sections were acidified in HCl for 30 min, and blocked in 300 mL·L^{-1} 3 mL, H_2O_2, for 10 min before digestion in proteinase K for 30 min, and then dehydrated with graded ethanol. After prehybridization at 37℃-40℃ for 2 h, the labeled cDNA probes were denatured in hybridization buffer at 95℃ for 10min,then -20℃ for 10 min, added on tissues which had been prehybridized at 37℃ overnight. Sections were washed with 2 × SSC, 1 × SSC, 0.2 × SSC, added Buffer I, and blocking water at room temperature for 20 min, and then rabbit anti-digoxin at 37℃ for 60 min, biotinylated goat anti-rabbit at 37℃ for 30 min, SABC at 37℃ for 30 min, finally added DAB to be colorated. After several washings, the sections were counterstained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and mounted the sections with gum for microscopic examination and photography.

Image pattern analysis and data processing After gray scale scanning and quantification the statistical analysis was performed. Comparison among groups was analyzed by *t* test, and the *P* value was used to judge the significant difference.

Detection of TIMP-1 by PCR method

The PCR primers of TIMP-1 are listed in Table 1.

Table1 TIMP-1, β-actin primer sections

	Primer	Nucleic acid sections	Position (bp)
TIMP-1	Positive strand	5'-TTCGTGGGGACACCAGAAGTC-3'	
	Antisence strand	5'-TATCTGGGACCGCAGGGACTG-3'	482
β-actin	Positive strand	5'-GGAGAAGATGACCCAGATCA-3'	
	Antisence strand	5'-GATCTTCATGAGGTAGTCAG-3'	234

RNA extraction The total RNA of liver was extracted with total RNA isolation system (produced by Promega Co.).

PCR amplification PCR was performed in 20 μL reactive volume containing 2 μL cDNA, 2 μL 10 × PCR buffer, 2 μL (2 mmol·L^{-1}) 4 × dNTP, 10 mmol·L^{-1} primer (2 μL TIMP-1, 2 μL β-actin), and 1U Taq DNA polymerase. The samples were subjected to 30 thermal cycles of 2min at 97℃ for predenaturation, 30s at 94℃ for denaturing, 30s at 56℃ for annealing, 50s at 72℃ for extension, and 7 min at 72℃ for final extension after the last cycle.

Quantitative analysis of PCR product Ten μL samples of PCR product was subjected to electrophoresis in 20 g·L^{-1} agarose gel with TAE buffer at 50V for 1h. After colorating with ethidium bromide and image forming, the quantitative analysis was performed. TIMP-1/ β-actin quotient is the indication of TIMP-1 expression level.

Pathologic grading of hepatic fibrosis

The pathologic grading of hepatic fibrosis used in our present paper was reported by Wang *et al*[43].

0: Normal liver without hyperplasia of collagenous fibers.

I: Slight extension of collagenous fibers from portal area or central veins.

II: Remarkable extension of collagenous fibers, without connecting each other and encysting the whole hepatic lobules.

III: Remarkable extension of collagenous fibers, connecting each other, and encysting the whole hepatic lobules.

IV: The hepatic lobules are encysted and separated by collagenous fibers. The normal structure of hepatic lobules is destroyed. The pseudolobules are formed, and it is dominant by big-square pseudolobuli.

V: The structure of hepatic lobules is fully destroyed, and the big square pseudolobules and small round ones occupy 50% respectively.

VI: The small round hepatic lobuli occupy almost the whole liver and the hyperplatic thick collagenous fibers are visible.

RESULTS

TIMP-1 immunohistochemical results

Image pattern analysis showed that the positive density value of the model group was the highest, and the colchicine treatment group was in the second place. There is no significant difference (P>0.05) between the above-mentioned two groups, and the density value of the experimental group is low (P<0.001, Table 2, Figures 1,2).

Table 2 Expression of TIMP-1 related antigen in the liver of rats

Group	*n*	TIMP-1
Normal group	10	59.8±20.3
Experimental group	6	98.7±25.7
Treatment group	6	396.1±58.4
Model group	6	481.1±61.0

The gene expression level of TIMP-1

The gene expression level of the model group was the highest, and the colchicine treatment group was the next. There was no significant difference (P>0.05) between the two above mentioned groups. The gene expression level of the experimental group was low (P<0.001, Table 3).

Table 3 Gene expression of TIMP-1 in the liver of rats

Group	*n*	TIMP-1
Normal group	10	0.3±0.1
Experimental group	6	0.6±0.1
Treatment group	6	1.7±0.4
Model group	6	1.9±0.5

Immunohistochemical study of collagen I, III

The hepatic content of collagen I, III of all the experimental animals was remarkably higher than that of the normal control group ($P<0.05$, $P<0.001$). Moreover, in all experimental animals, the hepatic content of collagen I, III of the model group was the highest, but that in the experimental group was the lowest (Table 4, Figure 3).

Table 4 Collagen I, III in the liver of rats

Group	*n*	Collagen I	Collagen III
Normal group	10	62±17	100±19
Experimental group	6	165±47	349±48
Treatment group	6	314±65	516±72
Model group	6	441±87	699±102

mRNA expression of procollagen I, III

There was distinct decrease of procollagen I, III expression in the liver of rats of the experimental group, it was significantly different compared with the model group ($P<0.05$). But there was no statistical difference (Table 5), between the experimental group and the treatment group.

Table 5 mRNA expression of procollagen I, III in the liver of rats

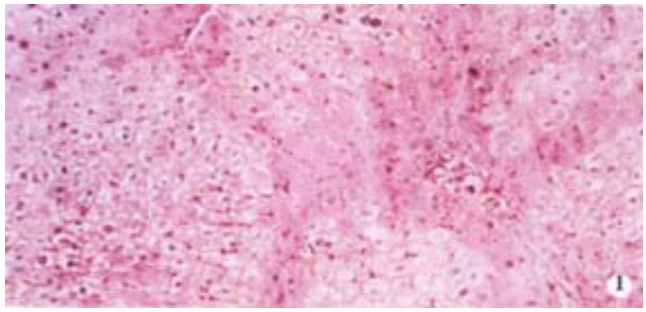
Group	*n*	Procollagen I	Procollagen III
Normal group	10	245.6±88.7	228.3±57.5
Experimental group	6	917.9±206.9	1050.9±271.4
Treatment group	6	896.8±198.1	977.8±221.4
Model group	6	1217.3±202.9	1484.5±249.1

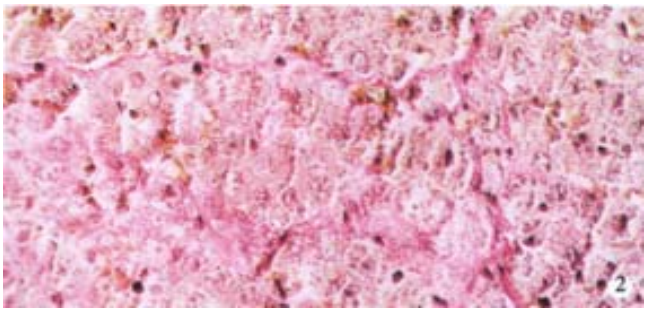
Pathologic grading of hepatic fibrosis

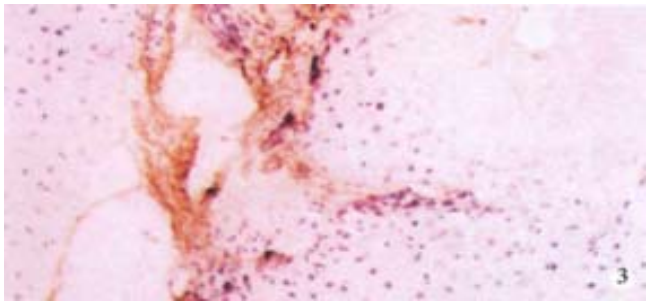
From Table 6, we can see the significant difference of pathologic grading between the control and other groups ($P<0.05$, $P<0.001$). The pathologic grading status was better in the experimental group than that in the treatment group (Figures 4,5). There was no significant difference between the treatment group and the model group.

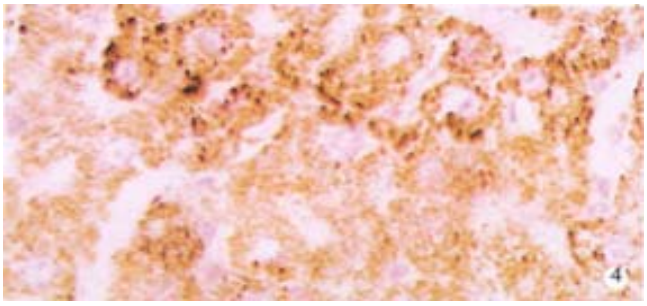
Table 6 The pathologic grading of hepatic fibrosis of experimental animal

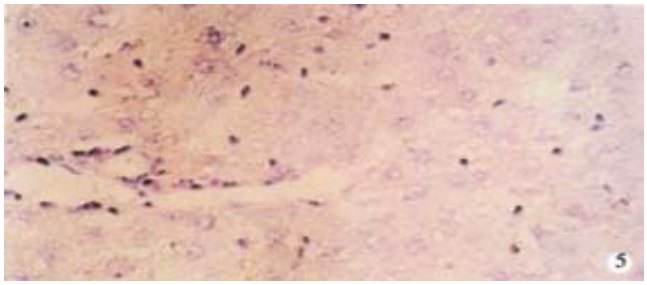
Group	*n*	Pathologic grading of hepatic fibrosis						
		0	I	II	III	IV	V	VI
Normal group	10	10	0	0	0	0	0	0
Experimental group	6	0	2	3	1	0	0	0
Treatment group	6	0	0	0	1	2	2	1
Model group	6	0	0	0	0	0	3	3











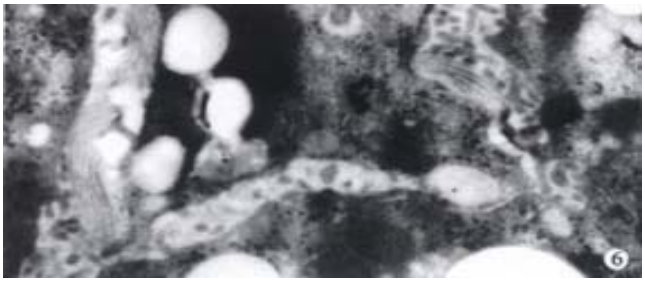


Figure 1 VG staining of rat liver (pseudolobuli were formed). ×100
Figure 2 Masson staining of rat liver. ×200
Figure 3 Collagen III in rat liver (immunohisochemistry). ×200
Figure 4 Protein expression of TIMP-1 in rat liver (immunohisochemistry). ×200
Figure 5 TIMP-1 protein expression of in rat liver after treated with asON. ×200
Figure 6 Activated HSC and lots of collagen fibers around HSC in the rat model of hepatic fibrosis EM 10K.

Electron microscopic observations

The activated hepatic stellate cell (HSC) and surrounding collagen fibers in the experimental group and the treatment group were observed. Neither deposition of collagen at the portal area in the experimental group, nor a lot of collagen deposition at the portal area were found in the treatment group. Compared with the model group, there was little change in the treatment group (Figure 6).

DISCUSSION

Nucleic acid complemented with DNA/RNA of genome is called antisense nucleic acid, including antisense DNA, antisense RNA, and ribozyme. According to the complementary principle, oligonucleotide can specifically bind to DNA/RNA of genome, therefore, the replication, transcription, translation of definite gene will be specifically inhibited and blocked. Such oligonucleotide is called antisense oligonucleotide (asON)[44]. AsON has the advantage of specificity, high potency and easy to be synthesized artificially. If asON is properly modified, it is possible to enhance the penetrating power, stability and bioavailability. Therefore, the purpose of this study mainly focused on exploring a possible way of reversing hepatic fibrosis and try to manufacture a new drug for anti-hepatic fibrosis through specifically inhibiting the gene and protein expression of TIMP-1 by asON phosphorthioate.

In our study, the expression level of TIMP-1 in the injured liver was found, and the expression appeared early with a major amplitude, especially in hepatic fibrosis and cirrhosis. The promoting effect of TIMP-1 in hepatic fibrosis and cirrhosis is stronger than TIMP-2[45-50], and 38% sequences of TIMP-2 are identical with TIMP-1, 12 cysteines of TIMP-2 are situated at the same sites, having similar spatial structure with TIMP-1, but they are non-glycosylated. There is no cross immunity reaction between TIMP-1 and TIMP-2[51-56]. TIMP-1 was chosen as the target gene. In our study, we found that the expression of TIMP-1 in the model group was four times higher than that in the normal control group. However, after asON phosphorthioate was injected through coccygeal vein, the gene and protein expression of TIMP-1 was remarkably lower than those in the other experimental groups ($P<0.001$). This result was demonstrated by the detection of TIMP-1 in liver by the immunohistochemical method and PCR. Our study clearly indicate that the asON phosphorthioate can be expressed *in vivo*, and is able to block the gene and protein expression of TIMP-1 in experimental hepatic fibrosis rats so as to enable the reversion of hepatic fibrosis.

In healthy human liver, the collagen type I, III accounts for about 80% of the total collagen of liver, while it rises up to more than 95% in fibrotic livers. The collagen type I covers about 60%-70% of the total collagen of fibrotic livers, and type III only 20%-30%[57-60]. MMP-1 is the main protease which can be inhibited by TIMP-1. Collagen I, III is the main target of MMP-1. MMP-1 has similar capacity of degrading collagen I, III. Therefore, collagen I, III are regarded as the important parameters to reflect the metabolism of collagen. Through the observation of the quantity of the collagen, we can judge the therapeutic effect of the anti-fibrotic drugs of the liver[61-63].

It was found that, in a same experimental animal treated with a same factor, the change of the hepatic content of collagen I, III showed a high uniformity ($r = 0.904$, $P<0.01$). The hepatic content of collagen I, III was lower in the experimental group (treated with as ON) and the treatment group with Colchicine than that in the model group. This suggests that the therapeutic factors have some effect in reversing the hepatic fibrosis. Immunohistological examination revealed that the content of collagen I, III was significantly lower in the experimental group than that in the treatment group with chlocoichne, while the mRNA levels of procollagen I, III were similar. In the liver of all experimental animals, both the hepatic content of collagen I, III and the mRNA level were significantly higher than the normal control group, which suggests the intervening factors do not reverse the hepatic fibrosis to normal within a period of time. This also shows the limitation of the therapeutic factor at present, which is demonstrated by the pathologic grading of hepatic fibrosis and the electron microscopic findings.

Histological examination revealed that asON therapy had better effect on hepatic fibrosis than colchicine. The asON is efficacious to reverse the hepatic fibrosis. However, colchicine had extremely limited effect on the therapy of hepatic fibrosis. The pathologic grading of hepatic fibrosis showed no remarkable difference as compared with the model group, although chlocoichne had some inhibitory effect on the rat hepatic content of collagen I, III and mRNA level.

Until now, the study on gene therapy for hepatic fibrosis and cirrhosis is limited to the animal models, and not yet applied to human beings[14,64]. The target gene is HGF[65] or TGF-β[66]. It has not yet been reported to make TIMP as the target gene. These developments are noteworthy, but there are many works to be done before it can be used in clinical practice[67].

To sum up, the animal experiment,

pathological examination and electronmicroscopic observation have proved that the asON phosphorthioate directed to TIMP-1 has some anti-hepatic fibrosis effect in the experimental immune hepatic fibrosis rat models. This result is quite heartening, but many studies should be done, especially the study of internalization of asON *in vivo*.

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Edited by Wu XN and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Hepatitis C virus in human B lymphocytes transformed by Epstein-Barr virus *in vitro* by in situ reverse transcriptase-polymerase chain reaction

Ji Lin Cheng,Bao Ling Liu, Yi Zhang, Wen Bin Tong, Zheng Yan and Bai Fang Feng

Subject headings hepatitis C-like viruses; herpes virus 4, human; B-lymphocytes; cells cultured

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Abstract

AIM To study persistence and replication of hepatitis C virus (HCV) in patients' peripheral blood mononuclear cells (PBMC) cultured *in vitro*.

METHODS Epstein-Barr virus (EBV) was used to transform the hepatitis C virus from a HCV positive patient to permanent lymphoblastoid cell lines (LCL). Positive and negative HCV RNA strands of the cultured cells and growth media were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) each month. Core and NS5 proteins of HCV were further tested using immunohistochemical SP method and in situ RT-PCR.

RESULTS HCV RNA positive strands were consistently detected the cultured cells for one year. The negative-strand RNA in LCL cells and the positive-strand RNA in supernatants were observed intermittently. Immunohistochemical results medicated expression of HCV NS3 and C proteins in LCL cytoplasm mostly. The positive signal of PCR product was dark blue and mainly localized to the LCL cytoplasm. The RT-PCR signal was eliminated by overnight RNase digestion but not DNase digestion.

CONCLUSION HCV may exist and remain functional in a cultured cell line for a long period.

Institute of Hepatology, People's Hospital, Medical Center of Beijing University, Beijing 100044, China
Ji Lin Cheng, received his M.D. from Beijing Medical University in 1999. He is currently undergoing postdoctoral training of the Second Medical University in Shanghai. Dr. Cheng studies mechanism and therapy related to chronic hepatic diseases.
Correspondence to: Ji Lin Cheng, Department of Gastroenterology, Xinhua Hospital of Shanghai Second Medical University, Shanghai 200092, China
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INTRODUCTION

Hepatitis C virus has a positive-strand RNA genome of about 10kb in length, which is grouped in the family Flaviviridae[1,2]. The viral particles are spherical with spike-like projections, and have morphological features similar to those of the Flavivirus[3]. The liver is the main target for replication of HCV *in vivo*[4,5], which occurs via minus-strand RNA as a replicative intermediate[7,8]. Recently, positive and negative strands have been detected in peripheral blood mononuclear cells (PBMC) isolated from HCV infected patients by both RT-PCR[6,7] and *in situ* hybridization[9]. PBMC are therefore suspected as a possible site of extra-hepatic replication of HCV. It has been recognized for almost 31 years that Epstein-Barr virus (EBV) is capable of transforming lymphocytes into immortal cell lines[10]. We detected HCV RNA of the cultured cells and growth media by reverse transcriptase-polymerase chain reaction (RT-PCR) each month. Antigens of HCV were further tested using the immunohistochemical and the streptavidin/peroxidase (SP) staining methods and *in situ* RT-PCR. Our results offer strong evidence for the persistence of HCV RNA in mononuclear blood cells.

MATERIALS AND METHODS

Human peripheral blood mononuclear cells

PBMCs were obtained from a female patient. PBMCs were whose serum tested positive for both anti-HCV antibodies and HCV RNA isolated from heparinized peripheral blood by using a diatrizoate-Ficoll (Eurobic) density gradient and washed three times in PBS before being resuspended in RPMI-1640 medium containing 20% fetal calf serum (FCS) (Gibco BRL).

Ebstein-Barr virus preparation

Viral stocks were prepared from the growth medium of a B95-8 cell line EBV-transformed marmoset lymphocyte cells. The viral stocks were centrifuged at 400 × *g* to sediment cells, frozen and thawed three times, and then passed through a 0.45 μm Millipore filter. They were determined to be free of mycoplasma and bacteria by culture.

Cell culture[11,12]

Before exposure to EBV, isolated PBMC populations were maintained for 13-24 hours at 2×10^6 cells/mL in 96-well plates. This procedure removed many phagocytic cells. Medium was RPMI-1640 supplemented with 20% heat-inactivated (56℃, 30 min) fetal calf serum, penicillin (50U/mL), and streptomycin (50 μg/mL). The cells were cultured at 37℃ in an atmosphere of 5% CO_2. Phytohemagglutinin A-M (PHA-M) (Difco) was dissolved in RPMI-1640, sterilized by passage through a 0.45 μm Millipore filter, and added to the lypmphocytes 24 hours before addition of EBV. Twenty-four hours after EBV transformation, the cultures were treated with Cyclosporin A (0.5 g/mL and 1.0 g/mL).

Stock of B lymphocytes transformed by EBV and its growth medium. After about one month, B lymphocytes transformed by EBV and subcultured were observed to have achieved immortalization. The culture medium was exchanged semi-quantity every week. The exchanged LCL was centrifuged at 400 × *g* for 5 minutes to sediment the cells. The cell pellets were placed in RPMI medium containing 20% FCS and 10% DMSO. The cells and supernatants were separately stored in liquid nitrogen. Before PCR, the fresh or stored sedimentary cells were washed ten times in DEPC-treated PBS. The last wash was collected and saved. The cells were diluted at 5×10^7 cells/mL.

RNA purification

Total RNA was extracted from 100 uL of culture supernatant or from 5×10^7 cells resuspended in 100 μL of DEPC-treated water by a single-step method as described by Chomczinsky[13].

Reverse transcription and nested PCR[14-17]

The synthesis of cDNA and the two rounds of PCR were performed using oligonucleotide primers from the highly conserved, untranslated 5'- region of the genome: P1 (Sense strand: 5'-CTGTGAGGAACTACTGTCTT-3', nucleotides 28-47), P2 (Antisense strand: 5'-AACACTACTCGGCTAGCAGT-3', nucleotides 229-248) for the first PCR round and P3 (Sense strand: 5'-TTCACGCAGAAAGCGTCTAG-3', nucleotides 46-65), P4 (Antisense strand: 5'-GTTGATCCAAGAAAGGACCC-3', nucleotides 171-190) for the second PCR round. Detection of the HCV positive strand: ten L of the RNA solution was denatured at 70℃ for 10 min and incubated in 42℃ for 40 min with 1U AMV and 50pmol the outer antisense oligonucletide primer (P2). Synthesis of cDNA was stopped by heating the samples at 95℃ for 10 min. Amplification of the DNA was performed using 10 uL cDNA solution and 50pmol one of the outer primers (P1). Thirty cycles of DNA amplification were carried out, followed by an extension step for 10 min at 72℃. Each cycle of PCR consisted of 95℃ for 60s, 55℃ for 90s and 72℃ for 120s. The second PCR was carried out in the same way with 5 uL of the first PCR mixture and 50pmol of each inner primer (P3 and P4).The amplified DNA was detected by 6% polyacrylamide gel electrophoresis and ethidium bromide staining. The size of the second DNA fragment generated by the PCR was 145 bp. Detection of the HCV negative-strand: 10 μL RNA solution was denatured at 70 ℃ for 10 min and incubated in 42℃ for 40 min with 1U AMV and 50pmol of the outer sense oligonucleotide primer (P1). The following PCR was as same as the detection of the HCV positive-strand.

Immunocytochemistry

Immunocytochemistry was performed according to streptavidin/peroxidase (SP) staining method [18-21] using antibodies against HCV Core and NS5. The anti-HCV Core monoclonal antibody was kindly supplied by L Haoying (Academy of Military Medical Sciences, Beijing, China). The antibody was produced by immunizing a mouse with synthetic peptide based on the sequence of the HCV genome. Monoclonal antibodies to HCV NS3 proteins were produced against a recombinant HCV NS5 protein and were kindly supplied by Zhan Meiyun (Institute of Virology, Chinese Academy of Preventive Medicine) and Gao Jianen (Institute of Hepatology, People's Hospital, Beijing Medical University). Histostain TM-SP kit was a ZYMED product (U.S.A.).

In situ RT-PCR[22-30]

LCL cells were carefully washed in sterile saline, resuspended in ice-cold 10% buffered formaldehyde solution and kept for 2 hours at 4℃. For each set of experiments, 2×10^6 cells were aliquoted in 0.5 mL Eppendorf tubes and treated with a 1% sterile saline solution of Triton-X100 (Sigma,St Louis, MO)at 4℃ for 10min with gentle shaking. Permeabilized LCL cells were then resuspended in 20 uL RT buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L $MgCl_2$) containing 10 mmol/L dithiotreitol, 200U cloned Moloney's murine leukemia virus RT (Gibco-BRL, Gaithersburg, MD), 40U RNase inhibitor (RNA guard; Pharmacia), 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 50pmol of the outer antisense oligonucletide primer (P2) of HCV. The tubes were incubated at 37℃ for 1 hour, 95℃ for 10 min to

inactivate residual RT activity, and immediately chilled on ice. After the RT step, LCL cells were washed again with ice-cold sterile saline, spun down, and resuspended in 25 uL of PCR buffer (50 mmol/ L KCl, 10 mmol/L Tris-Cl,2.5 mmol/L $MgCl_2$) containing 1 mmol/L each of dGTP, dATP, dTTP, dCTP, and 25pmol of both antisense (P2) and sense (P1) primers. After preheating the tubes for 7 min at 82℃, 1U Taq DNA polymerase (Promega) was added to each sample. Pre-melting at 95℃ for 5 min was followed by 20 cycles of 1min each at 94℃ (DNA denaturation), 55℃ (primer annealing), and 72℃ (primer extension). After the first PCR, LCL cells were washed again with ice-cold sterile saline, spun down, and resuspended in 25 uL PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-Cl, 2.5 mmol/L $MgCl_2$) containing 1 mmol/L each dGTP, dATP, dTTP, dCTP, and 25pmol of both antisense (P3, its N-end labelled biotin) and sense (P4) primers. The second PCR processes were the same as the first. At the end of suspension PCR, LCL cells were carefully washed 3 times again with ice-cold sterile saline and 1 time in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.4), with gentle agitation at 37℃. Cells (between 2×10^3 and 4×10^3) were spread on a glass slide coated with polytetrafluoroethylene. The slides were air dried and then washed in 2 × saline sodium citrate (SSC), buffer (0.3M sodium chloride and 0.03M sodium citrate). Amplification products were incubated with alkaline phosphatase-conjugated streptavidin (AP-SA) for one hour at 37℃. After incubation, slides were thoroughly washed with phosphate-buffered saline. The color was developed with nitroblue tetrazolium /5-bromo-4-chloro-l-3 indolyphosphate (NBT/BCIP) as the chromagen, which yields a dark blue color at the site of biotin binding avidin. The counterstain, nuclear fast red, stains nuclei pale pink and does not stain the cytoplasm.

RESULTS

Hepatitis C virus persistence in cell lines

We observed two distinct morphologies as early as 24 hours post-infection with EBV, indicating successful transformation of the virus into the B cells: ① blastogenesis became evident resulting in enlargement of the lymphocytes or ② there was increasing development of cell aggregates of proliferative lymphoblast cells (Figure 1). After one month, the EBV transformed lymphocytes exhibited huge, spherical cell bodies, a few projections on the body surface, and large, varying nuclei (Figure 2).

HCV of LCL and medium detected by nested RT-PCR

In order to observe the persistence of HCV in LCL, the appearance of viral plus-strands and minus-strands of the cell line were assayed in cell lysates and cultured media and the tenth wash of LCL each month during culturing continuously. The plus-strand HCV RNA in cell was successively detected during 12 months in the subcultured cells. The plus-strand HCV RNA in supernatants and the minus-strand HCV RNA in cells were intermittently positive RT-PCR(Table 1, Figures 3-5).

Table 1 Detection of HCV-RNA positive-strand and negative-strand by RT-PCR in EBVTB and their growth medium after incubation

	Incubation months											
	1	2	3	4	5	6	7	8	9	10	11	12
Cells (p/n)	+/+	+/-	+/-	+/+	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/+
Growth medium (p/n)	+/-	-/-	-/-	-/+	-/+	-/-	-/-	-/-	+/-	-/-	-/+	-/-

p = plus-strand, m = minus strand; +:PCR positive; -: PCR negative.

HCV core and NS3 antigens of immunocytochemistry positive in LCL

Expression of the virus-encoded proteins in the cells was tested by immunocytochemistry using a monoclonal antibody to synthetic HCV core peptide and recombinant HCV NS3. The immunocytochemical staining for both antigens was detected in the cytoplasm. The staining granular was seen as a brown lump in a side cytoplasm (Figures 6-8).

To verify the specificity of the immunocytochemical staining, the following controls were employed: ① negative control, the PBMC of the normal donors; ② staining with a mouse monoclonal antibody to surface antigen of hepatitis B virus as a negative control antibody; ③ PBS taking the place of specific antibody as a replacement test; ④ the normal human PBMC transformed by EB virus as a negative control; and ⑤ omission of incubation with horseradish-peroxidase-labeled streptavidin to test for endogenous peroxidase activity in LCL. The immunocytochemical staining reaction was absent in the negative controls and endogenous peroxidase activity was not detected in the cells under the current condition of immunocytochemistry.

HCV RNA signal of PCR product in situ RT-PCR

The positive signal of PCR product was dark blue and mainly localized to LCL cytoplasm (Figure 9). The specificity of the *in situ* cDNA product was checked in LCL cells. The RT-PCR signal was eliminated by overnight RNase digestion but not DNase digestion (Figure 10).

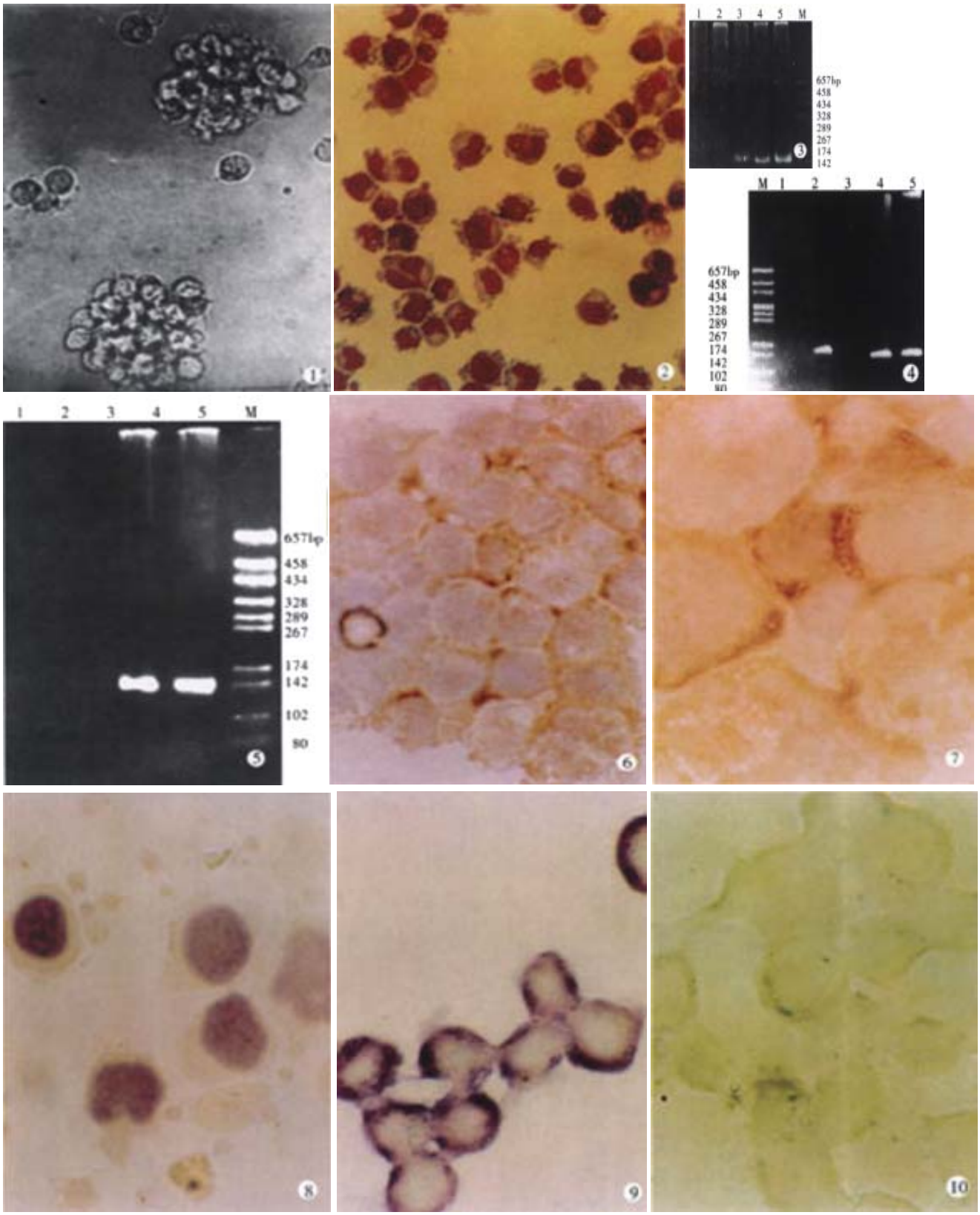

Figure 1 There was increasing development of cell aggregates of proliferative lymphoblast cells.
Figure 2 The immortal cell line of human peripheral blood mononuclear cells transformed by Epstein-Barr virus. The cell bodies are larger and sphereical. There are the false foot-like hairs or thorns on their surface. The cell nuclei are large and varied. The cells multiply and grow as cell regiments or clusters.
Figure 3 Identification of HCV RNA with RT-PCR in serum and PBMC with the chronic hepatitis C patient.
Lane 1: negative control; Lane 2: the last wash; Lane 3: HCV RNA positive serum; Lane 4: PBMC from hepatitis C patient; Lane 5: positive control. Lane M: pGEM-72f(+) / HaeIII markers
Figure 4 Identification of HCV RNA plus-strand with RT-PCR in the EBV transformed PBMC and the growth medium.

Lane 1: Negative control; Lane 2: LCL cells; Lane 3: the last wash of LCL; Lane 4: growth medium; Lane 5: positive control; Lane M: pGEM-72f(+) / HaeIII markers

Figure 5 HCV RNA minus strand with RT-PCR in the EBV transformed PBMC and the growth medium.

Lane 1: Negative control; Lane 2: growth medium; Lane 3: In cells; Lane 4: Positive control; Lane M:pGEM-72f(+) / HaeIII markers

Figure 6 Immunohistochemical SP method, DAB staining and hematoxylin counter staining of cell nuclei. NS3 antibody positive granules seen as a brown lump in the cytoplasm of LCL. ×400

Figure 7 Immunohistochemical SP method, DAB staining and hematoxylin counter-staining cell nuclei. Core antibody positive granules are seen as a brown lump with in the cytoplasm of LCL. ×400

Figure 8 Immunohistochemical negative control (anti-HBs replaced anti-HCV), a positive brown granule is not seen in the cytoplasm. ×400

Figure 9 The blue-black positive signals of HCV RNA are exhibited in the LCL cell plasma but not in the nucleolus. ×400

Figure 10 The blue-black signals are not exhibited in negative control cells *in situ* RT-PCR. ×400

DISCUSSION

Our results indicate that PBMCs from hepatitis C patients may be easily transformed into immortal lymphocytes and subcultured over a long period of time. We did not observe any signs of humoral or cellular immunity against LCL cells in the culture media. The presence of HCV in cells was not achieved by incubation with HCV-positive sera. HCV plus-strand or minus-strand RNA was detected in the cells, and the plus-strand HCV was detected in the growth media. Our results offer strong evidence for *in vivo* HCV infection of PBMC, and indicate that HCV may persist in the cells transformed by EBV and cultured *in vitro*. The detection of viral particles and HCV gene expressing proteins in the cells by immunohistochemistry[18-21] and *in situ* PCR[23-29] confirmed further that such cells may support complete HCV multiplication. Investigation of the hepatitis C virus life cycle and the evaluation of novel anti-viral strategies are limited by the lack of an efficient cell culture systems. Although chimpanzees can be successfully infected with HCV sera from human patients, they do not serve as a good animal model due to their limited numbers and high cost. Many researchers have reported using the HCV positive sera incubated with Molf-4[31], HPB-MA[31] and H9 cells[32] (the human T cell lines), fetal hepatocytes[33], bone marrow-derived B cell line[34], PBMC from a healthy donor[35], and daudi cells (a human B cell line)[22] as a cell model of *in vitro* HCV replication. These experiments have provided abundant material fowards the establishment of new satisfactory models. PBMCs have been suspected as a possible site of extra-hepatic replication of HCV and a bank of latent infection of HCV. It relates closely to relapse of chronic hepatitis C[36-41].

Just for this reason, PBMCs from hepatitis C patients were transformed by EBV into immortal cell *in vitro* and subcultured. We hope to establish a new cell model which is more representable of the true state of *in vivo* HCV infection.

The development of human cytogenetics demonstrates scientific progress on new methodological approaches. Thus, the real beginning of clinical cytogenetics was marked by introduction of the lymphocyte culture technique as an easily accessible source of mitotic cell. However, due to the limited life-span of cells, new blood sampling is necessary in cases of re-examination. This might be difficult or even impossible if patients are not available for different reasons. Our procedure is available for routine use and allows efficient transformation of peripheral B lymphocytes by EBV and thus the establishment of lymphoblastoid cell lines[42]. Compared to other methods of long-term cultivation, these cell lines have a number of advantages: ① the original material can easily be obtained from any hepatitis C patient. ② LCL is the ideal source for HCV molecular biology studies in humans as repeated HCV RNA preparations can be obtained without effort.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Carcinogenic potential of duodenal reflux juice from patients with long-standing postgastrectomy

Zhe Fu Ma[1], Zhong Yu Wang[1], Jun Ran Zhang[2], Peng Gong[1] and Hai Long Chen[1]

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Abstract

AIM To determine whether study on the carcinogenic potential of reflux juice from patients with remote gastrectomy could clarify the inherent relationship between duodenal reflux and gastric stump cancer.

METHODS A total of 37 reflux juice samples (13 BillrothI, 24 BillrothII) were employed in the present study. A two-stage transformation assay using BALB/c 3T3 cells was carried out to test the initiating or promoting activity of these samples.

RESULTS Two of 18 (11.1%) reflux samples exerted initiating activities, whereas 9/19 (47. 4%) samples enhanced the MNNG-initiating cell transformation, suggesting the duodenal reflux juice might more frequently possess the tumor-promoter activity ($P = 0.029$). In addition, there was no difference in initiating activities of the samples irrespective of surgical procedures ($P = 0.488$), while Billroth II samples exhibited stronger tumor-promoter activity than Billroth I samples ($P = 0.027$). Furthermore, the promoter activities were well correlated with the histological changes of the stomas ($r_s = 0.625$, $P = 0.004$), but neither their cytotoxicities nor initiating activities had this correlation (Probabilities were 0.523 and 0.085, respectively).

CONCLUSION The duodenal reflux juice from patients with remote postgastrectomy did have carcinogenic potential, and suggested that tumor-promoting activity should principally account for the high incidence of gastric cancer in gastrectomy patients. In contrast, it is difficult to explain the high stump-cancer incidence with the "N-nitroso compounds" theory-a popular theory for the intact stomach carcinogenesis, and it seemed to be justified to focus chemoprevention of this cancer on the tumor-promoting potential of reflux juice.

[1]Department of General Surgery, First Hospital, Dalian Medical University, Dalian 116011, China
[2]Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129, USA
Dr. Zhe Fu Ma, graduated from Shanxi Medical College in 1993, now Ph.D., M.D. in Department of Surgery, Dalian Medical University, majoring gastroenteric cancer, having 4 papers published.
Correspondence to: Dr. Zhe Fu Ma. Department of General Surgery, First Hospital, Dalian Medical University, Dalian 116011, China
Tel. 0086-411-4720334
Email. address: mazhefu@usa.net
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INTRODUCTION

Since gastric stump cancer was first described in 1922[1], it has been well established that the incidence of gastric carcinoma is increased in patients who have undergone a partial gastrectomy for peptic ulcer disease[2-6]. But the etiology and exact mechanism of gastric stump carcinogenesis are unclear. Decreased sensitivity of chief cells and parietal cells[7], alteration in gastrin level[8], hypoxia and hemodynamic changes[9], bacterial proliferation[10], and reflux[11,12] are the putative contributing factors. Among these, the excessive duodenal reflux induced by surgery seems to be the main risk factor, because the incidence of stump carcinomas is higher in Billroth II than in Billroth I[13,14], and most of the stump carcinomas are located near the stoma[15-17]. Nevertheless, what is the inherent association between the duodenal reflux and stump cancer To answer the question, a cell transformation assay was employed in our study to examine the carcinogenic potential, initiating and promoting activity, of reflux juices from patients with remote postgastrectomy, in terms of the common theory carcinogenesis is analytically considered to relate to the two stages, initiation and promotion[18-23].

MATERIALS AND METHODS

Sampling

Thirty-seven patients (10 women and 27 men; aged, 42-77 years) who underwent partial gastrectomy at least 10 years previously for benign ulcer disease received endoscopy. Each patient experienced either BillrothI or BillrothII procedures (Table 1). Endoscopy was carried out after 8 to 12 hours of fasting. The bile stained reflux fluid was aspirated in a sterile syringe, then sterilized by passing through a 0.22 μm Millipore filter and stored at -70℃ until analysis. The thirty seven patients were all eligible for this study (patients with histories of smoking were excluded from the study), and informed consent was obtained from each patient.

For the sake of enough reflux juice, samples from BillrothI or Billroth II patients were randomly extracted to make two almost equal sized groups to evaluate the initiating and promoting activity, respectively. At the end of the endoscopy, at least five biopsies in a circle around stoma were taken for histological assessment. Three grades were used which were, in ascending order of significance: chronic superficial gastritis (± minimal atrophic gastritis); atrophic gastritis/intestinal metaplasia; dysplasia[24].

Cytotoxicity assay

BALB/c 3T3 A31-1-1 cells 1×10^4 (one of three standardized cell lines generally recommended for the cell transformation assay)[25] were plated in each well of a 96-well plate covered with 100 μL DMEM (Dulbecco's modification of Eagle's medium, Gibco) supplemented with 10% FCS (fetal calf serum, Gibco) at 37℃ in a humidified incubator containing 5% CO_2 in air for 24 hours. The medium was then replaced by 100 μL medium containing reflux juice (15-20 doses designed serially per sample by a concentration gradient of 1.25% reflux juice), and further incubated for 24 hours. The culture was used as a negative control; 3-(4,5,dimethylthiazol-2yl)2,5-diphenyl tetrazolium bromide (MTT; Promega) was added (5 g/L) and the plates were incubated for a further 4 hours. The dye/medium in each well was carefully removed and 100 μL solubilization solution (Promega) was placed in each well for 1 hour. The plates were read at 570 nm in a microplate reader (Biorad 550). The mean absorbance was calculated and cell survival was expressed as the percentage absorbance of that in wells incubated with the negative control.

Transformation assay

Two-stage transformation was assayed by the protocol described by Hirakawa *et al*[26]. Only the volume of culture medium was changed from 5 mL to 4 mL. In the initial assay, actively growing cells (10^4 cells per 60 mm-diameter plastic dish) were plated. Cultures were incubated for 24 hours, reflux samples with graded concentrations (80% and 40% critical toxicity) were added for 72 hours (initiating phase), and 0.3 mg/L TPA (12-O-tetradecanoylphorbol 13-acetate) were present in the medium for 2 weeks 4 days after reflux juice was removed (promoting phase). The medium was then replaced with fresh, promoter-free medium and the culture was incubated for another 2 weeks. The culture medium was changed twice a week. Ten dishes were used for each sample in 2 independent tests. MNNG (N-methyl-N'-nitro-N-nitro-soguanidine) 0.5 mg/L and culture medium were used as positive and negative control, respectively. Finally, the culture cells were fixed and stained with Giemsa 5 weeks after plating. Type III transformed cell foci (deeply basophilic, criss-crossing, a dense layer formation and a random orientation of cells at the edges of foci[25] were counted. As for the transformation frequency (TF), the percentage of dishes with foci was calculated. In the promoting assay, 0.5 mg/L MNNG (dissolved in DMSO)was employed in the intiating phase, and reflux samples (25% toxicity) were added into the medium during the promoting phase. Twelve dishes were used for each sample in 2 independent tests. TPA 0.3 mg/L and culture medium were taken as positive and negative control, respectively. Other procedures of this assay were the same as described above.

Statistical analysis

One-way ANOVA was used to search for differences in the average TF values between BillrothI or Billroth II groups. In other sections, two-tailed Fisher's exact test was performed. A probability of $P<0.05$ was considered statistically significant and we used Bonferroni's method to get the nominal level for each comparison of TF between a sample and the culture control.

RESULTS

In the 2-stage transformation assay, without a known promoter TPA, a 3-day initiating treatment with a potent carcinogen MNNG at 0.5 mg/L had not significantly yielded an increasing number of transformed foci. While MNNG caused a very remarkable transformation with subsequent TPA (0.3 mg/L) promoting treatment (data not shown). The results suggested that the present assay system work well enough to examine whether an agent could exert an initiating or promoting activity. In addition, the doses of bile samples used in the cell transformation assay were selected based on the results of MTT (toxicity) assay. A small concentration gradient (1.25% reflux juice, v:v) was used in MTT assay to obtain the critical cytotoxicity more accurately. Because the toxicity of the samples were different (ranging from 75 to 250 μL aspirate/mL medium), a unified criterion (the same percent of each sample's own toxicity) was used to compare TF values among various samples effectively. In the initiating assay, the aspirate concentration was extended to a high dose, 80% critical toxicity, to ensure that our conclusions are free from false negative results. A low dose, 25% toxicity, was used in the promoting assay for the sake of the culture cells having to survive a long-term exposure to reflux juice (2 weeks). In addition, this dose-design method makes it feasible to study on the carcinogenic potential independent of the cytotoxicity of reflux juice.

Carcinogenic potential in the transformation assay

Six Billroth I and I2 Billroth II samples were randomly distributed into the initiating group. Two of 18 samples exerted significantly initiating activity compared with negative control group in the TPA-promoting cell transformation assay (Figure 1a). As

analysed by subsite, 1/6 Billroth I and 1/I1 BillrothII samples were positive. There was no marked difference in the average TF values between the two groups (Figure 1b) either at the high or low doses (data not shown for the low dose, 40% toxicity).

The residual samples, 7 Billroth I and 12 Billroth II, made up the promoting group. As shown in Figure 2a, there was an increased number of transformed foci in the MNNG-initiated target cells followed by applications of 9 of 19 samples. The nine positive samples included 2 BillrothI and 7 BillrothII aspirate. A significant difference in the average TF values was observed between the two groups ($P<0.05$, Figure 2b).

In comparison of the results between the initiating and promoting assay, the promoting positive rate (9/19, 47.4%) was statistically higher than initiating positive rate (2/18, 11.1%) ($P = 0.029$). Therefore, the reflux juice might usually exhibit the promoting activity and sparsely possess the promoting potential.

Histological grades with carcinogenic potential and cytotoxicity of reflux juice

A stronger correlation (Spearman's rank correlation coefficient = 0.625, $P = 0.004$) was found between the histological grades of stoma and the promoting activity, but not between histological grades and the initiating activity (Table 2). In addition, with the upward shift of histological grades, a small decrease in the toxic concentrations of reflux juice was observed in Table 1, which did not reach statistical significance.

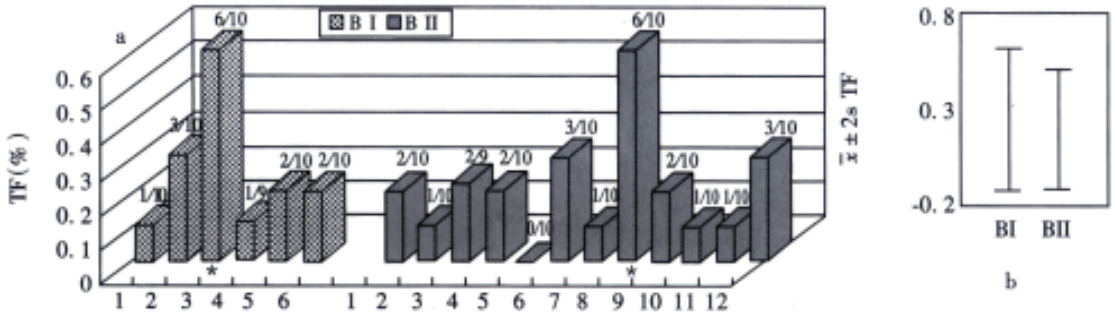

Figure 1 The intiating activity of reflux juice in tranformation assay. *Positive cases. TF of culture control was 7% (4/57). The average TF of BillrothI (BI) and Billroth II (BII) groups shown in Figure 1b.

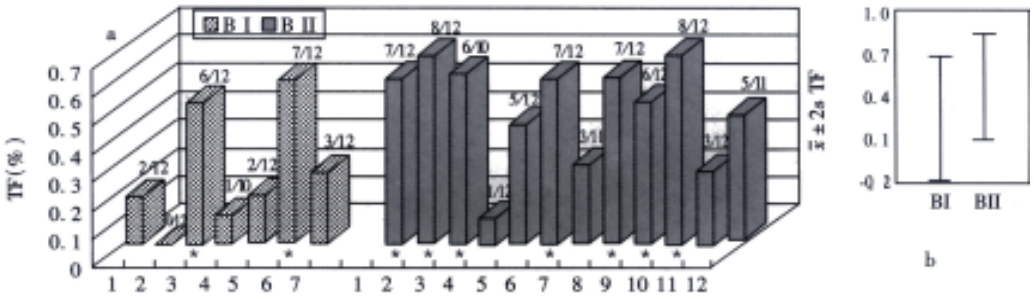

Figure 2 The promoting activity of reflux juice in tranformation assay. *Positive cases. TF of culture control was 7% (4/59). The average TF of BillrothI (BI) and Billroth II (BII) groups shown in Figure 2b.

Table 1 Operation types and critical toxicity of reflux juice in each histological grade

Histological grades	Billroth I	Billroth II	Critical toxic concentrations (%, v:v)	Correlation between histological grades and toxicity[a]
1 Chronic superficial gastritis	7	10	16.4±4.0	
2 Atrophic gastritis/intestinal metaplasia	4	7	15.8±5.0	
3 Dysplasia	2	7	12.9±4.6	r_s = -0.287, P = 0.085

[a]Two tailed spearman's rank correlation.

Table 2 Histological grades of stoma and carcinogenic potential of reflux juice in the cell transformation assay

Histological grades	TF (%)		Correlation coefficient[a]	
	Initiating	Promoting	Initiating	Promoting
1 Chronic superficial gastritis	20.1±18.4 (8)	25.3±18.7 (9)		
2 Atrophic gastritis/intestinal metaplasia	26.0±20.7 (5)	48.1±20.1 (6)	r_s = 0.161	r_s = 0.625
3 Dysplasia	20.4±7.10 (5)	56.7±10.6 (4)	P = 0.523	P = 0.004

[a]Two tailed Spearman's rank correlation. The number of the patients for each histological grade is inside the parentheses.

DISCUSSION

In present study, the duodenal reflux juice from patients with remote postgastrectomy exerted more frequently tumor-promoter activity compared to the initiating (mutagenic) activity. In addition, there was no difference in the initiating activity of reflux samples irrespective of surgical procedures. While the BillrothII aspirates exhibited a stronger tumor-promoter activity than BillrothI, in accordance with many epidemical findings, stump cancer preferred to the BillrothII procedure. All these results strongly suggested that partial gastrectomy - perse- should be responsible for the reflux promoting activity, implying an etiological role for the promoting activity of duodenal reflux juice in the pathogenesis of gastric stump cancer. It is just the tumor-promoter activity, a characteristic beyond what an intact stomach usually possesses, that may elucidate the high incidence of gastric cancer in postgastrectomy patients than in general population.

As for the etiology of gastric cancer, dietary factor has been emphasized principally since some procarcinogens, such as nitrates, can often enter the diet by vegetables, preservatives of food, even drinking water. Nitrite may derive from nitrate by the flora of the mouth or stomach (human saliva typically contains 6-10 mg/L nitrite and 15-35 mg/L nitrate[27], and further react with secondary amines to give rise to N-nitroso compounds which are strong carcinogens suspected of playing a role in upper gastrointestinal carcinogenesis[28] because of their spontaneous synthesis from dietary components and their ability to alkylate nucleic acids. This "N-nitroso compounds" theory has provided a potential explanation for some geographic regions at a very high risk for gastric cancer[29]. But whether this theory can work as well to interpret the high incidence of gastric cancer in the postgastrectomy patients is still a matter of dispute[30-34]. Although we did not measure the concentrations of N-nitroso compounds directly, the initiating assay can effectively detect the mutagenicity of the whole human reflux juice that may contain various mutagenic or carcinogenic substances inclusive of the N-nitroso compounds. When analysing our data from another angle, the initiating or mutagenic activity of reflux samples did not correlate to the histological grades of anastomotic area (Table 2), whereas the promoting activity significantly augmented with the progression of histological abnormalities. Thus, a causal role for the promoting activity of reflux juice in the pathogenesis of stump cancer was further suggested, implying that the tumor-promoter activity might principally account for the high incidence of gastric cancer in the long-standing postgastrectomy patients relative to the initiating activity. While this did not, of course, exclude that the initiating activity or mutagenic activity was indispensable to the stump carcinogenesis. But a perfect explanation for the high stump-cancer incidence seemed to be not available by the "N-nitroso compounds" theory, as it is different from the setting of intact stomach carcinoma. Rising gastric pH in the presence of bacteria after gastrectomy might not favor the formation of the mutagenic or carcinogenic compounds (e.g., N-nitroso compounds)[14,35], and those with a high concentration enough to take an initiating or mutagenic effect were seldom present in the reflux juice of postgastrectomy patients. In addition, as shown in Table 1, with the histological manifestations of the stoma, a slight increase in the non-specific cytotoxicity of reflux juice was observed. Although it did not reach the statistical significance, whether the result alluded to a synergic effect for the reflux toxicity in the gastric stump carcinogenesis deserved further studies.

What is the exact nature of the tumor-promoting species in the reflux juice Some substances exerting a persistent action (e.g., cell differentiating or proliferating[36-39]) must exist in the reflux juice. Unconjugated and secondary bile acids might be first candidates, which have been suggested to take a part in the colon cancer[40-46]. These bacteria-degraded bile acids also present in the gastric aspirate from remote postgastrectomy patients though consisting of a small portion of the whole reflux bile acids. Secondly, lysophosphatidylcholine (lysoPC), the product of phosphatidylcholine hydrolysis by phospholipase A2, has been suggested to play a role in the pathogenesis of gallbaldder cancer in the APBDJ (Anomalous Pancreaticobiliary Ductal Junction) patients[47]. LysoPC might also be produced in the stump stomach due to the reflux juice containing aberrant pancreatic juice and bile. It has been reported that lysoPC at much lower concentrations significantly enhanced the activation of protein kinase C (PKC)[48-50] and regulated cell differentiation[51] if diacylglycerol (DAG) was available. And the unconjugated bile acids happened to remarkably generate DAG[52]. Therefore, whether the above two factors could cooperate to make the reflux juice exhibit tumor-promoter activity This issue also warrants further investigations.

For the first time, we confirmed that the duodenal reflux juice from the long-standing postgastrectomy patients did exert the tumor-promoting activity and initiating activity, and further suggested that tumor-promoting potential should be mainly responsible for the high stump-cancer incidence. Simultaneously, our results demonstrated that a close relationship between the histological changes of anastomotic site and the reflux tumor-promoting activity, thus directly supporting to conduct endoscopic surveillance for postgastrectomy patients with precancerous lesions

(e.g., moderate and severe dysplasia) in stoma. In addition, to decrease the incidence of stump cancer effectively, it seems reasonable not only to perform reconstruction procedures (e.g., Roux en Yanastomosis) for those with severe duodenal reflux, but also to focus the chemoprevention of this cancer on tumor-promoting potential of the reflux juice.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Orthotopic transplantation model of human gastrointestinal cancer and detection of micrometastases

Jun Hui Cui[1], Uwe Krueger[2], Doris Henne-Bruns[2], Bernd Kremer[2] and Holger Kalthoff[2]

Subject headings mice, nude; gastrointestinal neoplasms/ pathology; neoplasm seeding; neoplasm metastasis

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Abstract

AIM To establish a relevant animal model of human gastrointestinal cancer, which can be used for repetitive investigations, so as to improve our understanding and management of carcinogenesis and cancer metastasis.

METHODS Intact tissues of human colorectal and pancreatic cancers were transplanted in nude mice. The biological characteristics of the original and the corresponding transplanted tumors were investigated by HE staining, PAS staining and immunostaining. The metastases in the livers and lungs of nude mice were investigated by immunostaining with biotinylated mab KL-1 and by RT-PCR using CK20 specific primers.

RESULTS There were totally 9 of 16 surgical specimens growing in nude mice subcutaneously and/or orthotopically (4 of 6 colorectal and 5 of 10 pancreatic cancer). Tumor cell content of the specimens and freezing of tissue specimens are important factors influencing the growth of transplanted tumor. In the group of fresh tumor tissues with greater than 50% tumor cell content, the success rate of the transplantation was 100% (3 cases of pancreatic cancer and 3 cases of colorectal cancer). The orthotopically transplanted tumors resemble the original tumor morphologically and biologically, including TAA expression such as CEA by immunohistochemistry, and CEA level in the serum of mice. Ki-67 labeling index and the expression of TAA especially K-ras, 17-1A and RA-96, are associated with the potential of tumor growth in nude mice. Micrometastases in the lungs and livers of tumor bearing mice can be detected by immunostaining with biotinylated mab KL-1 and CK20-specific RT-PCR.

CONCLUSION An orthotopic transplantation model for human colon and pancreatic cancer in nude mice has been set up. We have also established sensitive detection methods with CK-immunohistochemistry and CK20-RT-PCR to study xenotransplanted human cancer and its metastatic cancer cells in the liver and lung of nude mice. This study may be helpful in understanding the mechanism of cancer metastasis and in developing new diagnostic methods and therapeutic strategies for metastases including micrometastases.

[1]Department of General Surgery, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
[2]Department of General Surgery, Christian Albrechts University, Kiel, Germany
Dr. Jun Hui Cui, graduated from Zhejiang Medical University in 1984, earned master degree in 1990, studied in the Surgical Department of Kiel University and worked in the Lab of Molecular Oncology of Kiel University from 1994-1997, and achieved M.D. from Kiel University, Germany, now associate professor of surgery, specialized in colorectal oncology. Adviser of graduated student for master degree, having 20 publications published in key Chinese or English journals.
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Correspondence to: Dr. Jun Hui Cui, Department of Colorectal Surgery, the First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China
Tel. 0086-571-7236882, Fax. 0086-571-7072577
Email. Cjhui@mail.zj.hz.cn
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INTRODUCTION

Despite significant improvement in surgical techniques and apparently curative resection, recurrence and metastasis still occur and is the leading cause of solid malignant tumors[1-7]. Current standard diagnostic techniques are not able to detect early dissemination of cancer cells (micrometastases)[8-10], and conventional classification of tumor stages cannot account for the presence or absence of distant micrometastasis in the patients with small primary tumors. Thus, one of the most critical prognostic determinants for the subsequent clinical course is neglected in many patients[9,11-16]. Specimens from individual patients are often difficult to obtain for detailed analyses, but it will be much easier to investigate materials in animal models, for studying the various aspects of tumorigenesis, especially metastasis because any part of the tissues from the model can be taken for

detailed investigation.

Most models based on athymic nude mice have been playing an important role in evaluating many anti-cancer drugs. However, human tumors were transplanted subcutaneously and not at an orthotopically relevant organ site. The major problem of this model is that the transplanted tumors are located in an environment quite different from where most human tumors locate. Most subcutaneously transplanted tumors are surrounded by a pseudocapsule, and have little chance to invade and disseminate to the surrounding tissues and rarely metastasize, even when highly aggressive tumors have served as the source of the xenograft[17]. However, human tumor cells implanted orthotopically in the corresponding organs of nude mice can increase the metastatic capability of human tumor cells in nude mice[17-23]. As a relevant model of human gastrointestinal cancer, orthotopically transplanted tumors in nude mice can improve our knowledge about invasive growth and metastasis. Such tumor model would be available for repeated investigation of experimental diagnosis and treatment of early metastasis. It may help design more effective therapeutic strategies[6,19,22-24].

MATERIALS AND METHODS

Specimens and animals

Tumor specimens for transplantation were taken from primary lesions in pancreas, colon and rectum. Surgical tumor specimens which were directly taken from patients were aseptically removed during surgery and immediately transported and processed. Each specimen was separated into three parts: one part for transplantation of fresh specimen, one part for subsequent transplantation of DMSO-frozen specimen (mostly in 1d to 3d), and another part was stored at-80℃ for morphological, immunohistochemistry analyses and for RTPCR. Eight to twelve week old male or female athymic NMRI nude mice, which were obtained from the University Clinic Eppendorf, Hamburg, Germany, were kept in laminar-flow cabinets.

Surgical procedures of tumor transplantation

Each cancerous tissue (fresh or frozen tissue) was divided into small pieces about 2 mm in diameter. Mice were anesthetized with a mixture of 12 $g \cdot L^{-1}$ ketamin Curamed Phama GmbH) and 1.6 $g \cdot L^{-1}$ xylazin solution (Parke-Davis GmbH), The colon or pancreas was then carefully exposed and a tumor piece was then attached on the serosal surface of the colon or the wall of the pancreas, with 6-0 absorbable transmural suture. The colon or pancreas was then returned to the peritoneal cavity and the abdominal wall and the skin was closed with 6-0 absorbable suture. Immediately thereafter, another piece of the tissue was transplanted subcutaneously into the left frank of the respective mouse. For the purpose of convenience, the DMSO-frozen tissues were also transplanted into another 2-3 mice. Tumor pieces from each patient were transplanted into 2-3 mice in general.

Immunohistochemistry

A modified immunoperoxidase procedure, which was introduced in 1981 by Hsu *et al*, was used as follows. Tumor sections were air dried, fixed with acetone for 10 minutes prior to staining, transferred into PBS, blocked for 20 minutes with diluted normal horse serum, incubated for 30 minutes with primary antibody diluted in 10 $g \cdot L^{-1}$ BSA/PBS, washed for 5 minutes in PBS (if quenching of endogenous peroxidase was required, the sections were incubated for 30 minutes in methanol with 3 $mL \cdot L^{-1}$ H_2O_2), incubated for 30 min with a diluted biotinylated secondary antibody solution (biotinylated horse anti-mouse IgG 1:200 in 15 $mL \cdot L^{-1}$ horse serum/PBS). If the primary antibody was C-T84.66, biotinylated goat anti-human IgA+IgG+IgM (Jackson Inco. 1:1000) was used, washed for 5 min in PBS, incubated for 30 min with "VECTORSTAIN ELITE ABC Reagent", washed for 5 min in PBS, incubated for about 5 min in DAB solution, rinsed in tap water, counterstained and cleared, airdried and mounted. Positive and negative controls were included as mentioned above.

Monoclonal antibodies

mAb KL-1 and Biotinilated KL-1 anti-cytokeratin (IgG 1, kappa, 4 $mg \cdot L^{-1}$, Immunotech, Hamburg, Germany); CIP83 (5 $mg \cdot L^{-1}$, Kalthoff, Kiel, Germany) and chimeric-T84.66 (1:100, Neumaier, Hamburg, Germany) anti-CEA (IgG1, kappa); and MIB1 (2.5 $mg \cdot L^{-1}$) and Biotinilated MIB1(10 $mg \cdot L^{-1}$) anti-ki-67 (IgG 1, Dianova, Hamburg, Germany) were used both for investigating original and transplanted tumors. mAb CA-199, Ra-96 and 17. 1A (all 5 $mg \cdot L^{-1}$, Kalthoff, kiel, Germany) were used for investigating human original tumor only, Biotinilated KL-1 was also used for detecting micrometastatic cells in the livers and lungs of mice.

Detection of CEA level in mice serum

Serum from nude mice were measured using a Microparticle Enzyme Immunoassay (MEIA) for the quantitative measurement of CEA with the "IMx" immunoassay analyzer (Abbott Laboratories).

Micrometastases detected by CK20-specific RT-PCR

Livers and lungs of nude mice were cut into 10-20 μm slides with cryosection machine, then RNA was extracted using modified single-step RNA isolation

method with TRIzol (Gibco BRL, Eggenstein, Germany). The integrity of RNA was checked by gel-electrophoresis. RNA extracted from a pancreatic carcinoma cell line A818.4 was used as positive control, GAPDH (glycerdehyde-3-phosphate dehydrogenase) -RT-PCR was used to monitor cDNA synthesis. The detailed protocol of ck20-specific RT-PCR was described previously[25,26].

RESULTS

Mice were killed when transplanted tumors reached 1 cm or larger. There were 4 (67%) out of 6 colon cancer, 5 (50%) of 10 pancreatic cancer, totally 9 (56%) of 16 surgical specimens growing in nude mice sc and/or orthotopically. Tumor growth was observed in 0% of frozen tissues and in 63% of fresh tissues with paired samples of original tumors. When retransplanted with xeno-transplanted tumors, 39% of transplants of frozen tissues and 100% of fresh tissues grew in the mice (Table 1). In the group of fresh tumor samples with a tumor cell content ⩾50%, the success rate was 100% (6/6). While in the group with a cell content of <50%, the success rate was 40% (2/5), (Table 2). The expression of Ki-67, K-ras, 17.1A and Ra-96 antigens in human colorectal and pancreatic cancer with tumor growth in nude mice was higher than in those without tumor growth (Figures 1, 2). Almost the same morphology and CEA expressions were observed between original human tumors and corresponding xenotransplanted tumors of colon and pancreatic cancer (Figures 3-8). The serum CEA levels of mice are closely associated with the existence of tumors in nude mice (Table 3). Micrometastases in lungs and livers of tumor-bearing mice: 16 mice transplanted with colorectal, and pancreatic carcinomas from 7 cases of original tumors (5 of the 7 cases had corresponding orthotopic tumors) were investigated for liver and lung micrometastases. In the 16 mice, which were sacrificed around 13-14 weeks after transplantation, macroscopically invisible metastases were found in the lungs of 3 mice by KL-1 immunostaining and CK20-RT-PCR (2 colorectal and 1 pancreatic cancer). Additionally 2 liver metastases from colorectal cancer were detected by CK-20 specific RT-PCR only (Figures 9-11).

Table 1 Tumor growth of paired fresh (F) and DMSO-frozen (D) tissue: transplanted with original tumors and xenotransplanted tumors[a]

Type	TG / total (*n*)		Success rate (%)	
	F	D	F	D
Colon	3 / 3 (12 / 12)	0 / 3 (6 / 10)[b]	100 (100)	0 (60)
Pancreas	2 / 5 (8 / 8)	0 / 5 (1 / 8)	40 (100)	0 (13)
Total	5 / 8 (20 / 20)	0 / 8 (7 / 18)	63 (100)	0 (39)

[a]Figures in ( ) is the results of transplantation with xenotransplanted tumors; b1 mouse died after transplantation.

Table 2 Influence of tumor cell content on tumor growth of fresh surgical specimens

Tumor	TG / total (*n*)		Success rate	
	⩾50%	<50%	⩾50%	<50%
Colon	3 / 3	1 / 1	100	100
Pancreas	3 / 3	1 / 4	100	25
Total	6 / 6	2 / 5	100	40

Table 3 Serum CEA levels of mice with or without tumor

	With tumor($\bar{x} \pm s$, mg·L^{-1})	Without tumor($\bar{x} \pm s$, mg·L^{-1})	*P* value
Colon	19.33±28.77 (*n* = 17)	0.1 ± 0.06 (*n* = 7)	<0.005
Pancreas	2.85±1.89 (*n* = 11)	0.29 ± 0.2 (*n* = 7)	<0.005

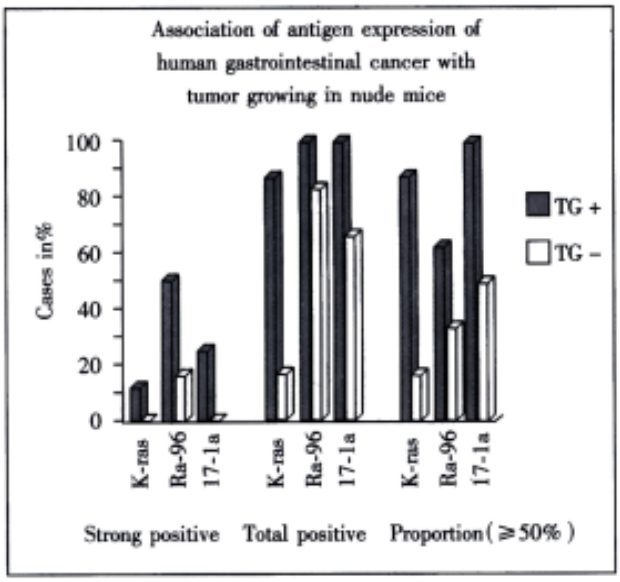


Figure 1 Ki-ras, Ra-96 and 17.1a antigen expression of original tumors.
Strong positive: with the positive intensity from "++" to "+++"
Total positive: with the positive intensity from "+" to "+++"
Proportion (⩾50%): ⩾50% of tumor cells in the section with positive staining
TG + (*n* = 8): = with tumor growth
TG - (*n* = 6): = without tumor growth

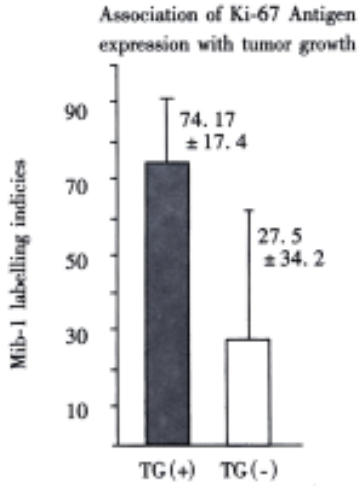
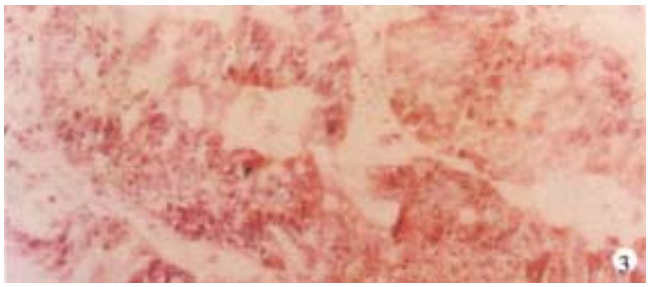
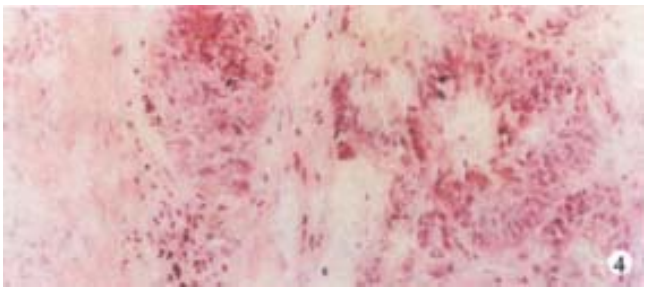
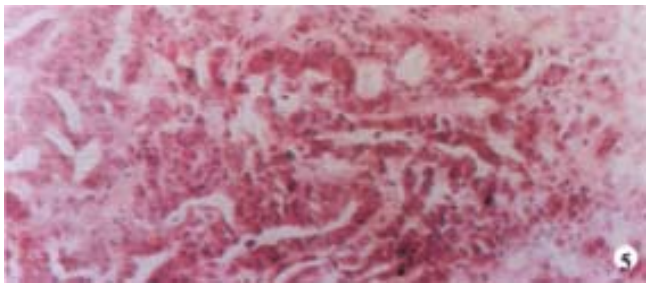
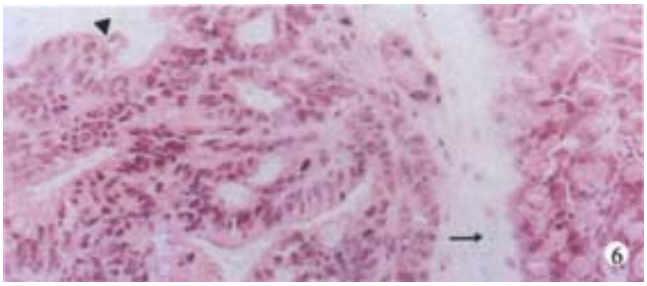


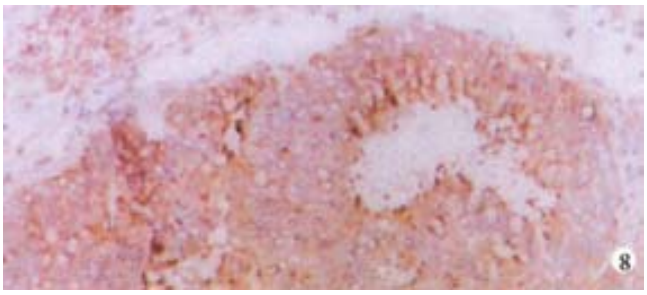
Figure 2 Ki-67 antigen expression of original tumors.
TG (+): 74.2 ± 17.4 (*n* = 9)
TG (-): 27.5 ± 34.2 (*n* = 7)
(*P*<0.005)

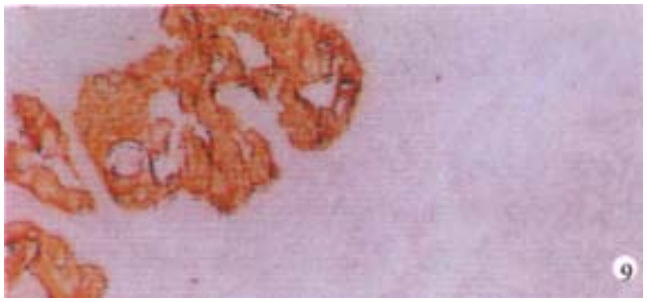












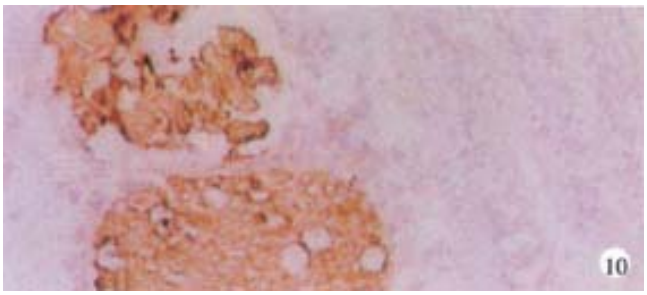


Figure 3 Human colon cancer. HE×20
Figure 4 transplanted orthotopic cancer. HE×20
Figure 5 Human pancreatic cancer. HE×20
Figure 6 Transplanted orthotopic pancreatic cancer. HE×20
Figure 7 CEA expression of human colon cancer, with immunostaining. ×20
Figure 8 CEA expression of transplanted orthotopic colon cancer, with immunostaining. ×20
Figure 9 Transplanted orthotopic colon cancer, with immunostaining staining. ×20
Figure 10 Metastatic colon cancer in the lung of nude mouse, with immunostaining. ×20

Figure 11 Specimens of 2 μg total RNA were subjected to CK-20 specific RT-PCR (485 bp product).
M. DNA molecular marker; 1:sc tumor (case A); 2:Orthotopic tumor (case A); 3:Liver (case A); 4:Lung (case A); 5:sc tumor (case B); 6:Orthotopic tumor (case B); 7:Liver (case B); 8:Lung (case B); 9:H2O; 10:A818.4 cell line (50 $\mu g \cdot L^{-1}$); 11:A818.4 cell line (50 $\mu g \cdot L^{-1}$) without reverse transcriptase.

DISCUSSION

Developing a relevant animal model of xenotransplantation of human tumors in nude mice could improve our understanding of the biological properties of human gastrointestinal cancer and lead to the development of new effective therapeutic concepts. Human tumor xenografts transplanted SC in athymic nude mice rarely give rise to metastases [24]. However, human tumor cells implanted orthotopically in the corresponding organs of nude mice can increase the metastatic capability of human tumor cells in nude mice[18,24]. Data have shown that desegregated tumor cell suspensions may not always express their fully malignant biological behavior[17,27]. Cell suspensions may have the possibility of seeding tumor cells "artificial" metastases in the surrounding tissues (i.e. peritoneal cavity) during the process of inoculation. Moreover, inoculation of desegregated tumor cell suspensions does not reflect the situation when metastatic cells spread from intact tissue growing in the human body.

Therefore, we have established an orthotopic transplantation tumor model for human tumors in nude mice with intact tissues of pancreatic, and colorectal cancer. The results showed similar morphology and biological behavior before and after transplantation in this model. Orthotopical transplanted tumors present many of the clinical manifestations of the biological behavior of gastrointestinal cancer in humans, including invasion, metastasis, and antigen expressions of CEA, CK, Ki-67, etc. CEA is a most commonly used tumor marker for gastrointestinal cancers. Recent studies showed that CEAmRNA RT-PCR or CEA immunostaining is frequently used in detection of micrometastases of gastrointestinal tract cancers [1,12,28,29]. CEA levels in serum play an important role in monitoring patients with gastrointestinal cancer [30-34]. We found that serum CEA level is obviously elevated in tumor bearing mice, suggesting that serum CEA level is a very useful marker in this model. KL-1 reacts with cytokeratins (CK), components of intracytoplasmic network of intermediate filaments (Ifs)[4,8,35], specifically reacts with epithelial tumors. Anti-CK mABs using immunocytochemistry or CK RT-PCR have been widely used for the detection of micrometastases in lymph nodes, venous blood and bone marrow[1,4,12,28,36-42]. It is satisfactory using KL-1 monoclonal antibody to detect micrometastasis in the lungs and livers of the mice in this study. Ki-67, aproliferation-associated antigen [43-46], is expressed in all phases of the cell cycle (G1, S, G2, and M) except for G0[47]. It is thought to be a useful predictor of aggressive tumor behavior and an indicator of patient survival[43]. Mib-1 is raised against recombinant parts of the Ki-67 antigen[48-52]. Our results showed that the original tumors with higher Mib-1 (Ki-67) labeling index had an increased tendency to grow in the nude mice.

Meanwhile, we established the sensitive method to detect disseminated tumor cells (micrometastasis). Sixteen tumor-bearing mice (with 5 orthotopic transplanted tumors) were used for investigating the metastasis of livers and lungs. There was no obvious metastases with naked eye and HE staining but 3 lung micrometastases with KL-1 immunostaining and CK20 RT-PCR, additionally 2 liver metastases with CK20 RT-PCR, suggesting that RT-PCR may be more sensitive than immunostaining. Many factors influence tumor growth in nude mice. On the one hand, tumor tissues are composed of cells with different biological characteristics[7]. On the other hand, different mice may have various reactions to transplantation.

Our data includes the frozen tumor tissue implantation, because the procedure is more flexible and convenient. Unfortunately, freezing of tissue specimens obviously reduced the success rate. However, for tumors with a good cellularity, freezing is a possible procedure. When xenograft tumor tissues were retransplanted (xenografts were taken from mice and frozen, then thawed and transplanted again), the tumor growth rate of pancreatic and colorectal frozen tumor tissues, was nearly 40% and only the colorectal tumors was 60%. This is likely due to the fact that xenograft tumor tissues contain more tumor cells and less damaged cells due to shorter storage time and better preservation. Tumor cell content is an important factor in our study. In the specimens with a tumor cell content greater than 50% (tumor cells vs stroma over 1:1), the success rate of fresh tumor tissues impliantation reached 100% (Table 3).

In this study, we found that TAAs, including C1P83, RA-96, 17.1A, K-ras and Ki-67 expression, were important indicators of tumor growing potential in nude mice.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Brief Reports

Inflammatory bowel disease in Estonia: a prospective epidemiologic study 1993-1998

Riina Salupere

Subject headings ulcerative colitis; Crohn disease; incidence

Salupere R. Inflammatory bowel disease in Estonia: a prospective epidemiologic study 1993-1998. ***World J Gastroenterol***, 2001;7(3):387-388

INTRODUCTION

The incidence of ulcerative colitis (UC) and Crohn's disease (CD) in Estonia in 1993-1998 was investigated prospectively. The mean annual incidence of UC was 1.7 per 100 000, and that of CD 1.4 per 100 000. This population-based study showed much lower incidence of UC and CD than those reported for western and northern Europe.

The incidence of ulcerative colitis (UC) and Crohn's disease (CD) have been studied by many doctors and every year new data[1,2] and reviews[3] published. A retrospective study of 1973-1992 revealed that UC (mean annual incidence 1.5 per 100 000) and CD (mean annual incidence 0.27 per 100 000) were not so common in Estonia as it is in other countries[4]. Estonia is a small country in northern Europe close to Scandinavian countries with the highest incidence rates of UC and CD[3]. And, therefore the aim of the present study was to prospectively estimate the incidence of UC and CD for 1993-1998.

MATERIALS AND METHODS

The population-based study included the total population within Tartu County and was based on both ambulatory and hospitalized patients at Tartu University Hospital: Department of Internal Medicine, Department of Pediatrics and Department of Surgery. Tartu County provided excellent conditions for the epidemiologic research as the advantages of small geographic size, a state-controlled health care delivery system, and universal accessibility to health care, also the accessibility of individual data and confidence in data quality. A rather stable population of Estonian origin (caucasian racial group) of the area consisted of 158516 inhabitants in 1993 and 151301 in 1998, i.e. approximately 10% of the population of Estonia. The diagnosis of UC or CD was based on endoscopic, radiological evidence or both, supported by histology from mucosal biopsies or surgical specimens using Lennard-Jones criteria. Only the patients with definite UC and CD in accordance with well-defined diagnostic criteria were included in the study. A thorough registration of the previously diagnosed UC and CD cases in 1973-1992 facilitated the confirmation of the new cases. The presence of antineutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescence was analyzed.

RESULTS

In 1993-1998 a total of 16 patients with UC and 13 patients with CD were diagnosed. The mean annual incidence of UC was 1.7 per 100 000. The mean age at the diagnosis was 41.2 years (range 16-68 years). The male to female ratio was 1.0. At the time of diagnosis 9 patients had proctosigmoiditis, 5 left-sided colitis and 2 pancolitis. Colonoscopy was done on 14 patients, barium enema on 2 patients.

The mean annual incidence of CD was 1.4 per 100 000. The mean age at the diagnosis was 36.0 years (range 5-69 years). The male to female ratio was 2.3. At the time of diagnosis 6 patients had colitis, 3 ileitis and 4 ileocolitis. Five patients were operated on due to CD. Colonoscopy was done on all patients.

IgG ANCA were detected in 7 of the 14 patients with UC and in 3 of the 11 patients with CD. The predominant ANCA staining pattern was perinuclear.

DISCUSSION

In this paper, the prospective study of 1993-1998 showed much lower incidence of UC and CD than those reported for western and northern Europe. At the same time, there seem to be no significant differences in the prevalence of ANCA compared with the data observed in different countries or in our earlier study. In our earlier study ANCA were detected in 49% patients with UC and 24% patients with CD[4]. Change over time in the incidence of CD (for 1973-1992 0.27 and for 1993-1998 1.4) may

Docent of Gastroenterology, Department of Internal Medicine, University of Tartu, Estonia
This work was supported by grants from the Estonian Science Foundation, No.1925 and No.3957.
Correspondence to: Riina Salupere, Department of Internal Medicine, University of Tartu, L.Puusepa 6, 51014 Tartu, Estonia
Tel. 372 7 318 658, 372 51 89712
Fax. 372 7 318 605, 372 7 318 607
Email. Riina.Salupere@kliinikum.ee
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suggest the bias in disease definition and case ascertainment or diagnostic access bias in the previous retrospective study or time trend in incidence.

The reasons for such a low incidence of UC and CD are not known. Therefore, several causes should be taken into account. For instance, altered concentrations of colonic microflora have been demonstrated in inflammatory bowel disease patients. It was hypothesized that this altered concentrations have been reported in association with a decrease in the potentially protective lactobacilli and bifidobacteria[5]. And, there are geographic variations in human gastrointestinal microflora, too. The intestinal microflora was compared in one-year-old Estonian and Swedish infants by quantitative culture of faecal samples[6]. The major differences in the composition of the microflora were high counts of lactobacilli and eubacteria in the Estonian and increased numbers of clostridia in the Swedish infants[6]. The causes for the differences in microflora of the Estonian and Swedish children remain unexplained. The current microflora of the Estonian infants appears to be similar to the prevailing microflora in infants of western Europa in the 1960s and 1970s[6]. Interestingly, epidemiological data generally do support steady and widespread increase in the incidence of CD during 1950-1990 in western Europa while the pattern for UC was stable[3]. Changes over time in the incidence of Crohn disease may suggest many possible factors including gastrointestinal microflora.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Expression, purification and immuno-characteristics of recombination UreB protein of *H. pylori*

Chao Wu[1], Quan Ming Zou[1], Hong Guo[2], Xiao Peng Yuan[1], Wei Jun Zhang[1], Dong Shui Lu[1] and Xu Hu Mao[1]

Subject headings *Helicobacter pylori*; urease; immunology; isolation; recombinant protein; purification

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INTRODUCTION

Helicobacter pylori (H. pylori) is associated with the development of chronic gastritis, peptic ulcer and gastric cancer and gastric MALT lymphoma[1-9]. *H. pylori* has many antigens, including urease, heat shock protein and vacuolating cytotoxin and so on, and urease is an important factor in the colonization of the gastric mucosa and suspected to cause damage to the gastric mucosa[10-14]. At the same time, urease is also one of the important protective antigens. It consists of two distict subunits with apparent molecular mass of 29.5 ku (UreA) and 66 ku (UreB), and urease B subunit is nontoxic, highly immunogenic, and an effective component of protective antigens[15-18]. In our study, Hp ureB gene was cloned in the fusion expression vector and expressed in *Escherichia coli*. We described the immunological characteristics of purified recombination UreB protein.

MATERIALS AND METHODS

Materials

pHp-UreB, plasmid that contains *H. pylori* urease B gene was constructed by Wu[19] (Third Military Medical University, Chongqing); plasmid PinPoint^{TM} Xa-3 (Promega, USA); *E.coli* JM109 (*SupE44endA*1 *hsdR*17*gyrA*96 *relA1thi* △ (*lac-proAB*)F'[*traD*36 *proAB*$^{+}$ *laclg lacz* △ M15]) was used as a host for analysis of urease B subunit expression; DNA Purification Kits (Sangon, Shanghai); a polyclonal antibody of rabbit directed against *H. pylori* was prepared by ourselves. Sera from *H. pylori* infected patients and sera from healthy people (Southwest Hospital, Chongqing); six-week-old female Balb/C mice were obtained from Laboratory Animal Centre, Third Military Medical University, Chongqing); mini-cycle^{TM}- PCR amplicon (PE Company, USA); UVP nucleic acid and protein analyser (UVP Company, USA); Bio-Rad mini-protein electrophoresis (Bio-Rad Company).

Construction of the expression vector

A 5' primer (CGTCAAGCTTATGAAA-AAGATTAGCAG) and a 3' primer (CGTCGATA-TCATCCTAGAAAATGCTAA) were used in a PCR with *Taq* polymerase to amplify the 1.7-kb fragment containing the sequences of ureB flanked by *Hin*d III and *Eco*R V restriction enzyme digestion sites. The amplification cycles were an initial denaturation step of 94℃ for 5 min, followed by 35 cycles of annealing at 55℃ for 2 min, extension at 72℃ for 2 min, and denaturation at 94 ℃ for 1 min, then extension at 72℃ for 2 min. PCR products were run on 10 g·L^{-1} agarose gels (containing 0.5 mg·L^{-1} ethidium bromide). The amplified product purified with DNA purification kits was digested with *Hin*d III and *Eco*R V, and ligated into the corresponding sites of PinPoint^{TM}Xa-3. The recombination plasmid named pPin-UreB was introduced into *E. coli* JM109 by $CaCl_2$ perforation.

Expression of the ureB gene

E.coli JM109 containing the expression plasmid pPin-UreB was grown in Luria-Bertani broth containing ampicillin(100 mg·L^{-1}) and biotin (2 μmol·L^{-1} final concentration). The culture was incubated at 37℃ and shaked at 200 r·min^{-1}, until the A600 was 0.8. Prior to adding 1 mmol·L^{-1} IPTG to cultures, a 1mL sample was taken (noninduced cell). Cultures were incubated for a further 5 h, at which time another 1 mL sample (induced cell) was taken. The noninduced and induced cell samples were later analyzed by sodium

[1]Department of Clinical Microbiology, Third Military Medical University, Chongqing 400038, China
[2]Department of Gastroenterology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China
Dr. Chao Wu, graduated from Third Military Medical University as a postgraduate in 2000, now a lecturer, specialized in diagnosis, prevention and therapy of *Helicobacter pylori* infection, having 6 papers published.
Supported by the National Major Science and Technology Projects, No.96-901-01-54.
Correspondence to: Chao Wu, Department of Clinical Microbiology and Immunology, Third Military Medical University, Chongqing 400038, China
Tel. 0086-23-68752315, Fax. 0086-23-68752316
Email. wuchao99@cta.cq.cn
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dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane by electroblotting. The membrane was incubated in TBST buffer for 60 min firstly, then in 15 mL TBST buffer containing 3 μL streeptavidin-alkaline phosphatase for 30 min at room temperature with gentle agitation. After washed with TBST for 5 min, the membrane was incubated with Promega's Western Blue R stabilized substrate for alkaline phosphatase at room temperature until the bands appear. Dark purple bands will indicate the location of the biotinylated protein species in the lanes containing cellular extracts.

Purification of the recombinant fusion protein

IPTG-induced cultures were spun at 8000 $r \cdot min^{-1}$ for 10 min at 4℃. Pellets were resuspended in cell lysis buffer (50 $mmol \cdot L^{-1}$ Tris-HCl pH 7.5, 50 $mmol \cdot L^{-1}$NaCl,50 $g \cdot L^{-1}$ glycerol) and sonicated on ice. Cellular debris were removed by centrifugation (10 000 $r \cdot min^{-1}$, 4℃, 15 min). The supernatant was added into the column containing SoftLink™- Resin, and the cell extract was captured efficiently. To elute the protein, adding a stabilizing buffer containing 50 $mmol \cdot L^{-1}$ biotin, when a volume of elution buffer equal to one-half the volume of resin in the column had been applied, stop the flow from the column. Wait 15min to allow for release of the fusion protein. The fractions containing higher concentration fusion protein were collected in the eluate. The purified fusion protein was cleaved by factor Xa protease at 37℃. The reaction products were added into SoftLink™- Resin Column again. In the process of elution, the purified UreB protein was collected.

Immunogenicity of recombinant UreB

Two groups of 10 Balb/C female mice (six week old) including controls were used as follows: ① NS control group was non-immunized mice that received NS; ② UreB group was the mice immunized with 200 μL NS containing purification rUreB protein (50 $g \cdot L^{-1}$) each time and once a week for 4 weeks under the skin of the back, and added in Freunds incomplete adjuvant for the first time. Thirty-five days after the immunization, blood was collected from the retro-orbital sinus and the antibody titer was measured with enzyme-linked immunosorbent assay (ELISA). The purified recombination UreB protein was used to coat 96-well microtiter plates (Corning-Coster Company, USA) and sheep anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was used in the assay.

Immunoreactivity of recombinant UreB

Microtiter ELISA plates were coated by incubating the plates with 5 $mg \cdot L^{-1}$ recombination UreB 0.1 mL diluted in phosphate-buffered saline (PBS) at 37℃ for 4 h. After washing plates twice with PBS, the remaining binding sites were blocked by incubating the plates with 10 $g \cdot L^{-1}$ bovine serum albumin (BSA) at 37℃ for 30 min (200 $mL \cdot well^{-1}$). The human antisera against Hp and control sera from healthy people were added to the coated well and incubated for 2 h at 37℃. After another washing procedure, HRP-conjugated sheep anti-human IgG antibody 100 μL diluted in PBS (working dilution, 1:5000) was added to each well and incubated for 1h at 37℃ and σ-phenylenediamine in PBS was used as the substrate. The enzyme substrate reaction was read with Spectra Classic spectrophotometer (Tecan, Austria) at 492 nm.

RESULTS

Construction of the expression vector

The PCR product amplified from plasmid pHp-UreB was analyzed under ultraviolet light after 10 $g \cdot L^{-1}$ agarose gel electrophoresis (Figure 1). The 1. 7kbPCR product was digested with *Hin*d III and *Eco*R V restriction enzyme and ligated into the corresponding sites of PinPoint™ Xa-3. The recombinant expression plasmid was named pPin-UreB. pPin-UreB was identified by digesting with *Hin*d III and *Eco*R V, and the 1.7kb ureB fragment was separated by electrophoresis in 10 $g \cdot L^{-1}$ agarose gel (Figure 2). Analysis of DNA sequencing, showed that the nucleotide sequence of ureB gene in plasmid pPin-UreB was the same as reported in the reference[19].

Expression of UreB gene and detection of the fusion protein

The plasmid pPin-UreB was induced to express *H. pylori* UreB protein by IPTG in *E.coli* JM109, and produced the fusion protein with predicted molecular masses of 79ku (Figure 3). The fusion protein on 100 $g \cdot L^{-1}$ polyacrylamide gel was transferred by electroblotting onto an NC membrane and was detected with Streeptavidin-Alkaline Phosphatase Detection System. The result showed that there was a positive band on the site of the fusion protein in pPin-UreB strain but not in control strains (Figure 4). Measured by UVP Protein Analyser, the biotinylated fusion protein was 150 $g \cdot kg^{-1}$ in the total bacterium protein.

Purification of recombinant UreB protein

The recombinant fusion protein expressed in *E. coli* was separated and purified by affinity chromatography with the SoftLink™- Soft Release Avidin Resin. The biotinylated fusion protein was cleaved into two parts with Factor Xa proteinase: UreB protein (66ku) and biotin tag protein (13ku).

With purification of column chromatography, the recombinant UreB protein was obtained and analyzed by SDS-PAGE and Western blotting. The purified UreB protein had predicted molecular masses of approximately 66ku and its purity was more than 95% (Figure 5).

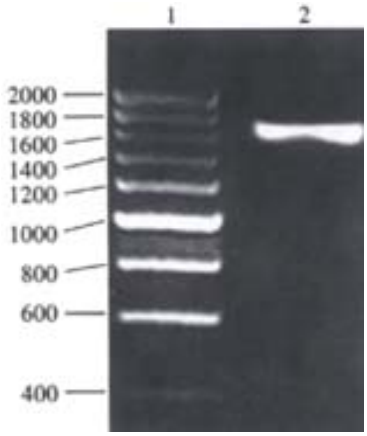

Figure 1 Analysis of the PCR product of *H. pylori* ureB gene by 1. 0% agarose gel electrophoresis.
1: 200 bp DNA ladder marker; 2: PCR product of ureB gene

Figure 2 Identification of recombinant plasmids pPin-UreB digested with *Hin*d III and *Eco*R V.
1: PCR product of ureB gene; 2: pPin-UreB plasmid digested by *Hin*d III and *Eco*RV; 3: PinPoint™Xa-3 plasmid digested with *Hin*dIII and *Eco*R V; 4:λDNA / *Hin*d III marker.

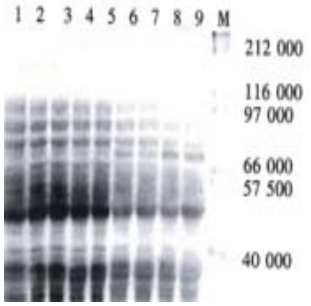

Figure 3 Analysis of expression product of recombinant plasmid pPin-UreB in *E.coli* JM109 by 10% SDS-PAGE.
1,2,3: *E.coli* JM109; 4: *E.coli* JM109 / PinPoint™Xa-3 before induction; 5: *E.coli* JM109 / PinPoint™Xa-3 after induction with IPTG; 6,7: *E.coli* JM109 / pPin-UreB before induction; 8,9; *E.coli* JM109 / pPin-UreB after induction with IPTG; M: Molecular weight marker (212, 116, 97, 66.2, 57.5, 40)×10^3.

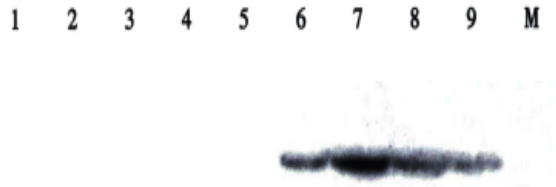

Figure 4 Analysis of recombinant fusion protein by Western-blotting.
1,2,3: *E.coli* JM109; 4: *E.coli* JM109 / PinPoint™ Xa-3 before induction; 5: *E.coli* JM109 / PinPoint™Xa-3 after induction with IPTG; 6,7:*E.coli* JM109 / pPin-UreB before induction; 8,9; *E.coli* JM109 / pPin-UreB after induction with IPTG; M: Protein molecular weight marker (212, 116, 97, 66.2, 57.5, 40)×10^3.

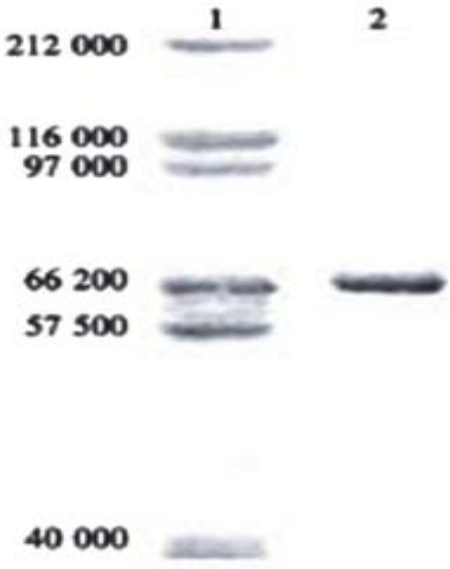

Figure 5 Determination of the purified rUreB by 10% SDS-PAGE.
1: Protein molecular weight marker; 2: The purified rUreB

Immunology character of recombinant UreB protein

Balb/C mice, immunized with recombinant UreB, generated anti-UreB antibody and the titer was detected to be 1:3000 with ELISA. However, in the mice of the control group no antibody was found. Fourty antisera against *H. pylori*and 20 control sera from healthy people were detected by ELISA with recombinant UreB protein, the coincidence ratio being 100%.

DISCUSSION

H. pylori urease is a nickel metalloenzyme, which hydrolyzes urea and is 50-100 g·kg^{-1} in total protein of the cell.*H. pylori* urease is a 550ku enzyme, consisting of two distinct subunits: UreA (29.5ku) and UreB (66ku). The ratio of subunits is approximately 1:1, suggesting a stoichiometry of

(29.5ku-66ku)6 for the native enzyme. The research of *H. pylori* urease genes suggest that ureA and ureB are structural genes, which are required for the synthesis and assembly of the 550ku apoenzyme [13], and these additional genes (ureC, ureD, ureE, ureF, ureG and ureH) are required for the expression of urease activity[13,20,21]. To express recombinant UreB protein (rUreB), we cloned *H. pylori* ureB gene by PCR and constructed the expression plasmid pPin-UreB containing *H. pylori* ureB gene. The purified rUreB was absent of urease activity in our study, which is different from what Li *et al*[22] reported. At the same time, the cloned ureB gene was sequenced and the sequence homology of nucleotide and predicted amino acid were 96.44% and 99.65% with those reported by Labigne *et al*[23]. The difference of nucleotide was due to the difference strains and gene diversity, but the obvious DNA homology suggested that the main antigenic determinants which encode *H. pylori* UreB protein were similar in difference strains[13,24,25].

Urease is the important antigen of *H. pylori*, which can stimulate significant immune response. These results suggested that the significant immunoprotection against *H. pylori* infection was induced after the purified native urease from *H. pylori* and recombinant urease were used to immune mice, and that the recombinant UreB but not rUreA protein produced immunoprotective response against *H. pylori* infection in mice after immunization separately[26-30]. In our study, the purified rUreB protein was used to immune Balb/C mice under the skin, and the antibody against rUreB was produced successfully. These demonstrated that the rUreB protein had obvious immunogenicity, and could be used in *H. pylori* vaccine research[31-33].

Individuals infected with *H. pylori* produce vast quantities of specific immunoglobulin G (IgG) antibodies in the serum[34-36]. Serum antibodies against *H. pylori* can be detected by a variety of methods including complement fixation, bacterial agglutination and immunofluorescence but enzyme-linked immunosorbent assay (ELISA) is usually used due to speed, simplicity and reproducibility [37-41]. *H. pylori* urease is a key protein used for detection of the organism by measuring serum antibody against the protein[13]. We discovered that the purified rUreB protein had a positive reaction with specific antibodies against *H. pylori* in sera of patients and negative reaction with the control sera from healthy people. The specificity and sensitivity of rUreB to *H. pylori* antisera of patients will be used in the diagnosis, assessment of curative effect and epidemiological investigation of *H. pylori*.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Transretinoic acid inhibits rats gastric epithelial dysplasia induced by N-methyl-N-nitro-N-nitrosoguanidine: influences on cell apoptosis and expression of its regulatory genes

Ru Tao Cui[1], Gan Cai[2], Zhao Bao Yin[2*], Yong Cheng[2], Qiu Hong Yang[1] and Tao Tian[3#]

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INTRODUCTION

Gastric epithelial dysplasia (GED) hypothetically is a straight-forward concept: dysplastic epithelium replacing the normal gastric epithelium of the stomach[1]. In the stomach, like any other segment of the gut, it is defined as an unequivocal non-invasive epithelial change[2,3]. The observation of gastric dysplasia as a cancerous lesion was recognized over a century ago, but it is only after the advent of gastroscopy that its clinical significance has been stressed[4-7].

All-trans retinoic acid can effectively reverse dysplasia in gastric epithelial cells, thereby inhibiting its progression to gastric cancer[8-10]. However, its mechanism is not yet clear. We used N-methyl-N-nitro-N-nitrosoguanidine (MNNG) to establish a rat model of gastric epithelial cell dysplasia, and to study the influence of all-trans retinoic acid. Changes in apoptosis and expression of Bcl-2, Fas and ICE were observed to investigate into insight of its mechanism.

[1]Liver Center, Beijing Friendship Hospital Affiliated to Capital University of Medical Sciences
[2]Department of Gastroenterology, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai China; and Department of Pathophysiology, Shanghai Medical University, Shanghai, China
[3]Pathological Department, Shanghai Medical University
*Now working at UCLA, USA
#Now working at University of Michigan, USA
Dr. Ru Tao Cui, graduated and got the bachelor degree from Shandong University of Traditional Chinese Medicine in 1994, got doctor degree from Shanghai University of Traditional Chinese Medicine in 2000, now studying as a postdoc, majoring gastroenterology, having 29 papers published.
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Correspondence to: Dr. Ru Tao Cui, Liver Center, Beijing Friendship Hospital, Capital University of Medical Sciences, Beijing 100050, China (The original address of this project: Department of Gastroenterology, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200021, China)
Email. cuirutao@yahoo.com.cn
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MATERIALS AND METHODS

Induction of gastric epithelial cell dysplasia and treatment with all-trans retinoic acid

Forty-five 8-week-old male Wistar rats weighing 120 g-140 g were housed in individual cages at a controlled temperature of 22℃, and a relative humidity of 50%. These animals were randomly divided into three groups each 15 rats. Group 1 served as blank control. Groups 2 and 3 were fed with MNNG to induce gastric epithelial cell dysplasia. Two grams MNNG (Fluka Co.) was dissolved in 2000 mL distilled water, placed in a brown bottle and kept at 4℃. The preserved MNNG solution was further diluted to the concentration of 1 g/mL for use as drinking water ad lib[11,12]. In addition, at one, three, five and seven weeks, 2 mL absolute alcohol was infused into the stomach of each animal. After 24 weeks, when gastric epithelial cell dysplasia had been induced, animals in group 2 were given 40 μg/kg all-trans retinoic acid (Shanghai No.6 Pharmaceutical Co.) through infusion into the stomach every day. Group 3 served as treatment controls and a placebo (distilled water) was given instead of all-trans retinoic acid. The animals were killed at 36 weeks.

The stomachs of the rats were cut along the greater curvature. Specimens were taken from the pyloric area and five paraffin sections were made for each rat following the conventional method. These sections were used for routine pathological examinations, apoptosis determination and measurements for the expression of Bcl-2, Fas and ICE. Pathological diagnoses and gradings were carried out according to the criteria set by the National Gastric Cancer Research Group[13,14].

Determination of apoptosis by TUNEL method

After dewaxing a 4 μm thick section, the fundamental steps of the procedure were: addition of proteinase K (20 μL/mL); incubated at 37℃; addition of Triton x-100 in 0.1% sodium citrate on ice, stood up; addition of 50 g TUNEL (Boehringer Co., Cat. no. 1684817); incubated at 37℃ in a humidified chamber; staining with diaminobenzidine (DAB); sealing the slide; and

examined under a light microscope. Apoptotic cells presented as brownish staining in the nucleus, although part of the cytoplasm also can be stained because of leakage of some nuclear DNA fragments. At least 500 cells were examined and the number of apoptotic cells per 100 cells calculated to arrive at the apoptosis index (AI)[15,16].

Expression of Bcl-2, Fas and ICE measured by immunohistochemical staining

Bcl-2 After dewaxing, the 4 μm thick section was stained using the Avidin-Biotion Complex (ABC) method[16]. The primary antibody used was 2 mg/L rabbit antirat Bcl-2 polyclonal antibody (Santa Cruz Co. USA, Cat. No. Sc-578) diluted 1:50.

Fas To detect Fas activity, sections were stained by ABC method. The primary antibody used was 2 mg/L rabbit antirat Fas polyclonal antibody (Santa Cruz Co. USA, Cat. No. sc-716G) diluted 1:50.

ICE To detect ICE activity, sections were stained by the ABC method. The primary antibody used was 2 mg/L rabbit antirat ICE polyclonal antibody (Santa Cruz Co. USA, Cat. No. sc-514) diluted 1:50.

Positivity control Slides of multiple gene-protein-positive gastric cancer specimens were used as positivite controls. If brown granules appeared on the nuclear membrane and in the cytoplasm of gastric epithelial cells, the specimen was considered to be Bcl-2 positive. If brown granules appeared on the cell membrane, the specimen was considered to be Fas positive; and if brown granules appeared in the cytoplasm, the specimen was considered to be ICE positive. The expression was graded according to the degree of staining: strongly positive (+++), moderately positive (++) and weakly positive (+). If more than 30% of cells in a slide were also considered positive. Those with strongly and moderately positive responses were considered to be overexposing the protein in question[17-20].

Probes

The following oligonucleotide probes were used in this study. Bcl-2, TGATACCAGCACTGGAGCAG, was synthesized by Shanghai Shenggong Company. Fas, CAGCCAGGAAAGATCAAACAGAGAGC, was bought from Fuzuo Company.

Northern-blot analysis

Total RNA was isolated from the gastric epithelial tissue by extraction of guanidine isothiocyanate and centrifugation in cesium chloride[21,22]. Poly(A) +RNA was selected by oligo(dt)-cellular chromatography[23]. Six micograms of poly(A) +RNA from each sample was electophoresed in 1% agarose gel containing 0.66 mol/L formaldehyde and ethidium bromide (0.66 mg/L).

After electrophoresis, the gels were photographed under UV light to confirm that approximately equal amounts of RNA were loaded. The gels were pretreated with 0.05N NaOH for 30 minutes at room temperature[24,25] and RNA was transferred onto nitrocellulose. Then, appropriate probes were labled with 32P deoxycytidine triphosphate using a Random Prime DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The blots were hybridized overnight at 62℃ (bcl-2) or 52℃ (Fas) in 59% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 mol/L NaCl, and 100 g/L of sonicated salmon sperm. Then the blots were washed in 2 × standard saline citrate (1×SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.4), and 0.5% sodium dodecyl sulfate four times at room temperature for 5 minutes and also washed (3 × 10 minutes) in 0.2 × SSC and 0.5% sodium dodecyl sulfate at 60℃. The blots were then exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70℃. The nitrocellulose filter was boiled in 0.1% SSC and 0.1% SDS for 30 minutes to strip off the radioactivity probes and rehybridized with another ^{32}P labelled Cdna prob in a similar manner[26]. The quantity of specificy transcripts in different lanes was determined by densitometric analysis of autoradiographs.

RESULTS

Histopathological changes of the gastric mucosa

The incidence of dysplasia in group 3 was significantly higher than that in group 2 (73.3% *vs* 26.7%, $P = 0.05$, Table 1).

Table 1 Histopathological changes and apoptosis of the gastric mucosa

Group	*n*	Modest dysplasia (*n*)	Modest and severe dysplasia (*n*)	Percentage (%)	Apoptotic index
Normal	15	0	0	0	8.3±3.1
DIM	15	3	11	73.3%	2.2±0.4[a]
DIMTR	15	4	4	26.7%[ab]	7.8±2.6[bc]

[a]$P<0.05$ *vs* normal; [b]$P>0.05$ *vs* normal; [c]$P>0.05$ *vs* DIM group.

Apoptosis

The apoptosis index was not significantly different in groups 1 and 2 ($P>0.05$), but there was a significant difference between groups 1 and 3 ($P<0.05$) and also between groups 2 and 3 ($P<0.05$).

Expression of apoptosis-associated proteins

Bcl-2 In group 1, two rats (13.3%) expressed Bcl-2 and one rat (6.7%) overexpressed Bcl-2. Expression of Bcl-2 was found in 10 rats (66.7%) and overexpression of Bcl-2 in five rats (33.3%) in group 3. Both the expression and overexpression

were significantly higher than that in group 1 ($P<0.05$). Group 2 had five rats (33.3%) expressing Bcl-2 and one rat (6.7%) overexpressing Bcl-2. Neither expression nor overexpression was significantly different from that of group 1 rats ($P>0.05$) but was significantly different from that of group 3 ($P<0.05$).

Fas In group 1, seven rats (46.7%) expressed Fas and two rats (13.3%) overexpressed of Fas. Group 3 had two rats (13.3%) with expression of Fas and two rats (13.3%) with overexpression of Fas. Expression of Fas was significantly different from that of group 1 ($P<0.05$), but overexpression was not ($P>0.05$). In group 2, six rats (40%) expressed Fas and four rats (26.7%) overexpressed Fas. Neither expression nor overexpression were significantly different from that of group 1 ($P>0.05$), but when compared with group 3, a significant difference was seen in the expression of Fas ($P<0.05$), yet not in overexpression ($P>0.05$).

ICE In group 1, three rats (20%) expressed ICE and no rats were found to be overexpressing this protein. Group 3 had two rats (13.3%) with expressing ICE and one rat (6.7%) with overexpressing ICE. Neither expression nor overexpression were significantly different from that in group 1 ($P>0.05$). In group 2, expression of ICE was seen in nine rats (60%) and overexpression was found in two rats (13.3%). Expression of ICE was significantly different from that in group 1 ($P<0.05$) but overexpression was not ($P>0.05$), and when compared with that of group 3, expression of ICE was significantly different ($P<0.05$) while overexpression was not ($P>0.05$).

Bcl-2 and Fas mRNA expression

The expression of bcl-2 mRNA increased and Fas mRNA was decreased in comparison of group 2 with group 1. The expression of bcl-2 mRNA was decreased and Fas mRNA was increased in comparison of group 2 with group 3 (Figure 1).

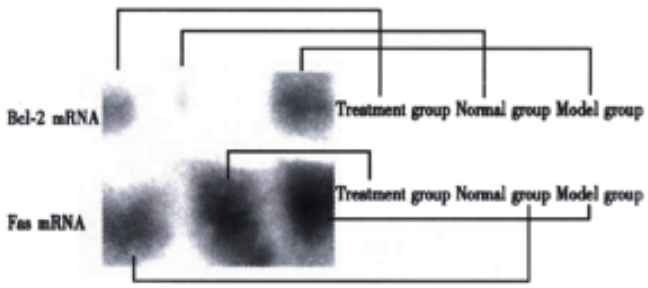

Figure 1 Bcl-2 and Fas mRNA expression.

DISCUSSION

With the introduction of fiberoptic endoscopy in the late 1960s and early 1970s, Nakamura and Nagayo in Japan were among the first to identify possible precancerous lesion on biopsical material and develop several categories for dysplasia[27,28]. In the West, Grundmann in 1975 quote for the first time the word dysplasia to describe exclusively precancerous gastric lesions[29]. Shortly after the WHO committee sanctioned this usage and detailed general diagnostic principles based on cellular atypia, abnormal differentiation and disorganised architecture[30,31]. There are differences between the Japanese and the Western criteria. A multicenter study is now under way to unify the diagnostic criteria[32-34]. So far there has been no unifed criteria of dysplasia among Chinese. We used the Japanese criteria in this study.

Is dysplasia reversible Several investigators have addressed the issue, but the cumulative results remain inconclusive. There are still coutroversies in the interpretation of atrophic changes and intestinal metaplasia resulting in wide discrepancy in the conclusions reached by different authors. Controlled, long-term prospective studies conducted in different ethnic and geographic settings are needed to provide sound evidence-based answers to the question of reversibility of atrophy, intestinal metaplasia, and epithelial dysplasia[6,35], trying to look for new drugs to reverse the dysplasia of great importance.

Normal gastric mucosal epithelial cells undergo apoptosis to clear up the senile cells and maintain the physiological balance of mucosal epithelial cells[36]. The homeostasis of gastric epithelial cells is maintained by the balance between cell proliferation and apoptosis. Alterations of these physiological cellular events in chronic pathological conditions of the stomach, as far as the proliferative pattern is concerned, an increase in the total number of epithelial proliferating cells and an abnormal distribution of the latter are frequently observed in chronic gastritis, gastric atrophy, intestinal metaplasia, gastric dysplasia and gastric cancer. Conversely, apoptosis has been found to be impaired in intestinal metaplasia, gastric dysplasia and cancer[37,38]. We consider the development of gastric cancer as a simple problem of balance, which is explained in the following formula: cumulative rate of epithelial cells' proliferation rate of epithelial cells' apoptotic rate of epithelial cells. When overaccumulation of gastric mucosal epithelial cells due to any reason, there is the possibility of development of gastric cancer. Thus, abnormal apoptosis might be one of the causes of gastric cancer development.

Apoptosis is modulated by regulatory genes. Bcl-2, Fas and ICE are the regulatory genes that have been predominantly studied. The statement of Bcl-2 can inhibit apoptosis, allowing the

proliferating cells to accumulate and inhibiting the removal of the malignant potential cells, thereby facilitating the development of cancerous change. Several scientists have studied the relation among bcl-2, gastric epithelial dysplasia and apoptosis and found that the expression abnormality leads to the apoptosis changed, the result in dysplasia and carcinoma[19,39-42]. The statement of Fas promotes apoptosis and his abnormality has relation with dysplasia too[43]. Interleukin-1-coverzyme can induce apoptosis in certain types of cells[44-46].

A few investigation of bcl-2 expression and dysplasia found that bcl-2 expression did not correlate with the presence or degree of dysplasia in either benign gastric mucosa or gastric carcinoma (GC) patients. bcl-2 protein is frequently expressed in GC[39,47]. The reason perhaps is the difference of rat's model and human gastric epithelial dysplasia, model induced by MNNG caused mainly by chemistrial carcinogenese, is not all the same with human.

Retinoic acid and its analog retinoid has a reverse effect on experimentally induced gastric mucosal precancerous lesions in rats[8-10,48]. The results of the present study further confirmed this. The results of the present study also revealed that retinoic acid can inhibit the overstatement of the Bcl-2 protein, promote the normal statement of the Fas protein and enhance the overstatement of the ICE protein, thereby promoting the apoptosis of gastric mucosal dysplastic epithelial cells. This may be one of the mechanisms by which retinoic acid reverses gastric mucosal precancerous lesions and the high statement of ICE may partly explain the side effects of retinoic acid.

The results of the present study reveal that in moderate and severe gastric mucosal dysplasia precancerous lesions, there is already abnormality in apoptosis and changes in associated genes. The increase in Bcl-2 statement, decrease in Fas statement and inhibition of apoptosis may be an important mechanisms in the progression of dysplasia to cancer.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Gastrin, somatostatin, and experimental disturbance of the gastrointestinal tract in rats

Yong Li Yao[1], Bo Xu[2], Wan Dai Zhang[1] and Yu Gang Song[1]

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INTRODUCTION

The field of gastrointestinal hormones has expanded at a dizzying rate[1-4]. Gastrointestinal hormones as regulatory peptides that appear to be major components of bodily integration and have important regulatory actions on physiological function of the gastrointestinal tract. The successful isolation of some gastrointestinal hormones and the development of sensitive methods for their detection have led to the unexpected finding that they also exist in the brain. Recent studies[5-8] have indicated that some disorders of gastrointestinal tract are related to gastrointestinal hormones. We explored this relationship by measuring concentrations of gastrin, somatostatin, G cell and D cell.

MATERIALS AND METHODS

We used healthy adult male Sprague Dawley rats weighing 220 g ± 30 g. The rats were housed in individual cages in a temperature-controlled room (23℃ ± 2℃) with a 12 h light dark cycle. The rats were fed standard rat chow (provided by Experimental Animal Center, First Military Medical University) and water ad labium. Rats were acclimatized to the environment for 7 days prior to the experiments.

Reserpine was obtained from the Hongqi Pharmaceutical Factory (lot No.960313). Si Junzi Tang, composed of ginseng, bighead, Fuling and Zhi Gancao, was prepared by routine method.

Fifty rats were randomly divided into five groups of ten rats each. Group A, the negative control group was injected with physiological saline, 0.5 mL·kg^{-1}·d^{-1},ip and distilled water, 2 mL, twice daily,ig, for 14 days. Group B was injected with reserpine, 0.5 mL·kg^{-1}·d^{-1},ip and distilled water, 2 mL, twice daily,ig, for 14 days. Group C was reserpine (0.5 mL·kg^{-1}·d^{-1}, ip) and Si Junzi Tang (2 mL, twice daily,ig), for 14 days. Group D was first injected with reserpine and distilled water for 14 days (similar to Group B). Group D was then injected with distilled water (2mL twice daily, ig) for 6 days. Group E was first injected with reserpine and water for 14 days (similar to Group B). Group E was then injected with Si Junzi Tang (2 mL, twice daily, ig) for 6 days.

We collected samples of body fluids (intestinal juice[9], gastric juice[10], plasma) and tissues (gastric antrum, jejunum, hypothalamus[11]) after treatment was finished for each group. All samples were stored at -70 ℃ until analysis. All gastroduodenal sections collected were fixed with neutral-buffered 10% formalin and embedded in paraffin.

Gastrin levels in samples were measured with radioimmunoassay kits from the Chinese Atomic Energy Research Institute of Immunotechnology. Somatostatin levels were measured with radioimmunoassay kits from Dong-Ya Research Institute of Immunotechnology. G cells and D cells of gastroduodenal mucosa were analyzed with immunohistochemical technique (using polyclonal antibody to gastrin and somatostatin) and the Quantimet 500 image analysis system (Leica, USA). Staining was performed using a streptavidin/peroxidase kit (SP™, ZYMED, USA). Immunostaining by replacing primary antibody with PBS was also conducted as a negative control. G cells and D cells of gastroduodenal mucosa were stained in continuous sections. Under magnification ×400, five randomly-selected cyclograms of each section were entered into the main computer of Quantimet 500 image analysis system from the photograph system. The main computer system analyzed graphs with corresponding software and obtained data of cell number, even square and even grey. The ratio of the cell number to square of G/D cells was calculated in continuous sections of G cells and D cells stained as follows: ratio of number of G/D cells = G cells number / D cells number; ratio of square on G/D cells = G cells square / D cells square.

[1]Institute of Gastrointestinal Diseases, Nanfang Hospital, First Military Medical University, Guangzhou 510515, Guangdong Province, China
[2]Department of Orthopedics Nanfang Hospital, First Military Medical University, Guangzhou 510515, Guangdong Province, China
Yong Li Yao, graduated from First Military Medical University with a master degree in 1998. She now works in the Institute of Gastrointestinal Diseases, Nanfang Hospital as a doctoral candidate majoring gastrointestinal diseases and gastrointestinal hormones, she has published 8 papers.
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Correspondence to: Yong Li Yao, Institute of Gastrointestinal Diseases, Nanfang Hospital, First Military Medical University, Guangzhou 510515, Guangdong Province, China
Tel. 0086-20-85141544
Email. xbyyl@fimmu.edu.cn
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Statistical analysis

Data were expressed as mean ± standard error of the mean. Experimental results were analyzed by analysis of variance and *t* tests for multiple comparisons. Statistical significance was determined at $P<0.05$.

RESULTS

Levels of gastrin and somatostatin in body fluids and tissues

The levels of gastrin in all samples tested from Group B (treated with reserpine alone) were 20%-60% less than Group A (controls). Levels of gastrin in Group D (treated with reserpine, the water) were also less than in Group A, though higher than in Group B. Levels of somatostatin were 50%-200% higher the Group B than in Group A. Levels of somatostatin were also higher than in Group A, though not as high as in Group B (Tables 1-4).

Table 1 Gastrin in body fluids (n = 10, $\bar{x} \pm s$, ng/L)

Group	Plasma	Gastric juice	Intestinal juice
A	168.40±38.01	48.72±12.55	147.36±31.47
B	109.46±40.88[a]	30.78±6.81[a]	96.58±14.36[a]
C	150.38±31.75	51.17±7.56[b]	171.19±25.28[b]
D	119.48±30.21[a]	33.03±6.64[ac]	122.57±31.61[bc]
E	179.84±64.77[bd]	54.29±7.05[bd]	79.01±33.31[abd]

[a]$P<0.05$ *vs* A,[b]$P<0.05$ *vs* B, [c]$P<0.05$ *vs* C, [d]$P<0.05$ *vs* D.

Table 2 Somatostatin in body fluids (n = 10, $\bar{x} \pm s$, ng/L)

Group	Plasma	Gastric juice	Intestinal juice
A	17.93±4.46	9.70±2.14	13.43±3.08
B	32.56±7.91[a]	29.21±4.58[a]	25.74±4.16[a]
C	18.24±4.02[b]	11.69±2.53[b]	15.30±3.31[b]
D	20.87±4.68[b]	22.56±4.99[abc]	24.16±4.50[ac]
E	16.98±3.53[b]	7.64±2.26[bcd]	13.85±2.19[bd]

[a]$P<0.05$ *vs* A,[b]$P<0.05$ *vs* B, [c]$P<0.05$ *vs* C, [d]$P<0.05$ *vs* D.

Table 3 Gastrin in tissues (n = 10, $\bar{x} \pm s$, ng/g)

Group	Gastric antrum	Jejunum	Hypothalamus
A	503.77±74.32	38.57±8.14	107.85±16.36
B	232.61±53.88[a]	22.47±3.02[a]	68.09±13.40[a]
C	493.75±91.20[b]	35.76±6.41[b]	102.79±12.29[b]
D	372.31±54.35[abc]	28.46±5.48[abc]	89.95±11.11[abc]
E	535.67±57.58[bd]	41.31±7.27[bd]	110.03±12.94[bd]

[a]$P<0.05$ *vs* A,[b]$P<0.05$ *vs* B, [c]$P<0.05$ *vs* C, [d]$P<0.05$ *vs* D.

Table 4 Somatostatin in tissues (n = 10, $\bar{x} \pm s$, ng/g)

Group	Gastric antrum	Jejunum	Hypothalamus
A	175.19±26.24	23.50±6.36	43.96±6.45
B	367.15±42.30[a]	47.31±10.97[a]	66.76±6.55[a]
C	207.23±34.08[b]	21.00±5.66[b]	45.56±5.57[b]
D	327.94±46.68[abc]	25.88±7.57[b]	59.45±6.02[abc]
E	184.94±57.58[bd]	17.61±5.12[b]	39.98±5.40[bd]

[a]$P<0.05$ *vs* A,[b]$P<0.05$ *vs* B, [c]$P<0.05$ *vs* C, [d]$P<0.05$ *vs* D.

Levels of both gastrin and somatostatin in Groups C (treated concurrently with reserpine Si Junzi Tang) and E (treated first with reserpine then Si Junzi Tang) were similar to those in Group A, indicating that Si Junzi Tang may provide a protective effect against the functional disturbance caused by reserpine.

Expression of G cells and D cells in gastroduodenal mucosa

G cells of gastric antrum were mainly located in the lower 2/3 of the mucosa and rarely in the upper 1/3. G cells appeared round, elliptical, fusiform, triangular or irregular. They differed from the typical endocrine cells of gastroduodenum in that they produced long cytoplasmic and processed the end with small bulbous expansions on the putative effector cells. G cells gave off long cytoplasmic and processed terminate on D cells and also on enterochromaffin cells (Figures 1 and 3). D cells of gastric antrum were mainly located in the lower 1/3 of the mucosa and rarely in the upper 2/3 (Figure 2). Appearance of D cells was similar to that of G cells. G cells and D cells of the duodenum were mainly distributed in the intestinal glands and their appearances were similar to those of the gastric antrum. The number and even square of G cells and D cells declined, whereas the even grey of G cells and the ratio of the number and square on G/D increased in functional disturbance of gastrointestinal tract (Tables 5-7).

Table 5 G cells and D cells of gastric antrum mucosa (n = 10, $\bar{x} \pm s$)

Group	G cells			D cells		
	Number	Square($\times 10^{-6}m^2$)	Even grey	Number	Square($\times 10^{-6}m^2$)	Even grey
A	103.60±12.33	98.05±7.06	125.41±2.29	15.67±5.21	70.49±8.96	124.04±3.14
B	40.33±5.53[a]	93.47±6.55	128.24±3.03[a]	5.70±1.32[a]	45.41±5.27[a]	115.38±2.62[a]
C	72.70±10.14[ab]	94.31±5.77	125.90±3.13[b]	11.20±2.55[ab]	61.05±7.11[ab]	123.72±3.10[b]
D	50.63±7.54[abc]	87.54±8.92[abc]	123.15±4.09[abc]	8.30±1.86[abc]	43.54±5.06[ac]	123.27±2.26[b]
E	88.03±10.37[abcd]	97.16±6.24[d]	125.39±2.22[bd]	13.83±3.11[abcd]	72.71±5.88[bcd]	124.47±2.46[b]

[a]$P<0.05$ *vs* A,[b]$P<0.05$ *vs* B, [c]$P<0.05$ *vs* C, [d]$P<0.05$ *vs* D.

Table 6 G cells and D cells of jejunum mucosa (n = 10, $\bar{x} \pm s$)

Group	G cells			D cells		
	Number	Square($\times10^{-6}m^2$)	Even grey	Number	Square($\times10^{-6}m^2$)	Even grey
A	63.57±10.16	81.99±9.75	125.44±3.14	7.80±2.58	60.56±7.26	125.82±1.70
B	29.43±7.11[a]	78.49±6.94	130.24±2.01[a]	3.13±1.14[a]	41.80±5.14[a]	111.29±3.26[a]
C	54.83±10.03[ab]	73.63±6.55[ab]	124.58±2.31[b]	6.77±1.98[b]	53.92±6.86[ab]	125.94±2.58[b]
D	35.37±9.16[abc]	62.18±6.07[abc]	122.34±2.56[abc]	5.10±1.92[abc]	44.52±6.02[ac]	123.80±2.96[b]
E	65.10±9.63[bcd]	77.44±7.69[ad]	125.80±2.45[bd]	7.53±2.26[bd]	61.77±10.27[bcd]	125.47±2.76[abcd]

[a]P<0.05 *vs* A,[b]P<0.05 *vs* B, [c]P<0.05 *vs* C, [d]P<0.05 *vs* D.

Table 7 Ratio of the number and square on G/D cells (n = 10, $\bar{x} \pm s$)

Group	Gastric antrum		Jejunum	
	Number (cells)	Square	Number (cells)	Square
A	6.48±0.77	1.42±0.24	8.43±1.57	1.39±0.20
B	7.20±0.54[a]	2.05±0.37[a]	9.82±2.71[a]	1.87±0.30[a]
C	6.47±0.69	1.55±0.26[b]	8.25±1.46[b]	1.38±0.21[b]
D	5.95±1.13[b]	2.01±0.30[ac]	7.33±1.16[b]	1.18±0.25[bc]
E	6.81±0.61[d]	1.29±0.13[abcd]	8.53±1.87[b]	1.24±0.25[b]

[a]P<0.05 *vs* A,[b]P<0.05 *vs* B, [c]P<0.05 *vs* C, [d]P<0.05 *vs* D.

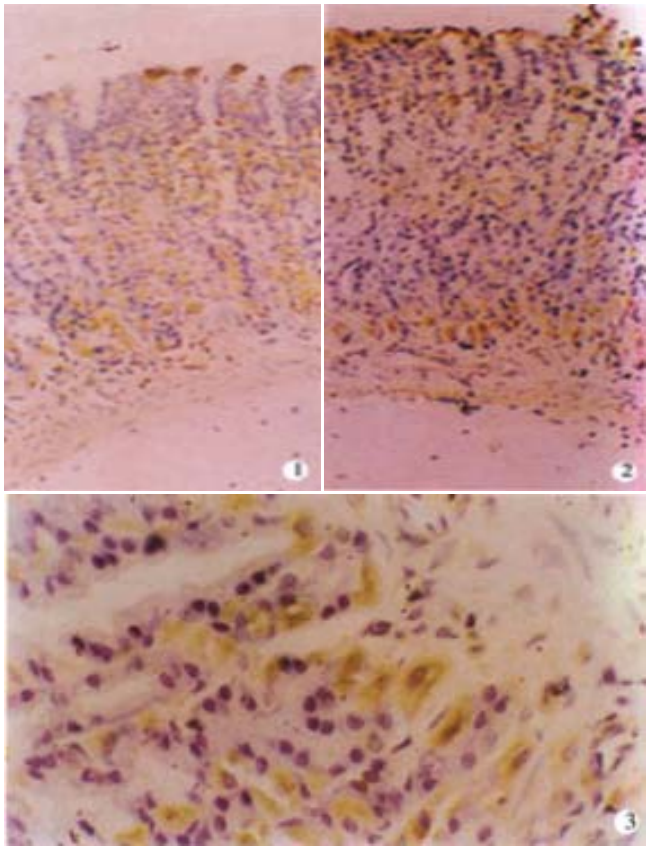

Figure 1 G cells of gastric antrum were mainly located in the lower 2/3 mucosa and rarely in the upper 1/3 mucosa. Magnification ×400

Figure 2 D cells of gastric antrum were mainly located in the lower 1/3 mucosa and rarely in the upper 2/3 mucosa. Magnification ×400

Figure 3 G cells appeared round, elliptical, fusiform, triangular or irregular. Magnification ×1000

DISCUSSION

Gastrin and somatostatin are both important gut hormones [12-21]. Edkins first discovered gastrin in 1905. However, the existence of gastrin was questioned until the middle of the 19th century. Gastrin is mostly distributed in the mucosa of gastric antrum, the mucosa of the jejunum, and the central nervous system. Gastrin has a wide range of biological actions[22-25]. The most potent actions of gastrin are stimulation of gastric acid secretion[26-29]and antral smooth muscle activity.

Somatostatin was originally isolated from extracts of sheep hypothalamus as a growth hormone inhibiting factor. In addition to its marked effect on GH secretion, the peptide[30-33] possesses a surprising range of biological effects paralleled by an equally wide but characteristic anatomical distribution. Somatostatin also appears to inhibit the secretion of many gastrointestinal hormones and may be an important regulator for gastrointestinal functions[34-37].

Upon functional disturbance of the gastrointestinal tract, we inferred[22-25] that the inhibition of the release of gastrin might lead to a decrease of basal and maximal gastric acid secretion and inhibition of the secretion of gastric proteinase and inner factor. We further anticipated a decrease in blood flow to the upper digestive tract mucosa, which would lead to a reduction in nutrition and proliferation of gastric mucosal cells. Somatostatin[30-33] inhibited not only secretion of basal gastric acid and gastric proteinase, but also the effect of gastrin on gastric acid secretion. Some studies have demonstrated that motilin[34] had a potent actions on the smooth muscle of the stomach, the duodenum, and the colon, which could enhance gastric emptying rate, colonic motility and nutrition absorption. We observed that

somatostatin inhibited the gastric emptying rate, colonic motility and nutritional absorption by inhibiting motlin secretion following administration of reserpine. We inferred that decrease of gastrin release, increase of somatostatin release, and mutual regulatory disturbance can lead to a functional disturbance of gastrointestinal tract. Treatment with Si Junzi Tang, appeared to regulate the levels of gastrin and somatostatin in body fluids and tissues. Following administration of Si Junzi Tang, both gastrin and somatostatin remained or returned to normal level in the experimental functional disturbance of gastrointestinal tract model. This result indicates that Si Junzi Tang may be used to treat disorders of gastrointestinal hormones secretion.

Somatostatin[38] is also produced by endocrine-like (D) cells of the gut and pancreas and by peripheral nerve cells. Its actions are not restricted to the hypothalamo-hypophyseal system, somatostatin may also inhibit the release of several gastrointestinal and pancreatic hormones and affect on gastric HCl and pancreatic enzyme secretion. The bulk of evidence[38-40] suggests that somatostatin is delivered directly onto the membranes of G cells and parietal cells. In agreement with the cytochemical observations of D cells processing termination on G cells. Studies on the isolated perfused stomach have shown that infusion of somatostatin antiserum results in a brisk increase of gastrin release to up to 70 per cent of maximally stimulated levels. These data suggest that G cells may be under tonic inhibitory control by somatostatin.

The reserpine induced functional disturbance caused the number of G cells, D cells, and the even square of D cells to decline, whereas the even grey of G cells evaluated and ratio of the number and square on G/D increased[39-40]. These data suggest that high levels of somatostatin detected in body fluids and tissues were partly due to the release of somatostatin by D cells. High levels of somatostatin resulted in a decrease of gastrin release. This might explain the changes observed in G cells and D cells.

The study confirmed that changes of gastrin and somatostatin are associated with gastrointestinal disorder, which is one of the important causes for pathogenesis of the gastrointestinal tract.

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Edited by Jason Carr

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Function of apoptosis and expression of the proteins *Bcl*-2, *p*53 and C-*myc* in the development of gastric cancer

An Gao Xu, Shao Guang Li, Ji Hong Liu and Ai Hua Gan

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INTRODUCTION

In China, the incidence and mortality of gastric cancer rank the second among all cancers. Recent studies indicate apoptosis could play a role in the development of cancer[1-20]. The aim of this study was investigat the insight of apoptosis and *bcl*-2, *p*53 and C-*myc* protein expression in the development of gastric cancer.

MATERIALS AND METHODS

Materials

All 122 specimens were collected by gastroscopy or surgical resection. Among these, 32 were chronic active gastritis, 29 were gastric ulcer, 17 were mild non-classic proliferation, 8 were severe non-classic proliferation, 6 were early gastric cancer, and 30 were progressive gastric cancer. The average age among those types of samples were 49.2, 46.3, 45.8, 50.3, 49.3 and 51.0, while the ratio of men to women was 20/12, 21/8, 11/6, 5/3, 5/1 and 21/9, respectively. There was no significant regularity among those samples after analyzed by statistics.

Reagents and methods

Apoptosis was detected using the TUNEL technique as reported by Ishida[1]. Cells in which the nuclear or cytoplasm was dyed brown were identified to be undergoing apoptosis. We observed five visual fields for each spechmen, and 100 nuclei were observed in each visual field. The average ratio of apoptosis cell was apoptosis index. Proteins *bcl*-2, *p*53, and C-*myc* were dyed by the ABC immunohistochemical method. Cells with obvious brown or deep brown after dying were defined to be positive.

RESULTS

The apoptosis index increased stepwise from chronic active gastritis to gastric ulcer and decreased from non-classic proliferation to early gastric cancer and progressive gastric cancer (Table 1). The expression of protein *bcl*-2 and C-*myc* increased progressively as follows: chronic active gastritis, gastric ulcer, mild non-classic proliferation, severe non-classic proliferation, and early gastric cancer. The expression of protein *bcl*-2 decreased when it developed into progressive gastric cancer while that of C-*myc* increased continually. Protein *p*53 was expressed only in severe non-classic proliferation gastric mucosa and gastric cancer.

The apoptosis index, C-*myc* and *p*53 expression of intestinal type were higher than that of diffuse type ($P<0.05$), while the *bcl*-2 expression was lower ($P<0.05$). The two types had the opposite outcomes (Table 2).

DISSCUSION

Apoptosis, programmed cell death, was first described by Kerr *et al*[21]. It is the programmed death of cells by fragmentation of DNA, cell shrinkage, and dilation of endoplasmic reticulum, followed by cell fragmentation and formation of membrane vesicles called apoptosis bodies[21-24]. Recent investigations have demonstrated that apoptosis plays a significiant role in the pathogenesis of tumors[25-30]. Screnhst have began to have realize that apoptosis, in concert with cell proliferation, is an important mechanism towards healthy tissues. Abnormal apoptosis contirbutes to the onset, development, and progression of cancer[2,31]. Stomach carcinoma is estimated to be one of the most frequent cancers worldwide[32-34].

According to Lauren, stomach cancer can be divided into adenocarcinomas of diffuse and intestinal types[35]. Ishida *et al*[1] reported the presence of apoptosis in gastric cancer tissue by using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. They pointed out that apoptosis played a decisive function in pre-cancer changes and participated in the

Research Laboratory of Digestive Disease, Huizhou Central People's Hospital, Huizhou 516001, Guangdong Province, China
Dr. An Gao Xu, graduated from Guangdong Medical College in 1984. He is an associate physician-in-chief, specializing in the research and treatment of gastrointestinal and liver tumors. He has published 24 papers and 1 book.
Supported by the Medical Research Foundation of Guangdong Province, No.1997423
Correspondence to: Dr. An Gao Xu, Research Laboratory of Digestive Disease, Huizhou Central People's Hospital, No.41 Elingbei Road, Huizhou 516001, Guangdong Province, China
Tel. 0086-752-2288160, Fax. 0086-752-2122977
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development of cancer, including epithelial hyperplasia which occurrs in the gastric mucosa. The apoptosis action in sick gastric mucosa cells decreased, cell life was prolonged, and cells were piled up. This may be the reason why gastric cancer developes, infiltrates and transfers. Kasagi *et al*[10] studied the apoptosis indexes of various levels of differentiation. There was some difference between tumor tissue of high and low differentiations (apoptosis indexes were 10.9% and 4.0%, respectively, $P<0.01$). The difference indicated gastric cancer which had a low differentiation was less likely to undergo apoptosis. Non-classic proliferation of gastric mucosa was considered to be a precancerous change. Mijic *et al* [36] reported that numeric densities of apoptosis cell are associated with tumor progression in human gastric carcinogenesis. We found that the apoptosis index decreased from mild non-proliferation to severe non-proliferation, early gastric cancer, and progressive gastric cancer. This indicated that during the development of gastric cancer, apoptosis was inhibited.

The mechanism of apoptosis modulation of gastric-intestinal epithelia is very complicated. Many genes and factors are involved. Various proteins or oncogenes and suppressor gene are involved in the process of apoptosis, including *p*53, *bcl*-2, *myc*, *ras*, Bax and the Fas/Fas system[37-41]. Bax protein expression has been identified in various human malignant tissues[7,15,42,43]. Research has indicated that *bcl*-2 is an inhibitor of apoptosis. Li *et al*[44] reported that abnormal *c-myc* and *bcl*-2 expression is an important factor in biological behavior of gastric carcinoma and can regulate apoptosis. Sundblad *et al*[8] found that the expression of *bcl*-2 increased in cells of gastric cancer. *Bcl*-2 appears to not only inhibit apoptosis, but the protein be an antagonist of apoptosis mediated by oncogenesis suppressor genes. When the expression of *bcl*-2 increased, cancer cells would resist the apoptosis induced by chemical drugs or γ-radiation during therapy. Our results indicates that when non-classic proliferation occurrs in gastric mucosa, the expression of *bcl*-2 increases significantly. Expression of *bcl*-2 reached the top at the early stage of gastric cancer and decreased in the progressive gastric cancer. *bcl*-2 might do some work both in the triggering of gastric cancer and developing of early gastric cancer. Although *bcl*-2 was a strong inhibitor to apoptosis, it could not induce the cancer alone. However, cancer has been associated with *bcl*-2 in combination with C-*myc*[45].

Gastric carcinogenesis is a gradually developed process which result from the sequential alteration of multigenes[1,46-48]. Gene *p*53 is an oncogenetic repressor. Its anti-cancer function has been realized by triggering apoptosis. If gene *p*53 is inhibited, apoptosis can not be induced. Ikeda *et al*[49] observed that the progression of gastric cancer is defined by a gradual increase of proliferation activity and constant occurrence of apoptosis. Furthermore, Ikeda reported that the naturally occurring apoptosis is induced predominantly via a *p*53-gene-independent pathway. The half life of the mutant protein *p*53 is prolonged when gene *p*53 is mutated. It is easy to detect by immunohistochemical methods. We observed that expression of *p*53 was mainly in progressive gastric cancer tissue. No *p*53 was observed in the tissue of benign stomach diseases and mild non-classic proliferation. This indicated that the mutation of *p*53 might be an event in the late gastric cancer. The cooperation of *bcl*-2 and C-*myc* could inhibit the biological function of *p*53 to suppress cell growth by keeping it in the cytoplasm. Meanwhile, the co-expression of *p*53 and C-*myc* would lead to cell apoptosis and inhibit oncogenesis[50,51]. The co-expression of *bcl*-2 and C-*myc* in the same carcinoma tissue indicates a higher level of malignancy and a lack of sensitivity to chemical therapy and radiotherapy. The prognosis is not favorable. On the other hand, the co-expression of *C-myc* and *p*53 indicates a low level of malignancy, less sensitivity to chemical therapy and radiotherapy, and a favorable prognosis. Therefore *p*53 status and the expression of *bcl*-2 by tumor cells might be good indicators of sensitivity to chemotherapy for patients with gastric cancer[52].

Table 1 Relationship between apoptosis, protein expression of *bcl*-2, *p*53,C-*myc* and each kind of gastric diseases

	Chronic active gastritis	Gastric ulcer	Non-classic proliferation		Gastric cancer	
			Mild	Severe	Early	Progressive
Sample numbers	32	29	17	8	6	30
Apoptosis index %	16.8±12.3	24.1±20.0[a]	19.3±16.4	15.7±15.2[c]	10.1±9.1[d]	6.3±6.0[e]
Bcl-2 positive	3	8	9	6	5	14
Numbers(%)	(9.4)	(27.6)[a]	(52.9)[b]	(75.0)[c]	(83.3)	(46.7)[e]
C-myc positive	5	6	6	4	3	20
Numbers(%)	(15.6)	(20.7)	(35.3)[b]	(50.0)	(50.0)	(63.3)[e]
P53 positive	0	0	0	2	2	19
Numbers(%)				(25.0)[c]	(33.3)	(63.3)[e]

*Compared with left itim: [a]$P<0.05$, gastric ulcer *vs* chronic active gastritis; [b]$P<0.05$, mild non-classic proliferation *vs* gastric ulcer; [c]$P<0.05$, severe non-classic proliferation *vs* mild non-classic proliferation; [d]$P<0.05$, early gastric cancer *vs* severe non-classic proliferation; [e]$P<0.05$, early gastric cancer *vs* progressive gastric cancer.

Table 2 Relationship between apoptosis, protein expression of *Bcl*-2, *P*53, *C-myc* and Lauren typing of gastric cancer

Lauren typing	Sample numbers	Apoptosis index %	Bcl-2 positive numbers(%)	C-myc positive numbers(%)	P53 positive numbers(%)
Intestinal	18	8.3±7.2	7(38.9)	13(77.7)	15(83.3)
Diffuse	12	5.1±4.9[a]	7(58.3)[a]	6(50.0)[a]	4(33.3)[b]

[a]$P<0.05$, Diffuse *vs* Intestinal; [b]$P<0.01$, Diffuse *vs* Intestinal.

Different pathological types of gastric cancer are associated with different physiological mechanisms. Intestinal gastric cancer had a higher level of differentiation and a closer relationship with gastric epithelial metaplasia as compared with diffuse type of gastric cancer. We observed intestinal type cancer more frequently among men and among older patients. Intestinal type cancer had a more favorable prognosis than diffuse type cancer. Also, the index of intestinal type of gastric cancer was higher than that of diffuse type ($P<0.05$), with lower expression of *bcl*-2 and higher expression of *C-myc* and *p*53. These results indicate that the pathogenic mechanism might be different between those two types of gastric cancer. Vollmers *et al*[53] had similar results, reporting that the gene expression modulation of apoptosis and the apoptosis indexes were different between type intestinal and diffuse gastric cancer. The apoptosis mechanism was different between those two types of gastric cancer.

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Edited by Jason Carr

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Preliminary results of Thymosin-a1 versus interferon-α treatment in patients with HBeAg negative and serum HBV DNA positive chronic hepatitis B

Lin Zhuang, Jing You, Bao Zhang Tang, Su Ying Ding, Kui Hua Yan, Dan Peng, Yan Mei Zhang and Lu Zhang

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious problem because of its worldwide distribution and possible adverse sequelae, such as cirrhosis and hepatocellular carcinoma. The World Health Organization estimates that HBV has infected more than 350 million people worldwide, and up to 20% of them will become chronic carriers and will be at significant risk for cirrhosis and HCC. The ultimate goal of the therapy for chronic hepatitis B is to prevent progression to cirrhosis and to prevent development of HCC. Various subgroups of hepatitis B surface antigen (HBsAg) positive patients with chronic hepatitis have been identified. Typical patients have hepatitis B e antigen (HBeAg) and HBV DNA in serum during the active phase of the disease and usually show remission if they seroconvert to antibody to HBeAg (anti-HBe). However, a subset of patients has been found to be HBeAg negative but instead to have anti-HBe and HBV DNA in serum. This form of hepatitis is characterized by a progressive and relapsing course with fluctuations of viral replication[1,2] and a poor response to interferon α (IFN-α) therapy[3-11].

Thymosin a1 (T-a1) is an immune modifier that has been shown to trigger maturational events in lymphocytes, to augment T-cell function, and to promote reconstitution of immune defects[12]. T-a1 has been shown to promote disease remission and cessation of HBV replication in patients with HBeAg-positive chronic hepatitis B without significant side effects[13,14]. Moreover, clinical trials using T-a1 in the treatment of patients with immunodeficiency or cancer indicate that this agent is nontoxic, enhances immune responsiveness and augments specific lymphocyte functions, including lymphoproliferative responses to mitogens, maturation of T-cells, antibody production, and T-cell mediated cytotoxicity[15,16]. Based on these observations, we conducted a randomized, controlled trial to compare the efficacy and the safety of T-a1 versus IFN-α therapy in anti-HBe and HBV DNA positive chronic hepatitis B.

MATERIALS AND METHODS

Materials

Forty-eight Chinese patients were enrolled in the study. All patients met the following criteria for entry: age between 18 and 60 years; presence of HBsAg in serum for at least 12 months; positive serum tests for anti-HBe and HBV DNA, documented on at least two occasions and at least 3 months apart during the 12 months before entry; aminotransferase levels higher than 1.5 times that the upper normal limit for at least 12 months; and liver biopsy taken within 3 months before enrollment showing chronic hepatitis. Eligible patients with evidence of cirrhosis were also included. Patients treated with immunosuppressive or antiviral therapy within 1 year before entry, and those with concurrent hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus infections, causes of liver disease other than HBV, intravenous drug abuse, pregnancy, malignancy, decompensated liver disease, chronic renal failure, or other serious medical illness that might interfere with this trial were excluded.

Thirty patients with the same virological and clinical characteristics, who were never treated with IFN-α and followed up for at least 12 months, were used as a historical control (HC) group to evaluate the efficacy of the therapies.

Methods

Forty-eight patients were randomly divided into two groups to receive either a 6-month course of T-a1 (Zasaxin, supplied by SciClone Pharmaceuticals Inc., San Mateo, CA) at a dose of 1.6 mg

[1]Department of Hepatology, Kunming Third Municipal People's Hospital, Kunming 650041, Yunnan Province, China
[2]Department of Infectious Diseases, The First Affiliated Hospital of Kunming Medical College, Kunming 650032, Yunnan Province, China
Lin Zhuang, graduated from Kunming Medical College in 1982, now associate professor of internal medicine, specialized in studies on gastroenterology and hepatology, having more than 40 papers published.
Correspondence to: Lin Zhuang, Department of Hepatology, Kunming Third Municipal People's Hospital, 319# Wu Jin Road, Kunming 650041, Yunnan Province, China
Tel. 0086-871-3516282
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subcutaneous injection twice a week or a 6-month course of IFN-α at a dose of 3-5 MU subcutaneous injection daily for fifteen days, then three times weekly. The patients assigned as a historical control group were followed up without specific treatment. All patients were assessed biweekly for the first 2 months of study, and then monthly for a total study duration of 12 months. Clinical and laboratory assessments consisted of a detailed history, including postinjection symptoms and physical examination; routine serum biochemical tests (serum alanine transaminase (ALT), aspartate transaminase (AST), r-glutamine transpeptidase (r-GT), alkaline phosphatase (AKP), albumin, globulin, bilirubin, etc.); complete cell count; markers of HBV replication and urine analysis. All biochemical and hematological tests were performed with routine automated techniques. HBV-markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb and lgm HBcAb) were detected by enzymelinked immunosorbent assay (ELISA) method. Serum HBV DNA was detected by polymerase chain reaction (PCR) method.

Responses were evaluated both at the end of the therapy and at the end of follow-up. A virological response was defined as sustained disappearance of serum HBV DNA, and a biochemical response as sustained normalization of serum ALT. At the end of the treatment and follow-up, a complete response was defined as HBV DNA clearance from the serum and normalization of ALT activity. Relapse was assessed on the basis of ALT flare and/or HBV DNA reappearance during the follow-up period.

Statistical analysis

Analysis of data was accomplished using Chi-square test. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Of the 48 patients enrolled in the study, 18 were randomized to receive T-a1 and 30 to receive IFN-α and all were followed up for 6- months. The three groups were not significantly different in age, sex, biochemical, histological, serological parameters and number of patients with histological evidence of cirrhosis.

The biochemical and virological modifications at the end of treatment and follow-up period in the two treated groups and the biochemical and virological events in the HC group are illustrated in Table 1. In the group receiving T-a1, serum HBV DNA was negative in 9 of 18 patients at the end of treatment. During the follow-up period, five other patients showed HBV DNA loss at the 2nd, 3rd, 5th (in 2 patients) and 6th month, respectively, whereas HBV DNA reappeared in two at the 2nd and 3rd month, respectively. In the group receiving IFN-α, 18 of 30 patients showed HBV DNA loss at the end of treatment. However, during the 6-month follow up, HBV DNA reappeared in 9 patients (in 2 patients at the 1st month, in 5 at the 2nd and in 2 at the 3rd month), while no one lost HBV DNA. In the HC group, HBV DNA became negative in 3 of 30 patients at the 6th (in 2 patients) and 12th month, respectively, whereas HBV DNA reappeared in one at the 8th month. HBV DNA loss was significantly higher in the T-a1 and IFN-α groups compared with the HC group both at the end of therapy (χ^2=11.98, P<0.01 and χ^2=19.21, P<0.01, respectively) and follow-up period (χ^2=19.58, P<0.01 and χ^2=5.46, P<0.05, respectively).

Table 1 Responses to treatment in patients with chronic hepatitis B

	T-a1 (n = 18)	IFN-α (n = 30)	HC (n = 30)
	After 6 mo of treatment		After 6 mo of follow-up
ALT normalization	7 (38.9%)	15 (50%)	3 (10%)
HBV DNA-negative	9 (50%)[b]	18 (60%)[b]	2 (6.7%)
ALT normal / HBV DNA-negative	6 (33.3%)	14 (46.7%)[b]	1 (3.3%)
	After 6 mo of follow-up		After 12 mo of follow-up
ALT normalization	12 (66.7%)[ab]	10 (33.3%)	2 (6.7%)
HBV DNA-negative	12 (66.7%)[ab]	9 (30%)	2 (6.7%)
ALT normal / HBV DNA-negative	10 (55.6%)[ab]	7 (23.3%)	1 (3.3%)

[a]P<0.05, *vs* IFN-α; [b]P<0.01, *vs* HC.

Serum ALT levels fell within the normal range in 7 of 18 patients given T-a1, in 15 of 30 patients in IFN-α group at the end of treatment and in 3 of 30 of the HC group after 6 months of follow-up. During the follow-up, ALT became normal in six patients receiving T-a1 and ALT flare occurred in one patient, whereas five patients of the IFN-α group showed ALT flare, and no one had normal ALT. In the HC group, ALT was normal in two patients between the 6th and 12th month of follow-up and ALT flare was seen in the three patients who had normal ALT during the first 6 months of follow-up. At the end of the study period, a complete response (ALT normalization and HBV DNA loss) was observed in 10 (55.6%) of 18 patients treated with T-a1, in 7 (23.3%) of 30 receiving IFN-α, and in 1 (3.3%) of 30 in HC patients (T-a1 *vs* IFN-α, P<0.05 and T-a1 *vs* HC, P<0.01).

Typical side effects of IFN-α treatment, such as flu-like syndrome, fatigue, irritability, and headache, were seen in most of the patients treated with IFN-α. However, no serious or long-term side effects were noted and no patients discontinued the treatment. Therapy with T-a1 was not associated with significant side effects. Only one patient reported local discomfort at injection sites. No systemic or constitutional symptoms were observed with T-a1 administrations.

DISCUSSION

Anti-HBe and HBV DNA-positive chronic hepatitis B is a clinical entity distinct from classical HbeAg positive chronic hepatitis B. This peculiar form of hepatitis B is usually characterized by a severe progressive outcome often leading to cirrhosis and only occasionally shows spontaneous remission. These patients present fluctuations of viral replication in which relapses of hepatitis and periods of biochemical remission and HBV DNA negativity may occur. IFN-α treatment does not appear to be as successful as in HBeAg positive disease[3-11]. Clinical studies suggested that IFN-α at the dosage ranging from 3MU to 9MU three times a week for 6months is able to suppress HBV replication in more than 50% of treated patients, but the relapse rate after treatment withdrawal is high[3-11]. In our study, a complete response was seen in 46.7% of the patients at the end of treatment and in 23.3% at the end of follow-up. Our results are similar to those previously reported, and the response rate is far from satisfactory.

The results of the present randomized, controlled trial have shown that T-a1 therapy at a dose of 1.6 mg via subcutaneous injection twice a week for 6 months is effective and safe in anti-HBe and HBV DNA-positive chronic hepatitis B, because nearly 60% of the treated patients became HBV DNA-seronegative 6 months after the end of therapy. This response rate is not only significantly higher than that of the spontaneous seroconversion rate (3.3% in this study), but also obviously higher than the response to IFN-α therapy alone (23.3%) assessed 6 months after the end of therapy. The study showed that, at the dose tested, T-a1 has the same efficacy as IFN-α in inducing clinical and virological remission. The response rate in terms of ALT normalization and/or HBV DNA loss was not significantly different in the T-a1 group as compared with the IFN-α group at the end of the treatment ($P>0.05$). But there was significantly difference in the response rate between the two groups at the end of the follow-up ($P<0.05$). The normalization of serum ALT and loss of HBV DNA were observed more frequently in the IFN-α group at the end of therapy and in the T-a1 group at the end of follow-up. Furthermore, in the T-a1 group, the response to the treatment was also observed during the follow-up period, but not in the IFN-α group. On the basis of these results and considering that ALT normalization and HBV DNA negativization may spontaneously occur in the untreated patients infected by the precore mutant virus, we retrospectively compared the two treated groups with a group of untreated patients followed for at least 12 months. The results showed that a significant higher rate of complete response occurred in the IFN-α group at the end of therapy and in the T-a1 group at the end of follow-up compared with the HC group.

It is noted that the benefit of T-a1 was not immediately apparent at the end of therapy. There was a trend for complete virological response to increase or accumulate gradually after the end of thymosin therapy. This trend was also reported in a multicenter American trial in which 5 of the 12 responders to T-a1 therapy showed a delayed response [17]. This is in contrast to therapy with IFN-α, in which responses usually occur during the first 4 months of treatment. These contrasting patterns of response were best demonstrated in a recent Italian study involving HbeAg negative, HBV DNA-positive, interferon naive patients with higher ALT level (181 ± 159U/L), in which the complete response (ALT normalization and HBV DNA loss) rate increased gradually from 29.4% at the end of therapy to 41.2% 6 months after the end of T-a1 therapy. In that study, the response to interferon therapy decreased from 43. 8% at the end of therapy to 25% 6 months after the end of therapy[18]. This trend of delayed effect of T-a1 was also reported by Chien *et al*. in patients with chronic hepatitis B recently[14]. The reasons for this delayed effect of T-a1 are not clear. The delayed response is not likely a result of direct antiviram effects similar to those of interferons. T-a1 may exert an immunoregulatory function that promotes the endogenous antiviral immune response, as previously suggested, improving the effectiveness and coordination of the host cellular immune mechanisms in clearing HBV infected hepatocytes.

It has been shown that patients treated with T-a1 have a higher peripheral blood helper T cell count (CD4) and IFN-α production by peripheral blood mononuclear cells during and after the end of T-a1 therapy[13]. In view of the immune mechanisms involved in the pathogenesis of liver injuries in chronic HBV infection, it is possible that T-a1 may activate viral-specific helper T cells and result in the amplification of the humoral immune response to viral proteins and the induction of viral antigen-specific cytotoxic T lymphocytes through secreting endogenous IFN-α, IFN-γ, interleukin-2, and tumor necrosis factor, and increase lymphocyte interleukin-2 receptor expression[19-28]. Moreover, T-a1 is able to act synergistically with endogenous IFN-α and IFN-β in stimulating natural killer activity[29]. Although T-a1 is not known to possess antiviral properties, a preliminary report showed that this agent is able to inhibit woodchuck hepatitis virus replication[30]. Hence, the delayed effect after the end of T-a1 therapy in the present study was possibly caused by the immunomodulating effect of T-a1 that induced persistently higher helper T-cell function. Because noncytolytic inhibition of HBV RNA, nucleocapsid particles, and replicative DNA intermediates by cytotoxic T lymphocytes has been described in the transgenic mouse model[31,32], it is also possible the viral clearance after T-a1 therapy,

particularly those without preceeding ALT flaring, may be mediated by noncytolytic antiviral effects of cytotoxic T lymphocytes. Clearly, further studies are needed to elucidate the possible mechanisms.

The tolerability of T-a1 was excellent and without side effects. This finding together with a lower number of weekly injections could favor better patient compliance.

In conclusion, the results of this trial indicate that, at the dosage tested, T-a1 is of potential interest in patients with anti-HBe and HBV DNA-positive chronic hepatitis B. Furthermore, compared with IFN-α, T-a1 is better tolerated and seems to induce a gradual and more sustained ALT normalization and HBV DNA negativization, so it might represent an alternative to IFN-α therapy. However, a response rate of 55.6% is still less than ideal. A more effective therapeutic approach, such as combination therapy using the immunomodulating effect of T-a1 and antiviral effect of interferon or nucleoside analogues (such as lamivudine, famciclovir, etc.), warrants further studies.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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A randomized controlled clinical trial on the treatment of Thymosin a1 versus interferon-α in patients with hepatitis B

Jing You, Lin Zhuang, Bao Zhang Tang, Wei Bo Yang, Su Ying Ding, Wu Li, Rong Xue Wu, Hong Li Zhang, Yan Mei Zhang, Shao Ming Yan and Lu Zhang

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious problem because of its worldwide distribution and possible adverse sequelae, such as cirrhosis and hepatocellular carcinoma (HCC)[1,2]. The World Health Organization estimates that HBV has infected more than 350 million people worldwide, and up to 20% of those infected with HBV will go on to become chronic carriers and be at significant risk for cirrhosis and HCC. The ultimate goals of therapy for chronic hepatitis B are to prevent progression to cirrhosis and to prevent development of HCC. Over the past 20 years, many antiviral or immunomodulatory agents, or both, have been used in patients with chronic HBV infection[3-9]. Among them, interferon alfa (IFN-α), the standard treatment for chronic HBV infection, has been shown to be effective, which induces an apparent initial response in approximately 40% of treated patients[10,11]. However, the response rate is far from satisfactory, particularly in Asian patients, the relapse rate after treatment withdrawal is high[12].

Thymosin-a1 (T-a1) is an immune modifier that has been shown to trigger maturational events in lymphocytes, to augment T-cell function, and to promote reconstitution of immune defects[13]. T-a1 has been shown to promote disease remission and cessation of HBV replication in patients with HBeAg-positive chronic hepatitis B without significant side effects[14]. Moreover, clinical trials using T-a1 in the treatment of patients with immunodeficiency or cancer indicate that this agent is nontoxic, enhances immune responsiveness and augments specific lymphocyte functions, including lymphoproliferative responses to mitogens, maturation of T-cells, antibody production, and T-cell-mediated cytotoxicity[15,16]. On the basis of these observations, we conducted a randomized, controlled trial to compare the efficacy and the safety of T-a1 versus INF-α therapy in patients with chronic hepatitis B.

MATERIALS AND METHODS

Materials

Fifty-one Chinese patients were enrolled in the study. All patients met the following criteria for entry: age between 18 and 60 years; presence of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the serum for at least 12 months; positive serum tests for HBV DNA, documented on at least two occasions, at least 3 months apart, during the 12 months before entry; aminotransferase levels higher than 1.5 times the upper normal limit for at least 12 months; and liver biopsy taken within 3 months before enrollment showing chronic hepatitis. Eligible patients with evidence of cirrhosis and severe hepatitis B were also included. Patients treated with immunosuppressive or antiviral therapy within 1 year before entry, and those with concurrent hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus infections, causes of liver disease other than HBV, intravenous drug abuse, pregnancy, malignancy, chronic renal failure, or other serious medical illness that might interfere with this trial were excluded.

Thirty patients with the same virological and clinical characteristics, never treated with IFN-α and followed up for at least 12 months were used as a historical control (HC) group to evaluate the efficacy of the therapies.

Methods

Fifty-one patients were randomly divided into two groups to receive either a 6-month course of T-a1 (Zadaxin, supplied by SciClone Pharmaceuticals Inc., San Mateo, CA) at a dose of 1.6 mg

[1]Department of Infectious Diseases, The First Affiliated Hospital of Kunming Medical College, Kunming 650032, Yunnan Province, China
[2]Department of Hepatology, Kunming Third Municipal People's Hospital, Kunming 650041, Yunnan Province, China
Jing You, graduated from Kunming Medical College in 1984, now associate professor of internal medicine, specialized in studies on gastroenterology and hepatology, having more than 40 papers published.
Correspondence to: Jing You, Department of Infectious Diseases, The First Affiliated Hospital of Kunming Medical College, 153# Xi Chang Road, Kunming 650032, Yunnan Province, China
Tel. 0086-871-5324888
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subcutaneous injection twice a week or a 6-month course of IFN-α at a dose of 3-5 MU subcutaneous injection each day for 15 days, then three times weekly. The patients assigned as a historical control group, were followed up without specific treatment. All patients were assessed biweekly for the first 2 months of study, and then monthly for a total study duration of 12 months. Clinical and laboratory assessments consisted of a detailed history, including postinjection symptoms and physical examination; routine serum biochemical tests (serum alanine transaminase (ALT), aspartate transaminase (AST), r-glutamine transpeptidase (r-GT), alkaline phosphatase (AKP), albumin, globulin, bilirubin, ets.); complete cell count; markers of HBV replication and urine analysis. All biochemical and hematological tests were performed with routine automated techniques. HBV-markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and Igm HBcAb) were detected by enzyme-linked immunosorbent assay (ELISA) method. Serum HBV DNA was detected by polymerase chain reaction (PCR) method.

Responses were evaluated both at the end of therapy and at the end of follow-up. A complete virological response was defined as a sustained loss of serum HBeAg in association with the disappearance of serum HBV DNA during the 12 months study. A biochemical response was defined as sustained normalization of serum ALT. At the end of treatment and follow-up, a complete response was defined as HBV DNA and HBeAg clearance from the serum and normalization of ALT activity. Relapse was assessed on the basis of ALT flare and/or HBV DNA/HBeAg reappearance during the follow-up period.

Statistical analysis

Analysis of data was accomplished using Chi-square test. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Of the 81 patients enrolled in the study, 30 had never been treated for HBV with IFN-α, and 2 were non-responders to earlier IFN-α therapy who received T-a1 in the study. Eighteen patients were randomized to receive T-a1 and 33 to receive IFN-α. All patients completed the 6-month follow-up. The three groups were not significantly different in age, sex, biochemical, histological, serological parameters and number of patients with histological evidence of cirrhosis.

The biochemical and virological modifications at the end of treatment and follow-up period in the two treated groups and the biochemical and virological events in the HC group are illustrated in Tables 1 and 2. In the group receiving T-a1, serum HBV DNA was negative in 10 of 18 patients at the end of treatment. During the follow-up period, five other patients showed HBV DNA loss at the 2nd, 3rd, 4th (in 2 patients) and 6th month, respectively, whereas HBV DNA reappeared in two (at the 3rd and 5th month, respectively). In the group receiving IFN-α, 22 of 33 patients showed HBV DNA loss at the end of treatment. However, during the 6 months of follow-up, HBV DNA reappeared in 9 patients (in 3 patients at the 1st month, in 4 at the 2nd and in 2 at the 3rd month), while no one lost HBV DNA. In the HC group, HBV DNA became negative in 3 of 30 patients (at the 5th, 6th, and 11th month, respectively), whereas HBV DNA reappeared in one (at the 7th month). HBV DNA loss was significantly higher in the T-al and IFN-α groups compared with the HC group both at the end of therapy (χ^2=28.97, P<0.01 and χ^2=23.99, P<0.01, respectively) and follow-up period (χ^2=22.49, P<0.01 and χ^2=9.27, P<0.01, respectively). The rates of seroconvertion of HBVe antigen antibody in the T-al, IFN-α and HC groups at the end of treatment were 33.3% (6/18), 45.5% (15/33) and 3.3% (1/30), respectively; and at the end of follow-up 55.6% (10/18), 27.3% (9/33) and 3.3% (1/30),respectively. Serum ALT levels fell within the normal range in 7 of 18 patients given T-al, in 16 of 33 patients in IFN-α group at the end of treatment and in 5 of 30 of the HC group after 6 months of follow-up. During the follow-up, five patients receiving T-al normalized ALT and one patient showed ALT flare, whereas six patients of the IFN-α group showed ALT flare, and no one normalized ALT. In the HC group, two patients normalized ALT between the 6th and 12th month of follow-up and an ALT flare was seen in the four patients who normalized ALT during the first 6 months of follow-up. At the end of the study period a complete response (ALT normalization and HBV DNA/HBeAg loss) was observed in 10 of 18 (55.6%) patients treated with T-al, in 9 of 33 (27.3%) receiving IFN-α, and in 1 of 30 (3.3%) in HC patients (T-al *vs* IFN-α, HC,P<0.01).

Table 1 Responses to treatment at the end of therapy *n*(%)

	T-a1 (n = 18)	IFN-α (n = 33)	HC (n = 30)
	(after 6 mo of follow-up)		
ALT normalization	7 (38.9)	16 (48.5)	5 (16.7)
HBV DNA-negative	10 (55.6)[b]	22 (66.7)[b]	2 (6.7)
ALT normal / HBV DNA and HBeAg-negative	6 (33.3)	15 (45.5)[b]	1 (3.3)

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

Table 2 Responses to treatment at the end of follow-up *n*(%)

	T-a1 (n = 18)	IFN-α (n = 33)	HC (n = 30)
	(after 12 mo of follow-up)		
ALT normalization	11 (61.1)[a]	10 (30.3)	3 (10)
HBV DNA-negative	13 (72.2)[ab]	13 (39.4)[b]	2 (6.7)
ALT normal / HBV DNA and HBeAg-negative	10 (55.6)[ab]	9 (27.3)	1 (3.3)

[a]P<0.05, *vs* IFN-α; [b]P<0.01, *vs* HC.

Typical side effects of IFN-α treatment, such as flu-like syndrome, fatigue, irritability, and headache, were seen in most of the patients treated with IFN-α. However, no serious or long-term side effects were noted and no patients discontinued the treatment. Therapy with T-al was not associated with significant side effects. Three patients reported local discomfort at injection sites. No systemic or constitutional symptoms were observed with T-al administrations.

DISCUSSION

The results of the present randomized, controlled trial have shown that T-a1 therapy at a dose of 1.6 mgvia subcutaneous injection twice a week for 6 months is effective and safe in patients with chronic hepatitis B, because nearly 60% of the treated patients became HBeAg- and HBV DNA-seronegative 6 months after the end of therapy. This response rate was not only significantly higher than that of the spontaneous seroconversion rate (3.3% in this study), but also obviously higher than the response to IFN-α therapy alone (27.3%) assessed 6 months after the end of therapy. The study showed that, at the dose tested, T-a1 has the same efficacy as IFN-α in inducing clinical and virological remission. The rate of response in terms of ALT normalization and/or HBV DNA and/or HBeAg loss was not significantly different in the T-a1 group compared with the IFN-α group at the end of the treatment (P>0.05). But there was significantly difference on the rate of response between the two groups at the end of the follow-up periods (P<0.05). The normalization of serum ALT and loss of HBV DNA and HBeAg were observed more frequently in the IFN-α group at the end of therapy and in the T-a1 group at the end of the follow-up. Furthermore, in the T-a1 group the response to the treatment was observed also during the follow-up period, but not in the IFN-α group. On the basis of these results and considering that ALT normalization and HBV DNA/HBeAg negativization may spontaneously occur in untreated patients, we retrospectively compared the two treated groups with a group of untreated patients followed for at least 12 months. The results showed that a significant higher rate of complete response occurred in the IFN-α group at the end of therapy and in the T-a1 group at the end of follow-up compared with the HC group.

It is noted that the benefit of T-a1 was not immediately apparent at the end of therapy. There was a trend for complete virological response to increase or accumulate gradually after the end of thymosin therapy. This trend was also reported in a multicenter American trial in which 5 of the 12 responders to T-a1 therapy showed a delayed response[17]. This is in contrast to therapy with IFN-α, in which responses usually occur during the first 4 months of treatment. These contrasting patterns of response were best demonstrated in a recent Italian study involving HbeAg negative, HBV DNA-positive, Interferon-naive patients with higher ALT level (181 ± 159 U/L), in which the complete response (ALT normalization and HBV DNA loss) rate increased gradually from 29.4% at the end of therapy to 41.2% 6 months after the end of T-a1 therapy. In that study, the response to interferon therapy decreased from 43.8% at the end of therapy to 25% 6 months after the end of therapy[18]. This trend of delayed effect of T-a1 was also reported by Chien *et al*. in patients with chronic hepatitis B recently[19]. The reasons for this delayed effect of T-a1 are not clear. The delayed response is not likely a result of direct antiviral effects similar to those of interferons. On speculating, T-a1 may exert an immunoregulatory function that promotes the endogenous antiviral immune response, as previously suggested, improving the effectiveness and coordination of the host cellular immune mechanisms in clearing HBV infected hepatocytes.

It has been shown that patients treated with T-a1 have a higher peripheral blood helper T cell count (CD4) and IFN-γ production by peripheral blood mononuclear cells during and after the end of T-a1 therapy[14]. In view of the immune mechanisms involved in the pathogenesis of liver injuries in chronic HBV infection, it is possible that T-a1 may activate viral-specific helper T cells and result in the amplification of the humoral immune response to viral proteins and the induction of viral antigen-specific cytotoxic T-ymphocytes through secreting endogenous IFN-α, IFN-γ, interleukin-2, and tumor necrosis factor, and increase lymphocyte interleukin-2 receptor expression[20-29]. Moreover, T-a1 is able to act synergistically with endogenous IFN-α and IFN-β in stimulating natural killer activity[30]. Although T-a1 is not known to possess antiviral properties, a preliminary report showed that this agent is able to inhibit woodchuck hepatitis virus replication[31]. Hence, the delayed effect after the end of T-a1 therapy in the present study was possibly caused by the immunomodulating effect of T-a1 that induced persistently higher helper T-cell function. Because noncytolytic inhibition of HBV RNA, nucleocapsid particles, and replicative DNA intermediates by cytotoxic T lymphocytes has been described in the transgenic mouse model[32,33], it is also possible that viral clearance after T-a1 therapy, particularly those without preceeding ALT flaring, may be mediated by noncytolytic antiviral effects of cytotoxic T lymphocytes. Clearly, further studies are needed to elucidate the possible mechanisms.

The tolerability of T-a1 was excellent and without side effects. This finding together with a lower number of weekly injections could favor better patient compliance.

In conclusion, the results of this trial indicate that, at the dosage tested, a 6-month T-a1 therapy is safe and effective in arresting HBV replication and

reducing lobular activity in patients with chronic hepatitis B. Furthermore, compared with IFN-α, T-a1 is better tolerated and seems to induce a gradual and more sustained ALT normalization and HBV DNA/HBeAg loss, so it might represent an alternative to IFN-α therapy. However, a response rate of 50% is still not sastifactory. A more effective therapeutic approach, such as combination therapy using the immunomodulating effect of T-a1 and antiviral effect of interferon or nucleoside analogues (such as lamivudine, famciclovir, etc.), warrants further studies.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Heat shock protein 72 normothermic ischemia, and the impact of congested portal blood reperfusion on rat liver

Chao Liu Dai[1], Zhen Long Xia[1], Makoto Kume[2], Yuzo Yamamoto[2], Kazuhiko Yamagami[2], Nobuhiro Ozaki[2] and Yoshio Yamaoka

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INTRODUCTION

From the technical aspect of liver surgery, control of bleeding during hepatic parenchymal resection is one of the most important procedures in hepatectomy. Pringle's maneuver, a temporary cross-clamping of the hepatoduodenal ligament, has often been used for this purpose[1]. This is the simplest and useful technique to reduce intraoperative blood loss. Unfortunately, this method has resulted in normothermic ischemiareperfusion injury of the liver. Many clinical observations indicate that prolonged intraoperative normothermic ischemia and succeeding reperfusion of the liver are the most significant disadvantages of Pringle's manever, and may cause postoperative functional disorder of the liver[2-6].

Although the process of ischemia reperfusion injury of the liver is complicated, it consists of three pathologic components: hepatic ischemia, portal congestion, and reinflow of arterial and congested portal blood. Ischemia-reperfusion injury is not merely an ischemic injury but is also related to reperfusion of blood. Moreover, it has been revealed that reactive oxygen radicals and some chemical mediators activated during recirculation play an important role in the development of liver injury after ischemia[7-11]. Because portal congestion is an extremely harmful factor that can induce bacterial translocation, generation of chemical mediators, and reactive oxygen radicals in the portal flow, it is conceivable that reinflow of congested portal blood containing activated pathogens may play a role in the development of ischemia-reperfusion injury of the liver. However, the influence of portal congestion on the development of liver injury during ischemia-reperfusion injury has not been well researched. Only a few authors have described the significance of the reperfusion of congested portal blood[12].

Heat shock protein 72 (HSP72) is produced in the liver after normothermic ischemia and reperfusion [13-15]. HSP72 is now being intensively investigated as one of the most fundamental stress proteins. The protein is produced in response to a variety of stress-induced stimuli and is considered to be an important factor which helps to maintain cellular homeostasis and increase the chance of pateint survival[16-23].

In the present study, we investigated the induction of HSP72 in the liver tissue after 15-minute Pringle's maneuver with or without portosystemic shunt in the rat model, to determine the influence of congested portal blood on the development of ischemia-reperfusion injury in liver.

MATERIALS AND METHODS

Male Wistar rats weighing 200 g to 300 g were housed in chip-bedded cages in a climate-controlled room (24℃ ± 1℃) under a 12-hour light-dark cycle. The rats were provided with ordinary rat chow and water *ad libitum*- according to the animal protection guidelines of Kyoto University. Rats were premedicated with an intramuscular injection of atropine sulfate (0.05 mg) and anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg rats weight) before all operative procedures. They were divided into two groups. In the first group (Group P), the abdomen was opened through a median laparotomy and the liver was detached from its ligaments. Total hepatic ischemia with portal congestion was produced by clamping

[1]Department of Surgery, The Second Clinical College of China Medical University, Shenyang 110003, Liaoning Province, China
[2]Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan
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Chao Liu Dai, M.D. graduated from Wenzhou Medical College in 1985, and received doctor degree from China Medical University in 1992. He is an Associated Professor and ViceDirector of the Department of Hepatobiliary Surgery, the Second Clinical College of China Medical University. He has published 40 papers.
Correspondence to: Chao Liu Dai, Department of Hepatobiliary Surgery, The Second Clinical College of China Medical University, No. 36 San Hao Street, HePing District, Shenyang 110003, Liaoning Province, China
Tel. 0086-24-23893501-512, Fax. 0086-24-23926097
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the hepatoduodenal ligament for 15 minutes with a microvascular clamp. In the second group (Group S), the abdomen was opened in the same way and the liver was also detached from its ligaments. Extracorporeal portosystemic shunt was achieved from the cecal branch of the superior mesenteric vein to the right external jugular vein, using a polyethylene tube (0.58 mm inner diameter×200 mm long, Dural Plastics Co., Australia) filled with 0.04 mL heparinized saline solution (250U/mL). The liver was subjected to warm ischemia by cross-clamping the hepatoduodenal ligament for 15 minutes with a microvascular clamp. However, portal congestion was prevented by the extracorporeal shunt. During these interventions, the abdomen was temporarily closed and an electric heater was placed under the animal to keep the rectal temperature at 36℃ ± 1℃. Then, declamping allowed reperfusion until sampling after specified periods.

Liver tissue was removed at 48 hours after each intervention and Western blot analysis was performed to detect HSP72 (monoclonal SPA-810, StressGen Biotechnologies Corp., Canada)[13]. To measure adenosine nucleotide concentration, liver tissue was sampled by freeze-clamping at 1 (n = 6), 3 (n = 6) and 48 hours (n = 6) after reperfusion. Enzymatic assay was performed to measure the concentration of adenine nucleotides in the liver tissue [24,25]. The energy charge potential (EC) was calculated according to the formula proposed by Atkinson: (ATP+0.5 ADP)/(ATP+ADP+AMP)[26], where ATP, ADP and AMP are adenosine tri, *di*, and monophosphate, respectively. Blood samples were taken from the aorta at 1 (n = 6), 3 (n = 6), and 48 hours (n = 6) after the end of each intervention. The serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) were measured at a biochemical laboratory. After sampling, rats were killed.

The values were expressed as mean ± standard deviation (SD). For the statistical analysis, Student's *t* test was performed for the biochemical parameters. A P value <0.05 was considered to be significant.

RESULTS

Comparing these two groups, HSP72 was markedly overexpressed in group P but its accumulation in group S was very slight (Figure 1).

In the comparison between AST, ALT and LDH levels in group P and group S (Table 1). There was a trend towards higher transaminase levels in group P, but the difference was not statistically significant (P>0.05).

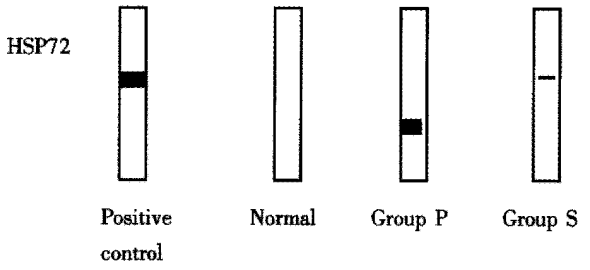

Figure 1 Heat shock protein (HSP) production in the rat liver after 48 hours of reperfusion in each group compared with that in normal liver (5 μg protein per lane). HSC70, which contains inducible HSP72 in approximately 10 per cent, was used as a positive control (0.2 μg protein per lane).

Table 1 Changes in AST, ALT and LDH (IU/L) in serum after reperfusion (n = 6)

Parameter	1 h	3 h	48 h
Group *P*			
AST	4832±1589	4896±3270	199±93
ALT	4860±2015	5218±3230	266±155
LDH	45235±21748	44619±29975	588±265
Group S			
AST	4147±1069	2731±1282	204±72
ALT	4230±1460	2689±1459	181±103
LDH	35752±15954	26327±21973	476±248

Results are expressed as means ± SD.
There was no statistically significant difference in AST, ALT and LDH at any time point examined between two groups.

There were no significant differences between the two groups at any time point examined (P>0.05, Table 2).

Table 2 Change in ATP levels (mmol/g wet wt tissue) and EC in liver tissue (n = 6)

Parameter	1 h	3 h	48 h
Group *P*			
ATP	1.49±0.69	1.91±0.42	3.08±0.42
EC	0.67±0.11	0.62±0.07	0.83±0.03
Group S			
ATP	1.60±0.55	2.22±0.81	3.00±0.22
EC	0.64±0.14	0.68±0.12	0.80±0.05

EC, energy charge. Results are expressed as means ± SD.
There were no significant differences in ATP concentration and EC level at 1 h, 3 h, and 48 h between two groups (P>0.05).

DISCUSSION

Heat shock proteins are intracellular proteins that have been well conserved during evolution. They are present in small amounts in normal cells and larger amounts in cells subjected to a variety of stressful stimuli. Heat shock stress, which induces the synthesis of HSPs, is closely related to the physiologic body temperature of the organism[16,27]. This is the reason by which special attention was paid to control the body temperature of the animals

during operations in this study to obviate the possible effects of body temperature changes. The rectal temperature of the animals was strictly controlled in the range of 36℃ ± 1℃ using an electric heating pad.

Schoeniger *et al*[14] reported that prolonged ischemia induced by Pringle's maneuver did not activate the HSP72 gene in the porcine liver until restoration of blood flow. They concluded that transcription of HSP72 mRNA was highly dependent on the generation of superoxide anions. This participation of superoxide anions in the activation of HSP72 gene partly suggests a contribution by the reinflow of portal blood, but the role of portal blood pooling was not a focus of their report. We observed that the reinflow of congested portal blood into ischemic liver after a shortterm Pringle's maneuver contributed to the production of HSP72 in the liver tissue. However, the liver subjected to the same period of ischemia produced less HSP72 when reperfusion was performed with non-congested portal blood. This observation suggests an adverse impact of congested portal blood on liver function after ischemia and reperfusion. In contrast, Tacchini *et al*[28] reported that inflow occlusion to the partial liver for a longer period (up to 60 minutes) also induced expression of HSP72 mRNA in the ischemic lobe in rat liver. In their experiment, portal congestion did not occur because of hemi-occlusion of the hepatic inflow. The discrepancy in the influence of congested portal blood in the production of HSP72 between their results and ours may be due to the difference in ischemic periods. When the ischemic stress is sufficient due to a longer insult, even non-congested blood may cause oxidative stress sufficient to provoke the activation of heat shock genes. However, when the ischemic derangement of hepatic parenchyma is not so severe, the nature of the blood used for reperfusion may have a marked impact on the results. These findings imply that portal pooling would act appreciably as a key stress factor to increase the production of HSP72, but it would not be an essential factor when the degree of pre-existing ischemia is already high.

Although the mechanism by which the reflow of congested portal blood induces HSP72 production is still unclear, the process seems to be complex. The mechanism most likely involves factors that operate in the ischemic reperfused liver. Active oxygen species and free radicals are known to activate heat shock genes[29,30]. It is likely that free radicals generated in the small bowel during portal congestion play a role in the development of liver tissue injury during reperfusion[31-38]. Kawamoto *et al*[7] reported that the temporary occlusion of the portal vein and hepatic artery both increased xanthine oxidase activity in portal plasma and increased the permeability of the intestine during reperfusion. Both of these were prevented when a portosystemic shunt was performed and splanchnic vascular congestion was prevented. Other potential causative factors in the production of HSP72 include endotoxin and various cytokines translocated from the intestines during portal congestion. Recently, participation of both endotoxin and bacteria in the production of HSP72 has been reported[17,27,39-41]. Pringle's maneuver for 15 minutes was observed to have significantly increased plasma endotoxin levels in the portal blood and enhanced bacterial translocation into the portal blood from the gut[9].

Our results also suggest that HSP72 might prove useful in evaluating the degree of hepatic ischemia-reperfusion injury. Levels of enzymes in serum (including AST, ALT, and LDH), ATP and EC in liver tissue have been commonly used to assess hepatocellular injury. In the present study, no significant difference was observed between the congested and non-congested groups at any time point examined. Thus, even the ATP concentration and EC cannot differentiate these two groups. Animals could fully recover from liver injury as long as the Pringle's maneuver lasted only 15 minutes, and no animals died as a result of it. However, the fact that the stress brought on by the reperfusion of congested portal blood was clearly greater than that produced by the reperfusion of non-congested blood, as demonstrated by the production of HSP72, implies that the accumulation of stress under a repeated or prolonged Pringle's maneuver would likely result in irreversible liver damage. This concept is also consistent with the report of Nitta *et al*[12]. They reported that conventional parameters or arterial ketone body ratio could be used to detect the ill-effect of congested portal blood during reperfusion when Pringle's maneuver was repeated four times, although they failed to detect it after a single 30-minute Pringle's maneuver in dogs. In this sense, the production of HSP72 seems to be more sensitive than conventional parameters to detect the given stress during ischemiareperfusion injury.

In conclusion, HSP72 induced in the liver tissue by a shortterm Pringle's maneuver was associated with the reinflow of congested portal blood into ischemic tissue. The influence of this congested portal blood upon reperfusion of ischemic tissue was detected by HSP72, but not by conventional biochemical parameters. HSP72 is suggested to be useful for determination of the degree of hepatic ischemia-reperfusion injury.

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Edited by Jason Carr

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Influence of IFNα-2b and BCG on the release of TNF and IL-1 by Kupffer cells in rats with hepatoma

Xian Yong Bai[1], Xiu Hong Jia[2], Ling Zhong Cheng[3] and Yun Di Gu[3]

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INTRODUCTION

Kupffer cells are residential macrophages in the liver, which play a critical role in the maintenance of normal liver function and in immunal surveilance of hepatocellular carcinoma (HCC) and other cancers[1]. The biological immune modulants have been used for treating patients with HCC and other cancers[2]. In our previous studies, the combined use of biological immune modulants showed better effects. The normal rats and hepatoma rats induced by DEN (Diethylnitrosamine) were treated by either IFNα-2b or BCG or both, the number of KCs and the amount of H_2O_2 released increased obviously, while the combined use of IFNα-2b and BCG showed the best results[3,4]. This study was focused on the influence of biological immune modulants on the release of TNF and IL-1 by KCs in rats with hepatoma.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 150 g-200 g, were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences. The rats were given drinking water containing 0.008% DEN for 12 weeks, then randomly divided into four groups, they were given IFNα-2b (Schering Plough Co. USA) at a dose of 16U/kg, BCG (Biological Product's Research Institute, Shanghai) at a dose of 0.1 mg/kg, and combination of the two intraperitoneally, the controls were given the same volume of normal saline at various survival periods (12th week, 16th week).

Isolation of KCs

Under anesthesia, the rats were exsanguinated and the livers were perfused *in situ* via the portal vein with Hank's balanced salt solution (HBSS). The livers were dissected free and passed through 60 μm brass screens into HBSS. Following centrifugation at 500 × *g* for 10 min (4℃), the pellet was resuspended in 30 mL HBSS containing 0.05% collagenase (TYPE I, Sigma) and 0.1% pronase (TYPE E, Sigma), and incubated for 30 min in a 37℃ agitating water bath, following centrifugation at 500 × *g* for 2 min to sediment the hepatocytes,an enriched nonparenchymal cell pellet was obtained by centrifugation of the supernatant at 500 × *g* for 35 min. Erythrocytes were lysed by incubation for 3-5 min with ammonium chloride (0.83%). The cells were washed twice with HBSS and resuspended in standard RPMI-1640. After 4 h incubation at 37℃ in 100 mm plastic petridishes, the nonadherent cells were removed by three successive wash with warm HBSS. The adherent cells, phagocytosing latex beads (Sigma) were designated as Kupffer cells. After 10 min incubation at 37℃ in Trypsin-EDTA (0.05%/0.02%), KCs were liberated from the plastic dishes by vigorous pipetting, washing, and counted. The purity of KC was >95% by latex bead ingestion and viability was >90% as indicated by trypan blue exclusion (0.4% trypan blue stain in 0.85% salin). The KCs of control hepatoma rats (at the 18th week) were isolated and treated with IFNα-2b (2500 U/mL), BCG (2 mg/L) or both respectively. The human hepatoma cell line SMMC-7721 cells and KCs were cocultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum at 37℃ in a humidified atmosphere containing 5% CO_2 for4 h. Supernatants were collected, and stored at -20℃ until use.

IL-1 activity assay

IL-1 activity in the culture supernatants was assayed by the enhancement of thymocyte proliferation to concanavalin A (ConA). Briefly, thymocytes were obtained from female C57BL/6N mice at 5-7 weeks of age, cell suspensions of thymocytes were prepared by pressing the thymus tissue through a 50 μm wire mesh, then filtering the tissue fragments through a 30 μm nylon mesh into RPMI-1640 medium supplemented with 2.4 g/L ConA. The

[1]Department of Histology and Embryology, Binzhou Medical College, Binzhou 256603, Shandong Province, China
[2]Department of Pediatrics, Binzhou Medical College, Binzhou 256603, Shandong Province, China
[3]Department of Histology and Embryology, Shanghai Medical University, Shanghai 200032, China
Dr. Xian Yong Bai, graduated from the Shanghai Medical University as a postgraduate in 1995, Professor of Histology and Embryology, specialized in hepatic tumor immunology, having 36 papers published.
Supported by the National Natural Science Foundation of China, No.39270379
Correspondence to: Dr. Xian Yong Bai, Department of Histology and Embryology, Binzhou Medical College, Binzhou 256603, Shandong Province, China
Tel. 0086-543-3323516, Fax. 0086-543-3322752
Email. bzbaixy@sina.com
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cells were resuspended to 1.5×10^6 cells/mL, and 100 μL of cell suspensions were placed in each well of 96-well microtiter plates, 100 μL of supernatants were added to each well and the plates were incubated for 72 h. Each cell culture well was plused 3.7×10^{10} μBq ^{3}H-thymidine during the final 8 h of incubation and harvested onto glass fibers, using an automatic cell harvester, ^{3}H-thymidine incorporation was determined by a liquid scintillation spectrometer, the enhancement of thymocyte proliferation of the unknown supernatant IL-1 levels was determined by the ratio of radioactivity incorporated in the experimental and control groups (SI).

TNF assay

The levels of TNF in culture supernatants were determined by a TNF specific ELISA obtained from Institute of Military Medical Sciences. The kit was composed of 96 determinations and the assays were performed exactly as recommended by the manufacturer. All samples were assayed in triplicate.

Statistical analysis

The results were presented as the mean of triplicates ± SEM, and statistical significance was assessed by Student's *t* test.

RESULTS

The influence of biological immune modulants on the release of TNF by KCs of normal rats *in vivo* (Table 1). The KCs of normal rats could release a little TNF and IL-1 when cocultured with human SMMC-7721 hepatoma cells, the TNF and IL-1 released by KCs of normal rats treated with biological immune modulants *in vivo* increased obviously. The effect of combined use of IFNα-2b and BCG exhibited the best effects, the combination of IFNα-2b and BCG increased the production of TNF by 3.5 times and the activity of IL-1 by 80%. BCG was better than IFNα-2b in increasing the release of IL-1, and IFNα-2b was better in increasing the production of TNF.

Table 1 The influence of biological immunal modulants on the release of TNF and IL-1 by KCs of normal rat *in vivo*

Groups	TNF (ng/L)	IL-1 (SI)
Control	56.2±10.5	1.40
BCG	79.4±17.6[a]	2.27[b]
IFNα-2b	134.9±51.4[b]	1.84[a]
IFNα-2b+BCG	199.5±71.5[b]	2.54[b]

n = 18, [a]P<0.05, [b]P<0.01 *vs* control.

The influence of biological immune modulants on the release of TNF and IL-1 by KCs of rat with hepatoma *in vivo* is shown in Table 2. The influence of biological stimulants on the release of TNF and IL-1 by KCs of rats with hepatoma *in vivo* was similar to those of normal rats. The combined use of IFNα-2b and BCG exhibited the best effect, the amount of TNF released by KCs treated with IFNα-2b and BCG increased by 5.6 times (12th week) and by 4. 5 times (16th week), the activity of IL-1 increased by KCs treated with IFNα-2b and BCG increased by 48% (12th week) and 78% (16th week).

Table 2 The influence of biological immunal modulants on the release of TNF and IL-1 by KCs of rats with hepatoma *in vivo*

Groups	2nd week		16th week	
	TNF (ng/L)	IL-1 (SI)	TNF (ng/L)	IL-1 (SI)
Control	50.1±9.4	1.57	79.4±10.6	1.58
BCG	234.4±63.1[b]	2.58[b]	148.5±47.2[a]	2.74[b]
IFNα-2b	251.2±82.1[b]	2.33[b]	166.0±30.5[a]	2.42[b]
IFN-2b+BCG	281.8±63.3[b]	2.80[b]	354.8±96.4[b]	4.08[b]

n = 18,[a]P<0.05, [b]P<0.01 *vs* control.

The influence of biological immune modulants on the release of TNF and IL-1 by KCs of rat with hepatoma *in vitro* is shown in Table 3. The influence of biological stimulants on the release of TNF and IL-1 by KCs of rats with hepatoma *in vitro* was similar to the result *in vivo*. IFNα-2b was better than BCG in increase of release of TNF by KCs, BCG gave better results than IFNα-2b in increasing the activity of IL-1 released by KCs. The combined IFNα-2b and BCG exhibited the best effects, the amount of TNF released by KCs treated with IFNα-2b and BCG increased by 123.8%, the activity of IL-1 released by KCs treated with IFNα-2b and BCG increased by 58%.

Table 3 The influence of biological immune modulants on the release of TNF and IL-1 by KCs of rats with hepatoma *in vitro*

Groups	TNF (ng/L)	IL-1 (SI)
Control	100.0±20.6	1.42
BCG	141.3±31.7[b]	1.80[a]
IFNα-2b	199.5±61.2[b]	1.64[a]
IFNα-2b+BCG	223.8±42.4[b]	2.24[b]

n = 18, [a]P<0.05, [b]P<0.01 *vs* control.

DISCUSSION

TNF and IL-1 are macrophage-derived cytokines, TNF is known to have cytotoxic and cytostatic effects on certain tumor cells, and with a pivotal role in inflammatory reactions and regulation of immunological response[5,6]. The actions of IL-1 have been elucidated in recent years, it is directly cytotoxic for some human tumor cells, and stimulates T cell proliferation by inducing production of interleukin 2 (IL-2) as well as increasing the number of IL-2 receptors on the T cell, and directly stimulate NK cell activity[7].

Kupffer cells are residential macrophages in the liver, and play an important role as scavenger cells in nonspecific elimination of gut endotoxins, immune complexes and viruses[8], especially the important role in immunal surveillance on HCC[9,10]. Kupffer cells have also been reported to release some cytokines when activated by biologically reactive substances, several reports have suggested that IFNγ regulates monocytic function, especially in the production of TNF and IL-1. Kawada had shown that IFNγ enhanced TNF production in the presence or absence of lipopolysaccharide (LPS), but suppressed IL-1 production by KCs[11]. Brandwein showed that IFNγ markedly inhibits LPS-stimulated IL-1 production by mouse peritoneal macrophages[12], but differed from the observations of Boraschi and Hart[13,14]. Amento *et al* have also observed that IFNγ did not stimulate IL-1 production by macrophages from the human monocyte cell line U937[15], and many reports had suggested that IFNγ augments both TNF and IL-1 production by macrophages only in the presence of LPS[12], this variable responses are undoubtedly related to the difference in both the interferon preparations and the responder cell types. Our study showed that IFNα-2b stimulated and enhanced TNF and IL-1 production by KCs in the absence of LPS, the difference may have resulted from the cell types and condition of macrophages, since KCs may always be exposed to endotoxins and activated to some degree [16]. In our previous studies, anti-tumor effects of KCs activated by various biological immunal modulants were different, but the combined use of various biological immunal stimulants showed the best effect [2]. Our previous studies of the mechanism of KCs function also showed that the combination of IFNα-2b and BCG exhibited the best results, the increase of the number and volume of KCs, the increase of the amount of H_2O_2 released by KCs[3,4]. The systematic study mentioned above, regardless of normal or hepatoma rats and both *in vivo* and *vitro* consistently confirmed the combined use of IFNα-2b and BCG was better than either one used alone in enhancing the activity of immune cells. These results suggested that the combined use of IFNα-2b and BCG to HCC patients should be advocated.

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Edited by Wu XN and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Inflammatory pseudotumor of the liver: 13 cases of MRI findings

Fu Hua Yan, Kang Rong Zhou, Ya Ping Jiang and Wei Bin Shi

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INTRODUCTION

Inflammatory pseudotumor of the liver, a rare benign lesion, is often confused with the malignant tumors. Until 1998, less than 80 cases had been reported in the world[1,2]. The accuracy of the pre-surgical diagnosis was low[3,4], and very few materials on the diagnosis using dynamic MRI were reported[5-7]. We collected thirteen cases of inflammatory pseudotumor of the liver were collected and proved by pathology with MRI studies. Our purpose is to describe and analyze the MRI findings of this lesion and find out the valuable signs suggesting the diagnosis so as to avoid unnecessary operations.

MATERIALS AND METHODS

Thirteen cases collected from September 1995 to December 2000 in our hospital (9 men and 4 women aged from 31-58 years, 41 years in average). More than half of the cases had no symptoms, and the others complained of upper abdominal discomfort, only three cases had fever of unknown causes. AFP and other liver lab testing were normal.

All of 13 cases underwent MR examinations with 1-5T Signa scanner (GE Medical Systems Milwaukee, WI), including T_1WI (TR/TE = 500-700 ms/14-16 ms), T_2WI SE sequence (TR/TE = 2000-4000 ms/60-90 ms) and FMPSPGR dynamic enhanced MRI(Matrix 256 × 128, thickness 7 mm, gap 3 mm, TR/TE Flip Angle = 100-150 ms/1.5-4.5 ms/60-90°). Four repeated acquisitions were obtained at 25-30, 60, 90 and 180s respectively following power injection of 15 mL-20 mL (0.15 $mmol \cdot kg^{-1}$) of Gd-DTPA (gadopentetic dimeglumine, Magnevist, Shering Pharmaceutical Ltd.) via an antecubital vein.

The MRI imagings of all cases were read and analyzed by two experienced doctors. Comparisons were made between preoperative diagnosis and results of surgical pathology.

RESULTS

A total of 16 lesions were identified by surgical pathology in the 13 cases, including three lesions in 1 case and single one in 12 cases. The size of the lesions was 0.8 cm-3 cm in diameter (2.1 cm in average). The shape of the lesions was also variable, being round, ovoid, lobular (seen in coalescence of small focus) and irregular.

On SE sequence T_1WI, 6 of 16 lesions were isointense (Figure 1A), the other 10 lesions were slightly hypointense (Figure 2A). On SE sequence T_2WI, 6 of 16 lesions were slightly hyperintense (Figure 2B), the other 10 lesions were nearly equal intensity of signal to that of normal parenchyma (Figure 1B). The lesions discovered on SE sequence MRI were not well defined and the conspicuity between the lesions and the parenchyma were much less than that seen on dynamic MRI imaging of portal venous and delayed phases.

On dynamic enhanced MRI, none of the lesions showed enhancement in the dominant arterial phase (Figure 1C, Figure 2C). Therefore, the lesions were not seen clearly in this phase. On the contrary, all the lesions were well defined in the portal venous phase or/and the delayed phase. In these phases, the following appearances could be found: ① peripheral enhancement of the lesion (12/16, 75%) (Figure 2D). ② Small nodular enhancement (6/16, 37.50%), located in the central area of the lesions like core (Figure 2D), or near the marginal area of the lesions which we described as stalactite. ③ Septum within the lesions which could be linear or thick and irregular in appearance were seen in 8 (50%) of 16 lesions, and enhanced in the portal venous phase or/and delayed phase (Figure 1D). ④ All findings on dynamic MR imaging were seen overlapped.

Department of Radiology, Zhongshan Hospital, Medical Center of Fudan University (Former Shanghai Medical University), 180 Fenglin Road, Shanghai 200032, China
Dr. Fu Hua Yan, graduated from Shanghai Medical University as a postgraduate in 1996, now an associated professor, a radiologist in chief specialized in the diagnosis of CT and MRI in hepatic diseases, having 22 papers published. She is the associated editor of the books Spiral CT and Body MRI.
Correspondence to: Dr. Fu Hua Yan, Department of Radiology, Zhongshan Hospital, Medical Center of Fudan University (the former Shanghai Medical University), 180 Fenglin Road, Shanghai 200032, China
Tel. 0086-21-64041990 Ext.2453
Email. yanfuhua@yahoo.com
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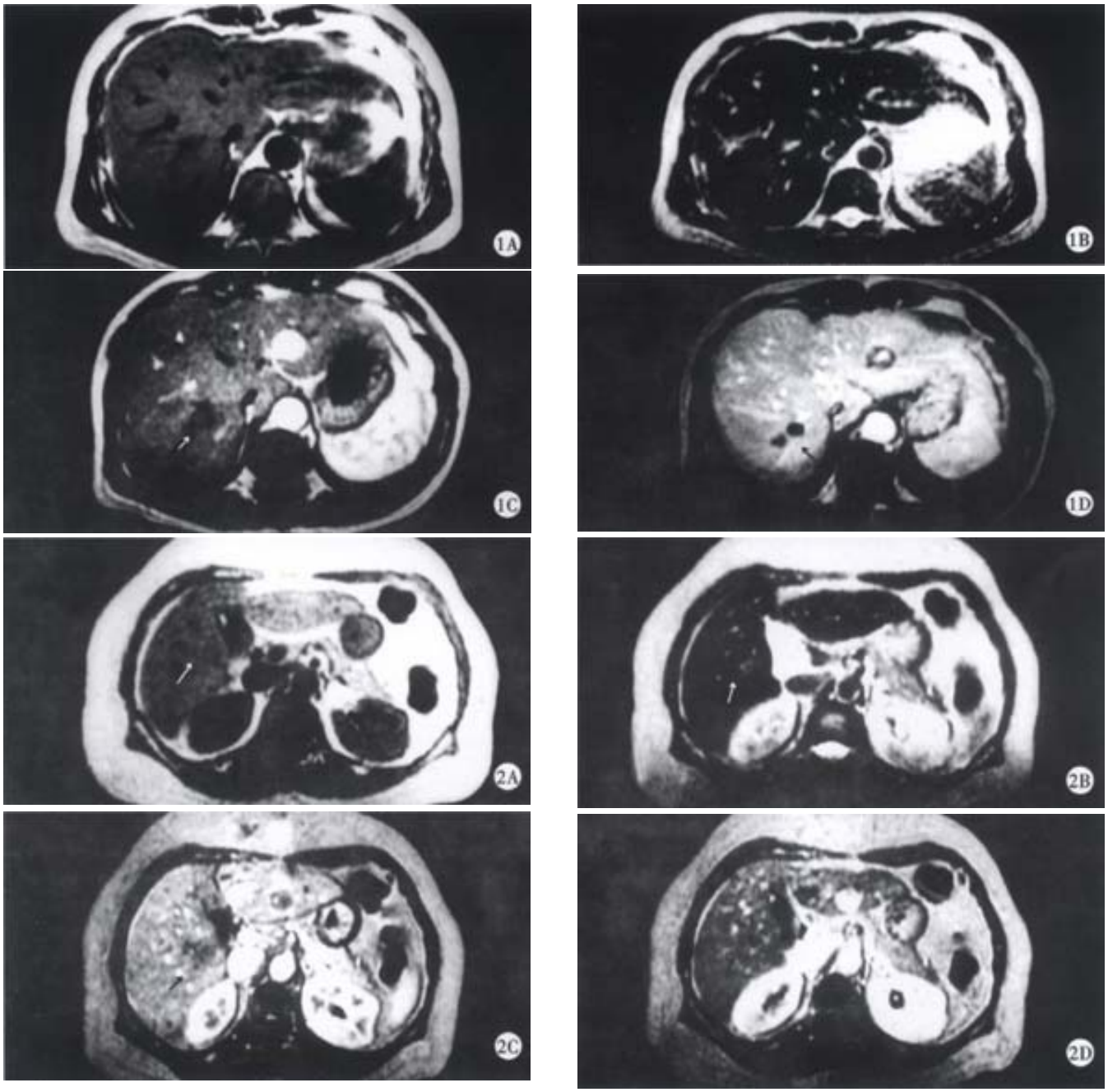

Figure 1 Inflammatory pseudotumor of the liver in the right posterior lobe. A SE T_1WI and B SE T_2WI could not show any lesions. C A hypointense lesion could be found in the right lobe (arrow), there is no enhancement in early arterial phase of dynamic contrast MRI. D portal venous phase of dynamic contrast MRI showed the enhanced septum (arrow).

Figure 2 Inflammatory pseudotumor of the liver in the right posterior lobe. A SE T_1WI showed a hypointense lesion(arrow). B SE T_2WI showed the lesion was slightly and inhomogeneously hyperintense(arrow) . C There is no enhancement in early arterial phase of dynamic contrast MRI, the edge of the lesion was not clear(arrow). D portal venous phase of dynamic contrast MRI showed the punctual core in the center and peripheral enhancement (arrow).

DISCUSSION

Correlation of pathology with MR imaging findings

Inflammatory pseudotumor of the liver is a rare benign disease, occasionally discovered by US, CT and MRI[5-9]. The etiology has not yet been very clear, and may be related to infection and immunogical compromise. Fibroblastic proliferation and chronic inflammatory cell infiltration constitute the characteristic features of the disease[3,10]. The mass is surrounded by the fibrocollageous stroma rich with capillary vessels, which may explain the peripheral enhancement in portal and delayed phases scans in the majority of cases due to the extravascular accumulation of contrast medium. Within the mass, there are less inflammatory cell components and more coagulative necrosis, so on SE T_2WI, most of the lesions were nearly equal

intensity of signal to that of normal parenchyma because of coagulative necrosis containing less free water. These are the characteristics of inflammatory pseudotumor of the liver. Six lesions showed slightly hyperintense on SE T_2WI, because of more inflammatory cell infiltration within the lesions. The larger area consisted of coagulative necrosis and cell components in the central region does not enhance as fibrosis in later phase and appears heterogeneous. It was not enhanced in early arterial phase since the lesion had no direct hepatic arterial blood supply.

Detection and differential diagnosis

Most of the lesions could be detected by sonography in our group, but as it was hard to be characterized, color Doppler ultrasound became mandatory supplementary modality, which could recognize the presence or absence of arterial blood flow to help determine the nature of the lesions.

SE sequence of MRI had more limitations in detection of the lesions since the difference of signal intensity was not conspicuous between the lesion and parenchyma on T_1WI as well as on T_2WI. Six of 16 lesions in this group were isointense on T_1WI and 10 of 16 lesions were also isointense on T_2WI, and the others had only slight or moderate difference with the normal hepatic parenchyma. So, contrast enhanced MRI was very important to avoid potentially missing detection of small lesions and it is very important to recognize the nature of the lesion and not to confuse with the malignant tumors, mainly hepatocellular carcinoma and metastases of the liver.

The key point to differentiate it from hypervascular HCC is the absence of enhancement in arterial phase. Attention should be paid to the differentiation from hypovascular HCC. The following findings on MRI might be helpful: ① peripheral enhancement of lesions on the later phase; ② the presentation of septum and core, which has never been seen in HCC; ③ variable or irregular shape of the lesions, coalescence of several small lesions in one case; ④ most of the lesions were isointense on T_2WI, but more than 90% lesions of HCC were hyperintense on T_2WI[11]. MRI with mangafodipir trisodium might help distinguish inflammatory pseudotumor of the liver from hepatocellular carcinoma[12]. But peripheral ring-like enhancement may mimic the metastatic lesions. The history of primary malignant tumors and the absence of fibrotic septum or linear appearance within the mass and the most of lesions were hyperintense on T_2WI could help the differentiation.

In summary, in view of rare occurrence, few cases of inflammatory pseudotumor of the liver have been accurately diagnosed preoperatively in the past. Since MRI was put into clinical application and with experiences accumulated, the accuracy of diagnosing this disease has been greatly improved.

In conclusion, MRI is very imperative to reveal the features of the lesions, such as no early arterial enhancement, peripheral enhancement, septum and small nodular enhancement occurred in portal venous and delayed phases.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Studies on mechanism of Sialy Lewis-X antigen in liver metastases of human colorectal carcinoma

Xiao Wei Li[1], Yan Qing Ding[1], Jun Jie Cai[1], Shao Qing Yang[2], Lian Bing An[3] and Dong Fang Qiao[3]

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INTRODUCTION

Sialyl Lewis-X antigen, correlated with carcinoma, is a group of carbohydrate antigen containing oligosaccharide expressed both on glycolipids and glycoproteins on cell surface of embryonic tissue and tumor tissue[1]. The SLeX antigen located on cell surface is synthesized principally by two enzymes, αl, 3fucosyltransfrease and α2, 3sialyctransferase. In adults, SLeX antigen is expressed principally on the surfaces of granulocytic cells and some tumor cells. It is a ligand of endothelial leucocyte adhesion molecules (ELAM-1, or E-Selectin) [2,3] and plays an important role in the adhesion of leucocyte to the vascular wall in inflammation and adhesion between tumor cells and blood endothelial cells in tumor metastases[4,5]. This study intends to observe the expression of SleX antigen and analyzes the correlation between the expression and metastatic potential by using cell lines of both Lovo[6] and HT29 [7] of human colon carcinoma. Our experiments have confirmed that both cell lines possess the capability of forming tumor and having the metastatic potential. Lovo cells obviously showed a greater hepatic metastatic potential in experimental nude mice than HT29 cells[8,9]. We employed *in situ* hybridization technique to detect the expression of SLeX antigen synthatase at mRNA level in both low and high metastatic potential of colon carcinoma cells. Meanwhile we used immunohistochemistry and flow cytometry to directly detect the expression of SLeX antigen at intracellular protein level within Lovo and HT29 cells. We observed morphologic features of interaction between tumor cells and blood vessel endothelial cells as well as the linkage of both cells in interaction by means of scanning and transmission electronmicroscope. We used a method of sealed colon carcinoma cells with SLeX monoclonal antibody to observe the adhesive potential between human colon carcinoma cells and umbilical vein endothelial cells and to observe the effects of this method on hepatic metastases of experimental nude mice. We expect to find out the mechanism of how SLeX antigen functions in human colon carcinoma hepatic metastases.

[1]Department of Pathology, Nanfang Hospital of the First Military Medical University, Guangzhou 510515, Guangdong Province, China
[2]The Northern Hospital of PLA, Shenyang 110015, Liaoning Province, China
[3]Department of Electronmicroscopy, First Military Medical University, Guangzhou 510515, Guangdong Province, China
Dr. Xiao Wei Li, graduated from the First Military Medical University with a MM degree in 1999, Physician in Charge of pathology, having 6 papers published.
Supported by the Key Programs of PLA, No.96Z028
Correspondence to: Professor Yan Qing Ding, Department of Pathology, The First Military Medical University, Tonghe, Guangzhou 510515, Guangdong Province, China
Tel. 0086-20-85141888 Ext.42148
Email. DYQ@fimmu.edu.cn
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MATERIALS AND METHODS

Materials

Cell lines, both Lovo and HT29 were provided by our laboratory. Endothelial cells were obtained by the perfusing and digesting human umbilical vein. Umbilical cord was provided by the Department of Gynecology and Obstetrics in Nanfang Hospital. BALB/C nude mice were provided by the Experimental Animal Center of the First Military Medical University.

Methods

Lovo, HT29 cells α1, 3Fuc-Toligonucleotide probe was detected by *in situ* hybridization ① Cell culture and manufacture of cell patch:Lovo and HT29 cells (using RPMI-1640 complete medium) were placed in an incubate trunk that contained 5% CO_2 at 37℃. The cells grown well and were digested by pancreatin to make up a 1×10^7/mL cell suspension, and then inoculated a drop of the cell suspension on cover slip of sterilized petridish and the complete medium was cultivated for 48 h, then removed the cover slip, washed thrice with 0.0l M, pH 7.2 PBS, fixed with acetone for 10min and finally washed with PBS for use. ② *In situ* hybridization: The cell patches were digested by 10 μg/mL protease K at 37℃ for 10 min and then washed with PBS buffer. 200 μL prehybri dization buffer was degenerated at 95℃ for 10 min, put into ice cubes to quench, then placed flat into a sealed moist case and incubated at 42℃ for 3 h. Five mL of α1, 3Fuc-T oligonucleotide probe of coragoxin with a density of 39.65pmol/μL was added into 200 μL pre hybridization liquid to form a hybridization liquid. The hybridization liquid was degenerated at 95℃ for 10 min. After being quenched in ice cubes, it was added into the cell sample, placed flat in a sealed moist case, and kept at 42℃ overnight. It was washed with 6 × SSC 45% aminic acid amine, and 2 × SSC and 0.2 × SSC

for 10 min respectively. It was added coragoxin Fab piece labeled alkaline phosphatase (1:125 dilute concentration) at 37℃ for 4 h, and then was washed by buffer. NBT/BCIP was added for coloration for 2 h. It was treated with 100% alcohol and xylene and sealed by neutral gum for later use. The control was done without oligonucleotide probe. Areas that showed positive α1, 3Fuc-T mRNA were dyed royal blue with royal blue grains. The deeper the color, the higher the positive degree. ③ Quantitative analysis and statistics: The quantitative analysis was done with Shen Hong's[10] aw and Leica-Q500M image analysis system. After 10 positive cells were taken randomly, we surveyed the gray degrees (Gα) and the gray degrees of their background (Gβ) in the positive areas. The positive units (PU = 100|Gα-Gβ|/256) were calculated with origin 50 software. Data were analyzed statistically with the *t* test of SPSS.

Immunohistochemistry

① Cell culture and manufacture of cell pieces: The procedure was identical to that of *in situ* hybridization. ② Immunohistochemistry LSAB: The approaches (omitted). The negative control was incubated with PBS on the cell piece without primary antibody. The SLeX protein expression areas were dyed brown. Deep brown was defined as strongly positive, while light brown as weakly positive. ③ Quantitative analysis and statistical method were the same as those in *in situ* hybridization.

Flow cytometry

① Cell culture was identical to that of *in situ* hybridization. ② Flow cytometry measure: The Lovo and HT29 cells digested with 0.25% panceatin, washed with PBS and centrifugated at 1000 rpm × 5 min were made into 1 × 10^7/mL cell suspension and then added SLeX monoclonal antibody (mouse anti-human IgM) (1:100 dilution). The negative control was processed with PBS instead of the primary antibody. Then the goat anti-mouse IgM labled FITC second antibody (1:60 dilution) was added at 4℃ for 30 min. All the suspensions were washed with PBS at 1000 rpm × 5 min, fixed with 1% polymeresatum at room temperature for 30 min, then re-washed with PBS and finally centrifugated at1000 rpm × 5 min for detection with EPICSEMTE model flow cytometry.

Adhesive test of tumor cells to human umbilical vein endothelium

① Tumor cell suspension preparation: The well-grown Lovo and HT29 cells were prepared into 1 × 10^7/mL cell suspension with complete culture medium. The suspension was divided into 4 groups: Lovo cell experimental group and its control group, HT29 cell experimental group and its control group. The experimental groups were added SLeX monoclonal antibody 10 μL (5 μg/mL) and the control groups PBS instead of SleX. They were pre-incubated at 37℃ for 30 min. ② Adhesive test of tumor cells to umbilical vein endothelium: The umbilical vein was cut open under sterile condition and was washed off red blood cells with PBS and then cut into 1 cm × 1 cm pieces, 0.5 cm thick paraffin was put onto the bottom of the 16-well culture plate which was to be rinsed 3 times with PBS after the paraffin congealed. The plate was exposed under ultraviolet light for 30 min, added a suitable amount of culture medium for immersion for 15 min. The luminal surface of blood vessel was put upwards and fixed with stainless needles on paraffin on the bottom of wells. The TNF-α (end density 1:500U/mL) was added at 37℃ for 4 h. After the culture was taken out, the prepared Lovo and HT29 cell suspensions were added into each well and incubated at 37℃ for 4 h in atmosphere 5% CO_2. The umbilical vein taken out was washed with PBS and fixed with 2.5% glutaraldehyde for 30 min and then washed again, dehydrated, dried at critical point, puffed gold and detected under a S-450 scanning electronic microscope. ③ Criteria of results and statistical method: During the adhesive test between tumor cells and umbilical vein endothelium, the tumor cells, which adhered to vascular endothelium in every vision field were counted by 500 × scanning electronmicroscope. Ten vision fields of each group were chosen randomly and their average values were calculated and statistically analyzed with *t* test.

Adhesive test between tumor cells and human umbilical vein endothelial cells

① Preparation of tumor cell suspension: as described above. ② Endothelial cell culture: 15 cm of fresh umbilical core from a healthy puerperal was washed off red blood cells with PBS. One end of the core was cramped and the 0.25% pancreatin was filled into the core from the other end. It was digested at 37℃ for 15 min. The digested fluid that contained the endothelial cells was drawn out and put into a centrifugal tube and centrifugated at 1000 rpm for 15 min and processed into the cell suspension with RPMI-1640 culture medium containing 20% calf serum. The suspension was inoculated onto a plastic cell petridish, and incubated with 100 μg/mL ECG at the atmosphere of 5% CO_2 at 37℃. The liquid was exchanged every 24 h-36 h. ③ Adhesive test of tumor cells to human umbilical vein endothelial cells: the well grown endothelial cells were digested with 0.25% panceatin, prepared into 1 × 10^3/mL cell suspension with complete culture medium, put onto 6 well petridish, added 100 μg/mL ECG, and kept at 37℃ for 20 h. After the 500U/mL TNF-α was added, it was kept at 37℃ for 4 h. One mL of Lovo cells and HT29 cells processed in experimental and control groups, was added respectively into each well. The petri dish was placed into an oscillator of 120 rmp,at room temperature for 30 min and washed twice with PBS. The cells were scraped and collected into the centrifugal tubes for centrifugation of 500 rmp × 10 min. After the upper clear liquid was removed, the cells were fixed with 2.5% glutaraldehyde

buffered, washed, fixed, dehydrated, embedded with gum, made into ultra-thin sections, colored with electronic stain for observation under a JEM1200-EX transmission electronmicroscope.

Effects of SLeX monoclonal antibody on liver metastases of experimental nude mouse

① Preparation of tumor cell suspension: The well-grown Lovo cells and HT29 cells were digested with 0.25% pancreatin, washed twice and diluted into the cell suspension with PBS after 1000 rpm × 5 min. The cell suspension concentration was regulated to 1×10^7/mL. The cell suspension was divided into 4 groups: Lovo cell, HT29 cell and their corresponding control groups. The experimental groups were added 10 μL SLeX monoclonal antibody while the control groups were added the same volume of PBS instead of SLeX. The cell suspensions were incubated at room temperature for 30 min. ② Influences of SLeX monoclonal antibody on liver metastases of experimental nude mouse: 365-week-old BALB/C healthy male nude mice were divided into 4 groups as described above. After the nude mice were anesthetized with 4% amine ketone intraperitoneal injection, their abdominal cavities were opened at 0.5 cm below left rib edge under sterile condition to expose their spleens. The cell suspension (0.3 mL) prepared was injected to the capsule below in the spleen and the abdominal cavity was closed. The mice were fed under SPF condition and killed after 4 weeks. Their spleens, livers, hearts, kidneys and lungs were taken out, dehydrated, transparented and paraffin-embedded. The tumor tissues visible were sliced while the tissues of which tumors were not found with naked eyes made serial sections before being HE dyed and observed under a light microscope. ③ Criterion of results and statistical methods: The liver metastases in each group was observed with naked eyes and under light microscope. The data obtained from the observation were tested with χ^2-test with SPSS software, with $P<0.05$ being significant.

RESULTS

In situ hybridization

αl, 3Fuc-T mRNA expressions in Lovo and HT29 cells showed negative results in the control groups while those were positive in the experimental groups. In the experimental groups, αl, 3Fuc-T mRNA in the Lovo cells were strongly positive, and their cytoplasm showed deep blue with visible royal blue fine particles (Figure 1), while αl, 3Fuc-T mRNA in the HT29 cells were weakly positive, and their cytoplasm showed light blue with visible royal blue fine particles (Figure 2). αl, 3Fuc-T mRNA expressions in Lovo cells were stronger than those HT29 cell, and their difference was significant ($P = 0.002$, Table 1).

Table 1 Expressions of αl, 3Fuc-TmRNA in two cell lines

	n	$\bar{x} \pm s$	*t*	*P*
Lovo cells	10	21.2055±7.7472	3.537	0.002
HT29 cells	10	10.7922±5.1653		

Immunohistochemistry

The SLeX expressions in Lovo cells and HT 29 cells were negative in the control groups but positive in experiment groups. The SLeX antigen expressions in Lovo cells were strongly positive, and their cytoplasm showed brown or deep brown (Figure 3); The SLeX antigen expressions in HT29 cells were weakly positive, and their membranes were brown (Figure 4). There was a significant difference between the expressions of the two ($P = 0.025$, Table 2).

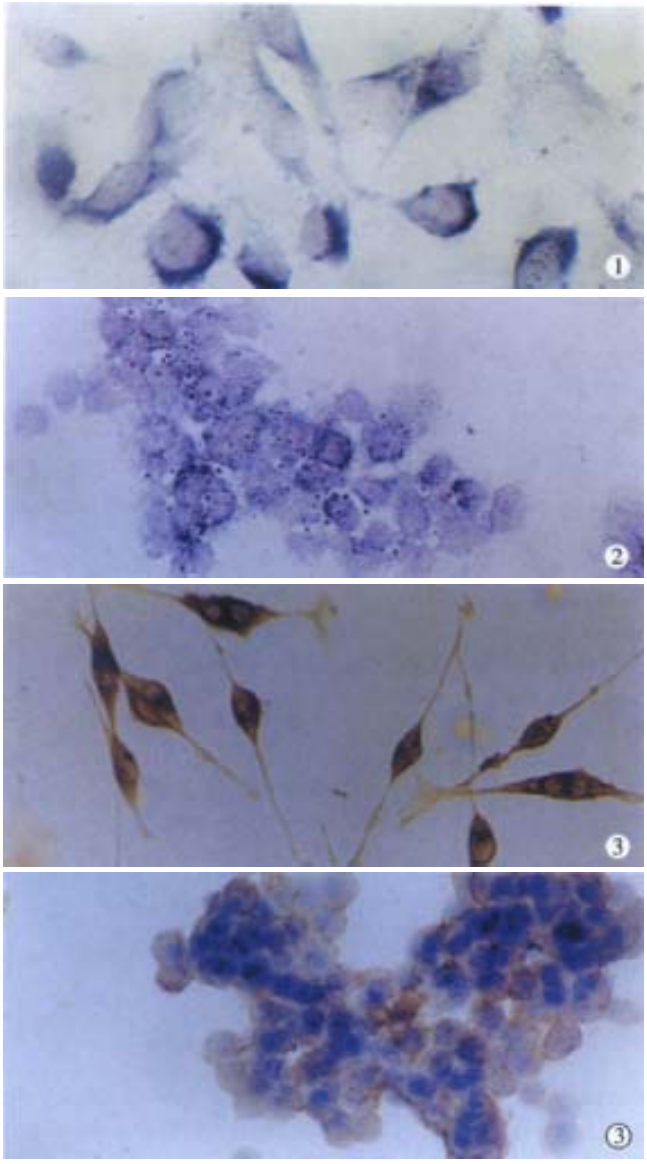

Figure 1 The expression of α1, 3Fuc-TmRNA in Lovo cells shows strongly positive staining, with deep royal blue cytoplasms and visible royal blue beads. *In situ* hybridization. ×400

Figure 2 The expression of α1, 3Fuc-TmRNA shows weakly positive staining, light royal blue with royal blue bead in cytoplasm of HT29 cells. *In situ* hybridization. ×400

Figure 3 The expression of SLeX antigen in Lovo cells shows strongly positive staining, with deep brown in cytiplasm. Immunohistochemistry. ×400

Figure 4 The expression of SLeX antigen shows positive or weakly positive staining, brown in membrane of HT29 cells. Immunohistochemistry. ×400

Table 2 Expressions of SLeX antigens in the two cell lines

	n	$\bar{x} \pm s$	*t*	*P*
Lovo cells	10	32.7633±10.8716	2.452	0.025
HT29 cells	10	21.9188±8.8016		

Flow cytometry quantitative detection

The Lovo cell and HT29 cell SLeX antigen expressions were positive. The ratio of positive SLeX antigen expressions in the Lovo experimental groups was 42. 9% while that in the control groups was 2.3%. The positive rates in the HT29 cell line were 23.3% and 2. 1% respectively in the experimental groups and their control groups. The percentage was greater in the experiment groups of Lovo cell line than that in the experiment groups of the HT29 cell line (Figures 5,6).

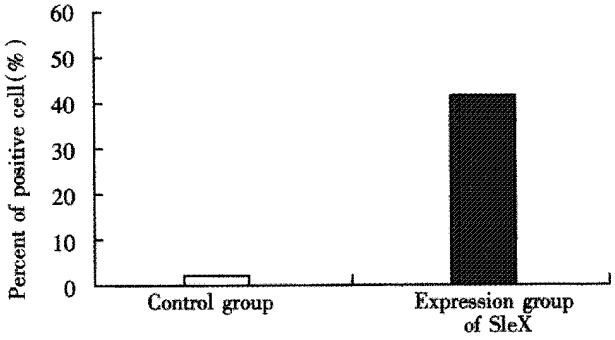

Figure 5 The detective result of Lovo cells. flow cytometry

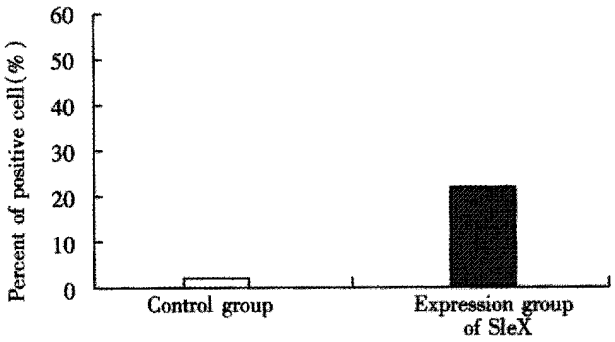

Figure 6 The detective result of HT29 cells. flow cytometry

Observation of the adhesion of tumor cells to umbilical vein endothelial cells under scanning electronmicroscope

Both Lovo cell and HT29 cell line had adhesive potential to umbilical vein endothelial cells in the control groups. The Lovo cell line showed greater adhesive potential to umbilical vein endothelial cells than the HT29 cell line. It could be observed under a 500 × scanning electronmicroscope after co-incubation for4h, a great deal of Lovo cells adhered to umbilical vein endothelial cells, and majority of them were clustered (Figure 7); and some of the HT29 cells adhered only to the surface of the umbilical vein endothelial cells, and only a part of them clustered (Figure 8). The adhesive potential was obviously even lower in the experiment groups. Under a 500 × scanning electronmicroscope after co-incubation for 4 h, the Lovo cells adhered to the umbilical vein endothelial cells were scattered and no clustering could be observed (Figure 9). The adhesive potential of the HT29 cells was weaker than that of the Lovo cells. Only a small amount of the HT29 cells adhered to the endothelial cells, and did not cluster (Figure 10). The cells adhered to the surface of the umbilical vein endothelial cells were fewer in the experiment groups than in the control groups. There was a significant difference between them ($P = 0.0001$, Table 3).

Table 3 The different adhesive potentials of tumor cells to umbilical vein endothelial cells with the tumor cells sealed

	n	Control groups $\bar{x} \pm s$	Experimental groups $\bar{x} \pm s$	*t*	*P*
Lovo cells	10	213±15.6702	124±16.7319	12.167	0.0001
HT29 cells	10	147±4.5522	69±7.3786	28.632	0.0001

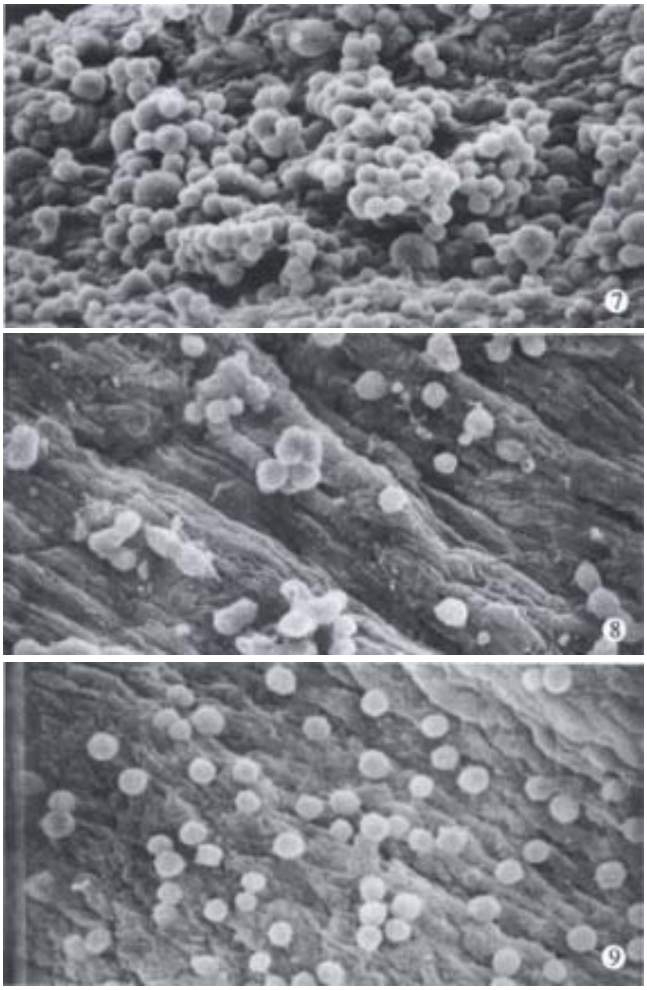

Figure 7 In control groups, a lot of Lovo cells adhered to the surface of umbilical vein endothelium, and tumor cells clustered. SEM, ×500

Figure 8 In control groups, The HT29 cells adhered to the surface of umbilical vein endothelium, a part of them clustered. SEM, ×500

Figure 9 In experimental groups, a small amount of the Lovo cells adhered to the umbilical vein endothelium, no clustering. SEM, ×500

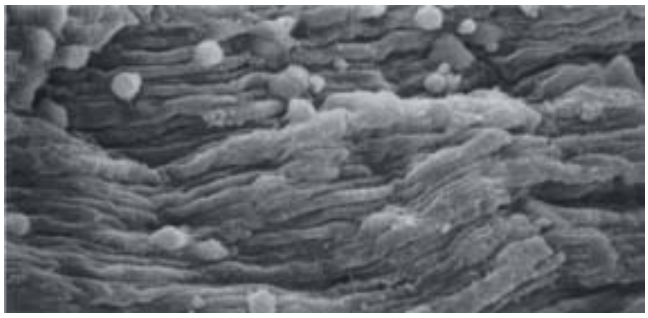

Figure 10 In experimental groups, a small amount of the HT29 cells adhered to the umbilical vein endothelium, no clustering. SEM, ×500

Observation of adhesive potential of tumor cells to umbilical vein endothelial cells under transmission electronmicroscope

In the control groups, there were a great deal of microvilli or interdigitations on the Lovo cell membrane, but few interdigitations on the HT29 cell membrane. The Lovo cells were linked to umbilical vein endothelium via interdigitations on the cell membrane (Figure 11). Some of the apophyses stretched into endothelial cell membrane; but the HT29 cells connected endothelial cell membrane directly via the cell membrane (Figure 12). No significant difference in the way of linking between the experimental groups and the control groups was observed.

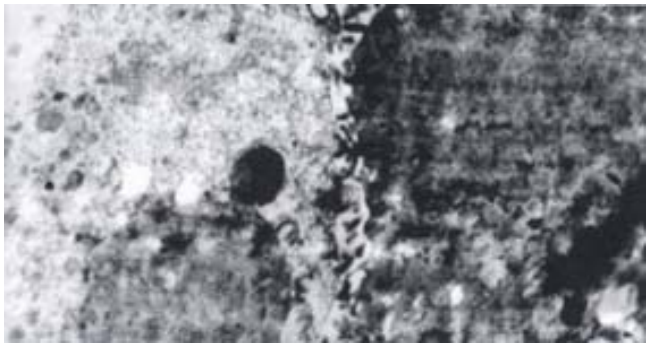

Figure 11 The Lovo cells were linked to umbilical vein endothelium via interdigitations on the cell membrane. TEM ×20K

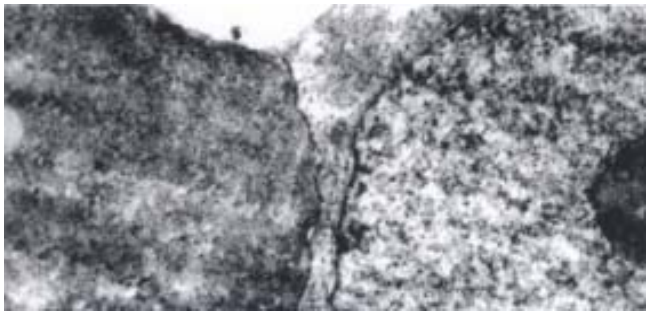

Figure 12 The HT29 cells connected endothelial cell membrane directly via the cell membrane. TEM ×24K

Effects of SLex monoclonal antibody on the liver metastases model of experimental nude mice

The ratio of liver metastases was 9/9 (100%) in the control groups of Lovo cells, while it was 2/9 (22%) in the experimental groups. The ratio of liver metastases in the experimental groups was less than that in the control groups with a significant difference between the two groups (P<0.001). The ratio of liver metastases was 4/9 (44.4%) in the control groups of HT29 cells, but 1/9 (11.1%) in the experimental groups. The ratio of liver metastases in the experimental groups was less than that in the control groups with significant difference between the two groups (P<0.002,Table 4).

Table 4 Effects of SLex monoclonal antibody on the liver metastases model of experimental nude mice

	Lovo cells		HT29 cells	
	Metastases	No metastases	Metastases	No metastases
Control groups	9	0	4	5
Experimental groups	2	7	1	8

Lovo cell groups χ^2 = 11.45, P<0.001; HT29 cell groups χ^2 = 2.498, P<0.002.

DISCUSSION

SLeX antigens are mostly expressed on the cell surface of epithelial tumors such as lung cancer, gastric cancer, colon cancer, ovary cancer[11,12]. The positive ratio in carcinoma of colon is 76%-90%[1,13]. The expression of SLeX antigen is mostly determined by two kinds of enzyme, αl, 3Fuc-T and α2, 3ST. The successive activities of the two enzymes existing in N-acethllactosamine synthesize the SLeX antigens. αl, 3Fuc-T is an important material to produce SLex antigens as well as to adjust and control the expression of SLeX antigens[14]. The increase of αl, 3Fuc-T in matrix plasma results in the increase of SLeX antigens on the cell surface[15]. The αl, 3Fuc-T and α2, 3ST synthesize the SLeX antigens which possess a strong affinity to Lectin in E-selectin and epidermal growth factor resulted with adhesion of tumor cells to the endothelial cells[3]. The SLex antigens on the cell surface in the colon carcinoma as a kind of ligands, their identification from and adhesion to E-selectin recipients on the endothelial cell surface of liver vascularitve are considered as the molecular basis of liver metastatic of colon carcinoma, and as a critical step in the liver metastatic process of colon carcinoma[5]. Modification of carbohydrate antigens on the tumor cell surface, such as SLeX antigen, will affect the ways of tumor cell metastases[4,5,16], such as adhesive potential[17], mobility[18,19], invasion[20], immunogenicity and other immune identification process[21,22] or other unknown cell behaviors which will affect the

metastasis potential of tumor cells. In other words, the structural changes of carbohydrates on tumor cell surface play a key role in the metastases of tumor cells[23]. The well known metastatic steps of tumor cells are: proliferation of tumor cells accompanied by angiogenesis of tumor → intrusion of tumor cells into basement membrane and then into blood vessels and lymphatic vessels → into blood circulation → detection in the target organ and adhesion to the capillaries → breaking through the capillaries and formation of minute metastatic spots → formation of new blood vessels and proliferation of metastatic spots[24]. There are different metastasis potential tumor cells in the primary area, for example, in colon carcinoma, SLeX antigens with high expression in tumor cells show high metastatic potential while the antigens with low expression in tumor cells show weak metastatic potential. The tumor cells with strong metastatic potential are more likely to fall off from the primary area and enter blood vessels and thus adhere to hepatic vascular endothelial cells so as to grow and form liver metastasis[25]. The reason is that SLeX antigens on the tumor cell surface as ligands of E-selectin on the endothelial cell surface of the capillaries induce adhesion between tumor cells and blood vessel endothelial cells of the target organ[26] and promote orientating tendency in motion and result in metastasis.

Our study demonstrated that the Lovo cell line with strong metastatic potential has high expression of SLeX antigen and high expression of SLeX antigen synthase, αl, 3Fuc-TmRNA while the HT29 cells line with weak metastatic potential has low expression of SLeX antigen and low expression of SLeX antigen synthase αl, 3Fuc-T. Our finding that SLeX antigen expression is closely correlated with colon carcinoma metastatic potential.

Our study also showed that Lovo cells with high expression of SLeX antigen had strong adhesive ability to vascular endothelial cells while the HT29 cell with low expression of SLeX antigen had weak adhesive potential. The connecting way to vascular endothelial cells of the Lovo cells with high expression of SLeX antigen was quite different from that of the HT29 cells with low expression of SLeX antigen. The SLeX monoclonal antibodies could effectively restrain the adhesion of tumor cells to vascular endothelial cells and well reduce the formation of liver metastases of experimental nude mice. We speculate that the reason for the decrease of adhesion is that SLeX monoclonal antibodies may have sealed carbohydrate antigens on the tumor cell membrane → site of SLex ligand and result in lower adhesive potential of tumor cells to vascular endothelial cells[27]. The results suggest that SLeX antigens play an important role in liver metastases of colon carcinoma. SLeX monoclonal antibody or its analogs may prevent the formation of liver metastases of colon carcinoma.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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The prognostic value of preoperative serum levels of CEA, CA19-9 and CA72-4 in patients with colorectal cancer

Chao Xu Zheng[1], Wen Hua Zhan[1], Ji Zong Zhao[2], Dong Zheng[3], Dong Ping Wang[1], Yu Long He[1] and Zhang Qing Zheng[1]

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INTRODUCTION

Carcinoembryonic antigen (CEA), originally described by Gold and Freedman[1] in 1965, is now an acknowledged member of immunoglobulin superfamily[2], with a role as an intracellular adhesion molecule[3]. Carbohydrate antigen 19-9 (CA19-9), obtained with a monoclonal antibody produced by immunizing a mouse with a colonic cancer cell line in 1979[4], is a ligand for E-selectin that plays an important role in the adhesion of cancer cells to endothelial cells[5,6]. More recently, tumor-associated glycoprotein 72 (TAG-72), has been identified with the monoclonal antibody B72.3[7]. This antigen has been found in a variety of human adenocarcinomas (colorectal, gastric, ovarian, breast, and lung), but rarely expressed in benign and normal adult tissue[8,9]. The CA72-4 antigen is an antigenic determinant of TAG-72 which is recognized by B72.3 and CC-49 monoclonal antibodies[10]. CEA, CA19-9 and CA72-4 represent the currently most useful tumor markers for gastrointestinal malignancies. Elevated serum levels of CEA, CA19-9 and CA72-4 have been found in many patients with colorectal, gastric, biliary tract, and pancreatic carcinoma[11-17]. Today, these tumor markers are primarily used in early diagnosis and monitoring of therapeutics[18,19], and determining the prognosis of patients who have undergone tumor resection. Since April 1996, these three tumor markers have been used as routine in our hospital for detecting colorectal cancer. The aim of this study was to assess their correlations with the tumor pathology and their prognostic value.

[1]Department of General Surgery, [2]Laboratory of Surgery,
[3]Department of Medicine, First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou 510080, Guangdong Province, China
Dr. Chao Xu Zheng, now working as a surgeon and lecturer in the Department of General Surgery, First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, who is a Ph.D. student, having 7 papers published.
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Correspondence to: Dr. Chao Xu Zheng, Department of General Surgery, First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, 58 Zhongshan 2nd Road, Guangzhou 510080, Guangdong Province, China
Tel. 0086-20-7755766 Ext.8720, Fax. 0086-20-87750632
Email. zcxzd@163.net
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MATERIALS AND MEHTODS

Patients'

A total of 107 patients with histologically diagnosed colorectal carcinoma were included in the study group. All had been treated surgically between April 1996 and March 1997. There were 74 men and 33 women. The mean age of the patients was 56.1 years (range: 26-81 years). The pathological findings (types and size of tumor, morphology, depth of invasion and number of positive lymph nodes) of the patients were reviewed. The patients were staged in accordance with Dukes classification. Ninety seven patients (90.7%) were followed up for at least 36 months or till death. Ten patients lost to follow up were excluded from the final assessment.

The control group included 155 healthy adults who received routine physical examination between December 1995 and July 1996 in our hospital, among whom, 72 were men and 83 were women. The mean age was 47.5 years (range: 20-89 years).

Serum assays for CEA, CA19-9 and CA72-4

Peripheral blood samples were obtained from the patients preoperatively and those from healthy subjects in the control group after fasted overnight. Sera were promptly separated and stored at -80℃ for use. Serum CEA was measured with a one-step, solid-phase enzyme immunoassay commercial kit (CEA EIA Kit, General Biologicals Inc., Taiwan). Serum CA19-9 was determined with a two-step, solid-phase enzyme immunoassay commercial kit (CA19-9 EIA Kit, Maxim Biotech Inc., USA). A solid-phase two-site radioimmunometric assay kit, CA72-4 RIA kit (Centocor Inc., USA), was used for determination of serum levels of CA72-4. The upper limit and cut off values of serum CEA, CA19-9 and CA72-4 in the control group 99% confidence interval were 5.0 μg/L, 31.0KU/L and

5 .0KU/L, respectively.

Statistical analysis

Statistical analysis was carried out using the SPSS 8.0 FOR WINDOWS statistical software package (SPSS Inc., USA). Chi-square test was used for univariate analysis between positive rates of serum levels of tumor markers and the pathological findings. The relation between recurrence rates and serum levels of these tumor markers was also evaluated by Chi-square test. The Kaplan-Meier method was used to calculate the cumulate survival rates and plot survival curves, and Log Rank test was used for the evaluation of statistical differences between the curves. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

The mean serum levels of CEA, CA19-9 and CA72-4 in 107 patients with colorectal cancer were 5.9 ± 10.1 μg/L (range: 0.1 - 70.0 μg/L), 61.5 ± 302.1KU/L (range: 0.1 - 5105.0 KU/L) and 8.4 ± 19.5 KU/L (range: 1.1-188.2 KU/L), respectively. The positive rates of serum CEA, CA19-9 and CA72-4 in these patients were 29. 9%, 25.2% and 32.7%, respectively.

Relationship between serum levels of tumor markers and pathological findings

The correlation between positive rates of serum tumor markers and the pathological variables is shown in Table 1. Positive rate of serum CA72-4 was significantly higher in poorly-differentiated colorectal cancer than in well and moderately-differentiated types ($P = 0.031$). There was no statistical significance between the positive serum tumor markers and the size and morphology of the colorectal cancer, but were well correlated with the depth of invasion. Both serum CA19-9 and CA72-4 were influenced by the number of metastatic lymph nodes. The results also suggested the more advanced the Dukes stage, the higher the positive rates of all these tumor markers.

Preoperative serum levels of CEA, CA19-9 and CA72-4 as prognostic indexes

Of the 97 followed up patients, 86 were classified as Dukes A, B and C stage according to the pathological findings. All underwent curative resection. The overall recurrence rate was 32.6%. In the serum CA19-9 or CA72-4 positive patients, the recurrence rates were significantly higher than that in patients with negative result (Table 2), while serum level of CEA did not correlate well with the recurrence rate ($P = 0.102$).

Among these 97 patients, positive serum CEA, CA19-9 and CA72-4 were found in 30, 25 and 31 cases, respectively. The overall 3-year survival rate was 67.0%. Survival curves of the patients according to positive and negative serum CEA, CA19-9 and CA72-4 are shown in Figures 1-3, respectively. Significant differences in survival curve were found between the positive and negative ones. The 3-year survival rate was 53.3% in serum CEA-positive patients and 73.1% in CEA-negative patients ($P = 0.042$, Figure 1); 48.0% in CA19-9 positive patients and 73.6% in CA19-9 negative patients ($P = 0.011$, Figure 2); 48.4% in CA72-4 positive patients, and 75.8% in CA72-4 negative patients ($P = 0.003$, Figure 3).

Table 1 Positive rates of CEA, CA19-9 and CA72-4 in accordance with pathologic findings

Pathological factors	No.of cases	CEA positive (%)	*P* value	CA19-9 positive (%)	*P* value	CA72-4 positive (%)	*P* value
Histological type							
Well, moderately	73	30.1		24.7		26.0	
Poorly	34	29.4	>0.05	26.4	>0.05	47.1	0.031
Tumor size (cm)							
≤5	57	31.6		22.8		28.1	
>5	50	28.0	>0.05	28.0	>0.05	38.0	>0.05
Gross morphology							
Polypoid, fungating	45	28.9		26.7		33.3	
Ulcerative	41	29.3		24.4		31.7	
Diffusely infiltrative	21	33.3	>0.05	23.8	>0.05	33.3	>0.05
Depth of invasion*							
Within sm	22	13.6		9.1		13.6	
dm, s	54	25.9	0.016	24.1	0.048	29.6	0.012
sp	31	48.4		38.7		51.6	
No. of positive lymph nodes							
0	61	29.5		14.8		21.3	
1-3	16	31.3	>0.05	31.3	0.011	37.5	0.008
>3	30	30.0		43.3		53.3	
Dukes stage							
A	8	12.5		0.0		0.0	
B	53	20.8		18.9		24.5	
C	33	39.4	0.041	30.3	0.019	39.4	0.003
D	13	53.8		53.8		69.2	

*sm: superficial muscularis propria; dm: deep muscularis propria; s: serosa layer; sp: serosal penetration to neighboring organs

Table 2 Recurrence rates in patients with positive and negative serum levels of CEA, CA19-9 and CA72-4 who underwent curative resection

Tumor markers		No. of cases	Recurrence rate (%)	*P* value
CEA	positive	24	45.8	
	negative	62	27.4	0.102
CA19-9	positive	19	52.6	
	negative	67	26.9	0.034
CA72-4	positive	24	54.2	
	negative	62	24.2	0.008

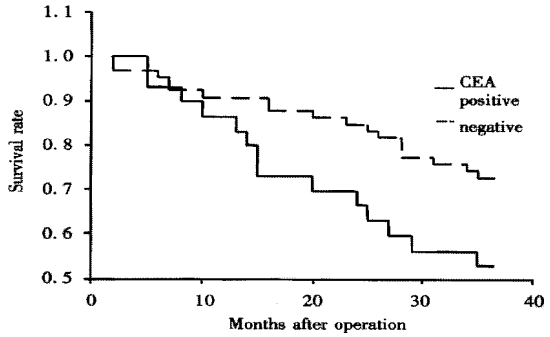

Figure 1 Survival curves of serum CEA-positive and CEA-negative patients. The difference in survival rates was statistically significant ($P = 0.042$).

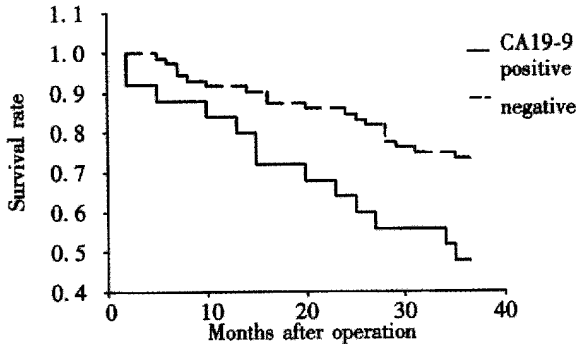

Figure 2 Survival curves of serum CA19-9 positive and CA19-9-negative patients. The difference in survival rates was statistically significant (P = 0.011).

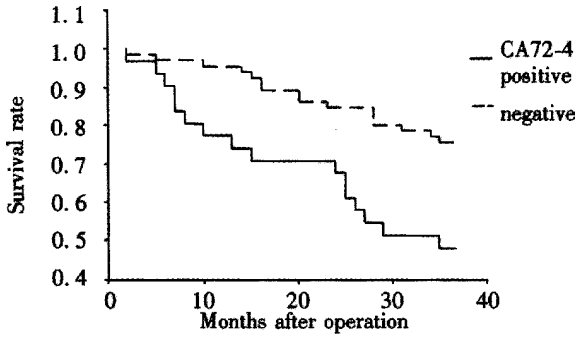

Figure 3 Survival curves of serum CA72-4 positive and CA72-4-negative patients. The difference in survival rates was statistically significant (P = 0.003).

DISCUSSION

Ideal tumor markers would be inexpensive screening and deliver early diagnosis in a large population at risk for cancer. Unfortunately, currently available serological tumor markers for gastrointestinal cancers have not been proven to be ideal. Although CEA, CA19-9 and CA72-4 are the tumor markers commonly used currently in colorectal cancer, all of them have low diagnostic sensitivity[11-13,17,20,21]. In this study, the overall positive rates of serum CEA, CA19-9 and CA72-4 were 29.2%, 25.2% and 32. 7%, respectively. For early (Dukes A and B) colorectal carcinoma, the positive rates of the above tumor markers were significantly lower than those with Dukes C and D (Table 1). The lack of sufficient sensitivity of serum tumor markers strongly restrict their applications as appropriate tools for screening and diagnosis[22,23]. Nevertheless, measurement of these tumor markers is still of importance in early detection of recurrence[24-26]. Careful examinations of abdominal-pelvic CT scan, abdominal ultrasonography, colonscopy and endoscopic ultrasonography can be complementary[27].

In this study, we investigated the correlation between preoperative serum revels of tumor markers and pathological following. The main aim was to reveal whether the following pathologic lesions as histological type, depth of invasion, lymph node metastion and staging of lesions, were related to the preoperative serum levels of tumor markers. Our data showed that the positive rate of serum CA72-4 was related to the histological type which was comparable with those reported by Ikeguchi *et al*[28], in which there was a close relationship between CA72-4 and proliferative activity of cancer cells. Although there was no significant correlation between these tumor markers and the size of the tumor or the morphology, serum CEA and CA19-9 correlated well with the depth of invasion and Dukes staging. In contrast to the study carried out by Sato *et al*[29], we found that serum CA19-9 was also influenced by the number of metastatic lymph nodes, whereas serum CA72-4 was related to the depth of invasion, lymph node metastasis and staging of the lesions.

An important consideration in assessing the clinical value of tumor markers is to find whether tumor marker assay can provide useful information on clinical outcome of the patients, in addition to the common prognostic factors. Previous reports demonstrated that elevated preoperative serum levels of CEA and CA19-9 were predictive of increased mortality in colorectal cancer[30]. Increased levels of CEA and CA19-9 were also associated with venous and lymphatic spread[31, 32]. As shown in our study and the reports of Forones and Tanaka[30], preoperative serum level of CEA was not a perfect predictor for recurrence in patients undergoing curative resection, but CA19-9 was proven to be a better indicator for recurrence. Positive CA19-9 expression on tumor tissue, and its positive pre and post-operative serum level, were associated with poor survival rates[33,34]. Our study showed that there was significant difference in 3-year survival curves between serum CEA-positive and CEA-negative patients, and also significant difference was found in serum CA19-9 positive and CA19-9 negative patients, which was similar to that reported by Sato *et al*[29]. CA72-4 was confirmed to have better sensitivity and specificity than CEA and CA19-9[35,36]. However, very few studies on the prognostic value of CA72-4 in colorectal cancer have been reported in the literature, except some concerning gastric cancer[16,28,37]. In the current study, in addition to CEA and CA19-9, we also investigated the prognostic value of serum level of CA72-4 in preoperative patients with colorectal cancer. We found that patients with positive serum CA72-4 had higher recurrence rate than those with negative CA72-4 (P = 0.008), and the 3-year survival rate much lower than that in CA72-4 negative patients (P = 0.003). High serum levels of tumor markers are often associated with more aggressive cancer, which usually has short disease free interval and short survival period. Both CEA and CA19-9 are molecules involved in intercellular

adhesion[3,5], hence, the cells expressing these molecules on their surfaces would possess a greater metastatic potential[38]. It was shown that in experimental metastasis model of colorectal carcinoma in nude mice, a systemic injection of recombinant CEA enhanced hepatic colonization of tumor cell *in vivo*[39], presumably by their increased hepatic retention[40]. Other authors reported that CA19-9 serves as a specific ligand for ELAM-1 and plays a significant role in the ELAM-1mediated binding of human cancer cells to activated endothelial cells, which indicates CA19-9 expressed on the surface of cancer cells may involve in the process of vascular invasion and metastasis[41]. Meanwhile, the function of TAG-72 (CA72-4) in the process of tumor progression remains unclear.

In conclusion, the measurement of preoperative serum levels of CEA, CA19-9 and CA72-4 will aid in predicting the prognosis of patients with colorectal cancer who have been treated surgically. Elevated serum levels of tumor markers indicate high risk of cancer recurrence and poor survival. Such patients should receive adjuvant therapy, at the earlier stages of this disease.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Neutron-induced apoptosis of HR8348 cells *in vitro*

Li Ping Wang[1], Ke Liang[2], Yu Shen[2], Wei Bo Yin[2], G. Hans[3] and Yan Jun Zeng[1]

Subject headings fast neutrons; X-rays; rectal neoplasms/pathology; tumor cells, cultured/radiation effects; apoptosis/radiation effects

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INTRODUCTION

To date, the major therapy for rectal carcinoma is extensive abdomino-perineal resection[1]. Unfortunately, after resection of rectal carcinoma, many patients still die of blood-borne metastases, usually in the liver or lungs, or local pelvic recurrence [2,3], which is the major cause of morbidity and mortality in patients with rectal carcinoma. Pre-or postoperative radiotherapy can reduce the incidence of local recurrence[4-7], but even with moderately high radiation doses, many patients are not locally controlled and have distant metastases[8,9]. The reason for this may be low intrinsic radiotherapy in at least some of the tumors.

Fast neutrons were introduced for cancer therapy in the 1970s. It has been reported that fast neutron therapy is effective for some carcinomas[10,11]. In addition, apoptosis induced by X-rays has been reported[12,13], but apoptosis induced by fast neutron rarely reported. Therefore, in the present study fluorescent staining of DNA is used to detect not only whether irradiation with fast neutron induces apoptosis, but also whether the difference in the level of apoptosis exists between X-rays and neutron induced Chinese rectal carcinoma cell lines. Furthermore, *p*53 and Bcl-2 gene expression were detected by immunohistochemical methods for Chinese human rectal carcinoma cell line with various doses of X-rays and neutrons, we could thereby compare the level of *p*53 and *Bcl*-2 gene expression induced by X-rays and neutrons.

[1]Biomechanics & Medical Information Institute, Beijing Polytechnic University, Beijng 100022, China
[2]Department of Gastrointestinal Surgery, Aalborg University, Denmark
[3]Cancer Institute (Hospital), Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100021, China
Li Ping Wang, graduated from Beijing Polytechnic University in 2000, major in tumor radiotherapy, having 3 papers published.
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Correspondence to: Yan Jun Zeng, Biomechanics & Medical Information Institute, Beijing Polytechnic University, No.100 Ping Le Yuan, Chao Yang District, Beijing 100022, China
Tel. 0086-10-67392172, Fax. 0086-10-67392297
Email. wcjd@public.bta.net.cn http://www. wd. org. cn
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MATERIALS AND METHODS

HR8348 cells taken from poorly differentiated adenocarcinoma of the rectum were grown as a monolayer in Dulbecco's Modified Eagle Medium supplemented with 100 mL·L^{-1} heat-inactivated fetal bovine serum and subcultured every other day. Culture conditions were 37℃ in a humidified atmosphere buffered by 50 mL·L^{-1} CO_2 in air and 56 g·L^{-1} sodium hydrogen carbonate, pH 7.4. Twenty-four hours after passaging, exponential cell cultures were irradiated at room temperature with a single dose of 6-Mv X-rays from Varian linear accelerator (Cancer Institute & Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College). The dose rate was about 250cGy·min^{-1}. The SSD (source-surface distance) was 100 cm. The PDD (percentage of depth dose) was 100%. The field of radiation was about 32. 6 cm × 30.5 cm. Cells were radiated with 0, 2, 4, 6, 8, 10Gy.

Fast-neutron-rays radiation conditions

The conditions of cells culture while cells were radiated by fast-neutron rays from electron cyclotron (High Energy Physics Institute, Chinese Academy of Sciences) were the same as by X-rays. The SSD was about was about 150 cm. The PDD was 93.68%.The field of radiation was about 18.0 cm × 18.0 cm. Cells were irradiated with 0, 0.67, 1.34, 2.01, 2.68, 3.34Gy (the same effects of radiobiology as one-third of the equivalent dose of X-rays).

Apoptosis assay

Cells were plated in 100 mm^2 tissue culture flask (10^6 cells/flask) for determination of apoptotic fraction. The cells were irradiated by various dosages of X-rays (0, 2, 4, 6, 8, 10Gy respectively) and fast-neutron-rays (0, 0. 67, 1.34, 2.01, 2.68, 3.34Gy respectively) in exponential phase, approximately24 h later. After replacement with fresh culture medium, the cell samples were exposed at room temperature to graded doses of X-rays or fast-neutron-rays. After radiation, the tissue culture flasks were incubated at 37℃ for 6, 24, 48 and 72 h respectively prior to the assessment of apoptosis. The cells (detached cells and attached cells) trypsiniszed were collected together, spread on glass slides and fixed with 900 mL·L^{-1} methonal and 100 mL·L^{-1} glacial acetic acid. Then, the slides were stained with 1 g·L^{-1} Hoechest 33 342 in PBS for 10 min, and rinsed briefly in water. The nuclear morphology was then examined by fluorescence microscopy.

Apoptotic cells were counted microscopically, according to published criteria[14-18]. The frequency was expressed as the number of apoptotic cells per 100 cells (apoptotic index). 1500 cells per data point were counted. Additionally, apoptosis was evaluated by gel electrophresis. Cells were washed once with PBS and digested overnight at 37℃ with 100 ug/mL proteinase K in20 mmol·L^{-1} TRIS-HCl (pH 8.0), 10 mmol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA and 10 g·L^{-1} SDS. DNA was extracted with phenol and chloroform, then precipitated with 1/10 volumes of 3 mol·L^{-1} sodium acetate and 4 volumes of ethanol, dissolved in TE buffer (pH 8.0) and digested with RNAase. DNA samples were electrophoresed on a 15 g·L^{-1} agrose gel, stained with ethidium bromide, and visualized with an UV illuminator. Moreover, the method of TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-biotin nick end labeling (TUNEL), was performed according to the instructions *in situ* cell death detection kit (Boehringer Mannheim, Germany).

Immunohistochemistry

The glass slides with cells were immersed in 3 mL·L^{-1} hydrogen peroxidase in phosphate buffered saline (PBS) for 5 minutes to block endogenus peroxidase activity. After washing with PBS, the slides were treated with 100 mL·L^{-1} normal goat serum to reduce nonspecific antibody binding. For antigen retrievals the slides were placed in an antigen retrieval solution (10 mmol·L^{-1} citric acid monohydrate pH 6.0) and heated in a microwave oven for 10 min. After washing with PBS the slides were incubated for 1h with anti-*p*53 and Bcl-2 monoclonal antibody. After further washing with PBS, slides were incubated with biotinylated rabbit anti-mouse second-stage antibody. After washing further with PBS, peroxidase-conjugated streptavidin was applied. Peroxidase activity was demonstrated by adding diaminobenzidine as a chromogen. The slides were counter-stained with hemotoxylin. Negative control slides were processed without the primary antibody. Positive control slides were processed from a nasopharyngeal squamous low-differentiated carcinoma previously shown to express high levels of p53 protein and Bcl-2 protein. Immunostained slides of both test and control slides were independently scored by two of the authors. In rare cases of disagreement, consensus was reached after discussion. For p53 protein, only nuclear staining was considered significant. For Bcl-2 protein, only cytoplasm staining was considered significant. A semiquantitative scale was used for grading of positive immunostaining, depending on the intensity of nuclear staining and the percentage of the stained nuclei.

RESULTS

Apoptosis in relation to time and dose

Increase of apoptotic cells above the spontaneous level (4.1%) was detected up to 2Gy (or 0.67Gy). Apoptotic index (percentage of apoptotic cells) increased with the increase of radiation dose and time after irradiation by X-rays and fast-neutron-rays. No cell apoptosis plateau level 72 h after radiation was observed. At the same point of time, the AI of cells induced by fast-neutron-rays was higher than AI of cells induced by X-rays. (Figure 1A, 1B,1C). Figure 2 represents the features of cell shrinkage, chromatin condensation and margination of cell apoptosis induced by X-rays and fast-neutron-rays respectively in Chinese rectal carcinoma cell line. We were able to visualize the characteristic DNA ladder pattern induced by X-rays and fast-neutron-rays (Figure 3). TUNEL clearly revealed a distinct pattern of nuclear staining (Figure 4).

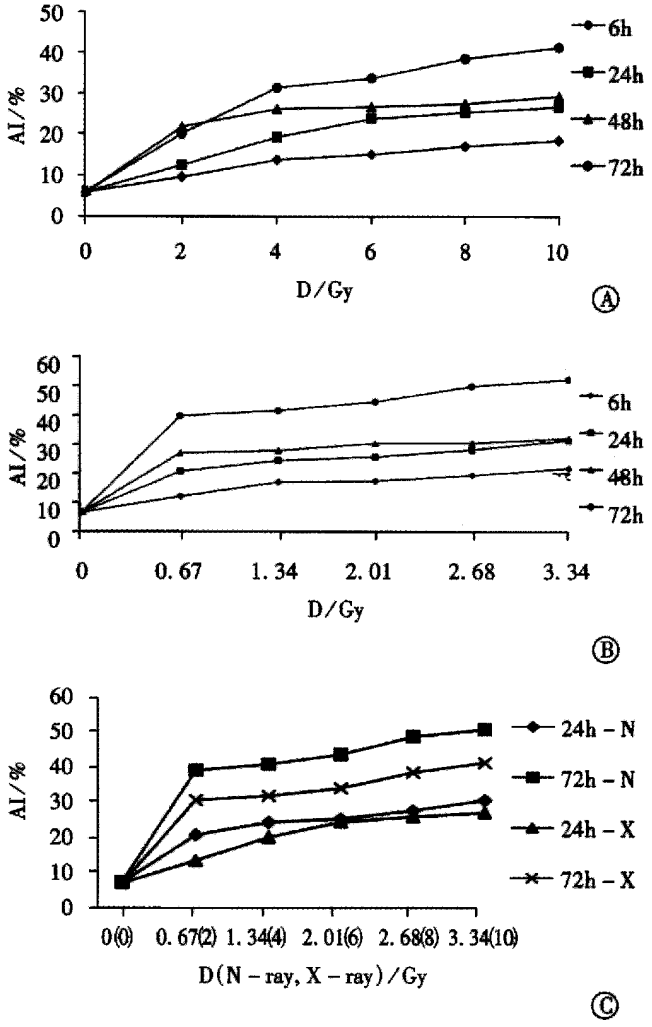

Figure 1 (A) AI at different time intervals after radiation of various doses of X-rays and (B) fast-neutron-rays. AI of cells induced by X-rays and fast-neutron-rays of various doses gradually increased with the increase of dose and time. (C) Comparison of AI between X-rays and N-rays. At the same point of time, AI of cells induced by fast-neutron-rays was higher than that induced by X-rays.

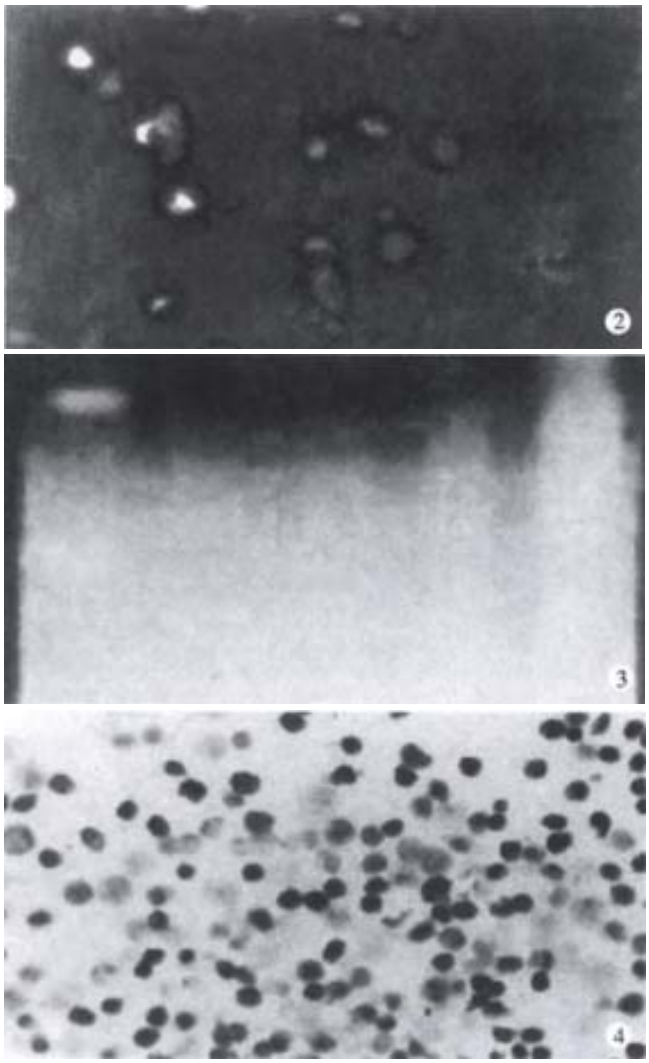

Figure 2 Hoechest 33 342 fluorescence staining of cells radiated by fast-neutron-rays shows the special morphological features of apoptotic cells: cell shrinkage, chromatin condensation, lunated margination.

Figure 3 Effect of X-rays on DNA fragmentation in HR 8348 cells. Cells were incubated for 6 h after radiation by X-rays. DNA fragmentation was examined by agrose gel analysis, as described in Materials and methods. A, 885 bp and 585 bp DNA marker; B, Control; C, 2Gy ; D, 8Gy; E, 10Gy.

Figure 4 Positive nuclear staining of cells irradiated by 8Gy X-rays with TUNEL was observed.

P53 and Bcl-2 immunostaining related to irradiation dose

High expression of p53 protein and Bcl-2 protein were observed in cells without radiation, and the percentage of positive cells was about 90%. After radiation with various dosages of X-rays, no apparent decrease in percentage of p53 positive cells (about 80%) was observed. However, the of p53 positive cells changed from deep yellow to light yellow. While the percentage and degree of Bcl-2 positive cells showed no apparent decrease when compared with p53 protein. As for fast-neutron-rays, it didn't lower the percentage or the degree of p53 and Bcl-2 positive cells significantly (Figure 5).

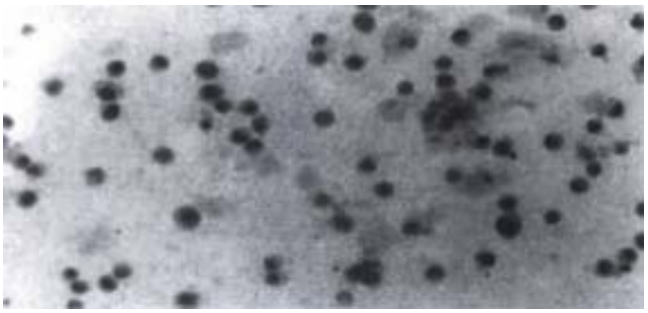

Figure 5 P53 positive nuclear staining of cells irradiated by 8Gy X-rays with immunohistochemistry staining was seen. Nuclei of cells appeared in light yellow.

DISCUSSION

Our results indicate that apoptosis induced by X-rays and fast-neutron-rays in Chinese rectal carcinoma cell line is time-dose dependent. Moreover, the level of apoptosis induced by fast-neutron-rays is higher than by X-rays, which suggests rectal cancer cells are more radiosensitive to fast-neutron-rays than to X-rays. Fast-neutron-rays is particulate radiation and high linear energy transfer (LET) radiation, with no charged particles. X-ray is electromagnetic radiation and low linear energy transfer radiation. These exists difference in radiosensitivity of cells between fast-neutron-rays and X-rays, because: ① anoxic cells exist in most tumor cells [19,20], and ionizing density causes the oxygen enhancement ratio (OER) of high LET radiation to be lower than that of low LET radiation, that is, the radiosensitivity of high LET radiation is less dependent on oxygen in cells than low LET radiation; ② fast neutrons are densely ionizing particles, with a high relative biological effectiveness relative to X-rays (RBE); and ③ sublethal damage repair produced by high LET radiation is lower than that by low LET radiation. So far, the molecular basis of their properties is not yet entirely understood. The term apoptosis was first used by Kerr, Wyllie and co-workers to describe a particular form of cell death, different from necrosis[21-23]. Much evidence has shown that apoptosis is an active process regulated by a series of proto-oncogenes and tumor suppressor genes associated with proliferation and differentiation, such as p53, Bcl-2, c-myc, ICE, Fas/APO-1 etc[24-27].

Of particulate interest is the Bcl-2 gene, which was discovered in 1984 through the cloning of the translocation breakpoint in nodular B cell lymphoma and located at 18q21. Later, the product of Bcl-2 was shown to be a mitochondrial internal membrane protein which had the unique property of blocking apoptosis induced in cultured immature B cells[28-30]. To date, the mechanisms of apoptosis blocking of Bcl-2 has been shown to be a complicated process, involving the regulation of a

growing list of proteins, which share sequence homology with Bcl-2, and the relevant genes[31,32]. One of these proteins, Bax, opposes the action of Bcl-2 and promotes apoptosis. It has been proved that apoptosis has been regulated by the proportion of Bcl-2 and Bax. The Bax's homologous dimer can induce apoptosis, but the rising Bcl-2 protein combining with Bax will form Bax-Bcl-2 heterogenous dimer that is more stable than "Bax-Bax". This will neutralize the Bax function of inducing apoptosis[33,34]. Therefore, only high expression of Bcl-2 protein is not sure to inhibit apoptosis. Our results indicate that downregulation of Bcl-2 protein expression did not appear with the increase in apoptotic index for cells irradiated by X-rays and fast-neutron-rays.

The tumor suppressor gene p53 is indispensable for normal development. It plays a central role in the cellular response to DNA damage from both endogenous and exogenous sources, providing a protection against tumorigenesis. Activation of p53 may result in a cell cycle delay, presumably to allow an opportunity for DNA repair to occur before replication or mitosis[35,36]. In some cell types, however, p53 activation results in apoptotic cell death as a means of eliminating irreparably damaged cells. The final outcome of p53 activation depends on many factors, and is mediated largely through the action of downstream effector gene transactivated by p53[37]. In our experiment, high expression of mt p53 protein appeared in cells without radiation. After being radiated with various doses of X-rays, downregulation of mt p53 protein occurred. In contrast, downregulation of mt p53 protein has not appeared in cells irradiated by various doses of fast-neutron-rays. Some evidence has shown that the difference of DNA dsb (double strand break) repair area exist[38], which suggests basic differences in the nature of the lesions induced by high and low LET ionizing radiations. Other data has provided clear experimental evidence for the existence of clustered DNA double-strand breaks and demonstrate that short DNA fragments may be produced by neutron radiation, an observation not made for damage by low-LET radiation[39]. Additionally, It has been reported that the DNA damage by fast neutrons was shown to be significantly greater than for the same absorbed dose of X-rays[40]. On these grounds, we infer that X-rays and fast-neutron-rays may lead to the different DNA damage, e.g. a single or double strand break in DNA, nuclear supercoils, or the differences in the nature of dsb (double-strand break) induced and the way they are repaired. The difference of DNA damage may lead to different pathway of apoptosis involved in the different regulation of relevant apoptotic genes. Fast-neutron-rays may induce the involvement of apoptotic gene other than p53.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Influence of whole peptidoglycan of bifidobacterium on cytotoxic effectors produced by mouse peritoneal macrophages

Li Sheng Wang[1], Hui Ming Zhu[1], Dian Yuan Zhou[2], Yu Lin Wang[1] and Wan Dai Zhang[2]

Subject headings bifidobacterium; whole peptidoglycan; macrophage; laser scanning confocal microscopy

Wang LS, Zhu HM, Zhou DY, Wang YL, Zhang WD. Influence of whole peptidoglycan of bifidobacterium on cytotoxic effectors produced by mouse peritoneal macrophages. ***World J Gastroenterol***, 2001;7(3):440-443

INTRODUCTION

Bifidobacteria are physiologically beneficial bacteria which are predominant in human intestine, and possess the most important functions. They play an important role in maintaining microbial balance of the intestine. Furthermore, their presence is thought to be an important indication of health of the body[1-4]. Whole peptidoglycan (WPG) is the major component in the cell wall of bifidobacterium, which is also a biological response modifier with nontoxic side effects. At present, many scholars have demonstrated that bifidobacteria can activate macrophages, T lymphocytes and natural killer cells to secrete many kinds of cytokines and important mediators[5-8]. In our report, the level of IL-6, IL-12 and TNF-α produced by macrophages is detected by employing laser scanning confocal microscopy, when these cells are stimulated by WPG of bifidobacteria bifidum. Simultaneously, the content of nitric oxide (NO) secreted from the macrophages is investigated by utilizing Griess reagent. Our goal is to explore the roles of these cytotoxic effector molecules on adjusting immune reaction of bifidobacterial WPG.

[1]Department of Gastroenterology, Shenzhen Municipal People's Hospital, Jinan University of Medical Sciences, Shenzhen 518020, Guangdong Province, China
[2]Chinese PLA Institute of Digestion, the First Military Medical University, Guangzhou 510515, Guangdong Province, China
Li Sheng Wang, graduated and obtained Ph.D, from the First Military Medical University in 1998, now working at Department of Gastroenterology, Shenzhen Municipal People's Hospital, Jinan University of Medical Sciences, having 35 papers published.
Supported by the Natural Science Foundation of Guangdong Province, No.994066
Correspondence to: Li Sheng Wang, Department of Gastroenterology, Shenzhen People's Hospital, Jinan University of Medical Sciences, Shenzhen 518020, Guangdong Province, China
Tel. 0086-755-5611620
Email. Wangls2001@163.net
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MATERIALS AND METHODS

Materials

Whole peptidoglycan It was extracted from the cell wall of bifidobacteria bifidum according to the method described by Sekine and his colleagues,donated by Dr. Hu, who worked at department of examination of Zhongjing Medical University. Simultaneously, it had been evaluated[9].

Experimental animals BALB/c nude mice were purchased from the Experimental Animal Center of the First Military Medical University, 6-8 weeks in age, 18 g-22 g in weight, and housed in the SPF animal room.

Methods

Division of the experimental group and management of WPG The experimental animals were divided into two groups: the WPG injection group: 10 nude mice, 2 mL of 100 $g \cdot L^{-1}$ thioglycollate was injected into the nude mice intraperitoneally on d1, 0. 25 mg WPG (equivalent to 1×10^9 bifidobacteria) was injected intraperitoneally from d 2-6 continuously; and the control group: the number of animals and procedures were similar to the WPG injection group on d1, 0.2 mL of PBS was injected intraperitoneally from d 2-6 continuously.

Collection and culture of peritoneal macrophages of nude mice All animals were killed on d7. Abdomen skin of the nude mice was sterilized routinely. Cells were obtained by sterile lavage of the peritoneal cavity with cold D-hanks balanced salt solution. The lavage fluid was centrifuged at 1000 rpm for 10 min. The supernatant was decanted. The cells thus obtained were twice washed, resuspended in RPMI 1640 culture medium containing free fetal calf serum, then adjusted to be $1 \times 10^9 \cdot L^{-1}$, and plated ($2 \times 10^5$ cells/well) in 96-well tissue culture plates. Nonadherent cells were removed by repeated washing after 2 h incubation at 37℃ in 50 $mL \cdot L^{-1}$ CO_2 in air. Adherent cells were macrophages. These cells were stimulated with 10 $\mu g \cdot L^{-1}$ LPS in RPMI 1640 containing 100 $mL \cdot L^{-1}$ FCS for 24 h. Supernatant was stored at -30℃ until assayed.

Detection of IL-6, IL-12 and TNF-α contents of peritoneal macrophages of nude mice Laser scanning

confocal microscopy was utilized to detect the content of IL-6, IL-12 and TNF-α. The concrete protocol was as follows: ① The peritoneal cell suspension of nude mice was harvested and added to the modified Petri plate, and the desired peritoneal exudate cells were adjusted to 1×10^5 in 100 μL·plate^{-1}. After incubated in a 50 mL·L^{-1} CO_2 incubator at 37℃ for 2 h, these plates were washed vigorously to eliminate nonadherent cells, and macrophages were obtained. Then 500 μLof RPMI 1640 medium containing 10 μg·L^{-1} LPS was added to each plate. After 24 h, the supernatants were discarded, and the macrophages were washed with phosphate buffered saline (PBS) 3 times for 5 min; ② 100 g·L^{-1} non-specific bovine serum album was added to the plates which were incubated for 10 min at room temperature; ③ The plates were rinsed with PBS 3 times for 5 min;④ 100 μL of rabbit anti-mouse IL-6, IL-12 and TNF-α monoclonal antibody (Genda Technology Corp. Canada) was added to the plates which were incubated for 60 min at 37℃ respectively; ⑤ The plates were rinsed with PBS 3 times for 5 min; ⑥ 100 μL of FITC labeled goat anti-rabbit IgG (Santa Cruz) was added to the plates which were incubated for 30 min at 37℃. The antibody was used at a dilution of 1 in 100. ⑦ The plates were rinsed with PBS 3 times for 5 min; ⑧ The fluorescent intensity of macrophages was detected by employing laser scanning confocal microscopy with an arousement wavelength of 488 nm and a radiation wavelength of 475 nm. For each plate, average fluorescent value from beyond 100 macrophages in different fields was investigated, which was chosen to be quantitative parameter.

Griess reagent Naphthylethylenediamine hydrochloride (0.1%) (Sigma) was prepared with distilled water, and 1% sulfanilamide (Sigma) was prepared with 5% H_3PO_4. The two reagents were mixed equally before utilization.

Detection of the level of NO After 1 mmol·L^{-1} sodium nitrite ($NaNO_2$) was diluted by means of doubling series, it was mixed with equal Griess reagent, then shaken for 10 min lightly at room temperature. At last, absorbance value (A) of triplicate samples was read on Bio-Rad 550 type microelisa reader using a test wavelength of 550 nm. One hundred microliter of each supernatant which was to be determining was mixed with 5 μL of 300 g·L^{-1} $ZnSO_4$ to remove protein, then centrifuged at 5000 rpm for 10 min. The supernatant was collected, and mixed with equal Griess reagent, then shaken lightly for 10 minutes at room temperature. Absorbance value (A) was read on Bio Rad 550 type Microelisa Reader using a test wavelength of 550 nm.

Statistic analysis

The statistical analysis between WPG injection group and control group was made by means of Student's *t* test.

RESULTS

NO content of produced by peritoneal macrophages of nude mice stimulated by WPG

A standard curve was made by analyzing the relationship between different concentrations of standard $NaNO_2$ and the corresponding A value assayed. Simultaneously, linear correlation and regression analysis was performed, and the linear regression equation was as follows: Y = 0.013 + 0.022X, r = 0.999. It suggested that there existed a fine correlation between the content of standard $NaNO_2$ and the corresponding A value. The content of NO was obtained correspondingly when the A value of different samples substituted the linear regression equation. By means of the statistical analysis,we found that the content of NO produced by peritoneal macrophages of nude mice in WPG injection group was significantly higher than in the control group (P<0.01, Table 1).

WPG influence on IL-6, IL-12 and TNF-α production of peritoneal macrophages of nude mice

The content of IL-6, IL-12 and TNF-α produced by peritoneal macrophages of nude mice was detected by employing laser scanning confocal microscopy, when WPG of bifidobacteria bifidum was injected into nude mice intraperitoneally. The results suggested that the macrophages present different color. The macrophages in WPG injection group appeared white, red, yellow and blue which was narrow in area relatively, while, the macrophages in control group were mainly blue and less yellow. Different color reflected different fluorescent intensities. Analyzing with the ACAS software of laser scanning confocal microscopy, showed that the fluorescent intensity, which reflected the content of IL-6, IL-12 and TNF-α emitting from peritoneal macrophages of nude mice in WPG injection group, was markedly higher that in control group (P<0.01, Table 1 and Figures 1,2).

Table 1 Influence of WPG on IL-6, IL-12, TNF-α and NO production by peritoneal macrophages of nude mice ($\bar{x} \pm s$, n = 10)

Groups	IL-6 / A	IL-12 / A	TNF-α / A	NO (μmol·L^{-1})
WPG	1956.48±265.32[b]	2603.24±395.72[b]	813.42±106.77[b]	53.21±6.40[b]
Control	931.56±189.70	1054.33±184.50	318.90±76.35	30.67±12.83

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

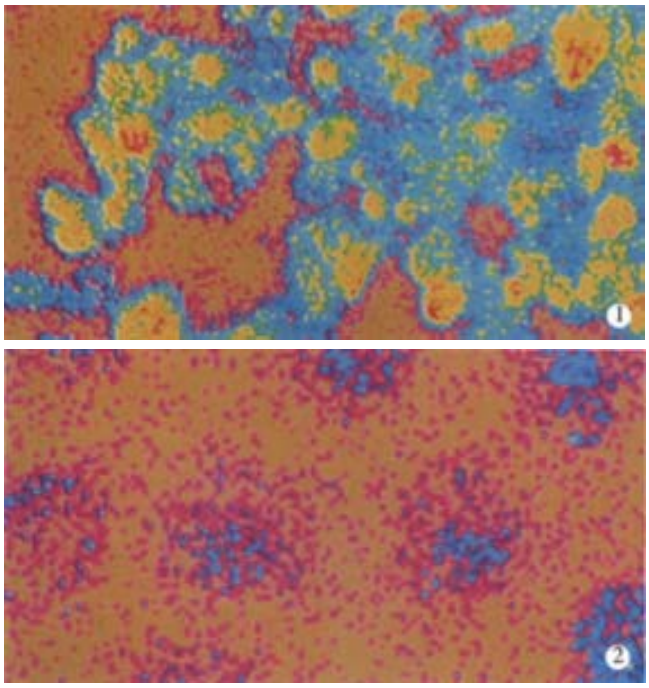

Figure 1 The fluorescence picture of IL-12 derived from the macrophages in WPG injection group.
Figure 2 The fluorescence picture of IL-12 derived from the macrophages in control group.

DISCUSSION

WPG of bifidobacteria bifidum is a bag-form structure which consists of polysaccharides and peptidoglycans. It preserved the integral structure of whole cells, and possessed some important biological characteristics, such as relaxation of senescence, antitumor, control of infection, antimutation, etc[10-14]. Furthermore, it could also activate macrophages of immune system of the body. Tejada-Simon and his colleagues demonstrated that WPG of bifidobacteria infantis could activate RAW 264.7 macrophage cell line to produce a lot of TNF-α and NO *in vitro*[8]. In our report, the fluorescence intensity of IL-1, IL-6 and TNF-α and the content of NO derived from the peritoneal macrophages of nude mice was significantly elevated when WPG of bifidobacteria bifidum was injected into nude mice intraperitoneally. It was indicated that WPG of bifidobacteria bifidum could activate macrophages to secrete a large amount of cytotoxic effector molecules.

IL-1, IL-6 and TNF-α was the important cytokines produced by activated macrophages. They could act many aspects of immune system. IL-6 could promote the differentiation and maturation of B lymphocytes, and stimulate these cells to secrete antibodies. Furthermore, it also could induce proliferation and activation of resting T lymphocytes directly[15,16]. IL-12 could induce the production of IFN-γ by resting and activated T lymphocytes and natural killer (NK) cells, and possess the ability to act as a LAK cell growth factor[17-20]. TNF-α could augment the antitumor ability of NK, CTL and LAK cells, and play an important role in adjusting the activation of T lymphocytes[21,22]. These cytokines had broard antitumor and antimetastatic activities *in vivo* and *in vitro* markedly[23-35]. NO was the signal molecules and effector molecules which had broard biological activities. It was also the important effector molecules that activated macrophages killing tumor cells and pathogenic micro-organisms[36-41]. The induction of these cytotoxic effector molecules may play an important role in antitumor immune reaction of WPG. It was widely acknowledged that WPG could obviously inhibit the growth of many kinds of tumors *in vivo*. Rhee and his colleagues demonstrated that WPG of bifidobacteria spp. Exhibited markedly antitumor activity against subcutaneously transplanted sarcoma 180 in mice [42]. Ishihara, *et al* reported that the volume of metastatic skin melanoma obviously decreased, when intralesional administration of WPG of bifidobacteria infantis was prepared. We had also found that WPG of bifidobacterium bifidum could induce apoptosis of the colorectal carcinoma transplantation neoplasms of nude mice, and inhibit its proliferation simultaneously[43]. In our report, WPG could activate macrophages to secrete a lot of IL-6, IL-12, TNF-α and NO. Because these important mediators present obvious antitumor activity, the cytotoxic effector molecules produced by activated macrophages may mediate the effect on antitumor of WPG.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Hepatocellular Carcinoma-Cause, Treatment and Metastasis

Zhao-You Tang

Liver Cancer Institute & Zhongshan Hospital of Fudan University
Correspondence to Zhao-You Tang, M.D., Professor of Surgery Chairman, Liver cancer Institute of Fudan University (previous Liver Cancer Institute of Shanghai Medical University) 136 Yixueyuan Road, Zhongshan Hospital, Shanghai 200032, China. zytang@srcap.stc.sh.cn
Tel: 0086-21-64037181, Fax: 0086-21-64037181
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Abbreviation **HCC-hepatocellular carcinoma, HBV-hepatitis B virus, HCV-hepatitis C virus, HGV-hepatitis G virus, TTV-transfusion transmitted virus, AFB1-aflatoxin B1, IFN-interferon, OLT-orthotopic liver transplantation, PH-partial hepatectomy, RCT-randomized controlled trial, TACE-transcatheter arterial chemoembolization, PEI-percutaneous ethanol injection, PMCT-percutaneous microwave coagulation therapy, RF-radiofrequency, AFP-alpha fetoprotein, VEGF-vascular endothelial growth factor.**

Abstract

In the recent decades, the incidence of hepatocellular carcinoma (HCC) has been found to be increasing in males in some countries. In China, HCC ranked second of cancer mortality since 1990s. Hepatitis B and C viruses (HBV and HCV) and dietary aflatoxin intake remain the major causative factors of HCC. Surgery plays a major role in the treatment of HCC, particularly for small HCC. Down-staging unresectable huge HCC to smaller HCC and followed by resection will probably be a new approach for further study. Liver transplantation is indicated for small HCC, however, some issues remain to be solved. Different modes of "regional cancer therapy for HCC" have been tried. Systemic chemotherapy has been disappointing in the past but the future can be promising. Biotherapy, such as cytokines, differentiation inducers, anti-angiogenic agents, gene therapy and tumor vaccine will probably play a role, particularly in the prevention of tumor recurrence. HCC invasiveness is currently the major target of study. Tremendous works have been done at the molecular level, which will provide clues for biomarker of HCC progression as well as targets for intervention.

Subject headings carcinoma, hepatocellular/etiology; carcinoma, hepatocellular/surgery; carcinoma, hepatocellular/drug therapy; liver neoplasms/etiology; liver neoplasms/surgery; liver neoplasms/drug therapy; human; review

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INTRODUCTION

Liver cancer is the 4th most common cause of death from cancer, the highest age-standardised mortality rate is in China ($34.7/10^5$, the 2nd cancer killer since 1990s), which alone accounts for 53% of all liver cancer deaths worldwide[1]. Recently, the incidence of HCC has been found to be increasing particularly in males in countries such as Japan, Italy, France, Switzerland, United Kingdom and the United States[2-4]. Clinical advances have mainly been made in the fields of medical imaging, surgery, regional cancer therapy and biotherapy. Rapidly growing knowledge in basic science appears at the molecular level, particularly in the study of HCC invasiveness. Although a lot of news from bench to bedside on the advances made in HCC has appeared, the overall dismal outcome of patients with HCC changed very little. In the United States, the relative 5 -year survival for liver cancer only increased from 4% (1974-76) to 6% (1986 -93) in white, and from 1% to 4% in black[5]. In Shanghai, the relative 5-year survival of liver cancer in 1988-91 was 4. 4%[6]. These indicate that there is still a long way to go in conquering HCC.

CAUSE AND PREVENTION

Viral hepatitis B (HBV) and/or C (HCV), aflatoxin and alcohol are major risk factors of HCC

However, the importance of these different factors varies in different geographic areas. HBV is more predominant in Chinese, Southeast Asian and African patients with HCC, whereas HCV is common in HCC patients in developed countries (Japan, France, Italy and others). The prevalence of hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV) in HCC patients were reported to be 63.2% and 11.2% respectively in China[7], which was similar to that reported in the past.Prospective studies showed that there is an additive effect of HCV and HBV infection on HCC development[8]. Cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC than patients infected by other HCV types[9-11]. An association was found between high serum alanine aminotransferase levels and more rapid development and high incidence rate of HCC in patients with HCV-associated cirrhosis[12]. In a transgenic mice, it was found that the core protein of HCV induces HCC[13].

Hepatitis G virus (HGV) and transfusion-transmitted virus (TTV) infection might not play an important role

Based on the data from China, Japan, Africa, United Kingdom and others, HGV might not play an important role in the development of HCC[14-18]. A case-control study also failed to support the hypothesis of an association between transfusion-transmitted virus (TTV) infection and HCC[19]. However, some authors claimed that HGV and TTV could not be completely excluded as causative agents[20-22].

In China, HBV and HCV (mainly HBV), aflatoxin and contamination of drinking water (such as microcystin, a promoter of hepatocarcinogenesis) remain as major risk factors of HCC, and alcohol should be added in northern China

A study showed that exposure to aflatoxin metabolite M1 (AFM1) can account for a substantial part of the risk of HCC[23].

Other risk factors have also been reported

In Japan, alcohol consumption and cigarette smoking were also risk factors of HCC, and synergism between them was observed[24,25]. In Italy, for attributable risk (AR) of HCC, heavy alcohol intake ranked first (45%), HCV second (36%) and HBV third (22%)[26]. The risk of dietary iron overload was 4.1 for HCC in black Africans[27], which is similar to that of haemochromatosis in Caucasians. A role of family history independent from and interacting with known risk factors for HCC was also reported, the odds ratio was reported to be 2.4[28].

HCC risk is high in individuals with both aflatoxin B1 (AFB1)-DNA adducts and HBsAg, suggesting a viral-chemical interaction

Furthermore, AFB1 exposure correlates with a specific mutation at codon 249 of the p53 tumor suppressor gene in HCC, indicating a molecular pathogenesis[29]. How the four major risk factors (HBV, AFB1, p53 mutation and male gender) for HCC interact to produce malignant liver tumors were also demonstrated in transgenic mouse models[30].

X protein of HBV is one of the target of how HBV induces HCC

The incidence of HCC was as high as 86% in a HBV-X transgenic mice[31]. It was also found that the structure of the X gene is modified in the majority of tumorous livers, suggesting a potential role of mutated X proteins in HBV-related liver oncogenesis[32]. Moreover, HBV-X might play a role in hepatic inflammation by up-regulating interleukin-6 production, which can eventually lead to HCC[33]. Transactivation of transforming growth factor alpha (TGF-alpha) gene by HBV preS1 was observed which provides a clue for understanding viral hepatocarcinogenesis[34]. Synergy between TGF-alpha and HBsAg in hepatocellular proliferation and carcinogenesis was also reported[35].

All of these indicate a multifactorial and multistep development of HCC. Interaction among HBV/HCV, aflatoxin, alcohol, and genetic susceptibility might be important.

The dawn of HCC prevention has been shown

Few approaches of HCC prevention have been emerged, namely: prevention of HBV infection using vaccine, avoid exposure to carcinogens and promoters by changing drinking water, and to prevent viral hepatitis B or C progressing to cirrhosis and HCC by interferon (IFN) therapy. Result from a universal hepatitis B vaccination program indicated that the incidence of HCC in children has declined[36]. A significant declining trend of HCC mortality rate ratios was observed in the vaccination group, but not in the reference group[37]. In Qidong County of China, after people changed their source of drinking water from pond-ditch water (microcystin was found) to deep-well water, the mortality rate of HCC stabilized and even decreased slowly[38]. An analysis of patients with chronic hepatitis, liver cirrhosis, chronic hepatitis bearing HCC and liver cirrhosis bearing HCC, found that the incidence of HCC in the control group was 10.4/100 person-year, while that in the IFN treated group was 1.2/100 person-year[39]. IFN decreased HCC incidence in patients with HBV related cirrhosis. The cumulative occurrence rates of HCC in the treated group and the untreated group were 17.0% and 30.8%, respectively, at the end of 10 years[40]. IFN therapy also decreased the development of HCV related HCC. HCC rates in the IFN treated and untreated groups were 7.6% and 12.4% at the 10th year respectively[41]. Patients with HCV-related cirrhosis also benefit from IFN treatment[42]. IFN therapy significantly reduces the risk for HCC, especially among virologic or biochemical responders of patients with chronic hepatitis C[43]. For those nonresponder, retreatment with IFN-alpha appeared to have the additional effect of suppressing the development of HCC in patients who had incomplete responses to the initial treatment, even when the HCV was not cleared with retreatment[44]. Currently, lamivudine or ribavirin, antiviral agent, is added to the treatment of HBV or HCV, however, long-term follow-up study is needed to evaluated whether this additional treatment will increase the efficacy of HCC prevention.

SURGERY OF HCC

Small HCC resection plays an important role to improve HCC prognosis

Small HCC resection has resulted in marked increase in 5-year survival rate from 20%-30% to 40%-60%. At the author's institution, the 5-year survival rate of 963 patients with small HCC ($\leqslant$5cm) resection was 65.1%, whereas it was only 36.1% for large HCC resection (n=1308); of the 368 HCC patients with 5-year survival, 198 (53.8%) patients received small HCC resection[45,46]. Early HCC with well differentiated cancer containing Glisson's triad has been recognized as an entity with a high rate of surgical cure, the 5-year survival was as high as 93%[47]. A comparison between subclinical HCC and symptomatic HCC revealed that operability was higher (26.8% versus 7.9%), and cumulative survival rate was also higher[48].

Makuuchi *et al* (1998) have performed 367 hepatectomies on 352 patients since 1990, the 5-year survival rate was 47.4%[49]. At the author's institution, HCC resection has been performed on 2119 patients between 1979-1998, the 5-year survival rate was 51.5%[50]. Recently, perioperative blood transfusion and diabetes mellitus were found to be prognostic factors after HCC resection[51,52]. An experimental study indicated that partial hepatectomy was associated with increased levels of TGF-alpha, TGF-beta, and basic fibroblast growth factor (bFGF) in the liver and accelerates local tumor growth[53].

Down-staging of unresectable huge HCC to smaller HCC followed by resection will probably be a new approach for further study

At the author's institution, the 5-year survival of 108 patients with this approach (down-staging by hepatic artery ligation, cannulation, cryosurgery, etc.) was 64.7%[45]. Another 65 patients with unresectable HCC down-staged by transcatheter arterial chemoembolization (TACE) followed by resection, the 5-year survival was 56.0%[54]. The 5-year survival rates

were similar to that of small HCC resection, which coincided with a reduction of median tumor size from 10.0cm to 5.0cm during the resection of this approach[55]. However, a well designed randomized trial is needed for a final evaluation.

Orthotopic liver transplantation (OLT) is a reasonable treatment for small HCC if partial hepatectomy (PH) is impossible

For decades, the role of OLT in the treatment of HCC has been unclear. In the early 1990s, it was accepted that small HCC was indicated for OLT. However, only retrospective data were available for the comparison between OLT and PH in the treatment of HCC. The 5-year survival rate of 422 HCC patients with OLT was 44.4%, and tumor histologic grade and tumor size (>5cm) were linked to recurrence-free patient survival[56]. A comparison between PH (n = 294) and OLT (n = 270) showed that survival was comparable, but operative mortality was lower in PH group, and concluded that HCC developing in a well compensated cirrhotic liver initially may be treated with PH, and OLT should be applied selectively to those patients with tumor recurrence and/or progressive hepatic failure[57]. A proper selection of candidates for PH gives better results than OLT, because of the increasing waiting time for OLT[58]. OLT is a reasonable treatment for patients with early stage tumors if PH is impossible. The oncological advantage of OLT compared with PH, however, is questionable[59]. As survival after PH and OLT for early stage HCC does not reveal a significant difference, resection of these tumors is still justifiable[60]. When compared with PH, OLT for resectable HCC offers substantial survival benefit among well-targeted subgroups of patients as long as an organ donor is available within 6 to 10 months time delay. However, the marginal cost-effectiveness ratios incurred by this strategy are higher than that of many other current medical interventions[61]. This might be of particular impact for developing countries where HCC is endemic.

NONSURGICAL THERAPIES FOR HCC

There is still a long way to go of nonsurgical therapies for HCC

Nonsurgical therapies for HCC generally include regional cancer therapies, radiotherapy, chemotherapy and biotherapy. Unfortunately, a systemic review of 37 RCTs to examine the effect of different treatments for non resectable patients indicated that only 3 modalities were minimally and uncertainly effective (embolization, tamoxifen and IFN)[62]. Another overview of 30 RCTs for unresectable HCC found that no treatment has clearly proven efficacy in survival. 5-Fluorouracil, adriamycin and transarterial chemotherapy were not associated with survival benefit at 1 year. The number of RCTs was insufficient to enable a conclusion to be reached for IFN and PEI (percutaneous ethanol injection). Controversy persists concerning tamoxifen efficacy[63].

Regional cancer therapy for HCC is one of the nonsurgical therapies that develops recently

Based on the advances of early detection and medical imaging, more HCCs can be diagnosed with small and localized lesions. As a result, regional cancer therapies have developed in the recent decades. Unfortunately, the number of RCTs was insufficient to make any conclusion as yet.

Transcatheter chemoembolization (TACE) is one choice of the treatment for unresectable but not far advanced HCC, particularly for patients with multifocal HCCs and with acceptable liver functions. However, some RCTs failed to demonstrate that TACE improve the survival with unresectable or advanced HCC[64,65]. A RCT found that the 4-year survival of intrahepatic-arterial ^{131}I-labeled lipiodol (2.2 GBq) was 10% when compared with 0% in TACE group (70mg cisplatin)[66]. Another RCT indicated that styrene maleic acid neocarzinostatin in Lipiodol was better than epirubicin in Lipiodol[67]. A comparison of planned periodic TACE and TACE based on tumor response found that the 3-year survival rates were 0% and 15% respectively in Okuda 2 stage, the mean time between the first and the third courses of TACE was 4 months and 14 months respectively, indicating the efficacy of TACE increased when it was used selectively and was repeated only when necessary[68]. The overall 5-year survival rate after TACE treatment is around 6%-8%. TACE resulted in prolongation of survival in patients with tumor volumes of less than 200mL, tumor-to-liver volume ratios of less than 5%, and iodized oil retention greater than or equal to 75%[69]. Complications of TACE were encountered in 4.4% of cases, of which, hepatic failure and down-staging of cirrhosis remain a problem[70].

Percutaneous ethanol injection (PEI) is a treatment choice of unresectable small HCC. The 4-year survival rate was 39% in 47 small HCC patients with cirrhosis, however, the 4-year recurrence rate was as high as 79%[71]. Local recurrence depends predominantly on the biologic characteristics of the tumor (histologic grade and intrahepatic recurrence), regardless of the efficacy of PEI[72]. For large (>5cm) HCC, PEI performed in a single session under general anesthesia was an alternative. In a series of 108 patients, the 4-year survival rates were 44% for single encapsulated HCC (5 cm - 8.5 cm), 18% for single infiltrating HCC (5cm-10cm) or multiple HCC and 0% for advanced disease, the mortality was 0.7% and major complications 4.6%[73]. A RCT study comparing 50% acetic acid and PEI indicated that local recurrence rate was lower and 2-year survival rates higher with acetic acid[74].

Percutaneous microwave coagulation therapy (PMCT) is an extension of PEI, the 5-year survival for patients with well-differentiated HCC treated with PMCT and PEI were comparable, however, among the patients with moderately or poorly differentiated HCC, 5-year survival with PMCT (78%) was better than with PEI (35%)[75]. PEI is difficult for small HCC on the surface of liver, however, PMCT can be performed safely in such patients[76]. After PMCT, a second biopsy on 19 patients showed complete destruction of tumor in 18 patients[77].

Radiofrequency (RF) hyperthermia is another mode of regional cancer therapy. Of the 73 HCC patients treated with RF and evaluated by CT, complete response rate was 10%, partial response rate 21%, and 5-year survival 17.5%[78]. A comparison was made between RF and PEI in the treatment of small HCCs. It was found that RF ablation resulted in a higher rate of complete necrosis (90% versus 80%) and requires fewer treatment sessions than PEI. However, the complication rate was higher with RF ablation than with PEI[79].

The inadequacy for complete control of cancer nodule is one of the major problems of regional cancer therapies. Therefore, surgery remains the choice of treatment for curatively resectable HCC with Child A cirrhosis until a RCT clarifies the situation. In general, TACE is a treatment choice

for multinodular and large unresectable HCC (a part of TNM Stage II, IIIA, IIIB and IVA; with Child A or Child B cirrhosis). The other regional therapies may be used on unresectable small HCC which is not multinodular.

Three-dimensional conformal radiotherapy will probably play a role for HCC treatment in the future

A pilot study indicated that three-dimensional conformal radiotherapy helped to avoid excessive exposure of the liver and adjacent organs and made it a safer treatment modality for unresectable HCC[80]. Selective internal radiation therapy using 90Y microspheres (median 3.0 GBq) was effective for selected cases of nonresectable HCC. There was a 50% reduction in tumor volume in 26.7% of patients after the first treatment, the nontumorous liver appeared more tolerant to internal radiation than external beam radiation. This treatment may help to convert nonresectable tumors to resectable ones[81]. At the author's institution, long-term follow-up study indicated that a combination of surgery and intrahepatic arterial infusion of ^{131}I-anti-HCC mAb improved survival of unresectable HCC[82].

Systemic chemotherapy for HCC has been disappointing in the past, but in the future can be promising

Neither complete response nor partial response was observed using paclitaxel (175mg/m^2 q3w) for unresectable HCC[83]. However, a phase II study with cisplatin, doxorubicin, 5-fluorouracil, and IFN-alpha in advanced unresectable HCC demonstrated that complete pathological remission was possible, partial response rate was 26%, no viable tumor cells were found in four out of nine resected specimens[84]. Based on the study of the expression of drug resistance-related genes in three human hepatoma cell lines, it was demonstrated that IFN-alpha modulated the mechanism of resistance to cisplatin in liver cancer[85]. Individual patient with complete remission of multiple HCC associated with HCV-related decompensated liver cirrhosis by oral administration of enteric-coated tegafur/uracil has been reported[86].

Biotherapy will play a role in the treatment of HCC in the future

However, the results were still controversial. Many RCTs of tamoxifen for advanced HCC were negative[87-90]. A lack of efficacy of antiandrogen treatment was found for unresectable HCC in a RCT[91]. Oral beta all transretinoic acid (50mg/m^2 t.i.d.) was also ineffective against HCC[92]. Interestingly, Octreotide, a somatostatin analogue, improves survival of inoperable HCC in another RCT[93]. Randomized controlled trial of interferon treatment for advanced HCC indicated that its administration prompts no benefit in terms of tumor progression rate and survival[94].

Gene therapy-"progress but many stone yet unturned[95]"

Gene therapy for HCC remains an attractive field. Experimental studies using cytokine genes (tumor necrosis factor, interleukin-2, interferon), suicide and *p*53 genes; using retrovirus, adenovirus and Epstein-barr virus as vectors; using AFP enhancer; using intraarterial administration, etc. have been reported[96-108]. Data from both the literature[109-112] and from the author's institution[113] demonstrated that human malenoma antigen (MAGE) gene expression is frequent in HCC, suggesting that HCC patients may be good candidates for specific immunotherapy (tumor vaccine) using MAGE encoded antigen. Dendrite cells are good candidates for this particular purpose[114].

METASTASIS OF HCC

Invasiveness of HCC has become a major target of recent research

The high recurrent rate in the liver with mainly intrahepatic metastatic spread remains a major obstacle to further improvement on the long-term survival after curative HCC resection. Therefore, research on the invasiveness of HCC has become a major target. Clinically, targets include prediction, treatment and prevention; in the laboratory, investigations include metastatic model, molecular events, angiogenesis, intervention, etc.

A routine biomarker for prediction of metastasis and recurrence is not yet available

Although many biomarkers have been tried, such as AFPmRNA, circulating VEGF and PD-ECGF[115,116], human macrophage metalloelastase gene[117], p27[118], p53 mutation[119], expression of p73[120], telomerase activity[121], etc.

Both pre- and postoperative chemotherapy or chemoembolization have not adequately proved to be effective for prevention of metastatic recurrence

Convincing evidence is lacking to support systemic preoperative chemoembolization in patients with initially resectable HCC[122]. Although many authors supported the strategy of postoperative chemoembolization, its effectiveness might be due to suppression of intrahepatic micrometastases rather than multicentric carcinogenesis[123]. Postoperative intraarterial chemotherapy has also been claimed to improve survival[124,125]. Recently, a RCT showed that postoperative adjuvant systemic chemotherapy using epirubicin and mitomycin C has a tendency to reduce recurrence rate[126]. However, two RCTs failed to demonstrate the effectiveness of postoperative adjuvant therapy. The adjuvant chemotherapy with epirubicin and carmofur after radical resection of HCC was not effective[127]. Postoperative chemotherapy using intravenous epirubicin and intraarterial iodized oil and cisplatin was associated with more frequent extrahepatic recurrences and a worse outcome[128]. Interestingly, a RCT revealed that oral polyprenoic acid prevents second primary HCC after surgical resection, and reconfirmed after longer follow-up study[129,130]. Recently, a RCT study indicated that a single 1850 MBq dose of intra-arterial ^{131}I-lipiodol increased the 3-year overall survival from 46.3% in the control to 86.4% in the treatment group[131].

The molecular basis of "HCC invasiveness" is similar to that of other solid cancers, its complexity represents as multi-genes involvement and multi-step process

Numerous papers have been published concerning the molecular basis of "HCC invasiveness" in the literature[132-150]. At the author's institution, studies concerning HCC invasiveness could be summarized into the followings: a Factors that positively related to invasiveness included: p16 and p53 mutation, H-ras, c-erbB-2, mdm2, TGFα, epidermal growth factor receptor (EGF-R), matrix metalloproteinase-2 (MMP-2), urokinase-type plasminogen activator (uPA), its receptor (uPA-R) and inhibitor (PAI-1),

intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), basic fibroblast growth factor (bFGF), etc. On the other hand, factors that negatively related to HCC invasiveness included: nm23-H1, Kai-1, tissue inhibitor of metalloproteinase-2 (TIMP-2), integrin α5, E-cadherin, etc. b The biological characteristics of small HCC was slightly better than that of large HCC c The following blood test have been tried with potential clinical implication: thrombomodulin, ICAM-1, PAI-1, VEGF, bFGF, etc. Serum ICAM-1 content was higher in patients with metastasis than those without metastasis. Loss of heterozygosity (LOH) at D14S62 and D14S51 (on chromosome 14q) in plasma DNA were also related to metastatic recurrence. The combination of several items that mentioned above increased sensitivity[151-164].

Comparison between primary HCC tumors and their metastatic lesions using comparative genomic hybridization (CGH) indicated that chromosome 8p deletion might contribute to HCC metastasis[165]. The presence of at least three novel tumor suppressor loci on 8p in HCC was reported[166], and DLC-1 might be one of the related tumor suppressor gene[167].

Metastatic human HCC model in nude mice (LCI-D20) and HCC cell line with metastatic potential (MHCC97) have been established[168,169]

Using corneal micropocket model in nude mice, the difference of angiogenesis induced by LCI-D20 and LCI-D35 (a low metastatic model) was also demonstrated[170]. Highly metastatic HCCs induced in male F344 rats and a transplantable lymph node metastatic mouse model of HCC were also reported[171,172]. These will provide a tool for the study of the mechanism and the intervention of metastasis.

Angiogenesis is closely related to HCC invasiveness

Vascular endothelial growth factor (VEGF) gene and protein expression are involved in the progression of HCC[173-175], and that VEGF 121 and 165 isoforms play a critical role in angiogenesis of HCC[176]. However, some author reported that VEGF might be associated with the angiogenic process of the cirrhotic liver, but not with the angiogenesis of HCC[177]. VEGF level increased after TACE, indicating that VEGF may be a marker for tumor ischemia[178]. Angiogenesis in HCC depends on the net balance between human macrophage metalloelastase (a potent angiogenesis inhibitor) and VEGF gene expressions[179]. Platelet-derived endothelial cell growth factor (PD-ECGF), another angiogenic factor, is also involved in HCC progression[180]. The enhanced gene expression of angiopoietin-2 may also contribute to the hypervascular phenotype[181]. Angiogenesis in HCC can be evaluated by CD34 immunohistochemistry[182,183]. At author's institution, using CD34 staining to measure microvessel density (MVD), we found that MVD was only useful for small HCC resection, the 5-year survival after resection of hypovascular type small HCC was double to that of hypervascular type, being 74.6% versus 34.7%[184]. As small HCCs increase in size and become increasingly dedifferentiated, the number of portal tracts apparently decreases and intratumoral arterioles develop. These findings may reflect changes in the hemodynamics as the HCC develops[185].

Experimental intervention of HCC metastases is progressing

Many approaches have been tried in preventing metastases, and anti-angiogenesis is one of the major target. For example, anti-angiogenic agent TNP-470, a derivative of fumagillin, was found to inhibited tumor growth and metastasis in nude mice bearing human HCC and suppressed the progression of experimentally-induced HCC in rats[186,187]. High-dose and long-term therapy with IFN-alpha inhibited tumor growth and recurrence in nude mice bearing human HCC xenografts with high metastatic potential in a dose-dependent manner, and the preventive effect was mediated by anti-angiogenesis[188]. However, its clinical significance has to be assessed by a RCT. Other experimental interventions for metastasis were also reported, such as matrix metalloproteinase inhibitor BB-94[189], 4-[3,5- Bis (trimethylsilyl) benzamido] benzoic acid (TAC-101)[190], antisense H-ras oligodeoxynucleotides[191], synthetic β peptide[192], etc.

In short, much has been done and much remains to be done. Well designed RCTs are needed for a more clear conclusion in many treatment modalities that are in debate. Some agents that have not been effective for advanced HCC may still be tried in the prevention of metastases and recurrence with a much smaller tumor burden. In the 21st century, prevention is doubtlessly of prime importance, however, detection of small HCC and studies on HCC invasiveness remain critical issues for further improvement of prognosis of HCC.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Fertility and pregnancy in inflammatory bowel disease

Elspeth Alstead

Correspondence to: Dr. Elspeth Alstead, Senior Lecturer and Consultant Physician, Department of Adult and Paediatric Gastroenterology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD, UK. e.m. alstead@mds.qmw.ac.uk
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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder affecting young adults in the reproductive years. It is common for both female and male patients with IBD to ask questions about IBD's effect on their relationships, sexual and reproductive function, in particular fertility, the outcome of pregnancy and its possible effects on the disease. An open discussion of the social situation and education targeted at these issues therefore forms an essential part of the management of any young person with IBD. The questions that are most commonly asked are summarised in Table 1. In order to answer these questions we need evidence. There are few large prospective case controlled studies to provide the information which is required but the available data, some of it from small observational studies, will be summarised in this chapter.

Table 1 Questions commonly asked by IBD patients

Sexual health	Will I be able to have normal relationships and a family?
Inheritance	Will my children inherit IBD?
Fertility	Will my fertility be impaired by IBD or its treatment?
Outcome of pregnancy	Will I have a normal, healthy baby?
Disease activity in pregnancy	Will my IBD flare up in pregnancy?
Drug and other treatments	I don't want to take any drugs during pregnancy?
Breast feeding	Is breast feeding advisable and safe

SEXUAL HEALTH

It is well established that general measures of quality of life are impaired in patients with IBD[1]. Sexual health is an important aspect of quality of life which is often overlooked in a routine gastroenterological consultation. Sexual problems in IBD often seem to be focused around three major factors: body image problems, difficulties with social relationships and impaired sexual function[2]. Crohn's disease (CD), in particular has been shown to have an impact on self-image, social relationships and sexual function[1]. Body image concerns are frequently found in IBD patients relating either to the direct physical effects of their disease such as weight loss, growth retardation as a result of chronically active disease in childhood, fistulae or perianal disease. The effect of surgery especially when a stoma is involved is associated with low self-esteem and poor body image. The side effects of steroids and other medications may lead to weight gain, hirsutism, skin changes and other features which promote feelings of unattractiveness. There is some evidence that psycho-social effects of stoma surgery performed in childhood, before puberty may be less severe than if such surgery is performed during the teenage and early adult years[3]. Partners of IBD patients with stomas have been found to be more likely to be able to accept the stoma than the patient themselves[4]. Psychological fears of loss of control of bodily functions and the fear of rejection by new or established partners in an intimate relationship all contribute to difficulties in social and sexual relationships. Counselling and practical advice and support is frequently helpful, but the need for it is not always identified in general gastroenterological practice.

Some studies have reported an increase in sexual difficulties including dyspareunia in women who have had surgery for IBD and there is some evidence that patients with IBD may delay or even defer pregnancy because of their disease[4,5]. Most of the reports of sexual dysfunction are in women with Crohn's disease. There are no specific reports of sexual dysfunction in women with ulcerative colitis (UC) who have not had previous surgery.

In men with IBD the risk of impotence after proctocolectomy is the main concern[4]. Advances in surgical technique have decreased although not eliminated post-operative sexual dysfunction, and this seems to apply to both conventional proctocolectomy and pouch surgery with a reported incidence of impotence of around 4%-8%[6]. It is important to remember that patients are often quite reluctant to discuss such delicate matters and tactful prompting and adequate time during the consultation is the key to their detection.

CONTRACEPTION

As there is clear evidence that the outcome of pregnancy is better in women with IBD who have quiescent disease at the time of conception, advice about contraception is keenly sought and opinions have differed over the years. In women who do not smoke and who have quiescent or mildly active Crohn's disease (CD), the use of a low dose combined oral contraceptive is not associated with increased disease activity compared with non-users[9,10]. There are no data about the thrombo-embolic complications of the oral contraceptive pill in IBD but this should be considered especially in patients with active UC.

INHERITANCE OF IBD

Individuals with IBD are often concerned that their children may inherit the illness. There is a familial increased risk in IBD which is stronger if the parent has CD. This risk also appears to be greater in Jewish families. The life-time risk factor for a child of a parent with CD is around 7%-9% of developing CD and about 10% for the development of IBD[11]. If both parents are affected the risk for any children may be up to 35%.

FERTILITY

Women with UC generally appear to have normal fertility, although one retrospective study from Scotland reported that women who had had surgery for IBD had increased infertility compared to the general population (25% *vs* 7%)[12]. Voluntary childlessness in people with IBD is still probably greater than in the general population however, possibly relating to fear of pregnancy or even inappropriate professional advice suggesting that pregnancy might be dangerous. In CD, fertility is probably normal in individuals with inactive disease[13,14]; however, fertility is impaired in women with active Crohn's disease. This relates directly to disease activity and fertility appears to revert to normal with the induction of remission. Women whose IBD develops before their first pregnancy have been shown to have fewer pregnancies than population controls. In women who had had a pregnancy prior to the onset of IBD, however, they seem to have a similar reproductive history to a control population[13].

Increasingly, men are concerned about fertility and other issues of reproductive health. Sulphasalazine has been known for many years to cause reversible semen abnormalities with impairment of fertility in up to 60% of men on the drug. This effect is reversed two months after withdrawing sulphasalazine. Men express concerns about the safety of immunosuppressive agents in terms of fertility and the risk of congenital abnormality, but there are no reliable data at all for guidance in this area[15].

PREGNANCY OUTCOME IN IBD

In recent years there has been an increased interest in the outcome of pregnancy in IBD and a number of epidemiological surveys and case controlled studies have been published. In UC, there is a large body of evidence suggesting that the frequency of spontaneous abortion, still birth and congenital abnormality are no different to that in the general population[16-19]. In quiescent Crohn's disease, pregnancy outcome, in terms of spontaneous abortion, still birth and congenital abnormality, is also no different from the general population[14,17]. Active Crohn's disease at the time of conception or during pregnancy significantly increases foetal loss and pre-term delivery and it appears that disease activity rather than medical treatment accounts for the adverse outcomes[20,21].

A large prospective population-based study looked at adverse pregnancy outcomes in 239773 single pregnancies in Sweden over a two year period. This included 756 pregnancies in women with IBD. This is the expected number of IBD pregnancies for this population[22]. No significant increase in the most serious adverse outcomes of still birth or infant death in the first year of life was found in the IBD mothers. There was also no significant increase in babies which were small for gestational age. There was however, a significantly increased risk of pre-term birth (odds ratio 1.81, 95% confidence intervals 1.06-3.07) at less than 33 weeks and for 33-36 weeks (odds ratio 1.48, confidence intervals 1.0-1.19), and of low birth weight (less than 1500g, odds ratio 2.15 confidence intervals 1.11-4.15). IBD patients also had an increased caesarean section rate (15% *vs* 10%). These estimates were not affected by adjustments for maternal age, parity and smoking. There was, however, not any information about whether the mothers experiencing pre-term delivery and low-birth weight babies had UC or CD, or whether these were women with active disease during their pregnancies. More recent studies from France and Denmark have confirmed this small increase in pre-term birth and low birth weight, particularly in CD[23,24].

INFLUENCE OF PREGNANCY ON IBD ACTIVITY

In any woman with quiescent IBD at the time of conception, the likelihood of a flare-up of IBD during pregnancy or the puerperium is no greater than in any other year of her life. Active UC at the time of conception is associated with continuing disease activity in about two thirds of pregnant women. Chronic activity will continue throughout pregnancy in about a quarter of these patients and in about 45% the activity may actually worsen[14]. This therefore constitutes a strong indication for aggressive medical treatment, since if remission can be induced by medical therapy, the course of pregnancy is similar to that in patients with quiescent disease at conception. About two thirds of women with active Crohn's disease at the time of conception will continue to have disease activity throughout the pregnancy and in about half of these there will be a deterioration during the pregnancy (Table 2). Therefore it is inadvisable to conceive when CD is active, but if conception occurs, an aggressive therapeutic strategy is indicated as there is clear evidence in Crohn's disease that disease activity is associated with pre-term birth and low-birth weight and some suggestion that early miscarriage may be increased.

MANAGEMENT OF IBD IN PREGNANCY

All the evidence suggests that maintenance treatment, certainly with aminosalicylates, should be continued throughout pregnancy and flare-ups of disease activity should be investigated and treated appropriately as in a non-pregnant patient. All pregnant women are very concerned about taking medication during gestation and it is essential if at all possible that these issues are broached and discussed well in advance of a planned pregnancy enabling informed discussion with the patient and her partner once she becomes pregnant.

Nutrition is extremely important in pregnancy, the average weight gain during a normal pregnancy being between 11 and 16kg. Folic acid supplementation is recommended for all pregnant women but in IBD, patients who may have folic acid deficiency or be taking drugs which interfere with folic acid metabolism, a dose of 5mg daily should be recommended rather than the usually advised dose of 400μg daily. It is extremely important to remember that early nutritional intervention is indicated in a woman with active disease who may not be gaining weight. Women with active CD in pregnancy have received an elemental diet as primary therapy with rapid resolution of symptoms[25] and supplemental feeding may be required in sick IBD patients who are failing to achieve the expected weight gain during pregnancy.

Proper investigation of gastrointestinal symptoms is not contraindicated during pregnancy and indeed it is important in order to ensure that appropriate treatment is advised. Blood

investigations are often difficult to interpret in pregnancy due to haemodilution, and therefore sigmoidoscopy and indeed colonoscopy may be indicated in some circumstances. Both these investigations have been shown to be safe in a small study[26]. Monitoring foetal heart rate during endoscopy has not shown any adverse effects and there has been no evidence of increased premature labour or foetal abnormalities following endoscopy in pregnancy. Radiographic imaging should obviously be avoided unless obstruction, perforation or toxic megacolon are suspected and if possible in this situation, plain abdominal films should be used rather than CT or barium studies which involve much higher radiation exposure. Ultrasound may be useful, for example to identify an intra-abdominal collection in patients with Crohn's disease.

Table 2 Influence of pregnancy on IBD activity
Meta-analysis data from Reference 17

INACTIVE disease at conception. Likelihood of relapse during pregnancy

	Ulcerative colitis	Crohn's disease
Number of pregnancies	528	186
Relapse	34%	27%

ACTIVE disease at conception. Pattern of disease activity in pregnancy

	Ulcerative colitis	Crohn's disease
Number of pregnancies	227	93
Better	27%	34%
No change	24%	32%
Worse	45%	33%

TREATMENT OF IBD IN PREGNANCY

Drug treatment

The safety (or risk) of drug therapy during pregnancy is of prime concern to any pregnant woman. In women with IBD, the most important factors in relation to treatment are to emphasise the importance of planned pregnancy when the disease is quiescent and the fact that, if conception occurs with active IBD, inducing remission with medical therapy carries less risk than continuing a pregnancy without treatment[26].

First line agents

Aminosalicylates and sulphasalazine have been widely used in pregnancy in IBD. They are safe in conventional doses and should be used for maintenance or induction of remission in the same way as in a non-pregnant individual. Both aminosalicylates and sulphasalazine are poorly systemically absorbed and there is little placental transfer from mother to foetus[27-29]. No evidence of teratogenicity has been demonstrated and the outcome of pregnancy has been shown to be similar to that in healthy women. There have however, been reports of nephrotoxicity in the foetus of a woman taking a high dose of mesalazine[30]. High dose aminosalicylates are not therefore advisable during pregnancy.

Corticosteroids are well tolerated in human pregnancy. They cross the placental barrier but there has been no convincing evidence of teratogenesis despite reports of cleft lip and palate in the past. Immune deficiency in the new-born infant is theoretically possible, but is very rarely reported in clinical practice. In IBD patients taking corticosteroids during pregnancy, no increase in foetal complications have been found compared to the general population[29]. It is, therefore, important to use corticosteroids in women with moderate to severe disease activity in pregnancy in the same way as in a non-pregnant patient.

Second line agents

Azathioprine and 6-mercaptupurine have never been demonstrated to be teratogenic in humans and do not have any effects on human interstitial cell function or gametogenesis in the doses used in clinical practice[31,32]. There is extensive experience of the use of these drugs in pregnancy in renal transplant recipients and in patients with systemic lupus erythematosus who are unable to discontinue immunosuppressive treatment, with very little evidence of adverse effect[33,34]. However, because of the theoretical possibility of teratogenesis in animals, gastroenterologists have been very cautious in advising discontinuation of azathioprine prior to pregnancy or even termination of pregnancy in women conceiving on azathioprine. In a small retrospective study on the use of azathioprine in pregnancy and IBD there were no serious adverse outcomes. All the women conceived while taking the drug and half of them continued to take it throughout gestation[35]. In a larger study, looking at pregnant women with IBD on 6-mercaptupurine, there were also no adverse outcomes of pregnancy[36], although in this study only a small number of patients actually continued to take the drug throughout their pregnancy.

In general therefore, if a patient is established and well on azathioprine or 6-mercaptupruine and it is felt to be essential to continue this drug to retain remission, after full discussion with the patient and her partner, it is reasonable to decide to continue treatment during pregnancy. It is essential that this decision is made by the patient who has been presented with the evidence. In view of the complications which may arise at the start of treatment with these agents, it is not advisable to commence treatment for the first time during pregnancy.

Cyclosporine has been used in patients with severe UC which has not responded to steroids in an attempt to avoid surgery which is said to carry a high risk of foetal mortality[37]. Cyclosporine is not teratogenic and has been extensively used in transplant recipients and lupus patients without increased adverse effects[38-41]. Cyclosporine is a highly toxic drug however, carrying the risk to the mother of hypertension, nephrotoxicity and hepatotoxicity and it would therefore appear to be undesirable in almost all circumstances except the avoidance of urgent colectomy in a patient with fulminant UC.

Methotrexate is mutagenic and teratogenic and is therefore contraindicated in pregnancy or immediately prior to conception. There are reports of women with IBD who have conceived while taking methotrexate, who had a high incidence of severe congenital abnormalities in the babies born from these pregnancies, with neural tube defects and other severe deformities[42,43]. In a woman who conceives on methotrexate and will not agree to a therapeutic abortion, however, the methotrexate must obviously be stopped immediately and high dose folic acid replacement is indicated.

Anti-TNF antibodies There are currently no data about pregnancy in patients receiving therapy. By definition this therapy is used for people with severe active CD, and for the present pregnancy should be discouraged during this treatment.There is no evidence that it is safe to continue with the pregnancy if conception occurs during treatment with anti-TNF antibody.

Antibiotics: Metronidazole has not been shown to have

adverse effects and has been used extensively in pregnancy by gynaecologists to treat bacterial vagionosis. There is not any evidence of increased risk of spontaneous abortion or congenital abnormality in humans[44,45]. Ciprofloxacin and other quinolone antibiotics have been suggested to be associated with musculoskeletal problems in foetuses in animal studies but this has not been substantiated in humans. Ciprofloxacin has been used during pregnancy with no increased incidence of spontaneous abortion or congenital abnormality and follow-up of the children born from these pregnancies is ongoing[46].

Surgery

Patients who have undergone previous surgical intervention for UC or CD do not appear to have any increase in problems during pregnancy compared to the general population. Patients who have undergone colectomy and ileostomy or ileal pouch operations can expect a normal outcome of pregnancy. In women with an ileostomy, stomal prolapse has been reported following hyperemesis. This can cause discomfort and require revision of the ileostomy post-partum. Opinions vary about the need for delivery by caesarean section following pouch surgery. Some centres have published data suggesting that vaginal delivery is appropriate. There are no published long-term studies of the effect of vaginal delivery on pouch function although one study demonstrated no short-term deterioration of pouch function[7,8]. Some surgeons advise elective caesarian section to avoid risk of sphincter damage.

Surgery for acute indications during pregnancy has been reported to carry a high risk of foetal loss and is generally felt to be inadvisable. Reports of a 60% risk of foetal loss for urgent colectomy in UC may be an overestimate in the 21st century. A small case report from Manchester recently reported six women who had surgery for intraperitoneal sepsis in Crohn's disease during pregnancy. Five healthy babies resulted from these pregnancies although one miscarriage occurred in a patient with a surgical complication[47].

Breast-feeding

Breast-feeding is the best option for mother and baby in most circumstances. Concerns about breast-feeding are related to worries about the secretion of drugs in breast milk. Sulphasalazine and the aminosalicylates are poorly absorbed from the bowel and very small amounts are excreted in breast milk. It is safe to breast-feed while taking these medications with the small reservation that it is inadvisable to take high doses of amino-salicylates as there is one report of renal impairment in a child of a mother on a high dose of mesalazine[37].

Prednisolone is also concentrated poorly in breast milk and the amount received by the infant is minimal[48]. If breast-feeding is deferred until 4 hours after taking steroids, this further decreases the dose to the infant. It is therefore considered safe to breast-feed while taking these first-line agents.

There are almost no data on the safety of breast-feeding while taking other agents used to treat IBD. Breast-feeding is not recommended by the manufacturers of azathioprine or 6-mercaptopurine. Many transplant recipients and patients who take these drugs for rheumatological disorders and who must remain on azathioprine have breast-fed without reports of ill effect to the baby. This is another situation where full discussion with the mother and her partner may allow them to make an informed decision on the basis of what little evidence is available.

CONCLUSION

The key to the management of the pregnant IBD patient is to have discussed the issues relating to reproductive health prior to conception. Counselling from teenage years in young patients with IBD will help them to understand the importance of planned pregnancy. Fertility can be expected to be normal except in women with active Crohn's disease. The outcome of pregnancy is usually normal but the risks are associated with active disease and more strongly with Crohn's disease. Disease activity is definitely associated with premature delivery and low birth weight. Drug treatment should be discussed in advance of pregnancy and it would seem logical that women should be encouraged to continue maintenance treatment with aminosalicylates or sulphasalazine during pregnancy. If an attack occurs it should be investigated and treated as in a non-pregnant patient, except that use of x-rays should be minimised. Patients maintained on azathioprine may wish to continue with the drug if it is important to retain remission after full discussion. A patient with quiescent IBD can expect normal fertility, normal outcome of pregnancy and there is no contraindication to breast-feeding. The management of IBD in pregnancy is a good example of the therapeutic partnership between patient and doctor. Education and communication are key, active disease is the greatest risk to the outcome of pregnancy and drug therapy may be necessary and if so is safe.

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Edited by Rampton DS and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Non-invasive investigation of inflammatory bowel disease

JA Tibble and I Bjarnason

Department of Medicine, Guy's, King's, St Thomas's Medical School, Bessemer Road, London SE5 9PJ
Correspondence to Prof. I Bjarnason, Department of Medicine, Guy's, King's, St Thomas's Medical School, Bessemer Road, London SE59PJ
Tel: 0044-207-7374000 Ext. 2159
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Abstract

The assessment of inflammatory activity in intestinal disease in man can be done using a variety of different techniques. These range from the use of non-invasive acute phase inflammatory markers measured in plasma such as C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) (both of which give an indirect assessment of disease activity) to the direct assessment of disease activity by intestinal biopsy performed during endoscopy in association with endoscopic scoring systems. Both radiology and endoscopy are conventional for the diagnosis of inflammatory bowel disease (IBD). However these techniques have severe limitations when it comes to assessing functional components of the disease such as activity and prognosis. Here we briefly review the value of two emerging intestinal function tests. Intestinal permeability, although ideally suited for diagnostic screening for small bowel Crohn's disease, appears to give reliable predictive data for imminent relapse of small bowel Crohn's disease and it can be used to assess responses to treatment. More significantly it is now clear that single stool assay of neutrophil specific proteins (calprotectin, lactoferrin) give the same quantitative data on intestinal inflammation as the 4-day faecal excretion of 111Indium labelled white cells. Faecal calprotectin is shown to be increased in over 95% of patients with IBD and correlates with clinical disease activity. It reliably differentiates between patients with IBD and irritable bowel syndrome. More importantly, at a given faecal calprotectin concentration in patients with quiescent IBD, the test has a specificity and sensitivity in excess of 85% in predicting clinical relapse of disease. This suggests that relapse of IBD is closely related to the degree of intestinal inflammation and suggests that targeted treatment at an asymptomatic stage of the disease may be indicated.

Subject headings inflammatory bowel diseases; permeability; NCAM; membrane glycoproteins

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INTRODUCTION

Distinguishing irritable bowel syndrome from inflammatory bowel disease

Gastroenterologists are often faced with the diagnostic difficulty of differentiating patients with the irritable bowel syndrome (IBS) from those with organic intestinal pathology, in particular inflammatory bowel disease (IBD). Many symptoms are common to both conditions including abdominal pain, bloating, excessive flatus and altered bowel habit while other clinical features such as a predominance of diarrhoea and rectal bleeding will increase the likelihood of organic disease. Although symptoms are a surprisingly good guide to a diagnosis, most clinicians proceed to and rely on laboratory tests to aid in the differential diagnosis. Certainly, fulfilling the ROME criteria[1,2] and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS. As a result a number of investigators[1,3,4] have recommended a straightforward approach to evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost effective management. Despite this the use of the Rome criteria has not been universal and is largely confined to use as entry criteria into research studies of patients with IBS. The concern for gastroenterologists is that some patients with organic intestinal disease will be incorrectly diagnosed if excess reliance is placed upon these criteria. They may therefore feel compelled to exclude all organic disease using invasive diagnostic investigations as objective evidence for there being no other significant pathology. This has significant implications for health care costs as well as exposing patients to the inherent risks associated with invasive procedures.

Managing inflammatory bowel disease

Once IBD is diagnosed the treatment involves induction and subsequently maintenance of remission based largely on clinical disease activity indices[5,6] and the physicians global assessment of well-being. The problem with the use of clinical disease activity scores is that they are a composite of quantitative subjective symptoms that are affected by non inflammatory processes such as fibrous strictures, fistulas and previous surgical intervention. As a guide to clinical decision making, many clinicians therefore use nonspecific laboratory tests to document relapse of disease and radiology and radio isotopic techniques to distinguish between actively inflamed disease and fibrotic strictures. In addition a number of blood tests (erythrocyte sedimentation rate (ESR), orosomucoid, C -reactive protein (CRP), platelet, and white cell counts, IL-6, TNF-α, IL-1β)[7-11] which reflect the systemic consequences of inflammation, have been proposed as predictors and/or markers of clinical relapse of IBD with varying degrees of success. However, the overall predictive values of these different variables in identifying patients at risk of relapse have in general been disappointing. This is possibly due to the fact that these measures are non-specific, affected by a variety of non-intestinal diseases[12] and most importantly do not measure the intestinal inflammation directly. Patients with clinically active IBD can have normal serological inflammatory indices while clinically quiescent disease may be associated with abnormal blood tests. In particular, there is a major discrepancy between severity of

symptoms and macroscopic evaluation of disease activity in patients with Crohn's disease limited to the colon.

Intestinal function tests

Although imperfect the above approach to diagnosis and management of patients with IBD remains the norm and in general it works well for the vast majority of patients. However, few would argue with the notion that there is scope for improvement. Where is such improvement to come from? Recently, investigators have turned to direct tests of intestinal function. Such tests provide new, direct and different information. They have the potential to be used as a diagnostic screen for intestinal disorders as well as providing prognostic information for the behaviour of the disease. At present there are three kinds of intestinal function tests that could fulfill the above promise, two of which (intestinal permeability and white cell scans) have a 20 year history. The third, namely direct assay of faeces for inflammatory markers, we suspect has the greatest potential. There follows a brief outline of how these tests can provide information that is not obtainable by other methods and their possible use in the day-to-day management of patients with IBD.

INTESTINAL PERMEABILITY

Permeability refers to that property of a membrane that enables passage of a solute by unmediated diffusion. The diffusion of a solute across a simple membrane is determined by the structure of the membrane (in terms of its composition, charge, thickness, etc.), the physicochemical properties of the solute (like molecular size, shape, charge and solubility) and its interaction with the media or solvent. Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered substances. The ideal permeability probe is water-soluble, non-toxic, non-degradable and not metabolised before, during or after permeating the intestine[13]. The probes should preferably not be naturally present in urine, be completely excreted in the urine following intravenous administration and be easily and accurately measurable. Fordtran *et al*[14] were instrumental in the development of ideas for assessing intestinal permeability in man but it was Menzies who introduced oligosaccharides as test substances for the non-invasive assessment of intestinal permeability[15] in 1974, and later formulated the principle of differential urinary excretion of orally administered test substances[16]. The importance of the differential urinary excretion principle is that it overcomes most if not all the problems associated with the use of a single test substance, where urinary excretion is dependent on a number of pre- and post-mucosal factors as well as intestinal permeability. The differential principle advocates that a nonhydrolyzed disaccharide (i.e. lactulose) and a monosaccharide (L-rhamnose or mannitol) are ingested together. As the pre- and post-mucosal determinants of their excretion affects the two test substances equally and the differential 5 hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables the urinary excretion ratio becomes a specific measure of intestinal permeability.

Tests of intestinal permeability were initially designed to allow reliable non-invasive detection of patients with untreated coeliac disease[16]. The tests have since come to be viewed as synonymous with assessing intestinal barrier function. In clinically active small bowel Crohn's disease the vast majority of patients (>95%) have an increase in the differential urinary excretion of ingested di-/mono-saccharides (lactulose/L rhamnose or mannitol) and half of those with Crohn's colitis are abnormal[13]. These figures are marginally improved with the use of 51CrEDTA, which requires a 24-hour, as opposed to a 5-hour urinary collection. The vast majority of patients with ulcerative colitis have normal small intestinal permeability when assessed by these methods. However, tests of intestinal permeability have not found widespread application as screening tests to discriminate between patients with Crohn's disease and IBS. The reason for this is probably that the urinary sugar analysis is time consuming and demanding, and there may be some concern that the tests lack specificity being abnormal in a variety of small intestinal diseases (Table 1). At first sight the test appears to identify a number of "clinically irrelevant" diseases, which usually translates into disease for which no treatment is available, but in practice the tests seem often to identify small intestinal pathology where none was previously expected, thus expanding the number of identifiable small bowel pathologies.

There have been attempts to use intestinal permeability as an index of disease activity in Crohn's disease. In general these have been disappointing because the degree of increase in the differential urinary excretion of lactulose/L-rhamnose or the excretion of ^{51}CrEDTA is dependent on localisation and extent of disease within the small bowel as well as activity of the inflammation[13]. Abnormalities in intestinal permeability may, however, be used as a predictor of imminent relapse of quiescent Crohn's disease. Three studies have now shown that, in patients with Crohn's disease in clinical remission, an increased intestinal permeability can predict those at significant risk of relapse of disease in the next few months[17-19]. The strength of this association is difficult to assess from the published studies. Nevertheless, less than 20% of those with normal intestinal permeability appear to relapse over the ensuing 6 months. Interestingly, elevated levels of IL-6 in serum, which can be viewed as a surrogate marker of intestinal inflammation, also has a predictive value for relapse of Crohn's disease[8], but receiver operating curve (ROC) analysis shows relatively low sensitivity and specificity (70 and 50%, respectively). The permeability ratio differs from such indices in that it is not based on concentrations of plasma proteins but rather represents functional changes in the intestinal mucosa, a direct consequence of intestinal inflammation.

The clinical implications of these findings are discussed later.

WHITE CELL SCANS AND FAECAL EXCRETION

Intense neutrophil recruitment to the intestinal mucosa is a feature common to inflammatory bowel diseases[20]. When a patient's own radiolabelled neutrophils are re-injected they migrate to sites of acute inflammation as well as to the liver, spleen and bone marrow[21]. Segal, Saverymuttu and Chadwick were instrumental in the introduction, validation and application of the 111Indium white cell technique for use in gastroenterology[20,22]. The technique visualises inflamed segments of bowel and quantitates the degree of inflammatory activity[20,23-26].

A number of studies have established that abdominal scans are abnormal in virtually all patients with active IBD; their accuracy in localisation of disease and distinguishing between actively inflamed and fibrous stricturing disease has implications for treatment. It was suggested that the technique could be used to discriminate, with an accuracy approaching 100%, between patients with IBD and IBS at the first outpatient visit. In practice this suggestion was not followed up with relevant research.

When combined with measurement of the 4 day faecal

excretion of labelled white cells for quantitation of the inflammatory activity the technique becomes a formidable tool for research and investigation. The faecal excretion of the labelled white cells quantitate inflammation accurately and can be used to document therapeutic efficacy of various treatments in IBD[25,27]. It has also been used to define a number of enteropathies (NSAIDs, alcohol, chronic renal failure, hypogammaglobulinaemia, HIV-AIDS, etc.) where none were suspected or impossible to demonstrate by techniques other than perhaps the intestinal permeability tests (Table 1)[28]. The method is not disease specific, resembling that of the permeability tests, but it is specific for intestinal inflammation. This is not a drawback as it is a simple matter to distinguish between the inflammatory activity in patients with IBD and the above enteropathies, colonic cancer, diverticulitis, etc., since patients with active IBD have excretion values often an order of magnitude higher than the others.

Table 1 Some conditions reported to be associated with increased intestinal permeability

Nonsteroidal anti-inflammatory drugs	Inflammatory bowel disease
Alcohol	Ankylosing spondylitis
Renal failure	Coeliac disease
Abdominal radiation	Intestinal ischaemia
Cytotoxic drug treatment	Hypogammaglobulinaemia
Abdominal surgery	HIV infection
Fasting	Endotoxinaemia
Total parenteral nutrition	Multiorgan failure
Food allergy	Diabetic diarrhoea
Multiple sclerosis	Scleroderma
Cystic fibrosis	Reactive arthritis
Recurrent abdominal pain of childhood	Intestinal infections / bacterial overgrowth
Neomycin	Whipples disease
Acute and chronic liver disease	Sarcoidosis

Why has the white cell technique not been universally adapted for use as a diagnostic screen in IBD, and to assess disease activity? It requires expensive labeling facilities including labelling cabinets. The labeling procedure is time consuming, taking over 2 hours. The cost of isotope and material is in excess of £200 (US $300) and the radiation dose is not trivial if abdominal scans are carried out, being equivalent to that of a barium enema[29,30]. A complete 4-day faecal collection is also demanding and unpleasant for patients, occasionally requiring hospital admission.

Other methods have attempted to build on this success. One such is 99mTc labeling of white cells[31]. This is purported to give superior quality abdominal scintigraphy (which is not clinically important), but does not allow late (>4 hours) scanning, because the label comes off and is excreted into the bowel independent of white cell excretion. Furthermore a faecal collection provides no quantitative information on intestinal inflammation (as the Technetium comes off the white cells and is excreted in faeces) and the labeling requires the same facilities as the white cells.

Newer techniques include E-selectin scanning[32]. This method is derived from the more conventional labelled white cell scintigraphy, but uses a labelled antibody to E-selectin, which is over-expressed in endothelial cells at sites of inflammation. It has the advantage of studying a more fixed entity that (unlike white cells) will not be shed at a variable rate into the bowel lumen and is applicable to the occasional patient with intestinal inflammation who is neutropenic.

In our opinion, the greatest impact that the white cell technique has had is that it ① emphasised that if a sensitive method is to be established for assessing intestinal function there are no shortcuts. Neurologists assess spinal fluid, respiratory physicians assess sputum, urologists urine and the gastroenterologist needs to come terms with the fact that faecal analysis is essential to obtain maximal information about the state of the intestine. ② emphasised that there is life beyond morphological assessment of the gut (x-ray and colonoscopic studies). ③ raised the possibility of dramatically changing our views on the treatment of IBD. Many patients with IBD in full clinical remission are shown to have significant intestinal inflammation[27,33]. At present treatment is non-specifically directed at maintaining remission (5-ASA, azathioprine, etc.). It seems highly probable that those patients with substantial inflammatory activity should be targeted for more aggressive therapy, in particular if they can be shown to be at significant risk of clinical relapse of disease. The analogy with the treatment of rheumatoid arthritis springs to mind. Here, first line treatment is directed to wards reducing the acute inflammatory component of the disease followed by a number of second line agents that can alter the natural history of the disease, reduce the frequency, duration and severity of relapses as well as reducing the joint damage.

FAECAL MARKERS

Faecal analysis is unpleasant but has been with us for a long time. Measure of electrolytes and osmolality helped in the differential diagnosis of diarrhoea in children. Faecal fats were a widespread screening test for steatorrhoea for a while and faecal occult bloods have become the yardstick for colorectal screening with which other methods need to be compared. An improvement on these techniques was the introduction of radioisotopically labelled compounds (labelled red blood cells, proteins, white cells) which provided quantitative and functional data and which was event specific (blood loss, inflammation, protein losing enteropathy, etc.) but non-specific for disease.

The inflamed hyperpermeable mucosa of patients with inflammatory bowel disease is associated with increased protein loss into the bowel lumen[34]. Studies using radiolabelled proteins have demonstrated that there is faecal protein loss in patients with active Crohn's disease and it may therefore be a useful marker of disease activity. Other studies have shown faecal α1 antitrypsin clearance to be a useful indicator of protein losing enteropathy[35] and that in patients with inflammatory bowel disease, 72 hour faecal clearance of α1 antitrypsin is a useful method for quantitating intestinal protein loss[36,37]. Faecal clearance of α1 antitrypsin correlates with that of ^{51}Cr-albumin, and moderate rectal bleeding does not affect the α1 antitrypsin determination[36]. Random faecal α1 antitrypsin levels have been shown to be as useful as more prolonged collection in measuring Crohn's disease activity[38] and correlated with several other laboratory measures that have been proposed as indicators of Crohn's disease activity[39].

Concerns about costs, radiation, and the need for prolonged faecal collections all worked against these techniques for routine use, although many remain very important for research studies. The idea then emerged that it might be possible to assay for cell proteins or substances that are specifically associated with a certain cell type and which would then provide information on a specific component of the inflammatory cascade. Ferguson's Edinburgh group was instrumental in expanding this idea[40]. Concerned about bacterial degradation of markers they used a whole gut lavage method involving ingestion of polyethylene-based purgatives

(Kleenprep or GoLitely) for obtaining clear liquid faecal samples for analysis. The analysis took to various markers, such as immunoglobulins, neutrophils-specific elastase, andhaemoglobin. Separate studies showed that Crohn's disease could be identified with ease, and that the method had a greater sensitivity for colorectal cancer than the conventional faecal occult blood technique. Ideally suited for research, the method has as yet not found wide application for routine screening purposes, possibly because of the drawback of patients needing to ingest large volumes of liquid.

Direct analysis of markers in faeces would be a major advance on this method. Here the problem is initially the bacterial degradation of the marker necessitating swift sample handling. One such marker, TNF, has been successfully used in children and in HIV infection in adults[41,42]. However, it is now clear that it is not necessary for the marker to be completely non-degraded, provided that the antibody (most of these assays are ELISA's or radio immunoassay) is directed at an epitope of the molecule which resists degradation. One such assay is that for lactoferrin[43]. Lactoferrin is a relatively specific marker for neutrophils, in which it is present in cytoplasmic granules.

Faecal calprotectin

The greatest experience with analysis of faecal proteins is with calprotectin[44-48]. It accounts for up to 50% of the neutrophilic cytosolic protein while being resistant to colonic bacterial degradation. It is easily measured in faeces by a commercially available ELISA.

Calprotectin was first isolated from granulocytes by Fagerhol *et al*[49] and named L1 protein, but was later named calprotectin upon identification of its calcium binding and antimicrobial properties[50]. The protein is a heterocomplex protein consisting of two heavy (L1H) chains and one light (L1L) chain[51] which are non-covalently linked[52]. Calprotectin appears to play a regulatory role in the inflammatory process[53] and functions in both an antimicrobial[50,54] and antiproliferative capacity[55-57]. It has both bactericidal and fungicidal properties with minimal inhibitory concentrations comparable to those of many antibiotics[50]. It is released from the cells during cell activation or cell death. The C-terminal sequence of the L1H chain has been shown to be identical to the N-terminus of peptides known as neutrophil immobilising factors (NIF)[58]. It has been suggested that NIF activity of the L1H chain depends upon its phosphorylation[59] and that such an activity of calprotectin could be important for the accumulation of granulocytes, while calprotectin released from dead neutrophils, macrophages and epithelial cells might exert antimicrobial activity, possibly by depriving microorganisms of zinc[60,61]. Calprotectin may inhibit metalloproteinases[62] which may also involve the deprivation of zinc suggesting that it may limit their participation as enzymatic cofactors for invading organisms. Interest in calprotectin as a marker for inflammation in the gut followed the realisation that 111 Indium labelled granulocyte scans could be used to both visualise and quantitate the acute inflammation in the gut of patients with inflammatory bowel disease[20,23]. These findings led to the idea that an increased influx of granulocytes into the intestinal mucosa in conditions of inflammation might give increased levels of proteins from such cells in faeces.

Others[63] have demonstrated that eosinophilic granulocytes are the main cellular source of calprotectin in the normal gut mucosa. However, relatively high levels of calprotectin are found in the stools of normal individuals- about six times the plasma levels (which are about 0. 5mg/L) . This is compatible with data suggesting that in normal individuals most circulating neutrophils migrate through the mucosal membrane of the gut wall and thereby terminate their circulating life[64]. Subsequent lysis within the gut lumen and release of cytosolic calprotectin thereby accounts for the median faecal levels of 2.0mg/L seen in healthy controls[46,65]. The diagnostic use of faecal calprotectin in a broad spectrum of intestinal diseases has been studied by a number of groups with remarkable agreement between the results to date.

Inflammatory bowel disease

It is almost possible to extrapolate all the findings obtained with the white cell faecal excretion technique to the calprotectin method. Both techniques correlate with histopathological assessment of disease activity in ulcerative colitis and there is a very good correlation between the 4-day faecal excretion of white cells and faecal calprotectin concentrations[33,45], a correlation which is maintained when single stool calprotectin concentrations are used as opposed to 1 or 4 day collections. The faecal calprotectin concentration has a narrow normal range with an upper limit of 10mg/L. As with the white cells, faecal calprotectin has potential as a screening procedure to differentiate between patients with IBD and IBS and it may be useful for documenting a fall in intestinal inflammation in response to successful treatment of disease. Calprotectin concentration is rarely within the normal range in patients with IBD despite full clinical remission and is therefore a highly sensitive method for detecting such patients irrespective of disease activity. In over 100 patients with Crohn's disease of varying severity and activity only 4 had normal calprotectin concentrations[33].

Since the method is so much simpler than the white cell technique, requiring only a single stool sample, extraction and an ELISA, it has potential as a screening test to distinguish between patients with IBD and IBS in an outpatient setting. One study in over 225 patients showed that a cut off of 30mg/L had a 100% sensitivity and 94% specificity for this purpose[33]. Another showed that this was also the case when over 600 unselected consecutive patients were studied. Indeed a patient presenting with positive ROME criteria and a normal faecal calprotectin has virtually no chance of having IBD[66]. As a result of these studies it is now our practice not to investigate such patients by radiology or colonoscopy with considerable cost saving implications. The white radiolabelled cell technique demonstrated reduced intestinal inflammation in response to 5-ASA treatment and elemental diets. We have shown (unpublished) that improvement in calprotectin parallels the improvement in the excretion of labelled white cells in response to treatment with elemental diets. These techniques prove to be much more reliable and reproducible than the changes in clinical disease indices. It seems likely that the assay of faecal calprotectin will become an integral part of the assessment of therapeutic efficacy of the acute inflammation in future treatment trials in patients with IBD.

Apart from screening and assessing response to treatment, the faecal calprotectin has a further major advantage over the white cell labeling technique in predicting relapse of IBD. It has been shown that, in patients with clinically quiescent IBD (ulcerative colitis and Crohn's disease), faecal calprotectin values above 50mg/L may be used to predict clinical relapse of disease within a few months with over 80% sensitivity[67]. Symptoms of inflammatory bowel disease often appear to be the direct consequence of the inflammatory process itself and often vary dependent upon

the location of the inflammation. Most patients with quiescent IBD have low-grade inflammation[27] and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity. Furthermore, as inflammation is a continuous process it may be that direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease.

The clinical implications of this, if substantiated, are considerable as it might offer targeted treatment at an earlier stage, with less side effects, to avert the relapse, as well as assessment of new therapeutic strategies to maintain symptomatic remission[68]. At present this is done with some degree of success with the rather indiscriminate use of sulphasalazine, 5-ASA and azathioprine, all of which are associated with side effects. However the calprotectin method offers guidance as to whom to treat at this stage and with what kind of vigour. Theoretically such treatment should lead to a dramatic reduction in the frequency and severity of clinical relapses with an improvement in the patient's quality of life.

In addition, the identification of patients at high risk of relapse will improve the design of clinical trials to assess the efficacy of therapeutic regimes designed to maintain patients in remission. In most such trials, patients studied tend to be a heterogeneous mix of those with high and low risk of relapse. This introduces possible bias when assessing the response to a particular treatment regime due to the imbalance of high risk patients in each treatment arm. Stratification by risk group using faecal calprotectin would reduce the possibility of such a bias. It is also possible that a lack of power in detecting a response to treatment may be due to the study of a large number of patients at low/intermediate risk of relapse, in whom all treatments may show the same efficacy, and therefore clinical trials studying a homogenous high risk group may be more powerful in detecting a difference in treatment efficacy.

Much work remains to be done, some is already on its way, but what is clear is that gastroenterologists need to move with the times and start thinking along the lines that rheumatologists do, that is, to implement treatments that alter the natural history of the disease. We are now in possession of tests that have the potential to revolutionise our approach to treatment of patients with IBD. There are some hurdles to overcome. The most frequent criticism of the "faecal" tests is that they are unacceptable to patients and unpleasant to work with.

The faecal calprotectin and lactoferrin methods are the first wave of techniques that allow non-invasive assessment of specific and selective cellular components of the intestinal inflammatory cascade. At present these are useful for a variety of purposes, outlined above, but it is likely that it will be possible to estimate the participation of other cells. Many other cells of the inflammatory cascade are numerically increased in biopsy specimens from patients with a variety of gastroenterological conditions. Some, such as mast cells and eosinophils, are thought to play a central role in mediating intestinal allergic reactions[69]. However, both types of cell are found to be activated in a number of other gastrointestinal inflammatory diseases such as inflammatory bowel disease, coeliac disease, eosinophilic gastroenteritis[69] and collagenous colitis[70], suggesting that both cell types may be involved in the pathogenesis of chronic intestinal inflammation. It may therefore be possible, as for neutrophils and calprotectin, to identify mast cell granule proteins, such as tryptase and chymase, in faecal samples and use them as markers of a specific component of the intestinal inflammatory response. The long-term objective might be to fully automate a faecal sample assay method that provides specific information on the activity of acute inflammation (neutrophils), chronic inflammation (T-cells) and allergy (mast cells).

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Edited by Rampton DS and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Acute variceal bleeding: general management

David Patch and Lucy Dagher

Liver Transplantation and Hepatobiliary Medicine, 9th Floor-Department of Surgery, Royal Free Hospital NHS Trust, Pond Street-Hampstead, London NW3 2QG
Correspondence to Dr. David Patch, Liver Transplantation and Hepatobiliary Medicine, 9th Floor Department of Surgery, Royal Free Hospital NHS Trust, Pond Street-Hampstead, London NW3 2QG
Tel: 0044-207-47226229, Fax: 0044-207-4726226
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TREATMENT STRATEGIES FOR ACUTE VARICEAL BLEEDING

Background

Acute variceal bleeding has a significant mortality which ranges from 5% to 50% in patients with cirrhosis[1]. Overall survival is probably improving, because of new therapeutic approaches, and improved medical care. However, mortality is still closely related to failure to control haemorrhage or early rebleeding, which is a distinct characteristic of portal hypertensive bleeding and occurs in as many as 50% of patients in the first days to 6 weeks after admission *et al*[2]. Severity of liver disease is recognised as a risk factor for both early rebleeding and short-term mortality after an episode of variceal bleeding[3]. Active bleeding during emergency endoscopy (e.g. oozing or spurting from the ruptured varix) has been found to be a significant indicator of the risk of early rebleeding[4,5]. Increased portal pressure (HVPG>16mmHg) has been proposed as a prognostic factor of early rebleeding in a study of continuous portal pressure measurement immediately after the bleeding episode[6], and recently Moitinho *et al*[7] have shown a failure to reduce portal pressure more than 20% from baseline is associated with a worse prognosis as well as early rebleeding.

Effective resuscitation, accurate diagnosis and early treatment are key to reducing mortality in variceal bleeding. The aims are not only to stop bleeding as soon as possible but also to prevent early re-bleeding. Early rebleeding, as with the peptic ulcer disease, is significantly associated with worsening mortality[5]. Thus treatments regime should be evaluated not only in terms of immediate cessation of haemorrhage, but also in terms of providing a bleed free interval of at least 5 days. This allows some recovery of the patient, and provides an opportunity for secondary preventative therapy to be instituted.

Diagnosis

Upper gastrointestinal endoscopy is essential to establish an accurate diagnosis, as 26%-56% of patients with portal hypertension and GI bleeding will have a non-variceal source[8], particularly from peptic ulcers and portal hypertensive gastropathy. Endoscopy should be performed as soon as resuscitation is adequate, and preferably with 6 hours of admission. This may need to be done with prior endotracheal intubation if there is exsanguinating haemorrhage or if the patient is too encephalopatic because of the substantial risk of aspiration.

Despite many authors and junior doctors proclaiming endoscopic prowess, it is the authors opinion that a definitive endoscopic diagnosis during or shortly after upper GI bleeding can be difficult, because the view can be obscured by blood. A diagnosis of bleeding varices is accepted either when a venous (non pulsatile) spurt is seen, or when there is fresh bleeding from the O-G junction in the presence of varices, or fresh blood in the fundus when gastric varices are present. In the absence of active bleeding (approximately 50%-70% of cases) either the presence of varices in the absence of other lesions, or a "white nipple sign" - a platelet plug on the surface of a varix[9,10], suggests varices as the source of haemorrhage.

When the diagnosis is in doubt, repeat endoscopy during re-bleeding is mandatory as it will show a variceal source in over 75% of patients[8]. Gastric varices are particularly difficult to diagnose, because of pooling of blood in the fundus. Endoscoping the patient on the right side, with the head up may help. If the diagnosis is still not made, splanchnic angiography will establish the presence of varices, and may display the bleeding site if the patient is actively haemorrhaging.

In the true emergency situation in which the patient is exsanguinating and varices are suspected on the basis of history and examination, a Sengstaken Blakemore tube (SBT) should be passed[11]. If control of bleeding is obtained, varices are likely to be the source of haemorrhage. If blood is still coming up the gastric aspiration port, then varices are less likely to be the cause of blood loss (although fundal varices are not always controlled by tamponade). In practice the position of the SBT has to be re checked and adequate traction applied. If there is still continued bleeding the diagnosis of variceal bleeding should be questioned or fundal bleeding suspected, and emergency angiography performed.

Therapeutic aims in acute variceal bleeding

The important point is to treat the patient and not just the source of bleeding (Figure 1). The specific aims are: ① Correct hypovolaemia; ② Stop bleeding as soon as possible; ③ Prevent early rebleeding; ④ Prevent complications associated with bleeding; ⑤ Prevent deterioration in liver function.

It is important to identify those at high risk of dying during the initial assessment. Individuals in this category should have early definitive therapy, the precise treatment regimen depending on availability Predictive factors for early deaths are: Severity of bleeding[12], Severity of liver disease[13], Presence of infection[14], Presence of renal dysfunction[15], Active bleeding[4,5], Early

rebleeding[5,6,10,16,17], Presence of cardiorespiratory disease, and Portal pressure[7].

Early rebleeding is also associated with many of the same factors associated with mortality including infections[18] and is a strong indication of increased mortality.

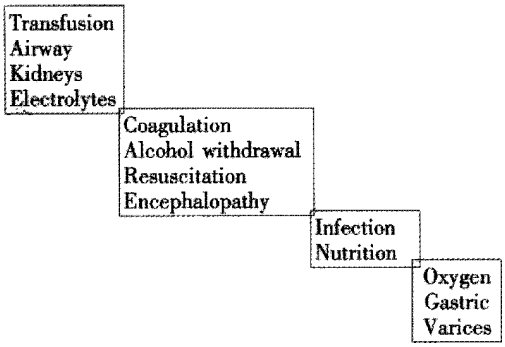


Figure 1 Take care in OGV.

Transfusion

Optimal volume replacement remains controversial. Following variceal bleeding in portal hypertensive animal models, return of arterial pressure to normal with immediate transfusion results in overshoot in portal venous pressure, with an associated risk of further bleeding[19]. This effect may not be relevant in clinical practice as volume replacement is always delayed with respect to the start of bleeding. Over-transfusion should certainly be avoided, and it is usual to aim for an Hb between 9-10g/dL, and right atrial pressure at 4 to 8mmHg, but fluid replacement may need to be greater in the presence of oliguria to be sure that the circulation is filled.

Large volume transfusion may worsen the haemorrhagic state, as well as lead to thrombocytopenia so that fresh frozen plasma and platelets need to be replaced. Optimal regimens for this are not known. It is reasonable to give 2 units of FFP after every 4 units of blood, and when the PT is >20 seconds. Cryoprecipitate is indicated when the fibrinogen level is less than 0.2g/L.

Platelet transfusions are necessary to improve primary haemostasis and should be used if the baseline count is 50×10^9/L or less. Platelet count may show little change following platelet transfusion in patients with splenomegaly. It is also routine to give intravenous vitamin K to cirrhotics, but no more than three doses of 10mg are required. Many cirrhotics have a background tendency of fibrinolysis. Transfusion of more than 15 units of blood results in prolongation of the prothrombin and partial thromboplastin time[20] in normal individuals, and occurs with smaller volumes of blood transfusion in cirrhosis.

Massive transfusion may cause pulmonary microembolism, and the use of filters is recommended for transfusions of 5L or more in normal humans[21]. Therefore, the routine use of filters in variceal haemorrhage could be considered, but rapid transfusions cannot be administered with these, limiting their application.

Further measures in patients who continue to bleed despite balloon tamponade include the use of desmopressin (DDAVP)[22] and antifibrinolytic factors. In stable cirrhotics the former produces a 2-4 fold increase in factors VIII and VWF, presumably by release from storage sites and may shorten or normalize the bleeding time[23]. However, in one study of variceal bleeding DDAVP in association with terlipressin was shown to be detrimental compared to terlipressin alone[24].

The use of antifibrinolytics has not been studied in variceal bleeding, although their role has been established in liver transplantation in our unit as well as others[25]. Their clinical utility should be established in clinical trials, and preferably when increased fibrinolysis has been documented. Recombinant factor VIII may be useful in variceal bleeding as it has been shown to normalize prothrombin time and bleeding times in cirrhotics[26].

PREVENTION OF COMPLICATIONS AND DETERIORATION IN LIVER FUNCTION

Infection control and treatment

Bacterial infections have been documented in 35%-66% of patients with cirrhosis who have variceal bleeding, with a incidence of SBP ranging from 7%-15%. However if only patients with ascites and gastrointestinal bleeding are considered, the incidence of SBP is very high. A recent meta-analysis has demonstrated that antibiotic prophylaxis significantly increased the mean survival rate (9.1% mean improvement rate, 95% CI: 2.9-15.3, P = 0.004) and also increased the mean percentage of patients free of infection (32% mean improvement rate, 95% confidence interval: 22 -42, $P<0.001$)[27](Figures 2,3). Finally our group has recently shown that bacterial infection, diagnosed on admission, is an independent prognostic factor of failure to control bleeding or early rebleeding[4]. These data may support a role of bacterial infection in the initiation of variceal bleeding[28]. All cirrhotics with upper gastrointestinal bleeding should receive prophylactic antibiotics whether sepsis is suspected or not. The optimal regimen is yet to be decided but oral or intravenous quinolones have been used.

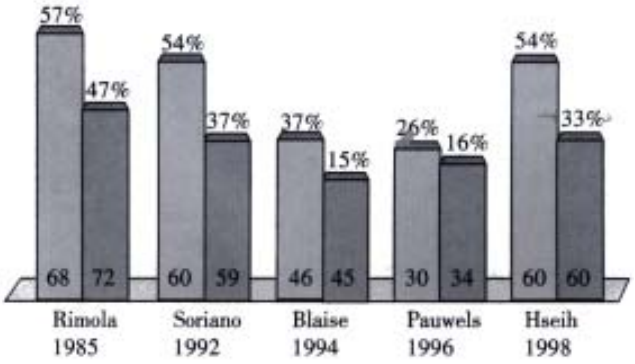


Figure 2 Antibiotics in GI bleeding in cirrhotics (Bernard 1999). Free of infection-mean improvement 32% ($P<0.001$).

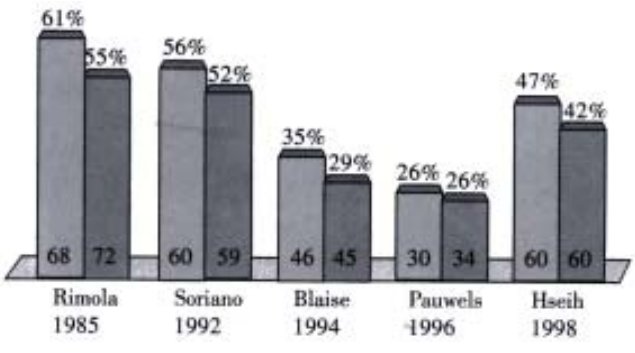


Figure 3 Antibiotics in GI bleeding in cirrhotics (Bernard 1999). Survival-mean improvement 9% (P = 0.0042).

Ascites and renal function

Renal failure may be precipitated by a variceal bleed, usually due to a combination of acute tubular necrosis, and hepatorenal syndrome (HRS) associated with deterioration in liver function and sepsis. HRS is associated with an over 95% mortality. Thus any iatrogenic precipitants must be avoided. The intravascular volume should be maintained preferably with Human Albumin Solution or blood initially. Normal saline should be avoided as it may cause further ascites formation. Catheterisation of the bladder and hourly urine output measurement is mandatory and nephrotoxic drugs should be avoided, especially aminoglycosides and non-steroidal drugs.

Dopamine was the first drug used due its vasodilator effect when given in subpressor doses. Dopamine is frequently prescribed to patients with renal impairment, and yet no studies have ever shown any convincing benefit[29,30]. It is our impression that occasionally a patient responds with an increase in urine output. It is therefore our practice to give a 12-hour trial of dopamine, and stop treatment if there has been no improvement of urine output.

Increasing ascites may occur shortly after bleeding, but should not be the main focus of fluid and electrolyte management until bleeding has stopped and the intravascular volume is stable. If there is a rising urea and creatinine, all diuretics should be stopped, and paracentesis performed if the abdomen becomes uncomfortable, re-infusing 8 gr. of albumin for every litre removed.

When the patient has stopped bleeding for 24 hours, nasogastric feeding can be commenced with low sodium feed. This avoids the need for maintenance fluid, and removes the risk of line sepsis. An unexplained rise in creatinine and urea may indicate sepsis. Evidence of sepsis should be sought by blood, ascitic, cannulae, and urine culture, and non-nephrotoxic broad-spectrum antibiotics commenced, regardless of evidence of sepsis. An undiagnosed delay in effective treatment of infection may increase mortality. In advanced cirrhosis, endotoxins and cytokines play an increasingly important role in advancing the hyperdynamic circulation and worsening renal function[31].

There is now increasing evidence for the use of vasopressin analogues in patients who develop renal impairment during the variceal bleeding episode, probably through maintenance of renal perfusion. Indeed, the beneficial effect of terlipressin with respect to bleeding and survival in trials to date may be through the prevention of this catastrophic complication[32-34].

Porto systemic encephalopathy

The precipitant factors include: haemorrhage, sepsis, sedative drugs, constipation, dehydration, and electrolyte imbalance. These should be evaluated and corrected. Hypokalaemia, hypomagnesaemia and hypoglycaemia may precipitate encephalopathy and should be aggressively corrected (e.g. a patient with ascites and a serum potassium of 3.0mmol/L is likely to require in excess of 100mmol over 24 hours).

As soon as the patient is taking oral fluid, lactulose 5mL -10mL QDS can be started. Phosphate enemas are also useful.

Alcohol withdrawal

It is important to be alert to the possibility of withdrawal from the patient's history. Encephalopathy and withdrawal may co-exist, and careful use of benzodiazepines may be required. Short acting benzodiazepines or oral chlormethiazole can be used. Intravenous preparations should be avoided, particularly of chlormethiazole, because of the risk of oversedation and aspiration.

Nutrition

Only a few cirrhotics are not malnourished[35], particularly with severe liver disease. This may be exacerbated in hospital, as often they do not want to eat, are "nil by mouth" because of investigations, and the food itself may be "unappealing".

A fine bore nasogastric tube should be passed 24 hours after cessation of bleeding to commence feeding. There is no evidence that this may precipitate a variceal bleed, and it allows treatment of encephalopathy in comatose patients and makes fluid management easier. It is extremely rare that parental nutrition is required.

Vitamin replacement: All patients with a significant alcoholic history should be assumed to be folate and thiamine deficient, and be given at least three doses of the latter intravenously. It is easier and more practical to assume all such patients are vitamin deficient rather than delay treatment whilst awaiting red cell transketolase activity levels.

Transfer of the patient with bleeding varices and use of balloon tamponade

Inter-hospital transfer should not be attempted unless the bleeding has been controlled, either with vasopressor agents/ endoscopic therapy or tamponade. If there is any suggestion of continued blood loss, and the source is known to be variceal, then a modified Sengstaken tube must be inserted prior to transfer.(i.e. with an oesophageal aspiration channel such as the Minnesota Tube).

DRUG THERAPY IN ACUTE VARICEAL BLEEDING

Pharmacological agents

The number of placebo controlled trials is relatively small, whilst in the case of octreotide there have been a large number of trials comparing with another form of therapy another drug, or sclerotherapy.

Vasopressin

Only 4 trials compared the efficacy of vasopressin with a placebo[36-39], and two of these studies used an intrarterial route of administration[38,39]. Using meta-analysis, there was a significant reduction in failure to control bleeding (Pooled Odds Ratio 0.22, 95%CI 0.12-0.43), but no benefit in terms of mortality.

Glypressin

The 3 trials are shown in Table 1. There has been criticism of these studies. The trial by Walker *et al* included other therapies, the timing of which is unclear, and the other two trials are hampered by insufficient patient numbers to avoid a type 2 error. These issues will be addressed in forthcoming trials.

A recent study in which terlipressin together with a nitrate patch or placebo was administered before arriving at hospital, based on reasonable evidence of bleeding varices, also showed a reduced mortality of glypressin in grade C patients. How this may have occurred deserves further examination, as there was no difference in blood pressure or early blood product requirements between the drug and placebo arms of the trial, and only three doses of the drug were given. Nonetheless, Terlipressin is a powerful splanchnic

vasoconstrictor, and may be preserving renal blood flow and hence preventing the development of hepatorenal syndrome, in a situation similar to the use of vasopressin anolgues in established hepatorenal failure.

It remains to be seen whether these data can be reproduced. Terlipressin is not licensed in the USA.

Table 1 Randomized studies of terlipressin

Study (ref)	Number of patients C/T	Child C %	Failure to control bleeding C/T	Death C/T
Terlipressin *vs* placebo				
Walker, 1986[40]	25/25	50	12/5	8/3
Freeman, 1989[41]	16/15	29	10/6	4/3
Soderlund, 1990[42]	29/31	33	13/5	11/3
Levacher, 1995[43]	43/41	81	23/12	20/12
POR (95% CI)			0.33 (0.19-0.57)	0.38 (0.22-0.69)
P value			0.0001	0.001

Somatostatin/Octreotide

The first 2 placebo controlled trials came to opposite conclusions. The trial by Valenzuela *et al* suggested that somatostatin is no more effective than placebo, when the end point was a bleed-free period of 4 hours[44]. Furthermore, the 83% placebo rate is the highest reported in the literature. In contrast, the trial by Burroughs *et al* reported a statistically significant benefit for somatostatin in controlling variceal bleeding over a 5 day treatment period, with failure to control bleeding seen in 36% of patients receiving somatostatin, compared with 59% of patients receiving placebo[45]. This emphasizes the problem of differences in end point selection, making meaningful comparisons difficult. The third study[46] also showed no effect of somatostatin, but it took 5 years to recruit 86 patients.

There has been only one randomised placebo controlled trial examining the efficacy of octreotide, and this has only been published in abstract. 383 patient admissions were randomised to 5 days octreotide or placebo. 58% of bleeding episodes in the drug arm were controlled compared with 60% in the placebo arm[45].

Trials comparing drug with drug have shown little statistical difference, though the side effect profile has generally favoured somatostatin/octreotide[47]. Trials in which drugs are examined in association with sclerotherapy are reviewed below.

Randomised controlled trials of emergency sclerotherapy in the management of acute variceal bleeding

Injection sclerotherapy, first introduced in 1939 and "rediscovered" in the late 1970's, rapidly became the endoscopic treatment of choice for the control of acute variceal bleeding. Paradoxically the best evidence for the value of sclerotherapy in the management of acute variceal bleeding has come from a comparatively recent published study by the Veterans Affairs Cooperative Variceal Sclerotherapy Group[48]. In this study sclerotherapy, compared to sham sclerotherapy, stopped haemorrhage from actively bleeding esophageal varices (91% in sclerotherapy arm compared to 60% in sham sclerotherappy, $P<0.001$) and significantly increased hospital survival (75% *vs* 51%, $P = 0.04$).

Today it is generally accepted that sclerotherapy should be performed at the diagnostic endoscopy, which should take place as soon as possible, because there is evidence that this is beneficial compared with delayed injection. Volumes of sclerosant should be small, 1-2 mls in each varix and should be applied in the distal 5cm of the oesophagus. More than two injection sessions are unlikely to arrest variceal bleeding within a 5-day period and are associated with significant complications rate including ulceration and aspiration[2]. Several sclerosing agents have been used for injection ie. polidocanol 1%-3%, ethanolamine oleate 5%, sodium tetradecyl sulfate 1%-2% and sodium morrhuate 5%. There is no evidence that any one sclerosant can be considered the optimal sclerosant for acute injection. As it has been shown that a substantial proportion of intravariceal sclerosant ends up in the paravariceal tissue and vice-versa there is no evidence that one technique is better than the other. One of the main shortcomings of sclerotherapy is the risk of local and systemic complications-although this varies greatly between trials and may be related to the experience of the operator.

Sclerotherapy vs drugs

There are 10 studies, including 921 patients: vasopressin was used in 1[49] terlipressin in 1[50], somatostatin in 3[51-53], and octreotide in 5[54-58], involving 921 patients. The evaluation of the treatment effect was performed at the end of the infusion of the drug (from 48hrs to 120hrs). The overall efficacy of sclerotherapy was 85% (range 73%-94%) in studies of 12 to 48h drug infusion[49,52-56,58] and 74% (68% -84%) in studies of 120hrs drug infusion[49,51,57]. There was significant heterogeneity ($P<0.05$) in the evaluation of failure to control bleeding in these studies was, which was mainly due to the different extent of benefit from sclerotherapy rather than different outcomes in individual studies: only two of the ten studies[55,56], reported that drugs were better than sclerotherapy but in neither did this reach statistical significance. Failure to control bleeding was statistically significantly less frequent in patients randomized to sclerotherapy (Der Simonian and Laird method: POR, 1.68 [95%CI, 1.07-2.63]). The NNT to avoid one rebleeding episode is 11 (95%CI, 6-113). Publication bias assessment showed that 9 null or negative studies would be needed to render the results of this meta analysis non-significant.

There was no significant heterogeneity in the evaluation of mortality in these studies: only two studies[54,56] reported a lower mortality in the drug arm but in neither was this statistically significant. Overall there were statistically significantly fewer deaths in patients randomized to sclerotherapy (POR, 1.43 [95% CI, 1.05-1.95]). The NNT to avoid one death is 15 (NNT, 15 [95% CI, 8-69]).

Finally the type of complications recorded in 8 studies[50-57] differed considerably, resulting in a significant heterogeneity ($P=0.04$). Four studies reported more complications in the sclerotherapy arm[51-54] while 3 reported more complications[50,55,57] in the drug arm and one found equal numbers in both arms[56]. The meta analysis showed a trend in favour of drug treatment but the result was not statistically significant (Der Simonian and Laird method:

POR, 0.71 [95% CI, 0.41-1.2]).

Sclerotherapy plus drugs vs sclerotherapy alone

This group comprised 5 studies[59-64] including 610 patients which compared sclerotherapy plus somatostatin, octreotide, or terlipress in with scleotherapy alone. Only three studies were placebo controlled[59,60,62]. Combination therapy was more effective (POR 0.42; 95%CI 0.29-0.6; failure to control bleeding sclerotherapy + drugs 22%, sclerotherapy alone 38% ARR 16, NNT=6, 95%CI 4-10). No effect on mortality was demonstrated. Only two studies provided data on complication[59,60]. There were no significant differences between arms.

Sclerotherapy vs variceal ligation

There are only two studies specifically designed to compare sclerotherapy with variceal ligation for the management of the acute bleeding episode[65,66]. Other data come from 10 studies of long term sclerotherapy versus variceal ligation[67-76]. There was no statistical heterogeneity (P=0.21) in the analysis of failure to control bleeding from the twelve studies[66-77], including a total of 419 patients. There was no difference between the two treatment modalities, although there was a trend in favour of variceal ligation (POR, 0.66 [95% CI, 0.36-1.18]). Short-term mortality was reported only in two studies[66,77] in both there was a trend in favour of variceal ligation but the result was not statistically significant. Finally only the two studies specifically designed to compare emergency sclerotherapy with variceal ligation[66,77] reported incidence of complications. Complications were less frequent in the variceal ligation arm in both studies and the result reached statistical significance in one[66].

Randomised controlled trials of emergency surgery in the management of acute variceal bleeding

Four randomised trials, performed during the previous decade, compared sclerotherapy to emergency staple transection[78-81]. Failure to control bleeding was reported only in two of these studies, with divergent results. Teres *et al*[80] reported that efficacy of transection in their study was only 71%, the lowest in the literature, compared to 83% in the sclerotherapy arm. In contrast in the largest study performed by Burroughs *et al*[81], a 5 day bleeding free interval was achieved in 90% of the transected patients (none rebled from varices) compared to 80% in those who had 2 emergency injection sessions. There was no difference in mortality between the two treatment modalities. Cello *et al* showed that emergency porta-caval shunt was more effective than emergency sclerotherapy (followed by elective sclerotherapy) in preventing early rebleeding (19% *vs* 50%)[78]. Hospital and 30 day mortality was not significantly different. Finally Orloff *et al* reported, in a small study[82], that portacaval shunt, performed in less than 8 hours from admission, was significantly better than medical treatment (vasopressin/balloon tamponade) in the control of acute variceal bleeding. Survival was also better in the shunted patients but the difference was not statistically significant.

Randomized controlled trials of tissue adhesives in the management of acute variceal bleeding

Two types of tissue adhesives (n-butyl-2-cyanoacrylate [Histoacryl] and isobutyl-2-cyanoacrylate [Bucrylate]) have been used for the control of variceal bleeding[83]. The adhesives could offer better immediate control of bleeding because they harden within seconds upon contact with blood[84]. However extra care must be taken to ensure that the adhesive does not come into contact with the endoscope and blocks the channels of the instrument. This can be prevented if the adhesive is mixed with lipiodol to delay hardening. Ideally, the sclerotherapy needle should be carefully placed within the varix prior to injection, to avoid leak of the adhesive[84]. Two randomised controlled trials compared sclerotherapy alone with the combination of sclerotherapy and Histoacryl for the control of active variceal bleeding[85,86]. The combined treatment was more effective than sclerotherapy alone in both studies. Two further studies compared Histoacryl to variceal ligation for the control of bleeding from oesophageal[87] or oesophagogastric varices[88]. The overall success rate for initial haemostasis of both treatment modalities was similar in these studies. However Histoacryl was superior to variceal ligation for the control of fundic variceal bleeding, but it was less effective for the prevention of rebleeding (67% *vs* 28%). Finally, in a recent small study[89], a biological fibrin glue (Tissucol) was more effective than sclerotherapy with polidocanol in the prevention of early rebleeding and had significantly lower incidence of complications. More studies are necessary to confirm these data and examine the potential risks of activation of coagulation, systemic embolism and transmission of infections with the human plasma derived fibrin glue.

Emergency transhepatic porto-systemic stent shunt

The first reports of TIPS used clinically were in patients with uncontrolled variceal bleeding. In this very ill group of patients TIPS was found to have a life saving role, stopping bleeding in over 90% of patients; over half of these were leaving hospital alive, figures that were unachievable before. Prolonged expensive postoperative ventilation on intensive care was avoided, a situation with a well-defined high mortality[90]. Perhaps as a consequence of this apparent efficacy, there have been no controlled trials of emergency TIPS against other forms of salvage therapy.

One of the biggest problems in discussing uncontrolled variceal bleeding is that there is no accepted definition for this clinical situation. At Baveno 2[91], the panel was unable to reach a consensus as to what this term meant and what its defining parameters were. A re-evaluation of this was recommended at Baveno 3[92] and justified by a prospective evaluation in France[93]. However the reduced efficacy of repeated sclerotherapy is well established, with bleeding control achieved in 70% after the first session and 90% after two sessions. Risk of aspiration, complications of sclerotherapy itself and the general deterioration of the patient render further endoscopy potentially hazardous, as established by Bornman *et al*[94] and at our unit[95] with no improvement in the control of bleeding. Thus with respect to esophageal varices, many use the definition of continued bleeding despite two sessions of therapeutic endoscopy within 5-day period of the index bleed. To this group of patients one

can add those who continue to bleed despite a correctly positioned Sengstaken Blakemore tube (approximately 10% of patients with Sengstaken tube[96], and those patients who continue to bleed from gastric or ectopic varices despite vasoconstrictor therapy.

The results of emergency TIPS are shown in Table 2. There is a predictable proportion of patients with Pugh's C cirrhosis and patients with bleeding gastric varices. In the largest series of salvage TIPS, gastric varices were shown to be no different in terms of bleeding characteristics and portal haemodynamics when compared with oesophageal varices[97].

Patients with bleeding varices that are inaccessible to an endoscope or respond poorly to sclerotherapy are well suited to TIPS. Typical cases include fundal varices, small bowel varices (classically around anastomotic or surgical resection sites[98,99] intraabdominal varices (punctured during large volume paracentesis[100], stomal varices[101,102] (usually in patients with inflammatory bowel disease and sclerosing cholangitis), and bleeding rectal varices[103]. These sites are also amenable to embolization via shunt. TIPS have been successfully placed in infants[104,105], and children[106-108] with similar efficacy.

The results of emergency TIPS are good, especially when compared historically with surgery[109], but the mortality in these series of patients with uncontrolled bleeding is high. There is a need to try to improve patient selection. A number of markers of outcome have been identified. Including the APACHE score[110], presence of hyponatremia and child C liver disease[111], hepatic encephalopathy before TIPS, presence of ascites and serum albumin[112]. Artificial neural network have been developed and validated[113], though many of these series have mixed patients having elective TIPS and those having the procedure as an emergency. The authors feel that this latter group of patients is likely to be different from patients having an elective procedure, with characteristics of haemodynamic instability, worse liver function, lower platelets counts, and higher serum urea concentrations.

At Royal Free Hospital a score system was developed on 54 patients undergoing emergency TIPS as salvage therapy[115]. A prognostic index was developed, based on six factors independently predicted death on multivariate analysis: presence of moderate/severe ascites, requirement for ventilation, white blood cell count, platelet count, serum creatinine and partial tromboplastine time. The score was validated in a further 31 patients and shown to be accurate in predicting mortality. The use of TIPS as a salvage therapy in patients who have uncontrolled variceal bleeding is likely to remain the most established indication for TIPS, and clinical experience to date suggest that this procedure will be required in 10%-20% of patients presenting portal hypertension related bleeding.

CONCLUSION

Today the therapeutic approach in cirrhotic patients with bleeding varices must include the prophylactic use of antibiotics, early endoscopic diagnosis and endoscopic therapy, probably combined use of vasoactive agents. The best evidence for an improvement in mortality is for terlipressin, but only data for 48-hour treatment exists; for somatostatin, which is an alternative, there is data for 5-days use. However mortality is unchanged in trials of endoscopic therapy versus endoscopic therapy combined with vasoactive agents. Further trials are indicated in this area. Although no randomized trials on emergency TIPS exist, this procedure is very effective in stopping bleeding and has virtually eliminated the need for emergency surgery, and reduced ITU stays. If new trials are to be done, comparison with glues or thrombin may be justified.

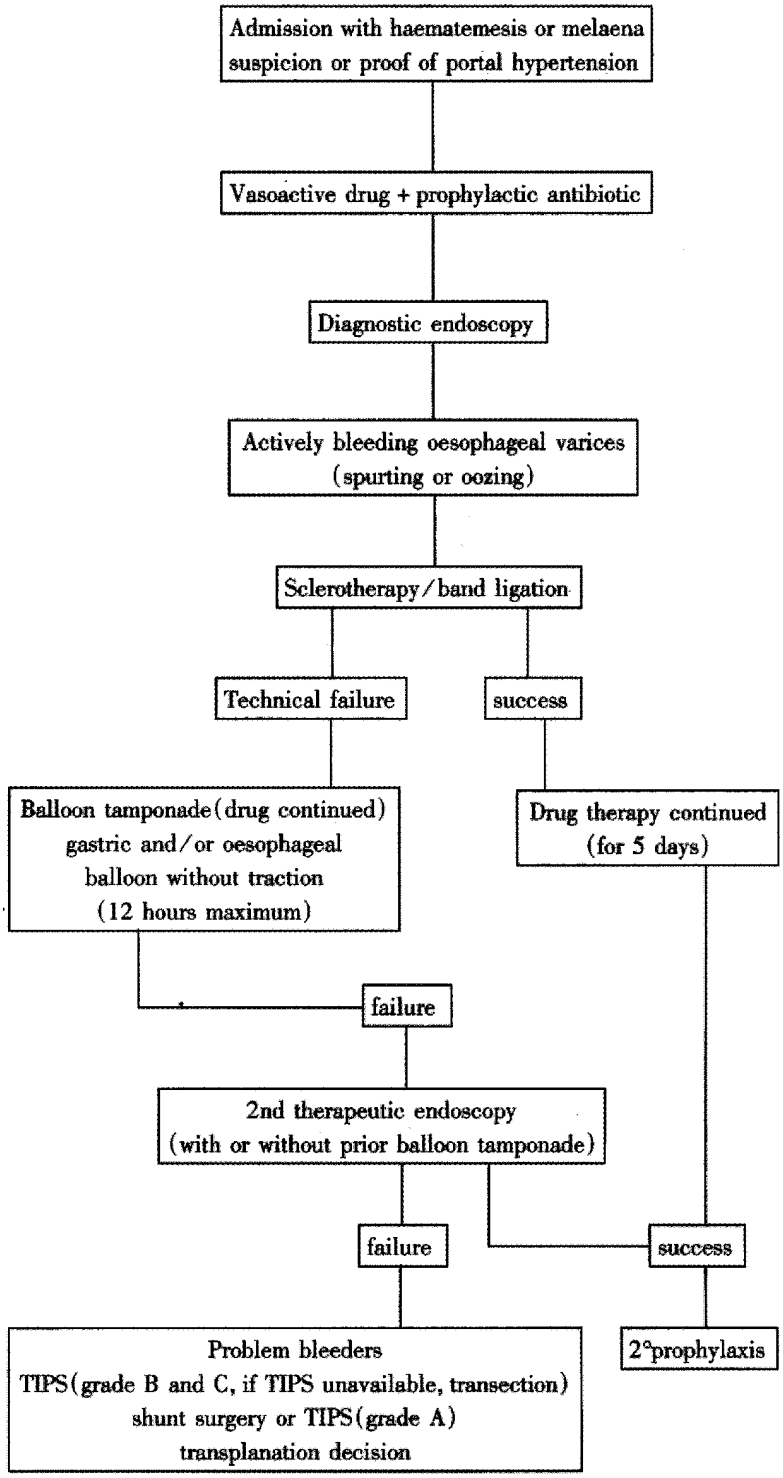
All studies should now adhere to consensus definition[92] so that the field can accumulate evidence for optimal treatment strategies.

Table 2 Reports of TIPS for acute variceal bleeding

Authors	Patients	Child score A / B	Source of bleeding, Gastric / Oesophageal / 42 days (%)	Immediate control of bleeding (in completed TIPS)	Early rebleeding (within 1mo)	Mortality
Other						
McCormick (1994)[116]	20	1 / 7 / 12	3 / 17 / -	20 / 20	6	11(55)
Jalan (1995)[109]	19	3 / 3 / 13	- / 19	17 / 17	3	8(42)
Sanyal (1996)[117]	30	1 / 7 / 22	4 / 26 / -	29 / 29	2	12(40)
Chau (1998)[97]	112	5 / 27 / 80	28 / 84 / -	110	15	41(37)
Gerbes (1998)[114]	11	1 / 3 / 7	8 / 11	10	3	3(27)
Banares (1998)[112]	56	11 / 22 / 23	19 / 37 / -	53 / 55	8	15(28)

Key Concepts:

- Aim is not only stop bleeding but prevent early rebleeding as this significantly impairs survival
- Prophylactic use of antibiotics is manadatory
- Vasoactive drugs administered before diagnostic endoscopy for at least 48 hours, and perhaps 5 days
- No more than 2 sessions of endoscopic therapy during the first 5 days of admission
- TIPS as salvage therapy for continued bleeding



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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Management of necrotizing pancreatitis

John Slavin[1], Paula Ghaneh[1], Robert Sutton[1], Mark Hartley[1], Peter Rowlands[2], Conall Garvey[2], Mark Hughes[2] and John Neoptolemos[1]

Departments of Surgery[1] and Radiology[2], Royal Liverpool University Hospital
Correspondence to Mr. J. Slavin, Senior Lecturer, Department of Surgery, Royal Liverpool University Hospital 5th floor UCD Building, Daulby Street, Liverpool, L69 3GA, United Kingdom, UK. jpss@liverpool.ac.uk
Tel: 0044-151-706-4177, Fax: 0044-151-706-5826
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Abstract

Infection complicating pancreatic necrosis leads to persisting sepsis, multiple organ dysfunction syndrome and accounts for about half the deaths that occur following acute pancreatitis. Severe cases due to gallstones require urgent endoscopic sphincterotomy. Patients with pancreatic necrosis should be followed with serial contrast enhanced computed tomography (CE-CT) and if infection is suspected fine needle aspiration of the necrotic area for bacteriology (FNAB) should be undertaken. Treatment of sterile necrosis should initially be non-operative. In the presence of infection necrosectomy is indicated. Although traditionally this has been by open surgery, minimally invasive procedures are a promising new alternative. There are many unresolved issues in the management of pancreatic necrosis. These include, the use of antibiotic prophylaxis, the precise indications for and frequency of repeat CE-CT and FNAB, and the role of enteral feeding.

Subject headings pancreatitis, acute necrotizing/drug therapy; pancreatitis, acute necrotizing/surgery; biopsy, needle; tomography, x-ray computered; enteral nutrition; human

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INTRODUCTION

Acute pancreatitis is common, the incidence in recent European studies varying between 20 and 70 cases per 100 000 population with an overall mortality of between 3% and 8%[1-6]. Most cases are secondary to gallstones or excess alcohol consumption. Activation of trypsinogen within pancreatic acinar cells is the critical initiating event[7]. This leads to autodigestion of the pancreas, with a localised and then systemic inflammatory response, which if marked leads to the development of multiple organ dysfunction syndrome (MODS) and death[8,9]. Approximately half of deaths from acute pancreatitis occur in the first week following an attack. In patients who survive the initial attack a proportion develop areas of pancreatic and peripancreatic necrosis. Secondary infection then leads to persisting sepsis, MODS, and accounts for the majority of the remaining late deaths[1,10].

IDENTIFYING PATIENTS WITH NECROSIS

Nearly all patients who suffer a mild attack of acute pancreatitis make a complete recovery[11]. About one third of patients with a severe attack, who develop organ failure during the first week, will however, subsequently develop pancreatic necrosis involving more than 30% of the gland. There are several methods that are routinely used to identify early those patients who are likely to develop organ failure and those who will be at risk of pancreatic necrosis. Specific clinico pathological scoring systems include those described by Imrie[12] and Ranson[13]. These, however, are only accurate 48 hours after hospital admission, when they correctly categorise around 80% of patients into mild and severe. An APACHE II score $\geqslant$9 on hospital admission correctly identifies around 85% patients who will suffer a severe attack[14]. Unfortunately the relative complexity of the APACHE II system limits its clinical use.

Plasma C reactive protein levels (CRP), greater than 150mg/L 48 hours after admission, are widely used to predict a severe attack of pancreatitis[15,16]. CRP levels do not however peak until seventy-two hours after onset of symptoms thus CRP levels, like the Imrie and Ranson scores are limited in predicting a severe attack during the first few hours following admission (Table 1). Plasma levels of other direct inflammatory mediators, such as interleukin-8 and interleukin-6 are elevated earlier in the course of an attack of acute pancreatitis and relate to the severity of the systemic inflammatory response[17]. Although the levels of these mediators are as accurate at the time of admission as the APACHE II score, the assay systems are not suitable for widespread clinical use. Urinary levels of trypsin activation peptide (TAP), the cleavage peptide released following the activation of trypsinogen, become significantly elevated with the onset of an attack and measuring TAP has been shown to be a valuable predictor of severe disease[18] and urinary TAP levels may ultimately form the basis of a simple bedside urine test (Table 1).

Intravenous contrast-enhanced computerised tomography (CE-CT) has also been used to predict the severity of an attack of acute pancreatitis[19]. Balthazar described a CT severity index, based on a combination of peripancreatic inflammation and degree of pancreatic necrosis as seen at initial CT study. Patients with a high CT severity index had 92% morbidity and 17% mortality; patients with a low CT severity index had 2% morbidity, and none died[20]. This type of scoring system using CT offers no advantages as compared to clinico-biochemical scoring systems for the prediction of severe disease[21]. Rather the value of CE-CT is in the detection of pancreatic necrosis and definition of its extent and distribution (Figure 1A)[22-25] as well as in helping to delineate any associated collections[26]. Serial CT scans also allow the progression of the disease to be followed and are an essential adjunct when surgical intervention is required.

Table 1 Prognostic accuracy of the APACHE II, the Imrie and Ranson scores, plasma CRP and urinary TAP levels[18]

Scoring System	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
Post-symptom 24 hrs					
Urinary TAP >35nmol/L	58	73	39	86	70
Plasma CRP >150mg/L	0	90	0	75	69
Plasma CRP >150mg/L or urinary TAP >35nmol/L	58	72	37	86	69
Plasma CRP >150mg/L and urinary TAP >35nmol/L	0	92	0	74	70
Post-symptom 48 hrs					
Urinary TAP >35nmol/L	81	71	42	94	73
Plasma CRP >150mg/L	65	73	37	90	72
Plasma CRP >150mg/L or urinary TAP >35nmol/L	86	60	35	94	65
Plasma CRP >150mg/L and urinary TAP >35nmol/L	60	85	50	90	80
Post-hospitalisation 24 hrs					
Urinary TAP >35nmol/L	68	74	44	89	73
Plasma CRP >150mg/L	47	82	42	84	74
Plasma CRP >150mg/L or urinary TAP >35nmol/L	74	66	38	90	68
Plasma CRP >150mg/L and urinary TAP >35nmol/L	40	91	57	83	79
APACHE II ⩾8	63	73	38	88	71
Post-hospitalisation 48 hrs					
Urinary TAP >35nmol/L	83	72	44	94	74
Plasma CRP >150mg/L	86	61	37	94	66
Plasma CRP >150mg/L or urinary TAP >35nmol/L	94	49	32	97	58
Plasma CRP >150mg/L and urinary TAP >35nmol/L	74	85	58	92	83
APACHE II ⩾8	56	64	30	85	63
Imrie Score ⩾3	77	75	44	93	76
Ranson Score ⩾3	89	64	38	96	69

PPV=positive predictive value; NPV=negative predictive value.

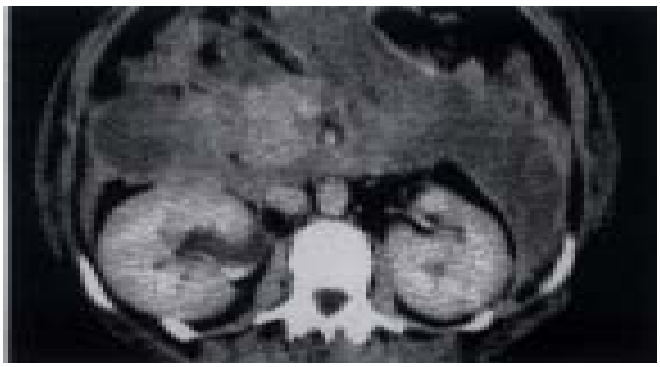

Figure 1A Extensive retroperitoneal pancreatic necrosis.

DETECTION OF INFECTION

In addition to the amount of pancreatic necrosis the outcome in severe pancreatitis is also determined by the presence or absence of infection within the necrotic tissue[27]. Clinical indicators that suggest the presence of infection include pyrexia, hypotension, continuing tachycardia, and a leukocytosis, but these features of sepsis syndrome are identical to those in patients with severe pancreatitis irrespective of the presence of pancreatic infection[28]. Beger *et al* studied 144 patients who underwent open necrosectomy. The proportion of patients who had demonstrable bacterial contamination at the time of necrosectomy increased from 24% during the first week to 36% in the second and peaked at 72% during the third week suggesting that infection is not immediate but that its frequency increases with time[29]. Table 2 shows organisms found within infected necrotic pancreas in their study, which was conducted prior to the routine use of prophylactic antibiotics. The profile of infecting organisms suggests origin from the gastrointestinal tract.

Table 2 Bacteria isolated from operative specimens taken at necrosectomy prior to the introduction of routine antibiotic prophylaxis, Beger *et al*, 1986[29]

Bacteria isolated	No. of patients
Gram - ve aerobic	
Escherichia coli	24
Enterobacter aerogenes	16
Pseudomonas aeruginosa	5
Proteus species	5
Klebsiella pneumonia	3
Citrobacter freundi	1
Gram - ve anaerobic	
Bacteroides species	5
Gram + ve aerobic	
Streptococcus faecalis	6
Staphlococcus aureus	4
Streptococcus viridans	1
Staplococcis epidermidis	1
Others	
Mycobacterium tuberculosis	1
Candida species	3

Several studies have shown that persistently elevated CRP is associated with infected pancreatic necrosis[30]. The presence of gas within an area of necrosis shown by CE-CT is highly suggestive of infection (Figure 1B), although it is desirable to detect the presence of infection before this becomes apparent. CE-CT guided fine needle aspiration, however, allows direct sampling of the necrotic tissue and subsequent microscopy and bacteriology (FNAB) will confirm the presence of infecting organisms (Figure 1C)[31,32].

Figure 1B Infection of pancreatic necrosis with gas forming organisms.

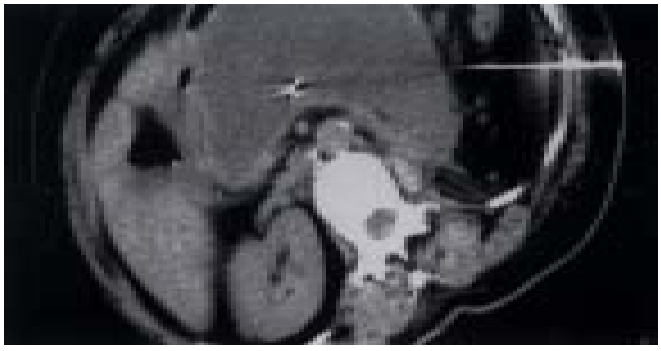

Figure 1C CE-CT guided fine needle aspiration for bacteriology.

The nature of the inflammatory response may also be modified by the presence of infection and recent studies have attempted to identify circulating factors that might confirm this. Serum procalcitonin is a potential marker for non-invasive prediction of infected necrosis[33]. Rau *et al* studied 50 patients with acute pancreatitis, 18 patients with oedematous pancreatitis, 14 patients with sterile necrosis, and 18 patients with infected necrosis. Levels of procalcitonin were measured in plasma during the first two weeks of admission. If levels reached 1.8ng/mL on at least two days during this time, sensitivity, specificity, and accuracy for the prediction of infected necrosis were 94%, 91%, and 92% respectively. This was not confirmed however in a more recent study[34].

PREVENTION OF PANCREATIC NECROSIS

Reducing the severity of the initial attack of acute pancreatitis might reduce the incidence and magnitude of pancreatic necrosis. Unfortunately at the present time, in the absence of effective intervention, management of the acute attack is predominantly supportive. One exception is the use of endoscopic retrograde cholangio-pancreatography and sphincterotomy in patients with predicted severe gallstone pancreatitis, which reduces the severity of an attack. Patients with severe acute pancreatitis due to gallstones need to undergo endoscopic sphincterotomy during ERCP, irrespective of the presence of acute cholangitis and ERCP should be undertaken within forty-eight hours of diagnosis[35-37].

ANTIBIOTIC PROPHYLAXIS

Prophylactic antibiotic use may reduce the incidence of septic complications particularly infection involving areas of pancreatic necrosis. In the 1970s three randomised placebo controlled studies assessed the role of prophylactic antibiotics in acute pancreatitis and found no effect on mortality or morbidity[38-40]. These studies, which were small, consisted almost entirely of patients with mild disease and without necrosis and thus no conclusions can be drawn.

In 1993 Pederzoli *et al* reported a multi-centre randomised study in which 74 patients with pancreatitis from all causes and with confirmed necrosis on CT at the time of admission were randomly assigned to imipenem or to no antibiotic[41]. The incidence of pancreatic sepsis, which was determined by fine needle aspiration or culture of intra-operative specimens, decreased from 30% in those untreated with antibiotics to 12% in the antibiotic treated group. There was, however, no significant difference in the rate of surgical intervention or mortality.

In a subsequent study from Finland, 60 patients with severe alcohol-induced necrotising pancreatitis as determined by CT and CRP estimation were randomly assigned to treatment with cefuroxime or to no antibiotic. One (3%) patient in the antibiotic treated group died compared to seven (23%) patients in the untreated group, ($P<0.05$)[42]. Surprisingly given the large difference in mortality there was no significant difference in the overall incidence of sepsis or the number of patients requiring surgery. Further, given the relatively small size of the study it is probable that there was heterogeneity in the randomisation as shown by the greater number of patients with fulminant pancreatitis on admission in the control group.

More recently, 60 patients with severe acute pancreatitis and necrosis affecting at least 50% of the pancreas, were randomly allocated to receive intravenous treatment for 2 weeks with pefloxacin, (30 patients), or imipenem, (30 patients), within 120 hours of onset of symptoms. The incidence of infected necrosis and extra-pancreatic infections was 34% and 44% respectively in the pefloxacin group and 10% and 20% in the imipenem group. Although imipenem proved significantly more effective in preventing pancreatic infections ($P<0.05$), there was no significant difference in mortality nor in the number of patients requiring surgery between the two treatments[43]. A feature of this last study and of other recent series[44,45] in which prophylactic antibiotics have been used is the increasing incidence of drug resistant or unusual organisms, including fungi, cultured from pancreatic tissue removed at necrosectomy. When such organisms are present the mortality following necrosectomy may be increased[46,47]. Thus the data imply that the use of prophylactic antibiotics promotes drug-resistant organisms and the growth of fungi. In the absence of further studies routine antibiotic prophylaxis in patients with acute pancreatitis cannot be recommended at present.

TRANSLOCATION OF GUT ORGANISMS

The gastrointestinal tract is thought to be the major source of organisms infecting necrotic pancreatic tissue. Increased translocation of bacteria and toxins is known to occur in acute pancreatitis[48,49]. Anaerobic bacteria are less likely to translocate from the gut lumen. Thus selective digestive decontamination (SDD) with appropriate antibiotics may change the intestinal flora to one that is less invasive. Between 1990 and 1993, 102 patients with severe pancreatitis from 16 centres in the Netherlands were randomized to selective digestive decontamination plus standard treatment or standard treatment alone[50]. There was a significant reduction in the incidence of gram-negative pancreatic infection in treated patients. Although deaths were reduced from 35% in the control group to 22% in the treatment group this difference was not significant. A short course of systemic antibiotics (cefotaxime) was used in the SDD group so that interpretation of the data with regard to the specific effects of gut decontamination as opposed to antibiotic prophylaxis is difficult[50].

Early re-introduction of nutrition via the gastrointestinal tract may also help to restore mucosal integrity and reduce translocation. A number of studies in patients with major trauma, surgery and burns showed that enteral nutrition significantly decreased the acute phase response and incidence of septic complications when compared with total parenteral nutrition[51,52]. In acute pancreatitis therefore early reintroduction of feeding via the gastro intestinal tract might also reduce the incidence of pancreatic infection.

Two randomized studies have compared enteral and parenteral nutrition in patients with severe acute pancreatitis. In the first study, 38 patients received enteral nutrition through a nasoenteric tube with a semi-elemental diet or parenteral nutrition through a central venous catheter. Patients who received enteral feeding experienced fewer total complications ($P<0.05$) and were at lower risk of developing septic complications ($P<0.01$) than those receiving parenteral nutrition. The cost of nutritional support was three times higher in patients who received parenteral nutrition[53].

In a second study from Leeds, 34 patients with acute pancreatitis received either parenteral or enteral nutrition for seven days and were then re-evaluated. The frequency of SIRS, sepsis, organ failure and the need for ITU admission was reduced in the enterally fed patients[54].

In a third study from Edinburgh, 27 patients with predicted severe acute pancreatitis were randomised to early introduction of enteral nutrition via a nasojejunal tube or conventional therapy, i.e. nil by mouth with re-introduction of oral intake with return of gut function. There were no significant complications as a consequence of enteral nutrition. The introduction of enteral nutrition did not affect the serum concentrations of IL-6 (P=0.28), soluble tumour necrosis factor-α receptor (P=0.53) or CRP (P=0.62) over the first 4 days of the study. Although there were no significant differences in intestinal permeability between the two patient groups at admission, by day four abnormal intestinal permeability occurred more frequently in patients receiving enteral nutrition (P=0.03).

Thus it can be concluded that enteral nutrition is safe in patients with severe acute pancreatitis and there is some evidence that it may be preferable to parenteral nutrition. The power of these three studies was too low to show any differences with respect to surgical intervention, incidence of pancreatic infection or mortality and the effect of nutrition route and timing on these outcomes requires further study.

NON-OPERATIVE TREATMENT OF PANCREATIC NECROSIS

Although there are isolated case reports of patients with pancreatic infection surviving with medical treatment alone[55] and limited success using percutaneous drainage[56,57], the presence of infection in necrotic pancreatic tissue is accepted to be an absolute indication for surgical intervention (Table 3). The situation in patients with extensive areas of sterile necrosis is less clear. Bradley *et al* reported on 38 patients with necrosis on CT who were initially treated medically and underwent FNAB if they remained persistently febrile. Infected pancreatic necrosis was demonstrated in 27 (71%) of the 38 patients with pancreatic necrosis who were treated by open drainage, with a mortality rate of 15%. All 11 patients with sterile pancreatic necrosis, including six with pulmonary and renal insufficiency, were successfully treated without surgery[58]. On the basis of this and subsequent studies sterile necrosis, should initially be managed non-operatively[59].

Table 3 Indications for surgical intervention

Absolute	• Presence of infected pancreatic necrosis shown by CE-CT or FNAB.
Relative	• In a patient with >50% pancreatic necrosis, failure to improve appreciably after 2 - 3 weeks, unexplained deterioration, or a suspicion of infected pancreatic necrosis even in the absence of firm evidence on CE-CT and FNAB. In a patient with >50% pancreatic necrosis, • prolonged illness with an unacceptably slow recovery

The optimal frequency of CE-CT imaging and FNAB has not been clearly established. In the recent study from Bern, all patients underwent contrast-enhanced CT within 24 to 48 hours of admission and this was repeated weekly in those patients whose clinical condition did not improve[45]. Fine needle aspiration under CT guidance with subsequent microscopy and bacteriological culture was undertaken to rule out infection in patients who developed signs of metabolic disorders, those with deteriorating function of lung, kidney or the cardio circulatory systems and those with persistent leukocytosis or fever (>38.5℃).

A second issue is the treatment of patients with sterile necrosis who remain unwell. In this group surgical intervention has been suggested for patients with persisting or advancing organ complications despite intensive care therapy[59]. In contrast in a recently published single-centre study, pancreatic infection, if confirmed by fine-needle aspiration, was considered an indication for surgery, whereas patients without signs of pancreatic infection were treated medically[45]. Eighty-six (42%) of the patients in this study had necrotizing disease, of which two thirds had sterile necrosis. The death rate was 1.8% (1/56) in patients with sterile necrosis managed without surgery versus 24% (7/29) in patients with infected necrosis (P<0.01). Two patients whose infected necrosis was not diagnosed in time died whilst receiving medical treatment. Thus, an intent to treat analysis (non-surgical *vs.* surgical treatment) produced a death rate of 5% (3/58) with conservative management versus 21% (6/28) with surgery. The authors concluded that non-surgical management, including early antibiotic treatment, should be used in all patients with sterile pancreatic necrosis[45]. In contrast other authors have observed a similar mortality in patients undergoing necrosectomy between those with sterile and those with infected necrosis[60].

TIMING OF SURGERY

Timing of surgery is critical. Necrosectomy is technically difficult during the first week but becomes progressively easier with time. One controlled trial has addressed the role of early surgery. Forty-one patients with pancreatic necrosis on CT were randomized to early necrosectomy (within 48 to 72 hours of onset) or late necrosectomy (at least 12 days after onset). Both groups continued with open packing and staged necrosectomies. Although the mortality rate (58% versus 27%) did not reach statistical significance, the odds ratio for mortality was 3.4 times higher in the early group and for this reason the study was terminated early[61]. Thus the contemporary management of patients with extensive necrosis involves repeated imaging using contrast-enhanced CT in association with fine needle aspiration for microscopy and bacteriology with immediate surgery if infection is detected.

OPEN NECROSECTOMY

Necrosectomy has traditionally been undertaken by an open route. Following laparotomy the lesser sac is opened if possible, the colon is mobilised downwards and the pancreas identified. Necrotic pancreas is debrided by blunt finger dissection and wide bore suction drainage. If opening of the lesser sac is not possible, direct access from the infracolic compartment via the left transverse mesocolon (space of Riolan) is an alternative. Adequate debridement is usually achieved with a single visit to theatre. Any associated fluid collections are drained by the most direct route. Large drains and irrigating catheters are left within the retroperitoneal area and continuous irrigation is continued post surgery[62]. The use of open packing with multiple visits to theatre prior to secondary closure over drains has been described but hospitalisation can be significantly reduced by using prolonged lavage rather than pre-planned multiple laparotomies. Mortality rates in recent series are generally between 20%-40%[45,58,60-67], but may be higher even in specialised centres[33].

Several developments have led to a reassessment of the role and the extent of surgery in acute pancreatitis. Percutaneous drainage has been advocated as a means of

treating pancreatic necrosis[56,57,68]. Unfortunately it is impossible to achieve adequate debridement of solid pancreatic debris by this route except in a minority of cases and it may lead to secondary infection in pancreatic necrosis that is initially sterile. Aggressive percutaneous drainage has been proposed as a means of treating infected pancreatic necrosis. A major reason for failure however is the variable amounts of infected solid material that cannot be removed. Indeed Paye *et al* [69] found percutaneous drainage to be largely inadequate requiring surgical intervention in the majority of cases.

In an attempt to reduce the high mortality from surgical necrosectomy less traumatic approaches than open laparotomy have been advocated. Fagniez *et al*[70] described a retroperitoneal approach for pancreatic necrosectomy through the left flank just anterior to the 12th rib. There was an overall mortality of 33% in 40 patients with severe pancreatic necrosis and 18% in the 22 patients in whom this was the only abdominal procedure performed. Similarly good results have been reported in three other small series[71-73]. Morbidity rates, including colonic fistulae and haemorrhage were, however, high.

Another factor that has led to the re-evaluation of the extent of surgery has been the concept of the two-hit response. This hypothesis states that many patients with a severe attack of acute pancreatitis are primed to mount an inappropriate and exaggerated inflammatory response to a second traumatic challenge[8,9]. Thus a subsequent hit, for example from an open procedure to debride the infected necrotic pancreas, may lead to an overwhelming systemic inflammatory response and death. This would account in part for the continuing high mortality that follows open surgical necrosectomy. Unfortunately patients liable to have such an abnormal response cannot be identified at present although markers of genetic susceptibility are being sought.

A new technique of minimally invasive pancreatic necrosectomy via a left loin approach, analogous to the open technique of Fagniez *et al*[70] was recently pioneered in Glasgow[74]. The advantages of this technique are two-fold. First the peritoneal cavity is not transgressed, and second, tissue damage is limited-thus reducing the magnitude of the systemic inflammatory response of the second hit. Mortality in the 10 patients treated by this technique was only 20%. We have used this technique in a further 14 patients with 3 (21%) deaths[75]. We believe that these results are encouraging and that in the future a significant proportion of patients with infected pancreatic necrosis may be managed by this technique.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Adjuvant therapy in pancreatic cancer

Paula Ghaneh, John Slavin, Robert Sutton, Mark Hartley and John P Neoptolemos

Department of Surgery, University of Liverpool, 5th Floor UCD Building, Daulby Street, Liverpool, L69 3GA, UK
Correspondence to Professor JP Neoptolemos, Department of Surgery, University of Liverpool, 5th Floor UCD Building, Daulby Street, Liverpool, L69 3GA, UK. j.p.neoptolemos@liverpool.ac.uk
Tel: 0044-151-706-4175, Fax: 0044-151-706-5798
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Abstract

The outlook for patients with pancreatic cancer has been grim. There have been major advances in the surgical treatment of pancreatic cancer, leading to a dramatic reduction in post-operative mortality from the development of high volume specialized centres. This stimulated the study of adjuvant and neoadjuvant treatments in pancreatic cancer including chemoradiotherapy and chemotherapy. Initial protocols have been based on the original but rather small GITSG study first reported in 1985**. There have been two large European trials totalling over 600 patients (EORTC and ESPAC-**1**) that do not support the use of chemoradiation as adjuvant therapy. A second major finding from the ESPAC-**1 **trial (**541 **patients randomized) was some but not conclusive evidence for a survival benefit associated with chemotherapy. A third major finding from the ESPAC-**1 **trial was that the quality of life was not affected by the use of adjuvant treatments compared to surgery alone. The ESPAC-**3 **trial aims to assess the definitive use of adjuvant chemotherapy in a randomized controlled trial of** 990 **patients.**

Subject headings pancreatic neoplasms/drug therapy; pancreatic neoplasms/radiotherapy; human; review

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INTRODUCTION

Pancreatic cancer is the 5th most common site of deaths due to cancer among all cancer sites in the Western world. Low cure rates ensure that the mortality is nearly as high as the incidence. It is responsible for 7 000 deaths per year in the UK[1], 40 000 per year in Europe and 28 000 in the USA[2]. The peak mortality ages are estimated to be between 65 and 72 years in Europe and 55 and 75 years in the USA[2]. The incidence of this deadly disease has been rising during the last century. In the past 20 years however, there have been vast improvements in the surgical management of patients with pancreatic cancer. The surgical procedures have been improved and become more standardized between centres and countries. The level of pre- and post-operative support for these patients has been optimized, particularly in established centres with a high throughput[3]. These measures have ensured that the outlook for patients with resectable disease has certainly improved, particularly in the short term. Extending patient survival still remains a problem. The overall five-year survival for all patents with pancreatic cancer is only 0.4%[4]. Patients who are suitable for resection have five-year survival rates of between 10% and 24%[5-8] and are virtually never cured. Therefore, even for the 10% to 15% of patients who undergo surgery, there appears to be no guarantee of cure or indeed long-term survival. These outcomes would suggest a role for the use of additional or adjuvant therapy to attempt to improve patient survival and quality of life.

RADICAL SURGERY

Japanese groups amongst others have been enthusiastic in pursuing radical resection as means of increasing disease free margins and thus hopefully improving patient survival. Radical surgery includes extensive lymph node dissection and retroperitoneal connective tissue clearance as well as pancreatic resection. The Japanese groups have suggested that these approaches are superior to conventional Kausch-Whipple resection but several studies have not shown significant survival advantages when compared with conventional resection[9-13]. Kayahara *et al* found that radical resection in patients with Stage I and II disease (Japanese classification) and clear margins (R0) resulted in a reduction of local recurrence but did not translate into improved survival because of hepatic metastases[14]. Interestingly highly detailed serial section analysis of presumed R0 specimens has revealed microscopic margin disease (R1) in up to 38% of specimens[14].

Difficulties are encountered when comparing survival figures of Western and Japanese studies because of the different staging systems used (UICC *vs* JPS respectively). This is because of the phenomenon of 'staging system migration' that may give apparently better survival for each stage in one system compared to the other even though there is no overall difference in survival. Satake *et al*[15] compared the Japanese and UICC staging systems in a large cohort of patients. Stage for stage the Japanese system revealed a better survival from Stage I to IV compared with UICC system. The overall five-year survival however, was the same (11%) because the systems had been analysed in identical patients.

The majority of radical resection studies have been non-randomized and performed in single institutions. The radical lymph node dissection allows more accurate staging of disease and these tumours will tend to be upstaged because of this. Thus it is necessary to examine overall group survival within the context of randomized studies by an intention to treat analysis. There has been one multicentre prospective randomized trial comparing traditional partial pancreatoduodenectomy with and without a more extensive lymph node dissection[16]. Eighty-one patients were randomized to receive a standard (n=40) or extended (n= 41) lymphadenectomy and retroperitoneal soft tissue clearance. The standard lymphadenectomy included removal of lymph nodes situated at the anterior and posterior

pancreatoduodenal, pyloric, main bile duct, superior and inferior pancreatic head and pancreatic body stations. The extended lymphadenectomy also included the removal of lymph nodes from the hepatic hilum, along the aorta from the diaphragmatic hiatus to the inferior mesenteric artery, laterally to both renal hila and clearance of the coeliac trunk and superior mesenteric artery. There was no significant difference of overall survival between the two groups. Patients who had lymph node positive disease demonstrated better survival following an extended resection compared to those who did not have an extended lymphadenectomy but must be regarded as a statistically invalid manoeuvre as this was a post-hoc subgroup analysis. In light of these findings the ultimate benefit of extended lymphadenectomy surgery still needs to be proven with further critical evaluation.

There appears to be no additional survival benefit associated with total pancreatectomy compared to Kausch-Whipple pancreatoduodenectomy and at the present time the pylorus preserving pancreatoduodenectomy has been shown to produce similar results to the more traditional Kausch Whipple procedure[17,18]. The latter approach is now the procedure of choice in most centres.

The lack of survival benefit associated with radical resection may be due in part to the pattern of disease recurrence in resected pancreatic cancer. Most tumour recurrences are local, peritoneal and hepatic[19-24]. The early appearance of hepatic metastases following resection almost certainly indicates the presence of hepatic micrometastases at the time of surgery. Microscopic peritoneal disease also tends to occur early in contrast to the relatively later presentation of local recurrence. Pancreatic cancer cells tend to spread within a range of peripancreatic tissues. Lymphatic infiltration and perineural invasion may be found in 90%-100% of resected specimens[25]. Reasons for recurrence following an apparently curative resection include residual retroperitoneal disease, perineural invasion, hepatic micrometastases and lymph node involvement. The pattern of relapse after surgery reflects the natural course of the disease without resection. The most commonly affected organs include abdominal lymph nodes (72%-83%), liver (64%-80%), peritoneum (40%-53%) and lung (27%-50%)[14]. An R0 resection in patients with no lymph node metastases cannot be achieved in more than about half of the patients undergoing resection. Kayahara *et al*[14] at post mortem examined 15 patients who had undergone radical resection. The local recurrence rate in this group of patients was 80%. The local recurrences were associated with perineural invasion, lymphatic invasion and soft tissue infiltration. High rates of local recurrence have been confirmed in numerous studies of patients who have undergone pancreatoduodenectomy and the majority occur within 1 to 2 years of surgery[23,24].

Identification of extrapancreatic disease at the pre-operative stage has improved due to accurate imaging techniques[26] and laparoscopy[27]. Peritoneal cytology has been shown to be positive in 58% of patients who may have unresectable tumours or have a limited postoperative survival[27]. The best predictors of outcome following surgery also reflect the causes of disease relapse. These include tumour stage (which also includes the lymph node status), grade of primary tumour and resection margin status[28-32]. Not surprisingly patients with stage I or II disease and negative resection margins tend to demonstrate the best survival.

The poor overall survival of patients with pancreatic cancer, even following optimal surgical intervention, and the pattern of disease progression and recurrence are clear indications for the use of additional treatment modalities.

CHEMOTHERAPY

Advanced pancreatic cancer

There have been many studies of chemotherapy in patients with advanced pancreatic cancer. Single agents and combination regimens have been used. At the present time there is no accepted standard chemotherapeutic agent for the treatment of pancreatic cancer. 5-fluorouracil (5-FU) remains the most effective and most frequently used single chemotherapy agent. 5-FU works partly by interference with enzymes such as thymidylate synthase and partly by incorporation of 5-FU metabolites into DNA and RNA. The response rates of -15% with a median survival of 3-5 months[33]. The addition of the modulator folinic acid has produced marginal survival benefit over 5-FU alone but this has not been a significant increase[33]. The addition of other modulators such as phosphonacetyl-L-aspartate (PALA), and interferon has also not produced significant improvements in survival[34,35]. Comparisons of 5-FU alone and 5-FU with a combination of other agents have not shown any advantage for the combination groups in randomized trials[33].

A new agent, gemcitabine has been compared to 5-FU in a randomized multi-centre phase III clinical trial[36]. Gemcitabine is a deoxycytidine analogue that is phosphorylated to an active form and competes with dCTP for incorporation into DNA. The study, in which over 70% of patients had stage IV disease, randomized 63 patients to receive gemcitabine and 63 patients to receive 5-FU. Median survival in the gemcitabine group was 5.7 months compared to 4.4 months in the 5-FU group but no patient survived beyond 19 months. The clinical benefit response was also significantly higher in the gemcitabine group[36]. Despite the fact that this is the only trial with a straight comparison between the two agents gemcitabine has been recommended as the drug of choice in the USA. Gemcitabine has also been combined with 5-FU in several phase II studies. It is generally well tolerated but can have unpredictable side effects such as neutropaenia, abnormal liver function tests and nausea and vomiting. In patients who have had previous radiotherapy to the mediastinum there have been unpredictable reactions[37].

There is good evidence from several randomized controlled trials comparing chemotherapy with a no treatment group that chemotherapy is of benefit in patients with advanced pancreatic cancer. Mallinson *et al*[38] demonstrated a median survival of 11 months for patients treated with 5-FU, cyclophosphamide, methotrexate, vincristine and mitomycin C compared to 2.2 months for the untreated control group. This regimen did not produce a significantly greater survival when compared to 5-FU alone in a much larger randomized control trial[39]. Another rather poorly controlled study compared 5-FU and carmustine to untreated controls[40]. There was no significant survival benefit associated with this regimen but the majority of patients in this study received only a single treatment and did not finish the course. A further trial of the combination of 5-FU, doxorubicin and mitomycin C (FAM) resulted in median survival of 33 weeks compared with median survival of 15 weeks in untreated control patients[41]. A recent study compared the use of 5-FU + folinic acid (+/- etoposide) with best supportive care and showed that the median survival in the treated group was 6 months compared to 2.5 months in the control group[42]. Moreover there was better overall

quality of life score for the treated patients.

Adjuvant chemotherapy

There have been only a few studies of adjuvant chemotherapy in pancreatic cancer and (up until the ESPAC-1 trial) there was only one randomized controlled trial comparing surgery and chemotherapy with surgery alone (Table 1)[43-46]. Splinter *et al* [43] reported no evidence of improvement or survival using a FAM regimen in 16 patients who had undergone pancreatoduodenectomy with a three year survival of 24% compared to a three year survival of 28% in 36 patients who had undergone surgery only. Patients from different time periods were included in the two groups and there were only nine patients with pancreatic ductal adenocarcinoma in the adjuvant group and 18 in the surgery only group. Baumel *et al*[46] reported adjuvant chemotherapy in 43 selected patients with a median survival of 12 months but there was no difference in median survival from those patients who underwent surgery only (12 months). Bakkevold *et al*[45] randomized 61 patients who had undergone pancreatoduodenectomy for pancreatic cancer or ampullary cancer to receive either six courses of FAM or no chemotherapy. There was a significant difference in the median survival rates between the two groups: 23 months. Unfortunately this did not translate into a significantly improved long-term survival however: the 5-year survival rates were 4% for the treatment arm versus 8% for the surgery only arm. There was also considerable toxicity encountered with the FAM regimen. Only 24 out of 30 patients randomized to treatment actually started therapy. Sixteen patients needed hospitalization after the first chemotherapy course and a total of 13 patients managed to complete all six cycles of FAM.

The European Study Group for Pancreatic Cancer (ESPAC) has commenced the ESPAC-3 trial with the objective of definitively defining the role of adjuvant chemotherapy following curative resection for pancreatic ductal adenocarcinoma. Two adjuvant regimens are being studied against a no chemotherapy control: ① 5-FU + folinic acid for 24 weeks versus ② gemcitabine for 24 weeks versus ③ observation. All patients will have undergone potentially curative resection for pancreatic ductal adenocarcinoma. A total of 990 patients (330 in each arm) will be recruited over the next few years and survival analysis will be completed after two years of follow-up. At the present time recruitment is underway from centres across Europe with further centres in Canada, Australia and New Zealand due to join.

RADIOTHERAPY (CHEMORADIOTHERAPY)

Advanced pancreatic cancer

External beam radiotherapy (EBRT) although used in the treatment of advanced pancreatic cancer, has never been compared with an untreated control arm in any randomized controlled trial. The most commonly used and probably the best radiosensitizer used with EBRT for advanced pancreatic cancer is 5-FU. Many retrospective studies of EBRT, usually in relatively small groups of selected patients report median survival times of 10 - 15 months with good palliation of symptoms[47,48].

The improved local control of disease achieved with EBRT has not translated into significantly longer survival times, so there have been various refinements in an attempt to enhance the effectiveness of radiotherapy. Wide field irradiation has been used to address the problem of hepatic micrometastases. A Radiation Therapy Oncology Group (RTOG) study of 79 patients who received pancreatic and hepatic irradiation resulted in a median survival of 8.4 months but at the expense of considerable toxicity[49]. Intraoperative radiation therapy (IORT) aims to deliver higher doses of radiation with greater precision and thus reducing the exposure of neighbouring organs. Experimentally, its effectiveness may be as high as five times the equivalent dose given by EBRT. In advanced pancreatic cancer the survival times achieved using IORT have not been encouraging (median survival -6 months) and it has been mainly used to boost EBRT[50,51]. A study comparing EBRT + IORT + 5-FU with EBRT +5-FU demonstrated no significant survival difference (12 and 13 months respectively)[50,51]. Complications encountered during IORT include, duodenal and gastric ulceration, vascular sclerosis and pancreatic abscess[52]. IORT offers good local control and pain relief but cannot be recommended as a standard treatment as it has not been possible to demonstrate any advantages over conventional therapy.

Adjuvant radiotherapy (chemoradiotherapy)

Adjuvant EBRT and IORT have been used alone and in combination in the adjuvant setting (Table 2)[21,32,53-60]. The majority of studies indicate that EBRT alone or in combination with IORT has a significant survival advantage over the use of IORT alone. In a randomized trial IORT was observed to reduce the local recurrence rate by 50% following surgery, but this did not translate into a significant survival advantage (3-year survival with IORT=7% *vs* no IORT 3%)[21]. In selected patients IORT and resection produced 3 and 5-year survival rates of 53% and 29%[61,62]. The use of IORT however requires specialised facilities and can be associated with severe complications.

EBRT (with concomitant chemotherapy) following resection is generally well tolerated but the degree of survival advantage, if any, is uncertain. To address this issue a multicentre Phase III trial organized by the European Organisation for Research and Treatment of Cancer (EORTC) compared chemoradiotherapy in patients following potentially curative surgery for pancreatic cancer with surgery alone[58]. Between 1987 and 1995, 218 patients were randomized to receive either chemoradiotherapy or no chemoradiotherapy following curative surgery for pancreatic or ampullary cancer. Ninety-three out of 110 patients randomized to treatment received a total of 40 Gy with concomitant continuous infusion of 5-FU. There were a total of 54 patients in the observation group and 60 patients in the treatment group with pancreatic ductal adenocarcinoma. There was no significant difference in median survival (with treatment 17.1 months *vs* 12.6 months with observation) and in five year survival [with treatment 20 (95% CI, 5-35)% *vs* 10 (0-20)% with observation]. Similarly there was no significant difference in survival between the treatment and observation groups in patients with ampullary cancer. This study showed that there was no survival advantage for adjuvant chemoradiotherapy for pancreatic and ampullary cancer but has been criticized because it was almost certainly underpowered.

REGIONAL THERAPY

Advanced pancreatic cancer

Regional therapy has been developed with the objective of

delivering high doses of cytotoxic drug to the tumour. The systemic side effects should be reduced with this approach. The coeliac and hepatic arteries and portal vein have all been used to deliver chemotherapeutic drugs to the tumour bed. Good control of hepatic metastases has been reported, with disease progression mainly due to local progression or peritoneal deposits. The combination of 5-FU, folinic acid and cisplatin produced median survival times of 9-14 months in selected patients[63,64]. It has also been reported that some apparently irresectable tumours have become resectable following regional therapy[64].

Adjuvant regional therapy

There have been several studies which have demonstrated improved survival in patients receiving regional chemotherapy following pancreatic resection largely in comparison with historical controls (Table 3)[65-68]. Link *et al*[69] found a median survival of 21 months in 18 patients who had undergone pancreatoduodenectomy for pancreatic ductal adenocarcinoma and then coeliac artery infusion of 5-FU, folinic acid, mitoxantrone and cisplatin compared to 9.3 months for historical controls. Disease progression occurred principally locally or in the peritoneum and was rarely detected in the liver. The rate of hepatic recurrence was greatly reduced using a combination of hepatic artery and portal vein infusion in patients with resected pancreatic, which in one study cancer produced a 54% three-year survival compared to 34% in historical controls[65]. Ozaki *et al*[66] found a 5-year survival rate of 32% patients treated with extended resection, IORT and hepatic artery and portal vein. The encouraging results of these small studies have prompted the ESPAC-2 trial, which is a multicentre, prospective randomized controlled Phase III trial. This study will compare adjuvant intra-arterial chemotherapy (cisplatin, 5-FU, folinic acid and mitoxantrone) and radiotherapy (Arm A) with surgery alone (Arm B) in patients who have undergone potentially curative resection for pancreatic ductal adenocarcinoma or ampullary carcinoma. The trial will recruit 110 patients into each arm and will be completed by 2007 aiming to provide a definitive answer to the role of adjuvant regional therapy for pancreatic cancer.

Table 1 Survival following surgery and adjuvant chemotherapy for pancreatic cancer

Series	Period	Number		Regimen	Median survival (months)	Actuarial survival (%)		
		Total	PDAC			1 year	3 year	5 year
Splinter *et al*[43]	1977-1984	36	18				28	
	1980-1984	16	9	5-FU / DOX / MMC			24	
Livingstone *et al*[44]	N / A	285	285	N / A				9
Bakkevold *et al**[45]	1984-1987	30	23	5-FU / DOX / MMC	23	70	70	4
		31	24		11	45	30	8
Baumel *et al*[46]	1982-1988	43	43	Not specified	12			
	1982-1988	527	527		12			

5-FU = 5-fluorouracil; DOX = doxorubicin; MMC = mitomycin C; * randomised cont rolled trial, PDAC = pancreatic ductal adenocarcinoma.

Table 2 Survival following surgery and radiotherapy for pancreatic cancer

Series	Year	Number	EBRT(Gy)	IORT(Gy)	Median survival (months)	Actuarial survival (%)		
						1 year	3 year	5 year
Willett *et al*[32]	1993	16 (nm)	40-50		21			29
		23 (pm)	40-50		11			0
Johnstone *et al*[53]	1993	26	45-55	20	18			
Zerbi *et al*[21]	1994	43		12.5-20	19	71	7	
		47			12	49	10	
Di Carlo *et al*[54]	1997	27			14			
		27		12.5-2017				
Dobelbower *et al*[55]	1997	14			6.5	15	0	0
		6		10-20	9	50	35	33
		14	50-67		14.5	64	28	0
		10	27-54	10-25	18	70	10	0
Farrell *et al*[56]	1997	14	60	12-25	16	62	22	15
Hishinuma *et al*[57]	1998	34	24 EBRT	13 EBRT + IOR	13	59		19
Klinkenbijl *et al**[58]	1999	54pdc			12.6			10
		60pdc	40		17.1			20
Mehta *et al*[59]	2000	52	45-54	8 IORT	32	75	38	
Lee *et al*[60]	2000	22					47	
		13	49				81	

EBRT = external beam radiotherapy; IORT = intraoperative radiotherapy; nm = nega tive resection margin; pm = positive resection margin; pdc = pancreatic ductal adenocarcinoma.

Table 3 Adjuvant regional therapy for pancreatic cancer

Series	Year	Number	Regimen	Median survival (months)	Actuarial survival (%)		
					1 year	3 year	5 year
Ishikawa *et al*[65]	1994	20	HAI + PVI			54	
Ozaki *et al*[66]	1994	24	IORT + HAI +/- PVI				32
Link *et al*[67]	1997	20	CAI	21			
		29		9.3			
Beger *et al*[68]	1999	24	CAI	23			54 (4 year)
		nd		10.5			9.5 (4 year)

HAI = hepatic arterial infusion; PVI = portal vein infusion; CAI = coeliac artery infusion

NEOADJUVANT THERAPY

The rationale for pre-operative therapy includes (a) the avoidance of long delays following surgery before starting adjuvant therapy and (b) an attempt to downstage the tumour and thereby increase the prospect of resection (Table 4)[70-77]. Pre-operative radiotherapy produced only modest increases in resectability and so chemotherapy was added in an attempt to improve the efficacy of this approach. Recent studies have reported resection rates as high as 60%[70-77] but not surprisingly those tumours >4cm-cm, encase the superior mesenteric artery or obstruct the superior mesenteric/ hepatic portal vein are less likely to be resected[71,73]. Pre-operative chemoradiotherapy may also increase the incidence of clear resection margins to as high as 90%, compared to the accepted norm of 60%-80%.

The effect of any long-term survival benefit from neoadjuvant treatment, if any, is not known due to the lack of randomized controlled studies. Twenty- four out of 53 patients with pancreatic ductal adenocarcinoma initially treated with 5-FU, mitomycin C and 50.4 Gy were able to undergo resection with a medi an survival of 15.7 months[74]. There was a significant level of toxicity associated with this regimen with two treatment related deaths. Another recent non-randomized study found a median survival of 19.2 months for patients with pancreatic cancer treated by pre-operative chemoradiation compared to 22 months for those treated by post-operative chemoradiation. Neither survival nor the pattern disease of recurrence was significantly different between the two groups[73].

COMBINATION THERAPY

Advanced pancreatic cancer

The combination of chemoradiation and follow-on chemotherapy may enable good local control with systemic destruction of the disease. The Gastrointestinal-Tumour Study Group (GITSG) randomized patients with advanced pancreatic cancer to receive either 60Gy EBRT (with radiosensitizing 5-FU) with or without follow-on 5-FU versus 40Gy EBRT with radiosensitizing 5-FU and follow-on 5-FU. The median survival times were 40, 23 and 42 weeks respectively, indicating a- likely valuable role for radiosensitizing (± follow-on)chemotherapy but not for increased radiotherapy[78]. Other combinations have been evaluated in the palliative setting[79]. A randomized trial of IORT versus IORT and methotrexate/5-FU produced a median survival of 4.8 and 8.5 months respectively[80].

Adjuvant combination therapy

The regimen originally adopted by the GITSG for patients with advanced pancreatic cancer was used in the adjuvant setting for a randomized trial in the 1970's . Forty-three patients were randomized to receive either 40Gy (with radiosensitizing 5-FU) then weekly 5-FU or surgery alone. The median survival in the treated group was 20 months compared to 11 months in the surgery only group and the two year survival rates were 42% and 15% respectively[81]. To increase numbers in the treatment group a further 30 patients received adjuvant therapy. The median survival was 18 months with a two year survival of 46%[82]. The number of patients that received treatment as part of the randomized study however was far too small for convincing conclusions to be drawn. The results were encouraging enough for other studies to adopt this protocol and investigate its role in the treatment of pancreatic cancer (Table 5)[81-88]. Yeo *et al*[84]compared three different regimens in selected patients who had undergone pancrea toduodenectomy: ① 40-45Gy EBRT plus follow-on 5-FU for 4 months (standard); ② 50-57Gy EBRT plus hepatic radiation plus 5-FU + folinic acid for 4 months (intensive); ③ no adjuvant treatment. The median survival was 21 months and the two-year survival was 44% for the group given standard adjuvant treatment, significantly better compared to 13.5 months and 30%, respectively in the no treatment group. There was no significant survival difference however between patients that had received the intensive treatment and those that had received no treatment. The main drawbacks to this study are the retrospective data that suffer from patient selection bias and no specification of patient performance status, which is an extremely important independent prognostic factor.

A phase III randomized controlled study organised by the Radiation Therapy Oncology Group (RTOG) in the USA is currently recruiting patients who have undergone resection for pancreatic adenocarcinoma. This study aims to compare 5-FU versus gemcitabine pre- and post- chemoradiotherapy all following surgery. The trial has already accrued the original 330 patient target but is still recruiting, presumably because of a lack of a significant therapeutic effect so far.

The UK Pancreatic Cancer Trials Group (UKPACA)[85] utilized the GITSG protocol for an open phase II study of 40 patients (34 with pancreatic ductal adenocarcinoma and 6 with ampullary tumour) who had undergone-pancreatoduodenectomy between 1987 and 1993 were recruited. Patients received 40Gy (with 5-FU as a radiosensitizer) plus 5-FU weekly for a maximum of 24 weeks. After a median of eight treatments there were no treatment related deaths and no hospitalizations even with a prolonged course of post-operative chemotherapy. The median survival for patients with pancreatic ductal adenocarcinoma was 13.2 months and the five-year survival rate was 15%.

The findings of these studies were instrumental in the design of the ESPAC-1 trial, which commenced in 1994. This trial was established to compare the effects of three adjuvant treatments with a control group. The four groups were: ① chemoradiotherapy (40Gy with radiosensitizing 5-FU); ② chemotherapy (5-FU plus folinic acid for six months); ③ combination chemoradiotherapy followed by chemotherapy; and ④ best supportive care. Patients were eligible following potentially curative resection for pancreatic ductal adenocarcinoma. Between February 1994 and April 2000 a total of 591 patients were randomized of which 541 had pancreatic ductal adenocarcinoma. A total of 61 centres recruited patients from UK, Ireland, France, Sweden, Spain, Italy, Germany, Switzerland, Greece, Hungary, Belgium and Austria. Randomization was stratified by resection margin status. Clinicians could randomize patients into a 2×2 factorial design (obse rvation, chemoradiation, chemotherapy or combination) or into one of the main treatment comparisons (i.e. chemoradiation *vs* control or chemotherapy *vs* control). Two-hundred and eighty five patients were randomized to the 2×2 factorial design, a further 68 patients were randomized to chemoradiation *vs* no chemoradiation and 188 patients were randomized to chemotherapy *vs* no chemotherapy. Tumour grade, size, nodal status and resection margin status were all significantly associated with survival. The overall results showed no benefit for chemoradiation *vs* no chemoradiation (median survival 15.5 months *vs* 16.2 months respectively). There was evidence of a survival benef it for

chemotherapy (median survival 19.7 months) compared to those patients who did not receive chemotherapy (median survival 14 months). The effect was reduced when taking into account whether patients had also received chemoradiation suggesting that chemoradiation may decrease the benefit of chemotherapy[89]. Moreover quality of life analysis showed no significant difference between any of the groups indicating that adjuvant therapy in pancreatic cancer is worthwhile, provided there is a significant prolongation of life[90].

The results of ESPAC-1 have provided for the first time a clear indication of a potential benefit for the use of chemotherapy in the treatment of pancreatic cancer. Even more importantly both ESPAC-1 and the EORTC trials have rejected the use of chemoradiation as adjuvant therapy in pancreatic cancer. Thus the focus of future studies such as ESPAC-3 will be on new and efficacious chemothe rapy regimens. This study is randomizing patients to three arms: 5-FU+folinic acid, gemcitabine and observation. The power of the study is such to detect a 10% difference in 2-year survival between any of the groups. Thus ESPAC-3 will establish (a) the benefit of adjuvant chemotherapy for pancreatic cancer and (b) if such, then the best form of chemotherapy.

Table 4 Neoadjuvant therapy for pancreatic cancer

Series	Year	Number	Regimen	Resection rate		Positive resection margin (*n*)	Median survival (months)	Actuarial survival (%)	
				n	%			3 year	5 year
Ishikawa *et al*[70]	1994	23	EBRT	17/23	74				22
Coia *et al*[71]	1994	27	EBRT + 5-FU + MMC	13/27	48	0/13	16	43	
Staley*et al*[72]	1996	39	EBRT + 5-FU + IORT	39/39	100	7/39	19		19 (4 year)
Spitz *et al*[73]	1997	41	EBRT + 5-FU	41/91	51	5/41	19.2		
Hoffman *et al*[74]	1998	53	EBRT + 5-FU + MMC	24/53	45		15.7		
White *et al*[75]	1999	25	5-FU + EBRT + MMC +CPP	5/25	20				
Wanebo *et al*[76]	2000	14	5-FU + EBRT +CPP	9/14	64				
Snady *et al*[77]	2000	68	EBRT+5-FU+STREP+CPP	20/68	29		32	32	
				48 NR	71		21	13	

EBRT = external beam radiotherapy; 5-FU = 5-fluorouracil; MMC= mitomycin C; IORT = intraoperative radiotherapy; STP = streptozocin; CDDP = cisplatinum; FA = folinic acid; DPD = dipyridamole; n = number; CPP=cisplatin; NR = not resectable.

Table 5 Results of combination therapy in patients who have undergone resection for pancreatic cancer

Series	Year	Number	Radiotherapy (Gy)	Chemotherapy	Median survival (months)	Actuarial survival (%)			
						1 year	2 year	3 year	5 year
Kalser *et al**[81]	1985	21	EBRT 40	5-FU	20	67	42	24	18
		22			11	50	15	7	8
GITSG[82]	1987	30	EBRT 40	5-FU	18		46		
Conlon *et al*[83]	1996	56	EBRT 45	5-FU	20		35		
Yeo *et al*[84]	1997	53			13.5		30		
		99	EBRT 40-45	5-FU	21		44		
		21	EBRT 50-57	5-FU + FA	17.5		22		
UKPACA[85]	1998	35	EBRT 40	5-FU	13	56	38	29	15
Abrams *et al*[86]	1999	23	EBRT	5-FU + FA	15.9				
Paulino *et al*[87]	1999	30	EBRT	5-FU	26				
		8	EBRT		5.5				
André *et al*[88]	2000	10	EBRT	5-FU + FA + CPP	17				

EBRT = external bean radiotherapy; 5-FU = 5-fluorouracil; FA = folinic acid; CPP = cisplatin

CONCLUSION

This is a very encouraging time for the treatment of pancreatic cancer. The results of these large European trials have at last given clear indications for future therapies of pancreatic cancer. By using this information, reasoned approaches are being developed to improve the treatment of patients with pancreatic cancer without sacrificing quality of life.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Experimental and clinicopathologic study on the relationship between transcription factor Egr-1 and esophageal carcinoma

Ming-Yao Wu, Mao-Huai Chen, Ying-Rui Liang, Guo-Zhao Meng, Huan-Xing Yang and Chu-Xiang Zhuang

Department of Pathology, Shantou University Medical College, Shantou 515031, Guangdong Province, China
Supported by the National Natural Science Foundation of China, No. 39670298.
Correspondence to Ming-Yao Wu, Department of Pathology, Shantou University Medical College, 22 Xinling Road, Shantou 515031, Guangdong Province, China. mywu@ncrc.stu.edu.cn
Tel: 0086-754-8900486, Fax: 0086-754-8557562
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Abstract

AIM **To observe the growth suppression effect of exogenous introduction of early growth response gene-1 (Egr-1 gene) on esophageal carcinoma tissue as well as on esophageal carcinoma cell line Eca109 and to explore the potential application of Egr-1 gene in gene therapy of tumor.**

METHODS **Eukaryotic expression vector of PCMV-Egr-1 plasmid was introduced into Eca109 cell line which expressed no Egr-1 protein originally with lipofectamine transfection method. The introduction and expression of PCMV-Egr-1 plasmid into Eca109 cell line was confirmed by G418 selection culture, PCR amplification of neogene contained in the vector, Western blot analysis and immunocytochemical analysis. The cell growth curve, soft agar colony formation rate and tumorigenicity in SCID mice were examined to demonstrate the growth suppression effect of exogenous Egr-1 gene on Eca109 cell line. The Egr-1 mRNA and Egr-1 protein were also detected in 50 surgical specimens of esophageal carcinoma by *in situ* hybridization and immunohistochemistry.**

RESULTS **Exogenous Egr-1 gene was introduced successfully into Eca109 cell line and expressed Egr-1 protein stably. The transfected Eca109 cell line grew more slowly than control Eca109 as shown by cell growth curves, the soft agar colony formation rate (4.0% *vs* 6.9%, *P*<0.01) and the average growth rate of tumor in SCID mice (35.5±7.6 *vs* 65.8±7.6, *P*<0.05). The expression level of Egr-1 mRNA and protein significantly increased in dysplastic epithelia adjacent to cancer rather than in cancer tissues (65.8% *vs* 20.0% by ISH and 57.9% *vs* 14.0% by IHC, *P*<0.01).**

CONCLUSION **Exogenous Egr-1 gene shows the strong effect of growth inhibition in Eca109 cell line. Egr-1 in the cancer tissue shows down-regulated expression that supports the inhibited function of Egr-1 in cancer growth and suggests Egr-1 may have an important role in gene therapy of esophageal carcinoma.**

Subject headings esophageal neoplasms/pathology; tumor cells, cultured; genes, immediate early; gene expression; transfection

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INTRODUCTION

Esophageal carcinoma is one of the most common malignant tumors in China[1-5]. Its pathogenesis and development are closely related with some of proto-oncogenes and their products[6-17]. Early growth response gene-1 (Egr-1 gene) is known as a member of immediate early gene (IEG) family characterized by rapid and transient expression in response to stimulation, modulating gene transcription positively or negatively depending on the cell types and playing an important role in the early cell growth. But its relationship with esophageal carcinoma is not well understood so far. In order to evaluate the growth inhibition in the cell line of esophageal carcinoma (Eca109) and analyze the expression of Egr-1 in the cancer tissue of esophageal carcinoma, the relationship between Egr-1 and esophageal carcinoma is explored by the methods of gene transfection, tumorigenicity in severe combined immunodeficient (SCID) mice and *in situ* hybridization.

MATERIALS AND METHODS

Human esophageal carcinoma cell line Eca109

Eca109 cell line from Chinese Academy of Medical Sciences was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 100mL·L^{-1} fetal bovine at 37℃ and 50mL·L^{-1} CO_2 incubator. The cancer tissue with adjacent mucosa and the esophageal mucosa in the upper surgical margin was from the same surgical specimen of 50 patients with esophageal carcinomas who had received neither chemotherapy nor radiotherapy before surgery. These were fixed in 40mL·L^{-1} neutral formalin with 1/1000 diethyl pyrocarbonate (DEPC, Sigma Chemical Co, USA) and embedded in paraffin, the sections were cut in 5μm.

Eukaryotic expression vector of PCMV-Egr-1 plasmid

The plasmid was donated by Dr RP Huang (Molecular Medicine, Northwest Hospital, WA, USA). The final construct contains the neogene (5.5kb fragment) driven by the respiratory syncytial virus (RSV) promoter and the Egr-1 gene (2.1kb fragment) driven by the human cytomegalovirus (CMV) promoter. The plasmid was confirmed by amplification, purification and tested by cutting endonuclease.

Gene transfection

The eukaryotic expression vector of PCMV-Egr-1 plasmid was transfected to human esophageal carcinoma cell line Eca109 by the lipofectamine (Gibcobrl Company, USA) according to the manufacturer's instructions. After transfection, the cells were trypsinized and reseeded at 1:2 ratio for selection culture with G418 at 600mg·L^{-1}. Four weeks later, the resistant colonies were formed.

Detection of transfected cell neogene with polymerase chain reaction

Extracting the cell DNA followed by performing polymerase chain reaction (PCR) to amplify 327bp of neogene with primer 1 (5'ACAAGATGGATTGCACGC AGG3') and primer 2 (5'TTCTCGGCAGGAGCAAGGTGAG3'). The cycling procedure was: denatured at 95℃ for 1 min, annealed at 55℃ for 1.5 min, extended at 72℃ for 1 min, and after 30 cycles, lengthened at 72℃ for 5 min. The untransfected Eca 109 cell line was used as a negative control.

Western blot and immunocytocheimstry

Cells (3×10^5) were harvested and dissolved by the addition of sodium dodecyl sulfate (SDS)-containing lysis buffer. The lysate was used for SDS-polyacrylamide gel electrophoresis on a 75g·L^{-1} gel. The proteins were electrophoretically transferred from the gel to NC membrane. The transferred membrane was treated with polycolonal antiserum against Egr-1 protein (1:200, Santa Cruz Biot Co, USA) followed by detection with peroxidase-labeled goat anti-rabbit (1:1000, Dako, USA). The resulting complexes were detected with the ECL reagent (Amersham Company, UK) according to the manufacturer's instructions. The Egr-1 protein was detected by immunocytochemistry.The untransfected Eca 109 cell line was used as a negative control.

Detection of biological feactures in transfected cells

Growth curve assays Cells (1×10^4) were seeded in each well of a 24-well plate and were allowed to grow for varying periods. The trypsinized cells were counted by cell counter plate. The growth assays were made by counting in triplicate on every other day of culture for up to 7d followed by constructing the cell growth curves.

Soft agar culture The anchorage independent growth was examined by seeding 1000 cells in 3g·L^{-1} agar medium into 60mm plates previously lined with 5g·L^{-1} agar medium. The plates (in triplicate and repeated twice) were cultured at 37℃, 50mL·L^{-1} CO_2 incubator for 14d. The average number of colony formation (any colony containing >50 cells was counted as a colony) and the colony formation rate (the number of colonies/the number of seeded cells) were calculated.

Tumorigenicity in SCID mice Two groups were divided randomly. The experimental group that consisted of 6 mice was used for Eca 109 cells with transfected Egr-1 injection. The control group of 4 mice was used for Eca 109 cells without transfected Egr-1 injection. Trypsinized cells (5×10^6) were injected into the subcutaneous sites on the shoulders of SCID mice. Animals were inspected at regular intervals for the appearance of visible tumors to measure the time of first appearance. Thirty days later, the mice were sacrificed and the tumors were carefully removed by blunt dissection. The tumors were weighed and their average growth rates were measured as mg·d^{-1}.

Egr-1 in situ hybridization

The expression of Egr-1 was detected by digoxigenin-labeled gene probe from a commercial kit (Boster Company, China) according to the manufacturer's instructions. The human breast tissue and the mouse brain tissue were used as the positive control. Either the sections detected with incubation solution instead of the probe or the sections digested with ribonucleases (RNase) (10mg·L^{-1}) before Egr-1 detection was designed for the negative control. The positive expression showed the brown staining signal in the cytoplasm.

Immunohistochemistry

Egr-1 was analyzed by using Egr-1 rabbit polyclonal antiserum with the SABC method according to the manufacturer's instructions (Boster Company, China) following treatment with 3.3'-diaminobenzidine (DAB) staining. The human breast tissue and the mouse brain tissue were used as the positive control. Negative control was designed using phosphate-buffered saline (PBS) instead of Egr-1 rabbit polyclonal antiserum in detection. The positive expression showed the brown staining signal in nuclei.

Statistical analysis

The data were statistically analyzed using Student's *t* test and the difference of results was analyzed by *U* test and χ^2 test.

RESULTS

Identification of plasmid

The eukaryotic expression vector of PCMV-Egr-1 plasmid was tested by PCR amplification, purification and restriction endonuclease and confirmed to be consistent with the plasmid map. With single digestion of *Sac* II, a 7.6kb band was obtained which represents the whole lengh of the plasmid DNA. With double digestion of *Sac* II combining with *Sma* I, both 5.5kb and 2.1kb bands were obtained which represented the fragment of the vector and Egr-1 gene fragment respectively (Figure 1).

Gene transfection

Eca109 was transfected with PCMV-Egr-1 plasmid by lipofectamine transfection method. Four weeks after G418 selection culture, the resistant colonies were formed. PCR

indicated that 327bp of neogene was shown in transfected Eca109 compared to the negative band in control Eca109 (Figure 2), demonstrating that PCMV-Egr-1 plasmid had been introduced into Eca109 cell and integrated into the genomic DNA.

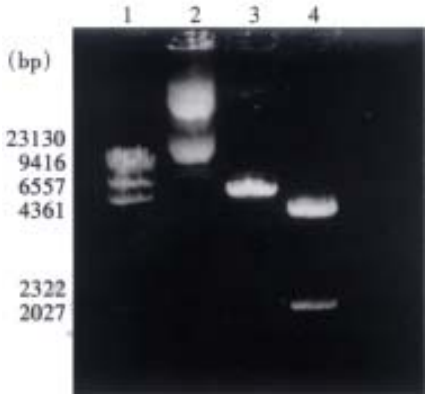

Figure 1 Identification of PCMV-Egr-1 plasmid.
1. Marker; 2. Uncut plasmid; 3. Cut with *Sac* II, showing 7.6kp fragment of whole plasmid; 4. Cut with *Sac* II and Sma I, showing 5.5kp fragment of vector and 2.1kp fragment of Egr-1 gene.

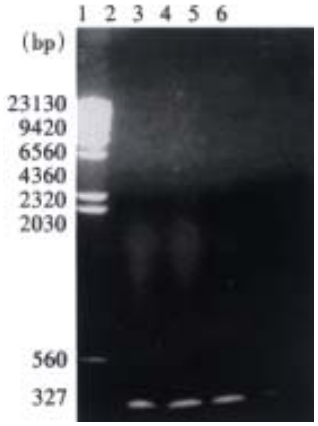

Figure 2 PCR amplification of neogene.
1. Marker; 2-5. For transfected Eca109, showing 327bp of neogene; 6. Negative control Eca109, no band was shown.

Detection of Egr-1 protein in transfected Eca109

Western blot The 80kd of Egr-1 protein was shown for transfected Eca109 compared to the negative band of the control Eca109.

Immunocytochemistry The transfected Eca109 was positively brown stained in the nucleus compared to the negative staining for control Eca109 (Figure 3). The results demonstrated that exogenous Egr-1 gene introduced into Eca109 expressed high level of Egr-1 protein.

Growth feature of transfected Eca109

Growth curves The transfected Eca109 grew much more slowly in DMEM medium than the control Eca109 cell (Figure 4).

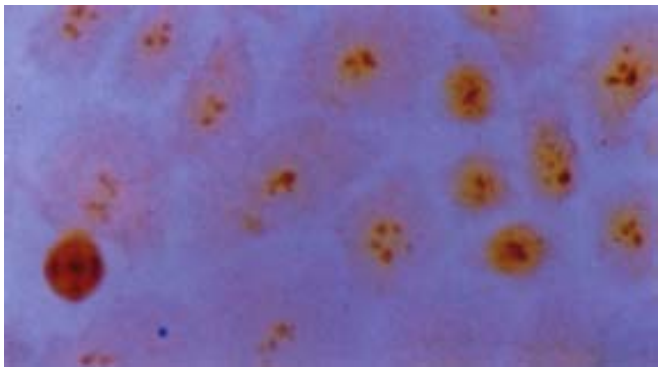

Figure 3 Positive Egr-1 protein in nuclei of transfected Eca109 cells. ICC ×400

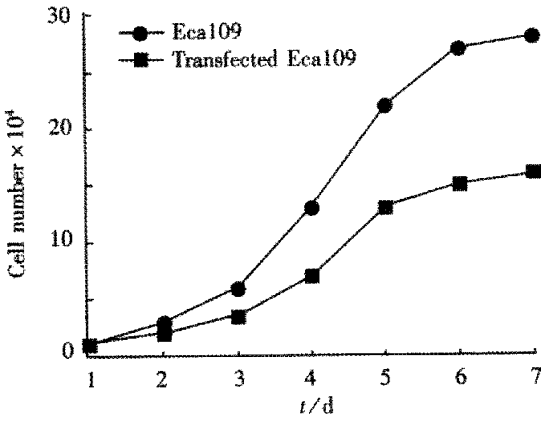

Figure 4 Cell growth curves showing lower growth rate in transfected Eca109 cell than in the control Eca109 cell.

Soft agar growth assay Small and few colonies were formed slowly in transfected Eca109 compared to large and numerous colonies formed quickly in control Eca109. The colony formation rates were 4.0% and 6.9% in transfected Eca109 and in control Eca109 respectively (Table 1) which demonstrated that the anchorage independent growth ability of Egr-1 expressing Eca109 was reduced.

Table 1 Soft agar assays in Eca109 cell line

Cell line	No. of seeded cell	No. of colonies				CFR
		1	2	3	Mean	
Eca109	1000	65	71	73	69±2.4	6.9%
Egr-1-Eca109	1000	40	43	39	40±1.2	4.0%[b]

[b]$P<0.01$, *vs* Eca109, *U* test.

Tumorigenicity in SCID mice The tumorigenicity test showed that the tumors started to appear on the 21st day after injection in the six SCID mice injected with transfected Eca109, and on the 14th day in four SCID mice injected with control Eca109. The tumors of Egr-1 expressing Eca109 grew slowly with an average growth rate of 35.5mg·d^{-1}, and the tumors of control Eca109 grew fast with an average growth rate of 65.8mg·d^{-1} (Figure 5, Table 2). The results demonstrated that the tumorigenicity in SCID mice of Egr-1 transfected Eca109 was inhibited.

Table 2 Tumorigenicity assays in Eca109 cell line

Cell line	Tumorigenicity rate	Growth rate of tumors(mg·d^{-1})						
		1	2	3	4	5	6	Mean
Eca109	4/4	83.3	70.0	46.6	63.3			65.8±7.6
Egr-1-Eca109	6/6	20.0	13.3	33.3	66.6	43.0	36.7	35.5±7.6[a]

[a]P<0.05, *vs* Eca109, Student's t test.

Egr-1 expression on esophageal tissues

In simple hyperplastic epithelia of esophageal mucosa, the expression of Egr-1 was found in the basal layer of the mucosa (Figure 6). In dysplastic epithelia, the expression of Egr-1 increased but significantly decreased in cancer tissues in which only a few cases of well-differentiated squamous cell carcinoma had the Egr-1 expression (Figure 7). The expression of Egr-1mRNA and proteins in the various pathological changes of esophagus are shown in Table 3.

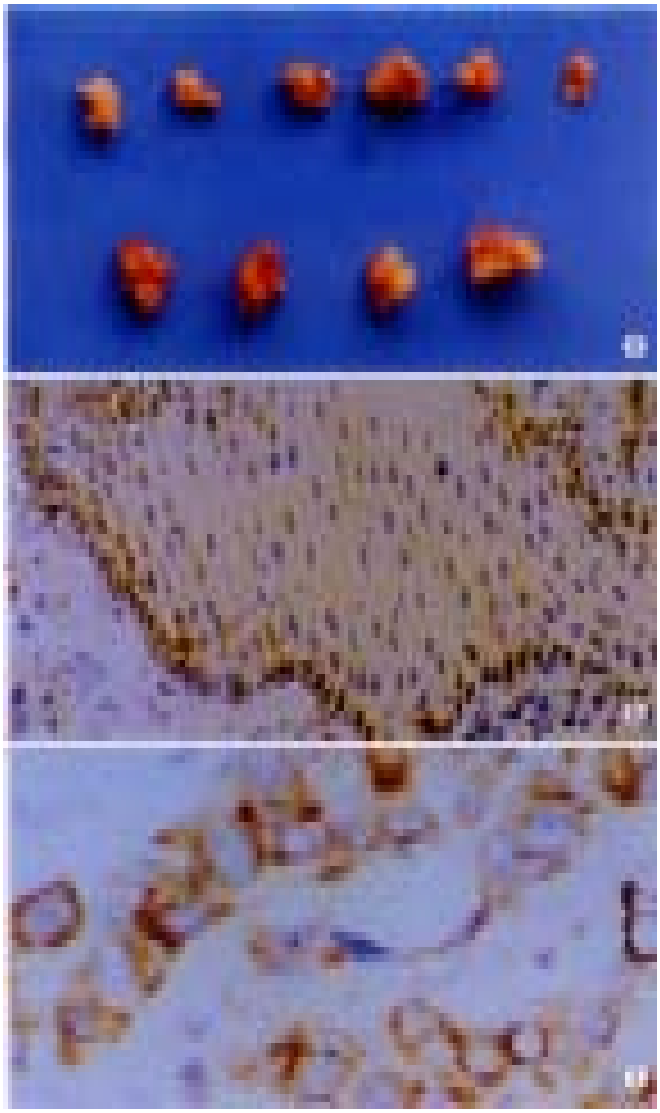

Figure 5 The tumors of transfected Eca109 are smaller than that of control Eca109, *in vitro*, 30 d after injection in tumorigenicity test in SCID mice.

Figure 6 Egr-1 protein expression in basal mucosal layer in simple hyperplastic epithelia of esophagus. IHC ×200

Figure 7 Positive Egr-1 mRNA in cytoplasm of esophageal squamous cell carcinoma. ISH ×400

Table 3 Expression of Egr-1mRNA and proteins in various pathological changes of esophagus, n(%)

Group	n	ISH	IHC
Simple hyperplasia	30	12 (40.0)	9 (30.0)
Dysplasia	38	25 (65.8)	22 (57.9)[a]
Esophageal cancer	50	10 (20.0)	7 (14.0)[b]

[a]P<0.05, *vs* simple hyperplasia, χ^2 test; [b]P<0.01, *vs* dysplasia,χ^2 test.

DISCUSSION

As we know, the oncogenes and tumor suppressor genes are involved in the pathogenesis and development of esophageal carcinoma[18-32]. Recent studies in dicate that Egr-1 gene as a tumor related-gene is correlated with the tumor development. It was reported that Egr-1 proteins were decreased and even disappeared in several kinds of cancer tissues. Furthermore, the Egr-1 mRNA expression was consistent with the expression level of Egr-1 protein which had been verified in the down-regulation of Egr-1 occurred at the transcription level in cancer cells[33]. Southern blot analysis indicated no deletion, no rearrangement or mutation of Egr-1 on DNA level. The exogenous introduction of Egr-1 could inhibit the growth of tumor cells accompanied in a dose-dependent manner, for example, the ability of anchorage independent growth and tumorigenicity in SCID mice of human HT1080 fibrosarcoma without original Egr-1 was significantly inhibited after the exogenous introduction of Egr-1. If the antisense Egr-1 was introduced to the cells, it would inhibit the endogenous Egr-1 expression and promote the malignant transformation of the cells. In the present study, eukaryotic expression vector of PCMV-Egr-1 plasmid was introduced into Eca109 cell line that expressed no Egr-1 protein originally with Lipofectamine transfection method. The introduction and expression of PCMV-Egr-1 plasmid into Eca109 cell line was confirmed by G418 selection culture by which colony formation persisted in next generations and by PCR amplification of neogene contained in the vector. Furthermore, the strong expression of Egr-1 protein in transfected Eca109 was detected by Western blot and immunocytochemistry, which verified the success of gene transfection. Growth inhibition of the transfected cells shown by the growth curves, the colony formation rates in the soft agar and tumorigenicity in SCID mice demonstrated that exogenous Egr-1 gene inhibited the growth of Eca109.

Recent studies suggest that the mechanism of suppression of tumor growth by Egr-1 is the Egr-1 protein with a zinc-finger domain which regulates the transcription of many downstream genes by binding to the GC rich element in the promoter region and modulates genes transcription and various biological effects[34-45]. Egr-1 can compete with transcription activator SP1 in binding to an overlapping consensus binding motif in the promoter region of GCE which commonly exists in oncogene and tumor suppressor gene and abolishes the function of SP1, leading to the transcription inhibition of downstream genes and growth inhibition of tumor cells[46,47]. In addition, the exogenous Egr-1 may inhibit the growth by binding to the GEE of TGF-β1 and activating the transcription of TGF-β1 and subsequently activating p21 gene or by down-regulating Bcl-2 gene to influence the tumor cells[48,49]. Some

studies indicated that the activation of some oncogenes e.g. wild type *p*53 gene, cell apoptosis, the TNF-α and the concentration of calcium may be involved in the mechanism of growth inhibition property of Egr-1[50,51]. On the whole, various and complicated mechanisms may be involved in the suppressive property of Egr-1 for tumor growth.

Egr-1 is one of the immediate early gene family which regulates the cellular growth and differentiation by activating Cyclin D1 to promote the cells from the G_0/G_1 phase into the G2/M phase[52]. The mechanism of down-expression of Egr-1 in cancer tissues is not clear up to now. It was hypothesized that the high concentration of Egr-1 proteins produced by overstimulation of Egr-1 interacted with the promoter region of EBS and subsequently the gene transcription was inhibited, which was concordant with the present results that showed strong expression of Egr-1 in dysplastic cells adjacent to esophageal carcinoma and weekly expression in cancer cells.

Since only downregulation expression without gene mutation occurred in Egr-1 DNA level, it was different from some tumor suppressor genes such as *p*3, *p*16 and Rb with mutation gene on DNA level. It is more convenient to introduce normal exogenous gene into the tumor cells than to repair mutation gene in gene therapy. The present study substantiates that exogenous Egr-1 as a target gene has a potential application in gene therapy of esophageal carcinoma.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Sleep deprivation increase the expression of inducible heat shock protein 70 in rat gastric mucosa

Xi-Zhong Shen[1], Marcel W.L. Koo[2] and Chi-Hin Cho[2]

[1]Department of Gastroenterology, Zhongshan Hospital, Fudan University, 136 Yixueyuan Road, Shanghai 200032, China
[2]Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Pokfulam, Hong Kong, China
Supported by the CRCG grant from the University of Hong Kong.
Correspondence to Dr. Marcel W.L. Koo, Department of Pharmacology, Faculty of Medicine, the University of Hong Kong, 5 Sassoon Road, Hong Kong, China. Wlkoo@hkusua.hku.hk
Tel: 0086-852-8199256, Fax. 0086-852-28170859
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Abstract

AIM **To investigate if sleep deprivation is able to increase the expression of inducible heat shock protein 70 in gastric mucosa and its possible role in mucosal defense.**

METHODS **Rats for sleep disruption were placed inside a computerized rotating drum, gastric mucosa was taken from rats with 1, 3 and 7d sleep deprivation. RT-PCR, immunohistochemistry and Western blotting were used to determine the expression of heat shock protein 70. Ethanol (500mL·L^{-1}, i.g.) was used to induce gastric mucosa damage.**

RESULTS **RT-PCR, Western blotting and immunostaining confirmed that the sleep deprivation as a stress resulted in significantly greater expression of inducible heat shock protein 70 in gastric mucosa of rats. After the 500mL·L^{-1} ethanol challenge, the ulcer area found in the rats with 7d sleep deprivation (19.15±4.2)mm^2 was significantly lower (P<0.01) than the corresponding control (53.7± 8.1) mm$_2$.**

CONCLUSION **Sleep deprivation as a stress, in addition to lowering the gastric mucosal barrier, is able to stimulate the expression of inducible heat shock protein 70 in gastric mucosa of rats, the heat shock protein 70 may play an important role in gastric mucosal protection.**

Subject headings sleep deprivation; heat shock proteins 70/biosynthesis; gastric mucosa; rats

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INTRODUCTION

Stress has been shown to induce gastric mucosal lesions and lower the effectiveness of the mucosa as a barrier[1-6]. In rats, gastric ulcers can be produced by cold-restraint stress[7-9] and it is frequently employed as a model for the study of the mechanisms of stress on ulcer formation. Cold-restraint stress, however, is not normally encountered in human subjects while sleep deprivation is a common experience among city dwellers. swift workers and medical professionals. It imposes stress on the body, and produces a variety of health problems[10-14]. Sleep deprivation is associated with poor congitive ability, and shortening of longevity[15]. Studies have found that the cognitive function of doctors after a night shift was considerably decreased. Sleep deprivation is also a major problem in the intensive care units and it has been suggested to affect the healing process of patients thus contributing to an increase in morbidity and mortality[16]. Sleep deprivation has been shown to induce typical dermatitis in experimental animals. Severe ulcerative and hyperkeratotic skin lesions localized to the paws and tails developed in rats deprived of sleep[17]. This effect of sleep deprivation may affect the epithelial linings of the gastrointestinal tract, because stress has been demonstrated to produce gastric mucosal lesions in rats[18,19]. Our previous works showed that the food and water consumption in sleep disturbed rats were not affected but they had a smaller percentage gain in body weight. The locomotion activities of sleep disturbed rats were similar to the controls, however, their adrenal weights were increased[20]. The mucosal epithelial cell proliferation rate was also suppressed by sleep disturbance. Although various factors have been proposed to account for this process, the precise mechanism of how sleep deprivation affects the gastric mucosa barrier, especially at the molecular level, still remains unclear. Previously, we used cDNA expression arrays to identify genes that abnormal expressed in gastric mucosa of sleep deprivation rats[20]. In this project, inducible heat shock protein 70, one candidate gene emerging from cDNA array for its potential significance was further analyzed.

MATERIALS AND METHODS

Rats and reagents

Male Sprague Dawley rats weighing 180g-200g were used in the experiments. They were housed in a temperature 22℃ ± 1℃ and humidity 65% - 70% controlled room with a day night cycle of 12h. The rats were given standard laboratory diet (Ralston Purina Co., Chicago, IL) and tap water *ad libitum*. Rats were starved for 24h and water withdrawn 1 hour prior to any oral or intragastric administration of agents in order to obtain a uniform distribution of those agents onto the gastric mucosa. All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, USA) unless specified otherwise. The present study has been examined and approved by the Committee on the Use of Live Rats for Teaching and Research of the University of Hong Kong.

Sleep disturbance

Rats for sleep disruption were placed inside a computerized rotating drum while the control animals were left undisturbed in a stationary drum. The drum was rotated 180° in 30s at 5 minutes intervals and was programed to switch off for 1h

every day at 13:00 to allow for an hour of undisturbed sleep. Sleep disturbance was continued for 1wk before the animals were killed.

Collection of gastric mucosa

Rats were killed by ether anesthesia followed by cutting off the abdominal aortic artery. The stomachs were removed rapidly, opened along the greater curvature, and rinsed with cooled normal saline thoroughly. A longitudinal section of gastric tissue was taken from the anterior part of the stomach and then fixed in 100mL·L^{-1} buffered formalin for 24h. It was cut into sections of 5μm and then used in immunostaining. Gastric mucosa was taken from the remaining part of the stomach by scraping with a glass slide on a glass dish on ice. They were wrapped by a piece of aluminum foil, immediately froze in liquid nitrogen and stored at -70℃ until assayed.

Detection of inducible heat shock protein 70 *mRNA expression by RT-PCR*[21-23]

Total RNA was extracted from gastric mucosa of rats by using Trizol reagent (Gibco BRL, Gaitherburg, MD). First-strand complementary DNAs were synthesized from 5μg RNA by using oligo dt primer and Thermoscript RT-PCR system (Gibco BRL, Gaithersburg, MD). The PCR cycle was performed for in ducible heat shock protein 70 and β-actin from the same complementary DNA sample using a PCR Thermal Cycler (Gene Amp PCR System 9700, The Perkin-Elmer Corporation, Norwalk, CT). The sequence of the oligonucleotide primers are as follows: sense inducible heat shock protein 70, 5'-TGCTGACCAAGATGAAG-3'; antisense inducible heat shock protein 70, 5'-AGAGTCGATCTCCAGGC-3'[24] and sense β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3'; antisense β-actin, 5'-CTCCTTAATGTCACGCACGATTTC-3'[25]. After-denaturation for 10 min at 95℃, 30 cycles of amplification were carried out followed by final extension of 10 min at 72℃, each step for 1 min. After amplification, 10μL of PCR products were electrophoresed in a 1% agarose gels containing 0.5μg/mL ethidium bromide.

Immunohistochemical detection of inducible heat shock protein 70 in gastric mucosa[26-30]

Fixed tissue sections (5μm) were mounted on Vectabond Reagent-coated slides, deparaffinized and rehydrated through xyline, graded ethanol to distilled water. After blocking endogenous peroxidase with 3mL·L^{-1} hydrogen peroxide in methanol for 30 min, sections were treated with 0.05mol·L^{-1} phosphate-buffered saline containing 30mL·L^{-1} normal horse serum and 3g·L^{-1} Triton X-100 for 30min, then the sections were rinsed with PBS, incubated with mouse anti-rat monoclonal antibody (StressGen Biotechnologies Corp. Victoria, Canada, Cat# SPA-810) at dilution of 1:200 overnight at 4℃ in a humidity chamber. The secondary, biotinylated antibody (LSAB kit, Dako, Denmark) was then applied for 30 min followed by rinsing with PBS. Staining was performed by the addition of streptavidin (LSAB kit, Dako, Denmark) for 30 min, rinsed in PBS and developed in 3, 3'-diaminobendine tetrahydrochloride for about 3 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted.

Detection of inducible heat shock protein 70 *proteins expression by Western blotting*

The gastric mucosa were homogenized at 4℃ in RIPA buffer (50 mmol·L^{-1} Tris-HCl, pH 7.5, 150 mmol·L^{-1} NaCl, 1g·L^{-1} sodium dodecyl sulfate, 5g·L^{-1} a-cholate, 2 mmol·L^{-1} EDTA, 10 g·L^{-1} Triton X-100, 100 mL·L^{-1} glycerol) containing 1 mmol·L^{-1} PMSF and 10 mg·L^{-1} aprotinin. After centrifuged at 10000×*g* at 4℃ for 20 min, the supernatant (50μg of total protein) were denatured and separated on 75g·L^{-1} sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA)[31,32]. The membranes were blocked with blocking buffer (50mL·L^{-1}) nonfat milk in wash buffer (20 mmol·L^{-1} PBS, pH 7.5 containing 100mmol·L^{-1} NaCl and 1g·L^{-1} Tween 20) for 1h at room temperature, and subsequently incubated at 4℃ with mouse monoclonal antibody against rat inducible heat shock protein 70 (StressGen Biotechnologies Gorp. Victoria, Canada, Cat # SPA-810) diluted in blocking buffer (1:1000). Membranes were washed 6 times and incubated with a rabbit-anti-mouse immunoglobulin G conjugated with the horseradish peroxidase (1:4000) (Bio-Rad Laboratories) for 1h . After six times additional washes, membranes were developed by a commercial chemiluminescence system (Amersham, Arlington Heights, IL) and exposed to X-ray film. Protein determinations were made with Bio-Rad protein assay kit with bovine serum albumin as a standard. Prestained molecular-weight standards (Bio-Rad) were used as markers.

Ethanol-induced gastric mucosal damage

Rats were starved for 24h before 1mL of 500mL·L^{-1} ethanol was administered orally to induce acute gastric mucosal damage[33]. Rats were killed 2h later by a sharp blow on the heads followed by cervical dislocation. The stomach was removed and opened along the greater curvature. The gastric lesion area (mm^2) was traced onto a glass plate and subsequently measured on a graph paper with 1mm^2 division[34]. The lesion index was calculated by dividing the total lesion area with the number of rats in each group.

Statistics

The data were statistically analyzed with the unpaired two-tailed Student's *t* test.

RESULTS

Sleep deprivation increase the expression of inducible heat shock protein 70

Total RNA and protein were isolated from rats' gastric mucosa at various times of sleep deprivation. As shown in Figure 1, RT-PCR results indicated that the expression of inducible heat shock protein 70 mRNA was low in normal gastric mucosa. Following the sleep deprivation, inducible heat shock protein 70 mRNA expression was elevated. The pattern of inducible heat shock protein 70 protein accumulation showed a similar trend to mRNA expression (Figure 2).

Inducible heat shock protein 70 immunohistochemistry in gastric mucosa

To further prove that inducible heat shock protein 70 is increased in gastric mucosa of sleep deprived rats, confirm their expression at protein level, and determine their cellular sources, immunostaining for iHsp 70 was performed on gastric mucosa of sleep deprived rats and normal rats. The results showed that inducible heat shock protein 70 staining was nearly absent in normal mucosa but in mucosa of sleep deprived rats was detected in epithelium (Figure 3).

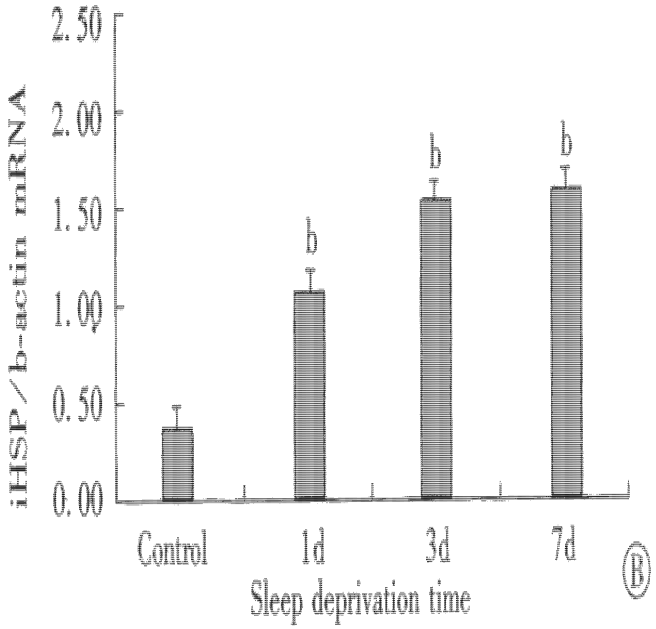

Figure 1 Effect of sleep deprivation in inducible heat shock protein 70 mRNA expression in gastric mucosa of rats. Inducible heat shock protein 70 mRNA was determined by RT-PCR.
(A). Gel photograph of PCR-amplified inducible heat shock protein 70 and β-actin cDNA derived from inducible heat shock protein 70 and β-actin mRNA. Lane 1: normal control; Lane 2: 1 day sleep deprivation; Lane 3: 3 days sleep deprivation; Lane 4: 7 days sleep deprivation.
(B). Bar graph showing the relative amount of inducible heat shock protein 70 mRNA quantified by densitometry and expressed as mean of inducible heat shock protein 70 mRNA: β-actin mRNA ratios. Error bars represent SE, n=8 for each group. $^bP<0.01$ *vs* control group.

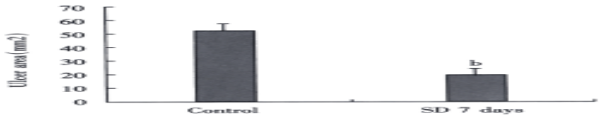

Figure 2 Western blotting analysis of inducible heat shock protein 70 from gastric mucosa of sleep deprivation rats. Lane 1 and 2: control; Lane 3 and 4: 1 day sleep deprivation; Lane 5 and 6: 3 days sleep deprivation; Lane 7 and 8: 7 days sleep deprivation.

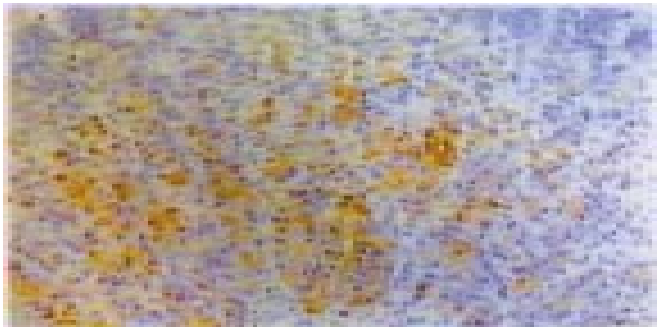

Figure 3 Inducible heat shock protein 70 immunohistochemistry in gastric mucosa of rats with 7 days sleep deprivation.

Sleep deprivation decrease ethanol induced gastric ulceration

After the 500mL·L^{-1} ethanol challenge, the ulcer area found in the rats with 7d sleep deprivation (19.2±4.2) mm^2 was significantly lower (P<0.01) then the corresponding control (53.7±8.1) mm^2, as shown in Figure 4.

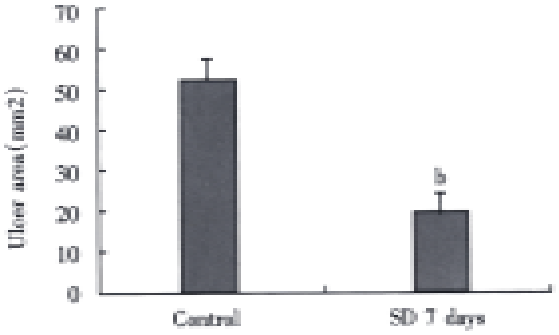

Figure 4 Effect of sleep deprivation in ethanol induced (50% ethanol 1mL p.o. for 2h) gastric ulceration in rats. Error bars represent SE, n=10 for each group. $^bP<0.01$ *vs* control group.

DISCUSSION

In our previously experiment, cDNA arrays were used to search for genes that were differentially expressed in gastric mucosa of sleep deprivation rats compared to gastric mucosa of control rats. More than 10 differentially expressed genes were found in total 588 genes, most of these were digestive enzyme related genes, one of the overexpression gene was inducible heat shock protein 70 gene[20]. A variety of chemicals, viruses, and noxious stimuli such as trauma, hypoxia, or ischemia trigger the heat shock response and the subsequent synthesis of heat shock proteins[35-43]. The results of our experiment indicated that sleep deprivation as a stress resulted in significantly greater expression of inducible heat shock protein 70 in gastric mucosa of rats, which was confirmed by RT-PCR, Western blotting and immunostaining. Substantial evidence showed that heat shock is capable of protecting cells, tissues and organs, and animals from a subsequent, normally lethal heating, as well as from other types of noxious condition[44]. The protective effect of heat shock is likely mediated by overexpressed heat shock protein 70, because there is a lag between heat shock and the development of protection correlated with the production of heat shock protein 70, and protection is affected when heat shock protein 70 production is inhibited by treatment with inhibitors[45-47]. Microinjection of anti-heat shock protein 70 antibody into fibroblasts to neutralize heat shock protein 70 increases the vulnerability of the cells to sublethal temperatures[48]. Furthermore, heat shock protein 70 also provides protection when induced by methods other than heat shock, such as rats that overexpress heat shock protein 70 induced by methods other than heat shock display protection of the lungs from sepsis-induced injury[49] and a reduction in hepatocyte apoptosis induced by tumor necrosis factor-α[40]. Transgenic mice that overexpress heat shock protein 70 demonstrate resistance to adverse effects of lethal heat or ischemia[41]. Similar evidence has been derived from the study of cultured cells after heat shock or heat shock protein 70 gene transfection to promote overexpression of heat shock protein 70[50,51]. Our results showed that sleep deprivation was able to decrease the gastric mucosa damage caused by 50% ethanol. Although we cannot completely preclude the possibility that the vascular, neural, and hormonal factors may be involved in this mucosa protective effect, our previous work showed that sleep deprivation decreased the gastric mucosal blood flow, hampered the ability of the gastric mucosa to repair itself by slowing down its cellular replication rate, and depressed gastric potential difference. In conclusion, our results lead us

to speculate that the heat shock protein 70 may play an important role in gastric mucosal protection.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Expression of vascular endothelial growth factor and its role in oncogenesis of human gastric carcinoma

Du-Hu Liu[1], Xue-Yong Zhang[1], Dai-Ming Fan[1], Yu-Xin Huang[2], Jin-Shan Zhang[3], Wei-Quan Huang[3], Yuan-Qiang Zhang[3], Qing-Sheng Huang[4], Wen-Yu Ma[4], Yu-Bo Chai[5] and Ming Jin[5]

[1]Institute of Digestive Disease, Xijing Hospital, [2]Department of Gastroenterology, Tangdu Hospital,[3]Department of Histology and Embryology, [4]Department of Microbiology,[5]Department of Biochemistry, Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
Correspondence to Du-Hu Liu, Institute of Combined Injury, Third Military Medical University, Chongqing 400038, China. liuduhu@163.net
Tel: 0086-23-68752000 Ext.71554(O), 68753270(H)
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Abstract

AIM **To establish the role of vascular endothelial growth factor (VEGF) in the oncogenesis of human gastric carcinoma more directly.**

METHODS **The expression of VEGF and its receptor kinase-domain insert containing receptor (KDR) in human gastric cancer tissue were observed by immunohistochemical staining. VEGF levels were manipulated in human gastric cancer cell using eukaryotic expression constructs designed to express the complete $VEGF_{165}$ complimentary DNA in either the sense or antisense orientation. The biological changes of the cells were observed in which VEGF was up-regulated or down-regulated.**

RESULTS **VEGF-positive rate was 50%, and VEGF was mainly localized in the cytoplasm and membrane of the tumor cells, while KDR was mainly located in the membrane of vascular endothelial cells in gastric cancer tissues and peri-cancerous tissue. In 2 cases of 50 specimens, the gastric cancer cells expressed KDR, localized in both the cytoplasm and membrane. Introduction of $VEGF_{165}$ antisense into human gastric cancer cells (SGC-7901, immunofluorescence intensity, 31.6%)) resulted in a significant reduction in VEGF-specific messenger RNA and total and cell surface VEGF protein (immunofluorescence intensity, 8.9%) (P<0.05). Conversely, stable integration of $VEGF_{165}$ in the sense orientation resulted in an increase in cellular and cell surface VEGF (immunofluorescence intensity, 75.4%) (P<0.05). Lowered VEGF levels were associated with a marked decrease in the growth of nude mouse xenografted tumor (at 33 days postimplantation, tomor volume:** 345.40±136.31 **mm^3) (P<0.05 *vs* control SGC-7901 group:** 1534.40±362.88 **mm^3), whereas up regulation of VEGF resulted in increased xenografted tumor size (at 33 days postimplantation, tomor volume:** 2350.50±637.70 **mm^3) (P<0.05 *vs* control SGC-7901 group).**

CONCLUSION **This study provides direct evidence that VEGF plays an important role in the oncogenesis of human gastric cancer.**

Subject headings endothelial growth factor/analysis; angiogenesis; solid tumor; stomach neoplasms/pathology; endothelium; vascular

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INTRODUCTION

It is known that malignant tumors depend on neovascularization for their growth and metastasis[1,2]. Recently, many studies have shown that the secretion and activation of various endothelial growth factors, called angiogenic factors, by tumor cells plays a crucial role in the formation of neovasculature[3-8]. VEGF is a powerful mitogen for vascular endothelial cells, both *in vitro* and *in vivo*. In addition, VEGF has the property of inducing vascular permeability *in vivo*. Three evidences for the central role played by VEGF in tumor angiogenesis were: ① the detection of high levels of VEGF expression in palisading cells around regions of necrosis in a number of solid tumor systems, coupled with the rapid induction of VEGF when tumor cells are grown under hypoxic conditions[9,10]; ② injection of antibodies against VEGF markedly reduced the *in vivo* growth of s.c. injected tumor cells, which are known to produce robust tumor angiogenesis[11]; and ③ introduction into tumor endothelial cells of a dominant-negative version of VEGF-R_2 by means of retroviral transfer markedly reduced tumor size[12]. The current study was designed to establish the role of VEGF in the oncogenesis of human gastric cancer more directly.

SUBJECTS AND METHODS

Immunohistochemistry

Resected specimens from 50 patients with gastric carcinoma who underwent gastrectomy at our institute were studied. The patients ranged in age from 27 to 76 years (average 56.3 years); 40 were men, and 10 were women. No patient had received chemotherapy or radiation therapy before surgery. Specimens were fixed in a 40g·L^{-1} formaldehyde solution and embedded in paraffin. Five μm thick sections were cut and mounted on glass slides. Immunohistochemistry was performed using the avidin-biotin complex method. Sections were dewaxed in xylene, taken through ethanol, and then incubated with 30mL·L^{-1} hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Sections were then washed in phosphate-buffered saline and incubated in 20mL·L^{-1} normal goat serum for 30 minutes to reduce nonspecific antibody binding. The antibody for VEGF and

KDR (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) were used respectively. Specimens were then incubated with a 1:500 dilution of anti-VEGF antibody overnight at 4℃, followed by three washes with PBS. Sections were then incubated with biotinylated goat antirabbit immunoglobulin G (Sino-American Biotechnology Co.) at a dilution of 1:100 for 2 hours followed by 3 washes. Slides were then treated with the complex of Reagent A and Reagent B (ABC kit, Sino-American Biotechnology Co.) for 2 hours at a dilution of 1:100 and were washed with phosphate-buffered saline 3 times. Finally, slides were incubated in phosphate-buffered saline containing diaminobenzidine and 300 $mL \cdot L^{-1}$ hydrogen peroxide for 10 minutes. Normal rabbit immunoglobulin-G was substituted for primary antibody as the negative control.

Plasmid constructs

pGEM-hVEGF is pGEM-3Zf(+) derivative Promega cloning vector plasmid containing the complete complementary DNA (cDNA) sequence of human $VEGF_{165}$ (this plasmid was generous gifts from Judith Abraham, Scios Nova, Inc.)[13]. The $VEGF_{165}$ cDNA was subcloned into the $pCDNA_3$ eukaryotic expression vector using the Eco-RI and *Xba* I or *Eco*R I and *Hin*d III restriction enzyme sites in sense or antisense orientation respectively. Restriction enzyme analysis (*Eco*R I, *Xba* I and *Hin*d III) and dideoxy sequencing method were used to confirm the orientation and quality of the VEGF cDNA in the $pCDNA_3$ vector, respectively.

Gastric cancer cell line culture conditions

Cell line SGC-7901 was derived from a moderately-differentiated gastric adenocarcinoma and has been characterized extensively[14]. The cells were routinely cultured in RPMI 1640 / 100 $mL \cdot L^{-1}$ NCS (heat-inactivated) and 2 $mmol \cdot L^{-1}$ L-glutamine in a humidified atmosphere of 50 $mL \cdot L^{-1}$ CO_2 at 37℃, supplemented with penicillin (100 $KU \cdot L^{-1}$) and streptomycin (100$mg \cdot L^{-1}$).

Liposome-mediated transfection

Human SGC-7901 cells were grown in RPMI 1640 / 100$mL \cdot L^{-1}$ NCS in 6-well tissue culture dishes to 50% confluence. The lipofect Amine-mediated transfection (Gibco/BRL) was performed as described previously using 2μg of recombinant constructs DNA (or $pCDNA_3$ vector alone)[15]. After 48h, they were trypsinized from the plates and dilute cells into selective medium containing Geneticin G_{418} (350$mg \cdot L^{-1}$). Cell death was observed after 3 days in culture, and discrete colonies were apparent by 10 days post-selection. Individual colonies were then isolated and grown in 24-well culture plates. Genomic DNA and total RNA were then isolated from these colonies, and PCR analysis and RNA dot blotting were performed. Clones demonstrated to express the sense and antisense-VEGF constructs were then recloned by growing single cell in 96-well plates.

Detection of expression constructs in genomic DNA by PCR

PCR was used to determine which human gastric cancer cell clones were successfully transfected with the sense or antisense-VEGF construct. PCR was performed on genomic DNA isolated from human SGC-7901 gastric cancer cells and individual clones of transfected cells using a sense primer(a) that corresponds to the 5' initial sequence of VEGF cDNA insert (5'GCACCCATGGCAGAAGGAGGAG 3') or an antisense primer (b) that corresponds to the 3' terminal sequence (5'TCACCGCCTCGGCTTGTCACATC 3') and a primer that corresponds to the SP_6 transcription start of the $pCDNA_3$ expression vector (5'GATTTAGGTGACACTATAG 3'). The PCR reaction was performed using standard protocols with 30 cycles of 50s at 94℃, 50s at 55℃ and 60s at 72℃. Appropriately sized amplification products were verified by agarose gel electrophoresis. Negative controls lacking target DNA or containing a nonhomologous plasmid routinely did not show amplification.

RNA dot blotting

Total RNA was isolated from parental and derivative cell lines using the Trizol reagent (GIBCO/BRL). The samples were serially diluted in diethyl pyrocarbonate-treated water containing placental RNase inhibitor (Boehringer Mannheim) to produce working stocks with a final concentration of 10$mg \cdot L^{-1}$ as spectrophotometrically. Denatured RNA was immobilized on nitrocellulose membranes (Boehringer Mannheim). "Run-off" digoxigenin (DIG) labeled riboprobes specific for the sense or antisense strands of VEGF was synthesized from appropriately linearized plasmid stocks using the DIG-RNA labeling kit (Boehinger Mannheim) and appropriate RNA polymerase (Promega) plus 10U placental RNase inhibitor. The specificity of each probe stock was verified by hybridization to originating plasmids and negative controls. The blots were prehybridized in DIG easy Hyb buffer (Boehringer Mannheim), then hybridized for 18 hours at 60℃ in buffer containing 100$\mu g \cdot L^{-1}$ of sense or antisense-specific DIG-labeled probe. After hybridization, the blots were washed extensively and the extent of hybridization was visualized colorimetrically using reagents from the DIG nucleic acid detection kit, Boehringer Mannheim).

Immunofluorescence staining

Confluent monolayers of parental and derivative cell lines were harvested in calcium and magnesium free Dulbeco's phosphate-buffered saline (CMF) supplemented with 2$mmol \cdot L^{-1}$ EDTA. These stocks were pelleted by centrifugation, resuspended in ice cold CMF with 10$g \cdot L^{-1}$ bovine serum albumin and 0.2$g \cdot L^{-1}$ sodium azide, quantitated for viability by trypan blue dye exclusion, and then diluted to a final total viable cells concentration of $1 \times 10^9 \cdot L^{-1}$. Each stock was then exposed to anti-VEGF for 1 hour at 4℃. The cells were washed three times with ice-cold CMF with 10$g \cdot L^{-1}$ bovine serum albumin, then exposed to a 1:20 dilution of fluorescein isothiocyanate- conjugated goat anti-rabbit IgG (Boehringer Mannheim) for 30 minutes at 4℃. The cells were washed twice in CMF, and extent of surface fluorescence for equal number subpopulations of cells was analyzed by fluorescence-activated cell-sorting (FACSTAR; Becton Dickinson) analysis.

In vitro growth rate

Human gastric cancer cells SGC-7901 and its transfected ones with sense or antisense VEGF were cultured at 4×10^4 cells and grown under standard culture conditions. Cell counts were performed on a hemocytometer, initially at 12h time points, and then every 24h for a total of at least 140h. The total number of cells from duplicate experiments was determined as a function of time (h), and the rate of division was calculated from the exponential phase of growth.

In vivo tumor analysis

Adult male or female (nu/nu) mice (5 - 8 weeks of age) received s.c. injections of SGC-7901 gastric cancer cells or SGC-7901 gastric cancer cells transfected with the sense-VEGF or antisense-VEGF constructs. Approximately 10^6 cells

resuspended in a volume of 100μL of serum free cell culture medium were s.c. injected into the dorsa of mice. The mice were monitored daily, and tumor sizes were determined by tridimensional calliper measurements. Tumor volume was calculated as 1/2×length×width 2 (mm^3). Mean tumor volumes were calculated from measurements performed on five mice in each of two individual experiments. The animals were killed 33 days later. s.c. tumors were removed from mice, formaldehyde fixed, and paraffin-embedded. Five μm thick sections were cut and mounted on glass slides. The sections were stained with hematoxylin and eosin[16-18].

RESULTS

VEGF and KDR expressions in human gastric cancer tissues

Among 50 formalin-fixed, paraffin-embedded surgically resected tissue specimens of gastric carcinoma, 10 specimens were composed of two different histological type cancer cells, most of which were poorly differentiated adenocarcinoma and mucinous cell carcinoma. Normal gastric mucosa was not immunoreactive with an anti-VEGF antibody. VEGF was mainly localized to the cytoplasm or the membrane of the carcinoma (Figure 1). Tumor cells that stained strongly for VEGF were observed more often in the invasive front than in the tumor center. Weakly positive VEGF staining was seen on some endothelial cells. VEGF expression was detected in 25 (50%) tumors. KDR was mainly localized in the cytoplasm or the membrane of vascular endothelial cells in gastric cancer tissue and peri-cancerous tissue (Figure 2). In 2 cases of 50 specimens, the gastric cancer cells expressed KDR, localized in the cytoplasm and membrane (Figure 3).

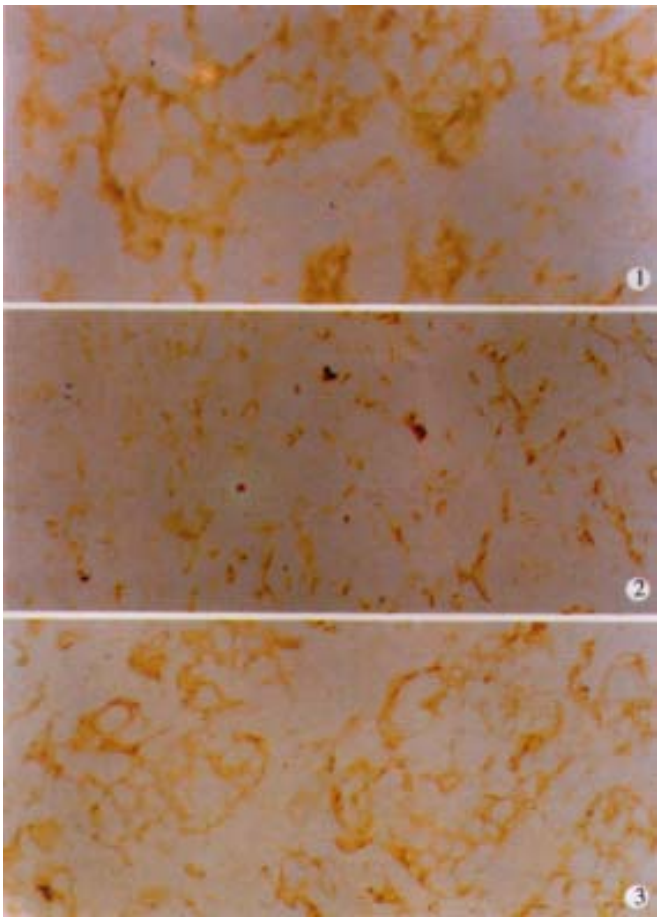

Figure 1 Immunohistochemical staining for VEGF in adenocarcinoma tissues of the stomach. The positive signal was found mainly on the surface and in the cytoplasm of gastric cancer cells. ×400

Figure 2 Immunohistochemical staining for flk-1/ KDR in undifferentiated cancer tissues of the stomach. The positive signal was found on the surface of endothelial cells. ×100

Figure 3 Immunohistochemical staining for flk-1/ KDR in adenocarcinoma tissues of the stomach. It was found mainly on the surface and in the cytoplasm of gastric cancer cells. ×200

Cloning of the sense and antisense VEGF Cdna

The $VEGF_{165}$ insert was cleaved from pGEM-3Zf(+) by restriction enzymes digestion and cloned into the same restriction sites of the eukaryotic expression vector, $pCDNA_3$. Restriction enzyme mapping and dideoxy sequencing assay performed on DNA from transformed clones demonstrated the presence of the $VEGF_{165}$ cDNA insert cloned in the sense and antisense orientation in the $pCDNA_3$ vector (Figure 4).

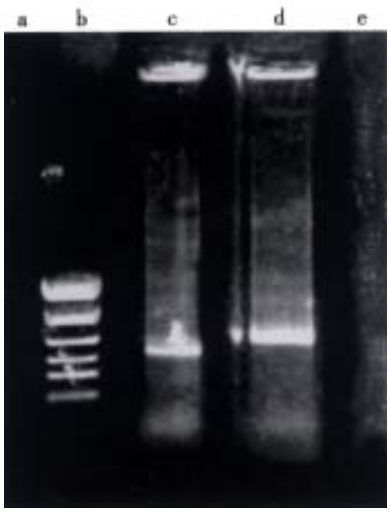

Figure 4 PCR analysis of SGC-s-hVEGF and SGC-as-hVEGF. a. SGC-s-hVEGF/SP_6+b; b. PCR marker; c. SGC-s-hVEGF/SP_6+a; d. SGC-as-hVEGF/SP_6b; e. SGC-as-hVEGF/SP_6+a

SGC-7901 gastric cancer cells expressing sense or antisense VEGF

Following transfection of human SGC-7901 gastric cancer cells with the sense or antisense VEGF construct (or vector alone control) and subsequent antibiotic selection, individual clones were isolated and grown in 24-well culture plates. Polymerase chain reaction (PCR) analysis of DNA isolated from these clones revealed that the positive clones had the sense or antisense VEGF construct. A selection of these clones were then re-cloned at the level of one cell/well, and the PCR analysis was repeated to confirm expression of the cDNA.

VEGF levels in SGC-7901 cells by stable transfection with sense and antisense VEGF constructs

To better determine whether VEGF plays a functional role in the oncogenesis of gastric cancer, gastric cancer cells were transfected with sense or antisense VEGF expression vector, and the effect on the growth of tumor was evaluated. Stable integration of expression constructs into G_{418}-resistant subclones was shown by PCR-based approach that utilized SP6 and VEGF-specific primer pairs and genomic DNA (see Methods). Introduction of the VEGF antisense construct into SGC-7901 cells resulted in a markedly reduction in the expression of VEGF-specific mRNA by dot blot analysis in SGC-7901/as hVEGF compared with the parental cell line.

Conversely, the expression of VEGF in mRNA level was enhanced in SGC-7901 cells transfected with the sense-VEGF construct. Alterations in total cellular VEGF after transfection with sense or antisense constructs were accompanied by similar changes in cell surface VEGF protein as determined by flow cytometer analysis (Figure 5).

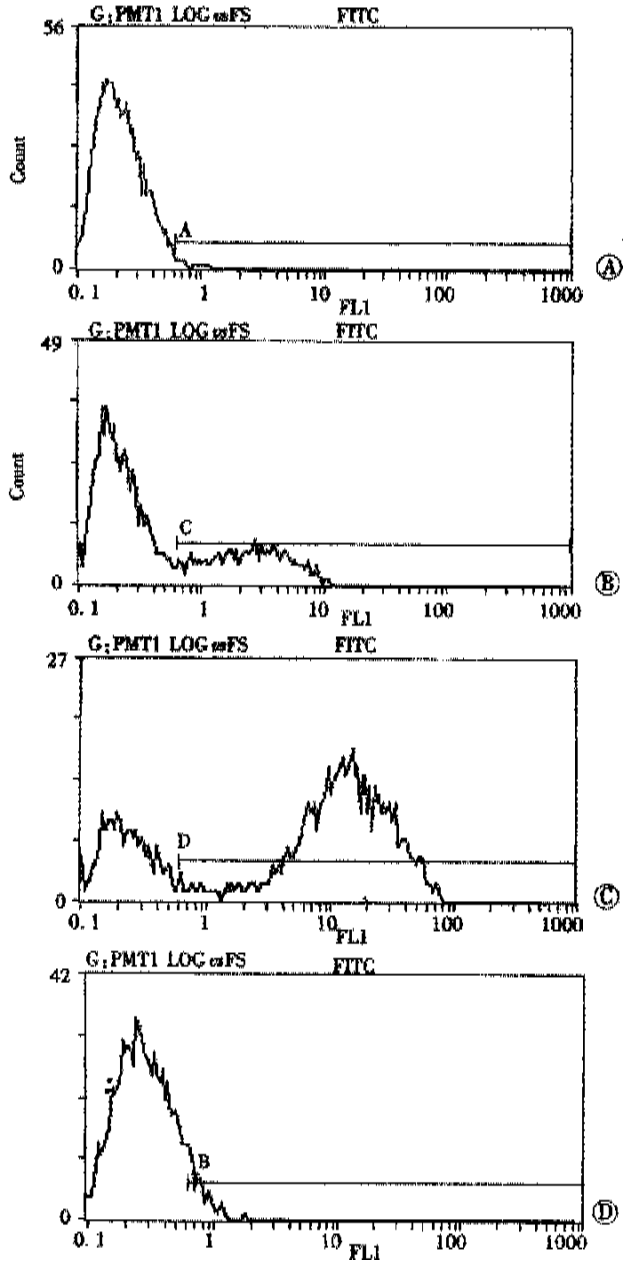

Figure 5 Changes in cell surface VEGF protein as deter mined by flow cytometer immunofluorescence staining analysis.
(A) Flow cytometric detection of VEGF changes of control. (B) Flow cytometric detection of VEGF changes of SGC-7901. (C) Flow cytometric detection of VEGF changes of SGC / s-hVEGF. (D) Flow cytometric detection of VEGF changes of SGC / as-hVEGF.

In vitro growth properties of parental and derivative cell lines

The sense-VEGF cell lines and antisense-VEGF cell lines appeared phenotypically indistinguishable from normal SGC-7901 gastric cancer cells and SGC-7901 transfected vector alone cells, and the growth rates of the derivative cell lines were identical to that of normal SGC-7901 cells (Figure 6).

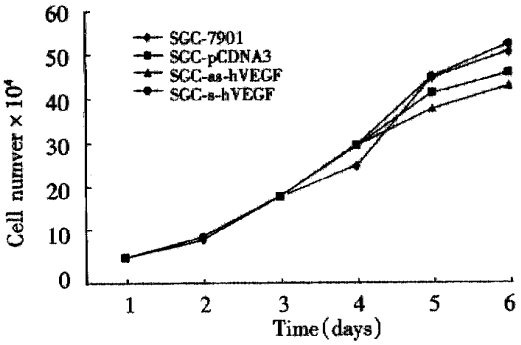

Figure 6 The growth curve of different transfectant.

In vivo growth of parental and derivative cell lines

Control human SGC-7901 gastric cancer cell, sense and antisense-VEGF cell lines were s.c. injected into nude mice, and tumor volumes were measured daily for the duration of the experiments. Tumor growth was detectable and measurable by 8 days postimplantation. At this time point, the parental cells, sense-VEGF cells, and antisense-VEGF cells had produced tumors of 13.25 ± 3.58 mm^3, 13.46±6.04 mm^3 and 12.46 ± 3.01 mm^3. There were no significant differences between their volumes(P>0.05). After 18 days postimplantation, however, the tumors from the sense-VEGF cell lines begun to grow more quickly than that from the parental cells, but the growth rate of tumors from the antisense-VEGF cell lines become slow. At 33 days postimplantation, sense-VEGF, SGC-7901, and antisense-VEGF cell lines produced tumors of 2350.50 ± 637.70 mm^3, 1534.40 ± 362.88 mm^3, and 345.40 ± 136.31 mm^3, respectively (Figure 7). At this time point, the tumor inhibiting rate was 77% through antisense inhibition. Sections of tumors were observed for their degree of tissue necrosis and vascularization. The results demonstrated that there was higher degree of necrosis in the tumors of the antisense-VEGF cell lines in comparison to tumors produced by control SGC-7901 cells, and the number of blood vessels observed in the tumors derived from the sense VEGF cell lines was higher.

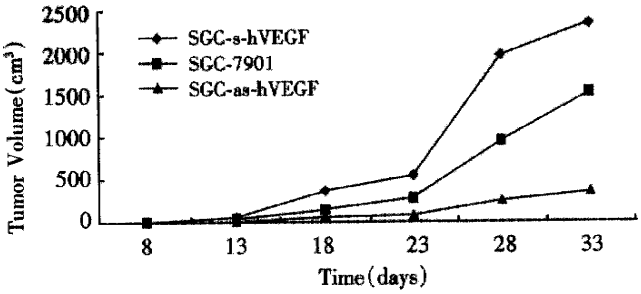

Figure 7 The volume of tumors in nude mice (n=5/ group) s.c. developing from s.c. injection totaling 1.4×10^6 cells / animal of the different transfectants.

DISCUSSION

Chemotherapy is the main treatment for patients with malignant tumor not to be resected, however, acquired resistance to chemotherapy is a major problem during cancer treatment. One mechanism for drug resistance is overexpression of the MDR (multidrug resistance)1 gene encoding the transmembrane efflux pump, P-glycoprotein (P-gp). In recent years, it has attracted much attention and has

been studied as a mechanism of multidrug resistance of tumors to anticancer drugs, however, the application of most agents with the capacity to reverse multidrug resistance (MDR) via modulation of the multidrug transporter P-glycoprotein (Pgp) was shown to be associated with toxic side-effects[19-21]. In addition to it, the heterogenerous of tumor may caused some trouble for choosing sensitive agents. Take altogether, many attempt were made to search for notoxic agents aiming at a common target. Neovascularization is critical for supporting the rapid growth of solid tumors. Tumor angiogenesis appears to be achieved by the expression of angiogenic agents within solid tumors that stimulate host vascular endothelial cell mitogenesis and possibly chemotaxis[22-25]. One such protein, vascular endothelial growth factor, or vascular permeability factor, is a selective endothelial cell mitogen and angiogenic agent induced by several growth factors and cytokines, and elevated expression of either the Ras[26,27], Raf[28], Src[29], or mutant P53[30] oncogenes and hypoxia[31-33] characteristic of rapidly growing solid tumors. VEGF was over expressed in many solid tumors, including breast[34], ovarian[35], lung[36], esophageal[37], and colon[38] cancer. These data suggest a potential role for VEGF in the oncogenesis of solid tumor.

VEGF-positive rate was 50% in human gastric cancer tissues, and it was mainly localized to the cytoplasm and membrane of the tumor cells, while KDR was mainly localized in the endothelial cells. This finding indicated that VEGF might have paracrine effect upon the endothelial cells to promote angiogenesis. In 50 specimens, of 2 cases in which the gastric cancer cells expressed VEGF and its receptor KDR, suggesting that VEGF might have an autocrine effect upon the gastric cancer cells themselves. Analysis of human gastric cancer tissue sections has shown that they are highly heterogeneous[39-41]. The cellular profile of each individual is also heterogeneous in that they contain cells at varying stages of malignancy and have growth factor/receptor expression profile that differ markedly[42-48].

The current study provides a more direct evidence that VEGF plays a role in the growth of gastric cancer. Reduction of VEGF mRNA and protein levels through antisense inhibition significantly lowered the growth rate of the tumors from antisense-VEGF cell lines. Conversely, elevation of VEGF levels after sense transfection resulted in a significant increase of the growth rate of the tumors from sense VEGF cell lines. These results provide strong evidence that VEGF plays an important role in the oncogenesis of the gastric cancer.

A potential therapy based on the interruption of paracrine and/or autocrine growth factor pathways that impinge upon the tumor cells themselves might well prove to be a successful antitumor approach[49-53]. Exploiting the ubiquity of tumor angiogenesis as a suitable target for therapy has been proposed previously to be an important concept for antitumor therapy[54]. This concept has recently been the subject of renewed interest in the development of new therapeutic strategies. Significant evidences are accumulating in favor of the notion that VEGF and its receptor play important roles in the development of solid tumors, such as those derived from gastric cancer origin.

Much indirect evidences indicate that VEGF is an important participant in tumor biology[11,12,55,56]. We now provide direct evidence that VEGF plays an important role in the growth of gastric cancer. VEGF might have mainly paracrine effect upon the endothelial cells to promote angiogenesis[57-62], and it might have an autocrine effect upon the gastric cancer cells themselves. The inhibition of VEGF is sufficient to control tumor growth *in vivo* by the suppression of tumor neovascularization[63-69]. The antisense VEGF strategy offers a new avenue of gene therapy development as an adjuvant treatment for human gastric cancer[70-76].

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Glutathione S-transferases M1, T1 genotypes and the risk gastric cancer: A case-control study

Lin Cai[1], Shun-Zhang Yu[2] and Zuo-Feng Zhang[3]

[1]Department of Epidemiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China
[2]Department of Epidemiology, Shanghai Medical University, Shanghai 200032,China
[3]Department of Epidemiology, UCLA School of Public Health, Los Angeles California, USA
Project supported by Natural Science Foundation of Fujian Province, China, No. C001009
Correspondence to Lin Cai, Department of Epidemiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China. zjcailin@pub5.fz.fj.cn
Tel: 0086-591-3569264
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Abstract

AIM **Glutathione S-transferases (GSTs) are involved in the detoxification of many potential carcinogens and appear to play a critical role in the protection from the effects of carcinogens. The contribution of glutathione S-transferases M1 and T1 genotypes to susceptibility to the risk of gastric cancer and their interaction with cigarette smoking are still unclear. The aim of this study was to determine whether there was any relationship between genetic polymorphisms of GSTM1 and GSTT1 and gastric cancer.**

METHODS **A population based case-control study was carried out in a high-risk area, Changle County, Fujian Province, China. The epidemiological data were collected by a standard questionnaire and blood samples were obtained from 95 incidence gastric cancer cases and 94 healthy controls. A polymerase chain reaction method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes in genomic DNA. Logistic regression model was employed in the data analysis.**

RESULTS **An increase in risk for gastric cancer was found among carriers of *GSTM1* null genotype. The adjusted odds ratio (OR) was 2.63 [95% Confidence Interval (95% CI) 1.17-5.88], after controlling for age, gender, cigarette smoking, alcohol drinking, and fish sauce intake. The frequency of *GSTT1* null genotype in cancer cases (43.16%) was not significantly different from that in controls (50.00%). However, the risk for gastric cancer in those with *GSTM1* null and *GSTT1* non-null genotype was significantly higher than in those with both *GSTM1* and *GSTT1* non-null genotype (OR = 2.77, 95% CI 1.15-6.77). Compared with those subjects who never smoked and had normal GSTM1 genotype, ORs were 1.60 (95% CI: 0.62-4.19) for never smokers with *GSTM1* null type, 2.33 (95% CI 0.88-6.28) for smokers with normal *GSTM1*, and 8.06 (95% CI 2.83-23.67) for smokers with *GSTM1* null type.**

CONCLUSIONS ***GSTM1* gene polymorphisms may be associated with genetic susceptibility of stomach cancer and may modulate tobacco-related carcinogenesis of gastric cancer.**

Subject headings glutathione transferase/genetics; genotype; polymorphism (genetics); stomach neoplasm/genetics; case control studies

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INTRODUCTION

Glutathione S-transferases (*GSTs*), a supergene family of detoxification enzymes, appear to form a protection mechanism against chemical carcinogenesis. In human tissues this family consists of four multigene classes, referred to as alpha, mu, pi, and theta. The *GSTM1* gene is classified into the mu class and the *GSTT1* gene belongs to the theta class. They detoxify reactive chemical species, such as polycyclic aromatic hydrocarbon epoxides by catalyzing their conjugation to glutathione. Genes coding for *GSTM1* and *GSTT1* proteins are polymorphic in humans and these genes are absent in 10% -60% of different ethnic populations[1,2]. Accumulating evidence indicates that susceptibility to cancer is mediated by genetically determined differences in the effectiveness of detoxification of potential carcinogens. Genetic differences are likely to be a major source of interindividual variation in susceptibility to cancer[3].

Gastric cancer is the most common cancer in whole China[4-8], especially in Changle County, Fujian Province, China[9,10]. Previous studies have shown that a number of environmental risk factors may play a role in a multistep and multifactorial process[11-13]. Tobacco smoking has been considered a potential risk factor for gastric cancer[14]. Few data have so far been reported on the risk of gastric cancer associated with genetic and environmental exposures. To evaluate the relationships between *GSTM1/GSTT1* and gastric cancer, a molecular epidemiological study was conducted in Changle County.

MATERIALS AND METHODS

Study subjects

Cases and controls were all residents in Changle County, China, which is one of areas with the highest rates of gastric cancer in the world. All primary gastric cancers (n=95) were histologically confirmed or diagnosed by operation between January 1996 to March 1998. Population controls (n = 94) were randomly selected from the same geographical region, and matched to cases by their gender and age. The field staff conducted face-to-face interviews. Cases and controls were interviewed in the same manner using a standard epidemiological questionnaire. Blood samples (5mL) were collected.

GSTM1 and GSTT1 Assay

DNA was isolated from peripheral white blood cells by proteinase K (Huamei Biotechnology, Inc.) digestion and phenol / chloroform extractions. The PCR reactions were performed in 50μL of a solution containing PCR buffer (1.5 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} KCl, 10 mmol·L^{-1} Tris-HCl, pH 8.3), 200 μmol·L^{-1} of each dNTP, 1 μmol·L^{-1} of each primer, 200ng of template DNA, and 2.5 unit of TAQ DNA polymerase (Promega). Primer sequences for *GSTM1* were 5'-GCTTCACGTGTTATGGAGGTTC-3' and 5'-GAGATGAAGTCCTCCAGATTT-3', which produced a 157 base pair band. The *GSTT1* primers were 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'-3, which produced a 480-base pair band. β-globin was used as an internal positive control, which was amplified with the following primers: 5'-CAACTTCATCCACGTTCACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3' and produced a 268-base pair band. The primers were synthesized by Sangon and PCR amplifications were carried out in a Thermal Cycler (Perkin Elmer 4800). Main cycling parameters were 94℃ for 8 min, followed by 35 cycles of 94℃ for 30s, 60℃ for 40s and 72℃ for 1 min with a final extension at 72℃ for 10 min. PCR products were detected by electrophoresis in agarose gels (2g·L^{-1} for *GSTM1* and 12g·L^{-1} for *GSTT1*).

Statistical analysis

The Chi-square method was used to test the frequencies of *GSTM1* and *GSTT1* genotypes. ORs and 95% CIs were calculated by logistic regression analysis controlling for possible confounding factors.

RESULTS

GSTM1 and *GSTT1* null genotypes are indicated by the absence of a 157bp band and 480 bp band, respectively. β-globin (268bp) indicating the presence of DNA is co-amplified in all the samples (Figures 1, 2).

Main characteristics of subjects

The main characteristics of cases and controls are presented in Table 1, the distribution of sex and age among cases and controls were not statistically significant (P>0.05).

Table 1 Main characteristics of cases and controls

	Cases (n=95)		Controls (n=94)	
	n	(%)	n	(%)
Age groups / yr				
<50	21	(22.1)	22	(23.4)
50 - 59	23	(24.2)	22	(23.4)
60 - 69	33	(34.7)	34	(36.2)
⩾70	18	(19.0)	16	(17.0)
Mean age	59 ± 11		58 ± 11	
Age range	32 - 78		34 - 79	
Gender				
Male	81	(85.3)	82	(87.2)
Female	14	(14.7)	12	(12.8)
Education				
College	1	(1.1)	1	(1.1)
High school	15	(15.8)	63	(67.0)
Elementary school	61	(64.2)	22	(23.4)
Illiterate	18	(19.0)	8	(8.5)

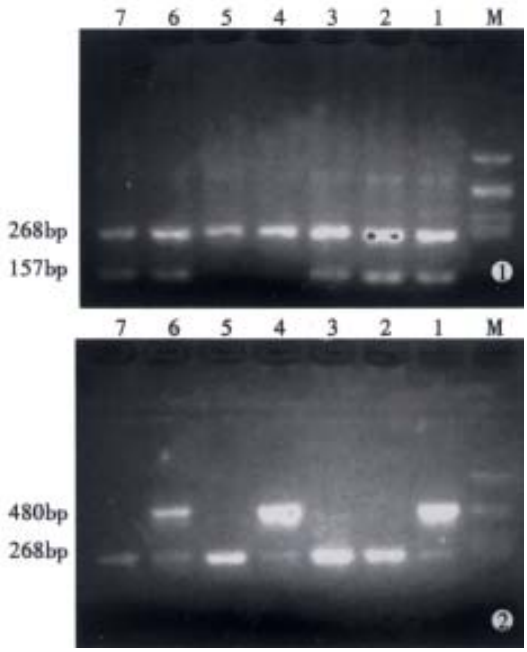

Figure 1 Agarose gel electrophoresis of PCR products. 157bp fragment: *GSTM1*; 268bp fragment: β-globin. Lane M: marker; Lanes 4 and 5:*GSTM1* null; Lanes 1, 2, 3, 6 and 7: *GSTM1* non null.

Figure 2 Agarose gel electrophoresis of PCR products. 480bp fragment: *GSTT1*; 268bp fragment: β-globin. Lane M: marker; Lanes 1, 4, and 6: nun-null; Lanes 2, 3, 5 and 7: *GSTT1* null.

GSTM1 and GSTT1 genotype frequencies in cases and controls

The results showed that GSTM1 null genotype distributed unevenly between gastric cancer cases and controls. The frequency of *GSTM1* null was significantly increased in gastric cancer cases compared with the general controls (χ^2 = 5.75, P=0.0165, Table 2).

Fifty percent (47/94) of individual in the controls exhibited the *GSTT*1 null genotype, and 43.2% (41/95) in gastric cancer cases. The frequencies of *GSTT*1 genotypes in cases and population controls were not significantly different (OR = 0.76, 95% CI 0.1 ~ 1.4). The odds ratio of gastric cancer associated with the combined genotypes of the polymorphisms of *GSTM*1 and *GSTT*1 are shown in Table 3. Persons who carried the *GSTM*1 null genotype and *GSTT*1 non-null had a higher risk of gastric cancer. The odds ratio was 2.77.

Table 2 Association between *GSTM1* and gastric cancer risk

	*GSTM*1 genotype				
	Nonnull	n	(%)	null	n	(%)
Contr		51	54.3		43	45.7
Case		35	36.8		60	63.2
Crude OR (95% CI)		2.03 (1.13-3.65)				
Adjusted OR[a] (95% CI)		2.03 (1.13-3.68)				
Adjusted OR[b] (95% CI)		2.47 (1.21-5.03)				
Adjusted OR[c] (95% CI)		2.63 (1.17-5.88)				

a: Logistic regression adjusted for age and sex; b: Adjusted for age, sex, cigarette smoking and alcohol drinking (yes / no); c: Adjusted for age, sex, cigarette smoking, alcohol drinking (yes / no), and fish sauce intake (continuous).

Table 3 Association between gastric cancer and combinations of *GSTM*1 and *GSTT*1 genotypes

*GSTM*1	*GSTT*1	Case		Contr		OR(95% CI)
		n	%	*n*	%	
Non-null	Non-null	21	22.1	30	31.9	1.00
Non-null	Null	14	14.7	21	22.3	0.95 (0.36~2.50)
Null	Null	27	28.4	26	27.7	1.48 (0.64~3.47)
Null	Non-null	33	34.7	17	18.1	2.77(1.15~6.77)

GSTM1 null genotype and smoking

Because *GSTM*1 may play an important role in the metabolism of tobacco smoke-derived carcinogens, the risk of gastric cancer associated with the polymophisms of metabolic enzymes may depend on the individuals' smoking status. We compared smokers with and without gastric cancer and found that the increased susceptibility to gastric cancer in smokers with *GSTM*1 null phenotype. The subjects which have been exposed to cigarette smoking and *GSTM*1 null genotypes had 8.06 fold risk to develop gastric cancer (Table 4).

Table 4 Risk of gastric cancer in relation to *GSTM1* genotypes by e smoking

Genotype	Smoke	Contr		Case		OR (95% CI)
		n	%	*n*	%	
Nonnull	No	28	29.8	12	12.6	1.00
Null	No	32	34.0	22	23.2	1.60 (0.62-4.19)
Nonnull	Yes	23	24.5	23	24.2	2.33 (0.88-6.28)
Null	Yes	11	11.7	38	40.0	8.06 (2.83-23.9)

DISCUSSION

Changle County is a hyperendemic area of gastric cancer. Familial aggregation of gastric cancer in this area has been reported in previous studies[15,16]. This familial tendency toward gastric cancer may result from a common environment shared by familial members of inherited genetic susceptibility[17]. Gastric cancer is a multistage process[18]. each caused by numbers of factors[19-31]. Environmental and host factors may all contribute to the etiology of gastric cancer[32]. The relationship between polymorphisms of genes involved in carcinogen metabolism and individual susceptibility to the mutagenic and carcinogenic actions of specific chemical exposure is a new field of research[33-35].

Recent studies reported genes that on code enzymes involved in the metabolism of carcioogens or environmental toxins may be related to an increased risk of cancer in some individuals[36,37]. GSTs are multifunctional proteins that catalyze many reactions between glutathione (GSH) and lipophilic compounds with electrophilic centers, including cytotoxic and genotoxic reactions[38]. Polycyclic aromatic hydrocarbons, N-nitrosomines, found in cigarette smoke and food, are potential human carcinogens[39,40]. Deficiency of detoxifying enzymes may affect the metabolic fates of these chemicals and raise cancer risks in exposed individuals[41]. The *GSTM*1 enzyme is involved in detoxifying a number of carcinogenic electrophiles, such as the epoxides of polycyclic aromatic hydrocarbons. Individuals with the homozygous *GSTM*1 null genotypes express no protein and are expected to have reduced abilities of detoxification of hazardous compounds, particularly epoxides.

In this study, *GSTT*1 gene deletion was not associated with gastric cancer. We observed evidence of a relationship between null genotype of *GSTM*1 and risk of gastric cancer. The *GSTM*1 genotype exhibited a higher frequency of gene deletions in cases than in controls. The finding suggests that *GSTM*1 may play a role in gastric cancer susceptibility. Gastric cancer, which is associated with exposure to smoking, may be more striking in individuals who carrying the null genotype *GSTM*1. This result suggests that intervention against smoking may be important for the prevention of gastric cancer in high incidence area because the *GSTM*1 is present in a majority of persons and the potential population impact may be important. However, these results should be considered preliminary. Larger studies will be needed to confirm potential gene-environment interactions.

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Preparation of single chain variable fragment of MG_7 mAb by phage display technology

Zhao-Cai Yu, Jie Ding, Yong-Zhan Nie, Dai-Ming Fan and Xue-Yong Zhang

Department of Gastroenterology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Correspondence to Prof. Xue-Yong Zhang, Department of Gastroenterology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. xyzhang@fmmu.edu.cn
Tel: 0086-29-3374576
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Abstract

AIM **To develop the single chain variable fragment of MG_7 murine anti-human gastric cancer monoclonal antibody using the phage display technology for obtaining a tumor-targeting mediator.**

METHODS **mRNA was isolated from MG_7 producing murine hybridoma cell line and converted into cDNA. The variable fragments of heavy and light chain were amplified separately and assembled into ScFv with a specially constructed DNA linker by PCR. The ScFvs DNA was ligated into the phagmid vector pCANTAB5E and the ligated sample was transformed into competent *E. Coli* TG1. The transformed cells were infected with M13K07 helper phage to form MG_7 recombinant phage antibody library. The volume and recombinant rate of the library were evaluated by means of bacterial colony count and restriction analysis. After two rounds of panning with gastric cancer cell line KATOIII of highly expressing MG_7-binding antigen, the phage clones displaying ScFv of the antibody were selected by ELISA from the enriched phage clones. The antigen binding affinity of the positive clone was detected by competition ELISA. HB2151 *E.coli* was transfected with the positive phage clone demonstrated by competition ELISA for production of a soluble form of the MG_7 ScFv. ELISA assay was used to detect the antigen-binding affinity of the soluble MG_7 ScFv. Finally, the relative molecular mass of soluble MG_7 ScFv was measured by SDS-PAGE.**

RESULTS **The V-H, V-L and ScFv DNAs were about 340bp, 320bp and 750bp, respectively. The volume of the library was up to 2×10^6 and 8 of 11 random clones were recombinants. Two phage clones could strongly compete with the original MG_7 antibody for binding to the antigen expressed on KATOIII cells. Within 2 strong positive phage clones, the soluble MG_7 ScFv from one clone was found to have the binding activity with KATOIII cells. SDS-PAGE showed that the relative molecular weight of soluble MG_7 ScFv was 32.**

CONCLUSION **The MG_7 ScFv was successfully produced by phage antibody technology, which may be useful for broadening the scope of application of the antibody.**

Subject headings antibodies, neoplasms/ biosynthesis; antibodies, monoclonal; stomach neoplasms/ immunology; bacteriophages/genetics

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INTRODUCTION

In our previous studies, MG_7 hybridoma cell line had been successfully prepared by immunization of mouse with KATOIII gastric cancer cells and hybridization of the B cells from the spleen of the immunized mouse with the murine myeloma cell line SP 2/0. This hybridoma cell line generates a kind of monoclonal antibody against gastric cancer which can specifically recognize an ascertained gastric cancer associated antigen[1]. MG_7 antibody was confirmed to be of great value and good potency in the targeting gene therapy of gastric cancer due to the overexpression of its corresponding antigen in a large proportion of patients with gastric cancer. But owing to its murine origin, like many other similar antibodies, MG_7 antibody can elicit human anti-mouse immunoreaction and thus its use in clinical practice is restricted[2,3]. One of the efficient solutions to this problem is to remove the constant region of antibody which makes main contribution to the immunogenicity of the murine antibody to human being. It has been proved that antibody devoid of constant region still maintains its capacity of specific antigen-binding affinity[4-10]. Additionally, antibody without constant region, termed ScFv, is a small molecule and comprises 1/6 of its original antibody in molecular mass. Therefore, ScFv can more readily penetrate into the solid tumor *in vivo* and be easily cleared up from the normal tissue. In the early 90's, the emergence of recombinant phage library represented a great breakthrough in the antibody technology which provides an economical means to prepare the ScFv/Fab of any desired antibody[11-19]. In the present study, the MG_7 recombinant phage antibody derived from MG_7 hybridoma was constructed and screened to prepare the MG_7 ScFv which might help establish an efficient strategy of targeting gene therapy in gastric cancer.

MATERIALS AND METHODS

Detection of antigen-binding affinity of MG_7 antibody

MG_7 hybridoma cells and KATOIII cells were cultured with RPMI 1640 (purchased from Gibco) supplemented with heat-inactivated $100mL\cdot L^{-1}$ fetal bovine serum at 37℃ under $50mL\cdot L^{-1}$ CO_2. MG_7 hybridoma cells were harvested at log phase and stored at -70℃ with aliquot of 10^6 for RNA isolation. Supernatant was collected for detection of antigen-binding affinity of MG_7 antibody by ELISA. KATOIII cells in log phase were transferred into a 96 wellplate and immobilized on the wall by centrifiguation at $1\,000\times g$ for 10 min, finally

fixed by 0.25mL·L^{-1} glutaraldehyde. Supernatant of 0.2mL was applied to each well and incubated at 4℃ overnight, and 0.1mL HRP-labeled goat anti-mouse (HRP-GAM) Ig was added into each well. The absorbance value (*A*) at 492nm of reactant in each well was measured after incubation for 1 hour at 37℃ and staining with OPD.

Construction of MG_7 recombinant phage antibody library

According to the protocol of svtotal RNA isolation system and polyAT tract mRNA isolation system (purchased from Promega), mRNA was isolated from MG_7 hybridoma cells and quantified by gel electrophoresis for following reverse transcription reaction. Subsequently, reverse transcription reaction was performed with 0.3μg mRNA, 2U reverse transcripase (purchased from Promega) mixed together for incubation of 1 hour under 37℃. PCR was conducted with a mixture of 10μg product of reverse transcription, 2U *Taq* DNA polymerase and 2μL V H/V_L primers mix (purchased from Promega) in a total volume of 50μL. The procedure of PCR was arranged in the following order: 95℃×5min; 94℃ ×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min. After precise quantification of PCR product by gel electrophoresis, 50ng of V_H and V_L product was respectively mixed with 50ng linker primer and 1μL *Taq* DNA polymerase to perform PCR (94℃×1min, 63℃×4min, 7 cycles). Subsequently,50ng RS primers (purchased from Promega) underwent another PCR (94℃×1min, 55℃×2min, 72℃×2min and 30 cycles; 72℃×10min). Two μL *Sfi* I and 0.5μg ScFv product were added into a sterile 0.5mL microtube and incubated at 50℃ for 4 hours. After being purified by PCR purification kit, 0.5μg *Sfi* I digested ScFv product mixed with 2μL *Not* I was incubated at 37℃ for 4 hours and purified again for later use. ScFv (150ng) and pCANTAB5E (250ng) mixed with 2μL T_4 DNA ligase was incubated at 16℃ for 16 hours. Ligated product was transformed into TG1 cell. Transformed product with aliquot of 100μL was placed onto SOBAG plates and incubated overnight at 37℃ to form bacterial clones.

Evaluation of volume and recombinant rate of phage antibody library

Colony count was adopted to exhibit the total number of clones formed on the SOBAG plates. Eleven clones were randomly singled out from the SOBAG plates and passaged into 5mL 2×YT-AG medium for an incubation of 12 hours at 37℃. Plasmid from each clone was respectively isolated and digested by *Eco* RI and *Hin* dIII. Gel electrophoresis was conducted with restriction digested product to examine the recombinant phagmid.

Panning and enrichment of MG_7 recombinant phage antibody

The initial recombinant phage antibody library was incubated for 1 hour at 37℃ with shaking at 250r·min^{-1}, and helper phage M13KO7 was added and incubated for another hour at 37℃ with shaking at 250 r·min^{-1}[20]. The culturing product was spinned at 1000×*g* for 10 min to precipiate the cells. Then the entire sample was gently resuspended in 10 mL 2× YT-AK medium. After an overnight incubation at 37℃ with shaking at 250r·min^{-1}, the culturing product was spinned at 1 000×*g* for 20 min and the supernatant which contained the recombinant phages was collected. Then, 2mL PEG/NaCl was added and placed on ice for 45 min for precipitation of recombinant phage clones. It was spinned at 10000×*g* for 20 min at 4℃ and the pellet was resuspended in 16mL 2×YT medium diluted with 14 mL blocking buffer containing 0.1g·L^{-1} sodium azide and incubated at room temperature for 15 min. Twenty mL of the diluted recombinant phage was then added to the flask which was coated with KATOIII cells and well blocked. The flask was incubated for 2 hours at 37℃, washed 10 times with PBS plus another 10 times with PBS containing 1mL·L^{-1} Tween20. Ten mL log-phase TG1 cells were added to the flask and incubated with shaking at 37℃ for 1 hour for reinfection. After two rounds of panning, reinfected TG1 cells with bound phages directly in the panning flask were plated for colony isolation.

Screening for MG_7 recombinant phages

Recombinant phages were rescued from individual clones and screened for MG_7 binding by ELISA. Microtiter wells were coated with KATOIII cells. Bound phages were detected by incubation with a 1:5000 dilution of conjugate (Pharmacia Biotech). And the detection was achieved by addition of TMB substrate. Clones reacted to KATOIII cells were referred to as positives.

Competitive test of positive selected MG_7 recombinant phages

Microtiter wells were coated with KATOIII cells as mentioned above. The supernatant of the selected positive MG_7 recombinant phages was applied into each well (100mL·well^{-1}) and then incubated for 1 hour at 37℃. After disposing of the supernatant, MG_7 antibody (100mg·L^{-1}, 50μL·well^{-1}) was added and incubated for 1 hour at 37℃. PBST was used to wash 5 times, and HRP-GAM Ig (1:1000 diluted, 50μL·well^{-1}) was added and developed by TMB to measure the absorbance value at 450nm. The inhibiting ratio of selected positive MG_7 recombinant phages with MG_7 antibody for binding of KATOIII cells was calculated by the following formula: Inhibiting ratio = 1- (value of sample/value of control)×100%

Detection of antigen-binding affinity of the soluble MG_7 ScFv

The positive phages were transfected into *E.coli* HB2151 cells for the production of a soluble form of the MG_7 ScFv. Five mL culturing product of transfected *E.coli* HB2151 cells with overnight induction of 1mmol·L^{-1} isopropyl β-D-thiogalactopyanoside (IPTG) was centrifugated at 1000×*g* to collect the sediment and the supernatant (containing extracellular soluble ScFvs). The sediment was given osmotic shock to prepare periplasmic extracts. Microtiter wells were coated with KATOIII cells, and ELISA test was made twice to detect the antigen-binding affinity of soluble MG_7 ScFv.

Measurement of the relative molecular weight of soluble MG_7 ScFv

Periplasmic extracts from transfected *E.coli* HB2151 cells induced by IPTG was adopted to measure the relative molecular weight of soluble MG_7 ScFv by SDS-PAGE.

RESULTS

Antigen-binding affinity of MG_7 antibody

The ELISA showed that the A_{492} absorbance of reactant with presence of MG_7 antibody was up to 0.65 (0.208 in control).

Amplification of V_H, V_L and ScFv gene

On electrophoresis, V_H product formed a band at 350bp and V_L at 320bp, and ScFv was successfully spliced together to form a fragment of 750bp, as shown in Figure 1.

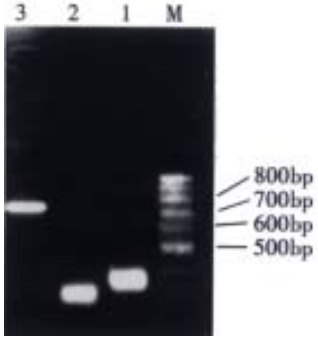

Figure 1 RT-PCR of V_H, V_L and ScFv fragment of MG_7 antibody. 1: V_H; 2: V_L; 3: ScFv; M: 100bp ladder

Volume of MG_7 phage antibody library

Colony counts showed that MG_7 phage antibody library consisted of 2×10^6 clones.

Recombinant rate of MG_7 recombinant phage antibody library

Eight of 11 random clones were found to release a 2.1 kb DNA fragment and confirmed to be recombinant phagmid by restriction analysis and gel electophoresis (Figure 2). The recombinant rate was 72.7%.

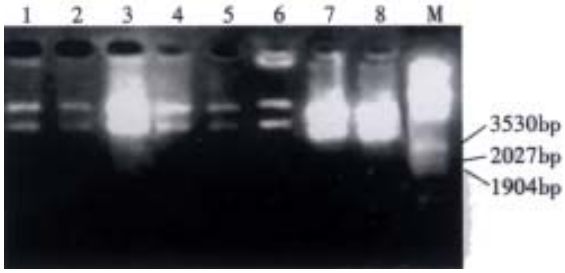

Figure 2 Enzymatic analysis of MG_7 recombinant phage antibody library with *Eco* RI and *Hin* dIII. 1-8: Recombinant clones from library; M: λ / *Eco* RI and *Hin* d III

Screening of MG_7 positive recombinant phages

Using ELISA assay, we yielded six strains of positive clones which had a good reaction with KATOIII cells (Table 1).

Table 1 ELISA results of screening from enriched phage displayed antibody library

Screening	Number of positive clones (A- value)						Neg. ctrl
	1	2	3	4	5	6	
First round	0.495	0.508	0.488	0.805	0.845	0.580	0.157
Second round	0.543	0.606	0.560	0.840	0.796	0.758	0.185

Results of competitive ELISA

Two strong positive clones were found to inhibit the binding of MG_7 antibody and KATOIII cells with the inhibiting ratio of 26.1% and 30%, respectively.

Antigen-binding affinity of soluble MG_7 ScFv

By means of ELISA assay, one of the strong positive clones exhibited the capacity of binding with KATOIII cells (Table 2).

Table 2 ELISA results of the soluble MG_7 ScFv for binding with KATOIII cells

ELISA	Number of strong positive clones (*A* value)		Neg. ctrl
	1	2	
First round	0.776	0.287	0.201
Second round	0.802	0.346	0.223

The relative molecular weight of soluble MG_7 ScFv

From Figure 3, an extra band on the lane of sample was visualized at M_r 32, as compared with the negative control. The relative molecular weight of soluble MG_7 ScFv was 31.

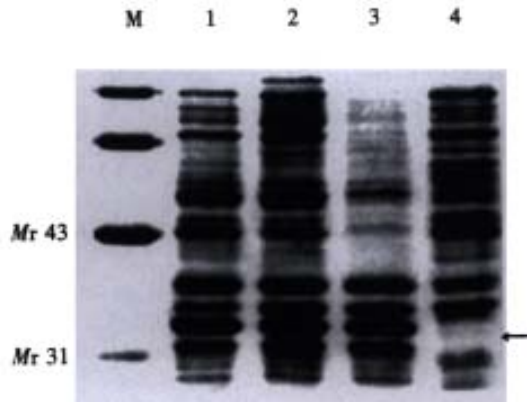

Figure 3 Measurement of the relative molecular weight of soluble MG_7 ScFv. 1-3: Periplasmic extracts; 4: Neg. ctrl; M: Low molecular mass protein marker

DISCUSSION

The phage antibody technique is one of the most remarkable achievements in antibody technology. With this technique, the repertoire of V_H and V_L genes are amplified and joined together by PCR and finally inserted into phagmid[20]. After transformation into *E.coli*, phage with the fusion of exogenous ScFv and pIII protein exposed on the surface was released from *E.coli* with the aid of the helper phage M13KO7. This technique uniforms the phenotype of ScFv to its genotype. By immunosorbance of the immobilized antigen, phage with functional ScFv can be bound and enriched. Subsequently, the desired phage harboring functional ScFv gene can be selected from the enriched phage antibody library by ELISA. The resultant ScFv can be solubly expressed in *E. coli* HB2151. The primary structural information of ScFv of antibody is accessible by DNA sequence of phagmid from the bound phage. Therefore, phage antibody has become an optimal measure to develop the ScFv of desired antibody[21-26].

It is well known that the immune system will be triggered and activated in response to the presence of certain antigens in patients with some kinds of diseases, such as tumor, infective diseases and autoimmune diseases[27-32]. The immune system will produce abundant B lymphocyte clones which can yield and secrete antibody directed against the disease associated antigens in these patients. Therefore, the B lymphocyte

population isolated from PBMC of these patients can be used as an ideal material source for construction of the recombinant phage antibody library[29-31]. Additionally, the B lymphocyte isolated from PBMC of immunized animals with given antigen is an alternative material source[20]. Besides the B lymphocyte population from patients or immunized animals, many kinds of antibody-producing hybridomas are also suitable as a kind of material source for construction of the recombinant phage antibody library[6,19]. Owing to the unraveling of biological functions over many antigen recognized by antibody from hybridomas, hybridomas are more favorable as material source for construction of recombinant phage antibody library.

In order to understand the quality of MG_7 hybridoma as a material source for construction of MG_7 recombinant phage antibody library, we detected the antigen-binding activity of MG_7 antibody in present study. ELISA assay showed that the *A* 492nm value of reactant with presence of MG_7 antibody was 0.65 which was over twice higher than that with absence of MG_7 antibody (0.208 only). It demonstrated that MG_7 hybridoma could secrete functional antibody and could be used as the source of mRNA to amplify the V_H and V_L genes of MG_7 antibody. Colony count and restriction analysis were conducted for evaluating the volume and quality of MG_7 recombinant antibody library. The large volume of MG_7 recombinant antibody library (2×10^6) and high recombinant rate (72.7%) confirmed that MG_7 phage antibody library comprised sufficient repertoire of recombinant clones for further research. ELISA assay and SDS-PAGE showed that the soluble MG_7 ScFv had antigen-binding activity and was M_r 31. Taken together, we have successfully constructed the MG_7 recombinant phage antibody library and prepared the phage-displayed/soluble MG_7 ScFv.

Gastric cancer is a highly prevalent neoplasm and is the first killer among various malignancies. In advanced cases, many current therapeutic approaches, including surgery combined with chemotherapy, appear to be palliative. These therapeutic approaches can not be targeted to and completely annihilate individual tumor cells, which leads to the failure of preventing metastasis and recurrence of many tumors. Besides, some kinds of therapeutic approaches, such as chemotherapy, can cause damage to both the tumor cells and normal tissue cells. Thus, introduction of a new way of targeting therapy for tumor is desperately needed to overcome these obstacles with the conventional approaches, such as surgery and chemotherapy[33-44]. Targeting therapy for tumors in the last decade has become a highlight in the field of tumor therapy. This therapy mediated by antibody still remains as a promising curative modality among the ways of tumor therapy and attracts worldwide attention[45-50].

Developing ScFv of the MG_7 is of great significance in both early diagnosis and treatment of gastric cancer. For instance, MG_7 ScFv fused with avidin can be used as a reagent in immuno-PCR for early diagnosis of gastric cancer. Additionally, a new immunotoxin with curative effect on gastric cancer can be developed by fusing the MG_7 ScFv and A subunit of ricin. MG_7 ScFv can direct the A subunit of ricin to MG_7 positive gastric cancer cells. Thus, the construction of MG_7 phage antibody library and subsequent preparation of MG_7 ScFv may be a step forward in seeking an efficient way for targeting therapy for gastric cancer.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Expression, deleton and mutation of *p*16 gene in human gastric cancer

Xiu-Sheng He[1], Qi Su[1], Zhu-Chu Chen[2], Xiu-Tao He[2], Zhi-Feng Long[1], Hui Ling[1] and Liang-Run Zhang[1]

[1]Oncology Institute, Nanhua University, Hengyang 421001, Hunan Province, China
[2]Oncology Institute, Center South University, Changsha 410078, Hunan Province, China
[3]Department of Gastroenterology, First People's Hospital of Changde City, Changde 415003, Hunan Province, China
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Correspondence to Dr. Xiu-Sheng He, The Oncology Institute of Center South University, Changsha 410078, Hunan Province, China. hxs59@hotmail.com
Tel: 0086-731-4805447, Fax: 0086-731-4485482
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Abstract

AIM **To investigate the relationship between the expression of *p*16 gene and the gastric carcinogenesis, depth of invasion and lymph node metastases, and to evaluate the deletion and mutation of exon** 2 **in *p*16 gene in gastric carcinoma.**

METHODS **The expression of P**16 **protein was examined by streptavidin-peroxidase conjugated method** (**S-P**)**; the deletion and mutation of p16 gene were respectively examined by polymerase chain reaction** (**PCR**) **and polymerase chain reaction single strand conformation polymorphism analysis** (**PCR-SSCP**) **in gastric carcinoma.**

RESULTS **Expression of P**16 **protein was detected in** 96.25**%** (77/80) **of the normal gastric mucosa, in** 92.00**%** (45/50) **of the dysplastic gastric mucosa and in** 47.54**%** (58/122) **of the gastric carcinoma. The positive rate of P**16 **protein expression in gastric carcinoma was significantly lower than that in normal gastric mucosa and dysplastic gastric mucosa** (***P***<0.05)**. The positive rate of P**16 **protein expression in mucoid carcinoma** 10.00**%** (1/10) **was significantly lower than that in poorly differentiated carcinoma** 51.22**%** (21/41)**, undifferentiated carcinoma** 57.69**%** (15/26) **and signet ring cell carcinoma** 62.50**%** (10/16) (***P***<0.05)**. The positive rate of p16 protein in 30 cases paired primary and lymph node metastatic gastric carcinoma: There was** 46.67**%** (14/30) **in primary gastric carcinoma,** 16.67**%** (5/30) **in lymph node metastatic gastric carcinoma. The positive rate of lymph node metastatic carcinoma was significantly lower than that of primary carcinoma** (***P***<0.05)**. There was of *p*16 gene mutation in exon** 2**, but** 5 **cases displayed deletion of *p*16 gene in exon** 2 **in the** 25 **primary gastric carcinomas.**

CONCLUSIONS **The expression loss of P**16 **protein related to the gastric carcinogenesis, gastric carcinoma histopathological subtypes and lymph merastasis. The mutation of *p*16 gene in exon** 2 **may not be involved in gastric carcinogenesis. But the deletion of *p*16 gene in exon** 2 **may be involved in gastric carcinogenesis.**

Subject headings gastric carcinoma; dysplasis p16/MTS1/CDK4I/CDKN2 gene; mutation deletion; expression; stomach neoplasms; genetics genes

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INTRODUCTION

Carcinogenesis and progression of human gastric cancer are related to the activation of proto-oncogenes and/or the inactivation of anti-oncogenes and they are the results of genetic alteration accumulated. A recently cloned new tumor suppressor *p*16 gene is located in 9p21, with the full-length of 8.5 kb. It consists of 2 introns and 3 exons, encoding P16 protein-whose molecular mass is 15840 M_r, a single strain peptide comprising 148 amino acid, participating in regulating the proliferation of normal cell growth negatively[1,2]. There was a high frequent loss of homozygosis of *p*16 gene in a variety of cancer cell lines such as gliocytoma, melanoma, breast cancer cell lines[2] and in certain primary cancer, for example, leukemia[3], gliomas[4], astrocytomas[5], bladder cancer[6], melanoma[7], oral squamous cell carcinomas[8], squamous cell carcinoma of head and neck neoplasm[9,10]. The frequency of *p*16 gene deletion and mutation is up to 75% in all kinds of human neoplasm, higher than that of the well-known p53 gene. Gastric cancer is common in China[11-30]. In this paper, S-P immunohistochemical staining was used to detect the expression of P16 protein in gastric cancer and precancerous lesions. PCR and PCR-SSCP methods were used to analyse the deletion and mutation of *p*16 gene exon 2. This study aims to evaluate the relationship between P16 protein and the carcinogenesis, progression, histological types as well as biologic behaviors in human gastric cancer, to find a new marker in early diagnosis and to discover the role of deletion and mutation of *p*16 gene in exon 2 in the carcinogenesis and progression of human gastric cancer.

MATERIAL AND METHODS

Specimens and treatment

All specimens were confirmed by pathology. Paraffin-embedded tissue were collected from the department of pathology and fresh resected specimens were from the First Affiliated Hospital of the Nanhua University, among which there were 50 cases of dysplasia of gastric mucosa and 122 cases of gastric cancer (25 cases were resected freshly from September 1995 to December 1996). In the 122 cases of gastric cancer, 29 were well-differentiated adenocarcinoma, 41 were poorly-differentiated adenocarcinoma, 26 were undifferentiated carcinoma, 16 were signet ring cell carcinoma and the other 10 were mucoid carcinoma. There were 81 men

and 41 women, 22 aged below 40 years, 69 aged from 41 to 59 years, and 31 were older than 60 years. The youngest was 15 years and the oldest 79 years (mean 56 years). Superficial muscles, were invated in 50 cases and deep muscles and the full layer in 72. Sixty-nine cases had lymph node metastasis, 53 had no lymph node metastasis. Thirty cases primary and lymph node metastasis cancer selected randomly were paired and compared. According to Borrmann's classification, 15 were type I, 43 were type II, 47 were type III and 17 were type IV. The 25 cases of fresh resected specimens included cancer, cancer-surroundings and normal mucosa selected far from cancer, were cut into 2 - 4 blocks under sterile conditions. Each block was 2-3 mm^3 and stored in -70℃ refrigerator for PCR and PCR-SSCP analysis. The rest tissues were fixed in 100 $mL \cdot L^{-1}$ neutral formalin, resected, dehydrated, cleaned and paraffin-embedded. All paraffin-embedded tissues were cut into sequential slices for 5μm and adhered to the glass which was processed by poly-lys previously.

Reagents and instruments

Rabbit-anti-human P16 protein multiple clonal antibody, streptavidin-peroxidase immunozator kit (S-P kit), and DAB were all bought from Maxim Company, USA. Protase K (Merk, USA), *Sma* I, agar gel, propylene acrylamide, N-N-sulmethyl bipropylene acrylamide, ammonium persulfate, xylene nitrile, bromophenol blue were bought from Shanghai Sangon Company. PCR primer synthesized by Shanghai Sangon, primer sequences of p16 gene exon 2[4].

Sense: 5'-TCT GAC CAT TCT GTT CTC TC-3'

Antisense: 5'-CTC AGC TTT GGA AGC TCT CA-3'

The fragment length of amplification was 384 bp. Primer sequences of β-active served as an internal control.

Sense: 5'-GCG GGG CGC CCC AGG CAC CA-3'

antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

The fragment length of amplification was 548bp.

Instrument Ultra low refrigerator (Japan) of -70℃, rotary sector (Germany), microscope (Japan), type 480 DNA amplificatory (PE,USA), type 901 ultraviolet spectrophometer (PE,USA), type DY-IIIB vertical eletrophores and all kinds of centrifuges(Beijing Liuyi).

METHODS

S-P immunohistochemical staining

Operated as the specification of sp kit, that was: paraffin-embedded tissue slices deparaffined hydrated→endogenous peroxidase blocked→added first antibody→then bridge antibody→added enzyme labeled S-P reagents→DAB colorized →hematoxylin stained→dehydrated→cleaned and paraffin-embedded→observed by microscope.

Genomic DNA extraction[31]

Frozen tissue of 0.5g was put into liquid nitrogen and powdered immediately, 10× buffer (10$mmol \cdot L^{-1}$ Tris-HCl pH 8.0, 0.1$mol \cdot L^{-1}$ EDTA pH 8.0, 5$g \cdot L^{-1}$ SDS) was added and span in 37℃ water for 1h at the same time, added protase K to the mixture at a final concentration of 100$mg \cdot L^{-1}$ in 50℃ water for 3h and readjusted the protase K as possible reaction. After the mixture lysed completely, 20$mg \cdot L^{-1}$ Rnase reacted in 37℃ water for 1h, saturated phony was put together and bugged slightly for 10 min, centrifuged and extracted up clean liquid transfer to a cleaned plastic tube, saturated phony processed repeatedly 3 times, added 1/10 volume 3$mol \cdot L^{-1}$ NaAc and 2-2.5 times cold ethyl, DNA precipitated by centrifugation, removed ethyl, DNA washed by 700$mL \cdot L^{-1}$ ethyl land centrifuged 3 times, dried, resolved with TE, A260/A280:1.8-1.9, stored at 0℃ for use.

PCR amplification

PCR was performed according to the reference[31] in 50μL reactive volume containing 0.1μg gDNA template, 200$\mu mol \cdot L^{-1}$ each of dCTP, DATP, dGTP, dTTP, 0.25$\mu mol \cdot L^{-1}$ primer, PCR buffer (Tris-HCl 10$mmol \cdot L^{-1}$, pH 8.3, $MgCl_2$ 1.5$\mu mol \cdot L^{-1}$, KCl 50$mmol \cdot L^{-1}$, gelatin 100$mg \cdot L^{-1}$) pre-denatured at 95℃ for 5 min and added 1.5μL of Taq DNA polymerase, 75μL of mineral oil. These samples were subjected to 30 cycles, including: 95℃ 1 min, 60℃ 1 min, 72℃ 1 min, and extended at 72℃ 5 min. Five μL of PCR product and appropriate bromophenol blue was added to the sample point container and electrophoresed at 20$g \cdot L^{-1}$ agarose gel containing 0.5$mg \cdot L^{-1}$ ethidium bromide at tank with 0.5×TBE liquid of electrophoresis, then observed and photographed with ultraviolet radiography.

PCR-SSCP analysis [31]

Five μL digested PCR product mixed with 5μL denatured dissolution (950$mL \cdot L^{-1}$ forman mide, 20$mmol \cdot L^{-1}$ EDTA, 0.05% bromophenol blue, 0.5$g \cdot L^{-1}$ xylene nitrile) denatured at 95℃ 5 min and colded on ice. Solution processed as above was added to the gel containing 80$g \cdot L^{-1}$ polypropylene acrylamide, vertical electrophoresed at 100 V for 4h and gel stained with silver: fixed in 100$mL \cdot L^{-1}$ alcohol for 10 min→oxidized in 100$g \cdot L^{-1}$ nitric acid for 3 min→drip washed for 1 min with double distilled water→stained in 12$mmol \cdot L^{-1}$ silver nitric acid for 20 min→drip washed for 1 min with double distilled water→showed appropriate color in 0.028 mol anhydrous sodium carbonate and 0.19$mL \cdot L^{-1}$ formalin→ended reducing response by 100$mL \cdot L^{-1}$ glacial acetic acid→drip washed with double distilled water→analysis results and photographed. P16 protein expression of confirmed positively cervix carcinoma served as positive control. PBS substituted with first antibody served as negative control.

Immunohistochemical determination

According to Gevadts' standard modefied slightly[32,33], nuclear or plasma stained brown-yellow as positive, (-) indicated no cell stained positive or only plasma stained or the number of nuclear stained positive less than 1 cell, (+) indicated the cells stained weakly or the number of stained cells less than 25%, (++) indicated the cells stained moderately or the stained cells covering about 26%-50%, (+++) indicated cells stained strongly or the number of stained cell more than 50%. The number of nuclear stained positively more than 2 cells per high time sight was considered to be positive. No folding, and no edging-effect fields were chosen to calculate 100 cells per 5 sights and evaluate the average number of positive cells. Positive cells discerned by two

researchers alone and decided on the disagreements together. No products of PCR amplification were loss of homozygosis of *p*16 gene, and abnormal traces found in PCR-SSCP were considered gene mutation.

Statistical analysis

Chiq-square test was used *P* value less than 0.05 was considered to be statistically significant.

RESULTS

P16 protein expression in gastric cancer

The positive rate of P16 protein expression in 80 cases of normal gastric mucosa was 96% (Figure 1), and in 50 cases of dysplasia mucosa was 90% (Figure 2). In these mucosa P16 protein expression could only be seen in partical adenoepithelial cells. We did not find staining in mucosal epithelial cells, matrix fibrocytes, lymphocytes and smooth myocytes. But in gastric cancer, the ratio was 48% (Figure 3). The positive rate of P16 protein expression in gastric cancer was lower than that in normal and dysplasia mucosa ($P<0.05$). There was no significant difference between the normal gastric mucosa and dysplasia mucosa ($P>0.05$, Table 1).

In the 122 gastric cancer, the positive rate of P16 protein expression was 38%, 51%, 58%, 62% and 10% in well-differentiated adenocarcinoma, poorly-differentiated adenocarcinoma, undifferentiated carcinoma, signet ring cell carcinoma and mucoid carcinoma, respectively. The P16 protein expression in mucoid carcinoma was lower than that in signet ring cell carcinoma, undifferentiated carcinoma and poorly-differentiated adenocarcinoma ($P<0.05$). The positive rate of P16 protein expression was 48% (24/50) in gastric cancer invaded superficial muscle layer and 47% (34/72) in gastric cancer invaded deep muscle and full layer. There was no apparent relevance between P16 protein expression and the depth of invasion ($P>0.05$). In 30 cases of paired primary cancer and lymph node metastasis cancer, the rate of P16 protein expression of the lymph node metastasis cancer was 17%(5/30), significantly lower than that of primary cancer, 47% (14/30), ($P<0.05$).

Deletion and mutation of p16 gene exon 2 in gastric cancer

In 25 fresh resected gastric cancer, there were 7 well-differentiated adenocarcinoma, 13 poorly-differentiated adenocarcinoma, 3 undif ferentiated carcinoma, 1 signet ring cell carcinoma and 1 mucoid carcinoma, cancer-arrounding and normal mucosa were taken at the same time. The PCR amplification showed no product in 1 case of well-differentiated adenocarcinoma, 1 case of poorly-differentiated adenocarcinoma and 1 case of mucoid carcinoma; little product found in 1 case of well-differentiated adenocarcinoma and 1 case of poorly-differentiated adenocarcinoma. There were products of PCR amplification in the rest 20 cases of gastric cancer, tumor adjacent tissue and normal mucosa. All experiments were repeated three times. The result was identical. No product of PCR amplification might indicate the loss of homozygosis of *p*16 gene, little product of PCR amplification was possibly loss of heterozygosis of *p*16 gene or loss of homozygosis of *p*16 gene, but contaminated with normal mucosa (Figure 4). Four of these 5 cases were P16 protein negative expression and 1 case expressed weekly by immunohistochemical staining. No gene mutation was observed in PCR-SSCP analysis after the PCR amplification products cut with *Sam* I (Figure 5, Table 1) (the location of restriction site, and the length of fragment are shown in Figure 6).

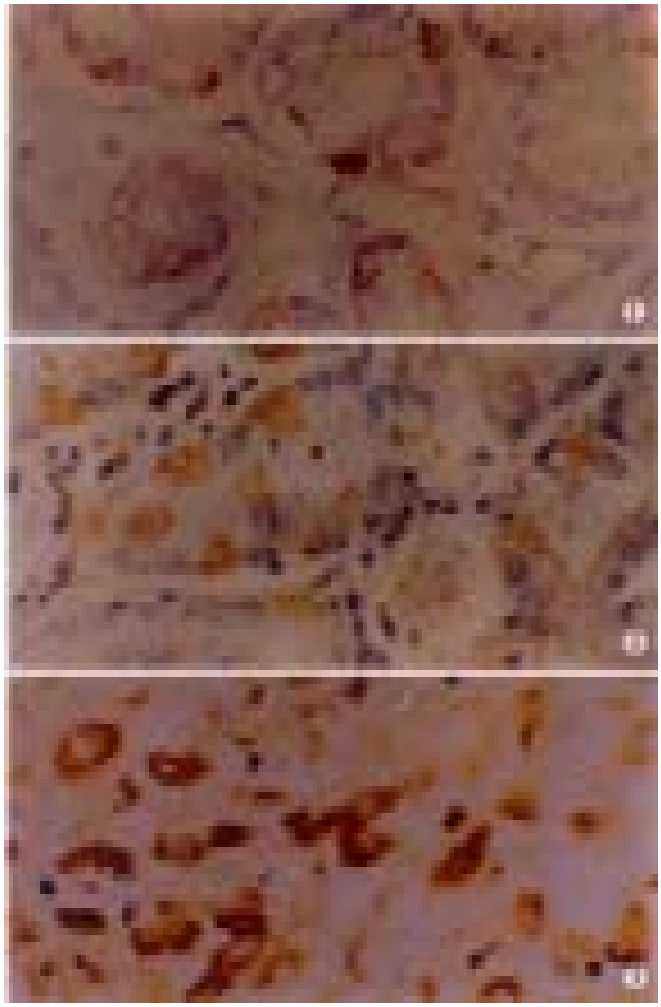

Figure 1 *p*16 protein expression in normal gastric mucosa. ×400
Figure 2 *p*16 protein expression in dysplastic gastric mucosa. ×400
Figure 3 *p*16 protein expression in gastric carcinoma. Immunohistochemic stain. Arrow shows the undifferentiated carcinoma positive cell. ×400

Table 1 P16 protein expression, p16 gene mutation and deletion gastric cancer

Hisological types	*n*	P16 protein				*p*16 gene		
		-	+	++	+++	Positive rate (%)	Mutation	Deletion
A. Normal gastric mucosa	80	3	41	20	16	96	0/25	0/25
B. Dysplasia gastric mucosa	50	5	12	19	14	92	0/25	0/25
C. Gastric cancer	122	64	13	20	25	48	0/25	5/25

A,B *vs* C, $P<0.05$. Mutation and deletion of p16 gene: B is tumor adjacent tissue.

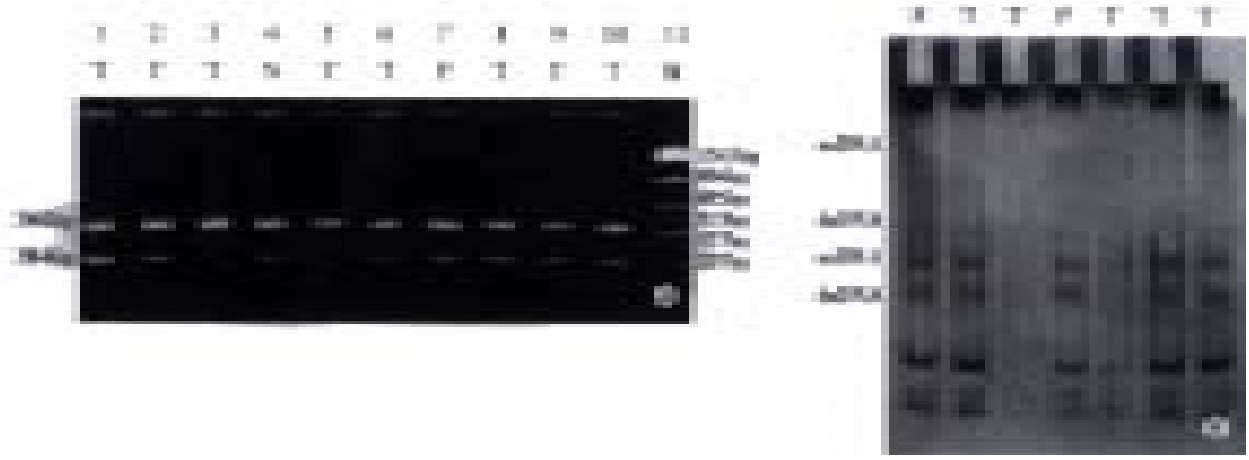

Figure 4 PCR amplification product in exon 2 of p16 gene.
Lines 1-4,5-6,8-10 (T: gastric cancer); Line 7 (P: tumor adjacent tissue); Line 4 (N: normal gastric mucosa); Line 11 (M: marker) Little PCR product in line 3 and no PCR product in line 5.

Figure 5 The exon 2 of p16 gene analyzed by SSCP. segment a 135bp, segment b 249bp.
Lines 2,3,5,6,7 (T: gastric cancer); Line 4 (P: tumor adjacent tissue); Line 1 (N: normal gastric mucosa).
No electrophoresis band on line 3, weak electrophoresis band on line 5, and no abnormal electrophoresis band in all lines.

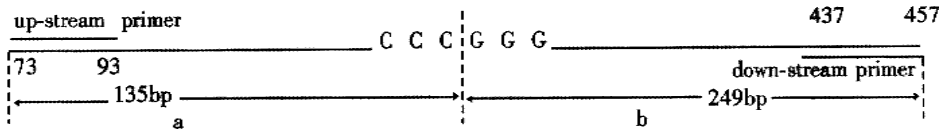

Figure 6 The length of PCR amplification of p16 gene exon 2 and the *Sam* I (CCCGGG) restriction site.

DISCUSSION

The *p*16 gene is a tumor suppressor gene that participates in the negative regulation of the cell growth and proliferation, and a hot spot in the molecular biological research of neoplasm since its discovery in 1993. The product of p16 gene-P16 protein is the inhibitor of CDK4. Its function is to default the activity of CDK4 by binding with CDK4 against Cyclin D_1 competitively, then inhibits the phosphation of Rb protein, transcription factor such as E2F when combined with the dephosphated Rb protein can not be released and activated, thus cells arrest in G_0/G_1 phase, resulting in cell dividing and proliferation suppressed. If p16 gene was abnormal, its function of negative regulation of cell growth would be lost. CDK4 combines to cyclin D_1 and PRb phosphates, lots of transcription factors would be released. The cell from G_1 phase enters into S phase rapidly, cell proliferates excessively and results in carcinogenesis and progression[1,2,34-36]. A lot of investigations show that there were P16 protein deletion and *p*16 gene abnormality in various primary cancers and cancer cell lines. The alteration forms of *p*16 gene were deletion, mutation[2,9,37-43], rearrangement, insertion[44-48], translocation[49-51]and hyper methylation of CpG islands presented in promotor sequence[52-58]. Such alterations consequently change the gene activity, cause abnormality, structure change of the product of *p*16 gene expression and the loss of its physiological functions. Recently, it has been reported that the exoteric *p*16 gene was transfected into the cancer cell which *p*16 gene deletes. The cancer cell restored *p*16 gene expression and cell growth was remarkably inhibited[59-67]. It has also been documented that the CpG island methylated of *p*16 gene cancer cell line was treated with 5-aza-2'-deoxycytidin. Cancer cell restored *p*16 gene expression and showed growth inhibition[68-71]. All these indicated that *p*16 gene and its product played important roles in the carcinogenesis.

This investigation showed that the positive rate of P16 protein expression in gastric cancer was remarkably lower than that in dysplasia and that in normal gastric mucosa ($P<0.05$). The result indicated that gastric carcinogenesis was probably related to the loss of P16 protein expression. But there was no significant difference between the normal mucosa and the dysplasia mucosa of the stomach ($P>0.05$). The positive rate of P16 protein expression in gastric cancer was not identical with other reports[72£¬73]. The cause was not clear. It was possibly related to the different standards of determination, reagents and some uncertain factors. However, the quantity of P16 protein expression increased from normal mucosa to precancerous lesions and gastric cancer ($P<0.05$). Following pathological lesions, P16 protein expression increased. This change may inhibit cell proliferation. The positive rate of P16 protein expression in mucoid carcinoma was significantly lower than in poorly-differentiated adenocarcinoma, undifferentiated carcinoma and signet cell carcinoma ($P<0.05$). The result suggested that the alteration of *p*16 gene was different in various histological types gastric cancer. The discrepancy of P16 protein expression exists in various histological types of lung and esophageal cancer[33,74]. There was difference of P16 protein expression and deletion of *p*16 gene in various differentiation types of gliomas. But the deletion of *p*16 gene concurred with the expression of P16 protein[4]. In 30 cases of primary gastric cancer paired with lymph node metastasis cancer, the positive expression rate of P16 protein in metastasis cancer was lower than in primary gastric cancer

($P<0.05$), which was in agreement with the reported results[75]. The result convincingly suggested that the P16 protein deletion might be related to gastric cancer metastasis and indicate P16 protein expression heterogentity in gastric cancer[76]. What was more intriguing that 2 neighboring lymph nodes metastasis cancer migrated from primary cancer had positive expression P16 protein. Expression of P16 protein is not only related to neoplasms metastasis but also related to prognosis and progression. Expression of P16 protein is low, clinical prognosis is bad[77-81]. We also investigated the relevance between various factors such as age, sex, the depth of invasion and Borrman classification and P16 protein expression in gastric cancer. There was no significant difference ($P>0.05$). The positive expression of P16 protein could merely be observed in partial adeno-epithelial cells of normal and dysplasia gastric mucosa, and weakly positive expression or undetectable in gastric mucosa epithelium cells, interstitial lymphocytes, fibroblasts and smooth muscle cells, which is contrary to some published files[82]. Nevertheless, others confer that the undetectability of P16 protein expression in neoplasm interstice[32], normal lung tissue[83] and normal urinoepithelial cells[82] might attribute to a paucity of P16 molecule in G_0/G_1[84] phase cells or short halftime of P16 protein[85].

Among some human neoplasms, *p*16 gene alterations always resided in exon 2[5]. There was no product of PCR amplification in 3 of 25 cases possibly due to the loss of homozygosis. Little product of PCR in 2 of 25 cases amplification might be the loss of heterozygosis or loss of homozygosis but normal mucosa contaminated. In the 5 cases, the expression of P16 protein was negative in 4 cases and weekly positive in one. The results manifested that 4 cases might be the loss of homozygosis and 1 case might be the loss of heterozygosis among the 5 cases of gastric cancer and the deletion of *p*16 gene is possibly related to the carcinogenesis and progression of gastric carcinoma. The rate of deletion in this study was slightly lower than that reported by others[74]. It was likely that only exon 2 was examined or inadequate for specimens or other unknown factors. Nevertheless, PCR amplification products were found in the rest 20 cases of gastric cancer, normal gastric mucosa and cancer-surrounding mucosa. No abnormal PAGE band and mutation of *p*16 gene was found by SSCP analysis digestion product of PCR amplification. We suggested that *p*16 gene was not involved in the carcinogenesis of gastric cancer, which coincided with other authors[86,87]. We also found that the frequency of *p*16 gene deletion was lower than that of deletion P16 protein expression. P16 protein was undetectable in normal and dysplasia gastric mucosa epithelial cells but in partial adenoepithelium. Some other uncertain mechanisms might exist in the regulation of *p*16 gene and the expression level of P16 protein[88-90], which require further studies.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Telomere erosion is independent of microsatellite instability but related to loss of heterozygosity in gastric cancer

Dian-Chun Fang, Shi-Ming Yang, Xiao-Dong Zhou, Dong-Xu Wang and Yuan-Hui Luo

Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
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Correspondence to Dian-Chun Fang, M. D., ph. D., Southwest Hospital Third Military Medical University, Chongqing 400038, China. Tel: 0086-23-68754624 or 68754124, Fax: 0086-23-65306499
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Abstract

AIM **To correlate the length of the telomere to microsatellite instability (MSI) and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.**

METHODS **Telomeric restriction fragment (TRF) length of gastric cancer was measured with Southern blot. LOH of APC, MCC and DCC genes, microsatellite instability (MSI) and frameshift mutation of hMSH6, TGF-βRII and BAX genes were analyzed by PCR-based methods.**

RESULTS **Sixty-eight cases of sporadic gastric carcinoma were studied for MSI using five microsatellite markers. MSI in at least one locus was detected in** 17 (25%) **of** 68 **tumors analyzed. Frameshift mutations of hMSH6, TGF-βRII and BAX were detected in** 2,6 **and 3 of gastric carcinomas respectively showing high MSI (**≥2 **loci, *n* =** 8**), but none was found in those showing low MSI (only one locus, *n*=**9**) or MSS (tumor lacking MSI or stable, *n*=**51**). Thirty-five cases, including all high MSI and low MSI, were studied for TRF. The mean TRF length was not correlated with clinicopathological parameters. No association was observed between TRF length and MSI or frameshift mutation. On the contrary, LOH at the DCC locus was related to telomere shortening (*P*<**0.01**). This tendency was also observed in APC and MCC genes, although there was no statistical significance.**

CONCLUSION **The development of gastric cancer can arise through two different genetic pathways. In high MSI gastric cancers, defective mismatch repair allows mutations to accumulate and generate the high MSI phenotype. In gastric cancers showing either low MSI or MSS, multiple deletions may represent the LOH pathway. Telomere erosion is independent of high MSI phenotype but related to the LOH pathway in gastric cancer.**

Subject headings gastric cancer; telomere restriction fragment; microsatellite instability; loss, heterozygosity

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INTRODUCTION

Eukaryotic linear chromosomes are capped by a special structure known as the telomere. In vertebrates, telomeres consist of several kilobases of tandem TTAGGG repeats bound by the related telomere-specific proteins, TRF1 and TRF2[1,2]. TRF1 regulates telomere length[3] and TRF2 maintains telomere integrity[4]. In most human cells, telomeres shorten with each cell division due to the incomplete replication of linear DNA molecules and the absence of telomere-elongating mechanisms[5]. It has been reported that telomeric repeats are often reduced in various human tumors as a consequence of many cell divisions of tumor cells, and the loss of telomeric repeats would cause additional senetic changes as a consequence of chromosomal instability.

The presence of telomeric DNA at the chromosomal termini is essential for genetic stability. Genetic instability may be classified into two different forms in which hypermutability occurs either by means of chromosomal instability or microsatellite instability (MSI)[6-10]. There is now increasing evidence that telomere shortening is involved in chromosomal instability. Indeed telomeres are thought to maintain the chromosome integrity during the cell cycle by allowing a proper segregation during cell division. Broken chromosomes that are uncapped become hotspots for different types of recombination. They may therefore undergo aberrant recombination, end-to-end fusions and bridge-fusion-breakage mechanisms. Being unprotected, the chromosomes are also susceptible to exonucleolytic degradation[11]. Telomeres would also prevent the activation of DNA-damage check-points[12]. Thus, without these essential structures, the chromosome is unstable.

MSI represents an important new form of genetic alteration characterized by widespread instability in repetitive nucleotide sequences. MSI has been found in the majority of tumors associated with hereditary non-polyposis colorectal cancer (HNPCC)[13,14] in which germ-line mutation occurs within the mismatch repair genes hMSH2, hMLH1, hPMS2 or hMSH6[15-17]. Mutations of the transforming growth factor type II receptor gene (TGF-βR II), and BAX gene are strongly correlated with MSI[18-20]. MSI is also a distinctive feature in about 10%-15% of sporadic colorectal tumors and to a varying degree in tumors of other organs, including the stomach[7,21-24]. Although alterations of telomerase activity have been reported in MSI and microsatellite stable (MSS) tumors[25,26], less clear, however, the relevance of progressive telomere shortening as a potential factor in MSI is less clear. The aim of the present study is to correlate telomere status with MSI and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.

MATERIAL AND METHOD

Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma patients in our hospital. Each specimen was frozen immediately and

stored at -80℃ until analyzed. A 5μm section was cut from each tissue and stained with hematoxylin /eosin in order to ascertain whether the cancer cells in tissues were predominant or not. Genomic DNA was isolated by standard proteinase-K digestion and phenol-chloroform extraction protocols. Of the 68 patients with gastric cancer, 45 were men and 23 were women with an age range of 30-76 years (mean age of 56.2 years at diagnosis). None of the patients included in the present series had a family history suggestive of HNPCC and had received chemotherapy or radiation therapy.

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed in 15μL of reaction mixture containing 10mmol·L^{-1} Tris-HCl (pH 8.3), 50mmol·L^{-1} KCl, 1.5mmol·L^{-1} $MgCl_2$, 200μmol·L^{-1} each deoxynucleotide triphoshate, 0.5μmol·L^{-1} of each primer, 0.75unit Ampli Taq polymerase (Perkin-Elmer, Norwalk, CT, USA), and 100ng genomic DNA. The reaction was carried out in a thermal cycler at 94℃ for 1min, 55℃-62℃ for 1min, and 72℃ for 1min, for 35 cycles with an initial denaturation step of 94℃ for 5min and a final extension step of 72℃ for 10min. The PCR products were then separated on 5% polyacrylamide, 7M urea denaturing gel, and visualized by autoradiography. MSI was defined as the presence of band shift in the tumor DNA that was not present in the corresponding normal DNA. Based on the number of mutated MSI markers in each tumor, carcinomas were characterized as high MSI if they manifested instability at two or more markers, low MSI if unstable at only one marker, and MSS if they showed no instability at any markers (microsatellite stable)[27-29].

To detect frameshift mutations in coding regions, repetitive mononucleotide sequences, the (A)10 tract of TGF-βRII, the (G)8 tract of BAX and the (C)8 tract of mismatch repair gene MSH6 were amplified using published primers, respectively[30-32]. The reaction condition consisted of 35 cycles at 94℃ for 1min, 55℃ or 56℃ for 1min and 72℃ for 1min using 0.5μCi[33p] dATP into 15μL reaction mixture. DNA denaturation, electrophoresis and autoradiographic procedure were done likewise with MSI analysis, except for TGF-βRII where 8% denaturing gel was used.

Southern blot analysis to estimate telomeric restriction fragment (TRF) length was based on previously reported methods[33]. High molecular weight DNA was prepared from each sample, followed by extraction with phenol chloroform isoamyl alcohol and precipitation with 3M sodium acetate and ethanol. Genomic DNA was digested with *Hin* fI (Promega, USA)at 37℃ for 6 hours and then 10μg of digested DNA was subject to electrophoresis on 1% agarose gels. After electrophoresis, the gel was denatured in 0.5M NaOH and 1.5M NaCl for 30 minutes and neutralized in 0.5M Tris (pH 8.0) and 1.5M NaCl for 30 minutes. The DNA was then transferred onto a nitrocellulose membrane (Promega, USA) overnight. The membrane was dried at 80℃ for 1 hour and subsequently hybridized to α-^{32} P-ATP end- labeled $(TTAGGG)_4$ probe. The membranes were autoradiographed on X-ray films for 24 hours. Each lane was scanned with a densitometer and the data was used to determine the mean TRF length as previously described[34].

The APC, MCC and DCC genes were investigated for LOH and PCR was carried out as described[35,36]. The priming regions were located within specific tumor suppressor genes at sequence, either a restriction fragment length polymorphism (RFLP) or a variable mumber of tandem repeats-type polymorphism (VNTR)[37]. Annealing temperature, extension time and the number of amplification cycles were optimized for each primer set. After amplification, PCR products were digested with appropriate restriction enzymes (for RFLPs) or not digested (for VNTRs) and electrophoresed on 1.5% agarose gels or 8% polyacrylamide gels, which were stained with ethidium bromide and photographed under UV light. When the intensity of one allele in cancerous tissue was less than 50% of the other allele in comparison with the ratio of intensity of the allele in the corresponding normal tissue, it was judged as LOH.

One factor analysis of variance, and Chi-square test with Yates' correction were used. A P value <0.05 was considered significant.

RESUTS

Alterations of electrophoretic patterns of PCR products of five microsatellite markers, TGF-βRII, BAX, and MSH3 genes were compared between tumor and normal DNA in each patient (Figures 1,2). MSI affecting at least one locus was observed in 17 (25%) of 68 tumors, among which eight (11.8%) had high MSI and nine (13.2%) had low MIS. A comparison of MSI status with frameshift mutation is shown in Table 1. The (A)10 frameshift mutation in TGF-βRII gene was detected in 6 of 8 gastric cancers with high MSI, whereas none of the low MSI or MSS tumors showed such a mutation. The mutation in the BAX (G)8 repeat site and hMSH6 (C)8 tracts was detected in 3 of 8 and 2 of 8 gastric cancers with high MSI, respectively, and no mutation was found in tumors with low MSI and MSS.

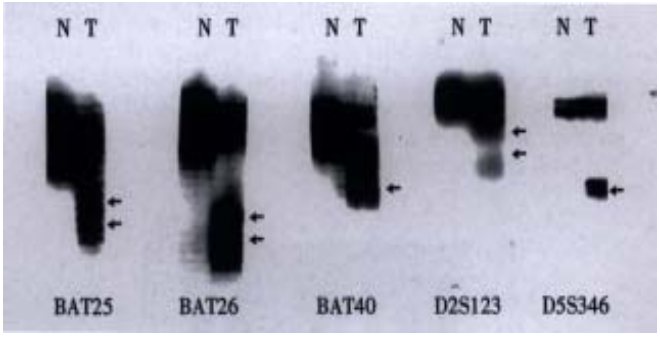

Figure 1 MSI in gastric cancer using 5 microsatellite loci (BAT-25, BAT-26, BAT40, D2S123, and D5S346). Arrows indicate variant conformers. (N: normal DNA pattern; T: tumor specimens containing variant conformers representing MIS)

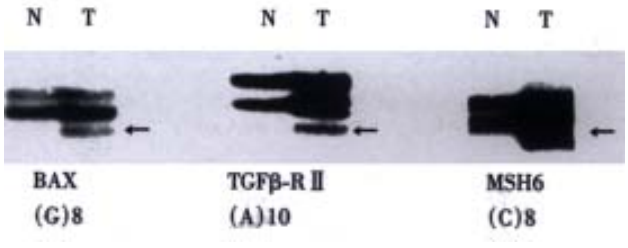

Figure 2 Frameshift mutations of hMSH6 TGF-beta RII, and BAX genes in gastric cancers. Arrows indicate conformational variants associated with frameshift mutations. (N: normal DNA; T: tumor DNA)

Table 1 Relationship between MSI status and frameshift mutation

MSI status	No. of cases	Frameshift mutations		
		MSH6	TGF β RII	BAX
MSI-H	8	2	6	3
MSI-L	9	0	0	0
MSS	51	0	0	0

Thirty-five gastric cancers, including all 8 with high MSI and 9 with low MSI, were examined for alteration in TRF length using the Southern blot technique. Since the signal of telomeric repeats is detected as a smear (Figure 3), we visually determined the peak of signal, which was confirmed using a densitometer. When the TRF length in the tumor was more than 20% shorter or longer than that in corresponding normal tissues, we classified the TRFs as shortened or elongated, as previously described[38]. Compared with the corresponding normal mucosa, 20 (57.1%) had considerable shortening (<80% of corresponding normal tissues), 12 (34.3%) had approximately the same length (between 80%-120%), and 3(8.6%) showed elongation (>120%). No correlation was found between mean TRF length and clinicopathological parameters in gastric carcinomas. However, a decrease of the mean TRF length with the age was observed except for the group aged from 50 to 59 years. The mean TRF length in the age group of 30 to 39 years was significantly longer than that in the group aged from 60 to 69 years or 70 to 79 years (P <0.05) (Table 2).

We compared the TRF length with MSI and frameshift mutation status. Table 3 shows the association of MSI and frameshift status to the TRF length of 35 gastric cancers. No relationship was observed between TRF length and MSI or frameshift mutation in gastric cancer.

Tissues from 35 patients were also studied for LOH of APC, MCC and DCC. In order to increase the assay sensitivity, three different sites, i.e., M2, M3 and VNTR were used for analysis of LOH at DCC genetic locus. LOH of APC, MCC and DCC were observed in 6 (27.3%) of 22, 7 (36.8%) of 19 and 18(56.3%) of 32 of informative cases, respectively (Figure 4). The relationship between LOH at various loci and the alteration in TRF length in primary tumors is shown in Table 4. LOH at the DCC locus were associated with telomere shortening (P<0.01). This tendency was also observed in APC and MCC genes, although there was no statistical significance.

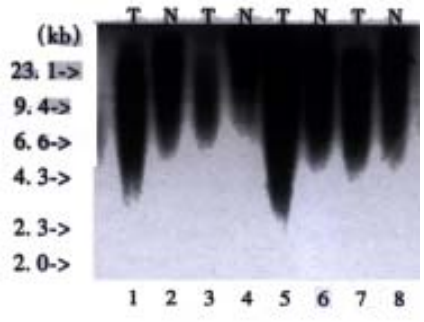

Figure 3 Southern blot analysis of telomere repeat arrays in DNA samples of patients with gastric cancer. DNA was digested with-*Hin* fI and hybridized with α-^{32} P APT end-labeled $(TTAGGG)_4$ probe.T =Tumour; N =Corresponding normal tissues.

Table 2 The relationship between the mean TRF length and clinicopathological pararmeters

Parameters	No. of cases	Mean TRF length ($\bar{x}$±s)
Gender		
Male	23	3.99±0.63
Female	12	4.32±1.00
Age(yrs)		
30-39	5	4.54±0.54
40-49	7	4.07±0.60
50-59	11	4.39±0.96
60-69	10	3.69±0.66[a]
70-79	2	3.40±0.07[a]
Size		
<5cm	12	3.80±1.20
>5cm	23	4.06±0.86
Differentiation		
Well and moderate	7	3.91±0.49
Poor	19	4.24±0.64
Mucinous	9	4.02±1.10
Clinical stage		
I and II	21	4.26±0.82
III and IV	14	3.84±0.68

[a]P<0.05 *vs* the age group of 30 to 39 years.

Table 3 Relationship between alterations of TRF length and MSI status or frameshift mutation

	No. of cases	Alterations in TRF length		
		Shortened (n=20)	Normal (n=12)	Elongated (n=3)
MSI status				
MSI-H	8	4	4	0
MSI-L	9	4	4	1
MSS	18	9	7	2
Frameshift mutation				
MSH3	35	1	1	0
TGF β RII	35	3	2	1
BAX	35	2	1	0

Table 4 Relationship between alterations of TRF length and loss of heterozygosity of APC, MCC and DCC genes

LOH	Alterations in TRF engh (LOH / Informative)		
	Shortened (n=20)	Normal (n=12)	Elongated (n=3)
APC	5 / 13	1 / 7	0 / 2
MCC	6 / 11	1 / 6	0 / 2
DCC	14 / 18[a]	3 / 11	1 / 3

[a]P<0.01.

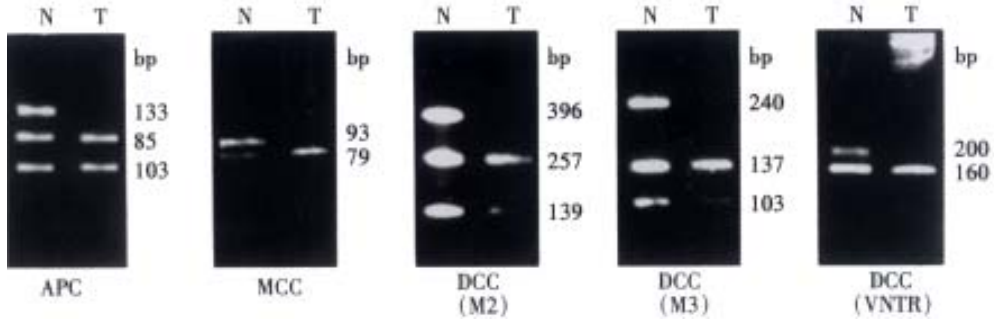

Figure 4 Representative LOH analysis of APC, MCC and DCC genes. (APC: Rsa1 RFLP in APC exon 11, loss of 133bp allele is seen in tumor DNA, MCC: A 14bp insertion / deletion polymorphism in MCC exon 10 gives rise to a 93 or 79 allele. Loss of the 93bp allele is seen in the tumor. DCC: Losses of 396bp, 240bp and 200bp alleles are seen at M2, M3 and VNTR polymorphic sequences in the tumor DNA)

DISCUSSION

In this study, 17(25%) of 68 sporadic gastric cancers had MSI in at least one locus. This finding is similar to previous studies[23,39]. There is now evidence that MSI cancer comprises distinctive high MSI and low MSI categories[40,41]. High MSI cancers are distinguished clinicopathologically and in their spectrum of genetic alterations from cancers showing low MSI and MSS cancers[40,41]. Our previous studies indicated that high MSI gastric cancers often show lower frequency of LOH of APC, MCC and DCC genes than low MSI and MSS cancers[41]. In our series of 68 gastric cancers, 8 were classified as high MSI, 9 as low MSI and 51 as MSS. Frameshift mutations of hMSH6, TGF-β RII and BAX were detected in gastric carcinomas with high MSI, but no mutation of these genes was found in those showing low MSI or MSS. These results indicate that these genes are mutational targets in high MSI tumor cells and support the notion that high MIS tumors identify an alterative pathway of tumorigenesis that has been proposed by Vogelstein and co-workers[42].

In the present study, 35 gastric carcinomas were examined for TRF length using the Southern blot. Telomere shortening was detected in 57.1% of the gastric carcinomas, indicating that telomere erosion may play a role in the gastric carcinoma development. It has been reported that telomere shortening was associated with a short survival and disease recurrent in prostate cancer[43]. In the current study, subdivision of the tumors according to telomere length did not reveal an obvious relationship between the shortening of telomere and tumor size, depth of invasion, node metastasis or clinical stages, indicating a limited role of the telomere shortening in predicting prognosis of gastric carcinomas. This finding is in agreement with the recently published data on renal cell carcinoma and colorectal carcinomas[44,45].

TRF reduction was demonstrated in various human malignant tumors[38,45-47]. In contrast to these results, it was also found that telomeres in some tumors are similar to, even elongated, as compared with normal tissues[36,48]. In this study, the mean TRF length was reduced in 20 of 35 tumors, similar in 12 cases and elongated in 3 cases, as compared with the corresponding normal mucosa. The discrepancy in TRF length may be related for several reasons. For tumor samples with TRF of normal length, there are at least three possibilities: ① The majority of cells in such tumors have not experienced many cell divisions nor experienced critical shortening of telomeric repeats; ② In most tumor cells, telomerase already activated, elongated the once shortened telomeric repeats back to normal length, as observed in HPV-immortalized epithelial cells *in vitro*[49]; and ③ The amount of tumor cells also affects TRF analysis. Therefore, with the predominance of normal cells in tumor specimens, telomere loss may be underestimated[48]. We found that the TRF length in tumors was progressively reduced with age, suggesting that patients' age should be considered in evaluating the TRF lengths of tumor. Finally, end-to-end chromosome fusion observed in some tumors could lead to telomere elongation[50].

It has been reported that telomerase activity and microsatellite instability are independent events in colorectal carcinogenesis[25]. To our knowledge, this is the first report on the relationship between MSI and telomere length. We did not find any correlation between telomere erosion and MSI, suggesting that the MSI pathway is independent of telomere erosion.

It has been found that chromosomal instability is correlated with telomere erosion and inactivation of G2 checkpoint function in human fibroblasts[12]. Alterations in the TRF length were often associated with LOH of the p53 gene and Rb gene, but less often with mutation of k-ras and p53 genes in lung cancer[38]. We found that LOH at the DCC locus was associated with telomere shortening. Although not statistically significant, LOH at the APC and MCC genetic loci tends to occur in telomere shortened gastric cancers, indicating that telomere erosion may be involved in the LOH pathway.

In summary, our data indicate that telomere shortening may play a role in gastric carcinogenesis. There are at least two distinct genetic instabilities in gastric tumorigenesis: one is the chromosomal instability (or suppressor pathway) and the other is microsatellite instability (or MSI pathway). The former, may include tumors with low MIS as well as MSS and accumulation of loss of tumor suppressor genes such as p53, Rb, APC, MCC and DCC plays an important role in their carcinogenesis; whereas the latter consists of a small subset of gastric cancer with high MSI, defective repair of mismatched bases results in an increased mutation rate at the nucleotide level, and consequent widespread MSI. Telomere shortening may be involved in the LOH pathway but independent of the MSI pathway. Our analysis of TRFs should further provide some clues to the molecular mechanisms underlying the profound genomic instability in the MSI and LOH pathway for gastric carcinoma.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Extraction and purification of TGFβ and its effect on the induction of apoptosis of hepatocytes

Xiao-Hui Si[1] and Lian-Jun Yang[2]

[1]Research Institute of Stomatology, the Ninth People's Hospital, Shanghai Second Medical University, Shanghai 200011, China
[2]Department of Pathology, Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
Correspondence to Dr. Xiao-Hui Si, Research Institute of Stomatology, the Ninth People's Hospital, Shanghai Second Medical University, Shanghai 200011, China.
Tel: 0086-21-63135412
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Abstract

AIM **To extract and purify the transforming growth factor β (TGF β), and to demonstrate its biological activity *in vivo* and induction of apoptosis of hepatocytes *in vitro*.**

METHODS **TGF β was isolated from fresh bovine platelets by acid/ethanol extraction method and purified with ion exchange and gel chromatography. The extracted TGF β as injected subcutaneously to mice, and its biological activity *in vivo* was observed** 72 **hrs post-injection by HE staining. The morphological changes were observed by HE staining and the occurrence of apoptosis was detected by TUNEL method after the human normal hepatic cell line QZG was treated with** 8μg·L^{-1}**TGFβ for** 12 **hrs *in vitro*.**

RESULTS **The molecular mass** 25 **ku TGF β protein was successfully extracted. It was able to induce localized granulation tissue formation *in vivo*. TGF β-treated hepatocytes showed obvious apoptotic morphological changes, including the pyknosis and dense-stained nuclei and cytoplasm, the fragmentary, annular or crescent nuclei, and the "bubbling" cytoplasm. Moreover, its apoptotic rate was significantly higher than that of the control group** (***P***<0.05)**.**

CONCLUSION **Biological active TGF β protein is extracted and purified successfully from bovine platelets, and it is able to induce the apoptosis of hepatocytes.**

Subject headings transforming growth factor beta/ isolation & purification; transforming growth factor beta/ pharmacology; liver/cytology; apoptosis

Si XH, Yang LJ. Extraction and purification of TGFβ and its effect on the induction of apoptosis of hepatocytes. *World J Gastroenterol*, 2001;7(4):527-531

INTRODUCTION

Liver diseases are very common in China[1-10], and apoptosis is the research focus in recent years[11-20]. Transforming growth factor β (TGFβ) is a kind of polypeptide growth factors that is extensively present in most tissues and cells. A variety of cell types, both nonneoplastic and neoplastic, synthesize TGF β and most of these cells have specific high-affinity receptors for TGF β[21-24]. It is a multifunctional molecule which is known to play an important regulatory role in cell growth, migration and differentiation[25], embryogenesis[26,27], tumorigenesis[28], wound healing[29-31], bone formation[32-35] and immunomodulation[36], acting by both autocrine and paracrine mechanisms. It is also suggested that TGF β may be responsible for some pathological process, such as scarring and fibrosis, renal diseases and immunosuppressant[37-39]. Furthermore, TGF β is able to induce many kinds of cells, including hepatic and hepatoma cells to undergo apoptosis[40,41]. The molecular mechanisms underlying TGF β induction of the apoptosis of hepatocytes is still unclear. In this study, TGF β was extracted from the fresh bovine platelets by acid/ethanol procedure and purified by ion exchange and gel chromatography, then its biological activity was detected *in vivo* and its induction of apoptosis of cultured hepatocytes was observed *in vitro*, and to provide the basis for the study of the relationship between TGFβ and the signal transduction of hepatocellular-apoptosis.

MATERIALS AND METHODS

Isolation of platelets

The fresh anticoagulant bovine blood was collected in bags containing 0.1 volume of 8.78g·L^{-1} NaCl and 22.50g·L^{-1} EDTA (pH 7.4). The blood was centrifuged at 2000r·min^{-1} for 15 min and the supernatant was recentrifuged at 4000r·min^{-1} for 15 min. Then the supernatant was discarded and the precipitated platelets was washed twice with PBS (pH 7.4) by centrifugation at 5000r·min^{-1} for 30 min[42,43]. All the centrifugations were carried out at 0℃.

Extraction procedure

TGF β was extracted by a modified acid/ethanol procedure of Roberts and others[44,45]. The platelets were suspended in acid/ethanol extraction solution containing 375mL of 950mL·L^{-1} ethanol and 7.5mL of concentrated HCl, plus 33mg phenylmethylsulfonyl fluoride(PMSF) and 1.9mg pepstatin A as protease inhibitors. The mixture was sonicated in ice-bath, extracted overnight at 4℃, and centrifuged at 15000r·min^{-1} for 40 min at 0℃. The supernatant was adjusted to pH 3.0 with concentrated ammonium hydroxide. Then 2 volumes of cold anhydrous ethanol (-20℃) and 4 volumes of cold anhydrous ether (-20℃) were immediately added. After the mixture stood at -20℃ for 48 hrs, the resulting precipitate was collected by centrifugation at 20000r·min^{-1} for 30 min at 0℃ and redissolved in 1mol·L^{-1} acetic acid. After extensive dialysis at 4℃ against 0.17mol·L^{-1} acetic acid in a dialyzing tube (molecular mass cutoff, 10000ku), the sample was then subjected to the next purification.

Ion-exchange chromatography

The above crude sample was centrifuged at 20000r·min^{-1} for 40 min at 0℃ to remove the small precipitate and the supernatant was then applied to a CM-Sepharose column

(1.6cm×11cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 0.17mol·L^{-1} acetic acid. The column was eluted successively with 0.17 mol·L^{-1} acetic acid, 41.02g·L^{-1} NaOAc, 82.04g·L^{-1} NaOAc and 4g·L^{-1} NaOH at a flow rate of 90mL·h^{-1} at room temperature. The fraction that eluted by 4g·L^{-1} NaOH was immediately neutralized by adding 100mL·L^{-1} acetic acid. The eluted fractions were collected respectively and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE).

Gel chromatography

The above fractions containing 25ku component were collected, concentrated with glycol polyethylene and extensive dialyzed at 4℃ against tridistilled water and then 1 mol·L^{-1} acetic acid. After being centrifuged at 20000r·min^{-1} for 10 min, the supernatant was applied to a Superdex 75 column (1.6cm×70cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 1mol·L^{-1} acetic acid. The elution was carried out at a flow rate of 36mL·h^{-1} with 1mol·L^{-1} acetic acid containing 11.69g·L^{-1} NaCl at room temperature. The eluted fractions were collected and analyzed by SDS-PAGE. The fraction containing the 25ku component was concentrated using glycol polyethylene. After extensive dialysis at 4℃ against tridistilled water and 0.17mol·L^{-1} acetic acid, the samples were stored at -20℃ for the following assays. The protein content was determined by Coomassie brilliant blue G-250 method and bovine serum albumin (BSA) was used as control.

Detection of TGF β activity in vivo[46]

Nine male Balb/c mice (10 days old) were injected subcutaneously each day in the back with the purified TGF β (0.5g·L^{-1}). After 72 hrs, the tissues at the injection sites were removed and fixed in 100mL·L^{-1} formalin and paraffin sections were then subjected to the routine HE staining. The other 3 mice were injected with equivalent BSA as controls.

TGF β induction of hepatocellular apoptosis

Human normal hepatic cell line QZG (purchased from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured routinely on cover slides. After having grown to logarithmic phase, 8μg·L^{-1} of TGF β was added and then for a further 12 hrs culture. BSA (8μg·L^{-1}) was added as control. The cells were subjected to HE staining. In addition, the cells were stained by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) method to detect apoptosis[47]. A random field of cells was chosen with a magnification of ×400, and adjacent non overlapping fields were counted until the minimum 500 limits for each slide was obtained. TUNEL index was expressed as the number of positive cells/the total number of cells.

RESULTS

Purification of TGF β

The crude TGFβ was purified by ion exchange chromatography on a CM-Sepharose column, as shown in Figure 1. SDS-PAGE analysis showed that peaks 2 and 3 contained the 25ku component. The peaks 2 and 3 fractions were collected and then purified by gel chromatography on a Superdex-75 column (Figure 2). SDS PAGE analysis showed that peak 2 predominantly contained 25ku component (Figure 3) . The protein content was 1.1g·L^{-1}.

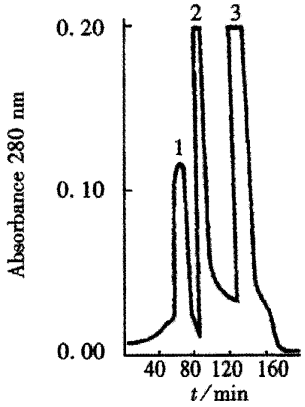

Figure 1 Ion-exchange chromatography of acid / ethanol extract on a CM Sepharose column.

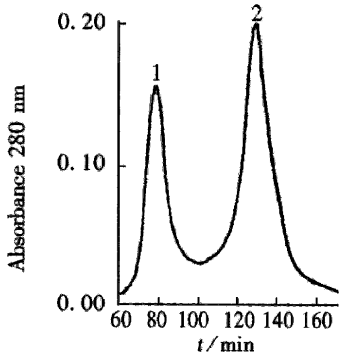

Figure 2 Gel chromatography of the CM-Sepharose peak 2 and peak 3 fractions on a Superdex-75 column.

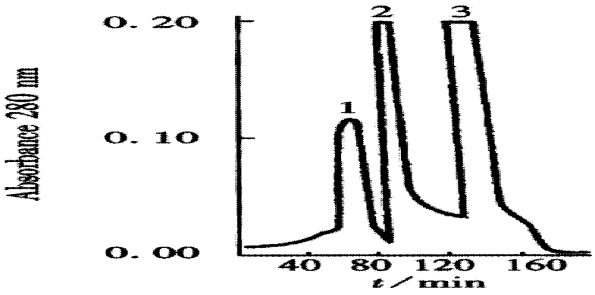

Figure 3 Molecular mass analysis of the extracted TGF β (SDS PAGE). Right Lane: marker proteins.

TGFβ activity in vivo

Seventy-two hrs after subcutaneous injection of TGF β, there was evident proliferation of fibroblasts and a few infiltration of inflammatory cells at the injection sites. The newly formed blood capillaries were also seen at the injection sites (Figure 4). The granulation tissue was confined at the injection sites. No obvious tissue change was observed in the control group.

Detection of apoptosis of hepatocytes

HE staining revealed that apoptotic hepatocytes exhibited the pyknosis and dense-stained nuclei and cytoplasm. Moreover, the nuclei demonstrated fragment, annular or crescent body, and the cytoplasm showed "bubbling" (Figure 5). TUNEL positive signals were olivine or yellow florescence in the nuclei. The TUNEL index of the TGF β treated group was 0.31 and distinctly higher than that of control group (0.09), (P<0.05)

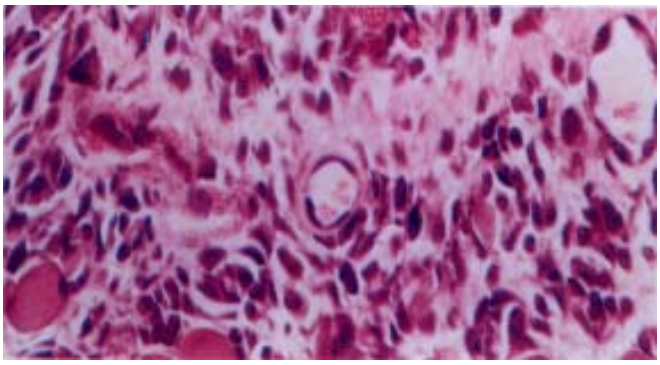

Figure 4 The granulation tissue formation after 72 hrs subcutaneous injection of TGF β. HE×400

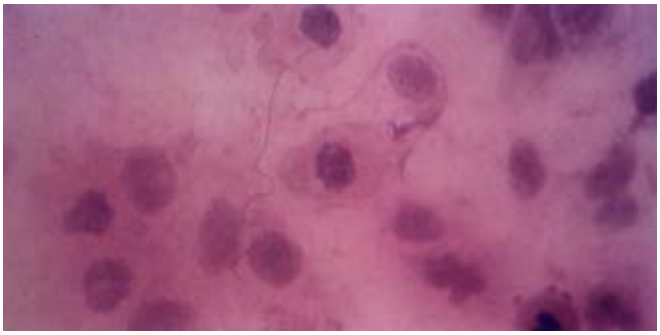

Figure 5 Hepatocytes QZG displayed apoptosis after being treated with 8μg/L TGF β for 12 hrs. HE×400

DISCUSSION

TGF β is a disulfide-linked homodimeric 25ku protein that consists of two identical 112 amino acid subunits, and only the dimer is biologically active. It is acid and heat stable and the presence of 18 half cystine residues in each dimeric molecular contributes to this stability[48-50]. There are at least five described subtypes of TGF β, encoded by distinct but closely related genes. TGF β 1, 2 and 3 have been found in many species, including humans; TGF β 4 has been found in chickens and TGF β 5 in amphibians[51] . Bone is the richest source of TGF β in the body, as it contains more than 200μg per kg of wet weight, whereas blood platelets represent the most concentrated source of TGF β (up to 20mg per kg of wet weight) and is released from β granules of platelets when blood clots[52,53]. Therefore, TGF β is generally extracted from the fresh platelets. It is suggested that TGF β is highly stable under acidic condition and can be activated by heating in boiling water for 5 min, or treatment with 1N acetic acid or 6M urea. The acid/ ethanol procedure is a practical way and previously used to extract biologically active polypeptides such as insulin, insulin like growth factor and platelet-derived growth factor[54,55]. Some other studies verify the effectiveness of this extraction procedure for isolation of TGF β from many tissues, including platelets, placenta and kidney[53,56,57]. Isolation of TGF β from platelets includes five steps: collection and washing of platelets, acid/ethanol extraction, ethanol/ether precipitation, ion exchange chromatography and gel chromatography. In the present study, crude TGF β was isolated from the fresh bovine platelets and then purified by ion exchange and gel chromatography. The SDS-PAGE analysis showed that the molecular weight of its main component was 25ku,which corresponded to that of standard TGF β protein.

Wound healing and tissue repair involve a complex series of biological events which include inflammation, cellular migration, fibroblasts proliferation, production of collagen and tissue remodeling. Growth factors have been reported to enhance the repair process in animal models by increasing the degree of cellularity, the rate of angiogenesis, and the amount of collagen accumulated[58,59]. TGF β has been studied in association with wound healing and the ability of TGF β to initiate a cascade of events leading to enhanced wound healing has been clearly demonstrated by many reports. Pierce and co-workers[60-65] have reported that TGF β significantly accelerate soft tissue repair by attracting fibroblasts into the wound and stimulating rapid synthesis, deposition and maturation of collagen *in vitro* and *in vivo*. Other major activities of TGF β are its abilities to promote the synthesis and deposition of various extracellular matrix (ECM) proteins and increase the expression of integrins and fibronectin, receptors that mediate cellular interactions with ECM proteins[66,67]. Different assay systems have been developed to measure the TGF β activities, including cell proliferation and inhibition assays, radio receptor assays, immunoassays and matrix formation or cell surface antigens expression assays[68,69]. In the present study, the analysis of activity *in vivo* demonstrated that there were fibroblast proliferation and blood capillaries formation after subcutaneous injection of the extracted TGF β. Moreover, the granulation tissue was only located in the injection sites and had no tendency to diffuse. That the action of the extracted TGF β *in vivo* led to granulation tissue formation suggested the successful extraction of TGF β and its participation in repair of tissue injury.

TGF β has been shown to either stimulate or inhibit proliferation in different cell types, and within same cell types, depending upon the stage of cell differentiation, *in vitro* condition and the presence of other growth factors. TGF β has a stimulatory effect on the proliferation of cells of mesenchymal origin, such as fibroblasts, osteoblasts and Schwann cells, yet is a growth inhibitor for cells of epithelial or neuroectodermal origin, including epithelial cells, osteoclasts, keratinocytes, T and B lymphocytes, endothelial cells and hepatocytes[70-72].

Apoptosis is a genetically and highly conserved process. Regulation of the balance between cell proliferation and apoptosis is essential for development and maintenance of multicellular organisms[73-77]. Previous studies suggest that TGF β is able to induce evidently apoptosis of hepatocytes and hepatoma cells *in vitro*. The animal experiments *in vivo* also manifest that the hepatocytes undergoing apoptosis have obviously elevated level of TGF β expression[78,79]. Furthermore, hepatoma cells which have a high apoptotic incidence rate, simultaneously demonstrate a high level of TGF β expression[80]. These studies suggest an involvement of TGF β in the initiation of apoptosis of hepatocytes. In our HE staining, normal hepatic cell line QZG showed remarkable morphological changes of apoptosis, including the pyknotic and hyperchromic cytoplasm and nuclei, and the fragmentary, crescent form or annular nuclei, and the “bubbling” cytoplasm, after being treated with exogenous TGF β. TUNEL staining also showed that the incidence rate of apoptosis was distincly higher in the TGF β treated group than that of the control group. The present study further supported the apoptotic induction of hepatocytes by TGF β, and verified the good biological activity of the extracted TGF β as well. Better understanding of the relationship between TGF β and the signal transduction of hepatocellular apoptosis requires further investigations.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Molecular mechanism about lymphogenous metastasis of hepatocarcinoma cells in mice

Li Hou[1], Ying Li[1], Yong-Hua Jia[1], Bo Wang[1], Yi Xin[2], Mao-Ying Ling[1] and Shen Lü[1]

[1]Department of Pathology, Dalian Medical University, Dalian 116027, Liaoning Province, China
[2]Department of Biochemistry, Dalian Medical University, Dalian 116027, Liaoning Province, China
Supported by the National Natural Science Foundation of China, No. 39470776
Correspondence to Shen Lü MD., PhD. Department of Pathology, Dalian Medical University, Dalian 116027, China. Houli72@163.net
Tel: 0086-411-4720226
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Abstract

AIM **To investigate the correlation between lymphogenous metastasis and matrix metalloproteinases (MMPs) activity and the expression of Fas ligand of tumor cells in lymph nodes.**

METHODES **Fifty-six inbred 615-mice were equally divided into 2 groups and inoculated with Hca-F and Hca-P cells. Their lymph node metastatic rates were examined. Growth fraction of lymphocytes in host lymph nodes was detected by flow cytometry. The Hca-F and Hca-P cells were cultured with extract of lymph node, liver or spleen. The quantity of MMPs in these supernatants was examined by zymographic analysis. The expression of Fas ligand, PCNA, Bcl-2 protein of Hca-F and Hca-P cells in the mice were examined by immunohistochemistry. The apoptosis signals of macrophages in lymph nodes were observed with *in situ* DNA fragmentation.**

RESULTS **On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes was as follows: in the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, while in Hca-P cells, the peak appeared on the 7th day post inoculation and then kept at a high level. With the extract of lymph node, the quantity of the MMP-9 activity increased (P<0.01) and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with the extracts of the liver and spleen. The expression of Fas Ligand of Hca-F cells was stronger than that of Hca-P cells (P<0.01). The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area were the same as that of Hca-P cells. *In situ* DNA fragmentation showed that the positive signals of macrophages were around Hca-F cells.**

CONCLUSION **Secretion of MMPs which was associated with metastatic ability of Hca-F and Hca-P tumor cells depends on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from being killed by host lymphocytes.**

Subject headings liver neoplasms/pathology; lymphatic metastasis; tumor cells, cultured; metalloproteinases/secretion

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INTRODUCTION

Metastasis is the most lethal attribute of a cancer[1,2]. Metastasis is a complex process which is made up of several steps[3]. Lymph nodes are often the first organ to develop metastasis[4,5]. Whether lymph nodes or other sites first develop metastases remain poorly understood. Lymph node metastases form a bridgehead for further metastatic spread. But its molecular mechanism is still unclear because of its complicated course. Anchorage on lymph node and escape from being killed by host immune cells in lymph node were two important steps. A mouse hepatocarcinoma cell line (Hca-F) with high lymphogenous metastatic potential and its syngeneic cell line (Hca-P) with low one were separated from hepatocarcinoma (HCC)[6] in mice. Matrix metalloproteinases (MMPs)[7] are a class of proteinases with variable substrate such as collagen, fibronectin and are related to the invasion-metastasis of hepatocellular cancer (HCC)[8]. Therefore, MMPs can be used as a marker of tumor cell infiltration in lymph node. The potential of tumor cells to induce apoptosis of host immune cells is to escape from being killed by immune cells. In this study, we detected the differences in the MMPs productions of Hca-F from Hca-P cells under various conditions, and different potentials of the carcinoma cells with different lymphogenous potentials to inhibit host immune reaction.

MATERIALS AND METHODS

Animals, cell lines and flow cytometry

Fifty-six inbred 615-mice maintained in our laboratory were equally divided into two groups. The Hca-F and Hca-P tumor cell lines preserved in our laboratory were inoculated at 2×10^6 in 28 mice subcutaneously in each group. On the 7th, 14th, 21st, and 28th day post-inoculation, two mice from each group were killed, and their lymphocytes were collected and detected for growth fraction with flow cytometry. The process of flow cytometry is as follows[9]: the lymph nodes were minced and centrifuged at 3000×g, and the supernatant was discarded. After repeated washing, cells were suspended in PBS. The lymphocytes at 10^5/100μL suspension were stained for 30 minutes by Propidum Iodide. Flow cytometry

was performed on a FACScan cytometer with LYSYII software. The fluorescence of 10^4 cells was analyzed for each sample. The other 40 mice were terminated on the 28th day post-inoculation, and their lymph nodes were H.E. stained and examined under microscope by paraffin sections. Therefore, the lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

Cell culture and zymographic analysis

The Hca-F and Hca-P cells cultured were put into different wells at 5×10^5, and then added 50mg extract of lymph node, liver or spleen respectively. The RPMI 1640 medium without fetal calf serum was placed into each well up to 1mL. The wells containing only Hca-F or Hca-P cells, and RPMI 1640 medium added only extracts of lymph node, liver or spleen up to 1mL served as controls. These cells were cultured at 37℃ for 24h. The supernatant of cultured cells was collected by centrifugation at 3000×*g*. MMP-2 and MMP-9 and their active type, and MMP-8 contained in supernatants of Hca-F and Hca-P with or without extracts of lymph node, liver or spleen were detected by zymographic analysis, according to the method described by Fridman *et al*[10]. The density and area of each band were measured using QuantiScan Software (Biosoft, USA).

Immunohistochemistry

The expressions of Fas-L (Santa Cruz, USA), proliferating cell nuclear antigen (Santa Cruz, USA, PCNA) and Bcl-2 (Santa Cruz, USA) protein in the tumor cells of inoculated area of Hca-F and Hca-P tumor cells, and metastatic tumor cells of lymph nodes of Hca-F were detected by standard immunohistochemistry[11]. The semiquantitative estimation of cancer cells stained was classified into 4 categories by assessing the percentage of stained tumor cells: 0, <2%; 1, 2%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75% cells.

In situ DNA fragmentation

We examined DNA fragmentation of the tumor cells of inoculated area of Hca-F and Hca-P cells, and metastatic tumor cells of Hca-F cells in lymph node, by the method of Zhu *et al*[12]. After desparaffin, these slides were pre-treated with $20mg\cdot L^{-1}$ proteinase K for 30 minutes, and then incubated with terminal deoxynucleotidy transferase and fluorescein labeled dUTP containing nucleotide mixture (TUNEL reaction mixture, *in situ* Cell Death Detection Kit/ POD, Boehringer Mannheim, Germany) in a humid atmosphere at 37℃ for 30 minutes. Each experiment set up by TUNEL reaction mixture without terminal transferase served as negative control.

RESULTS

Lymph node metastatic rate and flow cytometry

On the 28th day post-inoculation, the lymph node metastatic rate of Hca-F was 80%(16/20), whereas that of Hca-P was 25%(5/20). The growth fraction of lymphocytes from lymph nodes of mice transplanted with Hca-F and Hca-P tumor cells was examined using flow cytometry (Figure 1). For the Hca-F cells, the proliferating peak of lymphocytes appeared on the 14th day post-inoculation and then decreased rapidly, and for the Hca-P cells, the peak appeared on the 7th day post-inoculation and then kept at the high level.

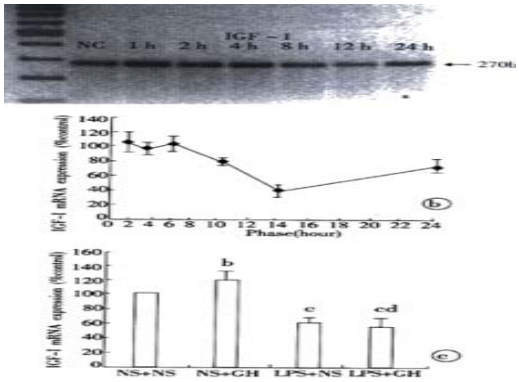

Figure 1 Changes of growth fraction of lmphocytes (G_2+S/G_1+G_2 +S+M) in draining lymph nodes of Hca-F/Hca-P burden mice.

Zymographic analysis

Both Hca-F and Hca-P cells produced a small account of MMP-9, but did not produce MMP-2 and MMP-8 (Table 1, Figure 2). Both Hca-F and Hca-P cells with extract of lymph node produced higher amounts of MMP-9 than Hca-F and Hca-P cells without extract of lymph node ($P<0.01$), and produced active MMP-9 and MMP-2. However, the Hca-F cells produce much greater quantities of activity of MMP-9, active MMP-9 and MMP-2 than Hca-P cells ($P<0.05$). The extract of lymph node did not contain any MMPs (Table 1, Figure 2). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of liver (Hca-F: 1767, Hca-P: 1564). The extract of liver contained activity of MMP-8 (1837). There was activity of MMP-8 in both Hca-F and Hca-P cells with extract of spleen (Hca-F: 2036, Hca-P: 1993). The extract of spleen contained the same quantity of activity of MMP-8 (1784). Therefore, we think that both Hca-F and Hca-P cells in the environment of liver and spleen did not produce activity of MMP-8 (Figures 3, 4).

Table 1 Activities of MMPs secreted from Hca-F and Hca-P cells under different conditions ($\bar{x}\pm s$)

Condition	MMP-2	Active MMP-2	MMP-9	Active MMP-9
Hca-F				
RPMI1640 medium	0	0	1256±157	0
Medium with lymph node extract	7364±2001	2009±901	12403±894	7297±1657
Hca-P				
RPMI1640 medium	0	0	2642±385	0
Medium with lymph node extract	2997±1990	1237±905	9086±686	3914±1253
Lmyph node extract	0	0	0	0

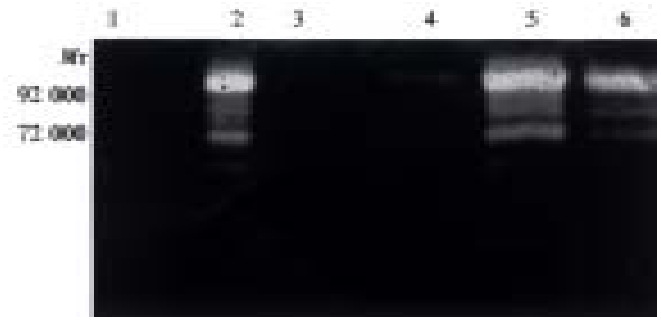

Figure 2 MMPs activity of Hca-F and Hca-P cells in RPMI 1640 with or without lymph node extract using zymographic analysis.
1. lymph node extract; 2. Type-four collagenase; 3. Hca-F cells; 4. Hca-P cells; 5. Hca-F cells in lymph node extract; 6. Hca-P cells in lymph node extract.

Figure 3 MMPs activity of Hca-F and Hca-P cells in liver extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in liver extract;
3. Hca-P cells in liver extract; 4. Liver extract.

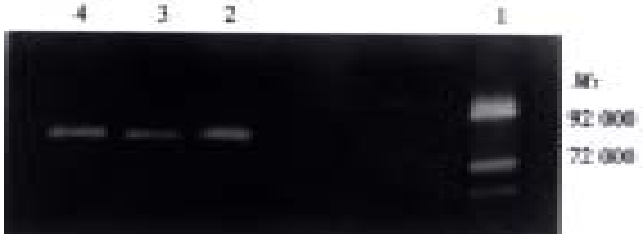

Figure 4 MMPs activity of Hca-F and Hca-P cells in spleen extract by using zymographic analysis.
1. Type-four collagenase; 2. Hca-F cells in spleen extract;
3. Hca-P cells in spleen extract; 4. Spleen extract.

Table 2 Analysis of Fas-L expression in Hca-F and Hca-P cells

Tumor	Grade				
	0	1	2	3	4
Primary tumor of Hca-F	0	0	0	8	12
Primary tumor of Hca-P	2	10	8	0	0
Metastatic tumor of Hca-F	0	5	9	6	0

Immunohistochemistry

The expression of Fas ligand protein of Hca-F cells was significantly higher than that of Hca-P cells ($P<0.01$, Table 2, Figure 5). The expressions of PCNA and Bcl-2 protein of Hca-F cells were as strong as those of Hca-P cells.

In situ DNA fragmentation

Few positive Hca-F and Hca-P cells were observed. Positive signals appeared in the macrophages around Hca-F cells (Figure 6).

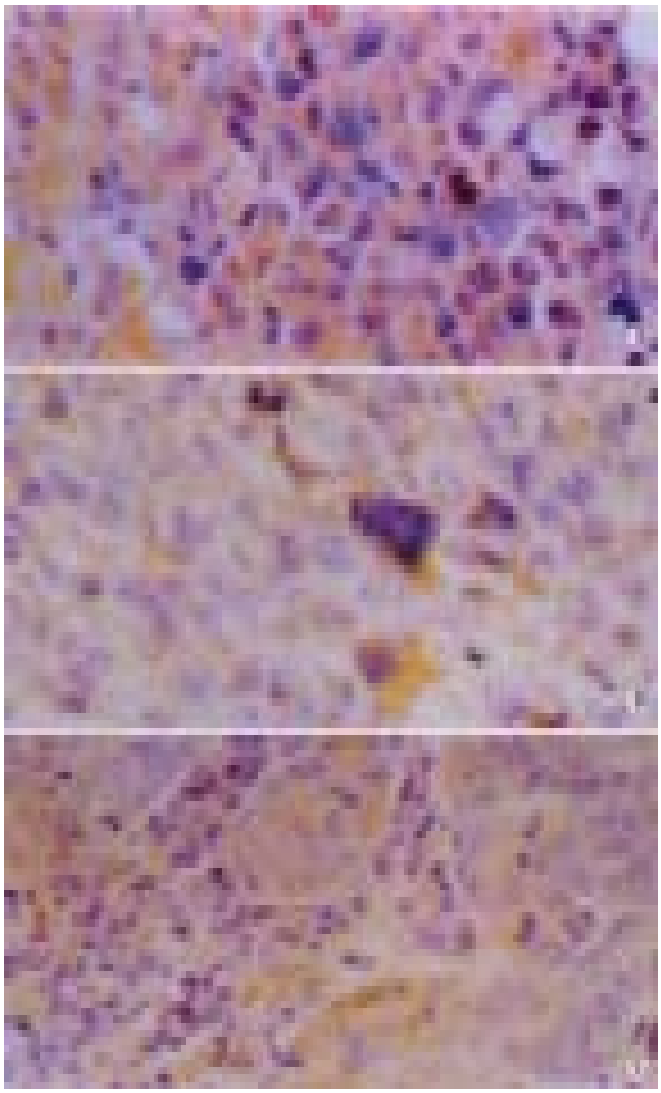

Figure 5 Fas-L expressed in Hca-F and Hca-P cells.
A: Fas-L expressed in Hca-F cells; B: in Hca-P cells;
C: in metastatic Hca-F cells.

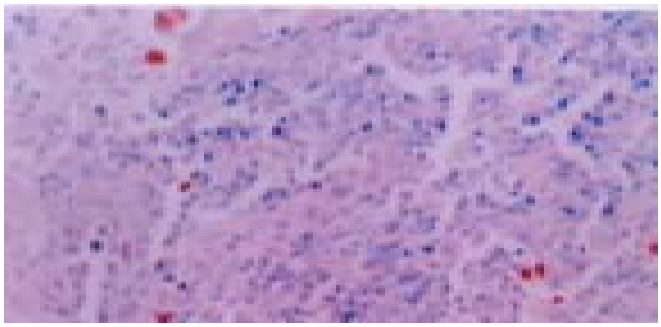

Figure 6 TUNEL of metastatic lymph node of Hca-F cells.

DISCUSSION

As early as a hundred years ago, the fact that tumor cells had organ-specific metastasis had attracted scientists' attention. Recent major discoveries concerning invasion and metastasis are identification of certain molecular mechanisms leading to organ-selective metastatization[13]. Cancer cell gene expression is regulated by interactions of tumor cells with host microenvironment, both in primary and secondary

lesions[14,15]. Whether the anchorage of carcinoma cells in lymph nodes is influenced by the specific environment of the lymph nodes remains unclear. Hca-F and Hca-P tumor cells have the potentials of specific lymphogenous metastasis. The matrix metalloproteinases[16] (MMPs) are a family of proteolytic enzymes, and the importance of MMPs in the processes of tumor invasion is now widely acknowledged in gastrointestinal cancer[17], breast cancer[18], colorectal cancer[19], and melanoma[20]. Inhibit the activity of MMPs can reduce the metastatic potential of cancer cells[21]. MMps digest collagen-containing structural barriers that cancer cells must pass in the step of cancer cell's anchoring to lymph node[22]. In this paper when the extract of lymph node was added, the quantity of the MMP-9 activity increased and active MMP-9 and MMP-2 were produced by both Hca-F and Hca-P tumor cells, which did not produce MMPs without the extract of lymph node or with extracts of liver and spleen. These results indicated that the secretion of MMPs of these tumor cells depend on the lymph node environment. In the environment with extract of lymph node, Hca-F cells with high lymphogenous metastatic potential produced much more MMPs than Hca-P cells with low one. So we can conjecture that Hca-F cells with high lymphogenous metastatic potential can easily receive the signal from lymph node and then start to infiltrate in lymph node to form metastatic focus.

The macrometastases were more proliferative than dormant micrometastases[23]. PCNA[24] functions as a cofactor of DNA-polymerase and is an important mark for evaluating the proliferation of colon cancer[25,26], gastric adenocarcinoma[27], lung cancer[28], ovarian cancer[29], thyroid carcinoma[30], and large intestine polyps[31]. We can use PCNA as an index of cellular proliferative status[32]. Bcl-2 proteins can extend cell survival by suppressing apoptosis[33] and are up-regulated in squamous cell carcinoma[34], breast cancer[35], lung cancer[36]. Bcl-2 proteins may promote metastasis in breast cancer[37] and melanoma[38]. The expressions of PCNA and Bcl-2 proteins could reflect accurately the status of cancer cells's growth. The expressions of PCNA and Bcl-2 protein of Hca-F cells in the tumors of inoculated area are the same as those of Hca-P cells. Therefore, the proliferating ability of Hca-F was equal to Hca-P, although their lymph node metastatic potentials were different.

A metastatic tumor in lymph node may form as long as the tumor cells escape the killing of lymphocytes. Fas/Apo-1, together with its protein-binding partner (Fas ligand), is a key regulator of programmed cell death and induces apoptosis when it binds FasL[39-42]. In this study, we found that growth fraction of lymphocytes in host lymph nodes was lower with Hca-F cells stimulation than with Hca-P cells. The result suggests that Hca-F cells may inhibit the growth and function of lymphocytes in lymph nodes. Tumor cell survives only by evasion of the immune system[43]. The Fas/FasL system is involved in the induction of apoptosis and mediates T-cell cytotoxicyty[44]. The expression of Fas ligand in many cancers plays an important role in establishing immunologically privileged environments that allow tumors to escape the host's immune surveillance, such as in esophageal carcinomas[45,46], lung cancer[47], melanoma[48], gastric carcinoma[49], intrahepatic cholangiocellular carcinoma[50] and promotes these cancers' metastasis. The expression of Fas ligand protein of Hca-F cells was stronger than that of Hca-P cells. Hca-F cells also produced Fos ligand in lymph node. Macrophages in lymph node are one of important antigen-presenting cells, and Fas ligand in tumor cells can combine with Fas in the membrane of macrophages to induce apoptosis and decrease their function. Because lymphocytes can not receive the signals from the macrophages, tumor cells in lymph nodes can escape the suppression of lymphocytes, then accomplish the metastatic process in the lymph node.

In a word, secretion of MMPs, which was associated with metastatic ability, of Hca-F and Hca-P tumor cells depend on the environment of lymph nodes. The increased expression of Fas ligand protein of Hca-F tumor cells with a high lymphogenous metastatic potential in lymph nodes may help tumor cells escape from the killing of host lymphocytes and shape up metastatic focus in lymph nodes.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection

Fu-Sheng Wang[1], Li-He Xing[1], Ming-Xu Liu[1], Chuan-Lin Zhu[2], Hui-Gang Liu[1], Hui-Fen Wang[2] and Zhou-Yun Lei[1]

[1]Division of Biological Engineering, [2] Fourth Department of Liver Diseases, Beijing Institute of Infectious Diseases, Beijing Hospital of Infectious Diseases, Beijing 100039, China
Supported by National Natural Science Foundation of China, No.39970831.
Correspondence to Dr. Fu-Sheng Wang, Division of Biological Engineering, Beijing Institute of Infectious Diseases, 26 Fengtai Road, Beijing 100039, China. fswang@public.bta.net.cn
Tel: 0086-10-66933332, Fax: 0086-10-63831870
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Abstract

AIM **To identify the property of dendritic cells (DCs) of peripheral blood monocytes (PBMC) in patients with chronic HBV infection.**

METHODS **Twenty patients with persistent HBV infection were included in this study,** 10 **healthy subjects being used as a control group. The peripheral blood mononuclear cells (PBMC) of T cell-depleted populations were incubated and induced into mature dendritic cells in the RPMI-**1640 **medium in the presence of cytokines GM-CSF, IL-**4**, FLt-**3**, TNF-α and** 100**mL·L^{-1} of fetal calf serum for a total of** 10-12 **days. The expressions of surface markers on DCs were evaluated using flow cytometric analysis. ELISA method was used to determine the cytokine levels of interleukin-**12 **(IL-**12**) and IL-**10 **in the supernatant produced by DCs. For detection of the stimulatory capacity of DCs to T cell proliferation, mytomycin C-treated DC were incubated with allogenic T cells.**

RESULTS **A typical morphology of mature DCs from healthy subjects and HBV-infected patients was induced in *in vitro* incubation, but the proliferation ability and cellular number of DCs from HBV-infected patients significantly decreased compared with healthy individuals. In particular, the expression levels of HLA-DR, CD**80 **(B**7-1**) and CD**86 **(B**7-2**) on DC surface from patients were also lower than that from healthy individuals** (0.46 ***vs*** 0.92 **for HLA-DR,** 0.44 ***vs*** 0.88 **for CD**80 **and** 0.44 ***vs*** 0.84 **for CD**86**, *P*<**0.05**). The stimulatory capacity and production of IL-**12 **of DCs from patients in allogenic mixed lymphocyte reaction (AMLR) significantly decreased, but the production level of nitric oxide (NO) by DCs simultaneously increased compared with healthy subjects** (86±15 ***vs*** 170±22 **μmol·L^{-1}, *P*<**0.05**).

CONCLUSION **The patients with chronic HBV infection have the defective function and immature phenotype of dendritic cells, which may be associated with the inability of efficient presentation of HBV antigens to host immune system for the clearance of HBV.**

Subject headings dendritic cells/pathology; hepatitis B, chronic/pathology; nitric oxide/analysis; interleukin-10/ analysis; interleuki-12/analysis

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INTRODUCTION

The infection of hepatitis B virus (HBV) causes 130 million HBV carriers, of them 23 million were patients with chronic hepatitis B in mainland China. As a result, a considerable number of the patients are developing progressive liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) each year[1]. The pathogenesis of developing chronic HBV infection is complex and unclear[2]. The dominant reason responsible for viral persistence within host is mainly lack of efficient antiviral immune response to the viral antigens, in particular, inability to produce specific CTL response, which is critical for complete elimination of virus within hepatocytes[3,4]. The mechanism for weak antiviral immune reaction in patients is not well understood. Dendritic cells (DCs) are one of the most potent antigen-presenting cells (APC) and play crucial roles in the enhancement or regulation of antiviral immune reactions. The previous reports showed the defects of DCs function in HBV-transgenic mice and in patients with HCC and chronic HCV infections[5-13], but there is little evidence of defect DCs function in chronic HBV-infected patients. In order to have more insights in this regard, we induced peripheral blood monocytes (PBMC) from HBV-infected patients and uninfected healthy donors into maturation of DCs, compared their morphologic characterization, expression of surface antigens, and their proliferation capability to stimulate AMLR for evaluation of the functions of DCs.

MATERIALS AND METHODS

Patients

Blood was obtained from each of the twenty patients who were positive for HBV antigens and serum HBV-DNA in this study after written consent had been obtained from them. All patients were negative for HCV infection and had no histories of other types of liver diseases. The diagnosis of chronic HBV infection is made in accordance with the standards for chronic viral hepatitis issued in the Fifth National Conference on Infectious Diseases and Parasitosis in China (Beijing, China, 1995)[14]. Ten age-matched healthy donors from the Blood Center of Chinese PLA 307 Hospital were assigned as controls. The clinical background of patients and donors are shown in Table 1.

Table 1 Clinical background of HBV-infected patients and healthy donors

Group	*n*	Gender M/F	Age (years)	ALT (nkat·L^{-1})	AST (nkat·L^{-1})	HBV DNA	HBsAg	Anti-HBs	HBeAg	Anti-HBe	Anti-HBc
Patients	20	17/3	29.5(12-47)	215±218	186±172	19	18	—	16	4	20
Donors	10	8/2	27.5(20-35)	20-40	20-40	—	—	6	—	—	—

—: Nagative results

Reagents

RPMI1640 medium, recombinant human IL-4, GM-CSF, fetal calf serum and TNF-α were all purchased from Gibco Co. (Jingmei Biological Co, Beijing). The Flt-3 ligand, mouse anti-human FITC-conjugated HLA-DR-FITC, CD-86-FITC, PE-conjugated CD80-PE, CD1α-PE monoclonal antibodies were obtained from BD-Pharmingen (Jingmei Biological Co, Beijing).

Culture of DCs

Fifteen milliliters of peripheral blood was drawn from each study subject in heparin-coated tube, PBMC were obtained by Ficoll Hypaque separation, washed twice, resuspended in RPMI1640 medium and incubated in 6-well culture plates at the concentration of 3×10^9 cells·L^{-1} in medium at 37℃ in 50mL·L^{-1} CO_2 atmosphere for 2h. Non-adherent lymphocytes were collected by careful rinsing in 37℃ warm and cryopreserved for their future use in antigen presentation assays. The adherent aggregates were kept for incubation in complete medium with 100mL·L^{-1} fetal calf serum (FCS) in the presence of 1×10^6 IU·L^{-1} GM-CSF, 1×10^6 IU·L^{-1} IL-4, and 50μg·L^{-1} Flt3-L respectively, and expanded over the next 7. At d7, 1×10^6 IU·L^{-1} TNF-α cytokine was added into the medium for 10-12 days incubation. The wells were fed every other day by aspirating 0.3mL medium and adding back 0.5mL fresh medium with cytokines. The protocol has been proven reproducible in 20 HBV-infected patients and 10 healthy donors[15].

AMLR stimulated by DCs

To evaluate the allo-stimulatory activity of DC, AMLR (allogeneic-mixed lymphocyte reaction) was performed[16]. After DCs had been treated with 50μg mytomycin C for 45 min at 37℃ and washed three times with 37℃ warm PBS (pH 7.0). The DCs were placed at 0.5×10^4, 1.0×10^4, 1.5×10^4 and 5.0×10^4 per well on 96-well flat-bottom culture plates, incubated in complete medium. T-cells were from the PBMC of a healthy donor by removing the CD8-positive cells, monocytes, B cells and NK cells with magnetic bead-tagged mouse monoclonal anti-human CD8, CD14, CD19, and CD56 Abs (BD-PharMingen). After the separation, the degrees of positivity of these cells in the samples were all less than 5% and those of CD4-positive cells were more than 95%, respectively. CD4 T cells at 2×10^5/well were mixed with DC and cultured for 4d at 37℃, 50mL·L^{-1} CO_2 atmosphere. The MTT method was used according to the manufacturer instructions.

Detection of NO IL-10 and IL-12

The levels of nitric oxide (NO) and cytokine production by DCs were determined according to a previous report with slight modification (Kakumu *et al*, 2000). In short, DC (1×10^9·L^{-1}), enriched from HBV-infected patients and uninfected healthy donors by cytokines, were cultured in RPMI1640 plus 100mL·L^{-1} FCS for 48h in the presence of *Staphylococcus aureus Cowan* I strain (SAC). After the end of the culture, supernatant was collected and centrifuged. The production of NO and cytokines in the culture supernatants was determined[16,17]. The levels of NO production in the sample was determined by assaying the stable end product NO^{2-} by Greiss reaction using a commercial kit (Griess Assay Kit NO kit-C; Wako), as described by Lu[18]. Aliquots of culture supernatants were incubated with Griess reagent (10g·L^{-1} sulfanilamide, 1g·L^{-1} nephthylethlenediamine, dihydrochloride, and 25g·L^{-1} H_3PO_4) 100 μL at room temperature for 10min. The color developed due to enzymatic reaction was determined with an ELISA reader at 540nm. Concentrations of NO in the sample were caliberated with a reference standard of sodium nitrite supplied with the kit and the levels of NO were expressed as μmol·L^{-1}.

IL-10 and IL-12 in the supernatants were estimated by an ELISA method using commercial kit (PharMingen, San Diego, CA) according to the instructions of the manufacturer. Samples were incubated on microtitre plates coated with the respective monoclonal antibodies (mAbs), followed by addition of a biotinylated second antibody. After removal of excess antibody, color development was finished by enzymatic reaction of strepavidin peroxidase, the intensity of which was directly proportional to the concentration of the respective cytokines in the samples. The amounts of cytokines in the samples were estimated by caliberating the absorbance (optical density, OD) values of the samples with A values of the standards, supplied with kits using an ELISA reader (Labsystems Multiskan MS, USA). The lowest levels of cytokines detectable by these kits were IL-10>15.0ng·L^{-1}, and IL-12>7.8ng·L^{-1}.

Flow cytometry

After 12d incubation, the mature DCs from patients and healthy donors were washed in PBS and directly stained with an optimum dilution of FITC conjugated specific antibody (PharMingen, San Diego, CA) or stained with an optimum dilution of primary antibody followed by FITC-conjugated antibody against HLA-DR, CD86 and PE-labelled antibody against CD80 and CD1a. Finally, FITC+ and PE-cells were counted in an FACScaliber (Becton Dickinson)[19,20].Subclass matched FITC-conjugated mouse IgG and FITC-conjugated secondary antibody alone served as controls.

Statistical analysis

The Student's *t* test was used to determine significant differences in mean values between two groups. Statistical significance was established at the $P<0.05$ level.

RESULTS

Proliferation and Morphology of DCs

The adherent cells, the progenitor cells of DCs were incubated in complete medium containing 100mL·L^{-1} FCS, Flt-3-L, GM-CSF and IL-4 at 37℃ in 50mL·L^{-1} CO_2 atmosphere. The proliferation of DCs could be observed from the third day

incubation. The number of proliferative DCs increased following the extension of incubation time, and reached top value at the 12th day, then gradually went down as shown in Figure 1. Under phase-contrast microscopy, the DCs display a typical morphology with many fine dendrites. These cells extend large, delicate processes or veils in many directions from the body, and display typical morphology of DCs. The yield of proliferation of DC populations increased around 45 times for the healthy donors and 30 times for HBV-infected patients after 12d incubation.

Surface markers on DC

Mature DCs 1×10^6 after 12d incubation were defined by the expression of dendritic cell-restricted markers. The analyses of flow cytometry showed that positive cells constituted between 56% and 80% of all cells recovered at the end of the culture; the purity was not different between patients and controls. The frequencies of $CD86^+$ cells in the dendritic cell population were 84%±10% for control donors (n = 10) and 44% ± 8% for patients with chronic HBV infection (n = 20), respectively, indicating a significant difference between the groups. In addition, the average ratio of $HLA\text{-}DR^+$, and $CD80^+$ surface molecules expressed in the dendritic cell population are shown in detail in Table 2. Figure 2 shows the fluorescence intensity of representative DCs from one patient and one healthy donor, and there was a high expression of surface markers on DCs from healthy donors, reflecting a significant statistical difference from the patient group.

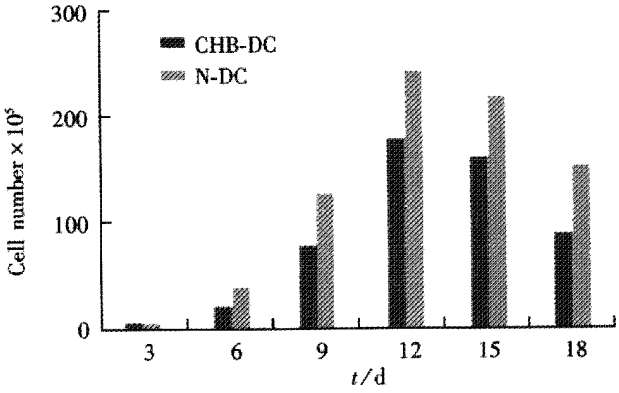

Figure 1 Proliferation of dendritic cell population in *in vitro* culture.

Table 2 Comparison of expression of DC surface markers from CHB patients and healthy donors (number fraction, $\bar{x}\pm s$)

Group	*n*	B7-1(CD80)	B7-2(CD86)	CD1[a]	HLA-DR
CHB-DC	20	0.44±0.08	0.44±0.08	0.25±0.07	0.46±0.09
Normal-DC	10	0.88±0.10[b]	0.84±0.09[b]	0.89±0.09[b]	0.92±0.09[b]

[b]P<0.001 *vs* CHB-DC.

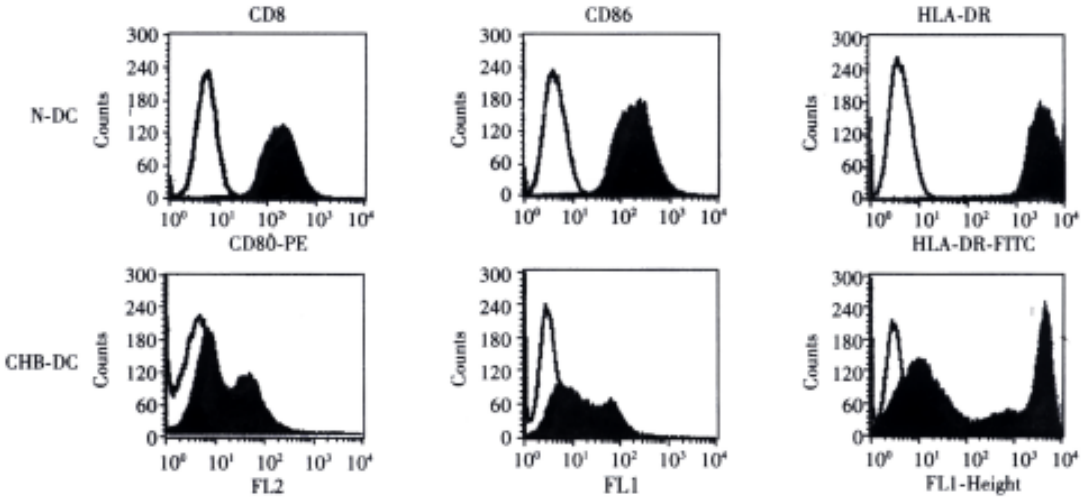

Figure 2 Comparison of fluorescent intensities of flow cytometric analyses on surface of dendritic cells (DCs) from a representative of HBV-infected patients (HB-DC) and a representative of uninfected healthy donors (N-DC). The green line is for signals from controls (DCs stained with mouse IgG_2 instead of specific antibody against surface marker of DCs). The green line with purple color represents the signals obtained from surface of DCs stained with corresponding specific antibody. Note the shift to the left in the peak with increasing expression of surface markers on DCs. Two other independent experiments gave similar results.

AMLR from HBV-infected patients

The T-cell stimulatory activity of DC populations in AMLR was expressed as stimulation index (SI) value, which is the ratio between the proliferative response (optical absorbance, OD) of T cells in the presence and the absence of DCs in the cultures at a T cell:DC ratio of 100:2.5, 100:5, and 100:25, respectively. Although the number of T cells was the same (2×10^5 cells/well) in all AMLR cultures, the SI went up significantly according to the increase of DCs number in AMLR. The SI values were between 2.1 and 4.8 for HBV-infected patients as compared with the values between 6.8 and 12.8 for healthy donors. The SI for HBV-infected patients was the lowest (SI=2.1) when the AMLR system contained 0.5×10^4 $well^{-1}$ of DCs. The results showed that HBV-infected patients tended to have significantly decreased T cell-stimulatory activity (P<0.01) as compared with the values for healthy donors.

Cytokine production by DC in AMLR

IL-12 is known to induce T cell proliferation and is the only

vital cytokine produced by DCs. Spontaneous IL-12 production of dendritic cells in the supernatants of dendritic cell population and AMLR are shown in Table 3. IL-12 produced by DC from patients (27±4ng·L^{-1}) was significantly lower than that from healthy donors (86±32ng·L^{-1}), indicating the statistical difference. Although the IL-12 in this AMLR was most likely produced entirely by the DCs, we decided to confirm this by culturing DCs for 48h and measuring the spontaneous production of IL-12 in culture by pure populations of DCs. Pure DCs from patients produced significantly lower amounts of IL-12 as against healthy donors. In addition, there was no statistical difference in IL-10 levels in AMLR supernatants of DCs from HBV-infected patients (18 ± 7 ng·L^{-1}) (Table 3) and normal controls (25±9ng·L^{-1}).

Table 3 Spontaneous cytokine production in pure DCs population and AMLR ($\bar{x}$±s, ng·L^{-1})

Dendritic cell	Supernatant from MLR culture		Supernatant from pure DCs population(ng·L^{-1})
	IL-12(ng·L^{-1})	IL-10(ng·L^{-1})	
CH-B	27±4	18±7	99±51
Normal	86±32[b]	25±9	218±104[b]

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

Increased NO production by DC from HBV-infected patients

As shown in Figure 3, the levels of NO in the AMLR supernatant of DCs from HBV-infected patients (168±35 μmol·L^{-1}) were significantly higher than that produced in AMLR of DCs from normal controls (90 ± 43 μmol·L^{-1}) (P<0.05). There was no correlation between the level of NO and the levels of transaminase (data not shown). NO produced by DCs is supposed to be one of the main products in AMLR supernatant. To have direct evidence of increased NO production by DCs, we cultured DC with SAC, a known inducer of NO. Pure DCs from HBV-infected patients produced significantly higher amounts of NO (170 ± 22 μmol·L^{-1}) than the normal controls (86 ± 15 μmol·L^{-1}, P<0.05, Figure 3). The significance of increased production of NO by DC in HBV infection might find the clinical implications of these observations.

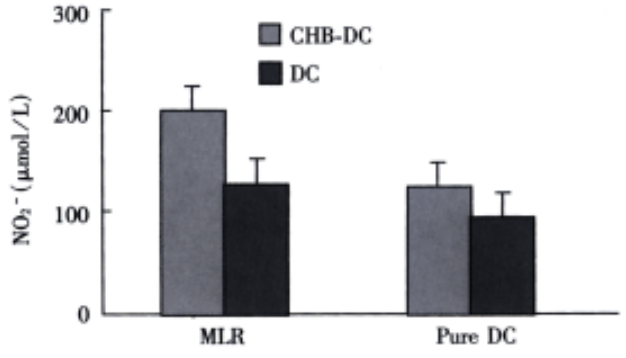

Figure 3 Increased NO production by DCs from HBV-infected patients. All the T cells (2×10^5/ well) were donated from a healthy volunteer in AMLR DC (1×10^4 well^{-1}) from patients with chronic hepatitis type B (CHB) and healthy donors were cultured for 5d. Pure population of DCs (1×10^9·L^{-1}) isolated by the cytokine enrichment method were cultured with SAC I strain 75mg·L^{-1} for 48h.

DISCUSSION

Though B and T lymphocytes are the main mediators of immunity, their function is under the control of DCs which is the sentinels of host immune system[21]. In this way, the property and its implications of DCs have become the focus of immunity against tumor and infectious diseases. Interestingly, the present culture techniques allowing the generation of large numbers of immunostimulatory DCs *in vitro* from human peripheral blood have made it possible to analyse the DC function in human diseases[7,10]. Though depletion and dysfunction of DCs were reported in some infectious diseases induced by HCV, HIV-1 and measles virus[6,20,22], dendritic cell function still remains uncertain in chronically HBV-infected patients. In particular, there appears to have few reports in the published literature about the effects of chronic HBV infection on DC function. These make us attempt to compare the stimulatory potential against allogenic CD4 T cells, levels of surface antigens, levels of interleukin-12, and degree of maturation of DCs from HBV-infected individuals with uninfected healthy donors in this study.

Flow cytometry showed that the expressions of cellular surface markers such as the co-stimulatory factors B7-1, B7-2 and CD1a and MHC II molecules HLA-DR on DCs significantly decreased in chronic HBV-infected patients compared to those in healthy donors. Furthermore, the T-cell stimulatory activities of DCs from patients were much lower than the DCs from uninfected healthy donors in AMLR.Simultaneously, there were notably low levels of cytokine IL-12 and high levels of NO produced by DCs from patients, in particular, NO was considered to have the suppressive activity on cellular enzymes and produce damage to normal cells. Taken together, our present study indicated that there is an immature phenotype and dysfunction of DC population in chronic HBV-infected patients in comparison with uninfected controls. It is necessary to study whether the dysfunction of DC population in patients is associated with the failure to mount an effective immune response for clearance of HBV.

Since IL-12 is an important cytokine to stimulate the proliferation of T lymphocytes, the decrease of IL-12 production was directly attributed to the low T-cell stimulatory of DCs from HBV-infected patients in AMLR[23]. The following reasons probably resulted in the low level of IL-12 production: ① IL-10 could inhibit the expression of class II and B7 molecules and suppress the IL-12 production of DCs[8]; ② there is a high level type I interferon in patients with HBV infection, which induces the down-regulation of IL-12 secretion of DCs[24]; ③ the previous study reported that the HBV itself and some cytokines induced by HBV infection may produce direct or indirect influences on transcriptions of IL-12 gene[25,26]; and ④ host genetic factors, such as the polymorphisms of both antigen-capturing receptor alleles and cytokine genes in DCs might explain HBV-infected patients mount dysfunction of DCs[27].

The maturation and efficient antigen-presentation of DCs are crucial for the initiation of immunity against viral infection[4]. The impairment of dendritic cell function suggested a role in the pathogenesis of chronic HBV infection, which has been probably attributed to the inability of the host to eradicate viruses[1]. It is unknown whether the HBV-infected DCs were eliminated by specific cytotoxic T lymphocytes (CTLs) if the DC is infected itself, which induced the dysfunction of DCs in these patients with chronic HBV infection or the HBV-infected DCs directly induce the T cell tolerance to HBV infection[27].

Decreased function of DC may allow the development of HBV infection, so that modulating the function of dendritic cells is considered to be beneficial to the production of

efficient immunity against viruses[28], but it must be made clear whether the dysfunction of DCs in patients is the result of direct injury of DCs from chronic HBV infection, or a protection from host itself to avoid the destruction of a large amount of HBV-infected hepatocytes induced by itself-inducing immune reaction, and or both In the early stage of HBV-infected patients, efficient antigen-presentation of DCs help the host to clear the virus[29], but in the late stage of chronic HBV-infected patients, induction of dysfunction of DCs or immune tolerance to HBV infection might also be helpful to host survival. Further studies are needed to clarify the mechanisms of depression of DCs function in HBV-infected patients[30,31].

The development of methods to generate a large number of DCs has facilitated their application for immunotherapy. Recent studies have demonstrated the safety and immunogenicity of DCs in humans and have begun to outline the durability, kinetics, and nature of the elicited T-cell responses. However, DC-based immunotherapy remains a challenge and several parameters need to be examined to optimize immune responses, in order to maximize the clinical efficacy against cancer and infectious diseases.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Abnormal β-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China

Jian Cui[1,2], Xin-Da Zhou[1], Yin-Kun Liu[1], Zhao-You Tang[1] and Maija H Zile[2]

[1]Liver Cancer Institute, Zhongshan Hospital, Medical Center of Fudan University, Shanghai 200032, China
[2]Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824 USA
Supported by National Ninth Five-year Plan of Medical Sciences of China (96-906-0105).
Correspondence to Jian Cui, M. D & Ph. D, Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824 USA. cuij@msu.edu
Tel: 1-517-353-9608
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Abstract

AIM **To study the abnormal expression of β-catenin gene and its relationship with invasiveness of primary hepatocellular carcinoma among Chinese people.**

METHODS **Thirty-four hepatocellular carcinoma (HCC) specimens and adjacent paracancerous tissues,** 4 **normal liver tissues were immunohistochemically stained to study subcellular distribution of β-catenin. Semiquantitive analysis of expression of β-catenin gene exon** 3 **mRNA was examined by RT-PCR and *in situ* hybridization. The relationship between expressions of both β-catenin protein, mRNA and clinicopathological characteristics of HCC was also analyzed.**

RESULTS **Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined displayed membranous type staining for β-catenin protein, occasionally with weak expression in the cytoplasm. While** 21 **cases** (61.8**%**) **of HCC examined showed accumulated type in cytoplasms or nuclei. The accumuled type Labling Index** (**LI**) **of cancer tissue and para-cancarous tissue was** (59.9±26.3) **and** (18.3±9.7) **respectively** (***P***<0.01)**. Higher accumulated type LI was closely related with invasiveness of HCC. Results of RT-PCR showed the β-catenin gene exon** 3 **mRNA Expression Index** (**EI**) **of** 34 **HCCs was higher than that of para-cancerous tissue and normal liver tissue. Using *in situ* hybridization, the signal corresponding to β catenin gene exon** 3 **mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous and normal liver tissues. Over expression of β-catenin exon** 3 **was also found to be correlated with high metastatic potential of HCC.**

CONCLUSION **Abnormal expression of β-catenin gene may contribute importantly to the invasiveness of HCC among Chinese people.**

Subject headings hepatocellular carcinoma; wnt pathway; β-catenin gene; metastasis

Cui J, Zhou XD, Liu YK, Tang ZY, Zile MH. Abnormal β-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China. *World J Gastroenterol*, 2001;7(4):542-546

INTRODUCTION

Hepatocellular carcinoma (HCC) is quite common in China. In recent years, great progresses have been made in the treatment of HCC, but the major problem is the high malignancy of HCC, that is, more than 50% of the patients receiving grossly radical treatment will suffer from recurrence within two years. So much effort has been put to investigate the molecular biological characteristics of HCC in order to lower the recurrence rate[1-20]. β-catenin is a ubiquitous intracellular protein which is important in both intercellular adhesion and Wingless/Wnt developmental signaling transduction pathway[21]. β-catenin plays an important role in the interactions between cadherins and other transmembrane receptor proteins, such as the epidermal growth factor receptor. In addition, it is also a signaling molecule and can activate gene transcription by forming a heterodimer with the T-cell factor/lymphoid enhancer-binding factor family of DNA binging proteins[22]. Previous studies have shown that β-catenin is involved in pathways that regulate cellular differentiation and proliferation. In the absence of growth or differentiation signals, cytoplasm β-catenin is rapidly turned over under the control of the APC protein and the GSK-3β, resulting in low level of cytoplasm β-catenin level in normal cells[23,24]. The presence of a wingless-Wnt signal in normal embryonic cells stabilizes β-catenin, which accumulates in the cytoplasm, where it binds to Tcf-lymphoid enhancer factor and triggers gene transcription. Abnormal expression and/or structural abnormalities of catenins are closely associated with tumor development for human esophageal, gastric and colon cancers[25,26]. Previous study has shown that E-cadherin expression was significantly lowed and is closely related with the metastatic potential of HCC[27], and abnormal β-catenin expression has been observed by immunohistochemistry in many malignant human tumors including HCC[28], so it is our logical thoughts whether abnormality of β-catenin gene existed and what its relationship with malignancy in HCC among Chinese people is because of the close relationship between E-cadherin and β-catenin.

MATERIALS AND METHODS

Tissue

Thirty-four HCC specimens and adjacent para-cancerous tissues, four normal liver tissues obtained from patients who underwent surgery in Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University were analyzed. The tissues were each cut into three parts: one was fixed in formalin, and then embedded in paraffin. Paraffin sections were stained with HE for histological examination of HCC and were also used for immunohistochemistry. One was immediately frozen by liquid nitrogen and stored at -80℃, which was to be used for DNA and RNA extraction. Genomic DNA was purified from all samples using standard proteinase K digestion and phenol/chloroform extraction. Total RNA was extracted using a Trizol reagent (Promega) according to

the protocol recommended by the manufacturer. And the last was rinsed in cold PBS, placed in OCT compound, and immediately frozen in liquid nitrogen, which was to be used for *in situ* hybridization.

Immunohistochemical staining

Immunohistochemical analysis was carried out with the avidin-biotin complex immunoperoxidase technique as described previously[29]. As the primary antibody, polyclonal human anti-β-catenin antibody (Sigma) was used at 500×dilution. As the secondary antibody, biotinylated anti-rabbit IgG (Dako) was used at 100× dilution. Staining was performed using avidin-biotin reagents, 3, 3'-diaminobenzidine, and hydrogen peroxide. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. All cases were divided into two groups according to immunostaining pattern. Cases with a membrancous staining pattern similar to that in normal hepatic cell were classified as membraneous or normal and cases with marked cytoplasmic and nuclear staining in addition to the membranous staining were defined as accumulated or abnormal. Cells from five randomized views were counted and the cell labeling index (LI) was arbitrarily defined as: (positive cells counted/all cells counted)×100.

RT-PCR

Primers for PCR were designed to amplify the consensus sequence for GSK-3 β phosphorylation in exon 3 of β-catenin gene, based on the published cDNA sequence of human β-catenin gene. To verify the validity of amplification, the primers were designed within the region of exon 3 of β-catenin gene, and the amplification was performed by direct PCR and RT-PCR respectively. Primers, F: AAAGCGGCTGTTA-GTCACTGG R: GACTTGGGAGGTATCCACATCC. PCR: PCR mixture, containing 100pM of primer A and B each, deoxyribonucleotide triphosphates at 200μmol·L^{-1} each, 1.5mmol·L^{-1} $MgCl_2$, 2U *Taq* polymerase (Promega) and 2μL DNA template was adjusted to 50μL by adding double distilled water. Then the mixture was overlaid with 50μL mineral oil and subjected to amplification for 40 cycles. Each cycle consisted of 95℃ for 60s, 55℃ for 45s, 72℃ for 45s. RT-PCR: Total RNAs were reverse-transcribed to obtain the cDNA that was going to be amplified. PCR was also performed under the above same condition except for adding 1μL cDNA to the PCR mixture. A 450bp fragment of β-actin mRNA was also amplified by RT-PCR as the internal control. The PCR products were identified first onto 20g·L^{-1} agrose gel and photographed. The photos of RT-PCR were scanned by optical density scanner (Shimadzu C-9000) and the gene expression index (EI) was arbitrarily defined as density Lum of β-catenin/density Lum of β-actin.

In situ hybridization

Cryostat sections (6μm) were obtained, dried for 2h at RT, and delipidated in chloroform for 5min. Sections were fixed in 40g·L^{-1} paraformaldehyde/PBS for 7min, rinsed in PBS for 3min, rinsed twice in 2× SSC for 5min, and prehybridized at 42℃ for 60min in 4× SSC/100g·L^{-1} dextran sulfate/1× Denhardt's solution/2mM EDTA/500g·L^{-1} deionized formamide/ 500mg·L^{-1} salmon sperm DNA. Hybridization was for 16h in 100μL of prehybridization solution and 20μg·L^{-1} digoxin labeled oligonucleotides (TGTTCC-CACTCATACAGGACTTGGGAGGTATCCACATCCTCTT CCTCAGGA). After hybridization, sections were rinsed twice in 2×SSC for 5min at 37℃, 3 times for 5min each in 60g·L^{-1} formamide and 0.2×SSC at 37℃ and twice for 5min each in 2×SSC at RT. Sections were then rinsed in 100 mol·L^{-1} Tris · HCl, pH 7.5/150 mol·L^{-1} NaCl for 5min, and treated with the same solution saturated with blocking mix for 30min, and then reacted with a 1:2000 dilution of alkaline phosphatase-conjugated sheep antidigoxigenin Fab fragments (750×10^3·L^{-1}) in the same solution. They were rinsed twice in 100mol·L^{-1} Tris · HCl, pH 7.5 and 150mol·L^{-1} NaCl for 5min each, then in 100 mol·L^{-1} Tris · HCl, pH 9.5/100 mol·L^{-1} NaCl/ 50mol·L^{-1} $MgCl_2$ for 10 min, and then reacted with 0.18g·L^{-1} 5-bromo-4-chloro-3-indolyl phosphate, 0.34g·L^{-1} nitroblue tetrazolium, and 240mg·L^{-1} levamisole (Sigma) in the same solution for 6h in the dark at RT. The reaction was stopped with 10mol·L^{-1} Tris · HCl (pH 8.0) and 1mol·L^{-1} EDTA. Sections were counterstained in nuclear methyl green, mounted with aqueous solution, and the final results of average density area and density lum of 500 signal positive cells were analyzed by a multifunctional true digital system (MTDS) using a computer. Albumin oligonucleotide probe and hybridization solution without probe were used as positive and negative control respectively.

RESULT

Immunohistochemical analysis

Immunostaining with polyclonal antibody was performed to evaluate the significance of β-catenin accumulation in HCC. Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined showed membranous type, occasionally with weak expression of β-catenin in the cytoplasm, but no β-catenin accumulation in nuclei was found. While for HCC, 21 cases (61.8%) showed accumulated type (Figure 1). The LI of accumulated type for tumor tissue and paracancerous tissue were 59.9±26.3 and 18.3±9.7 respectively (P<0.01), while the LI of membraneous type for tumor tissue and paracancerous tissue were 24.6±8.5 and 91.8±10.6 respectively (P<0.01, Table 1). When LI of accumulated type was analyzed according to the clinicopathological characteristics of HCC, close relationship could be seen with capsule, portal vein tumor thrombus, pathological grade, intrahepatic metastasis (Table 2) and postoperative recurrence (Figure 2).

β-catenin exon 3 mRNA expression

Since the primers were designed in such a way that the product was within β-catenin gene exon 3, direct PCR and RT-PCR were used separately to verify the amplification. Agrose gel electrophoresis showed that PCR and RT-PCR amplification products were both 132bp, which were the same as those of normal liver tissues, para-cancerous tissues and HCC tissues. None of amplification products showed fragment that was shorter. RT-PCR results showed the β-catenin exon 3 mRNA EI were (0.77±0.16) and (0.50±0.05) for HCC tissues and para-cancerous tissues respectively (P<0.05, Figure 3). In HCC, higher EI of β-catenin mRNA attempted to be seen in cancer with incomplete capsule, intrahepatic metastasis and portal vein thrombus (Table 2). Using *in situ* hybridization, we also found the signal corresponding to β-catenin exon 3 mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous tissues and normal liver tissues (Figure 4) and stronger signal of β-catenin mRNA was also closely related to incomplete capsule, intrahepatic metastasis and portal vein thrombus.

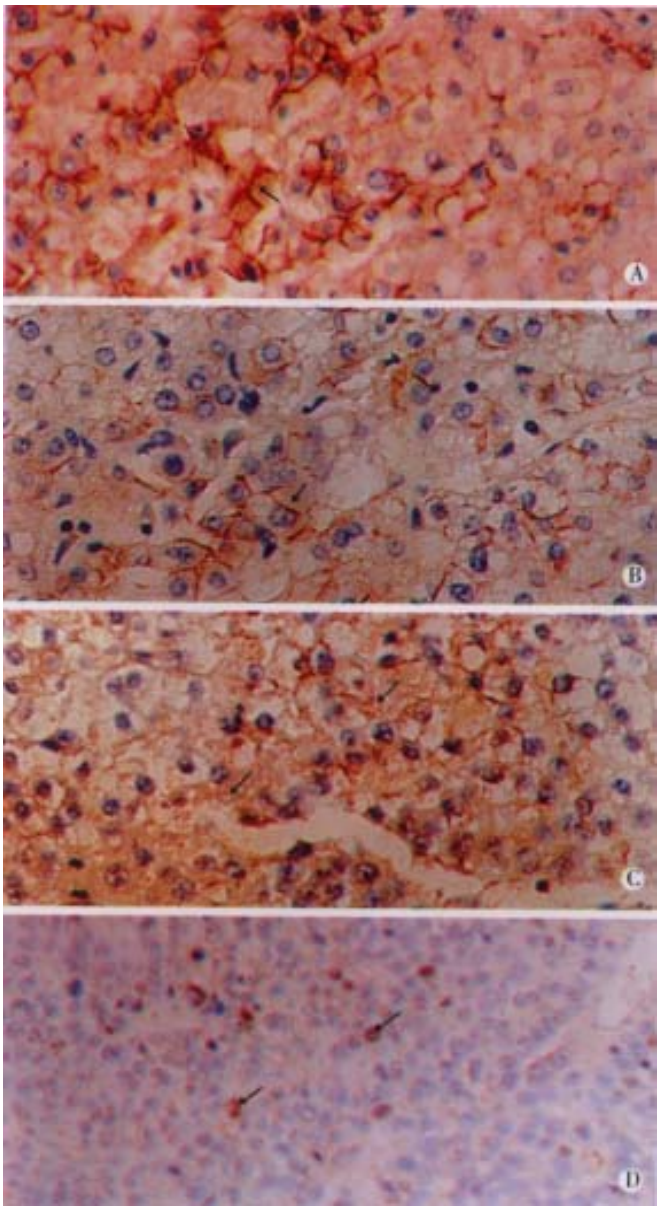

Figure 1 Immunohistochemistry of β-catenin.
A: In normal liver tissue, the staining was mainly positive on the cellular membrane (arrowpoint), with very weak cytoplasmic staining. ×200 B: Para-cancerous cirrhotic liver tissue showed membrane staining (arrowpoint) like normal liver tissue. C,D: For HCC, cytoplasmic and nuclear staining was dominant (arrowpoints), whereas membrane staining was rare. ×200

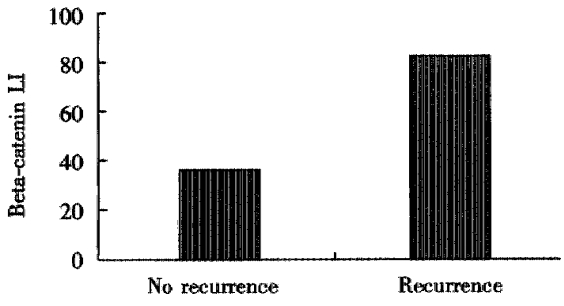

Figure 2 Labeling index (LI) of β-catenin.
Recurrent patient (n = 15) was much higher than that of non-recurrent patient (n=19) (84.9±17.4) *vs* (39.1±14.3).

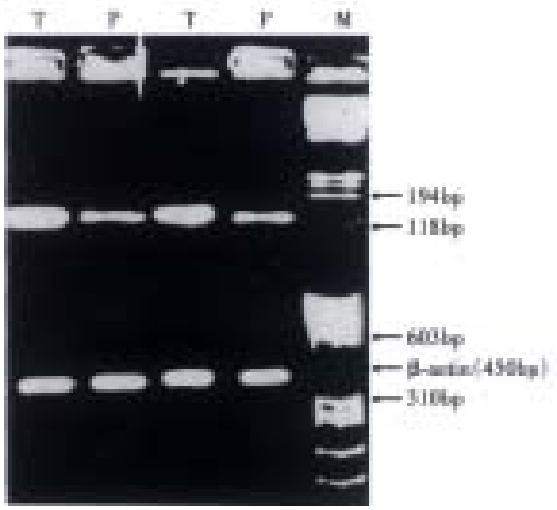

Figure 3 β-catenin mRNA expression index (EI).
HCC was higher *vs* para-cancerous tissue (P<0.05). P: para-cancerous tissue; T: HCC; M: nucleic acid molecular mass marker.

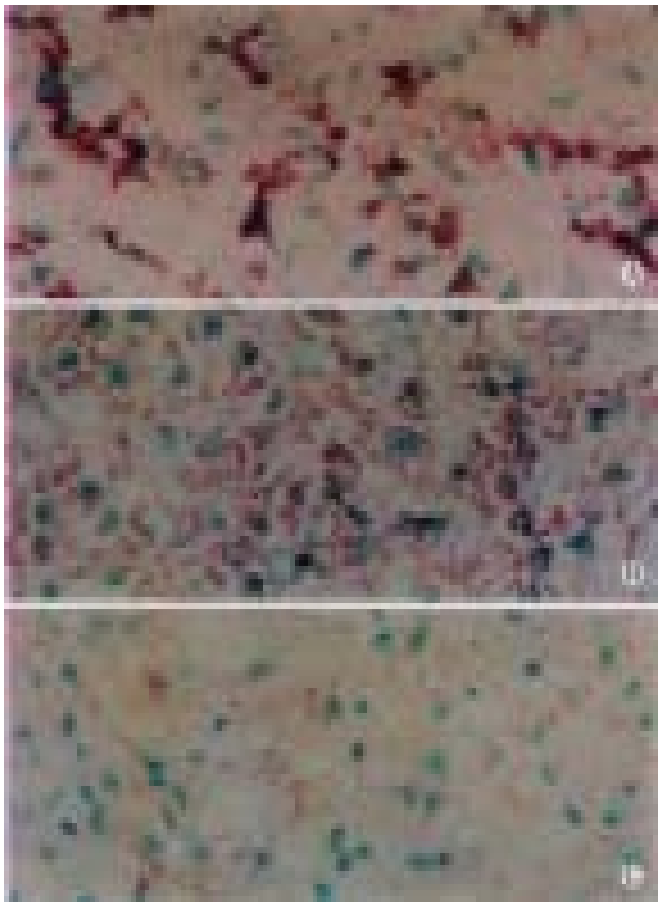

Figure 4 *In situ* hybridization of β-catenin gene mRNA. Stronger in HCC (A) *vs* para-cancerous cirrhotic liver tissue and (B) normal liver tissue (C).

Table 1 Labeling index for β-catenin accumulated type and membraneous type in HCC and para-cancerous tissues (n=34, $\bar{x}$±s)

Tissue	Membraneous	Accumulated
HCC	59.9±26.3	24.6±8.5
Para-cancerous tissue	18.3±9.7[b]	91.8±10.6[b]

[b]P<0.01 *vs* HCC.

Table 2 Relationship between labeling index of β-catenin accumulated type, expression index of β-catenin mRNA and clinicopathological characteristics of HCC

	n	LI of β-catenin accumulated type	EI of β-catenin mRNA
Male	31	58.4±14.2	0.8±0.2
Female	3	54.1±15.3	0.9±0.1
AFP ≤20ng/mL	9	49.3±17.2	0.8±0.1
AFP>20ng/mL	25	54.3±13.7	0.8±0.1
Tumor size			
≤5cm	15	58.7±20.4	0.8±0.2
5cm~10cm	7	54.4±21.3	0.8±0.2
>10cm	12	55.9±17.9	0.8±0.1
Capsule			
Complete	15	72.2±23.4	0.7±0.1
Incomplete	19	44.4±21.1[b]	0.9±0.1[a]
Intrahepatic Metastasis Yes	14	77.2±25.5	0.9±0.2
Intrahepatic Metastasis No	20	41.3±19.6[b]	0.7±0.1[a]
Portal vein thrombus Yes	19	79.8±14.9	0.9±0.2
Portal vein thrombus No	15	52.8±25.9[a]	0.6±0.2[a]
Edmondson's Grade II	19	39.7±20.0	0.7±0.4
Edmondson's Grade III	15	75.9±18.7[b]	0.8±0.2
Cirrhotic nodule ≤ 0.5cm	23	54.3±12.5	0.8±0.2
Cirrhotic nodule >0.5cm	11	62.2±16.6	0.8±0.1

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

DISCUSSION

Previous studies have shown that activation of the wnt pathway results in up-regulation of cytoplasmic β-catenin and its translocation to the nucleus, presumably via the binding of β-catenin to T-cell factor/lymphoid-enhancing factor family members[25,26,30]. Thus, as a first assessment, we examined the subcellular localization of β-catenin in 34 HCC specimens with the result that 61.8% of HCC specimens showed to be accumulated type, suggesting cytoplasmic stabilization of the protein. This showed that activation of Wnt pathway maybe of importance in the carcinogenesis of HCC among Chinese people. Although either β-catenin mutations involving the GSK-3 β phophorylation sites or inactivation of APC and some other factors are related to activation of the Wnt pathway in colon cancer and melanomas[31,32], loss of heterozygosity at the APC locus on chromosome 5 has been detected only at low frequency in human HCC, indicating that inactivation of APC may be infrequent[33]. So mutation of exon 3 of β-catenin gene is probably one of the most important factors activating Wnt pathway and thus causing β-catenin protein accumulated in the cytoplasms in HCC.

Although some studies have been made to investigate β-catenin mutation and abnormal Wnt pathway in HCC[34-41], no previous results have been reported concerning about the relationship between expression abnormality of β-catenin and clinocopathological features of HCC. Furthermore, research reports about the relationship between β-catenin abnormal expression and clinicopathological features of tumors such as colon cancer[42,43], melanoma[44,45], breast carcinoma[46,47], gastric carcinoma[48,49], and lung carcinoma[50,51] are rather various and some of the results were even totally contradictory. That is partly due to most of the previous immunohistochemical studies on β-catenin did not differentiate between membrane-associated type and intracellular accumulated type. Most tumors showed reduced β-catenin in the cytoskeletal fraction but increased β-catenin in the cytosolic fraction and truncated β-catenin protein which was encoded by mutational β-catenin gene was found bound weakly to β-catenin monoclonal antibody when compared with non-truncated β-catenin[52]. This is the reason why we chose polyclonal antibody instead of monoclonal antibody in our study. In this study we aimed to determine which type of expression abnormalities for β-catenin correlate with clinicopathological features and postoperative recurrence in HCC. Our results demonstrated that although great difference existed between cancer tissue and non-cancer tissue, we failed to show the LI of membraneous type to be correlated with the invasiveness of HCC (data not shown here). But, the LI of accumulated type was discovered closely related with the invasive characteristics of HCC, higher EI would predict high ability of invasiveness of HCC and thus a worse prognosis. This was different from another article about gastric carcinoma, which showed that membraneous type, instead of accumulated type, was related to the invasiveness and prognosis of the tumor[47].

Since abnormal expression of β-catenin protein can be caused by both β-catenin gene mutation and over expression, and in some HCCs, both strong membraneous type and accumulated type of staining could be observed, it is our logical thoughts to figure out whether over expression of β-catenin gene existed and what its relationship with the invasiveness of HCC was. This article is the first one to study the β-catenin gene expression in HCC at mRNA level. First we used RT-PCR to examine the expression of β-catenin gene exon 3 mRNA. Since RT-PCR was not very accurate in semi-quantitive analysis of gene expression, we chose *in situ* hybridization to reconfirm the results of RT-PCR. The results of them are the same, that is over expression did exist in HCC and it showed relationship with the invasiveness of HCC (data of relationship between *in situ* hybridization and HCC clinicopathological characteristics not shown). This could give some explanation why strong membranous and cytoplasmic distribution of β-catenin was observed on immunohistochemistry in some HCC while β-catenin gene exon 3 mutation was not observed. It was the accumulation of β-catenin, though apparently normal, that exceeded the capacity of E-cadherin combination and GSK-3 βdegradation, resulting in increase and stabilization of this protein in the cytoplasm.

Although we found that LI of β-catenin accumulated type was related with HCC recurrence, we were unable to find there was such relationship between β-catenin gene EI and HCC recurrence, either by RT-PCR or *in situ* hybridization. This implies that the LI of β-catenin accumulated type would be of greater value in predicting recurrence of HCC. From above we can see that abnormal expression of β-catenin protein, especially the accumulated type, which is closely related to the invasiveness of HCC among Chinese people. Further study should be carried out to confirm this and investigate what the other mechanism causing abnormal expression of β-catenin gene is.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

A study on pathogenicity of hepatitis G virus

Jia-Zhang Xu[1], Zhi-Guo Yang[1], Mei-Zhao Le[2], Mao-Rong Wang[1], Chang-Lun He[1] and Yun-Hua Sui[1]

[1]Institute of Hepatology, the 81st Hospital of PLA, Nanjing 210002, Jiangsu Province, China
[2]Department of Pathology, the 81st Hospital of PLA, Nanjing 210002, Jiangsu Province, China
Supported by the Science Foundation of Jiangsu Province, No. BK97173
Correspondence to Jia-Zhang Xu, Institute of Hepatology, the 81st Hospital of PLA, Nanjing 210002, Jiangsu Province, China
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Abstract

AIM **To study the pathogenicity of hepatitis G virus (HGV) and observe the genesis and pathological process of hepatitis G.**

METHODS **HGV-RNA in serum was detected by RT-PCR assay. The immunohistochemical assays of liver tissue were performed with HGV monocoloned antibody (McAb) expressed from the region of HGV NS5 nucleic acid sequence. The clinical and pathological data of 52 patients with hepatitis G were discussed. In animal experiment, the Chinese Rhesus monkeys were infected with the serum of a patient with HGV infection. And the dynamic changes in serology and liver histology of animals were observed.**

RESULTS **One hundred and fifty-four patients with HGV-RNA positive were selected from 1552 patients with various kinds of hepatitis. Of 154 patients with HGV infection, 52 were infected with HGV only, which accounted for 33.8% (52/154) and 102 with positive HGV-RNA were super-infected with other hepatitis viruses, which accounted for 66.2% (102/154). The clinical and pathological observation showed that the acute and chronic hepatitis could be induced by HGV. The slight abnormality of transaminases ALT and AST in serum of monkeys lasted nearly 12 months and histological results showed a series of pathological changes.**

CONCLUSION **HGV is a hepatotropic virus and has pathogenicty.**

Subject headings hepatitis virus G; pathogenicity

Xu JZ, Yang ZG, Le MZ, Wang MR, He CL, Sui YH. A study on patogenicity of hepatitis G virus. *World J Gastroenterol*, 2001;7 (4):547-550

INTRODUCTION

Hepatitis G virus (HGV/GBV) is a new type of hepatitis virus which was first identified by Simons and Linnen between 1995 and 1996[1,2]. It has been shown that HGV is a single-stranded, positive chain DNA virus which has world-wide distribution, and spread by blood circulation. HGV infection is mostly mixed with infections caused by other types of hepatitis viruses. The patients with single hepatitis G infection were rare. It makes the investigation difficult. Currently, the pathogenicity of hepatitis G virus is controversial. We report the results of our study below.

MATERIALS AND METHODS

Patients

One hundred and fifty-four patients with hepatitis G infection were collected from 1552 patients with different types of hepatitis admitted consecutively to our hospital during recent 4 years. The serum level of alanine trasaminase (ALT) in all investigated patients was twice that of the normal subjects. Fifty-two of these cases were simply HGV infected and 102 were super-infected with other types of hepatitis virus. The liver biopsies were performed in 42 of the 52 patients with single HGV infection.

Etiological assays for serum specimens

Serum specimens were tested for HGV-RNA by retro-polymerase chain reaction (RT-PCR), for HBV-M, anti-HAV IgM, anti-HEV IgG, anti-HCV IgG by enzyme-linked immunosorbent assay (ELISA), for HBV-DNA by PCR, and for HCV-RNA by RT-PCR. Reagents were obtained from Shanghai Meihua Company, DA-AN Genotype-Diagnostic Center of Zhongshan Medical University, Medical Institute of Nanjing Military Area, respectively. The primers of HGV were obtained from the Institute of Microbiology and Epidemiology of Chinese Military Academy of Medical Sciences.

Histological and immunohistochemical assays

The biopsied liver tissues from the patients were sent to 3 pathologists for histological examinations under light and electron microscopy to determine the degree of necrosis of the liver cells, inflammatory cellular infiltration and fibroplastic proliferation. As for immunohistological examinations, monoclonal antibodies (McAb) were obtained from HGV NS5 gene antigen, the labeled antigen and antibody were presented by the Institute of Microbiology and Epidemiology of Chinese Military Academy of Medical Sciences. The histochemical reagents with HCVAg (NS3) and HBsAg were obtained from Beijing Zhongshan Biotechnical Limited Company and Fuzhou Maixin Biotechnical Company Limited, respectively. The technical procedures were followed according to the requirements for use.

RESULTS

The relationship between HG and the other different types of viral hepatitis

One hundred and fifty-four cases of HG in our study were collected from 1552 cases of different types of viral hepatitis

and evidence of an HGV infection were confirmed by positive HGV-RNA twice in all 154 cases. The relationship between them is shown Table 1.

There were 52 patients with the simple HG infection (33.8%), and 103 of HG super-infected with other type of HV. Most of the cases were complicated with HCV and/or HBV infection (66.2%).

The clinical and pathological features of HGV infection

Of 52 cases of HG, the biopsies were performed in 42. The clinical and/or pathological diagnosis of 154 cases is shown in Table 2.

Table 1 The relationship between HG and the other viral hepatitis

Group	No. of cases	No. of positive HGV-RNA	%
HNA-E	583	52	8.9
HA	38	2	5.3
HB	713	69	9.1
HC	125	18	14.4
HB+HC	17	5	29.4
HA+HB	22	2	13.6
HB+HE	20	1	5.0
The others *	7	0	0
Total	1552	154	9.9

*The others include: HA+HC 2, HA+HE 1, HC+HE 3 and HA+HB+HE 1.

Table 2 The clinical and/or pathological diagnosis of 154 cases

Group	No.of cases	Acute hepatitis	Chronic hepatitis	Severe	Cirrhosis hepatitis
Sigle HG	52	2	49		1
Super-infected HG	102	80	4		18

It is difficult to identify what damages of the liver are predominant clinically and pathologically in the patients with HG super-infected with other HV infection. We had intensively observed the clinical manifestations and pathological lesions in single-infected cases of HG so as to better clarify the pathogenicity of HGV. Of the 52 cases, only 2 cases were acute hepatitis (confirmed by clinical and pathological evidence). Most of single-infected patients with HG had chronic hepatitis.

Case 1 A 46-year-old female with a history of surgery in 1988 was hospitalized with endometriosis. During the operation, blood transfusion was taken. Fifty days after transfusion, she felt weak and had a poor appetite, abnormal liver functions with ALT 180U/L. After treatment with biphenyl dimethyl dicaboxylate, the ALT level was lowered. But her liver function was continued to be abnormal (ALT: 66-120, AST: 57-108U/L) for 9 years. Serum examinations of pathogens performed in many large hospitals were negative. She was admitted to our hospital on Sept. 27, 1997. Laboratory examinations showed ALT 106U/L, AST-192U/L, normal level of TSB, A/G 1.17, and r- GP 22%. Serum tests for HA -E were negative. The test for HGV-RNA was positive. Liver biopsy was taken on October 18. Histological examinations showed hepatocyte swelling, acidophilic degeneration, piecemeal necrosis, obvious fibroplastic proliferation in the portal area and P-P bridging hepatic necrosis. The pathologic diagnosis showed chronic hepatitis: G 2-3, S 1-2. CT-displayed splenomegaly and occupied lesions in the liver. The patient was treated with α-Interferon at a dose of 3 million IU three times per week, for 6 months. Thirty days later, HGV-RNA became negative. Two months after cessation of the treatment, the HGV-RNA again became positive. The ALT continued to be abnormal. The patient had faint jaundice and symptoms of liver cirrhosis (Figure 1).

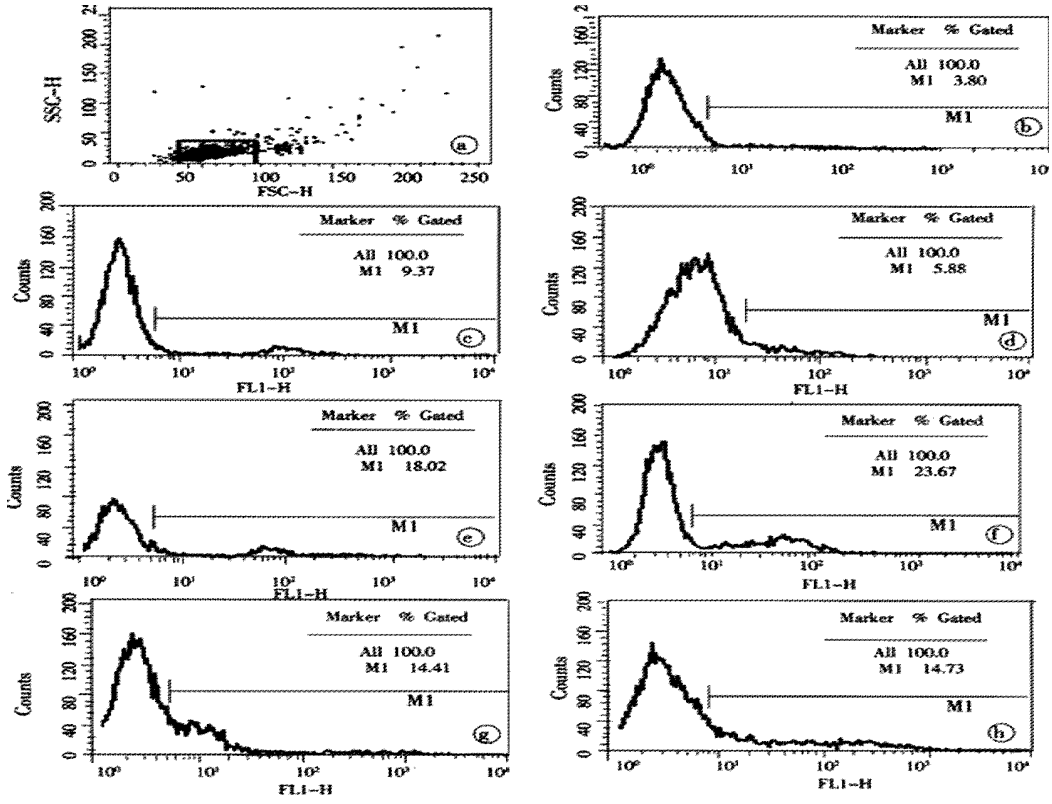

Figure 1 Dynamic changes of serum ALT, AST and HGV-RNA in patient Hu.

Case 2 An 8-year-old girl was admitted on July 9,1998 because of abdominal distension. Splenomegaly and a small amount of ascites were displayed by B super-sound examination. Repeated examinations showed positive HGV-RNA, ALT 60U, AST 120U/L, A/G 0.98, and r-GP 28%. CT showed uneven liver density with small nodular lesions, splenomegaly extended in an area of 8 costae. The clinical diagnosis was chronic hepatitis G infected from liver cirrhosis (Figure 2).

In our group, there were 2 patients with acute hepatitis G having following histological findings: cloudy hepatocyte swelling, partial vacuolation (Figure 3), punctate necrosis of liver cells, focal lymphocyte infiltration, and accidophilic degeneration of partial liver cells. In patients with chronic hepatitis G, the histological examinations of the liver tissue showed extended portal area, a moderate degree of lymphocyte infiltration, and piecemeal necrosis (Figure 4). A tendency of P-P bridge necrosis in the portal area could be found. In a case of acute hepatitits G, the electron micrographs showed shrinkage of liver cells with zigzag edges, extension of rough surfaced endoplasmic reticulum in hepatocytes, and proliferation of collagen fibrils extended into the cytoplasm in the damaged hepatocytes (Figure 5). In immunohistochemical assays, brown-yellow granules were found in the cytoplasm of hepatocytes stained by specific HGV McAb in either acute hepatitis G or chronic hepatitis G patients. The positive-stained granules were also seen in the nuclei of a few hepatocytes (Figure 6). Negative results were obtained by histochemical analysis with anti-HBs McAb and anti-HCV (McAb) expressed from HCV N3. All these results suggested that HGV could cause a series of histological damages of the liver.

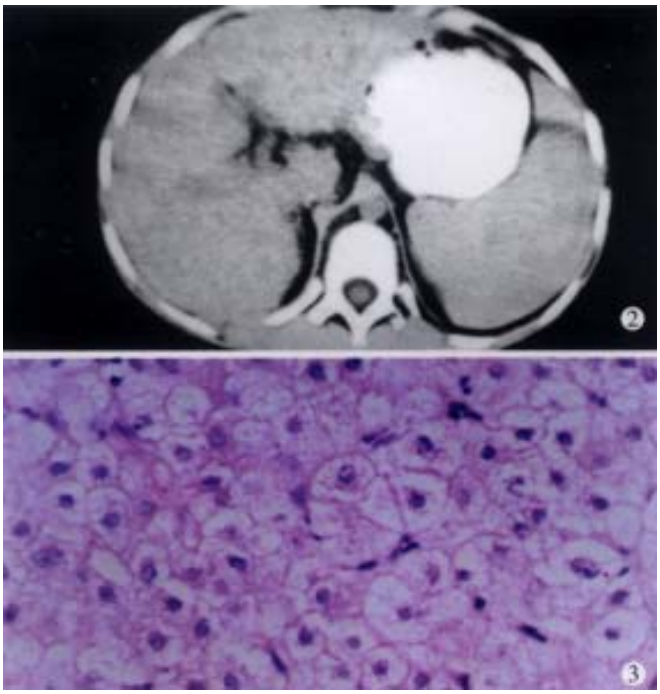

Figure 2 CT picture displays uneven liver density, small nodular changes and splenomegaly extended in an area of eight costae. EM× 15000

Figure 3 Histological changes of the liver showing cloudy swelling of hepatocytes.

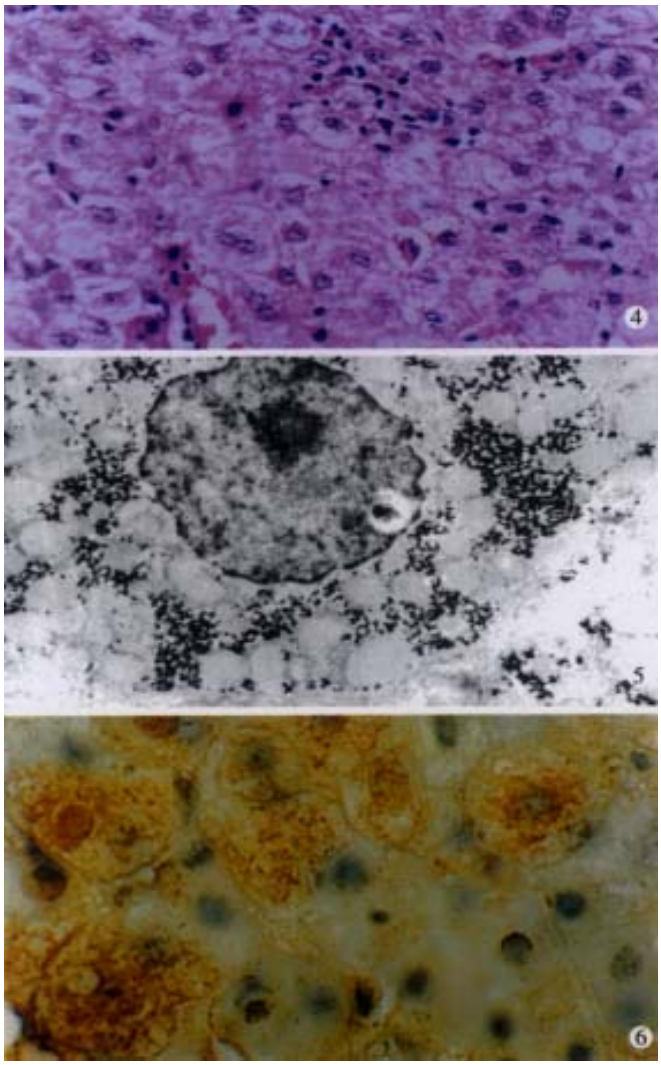

Figure 4 Histological changes of the liver in a patient with acute single-infected hepatitis G showing punctate necrosis, focal lymphocyte infiltration, and acidophilic degeneration of hepatocytes.

Figure 5 Ultrastructure of the liver tissues in a patient with acute simple HGV infection showing shirinkage of liver cells with zigzag edges, extension of rough surfaced endoplastic reticulum of hepatocytes, proliferation of collagen fibrils extended into the cytoplasm of hepatocytes. EM×15000

Figure 6 Immunohistochemical preperation stained by specific HGV McAb for HGVNS5 in a patient with single HG infection showing brown-yellow granules presented mostly in cytoplasm of hepatocytes, and partially in the nuclei. DAB staining, hematoxylin staining, BA staining. (oil)×1000

DISCUSSION

In recent years, the pathogenicity of hepatitis G virus has caused much controversies, because of the following aspects. ① HGV infection is usually mixed with the infections caused by other hepatitis viruses. The patient with single hepatitis G infection are rare; ② A body of evidence has been limited to serologic studies without sufficient histological evidence; ③ Lack of animal modes for the study of hepatitis G. A study on hepatitis G in our hospital was started earliest in China. In 1994, at "World Chinese Symposium on Hepatopathy" our report on clinical and pathological study in chronic hepatitis produced by "non-A, non-B and non-C agents" aroused great interests[3]. In 1996, we reported the paper "Clone of partial

HGV genes in Nanjing, China and analysis of its cDNA sequence", and established the method for determination of HGV-RNA by RT-PCR. The clinical and pathological characteristics of viral hepatitis G were described[4,5]. Of the 154 cases of HG collected from 1552 cases of different kinds of hepatitis in this paper, 52 were single HGV infection and 102 were super-infected with other hepatitis viruses, which accounted for 33.8% (52/154) and 66.2% (102/154) respectively. The clinical and pathological data from the simple HG patients in this study showed that: ① the clinical symptoms of HG patients vary in degree. It was mostly- sub-clinical; ② majority of patients had no jaundice, less hepatic damage than HB and a mild to moderate elevated ALT and AST level; ③ most of the patients were chronic. Of 52 patients with HG, there were 49 chronic cases, 1 cirrhosis and only 2 acute cases in our study. One of the two patients with acute HG had jaundice (TSB 106.7μmol/L) 30 days after blood transfusion, with the ALT level 535U/L and AST 116U/L; ④ histological changes were local necrosis and piecemeal necrosis in most patients, P-P bridge necrosis and a tendency of cirrhosis in a few patients. Electron micrographs of the two acute patients displayed proliferation of collagen fibrils extended into the cytoplasm of hepatocytes; ⑤ HGV antigen was generally present in cytoplasm and sometimes in the nuclei of the hepatocytes by immuohistochemical assay. We studied a Chinese Rhesus monkey intravenously attacked with the serum from a chronic hepatitis G patient only with positive HGV-RNA for one year. The dynamic changes of the serology and histology of the liver in the animal were observed before and after infection. It was found that the serological and histological changes of the animal were similar to that of the patients in our study[6]. Eighteen months after the infection, the monkey was dissected and its internal organs were taken for histological examination. The internal organs were normal except the liver tissue in which there were focal necrosis and slight piecemeal necrosis. The pathological diagnosis was confirmed to be chronic hepatitis. It is suggested that HGV would be a hepatotropic virus and had the pathogenicity to human.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Differential expression of a novel colorectal cancer differentiation-related gene in colorectal cancer

Xing-Guo Li, Jin-Dan Song and Yun-Qing Wang

Key Laboratory of Cell Biology, Ministry of Public Health of China, China Medical University, Shenyang 110001, Liaoning Province, China
Correspondence to Dr. Jin-Dan Song, Key Laboratory of Cell Biology, designated by the Ministry of Health of China, China Medical University, Shenyang 110001, Liaoning Province, China. jdsong@sun10.cmu.edu.cn
Tel/Fax: 0086-24-23256087
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Abstract

AIM **To investigate SBA2 expression in CRC cell lines and surgical specimens of CRC and autologous healthy mucosa.**

METHODS **Reverse transcription-polymerase chain reaction (RT-PCR) was used for relative quantification of SBA2 mRNA levels in 4 human CRC cell lines with different grades of differentiation and 30 clinical samples. Normalization of the results was achieved by simultaneous amplification of** β-actin as an internal control.

RESULTS **In the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82%). Markedly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts ($P<0.01$). SBA2 expression was significantly ($0.01<P<0.05$) correlated with the grade of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated cases. Of the 9 cases with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($0.01<P<0.05$).**

CONCLUSION **SBA2 gene might be a promising novel biomarker of cell differentiation in colorectal cancer and its biological features need further studies.**

Subject headings colorectal neoplasms/genetics; colorectal neoplasms/pathology; DNA, complementary; cell differentiation; gene expression; polymerase chain reaction

Li XG, Song JD, Wang YQ. Differential expression of a novel colorectal ancer differentiation-related gene in colorectal cancer. *World J Gastroenterol*, 2001;7(4):551-554

INTRODUCTION

Colorectal cancer (CRC) is increasing in China[1-10], and the treatment is still difficult in advanced stage[11-20]. Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel CRC differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human CRC cell line CloneA after modulation by sodium butyrate. We investigated the use of reverse transcription-polymerase chain reaction (RT-PCR) for the relative quantification of SBA2 expression in 4 human CRC cell lines with different grades of differentiation and in 30 surgical specimens of CRC and autologous healthy mucosa.

MATERIALS AND METHODS

Materials

The poorly-differentiated human CRC cell line CloneA, moderately-differentiated human CRC cell line CX1, and well-differentiated human CRC cell line CCL187 were obtained from Dana-Farber Cancer Institute, Harvard Medical School. The moderately-differentiated human CRC cell line LS174T was bought from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. These cells were cultured in Dulbecco's modified Eagle's medium containing $50mL \cdot L^{-1}$ calf serum at 37℃ with 100% humidity and $50mL \cdot L^{-1}$ CO_2. Tumor tissues were obtained from 30 patients (15 men and 15 women) at the time of surgery for removal of CRC. All cases were assessed by histopathology. Both tumor tissues and autologous healthy mucosa (at the distance >5cm from the neoplastic focus) were sampled. All specimens were snap frozen and stored in liquid nitrogen. Total cellular RNA was isolated with the TRIzol reagents (Gibco-BRL). The yield and quality of RNA preparation were determined by spectrophotometry.

Quantification of gene expression by semi-quantitative RT-PCR

Reverse transcription of 2μg of total RNA using 0.5μg oligo (dT)-15 primer was performed for 1h at 42℃ in 20μL of a reaction mixture containing 15U AMV reverse transcriptase (Promega), 5 mmol/L $MgCl_2$, 1 × RT buffer (10 mmol/L Tris-HCl, pH 9.0 at 25℃, 50 mmol/L KCl, 1 g/L Triton X 100), 1mmol/L dNTP mixture and 25U of recombinant RNasin ribonuclease inhibitor. The samples were then heated at 99℃ for 5 min to terminate the reverse transcription.

Primers used for amplification of β-actin specific sequence were residues 2217-2238 (TGTATGCCTCTG-GTCGTACCAC; sense-strand) and residues 2009-2030 (ACAGAGTACTTGCGCTCAGGAG; antisense-strand). PCR using these primers yields a 592 bp product. SBA2-specific sequence was amplified by the sense-strand primer (residues 51 - 72: GCTTGTCACGGCTTCTTACGAT) and the antisense-strand primer (residues 390 - 411: GCATAAGTGCTTCAGTGAGGAC), which yield a 361bp product.

Unless otherwise specified, 2.5μL of the reversely-

transcribed mixture was used as template DNA and amplified in a reaction volume of 25 μL containing 1 mmol/L $MgCl_2$, 1 ×RT buffer (10mmol/L Tris-HCl, pH 9.0 at 25℃, 50mmol/L KCl,1g/L Triton X100), 0.2μmo/L each of 5' and 3'primers for β-actin, 0.5μmol/L each of 5' and 3' primers for SBA2. After heat denaturation at 95℃ for 5min, 1.25U of thermus aquaticus DNA polymerase (Promega) was added to the mixture. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) in sequential cycles at 94℃ 30s; 68℃ 45s and 72℃ 1 min. After the last cycle, all samples were incubated for an additional 10 min at 72℃. Ten μL of PCR product were separated on 15g/L agarose gels, stained with ethidium bromide, and loaded onto 80g/L polyacrylamide gels, stained with DNA silver staining system (Promega). The gel was analyzed with the Electrophoresis Documentation and Analysis System 120 (Kodak 1D).

RESULTS

Linearity of PCR amplification

The yield of PCR product is proportional to the starting amount of the template only under conditions in which PCR amplification proceeds exponentially at a constant efficiency. To establish the optimal conditions for detection and quantification of SBA2 expression, the relative yield of PCR products was determined by terminating aliquots of starting reaction solution at sequential PCR cycles as shown in Figure 1. The resultant amplified sequences were analyzed and the signal intensities of the bands were plotted on a semilogarithmic scale against the cycle number to obtain amplification curves. Figure 1 shows that at higher cycle numbers (>28), the yield of the SBA2-specific product approaches the plateau while β-actin shows a tendency of saturation beyond 26 cycles. Before saturation at the plateau, however, good linearity is observed for both SBA2 and β-actin amplification over the range of cycles examined. Figure 2 compares the results obtained with successive dilutions of cDNA. The serial 1:2 dilutions were performed beginning with 5μL of reversely-transcribed mixture, as shown in the graph, and amplified 24 cycles. Thus the number of 24 cycles and the volume of 2.5μL of cDNA were chosen as the optimal parameters for the semi-quantitative RT-PCR.

If the efficiency of amplification is the same for each cycle (as indicated by the good linearity of the curves), the amount of cDNA produced can be predicted from the equation $cDNAn = (cDNA_0) \times (1+R)^n$, where cDNAn stands for the amount of product after n cycles, $cDNA_0$ is the initial amount of cDNA, and R is the efficiency of amplification. The efficiency of the amplification can be assessed from the following equation: $\log cDNAn = \log cDNA_0 + n \times \log (1+R)$. Within the exponential range, the linear regress equation for SBA2 is $y = 0.2612 \times x - 0.6179$ ($r = 0.9496$), for β-actin: $y = 0.2594 \times x - 0.4468$ ($r = 0.9754$), where y is the logarithm of cDNAn, x is the number of PCR cycles (n), r is correlation coeficient. The slope of the curve should be $\log (1+R)$ in the semi-logarithmic plot. Thus an R- value of 82% and 82% can be determined for SBA2 and β-actin respectively. Since the same amplification efficiency is observed for both the target and the internal control within the exponential range, the relative amounts of SBA2 mRNA can be determined by comparison with β-actin.

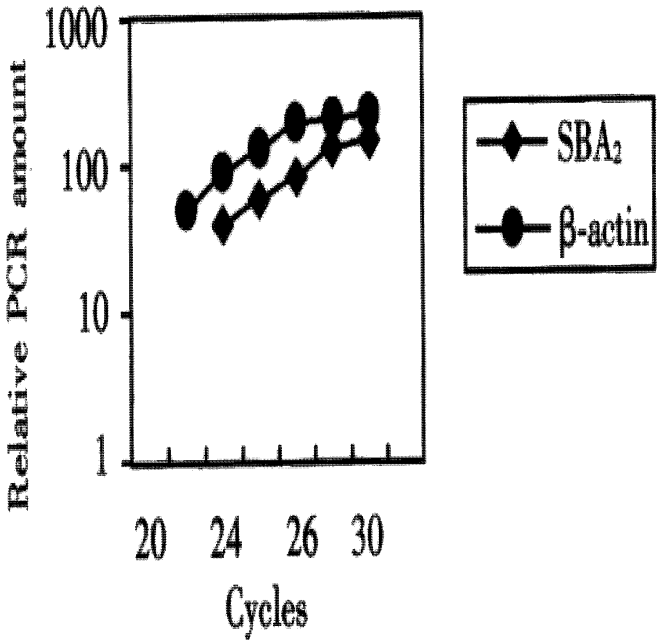

Figure 1 Detection of the exponential range by termination at sequential PCR cycles.
1-7: Cycles of 20, 22, 24, 25, 26, 28 and 30, respectively. M: Marker (100 bp DNA ladder).

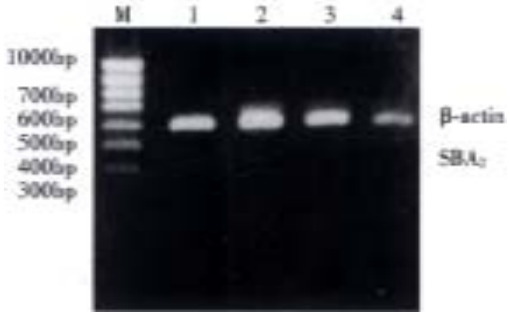

Figure 2 Detection of the exponential range by serial dilutions of cDNA.
1-4: cDNA of volume of 5, 2.5, 1.25, and 0.625μL, respectively. M: Marker (100 bp DNA ladder).

Patterns of SBA2 expression

In cultured cell lines and clinical specimens of CRC, the assay described above was used to investigate the levels of SBA2 mRNA in 4 human CRC cell lines and in 30 surgical samples of CRC and autologous healthy colonic mucosa. In the 30 samples analyzed, significantly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts (Wilcoxon test, $T = 6$, $P<0.01$).

Differential expression of SBA2 in clinical samples of CRC with different grades of differentiation was similar to that observed in 4 cell lines of CRC, as shown in Figures 3 and 4, respectively. Thirty clinical samples were assigned into 3 groups, depending on the grade of differentiation. The levels of SBA2 mRNA were significantly (q test, $0.01<P<0.05$) correlated with the degree of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated ones. Mean levels of SBA2 mRNA was calculated for each group. There was a significant difference between well-differentiated group (0.384 ± 0.024) and poorly-differentiated group (0.158 ± 0.014) ($q = 3.9564$, $0.01<P<0.05$), between moderately-differentiated (0.297

±0.015) and poorly-differentiated ($q = 3.2767$, $0.01<P<0.05$), in spite of no significant difference ($q = 0.0798$, $P>0.05$) between well-differentiated and moderately-differentiated.

We also analyzed the relationship between SBA2 expression and lymph nodes metastasis. Of the 9 patients with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($\chi^2=5.5622$, $0.01<P<0.05$).

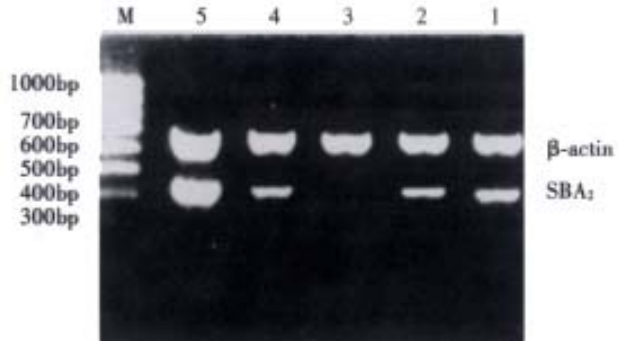

Figure 3 Relative quantification of SBA2 mRNA in colorectal tissues. 1-4: Moderately, moderately, poorly, and well differentiated CRC, respectively. 5: Healthy colorectal mucosa. M: Marker (100 bp DNA ladder).

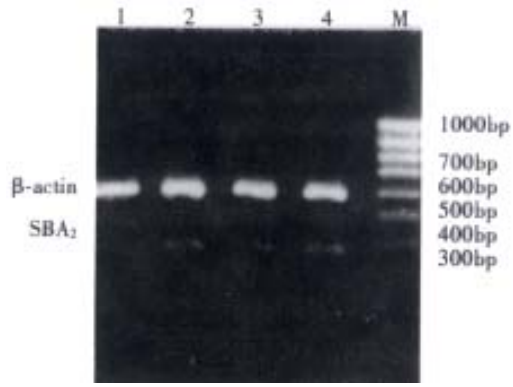

Figure 4 Relative quantification of SBA2 mRNA in cell lines of CRC. 1-4: Human CRC cell line CloneA, CX1, CCL187, and LS174T, respectively. M: Marker (100 bp DNA ladder).

DISCUSSION

We have developed an efficient protocol for relative quantification of gene expression in both clinical samples and cell lines of CRC by semi-quantitative RT-PCR. Linear evaluation of gene expression over a wide range was achieved by plotting the amplification curves of the sequence of interest and the β-actin sequence that served as an internal control. Determination of the optimal parameters of an exponential range was carried out by terminating the PCR at sequential cycles and by serially diluting the amount of cDNA. The amplification efficiency of PCR and the amount of sample cDNA have been verified by the simultaneous amplification of a sequence of a gene that serves as an internal control, such as, aldolaseA, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (G_3PD), dihydrofolate reductase[22]. However, the simultaneous amplification of a large amount of a sequence of an internal control can affect the efficiency of amplification of the sequence of interest[23]. A possible explanation of the competition is that the effect of an internal control sequence on amplification efficiency may depend on the gene sequences amplified[24]. For instance, the simultaneous amplification of a β2-microglobulin mRNA sequence suppressed the amplification efficiency of an *mdr* mRNA sequence, while amplification of β-actin sequence did not affect the amplification efficiency of a tax/rex sequence[25]. In our study, in the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82% for both). Thus β-ctin can be used as an internal control to normalize the relative levels of SBA2 mRNA in this experiment.

Histologic types reflect the biologic nature of the cancer, exercising the most decisive influence upon the diagnosis and prognosis. A variety of pathologic changes are all closely associated with the cellular differentiation in CRC, including gross typing, growth pattern, depth of infiltration and metastasis into lymph nodes[26,27]. Classic grading categorized CRC into three discrete classes: well, moderately and poorly differentiated. Such grading, however, is largely dependent on the subjective assessment of the histopathologist. Heterogeneity of differentiation in the same cancer specimen often leads to considerable inter-and intra-observer variation in grading[28]. Colorectal neoplasia develops in a mucosa that has alterations in proliferation, maturation, and differentiation[29]. Normally, the major zone of cell proliferation is at the base of the crypts (the lowest one third) with little extension to the surface of proliferating cells. As cells migrate from the crypts to the luminal surface, they become increasingly differentiated and mature so that by the time they have reached the surface they have lost their proliferative capabilities, finally leading to apoptosis (i.e. programmed cell death)[30].

Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel colorectal cancer (CRC) differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human colorectal cancer cell line CloneA after modulation by sodium butyrate. It consisted of 2470 nucleotides and an open reading frame (ORF) encoded 404 amino acid residues with a M_r of 44400. The deduced amino acid sequences showed significant homology to mouse SWiP-2 (96%), mouse WSB-2 (95%) and human WSB-1 (52%), which belong to a new family of the suppressor of cytokine signaling (SOCS). The SBA2 protein product may be a new member of SOCS protein family negatively regulating cytokine signal transduction. We have used RT-PCR for relative quantification of SBA2 mRNA levels in 4 CRC cell lines with different grades of differentiation and 30 surgical specimens of CRC and autologous healthy mucosa. Our assay showed significantly lower levels of SBA2 mRNA in tumors, as compared with coupled normal counterparts. Furthermore, differential expression of SBA2 in CRC with different grades of differentiation was observed both in clinical tissues and in cell lines SBA2 mRNA levels were correlated with the degree of differentiation in CRC, being relatively higher in well-differentiated samples and lower in poorly-differentiated ones. There was also a significant difference in the frequency of lower-level SBA2-expressing samples between patients with lymph nodes metastasis and those without metastasis. It was indicative of the close association of SBA2 with metastasis in CRC. The reduced levels of SBA2 mRNA in CRC and the correlation between SBA2 mRNA expression and the grade of differentiation in CRC suggest that SBA2 might be a

promising novel biomarker of cell differentiation of CRC, and that the biological features of SBA2 and its product in CRC and other tumors need further studies.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Relationship between plasma D(-)-lactate and intestinal damage after severe injuries in rats

Xiao-Qing Sun[1], Xiao-Bing Fu[1], Rong-Zhang[1], Yi Lü[1], Qun Deng[2], Xiao-Guo Jiang[1] and Zhi-Yong Sheng[1]

[1]Burn Institute, 304th Hospital, Beijing 100037, China
[2]Department of General Surgery, Chinese PLA 304 Hospital, Beijing 100037, China
Project supported by the Fund for National Outstanding Young Researchers of China, No. 39525024
Correspondence to Dr. Xiao-Qing Sun, Trauma Center, 304th Hospital, Beijing 100037, China. fuxb@cgw.net.cn
Tel: 0086-10-66867391, Fax: 0086-10-68429998
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Abstract

AIM **To explore the kinetic changes in plasma D(-)-lactate and lipopolysaccharide (LPS) levels, and investigate whether D(-)-lactate could be used as a marker of intestinal injury in rats following gut ischemia/reperfusion, burn, and acute necrotizing pancreatitis (ANP).**

METHODS **Three models were developed in rats: ① gut ischemia/reperfusion obtained by one hour of superior mesenteric artery occlusion followed by reperfusion; ② severe burn injury created by 30% of total body surface area (TBSA) full-thickness scald burn; and ③ ANP induced by continuous inverse infusion of sodium taurocholate and trypsin into main pancreatic duct. Plasma levels of D(-)-lactate in systemic circulation and LPS in portal circulation were measured by enzymatic-spectrophotometric method and limulus amebocyte lysate (LAL) test kit, respectively. Tissue samples of intestine were taken for histological analysis.**

RESULTS **One hour gut ischemia followed by reperfusion injuries resulted in a significant elevation in plasma D(-)-lactate and LPS levels, and there was a significant correlation between the plasma D(-)-lactate and LPS (r = 0.719, P<0.05). The plasma concentrations of D(-)-lactate and LPS increased significantly at 6h postburn, and there was also a remarkable correlation between them (r = 0.877, P<0.01). D(-)-lactate and LPS levels elevated significantly at 2h after ANP, with a similar significant correlation between the two levels (r =0.798, P<0.01). The desquamation of intestine villi and infiltration of inflammatory cells in the lamina propria were observed in all groups.**

CONCLUSION **The changes of plasma D(-)-lactate levels in systemic blood paralleled with LPS levels in the portal vein blood. The measurement of plasma D(-)-lactate level may be a useful marker to assess the intestinal injury and to monitor an increase of intestinal permeability and endotoxemia following severe injuries in early stage.**

Subject headings gut/injury; ischemia reperfusion/blood; burn/blood; acute necrotizing pancreatitis/blood; D(-)-lactate/blood; lipopolysaccharide/blood; intestinal permeability

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INTRODUCTION

Apart from their major functions of digestion and absorption of nutrients, the intestines also act as a barrier to prevent micro-organisms and toxins contained within the lumen from spreading to distant tissues and organs[1-7]. Failure of intestinal barrier function often occurs in many clinical conditions, including hemorrhage shock, severe burn injury, and the surgically critical illness, resulting in the increased intestinal permeability and subsequent translocation of bacteria or/and endotoxin from gut[8-12]. It is clear that increased gut permeability and bacteria with or without endotoxin translocation play a key role in the development of severe complications such as systemic inflammatory response syndrome (SIRS), sepsis, multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF)[13-20]. Therefore, it is important to know the intestinal injuries following a variety of insults (shock, burn injury, sepsis, and some critically surgical illness)[21,22]. D(-)-lactate is a product of bacterial fermentation. It is produced by many of the bacteria found in the human gastrointestinal tract[23]. Tissues in mammalian do not produce it and its metabolism is very slow[24]. In this study, we investigated the changes of plasma D(-)-lactate and lipopolysaccharide (LPS) levels and their correlation in gut ischemia/reperfusion, burn injury and acute necrotizing pancreatitis (ANP); and explored whether the changes of D(-)-lactate levels could be used as a predictor of increased intestinal permeability and endotoxemia following severe injuries.

MATERIALS AND METHODS

Animals

Male Wistar rats were used in this serial studies. They were housed in individual cages. The room temperature was maintained at 22℃ - 24℃ with a 12h light-dark cycle, and free access to a commercial laboratory rodent chow and fresh water were allowed. Twelve hours prior to experiment, the rats were fasted, but allowed free access to water.

Rat models of gut ischemia/reperfusion

Rats weighing 190g - 250g were divided into three groups. Gut ischemic group (n = 20): Animals were anesthetized with an intraperitoneal injection of 0.3mL 30g·L^{-1} pentobarbital sodium. Through a middle abdominal incision, intestinal

ischemia was produced by occluding the superior mesenteric artery for 1 and 1.5h with an automatic microvascular clamp. Animals were sacrificed at the end of gut is chemia. Gut ischemia/reperfusion group (n=50): superior mesenteric artery was occluded for 1h and then the vascular clamp was removed to produce gut reperfusion. Animals were sacrificed at 0.5, 1, 2, 6 and 24h after gut reperfusion. Sham-operated control (n=10): animals were treated identically omitting the superior mesenteric artery occlusion. Blood samples were collected aseptically from cervical artery and portal vein for D (-)-lactate and LPS assay before animals were killed at each time point.

Rat models of burn

Male Wistar rats weighing 190g - 250g were used. Animals were divided into two groups. In thermal group, they were subjected to a 30% total body surface area (TBSA) full-thickness scald burn injury (n=40). They were anesthetized with an intraperitoneal injection of 30g·L^{-1} pentobarbital sodium (60mg·kg^{-1}) and then the dorsal hair was shaved. A 30% TBSA full-thickness burn was created on the back of the rats in boiling water at 98℃-100℃ for 12s. Rats were resuscitated immediately after thermal injury with 50g·L^{-1} glucose saline solution (50mL·kg^{-1}) intraperitoneally. In the control group (n=10), rats were exposed to the room-temperature water. Animals in thermal group were killed at 3, 6 12 and 24h after burn. Blood samples were collected aseptically from cervical artery and portal vein before the rats were killed at each time point.

Rat models of acute necrotizing pancretitis (ANP)

Male Wistar rats weighing 270g-330g were randomly divided into two groups. In the ANP group (n=27), animals were anesthetized with 30g·L^{-1} pentobarbital sodium (60mg·kg^{-1}, ip). After medium laparotomy, the duodenum was mobilized and the pancreatic duct was identified at its duodenal junction. ANP was induced by a continuous inverse infusion of sodium taurocholate (50g·L^{-1}, 1mL·kg^{-1}) and trypsin (1.67×10^{5}U·kg^{-1}) into the main pancreatic duct. Animals were immediately given saline (50mL·kg^{-1}) subcutaneously after injury. In control group (n=6), animals were treated identically with infusion saline. Blood samples were taken aseptically from cervical artery and portal vein at 2, 8, 24 and 48h after injury.

D(-)-lactate determination

The plasma from systemic blood samples was obtained and subjected to a deproteination and neutralization process by acid/base precipitation using perchloric acid and potassium hydroxide. The protein-free plasma was then assayed for D (-)-lactate concentration by enzymatic-spectrophotometric method with minor modification[25].

Lipopolysaccharide (LPS) determination

The plasma from portal vein blood was also obtained and subjected to a deproteination and neutralization process by acid/base precipitation using perchloric acid and sodium hydroxide. The LPS levels of portal vein blood were assayed by the chromogenic limulus amebocyte lysate (LAL) test with a kinetic modification according to the test kit procedure[26].

Morphologic studies

Tissue samples of intestines were taken for morphologic study. Biospies were fixed in 100mL·L^{-1} neutral buffered formalin, embedded in paraffin, microtome sectioned at 4μm-6μm thickness, and stained with hematoxylin and eosin. Sections were examined under light microscope.

Statistical analysis

Data were expressed as means ± SD. The statistical significance of mean values between groups was evaluated by the Student's t test. The relationship between circulating systemic D(-)-lactate and portal vein LPS concentrations was determined by the calculation of Pearson correlation coefficient. P<0.05 was considered to be significant.

RESULTS

Kinetics of D(-)-lactate and lipopolysaccharide concentrations in plasma after gut ischemia/reperfusion in rats

One hour of gut ischemia alone induced a slight increase in systemic blood D(-)-lactate and portal vein blood LPS concentrations (Table 1). Either D(-)-lactate or LPS concentrations had a further significant increase at 0.5h-2h after gut reperfusion (P<0.05-0.01), and decreased to normal at 6h. Meanwhile, correlation analysis revealed a significant correlation between systemic blood D(-)-lactate levels and portal vein blood LPS concentrations (r = 0.719, P<0.05).

Table 1 The plasma contents of D(-)-lactate and lipopolysac charide in rats after gut ischemia / reperfusion insults (mean±SD)

Groups	Time (h)	No.	D(-)-lactate (mmol/L)	LPS(EU/L)
Sham-operated control		10	0.234±0.072	380±84
Gut ischemia	1	10	0.260±0.086	407±41
	1.5	10	0.269±0.092	453±129
Gut ischemia / reperfusion	0.5	10	0.489±0.179[b]	576±244[a]
	1	10	0.373±0.179[a]	611±278[a]
	2	10	0.253±0.062	562±167[a]
	6	10	0.237±0.044	335±73
	24	10	0.228±0.025	283±81

Compared with sham-operated control, respectively: [a]P<0.05; [b]P<0.01.

Alterations in plasma D(-)-lactate and LPS levels in thermal rats

Results presented in Table 2 indicated that there was a significant increase both in circulating blood D(-)-lactate and portal vein blood LPS concentrations at 6h after injury, and kept significantly increasing to the end of our observation period (72h, P<0.01). In addition, correlation analysis revealed that there was a strong positive correlation between plasma levels of D(-)-lactate and LPS after injury (r = 0.877, P<0.01).

Table 2 Changes in systemic blood D(-)-lactate levels and portal blood LPS content in thermal rats (mean±SD)

Groups	Time(h)	No.	D(-)-lactate (mmol/L)	LPS (EU/L)
Control group		10	0.275±0.175	118±37
Thermal group	3	10	0.371±0.123	159±83
	6	10	0.517±0.162[a]	347±111[a]
	12	10	0.619±0.208[a]	670±139[a]
	24	10	0.638±0.198[a]	396±57[a]

Compared with control group, respectively: [a]P<0.01.

Changes in plasma D(-)-lactate and LPS levels in ANP rats

In rats subjected to ANP, the levels of D(-)-lactate in systemic blood and LPS in portal vein blood began to increase at 2h after ANP (P<0.01) (Table 3), and peaked at 24h after injury. Furthermore, a marked correlation was noted between the changes in contents of plasma D(-)-lactate and LPS (r=0.798, P<0.01).

Table 3 Alterations in systemic blood D(-)-lactate levels and portal blood LPS content in ANP rats (mean±SD)

Groups	Time(h)	No.	D(-)-lactate (mmol/L)	LPS (EU/L)
Control group		6	0.157±0.044	105±7
ANP group	2	6	0.328±0.063[a]	301±131[a]
	8	7	0.507±0.157[a]	449±164[a]
	24	7	0.653±0.216[a]	611±210[a]
	48	7	0.448±0.112[a]	422±136[a]

Compared with control group, respectively:[a]P<0.01.

Gut pathology

Mucosal edema, necrosis, and the loss of the epithelium in mucosa, as well as vascular dilution, congestion, edema and inflammatory cell infiltration in the lamina propria were observed in small intestinal biopsies in three groups. The intestinal injury paralleled with the changes of plasma D(-)-lactate levels.

DISCUSSION

The present study showed that the intestinal damage caused by gut ischemia caused a slight increase in plasma concentrations of D(-)-lactate in systemic blood and LPS in portal vein blood. After gut ischemia followed by reperfusion, the plasma levels of D(-)-lactate and LPS significantly elevated, but declined to normal rapidly at 6h after reperfusion[27-29]. The intestinal damage mediated by burn injury or ANP displayed a more severe damage than that in gut ischemia/reperfusion. A remarked increase of plasma D(-)-lactate and LPS concentrations occurred at 6h, and 3h after insult, respectively, and persisting to the end of our observation. Moreover, the elevation of plasma D(-)-lactate levels in systemic blood was associated with increased plasma LPS contents in portal vein blood, and histological examination also exhibited intestinal injury in those three rodent models.

D(-)-lactate is produced by some bacteria including *Klebsilla*, *Escherichia coli*, *Lactobacillus species*, and *Bacteroides species*. It is an indigenous products in gut[24]. Normally, serum levels of D(-)-lactate in mammals are quite low. During the event that an ischemia/reperfusion insults, the mucosa is injured and intestinal permeability is increased, leading to an efflux of bacteria and the products of their metabolism[30-33], including D (-)-lactate into the circulation. Otherwise, the gut ischemic insult leads to a loss in normal host defenses against bacterial overgrowth, resulting in increased numbers of bacteria within the lumen of the infected intestine[34-36]. This bacterial proliferation would be expected to cause an increased bacterial metabolism with increased production of D(-)-lactate. Mammals do not possess the enzyme system to rapidly metabolize D (-)-lactate, thus, it passes through the liver with unchanged way and enters the peripheral blood early in the disease process. Thus, D(-)-lactate accumulation in the systemic circulation can generally be considered as a result of bacterial over growth and increase in gut permeability induced by some gastrointestinal disorders. Therefore, D(-)-lactate levels could be used as a predictor of intestinal injury. In fact, the elevation of plasma D(-)-lactate levels has been used as the predictor of bacterial infection in patients with short-bowel syndrome[37]. In rat model of acute mesenteric ischemia, D (-)-lactate was significantly elevated after gut ischemia, and the histopathological evaluation scores of intestinal injury were remarkably correlated to the plasma D(-)-lactate levels[38,39]. Recently, in clinical study, it has also been demonstrated that patients with mesenteric ischemia at laparotomy had significantly elevated D(-)-lactate levels in systemic circulation as compared with patients operated on for an acute abdomen or normal abdomen[40].

In conclusion, our data in these rat models suggest that the changes in D(-)-lactate concentrations paralleled with LPS concentrations, and correlated similarly with the intestinal histopathological alterations as well. Therefore, plasma D(-)-lactate in systemic circulation measurement would be a useful marker to evaluate intestinal injury and endoxemia following severe injuries.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Killing effect of TNF-related apoptosis inducing ligand regulated by tetracycline on gastric cancer cell line NCI-N87

Xiao-Chao Wei[1], Xin-Juan Wang[1], Kai-Chen[1], Lei Zhang[1], Yu Liang[1] and Xin-Li Lin[2]

[1]Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100083, China
[2]Protein Studies, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA
Supported by the National Natural Science Foundation of China, No. 39870850
Correspondence to Dr. Xin-Juan Wang, Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 38 Xue Yuan Road, Haidian District, Beijing 100083, China. Xinjuan@sun.bjmu.edu.cn
Tel: 0086-10-62091158, Fax: 0086-10-62015681
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Abstract

AIM **To clone the cDNA fragment of human TRAIL (TNF-related apoptosis inducing ligand) into a tetracycline-regulated gene expression system, the RevTet-On system, transduce expression vectors into a gastric carcinoma cell line-NCI-N87 and examine the effects of controlled expression of TRAIL *in vitro* on the gastric carcinoma cells.**

METHODS **The full-length cDNA of TRAIL was inserted into a vector under the control of the tetracycline-responsive element (TRE) to obtain the plasmid pRevTRE-TRAIL, which was transfected into a packaging cell line PT67. In addition, vector pRev-Tet On and pRevTRE were also transfected into PT67 separately. After hygromycin and G418 selection, the viral titer was determined. The medium containing retroviral vectors was collected and used to transduce a gastric carcinoma cell line NCI-N87. The resulting cell line NCI-N87-Tet On TRE-TRAIL and a control cell line, NCI-N87 Tet On-TRE, were established. TRAIL expression in the cell line was induced by incubating cells with doxycycline (Dox), which is a tetracycline analogue. The killing effect on gastric carcinoma cells was analyzed after induction.**

RESULTS **The recombinant plasmid pRev-TRE-TRAIL was constructed. After hygromycin or G418 selection, the producer cell lines PT67-TRE, PT67-TRE-TRAIL and PT67-Tet On were obtained, with titers of about 10^8 CFU·L^{-1}. By transducing NCI-N87 cells with retroviral vectors from these cell lines, stable cell lines NCI-N87-Tet On TRE-TRAIL (NN3T) and control cell line NCI-N87-Tet On TRE (NN2T) were established. The growth curves of the selected cell lines were the same with the wild type NCI-N87. When Dox was added, cell death was obvious in the test groups (29%-77%), whereas no difference was observed in control and wild type cell lines. With the addition of a medium from the test group, human leukemia cell line Jurkat was activated till death (83%), indicating the secretion of active TRAIL proteins from the test cells to the medium.**

CONCLUSION **With the use of the RevTet-On system, a regulated expression system for TRAIL was constructed. Using this system, the selected killing effect of TRAIL on gastric carcinoma cell line NCI-N87 could be observed.**

Subject headings TRAIL; Tet gene expression system; gastric carcinoma; stomach neoplasms/pathology; tumor cells, cultured; tumor necrosis factor; tetracycline; apoptosis; gene expression

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INTRODUCTION

TRAIL (TNF-related apoptosis inducing ligand, Apo2 ligand, Apo 2L), which belongs to the tumor necrosis factor (TNF) cytokine family, can induce rapid apoptosis in a wide variety of tumor cell lines[1-3]. TRAIL protein consists of 281 (human TRAIL) or 291 (mouse TRAIL) amino acids.The human TRAIL gene is located in chromosome 3(3q26). The mRNA distribution of TRAIL is broad[1,4-8]. Although the *in vivo* role of the TRAIL is not currently known, *in vitro* studies have found that TRAIL is capable of inducing apoptosis in a wide range of human tumor cells, but generally not normal cells[9-16]. TRAIL induces apoptosis by binding and cross-linking death-domain containing receptors[17-21], TRAIL-R1[22,23] (also known as DR4) and TRAIL-R2[24-26] (DR5). Apoptotic signaling occurs via recruitment of adapter proteins, which results in the activation of caspases[24,27]. Because of its selective killing effect on tumor, TRAIL is likely to be a drug in cancer treatment[28-30]. Therefore, we tested the effect of TRAIL expression in human gastric carcinoma cells, most commonly found in oriental population. In addition, the study may form a basis for the safety and effectiveness of TRAIL expression in gene therapy of cancer.

In gene therapy, it is important to regulate gene expression effectively. The tetracycline-controlled gene expression system (Tet system) is a tight control system[31]. It is based on the Tn10 specified tetracycline resistance operon of *E. coli*. In the Tet-Off system, the regulator unit, encodes a hybrid tTA (tetracycline-controlled transactivator) protein composed of the tetracycline repressor (tetR) fused to the herpes simplex virus (HSV) transactivator protein, VP16. The response unit, tetracycline-responsive element (TRE), is composed of the tetracycline resistance operon regulatory elements (tetO) embedded within a minimal cytomegalovirus (CMV) promoter. The expression of a gene inserted downstream of the promoter is highly dependent on tTA, which binds tetO sequences. With the addition of tetracycline, tTA protein dissociates from the TRE and the gene expression is inhibited. Conversely, the Tet-On system allows gene expression to be activated by the addition of

tetracycline. It is based on the reverse tTA (rtTA) in the regulator unit.

The RevTet-Off/On system uses the retroviral vectors. They have the characteristic of Tet system, and their gene transfer is more rapid and efficient, which can be used in gene therapy. In this study, TRAIL gene was cloned into the RevTet-On system, to achieve tight control of the expression of TRAIL protein in mammalian cells. The gene expression system on gastric carcinoma cell line NCI-N87 was constructed, and the killing effect of controlled-gene expression in the resulting cells was observed.

MATERIALS AND METHODS

Construction of retroviral plasmids

The RevTet-On System, which include pRevTet-On and pRev-TRE vectors and the RetroPack PT67 cell line, were purchased from Clontech, Palo Alto, California, USA. TRAIL gene was obtained by using PCR method from a human placenta cDNA library (Clontech). The PCR primers are: 5'-AAGCTTATGGCTATGATGGAGGTCCAGGGGGG-AC-3'and 5'-AAGCTTTTAGCCAACTAAAAAGGCCCCG-AAAAAACTGGC-3'. The resulting PCR product was cloned into an intermediate vector PCR-2 (Invitrogen, Carlsbad, California, USA) and then cut with *Hin* dIII, which was cloned into the same site in vector pLNCX, resulting in vector pLNCX-TRAIL. The Tet-regulated vector pRev-TRE-TRAIL was cloned in the same manner.

Cell culture

The retroviral packaging cell line PT67 and the murine fibroblast cell line NIH3T3 were maintained in Dulbecco's modified essential medium (DMEM) containing 100mL·L^{-1} fetal bovine serum. The human gastric carcinoma cell line, NCI-N87, and the human leukemia cell line, Jurkat, were maintained in RPMI 1640 medium containing 100mL·L^{-1} or 150mL·L^{-1} fetal bovine serum respectively.

Transfections, infections and determination of viral titer

The packaging cells PT67 were plated in a 60 mm plate at a density of 50%-80% 24h before transfection. Cells were washed with DMEM twice and incubated with 2mL DMEM before transfected with 10μg of plasmid DNA by lipofectin method (Life Technology, Gibco BRL). Four h-6h after transfection, 2mL DMEM (containing 200mL·L^{-1} FBS) was added. To obtain stable virus-producing cell lines, the packaging cells transfected with retroviral plasmids were plated in selection medium 48h later. The regulatory vector Tet-On carries the neomycin gene as a selectable marker. For G418 selection, cells were cultured in the presence of G418 (0.4g·L^{-1}, Gibco) for two- weeks. The vectors pRev-TRE and pRev-TRE-TRAIL carry the gene for hygromycin selection. These cells were selected in the presence of hygromycin B (0.06g·L^{-1}, Sigma) for two weeks. For transduction, NCI-N87 cells were plated 24h before infection. The medium from packaging cells containing virus were collected, filtered through a 0.45μm filter, and added to the NCI-N87 cells in the presence of Polybrene (4g·L^{-1}, Sigma). The medium was replaced 4h later. Three to six serial infections were performed to increase the efficiency of infection. Forty-eight hours after infection, the cells were subjected to G418 or hygromycin selection. For determination of viral titer, NIH3T3 cells were plated 24h before transduction in 6-well plates (2×10^5 cells per well). The cells were infected with filtered virus-containing medium (six 10-fold serial dilutions, 1 - 10^5). The cells were then incubated in G418 or Hygromycin 48h later, and the selection lasted about weeks until clear colonies appears.

Growth curve

The cells were plated in 96-well plates (5×10^4 cells per well), and counted by Typan blue dye exclusion method over a 24h period.

Induction of gene expression

The cells were added with the medium containing Dox (0.01 -10mg·L^{-1}, Sigma). After a period of time, the fraction of dead cell was calculated by Typan blue dye counting. The medium after induction was added in Jurkat cells (100mL·L^{-1}), the fraction of dead cell was calculated by Trypan blue dye.

RESULTS

Construction of retroviral vectors

To clone the TRAIL gene into the Tet system, we inserted the cDNA of TRAIL into the Tet response vector pRev-TRE, which was named pRevTRE-TRAIL. The desired recombinant plasmid orientation was confirmed by Hin dIII and *Ssp* I digestion. In this plasmid, the TRAIL gene is the downstream of the TRE, which can bind rtTA expressed by pRevTet-On. So, in combination with the pRevTet-On regulatory vector, the TRAIL gene can be inductively expressed at high levels in response to varying concentrations of tetracycline or doxycycline. Because of their retrovirus-mediated gene transfer, we can incorporate the Tet-controlled TRAIL protein expression system into mammalian cells.

Establishment of Tet-controlled TRAIL system

We transfected vectors into packaging cells PT67. Using G418 (pRevTet-On) and hygromycin selection (pRev-TRE and pRevTRE-TRAIL), we obtained the stable virus-producing cell lines, PT67-TetOn, PT67-TRE and PT67-TRE-TRAIL. Viral titers were 1-2×10^{8}CFU·L^{-1}, determined by titering NIH3T3 cells. After serial infections of NCI-N87 cells with viral-containing supernatants and antibiotic selections, two kinds of cell lines were established. One is the test group NCI-N87-TetOn-TRE-TRAIL (NN3T, Figure 1A), which is a Tet-controlled TRAIL expression cell line. The second is the control cell line NCI-N87-TetOn-TRE (NN2T, Figure 1B), which is the same with NN3T but without TRAIL expression.

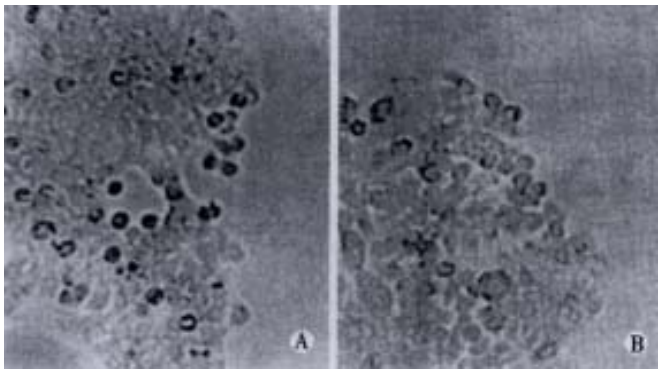

Figure 1 Morphology of cells. A: NN3T; B: NN2T

Killing effect of TRAIL induced by doxycycline

When we added doxcycline to cells, some cells died in the test group NN3T 48h later (Figure 2), whereas no clear death effect can be detected in cells in the control group (NN2T)

and wild type NCI-N87. Under normal conditions without Dox, the growth curves of these three types of cells showed no obvious differences (Figure 3). In order to test the possible secretion of TRAIL protein into the medium, the media from the test group and control group were removed and inoculated into the human leukemia cells Jurkat (sensitive to TRAIL-induced apoptosis). Obvious cell death was observed after one day culturing in the test group as compared with the control and the normal groups. From these results, we concluded that the regulated expression of TRAIL in the Tet system has killing effect on the gastric carcinoma cell NCI-N87.

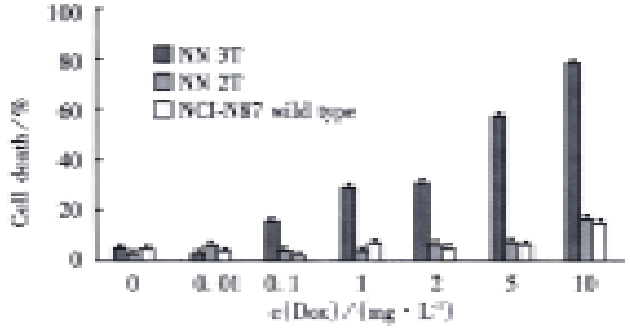

Figure 2 Killing effect of TRAIL regulated by doxcycline.

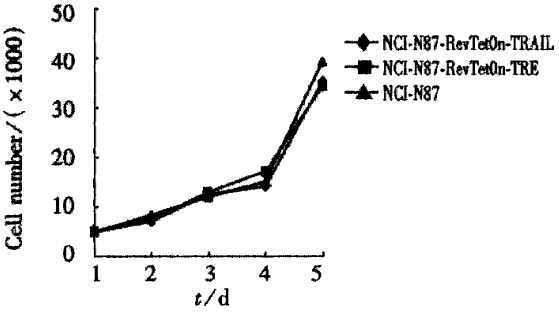

Figure 3 Growth curves of cells.

DISCUSSION

Tumor necrosis factor (TNF) is a prototypic member of the family of cytokines that interact with a corresponding set of receptors that form the TNF receptor (TNFR) family. Three of these ligands, CD95L, TNF and LTα, have received particular attention because they can induce apoptosis in transformed cells and activated lymphocytes[32-34]. The potential utility of systemically administered ligands is limited by their acute toxic effects on normal tissues *in vivo*, thereby limiting their potential widespread use in the treatment of cancer[35]. Previous studies have shown that TRAIL can induce apoptosis in a variety of tumor cell lines. In contrast to other members of TNF family, TRAIL mRNA is expressed constitutively in many tissues including peripheral blood lymphocytes, spleen, thymus, prostate, ovary, small intestine, colon and placenta[1,7,17,36-38], which suggests the existence of physiological mechanisms that can protect many normal cell types from induction of apoptosis specifically by TRAIL. A relatively high proportion (approximately two-thirds) of tumor cell lines tested so far are sensitive to the cytotoxic effects of TRAIL *in vitro*[1,7,11,39], indicating that TRAIL may prove to be a powerful cancer therapeutic factor.

Although TRAIL potently induces apoptosis in tumor cells and some virally infected cells, it has little or no detectable cytotoxic effects on normal cells, whereas this was first thought to be due to regulated expression of the TRAIL receptors[3,24,40-42], the fact that mRNA for both TRAIL and TRAIL receptors is often expressed in the same cells makes this explanation untenable[17,43]. Indeed, the identification of four distinct TRAIL receptors (TRAIL-R1, R2, R3, R4) has significantly increased the potential complexity of this receptor/ligand system[17,43,44]. Based on current information it seems mikely that multiple factors, both intra and extra-cellular, may function together to protect normal cells from the cytotoxic effects of TRAIL[17,43,45-48], but many questions remain unexplained.

Although the reason for determining the sensitivity of cells to TRAIL-induced apoptosis has not been understood, we still try to use TRAIL in cancer treatment to achieve the effectiveness and the safety of the therapy. In our laboratory, the cDNA sequence of TRAIL has been cloned from human placenta cDNA library. We have expressed the TRAIL in the *E. coli* by the pET system[49], after purification and refolding the antitumor activity of the protein has been examined[50]. On the other hand, a chief concern with the potential use of TRAIL protein in the treatment of tumors *in vivo* is the potential undesirable toxicities. Because of the recent report that some normal human cells were sensitive for the apoptosis induced by TRAIL[51], we inserted the gene into a mammalian expression vector to study its effect further in this experiment.

In most inducible mammalian gene expression systems (heavy metals, steroid hormones, or heat shock), induction is nonspecific and expression levels cannot be precisely regulated. In addition, these systems are generally leaky in the "off" state, and the inducing agent itself may be cytotoxic or have pleiotropic effects. In contrast, regulation of gene expression by the Tet system is very specific[31]. Furthermore, the levels of tetracycline or doxycycline required for the full range of gene expression are not cytotoxic and have no significant effect on cell proliferation or animal growth. Using retroviral vectors instead of DNA transfection to transduce the complete inducible system greatly expands the target cell types in which gene functions can be studied. This strategy may be useful for clinical applications in human gene therapy trials.

In this study, the effectiveness of TRAIL in killing human gastric cells was evident. It showed that the gastric carcinoma cell line was sensitized to TRAIL-induced cell death. In addition, the concentrations of doxycyline that constituted the effect were within a range of clinical security. We can believe that this effect will be safe to normal tissues with the specificity of TRAIL and regulation of the Tet system.

At the moment, malignant gastric carcinoma remains one of the difficult types of cancer to treat successfully, and with the incidence of gastric carcinoma increasing at China, it continues to be a leading cause of death in Japan and other countries of Asia. In our experiment, the expression system of TRAIL gene controlled by tetracycline was established in gastric carcinoma cell line NCI-N87, and its killing effect to the tumor cell was observed. This study clearly demonstrates the possible usefulness of Tet-controlled TRAIL expression system in gastric cancer gene therapy, and further studies of the mechanisms of TRAIL-mediated cytotoxicity are required to assess the potential use of TRAIL as an anti-cancer therapeutic *in vivo*.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Barrett's- metaplasia: clinical implications

Sauid Ishaq and Janusz A Jankowski

Epithelial Laboratory, Division of Medical Sciences, University of Birmingham, Edgbaston, B15 2 TH, UK
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Correspondence to Dr. Janusz A Jankowski MD PhD FRCP, Epithelial Laboratory, Division of Medical Sciences, University of Birmingham, Edgbaston, B15 2TH, UK.
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Abstract

The incidence of Barrett's metaplasia (BM) as well as Barrett's adenocarcinoma (BA) has been increasing in western populations. The prognosis of BA is worse because individuals present at a late stage. Attempts have been made to intervene at early stage using surveillance programmes, although proof of efficacy of endoscopic surveillance is lacking, particularly outside the specialist centres. The management of BM needs to be evidence-based as there is a lack clarity about how best to treat this condition. The role of proton pump inhibitors and antireflux surgery to control reflux symptoms is justified. Whether adequate control of gastroesophageal reflux early in the disease alters the natural history of Barrett's change once it has developed and or prevents it in patients with gastroesophageal reflux disease but with no Barrett's change remains unanswered. There is much to be learned about BM. Thus there is great need for carefully designed large randomised controlled trials to address these issues in order to determine how best to manage patients with BM.

Subject headings Barrett's esophagus/complications; metaplasia/complications; gastroesophageal reflux

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INTRODUCTION

The recent rise in the incidence of esophageal adenocarcinoma in parallel with an increased incidence of Barrett's metaplasia (BM) favours the theory that BM is a pre-cancerous lesion for adenocarcinoma. This BM is thought to occur as a result of prolonged and severe gastroesophageal reflux that can lead to chronic inflammation resulting in replacement of normal squamous epithelium in the distal oesophagus with specialised intestinal-type columnar epithelium containing goblet cells. Attempts have been made to treat acid reflux with medical treatment as well as anti-reflux surgery in the hope that it might prevent progression of BM. Surveillance strategies for established BM to detect early cancer have not been proven cost effective[1,2], although lesions identified during surveillance program have better prognosis. Despite extensive research, the natural history of BM is poorly understood. Many questions remain regarding the diagnosis of BM, its treatment, and the impact of surveillance strategies on early detection of cancer. It is important to realise the sheer scale of the problem as oesophagitis secondary to gastroesophageal reflux disease is one of most common conditions in the western world with up to 30% of adults complaining of heartburn at least once per month, a third of whom will develop oesophagitis. About 10% of patients with oesophagitis will progress to BM, of whom up to 5% will progress to cancer.

Using pooled data, the cancer risk in BM is about 1% (range 0.5% - 2%)[3]. Proposed clinical risk factors for cancer progression including chronicity of symptoms, length of Barrett's segment, gastroesophageal reflux and mucosal damage. For example gastroesophageal reflux symptoms are considered an independent risk predictor for cancer risk but may not discriminate low from high risk BM. Molecular changes in p53, p16, and *cyclin* D1 overexpression, decreased E-cadherin expression, and loss of heterozygosity of the adenomatosis polyposis coli (APC) gene have been detected[3]. These molecular changes have been evaluated in a clinical research setting but are not routinely used in clinical practice, although such genetic changes may become useful screening markers to monitor progression of BM and to identify individuals at risk of developing malignant transformation in the future. Thus, there is a pressing need for better understanding of BM and to develop strategies not only to prevent this pre-cancerous condition but also to identify high-risk individuals with this condition who are at risk of developing Barrett's adenocarcinoma. This article discusses issues that concern clinicians in the management of BM including definition, diagnosis, screening and surveillance of BM, as well as management of uncomplicated BM.

DEFINITION AND DIAGNOSIS OF BM

BM may be defined as visible columnar-lined oesophagus of any length above the esophago-gastric junction (OGJ) confirmed on biopsies with the presence of specialised intestinal-type columnar epithelium containing goblet cells. However, there is considerable disagreement in defining BM. One technical problem in this regard is the precise identification of the OGJ. Suggested endoscopic criteria for identifying OGJ include the point of flaring of the stomach from the tubular oesophagus[4] and confluence of the proximal margin of longitudinal gastric folds[5]. One has to realise that this location point of flare can shift during breathing as well as peristaltic activity in the oesophagus and prolapse of the gastric folds into the oesophagus could further confuse the situation. Distinction between long and short Barrett's segment is irrelevant as the tendency to develop high-grade dysplasia may be similar in short and long segment Barrett's[6].

To avoid confusion endoscopists should avoid such terminology and instead describe only what they see i.e. if columnar-lined oesophagus is present or not and if present whether it is continuous or tongues and islands are observed. The histopathologist often finds it difficult to differentiate between intestinal metaplasia occurring in the distal oesophagus and in the cardia, and thus should avoid the term Barrett's and instead report whether columnar epithelium is present and the presence or absence of intestinal metaplasia

and identification of squamo-columnar junction. It is essential that precise site of biopsies with reference to OGJ should be documented to help the histopathologist make an accurate diagnosis. Thus the diagnosis of BM becomes clinicopathological integrating both endoscopic and histopathological information to increase diagnostic accuracy. Occasionally (1% - 5% of cases), however, the pathologists see esophageal glands submerged under columnar-lined tissue corroborating the biopsy as being esophageal in origin (personal correspondence Prof N Shepherd, Gloucester Royal Hospital, UK) (Figure 1).

EPIDEMIOLOGY OF BM

The true prevalence of BM in the general public is unknown. Reported lifetime risk in adult general populations in western countries is 1%[7] based on post-mortem studies. In addition, the incidence of new cases is about 0.5%-2.0%/year[3] and increasing[8]. It mainly occurs in white people, with a male predominance (male: female 2.3:1)[9]. Recently its prevalence has been shown to be rising all over the world, including in the Far East[10]. There is no significant relationship between smoking, alcohol consumption and high body mass index in the genesis of this metaplastic change.

SCREENING AND SURVEILLANCE OF BM

It is not clear why only 10% of patients with oesophagitis progress to BM and why only a tiny fraction of these patients will go on to develop adenocarcinoma. In view of the low prevalence of BM in patients with gastroesophageal reflux as well as uncertainties regarding diagnosis and treatment, it is difficult to justify screening for Barrett's in patients with gastroesophageal reflux, although patients aged >45 with longstanding severe reflux symptoms (5 - 10 years) should have a one off screening endoscopy.Barrett's adenocarcinoma develops through stages of increasing dysplasia (Sandic 1998). If this theory is correct, then surveillance endoscopies biopsies of BM should permit early cancer detection and reduce mortality from Barrett's adenocarcinoma[11], as prognosis in early Barrett's adenocarcinoma is more favourable as compared to advanced disease[12]. Attempts to identify tumours at early stage with surveillance programmes has not been cost effective in few studies[1,2]. Surveillance of BM with no dysplasia at 2.5 years has been recommended, although its usefulness has never been validated in a randomised study. Using the Markov model based on UK NHS cost per life saved if the surveillance programme is based on two yearly endoscopy, surveillance is comparable to other health disciplines in terms of cost per cancer detected and cost per life saved. It is fair to say that for an expense of this order we should first have some convincing evidence of effectiveness of surveillance programmes. Until results of such studies are available we face the dilemma of telling patients that they have a pre-cancerous condition with 5% life-time risk of cancer and that we could offer lifelong surveillance endoscopy programme that cannot guarantee to detect every cancer that may develop.

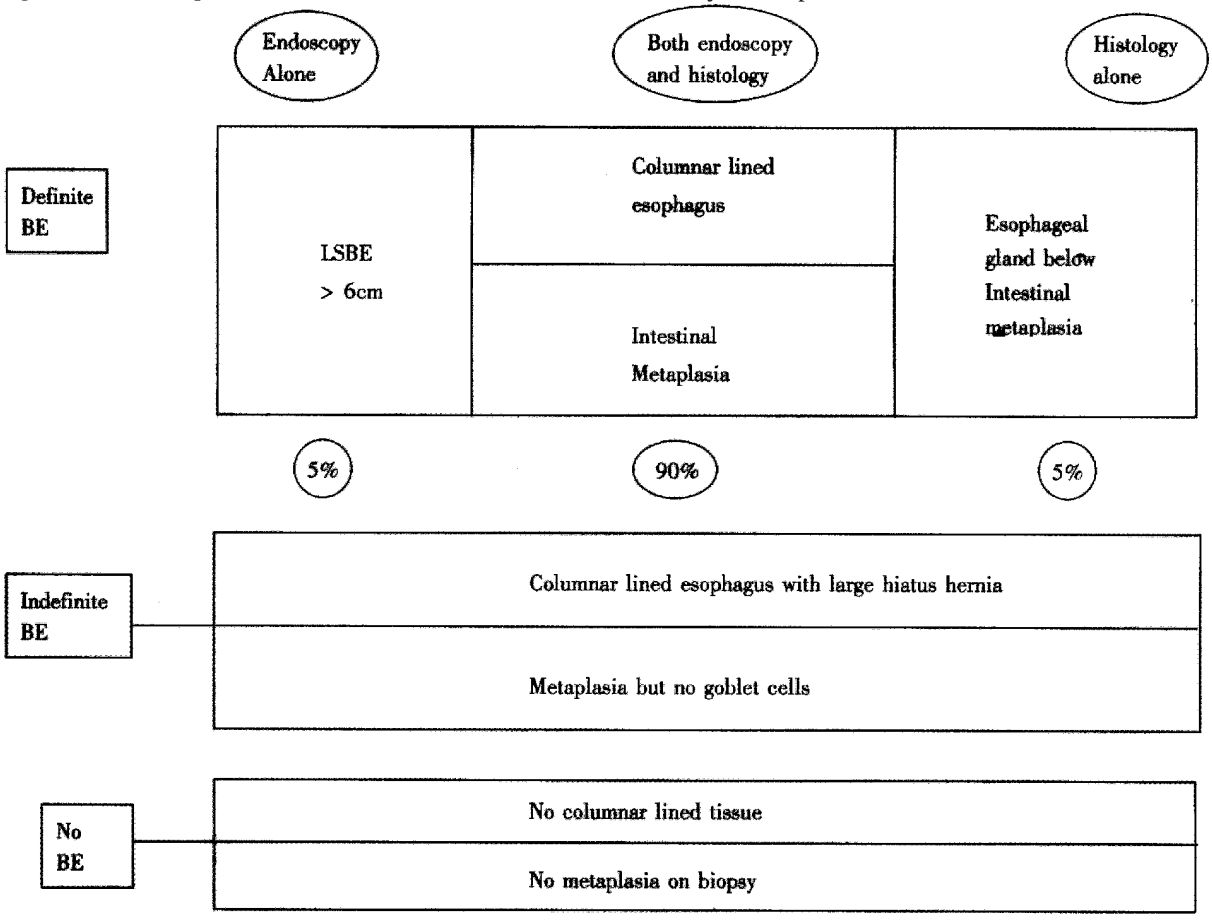

Figure 1 Diagnostic criteria for Barrett's esophagus (BE), based on endoscopic, histological criteria or both. LSBE (long segment Barrett's esophagus). Definite diagnosis can be made with endoscopy in 5%, histology alone in 5%, but in about 90% both endoscopy and histology is required.

MANAGEMENT OF UNCOMPLICATED BM (NO DYSPLASIA)

The symptoms of GORD, a presumed cause of BM, can be effectively eliminated with medical treatment or with anti-reflux surgery; however, regression of established BM does not occur with either intervention. On the other hand, many patients with BM will either have reduced or absent symptoms due to reduced sensation of Barrett's epithelium. The role of PPI's in BM is unclear. High dose PPI's are still proposed regardless of symptoms by many who justify that such an approach may be necessary to achieve regression of BM[13]. Opponents of this approach advocate no treatment of asymptomatic BM and are less convinced that acid suppression prevents complications. Until there is more clarity in the scientific evidence, PPI's remain an attractive treatment for the BM, especially if there is endoscopic evidence of esophagitis above the BM segment as is the case in about 40% -60% cases.

Competent fundoplication for BM has been advocated in patients with complications or intractable symptoms unresponsive to conservative medical treatment. If performed early, before the development of Barrett's changes, fundoplication is slightly more effective than medical treatment to prevent this metaplastic change. The effect of this anti-reflux surgery on the natural history of BM once it has developed is less clear. Until we have more data to determine the role of anti-reflux surgery in the setting of BM or GORD, it would be sensible to perform these procedures once medical treatment fails or on patients fully informed of the choice. In addition, because there is a 0.1%-0.2% mortality rate for fundoplication surgery, this detracts from its efficacy. In particular, if there is an estimated 5% lifetime risk of cancer in BM, only half will die of the cancer because co-morbidity such as cardiac diseases will usually kill them. Secondly, no treatment is ever 100% effective in preventing cancer, so if 50% of individuals could be prevented from getting cancer only 1.25% of the 5% at risk population would benefit from surgery. Therefore for every 9 patients benefiting from long term advantages of surgery, 1 or perhaps even 2 would die prematurely from the surgery itself.

Because of the inability to effect regression of Barrett's mucosa with medical or anti-reflux surgery, there has been renewed interest in the development of new modalities to eliminate this metaplastic change and hence reduce the cancer risk by destruction of Barrett's mucosa by endoscopic ablation with thermal (Laser, Argon Plasma Coagulator), chemical (photodynamic) or mechanical (surgical ultrasound) in a reflux free environment to prevent further damage. These techniques have shown healing by squamous epithelium regeneration in 66% - 100% patients[14], although nests of glandular epithelium may remain beneath the neo-squamous epithelium in up to 60% of patients that may progress to cancerous change. In view of this and high complication rates[14], many authors have debated the usefulness of these potentially hazardous therapies. Using NNT calculations, a reduction of absolute risk has not been established for any of these therapies. Efficacy of such treatment should thus be verified in controlled trials before their widespread use.

CONCLUSIONS

In spite of extensive research in this area it appears that we have more questions than answers. Our understanding of the natural history of BM is limited, as available data is not only insufficient but also either contradictory or subject to variable interpretation. Longstanding gastroesophageal reflux has been proposed to contribute towards BM. Whether adequate control of gastroesophageal reflux early in the disease alters the natural history of Barrett's change once it has developed, or prevents it in patients with GORD, remains unanswered. To date, we simply cannot estimate or eliminate either the cancer risk or BM itself. On the basis of evidence available, it is difficult to promote or reject Barrett's surveillance programmes on economic grounds alone. We must not forget that only 25% Barrett's adenocarcinoma patients are known to have BM before they develop cancer and that 75% of cancer patients present for the first time with this disease without any prior knowledge of GORD or BM. Thus there is a pressing need for more work to be done, in large randomised controlled trials, to unravel these issues before we will be able to treat this condition effectively.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Liver transplantation and artificial liver support in fulminant hepatic failure

Xiao-Feng Zhu, Gui-Hua Chen, Xiao-Shun He, Min-Qiang Lu, Guo-Dong Wang, Chang-Jie Cai, Yang Yang and Jie-Fu Huang

Transplantation Center, The Affiliated First Hospital, Sun Yat-Sen University of Medical Sciences, 510080 Guangzhou, Guangdong Province, China
Supported by the grant of Key Clinical Programme of China Ministry of Public Health, No.97040230
Correspondence to Dr. Xiao-Feng Zhu, Transplantation Center, Affiliated First Hospital, Sun Yat-Sen University of Medical Sciences, 510080 Guangzhou, Guangdong Province, China. Zhuxf@gzsums.edu.cn
Tel/Fax: 0086-20-87306082
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INTRODUCTION

Fulminant hepatic failure (FHF) is a severe disease with devastating consequences; the incidence is high in China. Before the availability of liver transplantation, the mortality rate was more than 80%[1,2]. The advent of liver transplantation revolutionized the outcome of FHF[3,4]. However, many patients were unwilling to accept liver transplantation until very late, hence most of them died because of donor shortage and urgency of the disease[5-7]. To overcome the problems, we performed orthotopic liver transplantation (OLT) in combination with artificial liver support (ALS) in the treatment of FHF in the past 2 years with satisfactory results. Our experience was reported below.

PATIENTS AND METHODS

Patients

All eight patients were male with a mean age of 40 (range 32 -49) all had hepatitis B with acute absolute liver failure on admission. These patients had a history of hepatitis for 7 days to 12 weeks, with acute onset of severe hepatic disfunction, rapidly progressive jaundice, abdominal distention, asthenia, ascites, coagulopathy and encephalopathy. Two of them were complicated with acute hepatorenal syndrome and acute necrotizing pancreatitis respectively. Seven patients had stage II-IV coma. All 8 patients received artificial liver support for 2-20 times before transplantation.

Artificial liver support therapy

All patients received artificial liver support treatment with Plasmaflo KM8800 (Kuraray Co. Japan). Plasma exchange (PE), hemodiafiltration (HDF) or bilirubin absorption were singly or jointly selected to treat the patients respectively[8]. On the first treatment, anconeus venous or femorali catheterization was established. Plasma exchange given was 2000mL - 4000mL, plasma transfusion or plasma substitute of 2500 mL - 4500 mL and albumin infusion of 20 g - 40 g each time. The rate of plasma seperation and the flow rate of plasma exchange were controlled at the speed of 20-30mL/ min and 60 - 100mL/min, respectively. The whole course took 3-5 hours.

Procedure of liver transplantation

The 8 patients underwent orthotopic liver transplantation under veno-venous bypass[9,10], of whom 7 underwent standard orthotopic liver transplantation[11] and 1 modified Piggyback liver transplantation[12]. The transplantation was successfully performed with a median anhepatic phase of 78 minutes and operation lasted averagely 5 hours and 30 minutes, but the 2 patients with preoperative renal failure had oliguria during operation. An average bleeding of 5600mL (2000mL-10000mL) was recorded during the operation and the blood lost was collected with Cellsaver.

RESULT

Recipient's survival

All patients became conscious soon after liver transplantation and one in stage IV coma also awoke 2 hours postoperatively. Six of 8 recipients have survived for 2-20 months with good hepatic function, of whom 3 had returned to normal work for more than 18 months. Three days after transplantation, 2 patients died of multi-organ failure (MOF), one with acute necrotic pancreatitis that was unnoticed. Acute rejection occurred in one patient who recovered after anti-rejection treatment of methylprednisolone.

Effect of artificial liver support on FHF

After treatment with artificial liver support the ascites and coagulopathy, decreased serum bilirubin declined, encephalopathy relieved and hepatic function improved (Table 1).

Table 1 Changes of hepatic function before and after ALS

Treatment	N	ALT(U/L)	AST(U/L)	TBil(μmol/L)	IBil(μmol/L)	PT(second)
Before	8	125.4+55.9	132.0+42.9	559.2+209.3	310.8+151.8	40.9+6.7
After	8	120.3+35.5	119.0+29.6	423.7+157.0	252.3+118.5	36.7+6.2
t		0.574	1.336	6.187	4.206	1.959
P		>0.50	>0.20	<0.001	<0.005	>0.05

DISCUSSION

Fulminant hepatic failure progresses rapidly with high mortality and liver transplantation has emerged as an effective therapy for whom do not responded to the standard treatment[13-16]. All patients with FHF must be considered as potential transplantation candidates[17,18]. At King's College Hospital in England, the criteria used for liver transplantation are dependent on the cause of FHF[19,20]. In patients with paracetamol-induced FHF, a pH of less than 7.3 at 24 hours or more after overdose, with concurrent presence of a serum creatinine level greater than 300 μmol/L (>3.4 mg/dL), hepatic encephalopathy of grade III or IV, and a prothrombin time greater than 100 seconds are considered indications for liver transplantation. In non-paracetamol-induced FHF, the decision is based on the occurrence of three of the following: a prothrombin time greater than 50 seconds; jaundice proceeding to encephalopathy more than 7 days; non-A, non-B hepatitis or drug-induced hepatitis; age younger than 10 years or older than 40 years; bilirubin level greater than 300 μmol/L (>17.5 mg/dL); or an isolated finding of prothrombin time of greater than 100 seconds. The indications of 8 patients in our group met with the criteria of King's College Hospital. But in China, where brain death has not been accepted as a criterion for human death, most of the patients with FHF died before the organ became available because not only of the organ donor shortage but also of the rapidity of the course. Thus, it is necessary to develop artificial liver support system as a bridge to cross over to liver transplantation[21,22]. In our study, 8 patients with FHF underwent artificial liver support, then 6 of them survived the most critical period and returned to normal life. Liver transplantation plus artificial liver support creates a new avenue for treatment of FHF.

Artificial liver support system can remove the toxic substances by way of plasma exchange[23], hemodialysis[24], hemo-infiltration[25] and absorption[26], in order to substitute the hepatic function of detoxication[27-31]. Perhaps ALS can remove the toxic substances causing encephalopathy, improve the patients' consciousness, prevent and treat multi-organ failure[32,33]. Why ALS is effective in preventing brain edema is also unelucidated, perhaps by change in permeability of the blood-brain barrier and by raising the osmolality of neural cells[34-36]. ALS, particular the hemodialysis, may prevent the brain edema[37-41]. Our experience showed that pretransplant ALS may not only help the patients to tide over critical period but also increase the chance for liver the transplantation

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Protective effects of cyclosporine A on T-cell dependent ConA-induced liver injury in Kunming mice

Xiu-Li Zhang, Qi-Zhen Quan, Zi-Qin Sun, Yao-Jun Wang, Xue-Liang Jiang, Dong-Wang and Wen-Bo Li

Department of Gastroenterology, General Hospital of Jinan Military Command, Jinan 250031, Shandong Province, China
Correspondence to Xiu-Li Zhang, Department of Gastroenterology, Chinese PLA Gerneral Hospital of Jinan Command Area, Jinan 250031, Shandong Province, China. xiuliz@jn-public.sd.cninfo.net
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Zhang XL, Quan QZ, Sun ZQ, Wang YJ, Jiang XL, Wang D, Li WB. Protective effects of cyclosporine A on T-cell dependent ConA-induced liver injury in Kunming mice. *World J Gastroenterol*, 2001;7(4):569-571

INTRODUCTION

The T-cell dependent specific liver injury in mice induced by concanavalin A (ConA) is a newly established experimental liver injury model, which is considered more eligible for the study of pathophysiology of several human liver diseases, such as viral hepatitis and autoimmune hepatitis[1-9]. T cell activation and several cytokines release had been proven to play a critical role in ConA-induced liver injury[10-19]. Cyclosprine A (CsA), an effective in hibitor of activation of T lymphocyte, has been used widely in clinical treatment, especially in autoimmune diseases and organ transplantation[20-25]. In this study, we investigated the possible effect of CsA on ConA-induced liver injury in Kunming mice.

MATERIALS AND METHODS

Materials

Male Kunming mice were purchased from the animal experimental center of the Second Military Medical University, weight range 17g-21g, free access to water and food prior to the experiment. ConA and CsA were purchased from Dongfeng Ltd Shanghai and Sandoz Ltd respectively.

Methods

All the fifteen Kunming mice were divided into three groups randomly. ConA at a dose of 40mg·kg^{-1} was administered through the tail vein as a solution in pyrogen-free PBS at a volume of 300μL, which was the ConA group. CsA was injected subcutaneously twice at a dose of 130mg·kg^{-1} 15 and 1h before ConA challenge, which was used as the CsA group. PBS only in the corresponding volume served as controls.

Eight hours after ConA administration, the Kunming mice were sacrificed by cervical dislocation. Blood samples were obtained by puncture of heart with 25g·L^{-1} heparin. Liver specimen was fixed immediately in 100 mL·L^{-1} formalin/PBS for histological examination with HE stain. The degree of liver injury was assessed by determination of serum alanine aminotransferase (ALT) activity, serum TNF-α was determined by radioimmunoassay.

Statistics

The results were analyzed by Student's *t* test. The data were expressed as $\bar{x} \pm s$, and $P<0.05$ was considered to be significant.

RESULTS

ConA-induced liver injury in Kunming mice

Eight hours after ConA administration, two out of the five experimental mice were found dead in ConA only group, with elevated serum ALT 22 261 ± 2 523 nkat·L^{-1}. The concentration of serum TNF-α also increased significantly in ConA only group, increased more significantly than that of PBS only group 647±183ng·L^{-1} (Table 1).

Histological examination of liver specimen from ConA-treated mice showed diffuse cloudy swelling of the cytoplasm, spotty and necrotic foci were frequently present, severe agglutination of erythrocytes in the sinusoids of the liver were also observed. Lots of infiltrated lymphocytes in the portal area were the characteristic of this new liver injury model (Figure 1), indicating that lymphocyte may play an important role in the pathogenesis of ConA-induced liver injury, whereas no obvious tissue damage was found in the lung or kidney.

CsA protection

When pretreated with CsA (CsA group), serum ALT activity declined significantly (730 ± 266) nkat·L^{-1}, and the serum TNF-α was below the detective level (Table 1). No obvious hepatic necrosis or lymphocytes infiltration in the portal area was observed under light microscopy in the CsA group (Figure 2).

Table 1 ConA-induced liver injury in Kunming mice (n=5, $\bar{x}$±s)

	ALT- nkat·L^{-1}	TNF-α ng·L^{-1}	Dead
Con A only	22 261±2 523[b]	1230±240[b]	2
PBS only	647±183		0
CsA	730±266		0

[b]$P<0.05$, *vs* PBS only or CsA.

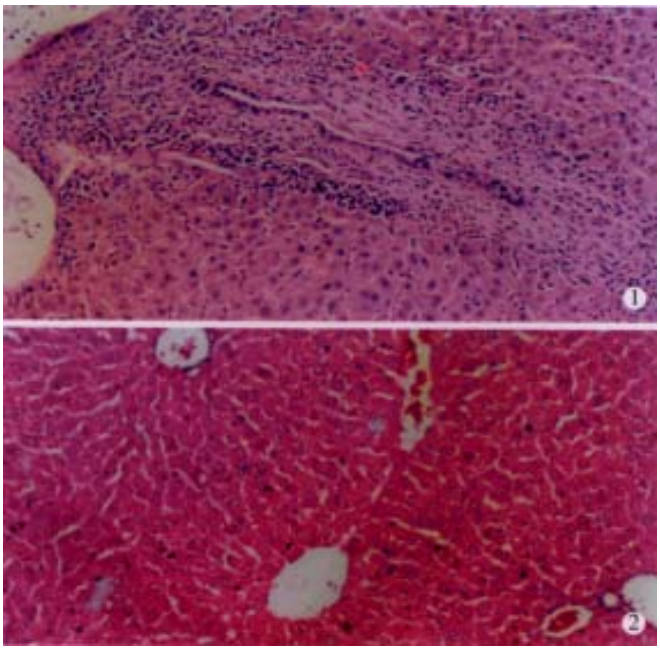

Figure 1 ConA (40 mg·kg^{-1}) induced liver injury. Hepatocyte necrosis and infiltration of lymphocytes in the portal area. HE×66
Figure 2 CsA pretreated, no obvious hepatocyte necrosis or infiltration of lymphocytes was observed. HE×66

DISCUSSION

ConA-induced specific liver injury in mice is a newly developed experimental animal model, which has been closely studied in the pathogenesis of the liver injury in recent years. T lymphocyte activation, cytokines release such as TNF-α, interferon-γ, and interleukines have been discovered to be involved in the pathogenesis of this liver injury model, especially the activation of T lymphocyte and the subsequent release of TNF-α are considered to play a much more important role in this experimental liver injury. The pathological process of ConA-induced liver injury was similar to what seen in several human liver diseases, such as viral hepatitis, and at least three types of autoimmune hepatitis. CsA is a specific inhibitor of T lymphocyte by inhibiting the transcription[21,23], and whether CsA has any protective effect on ConA-induced Kunming mice liver injury by inactivation of T lymphocytes has to be studied In our experiment, the ConA-induced specific liver injury was successfully duplicated in Kunming mice. The results (Table 1) showed that eight hours after ConA administration, the serum ALT activity was significantly increased compared with that in the control group (PBS only group). At the same time, two mice (2/5) died within eight hours. When pretreated with CsA, no death occurred, and the serum ALT level also declined significantly as compared with that of ConA group 730 ± 266 *vs* 22 261± 2 523 nkat·L^{-1}, P<0.01. The experimental results showed that CsA had potential protective effect on the ConA-induced liver injury in Kunming mice.

TNF-α has been proven to be the key cytokine in the destruction of hepatocyte in human liver diseases or liver injury animal model, such as acute and chronic viral hepatitis, especially in fulminant liver failure[26-41]. Anti-TNF antibody resulted in complete protection of ConA-induced liver injury in Balb/C mice[13]. We found that TNF-α increased significantly within eight hours when treated with ConA, but when pretreated with CsA before ConA administration, serum TNF-α became undetectable, hence the reduction of TNF-α might be due to the partial protective effects of CsA. Besides destruction of hepatocytes seen in the liver specimen of ConA group, lots of infiltrating lymphocytes in the portal area were also observed (Figure 1). When pretreated with CsA (CsA group), there was absence of lymphocytes infiltration in the portal area (Figure 2). The histological results gave a direct evidence that the protective effect of CsA in ConA-induced Kunming mice liver injury is through abrogating the activation of the T lymphocytes. The decline of serum TNF-α in CsA group may be a subsequent to T lymphocyte inactivation. $CD4^+$ lymphocyte was identified as the effector cells in the ConA-induced liver injury[3], however, in view of the fact that TNF-α synthesis is substantially higher in macrophages than in T lymphocytes, it seems likely that activated T lymphocytes might stimulate the macrophages to release TNF-α[15], but details of the concrete process worth further studies.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Prevention of grafted liver from reperfusive injury

Kai Ma[1], Yang Yu[2], Xian-Min Bu[1], Yan-Jun Li[1], Xian-Wei Dai[1], Liang Wang[1], Yang Dai[1], Hai-Ying Zhao[1] and Xiang-Hong Yang[3]

[1]Department of General Surgery, Second Clinical College, China Medical University, Shenyang 110003, Liaoning Province, China
[2]Department of Physiology, Shenyang Physical Education College, Shenyang, Liaoning Province, China
[3]Department of Pathology, China Medical University, Shenyang, Liaoning Province, China
Project supported by the Natural Science Fund of Liaoning Province, No.962280
Correspondence to Dr. Kai Ma, Department of General Surgery, Second Clinical College, China Medical University, Shenyang 110003, Liaoning Province, China
Tel: 0086-24-23893501 Ext.512
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INTRODUCTION

The incidence of primary non-function (PNF) of grafted liver in the early postoperative stage is 2% - 23%[1-4], its main cause is the ischemic-reperfusive injury[5,6]. In this experiment, anisodamine was added into the preserving fluid and the grafted liver was rewarmed at different temperatures to protect the cell membrane and prevent ischemic-reperfusive injury.

METERIALS AND METHODS

Selection and grouping

Twenty male Wistar rats (270g - 330g in weight, 10 - 12 weeks in age) were used in the experiment. The rats were divided into 2 groups, 10 in each group, and the action of anisodamine was studied. In the experimental group, 40mg anisodamine was added into 1 liter of preserving fluid, no anisodamine was used in the control group. The rats were divided into 4 groups, 5 in each group, and the action of rewarming was studied. Before reperfusion, the 12℃, 20℃, 28℃ and 36℃ of gelofusine were injected into the portal vein respectively to rewarm the grafted liver.

Establishment of animal model[7-9]

Make a midline epigastric incision, dissociate the liver fully, incise the infrahepatic inferior vana cava (IVC), input a shaped three way stopcock, make its upper end 5mm higher than liver, ligate the both ends of IVC incision, and obstruct hepatic artery[10]. Cut off the portal vein, connect its distal end with one opening of the three-way stopcock, shunt the portal-cava vein provisionally, and reverse the blood of IVC and portal vein to the heart through the duct. Inject proximately 5 mL saline mixed with 1 mL heparin. Ligate the suprahepatic inferior vana cava provisionally, release the ligature above the IVC incision, wash the liver through portal vein, make the preserving fluid flow outside the duct. Wash the liver at low temperature for 4 hours within the body, maintain the pressure at 90 - 100 cm H_2O, and velocity at 8-12mL/min. The preserving fluid was the lactic Linger's fluid composed of 10mL dethomaxone, 100mg ATP and 100U/L insulin. After managing the experimental factors, take out the three-way stopcock, connect with the portal vein, release the occlusion of hepatic artery, repair the IVC incision, and restore the hepatic reperfusion[11].

Collection and test of samples

Liver tissue of 500mg was resected before the obstruction of blood and reperfusion, and 30min and 60min after reperfusion respectively. Superoxide dismutase (SOD)[12,13] and lipid peroxidase (LPO) were tested[14], and the morphologic changes were observed under microscopy and electric microscopy synchronically[15,16].

Statistical analysis

Data were presented as the mean ± SE. The *t* test was applied between two groups and variance analysis between multi-groups. $P<0.05$ values were regarded as significant.

RESULTS

Effect of anisodamine on the changes of oxygen-derived radicals (Table 1)

Table 1 Effect of anisodamine on change of oxygen-derived radicals

Groups	LPO(nmol/100mg)		SOD(nu/mg pr)	
	EG(10)	CG(10)	EG(10)	CG(10)
Pre-obstruction of blood	48.50±2.53	53.80±2.19	109.70±4.23	105.00±7.33
Pre-reperfusion	61.10±5.12	72.30±2.44	100.20±5.66	97.60±6.35
30' post-reperfusion	164.40±10.55	273.30±14.61[b]	72.50±5.60	55.10±6.47[b]
60' post-reperfusion	142.40±11.35[b]	242.40±11.92[b]	61.50±6.99[b]	43.10±6.61[b]

[b]$P<0.01$ *vs* control group.

Effect of rewarming on LPO and SOD of grafted liver (Tables 2 and 3)

Table 2 Effect of rewarming on LPO of grafted liver

Temperature of rewarming	*n*	Pre-obstruction	Post-rewarming	30'post-reperfusion[b]	60'post-reperfusion
12℃	5	51.25±536	71.00±14.72	245.00±44.63	195.25±38.14
20℃	5	51.00±6.92	68.00±11.95	211.25±37.49	192.25±10.08
28℃	5	55.50±11.24	70.00±13.01	206.25±38.80	180.25±38.54
36℃[a]	5	50.00±7.22	1.75±7.55	190.50±25.34	175.50±18.65

[a]$P<0.05$ *vs* the other groups; [b]$P<0.01$ *vs* the post-rewarming group.

Table 3 Effect of rewarming on SOD of grafted liver

Temperature of rewarming	*n*	Pre-obstruction	Post-rewarming	30'post-reperfusion[b]	60'post-reperfusion
12℃	5	105.00±10.02	87.25±14.00	52.75±13.90	44.50±10.74
20℃	5	103.20±13.64	90.75±10.46	64.50±8.21	55.50±7.35
28℃	5	108.23±6.89	92.50±5.98	65.50±4.50	56.50±4.65
36℃[a]	5	112.50±8.24	90.25±9.64	72.50±10.44	64.50±10.10

[a]$P<0.05$ *vs* the other groups; [b]$P<0.01$ *vs* the post-rewarming group.

Morphologic change of liver cells

Observation under microscopy No obvious changes in HE stain between the post-rewarming groups, the hepatic tissue swelled when rewarmed at 4℃ at 30min and 60min post-reperfusion. Light red granules could be seen in the cellular plasm, no obvious changes after the rewarming at 28℃ and 36℃. At 60min post-reperfusion, the effect was better in anisodamine group than in the other groups.

Observation under electric microscopy The chrodrosome of hepatic cells swelled slightly after rewarming and the structure was roughly normal. At 30min post-reperfusion, the chrodrosomes of hepatic cells swelled, being destroyed partially and impaired in structure and the endoplasmic reticulums dilated in the 4℃, 12℃ and 20℃ rewarming groups. The injury was more serious at 60min post-reperfusion. Accasionally, the chrodrosomes swelled slightly and the ridges decreased. At 60min post-reperfusion, the chrodrosomes of hepatic cells swelled, and were impaired obviously, and the endoplasmic reticulums dilated in the non-anisodamine group. The results of anisodamine group were better evidently than the other groups. The injury of hepatic cells was the most slight in the 36℃ rewarming group.

DISCUSSION

Oxygen-derived radical and malmicrocirculation were the main causes of postoperative primary nonfunction of grafted liver[15,17]. Resent studies found that anisodamine can stabilize cell membrane and resist oxygen-derived radical[18-21], thus protecting cells from injury. Up to now, there has been no report about application of anisodamine in liver transplantation. This study deals with the protective action of anisodamine during the low temperature preserving period. The results showed that anisodamine had no obvious influence on LPO and SOD during the low temperature preserving period, yet it may reduce the production of LPO and stop the decrease of SOD after reperfusion[22]. At the time of ischemia-reperfusion, the increase of intracellular Ca^{2+} activates Ca^{2+} dependent proteinase, which can change xanthine dehydrogenase into xanthine oxidase (XOD). Rich oxygen supply accompanying with reperfusion oxidates xanthine and hypoxanthine into uric acid under the action of XOD, meanwhile produces- lots of oxygen-derived radicals[23,24]. Anisodamine is the antagonist of Ca^{2+}, it may inhibit the change of xanthine dehydrogenase into xanthine oxidase, thereby the anti-oxygen-derived radical action of anisodamine may reduce the peroxide injury of lipid of cell membrane, and relieve the reperfusive injury of grafted liver[25].

The study found that the production of LPO and decrease of SOD occurred chiefly after reperfusion. With the increase of LPO, SOD decreased gradually, indicating that SOD may antagonize LPO[26]. Pathologic observation verified that the injury of hepatic cells became more serious with the lasting of reperfusion, indicating that peroxide action of lipid caused by oxygen-derived radicals mainly occurred after reperfusion. Oxygen-derived radicals may lead to peroxide reaction of lipid, and the lipid radicals cava cause further decrease of mobility and increase of the permeability of cell membrane, swelling of the chrodrosome, release of lysosome, and serious injury of tissues[27]. We reckoned that the oxygen-derived radicals after reperfusion may damage the grafted liver, which is a chief cause of post-operative primary non-function of grafted liver.

In 36℃ rewarming group, the level of LPO was obviously lower and the activity of SOD higher than that in other groups. There was no evident morphologic change under microscopy in the 28℃ and 36℃ rewarming groups, and the change under electric microscopy was slight. It indicated that rewarming to grafted liver preserved in the low temperature fluid reduced the production of oxygen-derived radicals, and relieved the injury of grafted liver. Low temperature may decrease the activity of ATPase and the function of K^+ Na^+ and Ca^{2+} pumps in cell membrane, impair the electrolytes[28,29]. Reperfusion may lead to anomaly of Ca^{2+} and production of oxygen-derived radicals. Rewarming may improve the activity of ATPase and restore the function of pumps, therefore decreasing the intracellular concentration of Ca^{2+} and inhibiting the production of oxygen-derived radicals[30], and protecting the cells of grafted liver[31]. This study showed that morphologic change of hepatic cells was slighter in the 28℃ and 36℃ rewarming groups than in other groups. There was no significant difference between the 28℃ and 36℃ groups. Less oxygen-derived radical was produced in the 36℃ group than in other groups. Therefore, we think that it is a favorable choice for liver transplantation to apply anisodamine during the low temperature preserving period and

rewarm the grafted liver before reperfusion at 36℃[32].

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Differentially expressed genes in hepatocellular carcinoma induced by woodchuck hepatitis B virus in mice

Xiao-Yan Cao[1], Jie Liu[1], Zhao-Rui Lian[2], Marcy Clayton[2], Jia-Lu Hu[1], Ming-Hua Zhu[3], Dai-Ming Fan[1] and Mark Feitelson[2]

[1]Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an 710033, China
[2]Department of Pathology & Cell Biology, Thomas Jefferson University, Philadelphia, PA19107 USA
[3]Department of Pathology, Second Military Medical University, Shanghai 200433, China
This work was supported by NSFC grant 30024002
Correspondence to Jie Liu, Institute of Digestive Diseases, Xijing Hospital, Xi'an 710033, Shaanxi Province, China. jliu5@pub.xaonline.com
Tel: 0086-29-3375229, Fax: 0086-29-2359041
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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major causes of death in the world. The mechanism of carcinogenesis is unknown, although it is widely accepted that HBV and HCV are closely related to liver cancer[1-5]. Previously, a variety of studies have described the differences in gene expression which distinguished tumor from nontumor[6-11]. Cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer.

Traditionally, several methods were used to clone the new genes, which means to compare two population of mRNA and obtain clones of genes that expressed in one population but not in the other. Although these methods have been successful in some cases, they require many rounds of hybridization and are not well suited for the identification of rare messages. The suppression subtractive hybridization is a latest method employed in the gene cloning, which is a unique method based on selective amplification of differentially expressed sequences and overcomes technical limitation of traditional subtraction methods[12-14]. Hence, the purpose of our study is to find the differentially expressed genes in liver tumor and nontumor tissues induced by woodchuck hepatitis B virus using suppression subtractive hybridization.

MATERIALS AND METHODS

Patient samples

The tumor and nontumor tissues induced by woodchuck hepatitis B virus were obtained from Department of Pathology & Cell Biology, Thomas Jefferson University, Philadelphia, USA. The other HCC and surrounding nontumor liver tissues used for analysis were obtained from the patients who had undergone surgery for the removal of their tumors in Xijing Hospital. Fresh frozen blocks and -80℃ snap frozen paired liver and tumor samples from individual patients were collected, and were then made available for RNA extraction and *in situ* hybridization.

Total RNA and mRNA extraction

Total RNA and mRNA were extracted separately from tumor and nontumor tissues by using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA) and the quality of extraction was determined by assaying 18S and 28S rRNA with agarose gel electrophoresis and ethidium bromide staining.

RT-PCR and adaptor ligation

The reverse transcriptase PCR was started with 2μg poly-(A) + RNA isolated from tumor and nontumor tissues. Two adaptors were ligated to the fraction of *Rsa* I digested cDNA generated by RT-PCR. The sequence of two adaptors is as follows:

Ad1: 5'-CTAATACGACTCAC-TATAGGGCTCGAGCGGCC-GCCCGGGCAGGT-3'
Ad2: 5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGG-TGCGGAGGGCGGT-3'

cDNA subtraction and suppression PCR

The cDNA from tumor was referred to as tester, and the reference cDNA from nontumor as driver. The tester and driver cDNA were digested with *Rsa* I to obtain shorter, blunt-ended moleclules. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adaptors. The driver cDNA had no adaptor. Two hybridizations were then performed. In the first hybridization, an excess of driver cDNA was added to each sample of tester for equalization and enrichment of differentially expressed gene. During the second hybridization, templates for PCR amplification were generated from differentially expressed sequence. The entire population of molecules was then subjected into PCR to amplify the desired differentially expressed genes. In the first PCR, only differentially expressed genes were amplified exponentially because of using suppression PCR. The second PCR was performed using nested primer which matched the sequence of adaptors to reduce the background and further enrich the differentially expressed genes.

Sequencing and GeneBank search of cloned genes

Following agarose gel electrophoresis, the unique fragments were eluted from the gels (using Qiagen gel extraction kit,

Qiagen, Inc. Valencea, CA, USA) and cloned into pT7Blue (R) T vector (Novagen, Medison, WI, USA). Positive clones were selected by blue-white phenotype. Recombinant DNAs were isolated from minipreps of individual clones, and digested by *Rsa* I to check insert size, and then both strands were individually analysed by sequence analysis in the DNA sequence facility at the Kimmel Cancer Institute of Thomas Jefferson University in USA. The sequences obtained were compared with those in GeneBank using the FASTA command in the GCG software package for homology to known genes.

In situ hybridization (ISH)

The gene fragments obtained from PCR select cDNA subtraction were used as probes for *in situ* hybridization (ISH). ISH was conducted to verify that the subtraction hybridization procedure yielded probes whose expression was different between tumor and normal tissues. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according the instruction provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

RESULTS

PCR selected cDNA subtraction, cloning, sequencing and GeneBank search

PCR select cDNA subtraction generated totally 14 differentially expressed genes in tumors as compared with nontumors. Among them, 8 cDNA fragments from both tumor and nontumors had considerable homology with known genes in GeneBank (Table 1). Five genes from tumor and one gene from normal liver tissues had no homology as compared to those in the GeneBank, which implied that these may be new genes. PCR select cDNA subtraction was also performed with HBV virus X gene transfected HepG2 cells and control HepG2 cells. Ten genes were differentially expressed in HepG2X compared with HepG2 cells(data not shown). Interestingly, three genes cloned from the tumor tissue of woodchuck mouse liver shared considerable homology with sequences independently found to be upregulated in HBV-X [+] cells, suggesting that the different expressions of HBxAg effector can be independently observed in the tumor and nontumor tissues induced by woodchuck hepatitis B virus.

Table 1 Differentially expressed genes in tumor and nontumor liver induced by woodchuck hepatitis B virus

Clone	GeneBank search	
	Match	% homology
Tumor[a]		
T8[b]	Human chromosome 1(UT751,L1637).	54% in 280bp overlap
T18	Unknown protein, uterine endometrium(x7723)	60% in 151bp overlap
T19	Ribosomal protein L35A(x03475)	88% in 91bp overlap
T22	Human T cell receptor beta chain(L166059)	61% in 97 bp overlap
T6	None	
T7[b]	None	
T11[b]	None	
T24	None	
T25	None	
Nontumor[a]		
N7	Human aminopeptidase N(x13276)	93% in 54 bp overlap
N10	Human IFN receptor gene (U10360)	79% in 271 bp overlap
N11	Human glutathione S-transferase(L02321)	75% in 248 bp overlap
N13	Beta-2 glycoprotein 1 from HepG2(S80305)	79% in 159bp overlap
N8	None	

[a]The clones represent fragments of genes whose expression is activated (T6,T7, T11, T18, T19, T22, T24, T25) or suppressed (N7, N8, N10, N11, N13) in HCC compared to nontumor cells.
[b]Probes whose sequences share considerable homology with sequences independently found to be upregulated in HbxAg[+] cells.

Validation and in vivo expression patterns of these genes

The cDNA fragments obtained from subtraction hybridization of tumor and nontumor tissue were then used as probes for *in situ* hybridization.In all cases, the probes from tumor showed transcripts that were preferentially expressed in tumor tissue compared with nontumor tissues. In contrast, the genes from nontumor tissue demonstrated strong hybridization in normal tissues, but little or no signal in tumor tissues.

DISCUSSION

Hepatocellular carcinoma is one of the major causes of the death in the world[15-20]. Although many researchers worked on HCC, the mechanism is still unclear[21-46]. It is widely accepted that HBV is closely associated with HCC, especially HBxAg. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[47-49]. The site of HBV integration is scattered throughout the host genome[50], making it unlikely that HBV brings about hepatocellular transformation by cis acting mechanisms in most cases. With regard to virus sequences, integration commonly occurs within a small region near the end of the virus genome[51], which is consistent with the hypothesis that transformation may be associated with the expression of one or more virus proteins from the integrated templates acting in trans. Integrated fragments of HBV DNA have been shown to make a truncated preS/S and or HBX polypeptides, both of which have trans-activting activities[52-56]. However only HBxAg transforms a mouse hepatocyte cell line in culture[57,58], and gives rise to liver tumors in at least one strain of transgenic mice[59-61]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[62-66]. HBxAg is more consistently expressed than preS in the liver of infected patients. In addition, the findings that HBxAg binds to and inactivates the tumor suppressor p53 both *in vitro* and *in vivo*[67-69], and that it may bind to and alter the function of other transcriptional factors in the cells[70], implied that HBxAg

function is important to the pathogenesis of HCC. There is some evidence that HBxAg naturally trans activates the insulin-like growth factor-1 (IGF-1) receptor[71], and may also stimulate the production of IGF-11[72], both of which may help sustain the survival and/or growth of tumor cells.

Because lots of factors are involved in the development of HCC induced by HBV and the mechanism need to be further elucidated, the new genes, especially the functional genes directly related with tumor are still worth being found in the liver tissues infected by HBV. Using the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor induced by woodchuck hepatitis B virus. The use of these fragments as probes for *in situ* hybridization of tumor and nontumor tissues verified that the PCR-selected cDNA subtraction actually yielded differences in the gene expression that distinguished tumor from nontumor, and that its differential expression may be relevant to the pathogenesis of HCC. Because of hepatitis B virus is closely associated with the development of chronic liver diseases, such as hepatitis and cirrhosis, as well as with the development of HCC, it is not known whether these differences are associated with HBxAg associated trans-activation[73-77], its inhibition of protesome function[60], its ribo/deoxy APTase[78], or AMP kinase activation[79], and/or its ability to alter signal transduction pathways[80]. However, experiments are in progress to firmly address these issues.

The results of this study showed that the up-regulation of multiple genes in tumor had considerable homology with known products from GeneBank, suggesting that the function of these genes is likely to positively regulate cell growth, while several genes generated from normal tissues suggests that these genes may be the negative regulators for cell growth. In addition, five genes from tumor and one gene from normal liver tissues had no homology as compared with entries in GeneBank, which implied that these may be new genes, and that it is very important to clone the full-length genes of these cDNA fragments to do the functional analysis. This kind of experiments are already on the way.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Cloning of differentially expressed genes in human hepatocellular carcinoma and nontumor liver

Xiao-Yan Cao[1], Jie Liu[1], Zhao-Rui Lian[2], Marcy Clayton[2], Jia-Lu Hu[1], Ming-Hua Zhu[3], Dai-Ming Fan[1] and Mark Feitelson[2]

[1]Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
[2]Department of Pathology & Cell Biology, Thomas Jefferson University, Philadelphia, PA19107 USA
[3]Department of Pathology, Second Military Medical University, Shanghai 200433, China
This work was supported by NSFC Grant 30024002.
Correspondence to Jie Liu, Institute of Digestive Diseases, Xijing Hospital, Xi'an 710033, Shaanxi Province, China.
Tel: 0086-29-3375229, Fax: 0086-29-2359041
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INTRODUCTION

The mechanism of hepatocellular carcinoma (HCC) is still unclear, although some genes have been found to play a role in the transformation of liver cells, and a variety of studies have described differences in gene expression which distinguished tumor from nontumor[1-6]. The new genes, especially the functional genes directly related with tumor are still worth being found.

The purpose of our study is to find the different genes between human liver tumor and normal tissues using suppression subtractive hybridization.

MATERIALS AND METHODS

Patients samples

HCC and surrounding nontumor liver tissues used for analysis were obtained from the patients who had undergone surgery for the removal of their tumors in Xijing Hospital. Fresh frozen blocks and -80℃ snap frozen paired liver and tumor samples from individual patients were collected, and were then made available for RNA extraction and *in situ* hybridization.

PCR selected cDNA subtraction, cloning, sequencing and identification of cloned gene fragments

The difference in gene expression between human tumor and nontumor tissues were evaluated by a commercially available subtraction hybridization approach (the PCR selected cDNA subtraction kit from Clontech, Palo Alto, CA, USA) according to the instruction provided by the manufacturer. Briefly, we got total RNA and mRNA from tumor and nontumor tissues using the Qiagen RNeasy Kit (Qiagen, Inc. Valencea, CA, USA), and then both mRNA (2 μg each) were converted into cDNA. We refer to the cDNA from tumor as tester, and the reference cDNA from nontumor as driver. The tester and driver cDNA were digested with *Rsa* I to obtain shorter, blunt-ended moleclule. The tester cDNA was then subdivided into two portions and each ligated with different cDNA adapters. The driver cDNA had no adaptor. Two hybridization was then performed. In the first hybridization, an excess of driver cDNA was added to each sample of tester for equalization and enrichment of differentially expressed gene. During the second hybridization, templates for PCR amplification were generated from differentially expressed sequence. The entire population of molecules was then subjected into PCR to amplify the desired differentially expressed genes. In the first PCR, only differentially expressed genes were amplified exponentially because of using suppression PCR. The second PCR was performed using nested primer to reduce any background and to further enrich differentially expressed genes. The cDNA fragments were directly inserted into a T/A cloning vector(Novagen, Medison, WI, USA), and homology analysis was undertaken within GeneBank. On the other hand, we used normal tissues as the tester and tumor as the driver to do PCR select cDNA hybridization. The procedure was as above.

In situ hybridization (ISH)

The gene fragments obtained from PCR select cDNA subtraction were used as probes for *in situ* hybridization (ISH). ISH was conducted to verify that the subtraction hybridization procedure yielded probes whose expression differed in tumor compared to normal tissue. ISH was carried out using the Oncor ISH and digoxigenenin/biotin detection kits according to the instruction provided by the manufacturer (Oncor, Gaithersburg, MD, USA).

RESULTS

PCR selected cDNA subtraction, cloning, sequencing and GeneBank search

PCR select cDNA subtraction generated totally 19 differentially expressed genes in tumors and nontumors. Among them, 14 cDNA fragments had considerable homology with known genes in GeneBank (Table 1). For example, T2 and T3 had homology with ribosomal protein and elongation factor EF-1α, suggesting that these genes may stimulate cell growth. N1 from normal tissues had homology with interferon gamma gene, suggesting that this gene may be a negative regulator for cell growth. Interestingly, one gene from tumor

and three genes from normal liver tissues had no homology as compared with those in GeneBank, which implied that these may be new genes.

Validation and in vivo expression patterns of these genes

The cDNA fragments obtained from subtraction hybridization of tumor and nontumor tissues were then used as probes for *in situ* hybridization. In all cases, the probes from tumor showed transcripts that were preferentially expressed in tumor tissues as compared with nontumors. In contrast, the genes from nontumor tissues demonstrated strong hybridization in normal tissues, but little or no signal in tumor tissues.

Table 1 Differentially expressed genes in human tumor and nontumor liver

Clone	GeneBank search	
	Match	% homology
Tumor		
T1	Retinoblastoma gene (L11910)	75% in 193 bp overlap
T2	Ribosomal protein L7(L16588)	87% in 209 bp overlap
T3	Elongation factor EF-1α (J04617)	85 % in 157 bp overlap
T4	2-oxoglutarate dehydrogenase (D10525)	89% in 258 bp overlap
T5	Proteasome activator HPA28 subunit β (D45348)	93% in 204 bp overlap
T6	Ribosomal protein S2 (X57432)	89% in 195bp overlap
T7	Rab geramylgeranyl transferase-α Subunit(Y08200)	90% in 110 bp overlap
T8	Nuclear-encoded mitochondrial NADH-ubiquitinone reductase	93% in 197 bp overlap
T9	None	
Nontumor		
N1	Interferon gamma gene (L07633)	88% in 308bp overlap
N2	None	
N3	V-fos transformation effector protein	92% in 200bp overlap
N4	Sigma-1 receptor (266537)	75% in 123bp overlap
N5	Glycoprotein gll gene (D00464)- 3'flanking region	62% in 549bp overlap
N6	None	
N7	RABAPTIN-5 protein(X91141)	86% in 110bp overlap
N8	Dishevelled-3 (DUL3) protein	89% in 72bp overlap
N9	None	
N10	None	

DISCUSSION

Hepatocellular carcinoma is one of the major causes of death in the world[7-10]. The mechanism of carcinogenesis is unknown, although it is widely accepted that hepatitis B virus (HBV) and hepatitis C virus (HCV) are closely related to liver cancer, especially hepatitis B virus X antigen[11-14]. A common feature of HBV infection is the integration of HBV DNA, in whole or in part, into host chromatin[15-17]. The sites of HBV integration are scattered throughout the host genome[18], making it unlikely that HBV brings about hepatocellular transformation by cis-acting mechanisms in most cases. With regard to virus sequences, integration commonly occurs within a small region near the end of the virus genome[19], which is consistent with the hypothesis that transformation may be associated with the expression of one or more virus proteins from the integrated templates acting in *trans*. Integrated fragments of HBV DNA have been shown to make a truncated preS/S and or HBX polypeptides, both of which have trans-activting activities[20-24]. However, only HBxAg transforms a mouse hepatocyte cell line in culture[25,26], and gives rise to liver tumors in at least one strain of transgenic mice[27-29]. Independent work has also shown that HBxAg stimulates the cell cycle, perhaps by the activation of a number of signal transduction pathways[30-34]. The expression of HBxAg is more consistent than that of preS in the liver of infected patients. In addition, the findings that HBxAg binds to and inactivates the tumor suppressor p53 both *in vitro* and *in vivo*[35-37], and that it may bind to and alter the function of other transcriptional factors in the cells[38], implied that HBxAg function is important to the pathogenesis of HCC. There is some evidence that HBxAg naturally trans-activates the insulin-like growth factor-1 (IGF-1) receptor[39], and may also stimulate the production of IGF-11[40], both of which may help sustain the survival and/or growth of tumor cells.

Because the mechanism of HCC induced by HBV still need to be elucidated, cloning of the genes, especially the genes associated with HBV and HCV, is still very important to account for the development of liver cancer. By the newly created method, which is the suppression subtractive hybridization, we identified the difference in gene expression which distinguished tumor from nontumor. The use of these fragments as probes for *in situ* hybridization of tumor and nontumor tissues verified that the PCR-selected cDNA subtraction actually yielded differences in the gene expression that distinguished tumor from nontumor, and that its differential expression may be relevant to the pathogenesis of HCC. It is not known whether these differences are associated with HBxAg associated trans-activation[41,42], its inhibition of protesome function[43] its ribo/deoxy APTase[44], or AMP kinase activation[45], and/or its ability to alter signal transduction pathways[46], because hepatitis B virus is closely associated with the development of chronic liver diseases, such as hepatitis and cirrhosis, as well as with the development of hepatocellular carcinoma (HCC)[47-60]. However, experiments are in progress to firmly address these issues.

The results of this study showed that the up-regulation of multiple genes in tumor which have considerable homology with known products from GeneBank, for example, ribasomal protein and elongation factor EF-12, suggesting that the

function of these genes is likely to positively regulate cell growth. Several genes are generated from normal tissues and one has >88% homology with interferon gamma gene, suggesting that these genes may be the negative regulators for cell growth. In addition, one gene from tumor and three genes from normal liver tissues had no homology, as compared with entries in GeneBank, which implied that these may be new genes, and that it is very important to clone the full-length genes of these cDNA fragments to do the functional analysis. This kind of experiments are already on the way.

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Edited by Wu XN and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Identification of the epitopes on HCV core protein recognized by HLA-A2 restricted cytotoxic T lymphocytes

Hong-Chao Zhou[1], De-Zhong Xu[1], Xue-Ping Wang[1], Jing-Xia Zhang[1], Ying-Huang[1], Yong-Ping Yan[1], Yong Zhu[2] and Bo-Quan Jin[2]

[1]Department of Epidemiology, the Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
[2]Department of Immunology, the Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
Supported by the National Nature Science Foundation of China, No. 39800121
Correspondence to Dr. Yong Zhu, Department of Immunology, the Fourth Military Medical University, Xi'an 710033, China. juliu@fmmu.edu.cn, Shaanxi , http: //www.fmmu.edu.cn
Tel: 0086-29-3374871 Ext.17, Fax. 0086-29-3374868
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Abstract

AIM **To identify hepatitis C virus(HCV) core protein epitopes recognized by HLA-A2 restricted cytotoxic T lymphocyte (CTL).**

METHODS **Utilizing the method of computer prediction followed by a 4h ^{51}Cr release assay confirmation.**

RESULTS **The results showed that peripheral blood mononuclear cells (PBMC) obtained from two HLA-A2 positive donors who were infected with HCV could lyse autologous target cells labeled with peptide "ALAHGVRAL (core 150-158)". The rates of specific lysis of the cells from the two donors were 37.5% and 15.8%, respectively. Blocking of the CTL response with anti-CD4 mAb caused no significant decrease of the specific lysis. But blocking of CTL response with anti-CD8 mAb could abolish the lysis.**

CONCLUSION **The peptide (core 150-158) is the candidate epitope recognized by HLA- A2 restricted CTL.**

Subject headings hepatitis C virus; cytotoxic T lymphocyte; HLA-A2; epitope

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INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus responsible for the majority non-A non-B hepatitis[1,2]. More than 50% - 60% of acute infection lead to chronic disease, and once chronicity is established, spontaneous recovery is exceptional. The related mechanism is still unknown[3-5]. Recent studies demonstrate that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) of patients with chronic hepatitis C recognize epitopes from different regions of both structural and nonstructural HCV proteins[6-12]. Some scholars speculate that CTL-mediated cellular immune response probably plays an important role in viral clearance[13,14].

$CD8^+$ CTL interact through their polymorphic T cell receptor with HLA class I molecules containing endogenously synthesized peptides of 9-11 on the surface of infected cells. The presence of allele-specific amino acid motifs has been demonstrated by sequencing of peptides eluted from MHC molecules. Among the best studied motifs is that of HLA-A2, which is prevalent in a high percentage of population. Several reports[7,8,15-20] described the method of using HCV derived synthetic peptides containing the HLA-A2.1 binding motif to identify and characterize the HLA-A2 restricted CTL in the peripheral blood of patients with chronic HCV infection. We[21] have designed a computer programme to score the reported HCV peptides. Our results revealed that all the reported peptides were with a relative high score of 144 points or higher. Based on the previous study, we attempted to identify the epitopes recognized by the HLA-A2 restricted CTL on HCV core protein utilizing the method of computer prediction followed by 4h ^{51}Cr release assay.

MATERIALS AND METHODS

Materials

Subjects Six patients with chronic hepatitis C and 2 healthy controls were selected from among those monitored at Xijing blood center. Table 1 summarizes patient characteristics and history of treatment. All subjects had not received any antiviral treatment for at least one year.

Table 1 HLA-A and serology of patients studied for CTL response to HCV epitopes

Subjects	HLA-A	Anti-HCV	HCV-RNA
Experiment			
Li	A2A31	+	+
Zhang	A2A11	+	+
Tang	A2A33	+	+
Zhang	A2A11	+	+
Patient control			
Li	A11	+	+
+	A3A33	+	
Health control			
Zheng	A2A11	-	-
Wang	A2A24	-	-

Note: All subjects received no treatment and had been followed-up for one year.

HLA typing HLA typing of PBMC from patients and from normal donors was determined by microcytotoxicity, using trays (One lamda, Canoga Park, CA). The HLA haplotypes of subjects participating in this study are shown in Table 1.

Merthods

Prediction of candidate HLA-A2 restricted CTL epitopes Based on previous study, we use our computer programme to predict HLA-A2 restricted CTL on HCV C protein. In brief, a

computer programme with the function of finding peptides containing HLA-A2 allele-specific peptide motif was written in C language. The HCV cDNA is translated into HCV amino acid sequence from which the peptides was chosen, and the selected peptides include those with a length of 9-11 amino acids, a leucine (L), isoleucine (I) or methionine (M) at position 2 and a leucine (L) or Valine (V) at the last position. According to Nijiman's score system, we scored six points for an anchor residue, four points for a strong and two points for a weak residue. The score for a given peptide is obtained by multiplication of the scores for each amino acid position. Predicted candidate CTL epitopes with scores of 144 or higher.

Synthetic peptides Peptides YLLPRRGPRL (core35-44), NLGKVIDTL (core 118 - 126), DLMGYIPLV (core 132 - 140) and ALAHGVRAL (core 150 - 158) were selected from the predicted results and synthesized in automated multiple peptide synthesizer (American Research Genetics, Inc). All peptides were >90% pure and diluted to $1g \cdot L^{-1}$ with RPMI1640 medium before use (Gibco, Grand Ialand, N.Y.).

CTL generation PBMC from donors were separated on Ficoll-Hypaque density gradients (Shanghai Huajing, Inc), washed three times in phosphate-buffered saline (PBS), resuspended in RPMI1640 medium (Gibco, BRL.) supplemented with L-glutamine ($10g \cdot L^{-1}$), penicillin ($5 \times 10^4 U \cdot L^{-1}$), streptomycin ($50mg \cdot L^{-1}$) and Hepes ($5mol \cdot L^{-1}$) containing $100mL \cdot L^{-1}$ fetal calf serum (FCS) and plated in 24-well plates at 4×10^6 cells per well. PBMC were stimulated with concanavalin A (ConA, 20μg per well) during the first week. On d3, 1mL of complete medium supplemented with rIL-2 at 2×10^3 $U \cdot L^{-1}$ final concentration was added into each well. On d7, the cultures were restimulated with the peptides plus rIL-2 and irradiated (30Gy) autologous PBMC feeder cells, and the cultured PBMC were restimulated five days later with the original peptides plus rIL-2. On d16, the stimulated cells were used as effectors in CTL assay.

Preparation of autoguous B lymphoblastoid cell line After Ficoll-Hypaque separation, PBMC were suspended in the RPMI1640 medium containing $200 mL \cdot L^{-1}$ FCS and then plated in 24-well culture plate at a concentration of 2×10^6 cells per well. EBV-transfected B cell lines were established by culturing 2×10^6 PBMC with $100g \cdot L^{-1}$ of cyclosporin A and 1 mL B95-8 EBV culture supernatant (provided by Dr. Jin, the Fourth Military Medical University, Xi'an). After transformation, the lymphoblastoid cell lines (B-LCL) were maintained in RPMI1640 medium with $200 mL \cdot L^{-1}$ FCS, with media change twice each week. The cell lines were maintained at 37℃ in a humidified chamber with $50 mL \cdot L^{-1}$ CO_2 and used as targets.

CTL assay Target cells were incubated overnight with synthetic peptides at $200 mg \cdot L^{-1}$, and then were labeled with 3.7MBq 1-Cr for 1h and washed three times with PBS. Cytotoxity activity was determined in a standard 4h Cr release assay using U -bottom 96 welL plates containing 5000 autogenous targets per well. All assays were performed in triplicate with effector target cell (E/T) ratios of 100:1, 50: 1, and 1:1. Maximum release was determined on the basis of lysis of labeled target cells with $50g \cdot L^{-1}$ Triton X-100. We examined spontaneous release by incubating target cells in the absence of effector cells. It was less than 25% of the maximum release. Percent cytotoixity was determined by the formula: 100×[(experiment release-spontaneous release)/ (maximum release-spontaneous release)].

Blocking of CTL response by antibodies CTL responses were tested in the presence of anti-CD8 or anti-CD4 monoclonal antibody added to the 96-well plates at the indicated concentrations used for the CTL assay.

RESULTS

Prediction of HLA-A2 restricted CTL epitopes on HCV protein

Seven high-scoring peptides (≥144 points) were selected from HCV C protein using our computer programme. Among them, peptide ① and peptide ④, namely peptide YLLPRRGPRL and peptide DLMGYIPLV, have been reported to be epitopes recognized by HLA-A2.1 restricted CTL. Predicted peptide ⑦, namely FLLALLSCL (core 177-185) was almost the same as the reported peptide LLALLSCLTV (core 178-187). The rest predicted peptides have not been proved to be epitopes recognized by HLA-A2 restricted CTL. Four peptides (peptide ①,②,④,⑤), were selected randomly from the seven predicted peptides to be used in CTL assay (Table 2).

Table 2 Predicted results of epitopes recognized by HLA-A2 restricted CTL on HCV C protein

No	Peptide sequence	Peptide site	Score
①	YLLPRRGPRL	35-44	144
②	NLGKVIDTL	118-126	576
③	TLTCGFADL	125-133	144
④	DLMGYIPLV	132-140	576
⑤	ALAHGVRAL	150-158	576
⑥	NLPGCSFSIFL	168-176	288
⑦	FLLALLSCL	177-185	144

A: Reported epitopes recognized by HLA-A2.1 restricted CTL; B: Almost consistent with the reported epitope LLALLSCLTV (core 178 -187)

Screen of HCV peptide-specific response

In this experiment PBMC from 8 subjects were stimulated individually with the four peptides from HCV C protein, and cultures were tested after 16d of expansion for peptide-specific CTL activity. Two of the four donors of HLA-A2 and HCV RNA positive responded to peptide ⑤. After induced by peptide ⑤, the two donors' PBMC can lyse autologous target cells pulsed with peptide ⑤ and the specific lysis was 37.5% and 15.8%, respectively (Table 3). Treatment of the CTL specific for peptide ⑤ with anti-CD8 mAb, but not anti-CD4 mAb, plue complement markedly reduced cytotoxic activity on target cells (Figure 1).

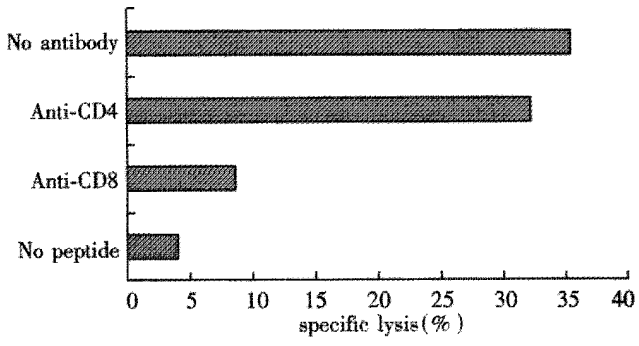

Figure 1 Blocking of CTL activity of Mr.Tang with mAb.

Table 3 CTL activity induced by predicted peptides on HCV C protein

Subject	HLA type				^{51}Cr-release (%lysis)			
	A		B		①	②	④	⑤
Experiment								
Li	2	31	51	16	-2.6	9.6	0.5	NT
Zhang	2	11	8	27	0.4	NT	2.4	6.9
Tang	2	33	44	55	-3.8	5.1	3.3	37.5
Zhang	2	11	62	35	-1.1	9.8	8.4	12.2
Healthy control								
Zheng	2	11	62	39	-2.9	8.0	6.8	-0.2
Wang	2	24	61	46	0.2	NT	3.7	10.0
Patient control								
Li	11		62	35	-2.6	2.6	2.3	-1.8
Zhu	3	33	17	35	-2.5	1.6	0	2.5

CTL activity induced by peptides at E/T ratio of 50:1; NT: No test; ①, ②, ④ and ⑤ represent YLLPRRGPRL (core35 - 44), NLGKVIDTL (core118-126), DLMGYIPLV (core132-140) and ALAHGVRAL (core150-158), respectively.

DISCUSSION

CTL mediated cellular immune response probably plays an important role in anti HCV infection. Many researchers reported[22-30] that CTL specific for HCV were discovered in PBMC and liver infiltrated lymphocytes of patients infected with HCV, and that the epitopes recognized by CTL were identified. Owing to the fact that HLA-A2 exhibits a high gene frequency in populations, Cerny *et al*[7] focused their study on the epitopes recognized by HLA-A3 restricted CTL and have determined several epitopes on every protein region recognized by CTL. However, because of the great work and high cost, it is quite difficult to manipulate in general laboratory. In this study, we attempted to identify the HCV peptides containing HLA-A2 binding motifs, and to confirm the prediction via 4h ^{51}Cr release assay.

Prediction of candidate epitopes recognized by HLA-A2 restricted CTL

According to the reference[31-32], determining epitopes recognized by CTL included two steps: synthesis of many peptides with multi-peptide overlapping method, and identification of the peptides with experimental means. Up till now, peptides YLLPRRGPRLL (core35 - 44), DLMGYIPLV (core132 - 140), and LLALLSCLTV (core178 -187) were determined by using this method. The method is direct and reliable, but difficult to manipulate. We analyzed HCV core protein using computer programme. The results demonstrated that there were only 7 peptides with scores of 144 or higher. Of those[21], two peptides, YLLPRRGPRL (core35 - 44), DLMGYIPLV (core132 - 140) were ever reported in other studies, which could be recognized by HLA-A2 restricted CTL. Another peptide, FLLALLSCL (core177 -185) was in agreement with the reported peptide LLALLSCLTV (core178 - 187). Purposeful study of the 7 peptides would simplify experimental processes and save cost. Of those, 4 peptides (Nos ①,②,④,⑤), were synthesized and applied in CTL assay.

Activation of CTL in peripheral blood of donors by synthsized peptides

Four synthesized peptides of the HCV core protein were tested using CTL assay. Four donors were positive for HLA-A2. Among donors positive for HCV RNA, 2 donors' PBMC were found to have lysed autologous target cell-labeled with peptide ⑤. The specific lysis rate was 37.5% and 15.8% respectively. The other 3 peptides didn't show obvious CTL induction action. CTL response was very weak in two healthy and HLA-A2 positive donors, and also in two HCV RNA+ HLA-A2- donors.

According to the reference[33], the lysis might be considered specific with the lysis rate $\geqslant$15%. The specific lysis rate was up to 37.5% in Tang with effector / target cell (E/T) ratio of 50:1, and 15.8% in Zhang with E/T ratio of 100:1. Blocking of the CTL response with anti-CD4 mAb did not decrease the specific lysis significantly. But blocking of the CTL response with anti-CD8 mAb could abolish the lysis. It indicated that[34-40] the lysis was mediated by CD8$^+$ T cells rather than CD4$^+$ T cells, and that the epitope ⑤ was probably the candidate epitope recognized by HLA-A2 restricted CTL.

Although 3 peptides, including 2 reported in other studies, YLLPRRGPRL (core35 - 44), DLMGYIPLV (core132-140), didn't demonstrate obvious CTL induced activity, we could not draw a conclusion that they were not HLA-A2 restricted CTL recognized epitopes. The two reported epitopes were recognized by HLA-A2.1 restricted CTL, but in this study, we did not determine the HLA-A2 subtypes. Various subtypes of HLA-A2 restricted CTL probably recognized different epitopes[41-50]. Another pssible reason is that HCV protein sequence of HCV-infected patients might not be in complete accordance with the synthesized peptides. To clarify the reasons, further study is still necessary.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Treatment of malignant digestive tract obstruction by combined intraluminal stent installation and intra-arterial drug infusion

Ai-Wu Mao[1], Zhong-Du Gao[1], Jia-Yu Xu[2], Ren-Jie Yang[3], Xiang-Seng Xiao[4], Ting-Hui Jiang[1] and Wei-Jun Jiang[1]

[1]Department of Interventional Radiology, Shanghai S. T, Luke's Hospital, 768 Yu Yuan Road, Shanghai 200050, China
[2]Department of Gastroenterology Rui Jin Hospital, Shanghai Second Medical University, [3]Department of Tumor Hospital, Beijing Medical University
[4]Department of Imaging Chang Zheng Hospital, Shanghai Second Military Medical University
Correspondence to Ai-Wu Mao, Department of Interventional Radiology, Shanghai S.T, Luke's Hospital, 768 Yu Yuan Road, Shanghai 200050, China.maw1@elong.com.
Tel: 0086-21-62524259 Ext. 3397, 3019
Fax: 0086-21-62113884
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Abstract

AIM **To study the palliative treatment of malignant obstrution of digestive tract with placement of intraluminal stent combined with intra-arterial infusion of chemotherapeutic drugs.**

METHODS **A total of** 281 **cases of digestive tract malignant obstruction were given per oral** (**esophagus, stomach, duodenum and jejunum**), **per anal** (**colon and rectum**) **and percutaneous transhepatic** (**biliary**) **installation of metallic stent. Among them,** 203 **cases received drug infusion by cannulation of tumor supplying artery with Seldinger's technique.**

RESULTS **Altogether** 350 **stents were installed in** 281 **cases, obstructive symptoms were relieved or ameliorated after installation. Occurrence of restenotic obstruction was** 8-43 **weeks among those with intra-arterial drug infusion, which was later than** 4-26 **weeks in the group with only stent installation. The average survival time of the former group was** 43 (3-105) **weeks, which was significantly longer than** 13 (3-24) **weeks of the latter group.**

CONCLUSION **Intraluminal placement of stent combined with intra-arterial infusion chemotherapy is one of the effective palliative therapies for malignant obstruction of the digestive tract with symptomatic as well as etiological treatment.**

Subject headings digestive tract disease; treatment; stent; therapeutic embolization chemotherapy; infusion, local

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INTRODUCTION

Intraluminal installation of stent in digestive tract is being more and more widely used[1-31]. Drug infusion via supplying artery and embolization therapy have been used in clinical practice as an effective way of suppressing the growth of malignant tumors[32-43] But up to now there have been few reports of the combined use of the two in the treatment of malignant stenotic lesions[44,45]. We have used the combined therapy in cases of malignant stenosis of digestive tract and have obtained good results in relieving symptoms, improving quality of life and prolongation of survival time. We managed 281 cases from October 1996 to May 2000 and the therapeutic results are reported below.

MATERIAL AND METHODS

Patients

A total of 281 patients with malignant obstruction of digestive tract were treated. Among them, 182 were males and 99 females, age ranged from 20 to 93 years, with an average of 65 years. The cause of obstruction was stenosis or obstruction of the digestive tract by infiltration or pressure of the malignant tumor. All cases belonged to late stages of tumor, impossible for surgical resection. One hundred and seventy-seven cases had operative histories of primary tumor resection, with reconstruction of digestive tract, biliary endostomy, bypass drainage or laparatomy, etc, 6 had histories of four operations, 83 cases of one operation . The primary disease included carcinoma of esophagus, 79 cases; stomach cancer 55 cases; carcinoma of liver, 25 cases; carcinoma of pancreas, 53 cases; colorectal cancer, 39 cases; cholangiocarcinoma, 10 cases; and other carcinomas, 20 cases. Pathological types: adenocarcinoma, 96 cases; squamous cell carcinoma, 74 cases; and others, 111 cases. Among the 281 cases, site of stenosis and obstruction at esophagus, 119 cases; at stomach (including gastrointestinal stoma), duodenum and jejunum, 76 cases; colorectal segment, 28 cases; and biliary tract, 58 cases. Some cases had more than two sites of stenosis or obstruction. They were divided in to groups A and B by patient or close relative's choice. Group A (203 cases) received treatment by installation of intraluminal stent combined with intra-arterial cannulation for drug infusion via supplying artery. Group B (78 cases) received only installation of intraluminal stent and again according to their choice supplemented with intravenous chemotherapy, radiotherapy or traditional Chinese medicinal treatment. Those with impaired liver and /or kidney function due to the cancer, or very poor general condition not suitable for intravenous chemotherapy and radiotherapy were advised to join group A. In group A, there were 132 males and 71 females, aged 20 - 93 years, averaging 61 years. Of them, 82 cases could not meet the requirements of routine radiotherapy and chemotherapy such as blood routine, liver and kidney

function or abnormalities of some other tests. In group B, there were 50 males and 28 females, age ranged from 28 - 87 years, averaging 58 years. Fourteen cases could not meet the requirements of routine radiotherapy and chemotherapy. Karnofsky quality of life evaluation in group A was 10-80 (average 36.2) score and 20 - 80 (average 40.2) score in group B.

Metallic intraluminal stent

Woven type nickel-titanium alloy stent was mainly used. The tubular meshwork, the whole some structure somewhat like wallstent was woven by single fine threads of nickel-titanium alloy of 0.20mm-0.32mm in diameter. Membranous stent was coated with polyurethane or silica gel on the mesh of the stent main body. For biliary tract, straight-neted duct stent was used without trumpet ends. The diameter of the tube is 0.8cm - 1.6cm, and the length 6cm - 10cm. The stent used for esophagus, stomach, duodenum and jejunum is of the type with trumpet opening on one end or both ends. The proximal end is basin-trumpet in shape, and the distal end cup or mushroom in shape. The tubular diameter of the stent main body is 1.1 cm - 1.5 cm at cervical segment, 1.8 cm - 2.8 cm at thoracic and abdominal segment of the esophagus. At stomach body, it is 2.0cm-2.5cm, and at duodenum and jejunum 1.8 cm - 2.0 cm; the length of the stent is 3.5 cm - 14cm. The stent for the use of colon and rectum has both ends of ball or mushroom shape trumpet opening. The tubular diameter of the stent main body is 2.0cm - 2.2cm at transverse colon, 2.5cm - 2.8cm at descending colon, and 2.5cm - 3.2cm at sigmoid colon and rectum; the length of the stent is 8cm-14cm. The width of the trumpet opening of all stents is 0.4cm-0.7cm. Some stents are specially made for specific requirements. Other stents we have used are home made"Z" shape esophageal stent, Wallstent for biliary and esophageal use (Schineder) and Memotherm biliary stent (Agmidel).

Transporter and auxilliary instruments

Wallstent and Memotherm stents were provided with a disposable coaxial two duct type transporter. Home-made "Z" shape stent was fixed with a sheath tube type pusher. Home made biliary and esophageal stents for cervical segment were used together with a sheath tube type pusher of Cook Company. For other stents we used self-made posterior positioned coaxial three ducts type transporter. Other auxilliary instruments included 6F Corber transmitting catheter, 2600mm long Torumo Radifocus guide wire, 2600mm-3000mm long soft head super-hard guide wire, 1300mm long exchange catheter, bulb dilating catheter of 25mm in diameter and double-channel imaging catheter made from used bulb catheters. Gastroscope, colonoscope, microwave or diathermic apparatus were also available.

Treatment modalities

Installation of intraluminal stent. Stent was placed perorally to esophagus, stomach, duodenum and jejunum. Radiopaque guide wire was inserted via gastroscope or perorally. The pusher attached with the stent was introduced by the guide wire so that the stent could be slowly released after passing through the stenosed segment. For the installation of stent in duodenum and jejunum, super-hard guide wire was introduced via exchange catheter, and the stent was put by the pusher introduced by the hard guide wire. Biliary stent was installed by percutaneous puncture and insertion method. The puncture needle was inserted percutaneously and transhepatically into biliary tract. Radifocus guide wire was introduced through the puncture needle along the intrahepatic biliary tract into the common bile duct, and then through the stenosed segment deep into the small intestine. Through exchange soft head hard guide wire, sheath tube or coaxial two ducts type transporter put the stent into the stenosed segment. Colonic and rectal stent was inserted through anus. Radifocus guide wire was inserted through the stenosed segment by the catheter or via colonoscope. Exchange catheter was introduced and replaced the super-hard guide wire. The hard guide wire introduced the coaxial two ducts type transporter that releases the stent.

Intra-arterial drug infusion

Two to eight days before the installation of the stent or 0d-4d after it, chemotherapeutic infusion via cannulation of the supplying artery of the tumor was given. The Seldinger's technique was used. According to the primary lesion or the infiltration field of the metastatic site, the supplying artery of the tumor was chosen as the target artery. Generally, the main trunk of the supplying artery was chosen for cannulation or multiple target points were chosen for cannulation at one time. The target arteries chosen for cannulation are: external jugular artery, inferior thyroid artery, or subclavicular artery bronchial artery, esophageal propriae artery, intercostal artery, hepatic artery (or coeliac artery trunk), superior mesenteric artery, inferior mesenteric artery, internal iliac artery, etc. The perfusing drugs used with dosage calculated according to surface area of the body were epirubicin 25mg·m^2, carboplatin 200mg·m^2, 5-Fu 500mg·m^2 forming triad drug group for use. For patients with abnormal liver and /or kidney functions (all caused by the malignant factors), dosage will be decreased accordingly. For those patients having normal routine profiles with better general condition, VM26 50-100mg·m^2 was added. For those with impaired cardiac function, adriamycin was substituted by pirarubicin 300mg·m^2. For some suitable patients emulsified 40% lipiodol and chemotherapeutics were given for superselective emolization. Patients with arterial venous fistula were first given gelfoam strips to block the fistular tract and then drug infusion. The second treatment was administered three weeks afterwards, and the third treatment was given after another 3 -5 weeks. From then on, the interval could be prolonged to 1.5-5 months.

RESULTS

Installation of stent (Table 1)

A total of 350 stents were installed in 281cases. Among them 144 were esophageal stents (23 in cervical segment, 69 in thoracic segment and 52 in abdominal segment of esophagus). Ninety-eight stents were put in stomach duodenum and jejunum (21 in gastric body and pyloric region, 22 in gastrointestinal anastomotic oriface, 49 in descending and horizontal part of duodenum and 6 in proximal jejunum); 30 in colon and rectum (9 in transverse colon, 5 in descending and 16 in sigmoid colon and rectum). Seventy-eight were biliary stents. Among the 144 esophageal stents, 142 were home-made woven type tubular mesh stents (127 with attached membrane and 15 without membrane), one was home-made "Z" type stent with attached membrane. There

was one wallstent without membrane. Among 98 stents used in stomach, duodenum and jejunum, 97 were home made (35 with attached membrane, 62 without). One Wallstent without membrane was used. Thirty stents used in colon and rectom (20 with membrane and 10 without) and 71 of 78 biliary stents were all home made. There were 4 Wallstents and 3 Memotherm stents. Biliary stents were all without membrane. The distribution of stents installed in groups A and B are shown in Table 1.

Drug infusion (Table 2)

Group A (203 cases) had received cannulation of supplying artery with infusion of chemotherapeutics (a total of 708 times). The minimum was one time (9 cases) and maximum was 14 times (one case). The average time was 3.49. Table 2 shows different patients undergoing drug infusion. Among the nine cases receiving treatment only once, which was refused by their relatives in 5 cases, and discontinued due to deterioration of their general condition in 2 cases, and the remaining 2 cases died. Three out of the 203 cases had received lipiodol emulsion embolization for 7 times, 11 cases had gelfoam embolization for 18 times. Besides occational hematoma at the puncture site, few cases complained of pain at the site of drug infusion and embolization treatment and 37% of cases had reversible lowering of blood counts, and no other arterial interventional procedure related complication was noted.

Clinical symptomatic impro ement

Among 119 cases with installation of esophageal stent, 18 had stent in the cervical segment and they all could take low residue regular diet. In 101 cases with stent in the thoracicor abdominal segment, those with simple stenosis basically restored to normal meals. Among 13 patients with esophagotracheal or esophagothoracic fistula, 11 could take normal meals after complete obliteration of the fistular tract, one still had some irritating cough while eating. Another case was a patient who developed esophageal stenosis with esophagothoracic fistula after unilateral pneumonectomy. Four days after installation of stent and obliteration of the fistula, the patient developed obvious irritating cough during eating. Imaging demonstrated proximal orifice of the stent protruded into the thoracic cavity forming another fistular tract. Among 76 patients with stent in stomach, duodenum or jejunum, 71 restored to normal meals and 5 had the obstructive symptoms improved, but could only take liquid diet. Twenty-eight cases with stent in colon or rectum had immediate relief of intestinal obstructive symptoms after installation, one of them who had colonic pelvic fistula had no more exudative leakage from the fistula tract after the installation. A case of rectal vaginal fistula had much less fecal exudation from vagina after installation. In 58 cases with installation of stent into the biliary tract, jaundice was significantly decreased, 39 cases had serum bilirubin returned to normal within a week. Nineteen cases had serum bilirubin dropped below 70mmol/L. Clinical symptomatic improvement were similar between groups A and B shortly after treatment. Among 82 cases in group A whose conditions could not reach the requirements of laboratory profiles for routine radiotherapy or chemotherapy, 46 cases fulfilled the requirements after two drug infusions. Among 14 cases of group B, only 3 cases approached the requirements after 1-2 months. As the disease progressed, the number of patients whose original profiles were normal but became abnormal when the disease got worse, was significantly larger in group B than in group A. Comparison of Karnofsky life quality evaluation between the two groups at 4 - 6 week after installation of stent showed that the average score in group A increased from 36.2 to 53.2, while in group B, from 40.2 to 46.2. There was significant difference between the two groups.

Maintenance of efficacy and survival time

In group B, 23 restenotic obstructions occurred 4-26 weeks after treatment in 15 (23.1%) cases. In group A, 29 (14.3%) had 38 restenotic obstructions 8-43 weeks after treatment (Table 3). The survival time in group A was 3-105 weeks, averaging 43 weeks, while 3-24 weeks in group B averaging only 13 weeks. The survival time of group A was more significantly prolonged than group B (Table 4).

Table 1 Installation of stents

Site	Group A		Group B		Total	
	No.of cases	No.of stents	No.of cases	No.of stents	No.of cases	No.of stents
Esophagus	81	95	38	49	119	144
Stomach, small intestine	55	64	21	34	76	98
Colon, rectum	19	20	9	10	28	30
Biliary tract	48	53	10	25	58	78
Total	203	233	78	116	281	350

Table 2 Drug infusion

Site	*N*	Infusion (*n*)	Total infusion times	Max. per case	Min. per case	Average per case
Esophagus	119	81	310	12	2	3.83
Stomach, small intestine	76	55	229	14	1	4.16
Colon, rectum	28	19	39	8	1	2.94
Biliary tract	58	48	130	9	2	3.65
Total	281	203	708			3.49

Table 3 Maintenance of efficacy (groups A / B)

Site	*n*	Restenosis		Restenosis (wk)
		n	No.of times	
Esophagus	81 / 38	15 / 10	20 / 12	12-35 / 04-17
Stomach, small intestine	55 / 21	06 / 04	07 / 05	17-42 / 07-16
Colon, rectum	19 / 09	02 / 00	02 / 00	26-43 / 0
Biliary tract	48 / 10	06 / 04	09 / 06	8-33 / 05-26
Total	203 / 78	29(14.3) / 15(23.1)	38 / 23	08-43 / 04-26

Table 4 Comparison of survival time (groups A / B)

Sites	Cases	Survival (*n*)	Death (*n*)	Survival (wk)	
				Range	Average
Esophagus	81 / 38	23 / 03	58 / 35	5-103 / 5-20	36 / 14
Stomach, small intestine	55 / 21	09 / 02	46 / 19	4-105 / 2-17	38 / 11
Colon, rectom	19 / 09	02 / 00	17 / 09	3-98 / 3-13	33 / 08
Biliary tract	48 / 10	06 / 01	42 / 09	6-54 / 3-24	30 / 18
Total	203 / 78	40 / 06	163 / 72	3-105 / 3-24	43 / 13

$P<0.05$, for all sites.

DISCUSSION

Significance and technical difficulties

Digestive tract is the necessary passage of alimentation digestion, absorption and excretion of waste metabolites. Obstruction of digestive tract interferes with food intake, bowel movement, or causes obstructive jaundice. All these seriously affect the quality of life of the patients and even accelerate death. In 1983, Frimberger first reported the use of metallic stent to treat esophageal stenosis[46]. Domschke in 1990 successfully used self-expansible woven mesh type metallic stent to treat a case of esophageal malignant obstruction[47]. Thus it was made possible the relief of obstruction of digestive tract by non-surgical procedures. Karnel *et al*[48], Goldrn *et al*[49], Keymling *et al*[50] and others had respectively tried to use metallic intraluminal stent in treating colonic, biliary and duodenal obstruction. Their success had laid the basis of the expanded use of digestive tract intraluminal stent. As intraluminal installation of stent in digestive tract is a non-surgical method with mini-invasive technique to render the stenosed digestive tract becoming patent again, thus providing a new approach in the palliative treatment of digestive tract malignant obstruction. Comparing with the conventional surgical operation, treatment by intraluminal installation of stent has the characteristics of mini-invasion, fast, effect, good clinical sutclins and repeatablity of the procedure. This avoids the damage caused by surgical operation. For those with no indication for operation or too weak to stand the operation, treatment with intraluminal installation of stent can provide palliation to the patients with symptomatic relief and improved quality of life.

Compared with vascular lumen and other non-vascular lumens, the various lumens at different parts of the digestive tract have special histologic structures and functional characteristics. For example, the cervical segment of esophagus has strong contractive force and is very sensitive to foreign body. Duodenum and jejunum are quite distant from mouth, their lumens are tortuous with frequent peristalsis. Colon has a haustral structure with a strong group contractile force. The peculiar tissue structure and functional characteristics make the procedure of installation of intraluminal stent somewhat difficult. So, up to now, the clinical use of installation of digestive tract intraluminal stent is still limitted to esophagus and biliary tract. We have chosen nickel-titanium alloy in the form of single fine threads woven longitudinally and transversely into a flexible mesh tabular stent according to the common feature of digestive tract structure and function. For the installation of stent in the high position cervical segment of esophagus, we made tolerance dilatation test and used small caliber so as to ameliorate foreign body or pain sensation, thus succeeding in putting the stent in the cervical segment of esophagus. We used high hardness, small friction and elongation coefficient, not easily restored once deformed material, polytetrafluroethylene, to make coaxial duct and put it in between the external and internal ducts made of polyethylene, which is soft, easily to be restored with big friction force. The coaxial duct type transporter was made in this way that can be introduced into tortuous intestinal tube to release the coaxial duct type transporter of the stent. We also used endoscope to help the insertion of the guide wire. Super-hard guide wire helps enlarge the turning angle of the colonic loops. All these measures solve the difficulty encounted in the remote release of the stent. This not only increased the success rate of installation of duodenal and proximal jejunal intraluminal stent orally[11], but also facilitated the installation of stent at high level transverse colon via anal route[17]. At the same time, we selected big caliber with high degree hardness double bulb shaped or mush room shaped trumpet orifice stent. This not only increased the expanding force but also increased the compliance of the connecting segment between the terminal opening of the sent and the normal intestinal tract. The therapeutic effect of colonic and rectal stent installation was thus elevated and its complication reduced.

Drug infusion via supplying artery of tumor

Intraluminal stent treatment can build up the basis for the patients for further treatment by the relief of digestive tract obstructive symptoms and improvement of life quality of late tumor cases. The advanced patients with tumor can rarely tolerate the toxic or side effect of traditional radio-and/or chemotherapy. In fact, the sensitivity toward traditional radio- and chemotherapy in absolute majority of patients with solid tumor of digestive tract is rather poor. As

chemotherapeutics have killing and injurious effect to most tumor cells, the difference of therapeutic effect is mainly determined by whether the drug in the target organ can reach effective antitumor blood drug concentration or not. The general effect of traditional chemotherapy makes it difficult to reach an effective blood drug concentration in the target organ of gastrointestinal tract at safe dosage. As a result, clinical efficacy is low, while toxic and side effects are severe. Interventional chemotherapy by drug infusion of supplying artery can make the blood drug concentration in the vascular network of the tumor area reach an effective antitumor level by relative safe dosage, thus decreasing toxic and side reactions and increasing therapeutic efficacy. Intraluminal installation of stent in combination with drug infusion of supplying artery can release obstruction, improve life quality and at the same time inhibit growth of the malignant tumor. In the present study, the average survival time of our group B patients was 13 weeks, close to that reported by Turegano-Fuentes *et al*[51], Cwikiel *et al*[52] and P Scott Mackie *et al*[53]. The average survival time was 44 weeks in group A, significantly longer than the former. The restenosis was also more significantly prolonged in group A than in group B. This showed that interventional chemotherapy by arterial cannulation can produce in certain extent inhibition of the malignant tumor growth by its therapeutic effect. In conclusion, intraluminal stent therapy in combination with intra-arterial cannulation of interventional chemotherapy can be considered as an effective therapy with regard to symptomatic and etiological treatment and should be used more widely in the near future.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• MONOGRAPH •

Studies on gallstone in China

Jing-Sen Shi[1], Jing-Yun Ma[2], Li-Hong Zhu[2], Bo-Rong Pan[2], Zuo-Ren Wang[2] and Lian-Sheng Ma[2]

[1]Research Laboratory of Hepato-biliary Surgery, First Hospital of Xi'an Jiaotong University
[2]The WJG Press, P.O. Box 2345, Beijing 100023, China
Correspondence to: Lian-Sheng Ma, The WJG Press, P.O. Box 2345, Beijing 100023, China
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INTRODUCTION

Gallstone is one of the common primary diseases of bile system. Chinese researchers have done comprehensive and thorough studies on it, but there are still some problems we have not solved. It is necessary to review the achievements we have made in this field recently, to summarize the experiences and find the tendency so as to provide a sound foundation for the researches in the new century. Due to the popularization of molecular biological research methods, a rapid development of modern imaging techniques and medical equipment, the basic and clinical studies of cholelithiasis have set foot on the fast lane. Studies on the cause of cholelithiasis formation and its prevention have covered areas from epidemio logical investigation at macroscopical level to molecular biological researches at microcosmic level. Clinical studies include prevention and treatment of cholelithiasis with traditional Chinese medicine, popularization of micro-injury surgery, treatment of complicated calculus of bile duct, and other aspects.

PRESENT SITUATION

Studies on pathogenesis of gallstone

Study on the cause of cholelithiasis formation is an important field of studies on gallstone over a long period of time in China, and it will hold a very import ant position in the future. Formation of gallstone is a complicated pathologic process, which involves many factors. Researches in this field consist of epidemi ological investigation, studies on components of gallstone, biliary elements which cause stone formation, anatomy and physiology of bile system, etc.

Han *et al*[1]found that formation of gallbladder stones is related to age, metabolic disturbance of fat and damage of gallbladder emptying function in a case-control study on 66 cases of asymptomatic gallbladder stones. To know the morbidity of gallbladder stone in the young is essential to the study of the pathogenecity and prevention of cholelithiasis. Shi *et al*[2] found a morbidity of gallstone of 0.94% in 522 young students by an epidemiological investigation, which is significantly lower than in adults. By ultrasound scanning study, we also found that the gallbladder volume of young female is smaller than that of male (P<0.01), and the rate of cystic contraction 1 hour after meal is also lower than in male. The result supports that the gallbladder contractility of female and fatty is weakened significantly, which is one of the key factors of accumulation of bile and inducement of gallstone. Kuang *et al*[3] studied the relation between asymptom HBV carrier and gallstone, and found that serum HBV-M positive detectable rate in patients with gallbladder stone is higher than normal control group. He thinks that infection of HBV could be an inducer of gallstone formation. Zhu *et al*[4] investigated the incidence of gallstone in 672 fatty liver patients diagnosed by B-ultrasonography and 14610 non-fatty-liver subjects simultaneously, and found that the incidence increases in fatty liver patients obviously because of metabolic disturbance.

Wu *et al*[5] used X-ray diffraction analysis to measure human gallbl adder bile stone. The results are as follows: particulate gallbladder bile stone is chiefly composed of crystalline material containing cholesterol; sandy bile stone is mainly made up of noncrystalline material containing bile pigment or porphobilinogen. They compared the results with those in the early 1980's and found that the proportional number presented a rise in cholesterol bile stone. They thought that the main reason is the increase of cholesterol intake, metabolic disturbance of cholesterol, and descending incidence of biliary ascariasis and bacterial infection. They also determined bilirubin level by spectrophotometry and determined cholesterol contents by TLc scanning in 31 cases of human biliary calculus, and found that 31 cases of gallstone were all mixed bilestones primarily containing pure cholesterol or cholesterol bilestones[6,7]. Shi *et al*[8,9] studied black bilestones by Fourier Transform Infrared Spectroscopy and found that known organic constituent and inorganic constituents of black stones were the same, but their contents are varied. They think that black stone contains quite a few of protein, and that might be the key constituent to form black stone.

The study on the relationship between metabolism of bile acid and formation of gallstone[10-12] is the subject causing great attention of scholars all the time. It had been shown that the ability of diffluence was determined by the ratio of choleserol, bile acid and lecithin. In the bile of patients who suffered from cholesterol calculus, bile salts, total bile acid pool, and contents of chenodeoxycholic acid decreased. At the same time, contents of cholyglycine rose while cholyltaurine reduced, and proportion of conjugated deoxy-cholanic acid increased. All above factors promote cholesterol crystal nucleation and gallstone formation. Uniform recognition about the mechanism of bile acid dissolving bilirubin has not been obtained, but most of scholars believe that reduced bile acid is one of the key factors in gallstone formation. These results provide a solid theoretical basis to study the cause of cholelithiasis formation, prevention of gallstone and litholytic therapy. However, because some mechanisms have not been pinpointed, the effects of clinical application are not satisfactory. Lu *et al*[13] found that fever had obvious influence on biliary elements in a study on guinea pigs. All the animals in fever group had significantly higher concentrations of total bile protein and bilirubin than that of the control groups, which is prone to gallstone formation.

Bacterial infection is very important in the forming of the gallstone. Recently, there are many research reports[14-17]

about the function in the for ming of the gallbladder stone. The content of aerobe, anaerobe, L-bacterium, and *Helicobacter pylori* of the gallbladder tissue, bile, gallstone and portal vein have been tested through the use of the immunohistochemistry and the proliferation of PCR-DNA. These results indicated that bacterial infection and the formation of gallbladder stone and the chronic inflammation of bile duct system are closely related. According to the bacterial enzymology, the formation of bilirubin gallstone is related to the content of exterior β-glucuronidas -G, which result from the proliferation of bile duct bacterial infection, the interior environment (the best pH is 7.0), and the content of inhibitor. But according to the research of Yang *et al*[18], the interior β-glucuronidas-G, as a kind of active enzyme in human body, is one of the related factors of bilirubin gallstone.

The research of the relation between the contracting function of gallbladder and incidence of gallstone is a very important content of the study on cause of gallstone formation. Ma *et al*[19] found that the gallbladder emptying rate is not normal in half of the gallstone patients through the observati on of 100 cases of gallbladder stone patients. He considers that the function of gallbladder is mainly affected by its thickness, the location of the gallstone and the shape of the gallstone. Zhao *et al*[20] also holds the vie w that gallbladder emptying rate in gallstone patients' is below the normal, and especially in the pregnant women it is lower than in the men of the same age. Xu *et al*[21] have done a research on the change of the contractive function of gallbladder after gastrectomy, and the result showed that and incidence of cholestasis, and gallstone rose because of cut of liver rami of nervus vagus, gastrointestinal reconstruction and decrease of secretion of cholecystokinin.

In recent years, many researchers have done experiments to explore the relation between the disease of gallbladder stone and human gene group with molecular biological technology, and that made the study enter into gene level. Jiang *et al*[22] used PCR-RFLP to analyse the relation between variety of *Xba* I in Apo B Gene and blood fat. The research shows that the X^+ allele of Apo B gene is associated with high serum cholesterol and it may be regarded as a high-risk gene of cholesterol gallstone formation. Shuai *et al*[23] explored the relation between the expression of CCK-A receptor of gallbladder and gallbladder hypomotility in patients with gallstone. He thought that the down-regulation of gene expression of CCK-A receptor plays an important role in gallbladder hypomotility in patients with gallstone.

The relation between the change of interior or exterior hormone level and the formation of gallstone attracts the attention of scholars. Through case-control study, Luo *et al*[24] concluded that the use of steroid contraceptives in gallbladder stone women patients aged 20-44 years and the absorpt ion quantity of heat are the main factors of the formation of gallbladder stone. Steroid contraceptives containing estrogen and progesterone, are the reflection of the effect of exterior hormone on gallstone formation. With the help of radioimmunology, Wang *et al*[25] have experimented on the alteration of ATCH, Cor and thyroxine of the gallbladder stone. The results showed that the formation of cholecystolithiasis was related to the increase of Cor, and the decrease of T3 in serum. They[26] also proved that the cholelithiasis patients have an obvious increase in level of tyrosine peptide and cholecystokinin in serum in another experiment. Han *et al*[1] found that the content of the insulin in gallstone patients was much higher than in non-gallstone patients, showing that the level of insulin was related with formation of gallstone.

Research on prevention and treatment of gallstones

Research on prevention and treatment of gallstones can be divided into combined treatment by traditional Chinese medicine and modern medicine, surgery, litholytic and lithotriptic therapy. An important part of non-surgical treatments of gallstones is litholytic and lithagogue one. Great achievements have been made in extrinsic experiments of dissolving gallstones with different solvents for different components of gallstones. However, the outcome of clinical applications is not satisfactory as far as cholesterol or bile pigmental stone, litholytic treatment by oral drug in particular is concerned. Another important cause of the limited clinical application is the toxic and side effects of the litholytic. Many scholars put much emphasis on studying extrinsic lithodialysis and direct perfusion of litholytic into the biliary tract[27-30]. The application of traditional Chinese medicine is given much attention in China. Litholytic and lithagogue treatment with Chinese herbs in clinical application is the most vigorous research field in gallstone research in China besides the researches on gallstone formation. The drugs selected can change the components of stone-forming bile, dissolve the gallstones formed, improve contractility of the gallb ladder, relax sphincter of the bile duct and exert anti-inflammatory and antibacterial functions. Several medical institutions of our country[31-37] have carried out basic and clinical experimental researches on their prepared patent Chinese drugs with litholytic and lithagogue functions. They proved that some formulae can relieve the depressed liver, normalize the function of the gallbladder, promote blood circulation to remove stagnancy, heat and dampness, and achieve purgation. Many clinicians[38-43] have made inductions of indications and specific methods for treating cholelithiasis with Chinese herbal drugs and combined traditional Chinese and modern medicine, making its application more rational and effective. Zhou *et al*[44] made extrinsic CT scan of cholelithiasis cases, compared the results with the chemical components of the stone, and proved that CT imaging and value can demonstrate the chemical type of gallstones. He suggested that 40HU, the critical value of judging cholesterol calculus can provide a valuable basis for non-surgical treatment, especially litholytic method.

With the popularization of laparoscopy, the age of micro-traumatic surgery has come and great changes have taken place in surgical operation and surgical ideology. Cholecystectomy has entered the stage of laparoscopy in developed countries. This tendency is becoming obvious in China. The surgical treatment of gallstones with clinical application of celioscope[45-53] consists of peritoneoscopic cholecystectomy, peritoneoscopic choledocholithotomy, endosco pic papillectomy, and application of choledochoscopy. Indications of laparoscopy for gallstone treatment are not limited to pure gallbladder stone now. Choledoc holith, acute cholecystitis, and acute pancreatitis are no longer the contraindications of laparoscopic cholecystectomy (LC). Among them, choledocholith could be treated by two methods at least. One is sphincterotomy of Oddi before LC, The other is laparoscopic choledochotomy. Sphincterotomy performed with endoscope is a simple therapy for calculus of common bile duct with a diameter smaller than 3 cm and strictur e of terminal common bile duct shorter than 3 cm. Undoubtedly, application of all kinds of choledochoscopy has improved the rate of pre- and intra-operative diagnoses of biliary diseases, reduced the rate of residual bile stone, and could help patients avoid a second operation. Therefore, their application is welcome by clinicians increasingly.

Much concern is being taken in research on surgical treatment of hepatic calculus[54-56]. As Huang pointed out, 'early-stage systemic lobectomy of liver or segmental hepatectomy' is drawing a highly

increasing attention in treating hepatic calculus, especially in early stage cases that are diagnosed by CT scanning. It is thought that the treatment procedure could achieve the aim of maximal clearance of lesions and reservation of functional liver tissues. Furthermore, most scholars[57] think that irritating of hepatic calcul us and secondary infection could result in mucosal ulcer of bile duct, or even metaplasia leading to the development of hepatic cholangiocarcinoma finally, although there are disputes about question. Therefore, the reports of present clinical studies make lobectomy of liver the key point in surgical treatment of hepatic calculus although removing obstruction, getting rid of calculus and making drainage are the principles that should be followed. Introperative choledochoscopy, intraoperative ultrasound, intraoperative fast pathological examination and other imaging and diagnostic metho ds are key measures in treating hepatic calculus.

Operations of biliary tract, especially cholecystectomy, have been widely perfor med in grass-root-level medical institutions, but many complications and seque lae have resulted from inappropriate handling of indications, inappropriate choice of operative methods, and malpractice during operation, even making another operation necessary. With aging of the population, more and more studies[12,49] have been conducted on prevention and treatment of gallstones of elderly people. As to the chemical cholecystotomy started in the 1980s, Sun Shuming[58] concluded that chemical excision is very safe after uhe experiment performed on pigs. The clinical study of lithotriptic therapy[53,59,60] shows that extracorporeal shock wave lithotomy or lithotomy through sinus tract of skin after operation is effective to cure gallstone. However, these techniques and their values still need further improvement.

COMMENT AND PROSPECTIVES

Researches on causes of gallstone have been carried out for a long time with many conclusions drawn about cholesterol lithiasis and bilirubin lithiasis. Take the former as an example, almost all the cases experience three courses: cholesterol saturating, unbalance between causing and anti-causing factors; and dynamic disfunction of bile duct. Experts have worked over all aspects of that and obtained valuable findings. New research trend in this field is to discover its molecular biological and genetic nature with sophisticated research approaches. And we can expect that research in this field will become a hotspot in the up coming years, with the deepening of human genome Project. According to the status in quo, many risk factors causing the formation of gallstone have already been discovered, but in our opinion, emphasis must be laid on nation-wide big sample clinical epidemiological surveys so as to find effective measures to prevent gallstone occurrence based on integrating the basic research with clinical practice.

There is a close relationship between the research on prevention of gallstone and litholytic therapy and study on the cause of cholelithiasis formation. An effective litholytic therapy using traditional Chinese medicine could be expected if we take good advantage of the dissolving measures used abroad. But more precise basic studies on this issue are needed in China. We anticipate that, just like what has happened to other traditional Chinese medicine, researches focusing on traditional medicine curing gallstone will shift from simple summarization of clinical cases to strict double-blind case-control study using monomer components purified from raw Chinese medicine through high-tech settings.

Surgical treatment still is a consequence in gallstone treatment. As far as gall stone is concerned, laparoscopic cholecystectomy will play a major role in the treatment of the disease for a relatively long time. At the same time, more reports on the issue will appear. Consequently, laparoscopy will be widely utilized in all branches of surgery. Basic and clinical researches on complications of this therapy, such as bile duct injury, will be further strengthened. As to the therapies for hepatic calculus, early-stage systemic lobectomy of liver or segmental hepatectomy will be commonly recognized, and the idea about curing the cases of this type will shift from treating the complications to radical surgical treatment. Furthermore, choledochoscopy, which belongs to micro-injury surgery, will play an more important role. Nevertheless, effect of lithotriptic therapy and chemical cholecystotomy in curing lithiasis should be further evaluated.

The present faults of some gallstone studies in China are the echo at low level and the lack of innovation. Therefore, revolutional progresses are difficult to achieve. The key to better the current situation in a short time is to put more stress on prevention and treatment of gallstone, especially on issues about the integration of theory and application of basic findings to clinical practice.

SUGGESTIONS

To summarize, through the application of molecular biological techniques, studies on causes of gallstone and their mechanism at molecular and genetic level should be enhanced. A good theoratic basis for prevention of gallstone could be anti cipated. In the research on prevention of gallstone and litholytic therapy, especially the application of traditional Chinese medicine, more emphasis should be laid on the basic researches of mechanism and strict double blind case-control studies. Micro-injury surgery should be especially stressed. At the same time, clinical case-control studies with vast specimens are expected to guide the gal lstone therapy. As it is known that improved health situation and changing the diet structure will make the incidence of cholesterol lithiasis in gallstone increase in the coming years, researches on this issue will be strengthened. In regard to prevention of hepatic bile duct stone, more importance should be attached to researches on indication and complications of early stage systemic lobectomy of liver or segmental hepatectomy and prevention of the recrudescence of gallstone after surgery.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Metastatic human hepatocellular carcinoma models in nude mice and cell line with metastatic potential

Zhao-You Tang, Fan-Xian Sun, Jian Tian, Sheng-Long Ye, Yin-Kun Liu, Kang-Da Liu, Qiong Xue, Jie Chen, Jing-Lin Xia, Lun-Xiu Qin, Hui-Chuan Sun, Lu Wang, Jian Zhou, Yan Li, Zeng-Chen Ma, Xin-Da Zhou, Zhi-Quan Wu, Zhi-Ying Lini and Bing-Hui Yang

Liver Cancer Institute of Fudan University and Zhongshan Hospital, Shanghai 200032, China
Correspondence to: Zhao-You Tang, M.D., Professor of Surgery, Chairman, Liver Cancer Institute of Fudan University (previous Liver Cancer Institute of Shanghai Medical University) 136 Yixueyuan Road, Zhongshan Hospital, Shanghai 200032,
China.zytang@srcap.stc.sh.cn
Telephone: +86-21-64037181, Fax: +86-21-64037181
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Abstract

Metastatic human HCC model is needed for the studies on mechanism and intervention of metastatic recurrence. By using orthotopic implantation of histologically intact tissues of 30 surgical specimens, a patient-like metastatic model of human HCC in nude mice (LCI-D20) and a low metastatic model of human HCC in nude mice (LCI-D35) have been established. All mice with transplanted LCI-D20 tumors exhibited extremely high metastatic ability including spontaneous metastasis to liver, lungs, lymph nodes and peritoneal seeding. Remarkable difference was also found in expression of some of the invasiveness related genes and growth factors between the LCI-D20 and LCI-D35 tumors. PAI-1 increased gradually following tumor progression in LCI-D20 model, and correlated with tumor size and AFP level. Phasic expression of tissue intercellular adhesion molecule-1 in this model was also observed. Using corneal micropocket model, it was demonstrated that the vascular response induced by LCI-D20 tumor was stronger than that induced by LCI-D35 tumor. Similar report on metastatic human HCC model in nude mice and human HCC cell line with metastatic potential was rarely found in the literature. This LCI-D20 model has been widely used for the studies on intervention of metastasis, including anti-angiogenesis, antisense approach, metallopro teinase inhibitor, differentiation inducer, etc. It is concluded that the establishment of metastatic human HCC model in nude mice and human HCC cell line with metastatic potential will provide important models for the *in vivo* and *in vitro* study of HCC invasiveness, angiogenesis as well as intervention of HCC recurrence.

Subject headings hepatocellular carcinoma; metastasis; metastatic model; nude mice; cell line; experimental intervention; angiogenesis

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INTRODUCTION

Liver cancer is the 4th most common cause of death from cancer and the 3rd most common in men. The highest age-standardised mortality rate was in China (34.7/100000), which alone accounts for 53% of all liver cancer deaths worldwide[1]. Surgical resection has been accepted the best treatment forhepatocellular carcinoma (HCC), the most common type of primary liver cancer in China. However, recurrence and metastasis remain the major obstacles for further prolonging survival after resection. Even after curative resection of small HCC, the recurrent rate remained high[2,3]. Therefore, studies on metastasis and recurrence will be an important issue in the 21st century. To this end, metastatic human HCC mod el in nude mice and cell line with metastatic potential are needed for the studies on mechanism, angiogenesis and intervention of metastatic recurrence.

Brief review of literature

In 1963, the first human HCC cell line (BEL-16) was established by Chen[4]. At the authors' institution, the human HCC model in nude mice (LTNM) was established in 1982, but metastasis was not found in this model[5]. Human HCC nude mice model and human HCC cell line with metastatic potential were rarely reported in the literature.

Hepatocellular carcinoma cell line

In the recent three decades, a good number of human HCC cell lines have been established. Shen *et al*[6], after the establishment of the first human HCC cell line in 1963, reported a series of human HCC cell line (BEL-7402, BEL-7404, BEL-7405) in the ensuing years. In 1973, Alexander *et al*[7] established the famous human HCC cell line (PLC/PRF/5) which produces HBsAg. Dong[8] established the human HCC cell line (SMMC-7721) in 1977, which remains one of the human HCC cell line that currently used in China.

Many human HCC cell lines have been established for the studies on etiological factors of HCC, such as hepatitis B virus (HBV)[9-14], hepatitis C virus (HCV)[15], hemochromatosis[16] , thorotrast[17], for the study of alpha fetoprotein (AFP)[18,19], and for other studies[20-30]. Unfortunately, of the above human HCC cell lines, metastatic potential was rarely mentioned or demonstrated.For animal HCC cell lines, the establishment of such cell lines in rat[31], in woodchuck[32-34], and in chicken[35] have been reported.

Hepatocellular carcinoma cell line with metastatic potential

Human HCC cell line with metastatic potential was rarely reported in the literature. Besides Tian *et al*[36,37] at the authors' institution reported two paper in 1998 and 1999, only one paper has yet been reported. Seki *et al*[38] (1999) established a human hepatocellular carcinoma cells with metastasis to lymph nodes.

Again, very few papers have been reported concerning animal HCC with metastati c behavior. Ogawa *et al*[39] (2001) reported the establishment of rat HCC cell lines with differing metastatic potential in nude mice. Reichner *et al*[40] (1996) reported that interleukin-6 production by rat hepatocellular carcinoma cells is associated with metastatic potential.

Hepatocellular carcinoma model in animal

In 1976. Shimosato *et al*[41] reported the establishment of a series of human tumors in nude mice including HCC. The same group has used the human HCC nude mice model for the study of alpha fetoprotein in relation to tumor growth [42]. As had mentioned, at the authors' institution, a human HCC model in nude mice has been reported in 1982[5]. In 1995, Liu *et al*[43] established a nude mice xenograft model from human HCC. In 1996, Leveille-Webster *et al*[44] established an intrahepatic xenografts of human HCC in severe combined immunodeficiency mice for the study of multidrug resistance.

For animal HCC model, Qian *et al*[45] (1987) established a transplan table HCC model in 615-strain mice (H 615).

Hepatocellular carcinoma model in animal with metastatic behavior

In this paper, we need to focus to human HCC model in nude mice with metastati c behavior. In 1993, Aruga *et al*[46] reported the establishment and characterization of liver metastatic model of human hepatoma in nude mice, metastasis was mainly found in the liver of this subcutaneous tumor model. Sun *et al*[47,48] (1995 and 1996) at the authors' institution reported the first patient-like metastatic human HCC model in nude mice with 100% of spontaneous metastasis to lung, lymph node and liver. Peng *et al*[49] (1996) established a human HCC model in nude mice using orthotopic transplantation, and malignant behavior (invasion of abdominal cavity) was observed. Tao *et al*[50] (1998) established a human HCC nude mice model using SMMC-LTNM tumor transplanted into abdominal cavity and liver, the lung metastatic rate was 59%. Genda *et al*[51] (1999) reported the construction of metastatic models using orthotopic implantation of human HCC cell lines into the livers of SCID mice, two of the 5 cell lines injected showed vascular tumor thrombi and intrahepatic metastasis. Zheng *et al*[52] (2000) establ ished an orthotopic transplantation tumor model from the subcutaneous model of human HCC in nude mice, the spontaneous metastatic rate was 57.8%. Shi *et al*[53] (2001) established a human HCC model in nude mice with high metastatic rate in lymph node.

For animal HCC, Masui *et al*[54] (1997) reported a highly metastat ic HCC in male F344 rats induced by chemical carcinogens. Li *et al*[55] (1998) established a lymph node metastatic model of mouse HCC Hca-F cells in C3H/Hej mice.

A synopsis of related studies at Liver Cancer Institute of Fudan University

At the authors' institution, studies on recurrence and metastasis of HCC have been conducted since 1993[56-60]. Because either metastatic human HCC model in nude mice or human HCC cell line with metastatic potential was not availuable at that time, therefore, efforts have been made for the establishment of such model and cell line. At the authors' institution, the establishment of metastatic human HCC model in nude mice was reported in 1995 (in Chinese) and 1996 (in English)[47,48] and human HCC cell line with high metastatic potential was reported in 1998 (in Chinese) and 1999 (in English)[36,37]. These might probably be the first metastatic human HCC model in nude mice and cell line with metastatic potential. A Synopsis on the establishment and studies of these models at the Liver Cancer Institute of Fudan University is reported herein.

Establishment of metastatic human HCC in nude mice

In 1988, development of *in vivo* models for studies of brain metastasis has been reported in Fidler's group[61]. In early 1990s, "metastatic models constructed in nude mice by orthotopic transplantation of histologically inta ct patient specimens" has been used in Hoffman's group, and several such models including lung cancer, pancreatic cancer, ovarian cancer, etc have been reported[62-64]. However, patient-like human HCC model in nude mice with metastatic behavior was not found.

At the authors' institution, by using orthotopic implantation of histologically preserved metastatic tumor tissues of 30 surgical specimens, a highly metastatic model of human HCC in nude mice (LCI-D20) has been established. This model was obtained through *in vivo* clonal selection by repeated "lung foci to liver". All mice with transplanted LCI-D20 tumors in the liver exhibited 100% transplantability and metastatic ability as well as various manifestations of tumor behaviour in HCC patients. These included: local growth, regional invasion , spontaneous metastasis to liver, lungs, lymph nodes and peritoneal seeding. The high metastatic ability maintained up to 120 passages. Histological characte ristics of LCI-D20 tumor were similar to those of the original tumor. Karyotype analysis revealed heteroploid cells. Expression of AFP and HBxAg was shown using immunohistochemistry. The duration between two passages was around 20 d. At the same period, using orthotopic implantation of histologically preserved metastatic tumor tissues, a low metastatic model of human HCC in nude mice (LCI-D35) has also been established as a control. Invasion to the liver and peripheral organs was not found. Pathological findings revealed no metastasis in the liver, lung and lymph node. The duration between the two passages was around 35d. The biological characteristics of this LCI-D35 model remained unchanged up to 59 passages. Karyotype analysis revealed diploid cells[47,48].

Biological characteristics of LCI-D20 and LCI-D35 models

Remarkable difference was found between the LCI-D20 and LCI-D35 tumors: ① High expression of some of the invasiveness related genes, such as c-*fos*, c-*jun*, N-ras, H-ras and P53 mutation was found in LCI-D20 tumor but not in LCI-D35 tumor[65]. ② Using comparative genomic hybridization (CGH) technique, we have demonstrated that chromosome 8p deletion was associated with HCC metastasis[66]. When comparison was made between LCI-D20 and LCI-D35 using CGH, it was shown that 8p deletion remains one of the important alterations[67]. ③ Corneal micropocket model has been employed to investigate angiogensis, it was found that the vascular response induced by high metastatic model LCI-D20 was stronger than in low metastatic model LCI-D35[68]. ④ N-Acetylglucosaminyltra nsferase V (GnT V) activity was much higher in LCI-D20 model when compared with LCI-D35, indicating the close relation between GnT V activity and HCC metastas is[69].

It was observed that both serum and tissue PAI-1 content increased gradually following tumor progression in LCI-D20 model, PAI-1 correlated with tumor size and AFP level and provided potential clinical impact as prognostic marker[70]. Phasic expression of tissue intercellular adhesion molecule-1 (ICAM-1) in this model was also observed, ICAM-1 increased

with the progressio n of LCI-D20 tumor, and markedly increased when metastasis occurrs[71].

Establishment of human HCC cell line with high metastatic potential

Metastatic behavior was not reported in human HCC cell lines that commonly use d in China when inoculated into nude mice, such as BEL-7402[6], PLC/PRF/5[7] and SMMC-7721[8].

In order to conduct in vitro study for metastatic recurrence, a human HCC cell line with metastatic potential (MHCC97) was established from a subcutaneous xenograft of the above LCI-D20 tumor using alternating *in vivo* and *in vitro* cultivation. The MHCC97 cells appear as polygonal epithelial cells. The doubling time was 31 h. Karyotype analysis revealed that the number of chromosome was 59-65, the median range of chromosome number was 60-61, which accounted for 73%. Aberrant chromosomes i(1)(q) and der(4) (pter→q35::?) were its chromo some markers, which might be related to carcinogenesis and progression of HCC. Secretion of AFP was demonstrated in MHCC97 cells. HBsAg and HBxAg were detected using PCR. Upon intrahepatic inoculation in nude mice, the xenograft grew and metastasized to the lungs, with metastatic rate up to 100% at 5th week. The cancer cells of lung foci were AFP positive. The latency period of tumor nodule formation after inoculation was 15d-20d. Invasion to the liver, diaphragm an d abdominal wall was observed after intrahepatic inoculation. The biological cha racteristics remained stable after in vitro passages for 2 years. The MHCC97 cell line was preserved in liquid nitrogen at the 120 passages. RT-PCR products for integrin α5 and β1, uPA-R, VEGF and nm23-H1 mRNA from MHCC97 cell line were positive. Immunostaining showed strongly positive for c-Met, uPA-R in both of xenografts and lung me tastatic lesions. However, integrin α5 and β1 were positive only in xenograft but not in lung metastatic lesions. E-cadherin was not expressed either in xenograft or in the metastatic lesions. Mutation of p53 at codon 249 was also observed in MHCC9 7 cells, but not in LCI-D35 cells (low metastatic model), indicating p53 mutati on might relate to HCC metastasis[36,37,72].

Experimental intervention for metastasis using LCI-D20 model

The LCI-D20 model, a patient-like metastatic human HCC model, is a useful model for the studies of experimental intervention. Two kinds of approach have been used: ① Studies on a well established model to observe the tumor inhibition rate and lung metastatic rate. ② Studies on intervention after curative resection of the established liver xenograft, which mimicked to that of curativ e resection of HCC in patient, to observe the recurrent rate and lung metastatic rate.

Several anti-angiogenic agents have been studied in nude mice bearing LCI-D20 tumor. ① Suramin was shown to inhibit tumor growth and metastasis of human HCC in nude mice, when compared with control, the tumor volume (cm^3) was 7.5 *vs* 10.8, and lung metastasis found in 62.5% and 100% respectively[73]. ② Inhibitory effect of the angiogenesis inhibitor TNP-470 on tumor growth (tumor weight being 0.97 g *vs* 2.04 g) and lung metastasis (being 8% *vs* 50%) was also demonstrated in LCI-D20 nude mice model[74]. ③ Gene transfer of dominant-negative flk-1 mutant has been studied and showed inhibition of angiogenesis, growth and metastases in LCI-D20 model, the tumor was 10 folds smaller than the control, lung metasta sis being 20% *vs* 100%, and vessels were hardly visible as compared with rich neovascularization in the control[75]. ④ Endostatin, a potent anti-angiogenic agent, has been shown to inhibit tumor growth in LCI-D20 model, and the combination with cisplatin enhanced the response, the mean tumor volume (mm^3) was 8376 for control, 3777 for endostatin, 1629 for cisplatin, and 463 for endostatin + cisplatin[76]. ⑤ Cytostatic calcium influx inhibitor carboxyamido- triazole (CAI) was also proved of effect for anti-angiogenesis[77]. ⑥ Recently, interferon (1b was proved effective to prevent the recu rrence in the liver and inhibit lung metastasis after resection of liver tumor in a dose-dependent manner. The mechanism was mediated by anti-angiogenesis[78].

The following approaches have also been tried for the intervention of metastas is in the LCI-D20 model. ① Antisense H-ras: When antisense H-ras oligodeoxyn ucleotides (ODNs) was used, specific inhibition of H-ras expression observed. Antisense H-ras ODNs induced apoptotic cell death, inhibited the growth rate of LCI-D20 cells in vitro and *in vivo*, and alter *in vivo* tumorigenesity (being 50% *vs* 100%) and metastatic potential (lung metastases being 0% *vs* 100%)[79]. ② Heparin is structurally and functionally similar to that of heparan sulfate, metabolite of suramin, therefore the role of heparin on metastasis was studied in LCI-D20 model. It has been demonstrated that hepa rin inhibited tumor growth (tumor size being 1.50±0.61 cm *vs* 2.98±0.50 cm in the control), inhibited lung metastas is (being 20% *vs* 60%) and prolonged survival (50 days survival being 60% *vs* 0)[80]. ③ Metalloproteinase inhibitor-BB94: Effect of BB-94 on tumor growth and metastasis in the LCI-D20 model was also observed, the tumor weight being 2.27g *vs* 3.13 g, lung metastasis being 44% *vs* 100%, and survival on day 45 being 100% and 56%[81]. ④ PD-ECGF that expressed in HCC, and particularly in tumor thrombus, is able to convert more prodrug (such as Furtulon and Xeloda-Capecitabine) into 5-Fu. Using capecitabine, prevention of lung metastasis as well as inhibition of tumor growth was observed in nude mice model of LCI-D20, thus will of potential as "targeting chemotherapy"[82]. ⑤ ICAM-1 is closely related to HCC metastasis. It was demonstrated that β peptide (a polypeptide designed by authors' institution, which is able to block ICAM-1) can inhibit recurrence in the liver and lung metastasis in LCI-D20 model after resection of tumor at early stage and advanced stage. However, the metastatic recurrent rate in the liver after resection in the early stage was lower than that after resection in the advanced stage, being 0%(0/5) versus 60%(3/5), and 100%(5/5) in the control[83]. ⑥ Retinoid acid was not effective in controlling tumor growth and metastasis in this particular LCI-D20 model.

The conclusion is that the establishment of metastatic human HCC model in nude mice and human HCC cell line with metastatic potential will provide an importan t model for the *in vivo* and *in vitro* study of mechanism of HCC metastasi s, angiogenesis as well as intervention of HCC recurrence after resection.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Liver transplantation in the UK

SR Bramhall, E Minford, B Gunson and JAC Buckels

The Liver Unit, Queen Elizabeth Hospital, Birmingham, UK
Correspondence to: SR Bramhall, Department of Surgery, Queen Elizabeth Hospital, Birmingham B15 2TH,
UK. S.R.Bramhall@bham.ac.uk
Telephone: 0121 627 2276, Fax: 0121 472 1230
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Abstract

Introduction: **This paper provides a review of the practice of liver transplantation with the main emphasis on UK practice and indications for transplantation.**
Referral and Assessment: **This section reviews the process of referral and assessment of patients with liver disease with reference to UK practice.**
Donor Organs: **The practice of brainstem death and cadaveric organ donation is peculiar to individual countries and rates of donation and potential areas of improvement are addressed.**
Operative Technique: **The technical innovations that have led to liver transplantation becoming a semi-elective procedure are reviewed. Specific emphasis is made to the role of liver reduction and splitting and living related liver transplantation and how this impacts on UK practice are reviewed. The complications of liver transplantation are also reviewed with reference to our own unit.** ***Immunosuppression:*** **The evolution of immunosuppression and its impact on liver transplantation are reviewed with some reference to future protocols. Retransplantation: The role of retransplantation is reviewed.**
Outcome and Survival: **The results of liver transplantation are reviewed with specific emphasis on our own experience.**
Future: **The future of liver transplantation is addressed.**

Subject headings liver transplantation; review; Great Britain; human

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INTRODUCTION

Recent years have seen dramatic changes in the practice of liver transplantation. In 1980 in Europe fewer than 30 liver grafts were performed compared to over 3000 in 1995 (Figure 1). During this period liver transplantation has evolved from a rare procedure in patients with end-stage liver disease, to a semi-elective operation with current predictable success rates of approximately 90% in patients with chronic disease.

In the early period of liver transplantation it was reserved for patients with end stage chronic liver disease or unresectable primary liver malignancy, but in recent years there has been a considerable broadening of the accepted indications. Improving results have led to liver transplantation becoming a semi-elective procedure with both quantity and quality of life being of major concern. Patients who may not be in immediate risk of death from liver decompensation but have significantly impaired quality of life are now considered as candidates.

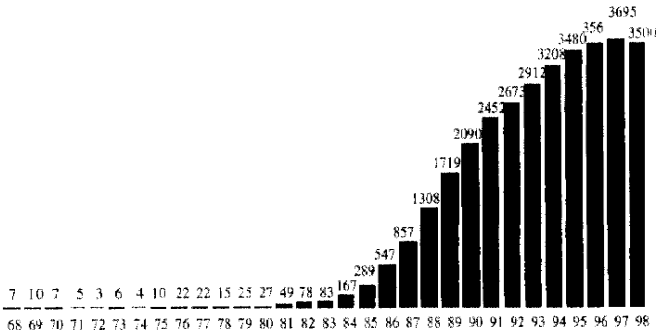

Figure 1 Evolution of european liver transplantation.

The current indications can be classified into four broad groups; chronic liver failure, acute liver failure, primary hepatic malignancy not treatable by conventional resection and inborn errors of metabolism due to a liver based enzyme defect but without parenchymal liver disease (Table 1).

Chronic liver failure is the most common indication for liver replacement and can be caused by a wide variety of diseases including autoimmune, viral, congenital and alcohol induced liver disease. Primary biliary cirrhosis (PBC) is the commonest indication for liver transplantation in the UK. Several studies on survival in PBC have led to the development of a prognostic index that is helpful in planning the timing of liver replacement[1]. Primary sclerosing cholangitis (PSC) is a condition that is usually found in patients with inflammatory bowel disease. Progression of PSC is less predictable than PBC but approximately 30% of patients with PSC will develop cholangiocarcinoma that is usually incurable at diagnosis. Autoimmune hepatitis (AIH) is less common than PBC and PSC but immunosuppressive therapy can delay progression. Excess immunosuppression prior to transplantation however, may increase the morbidity and mortality associated with liver replacement and the optimal timing of liver replacement is a finely balanced decision.

An estimated 300 million people worldwide carry the hepatitis B virus (HBV). In Western Europe and North America the carrier rate is low (0.5%) and is mainly confined to high-risk groups including intravenous drug users, homosexuals and immigrants from high prevalence areas. HBV is a significant problem however, because of the risks of early recurrence after liver replacement. Patients who are HBV-DNA positive at time of transplant develop rapid recurrence with early death. The results of trials of antiviral therapy using agents such as lamivudine and HBV specific immunoglobulins prior to transplantation suggest that viral replication can be suppressed prior to and post liver replacement with encouraging early results[2-5]. Hepatitis C

virus (HCV) is an increasing public health problem. Most patients seen in the UK have become infected following transfusion of blood products or from intravenous drug abuse. The development of cirrhosis following HCV infection is slow but with a significant risk of subsequent hepatocellular carcinoma development[6]. Recurrence of HCV after transplantation is common but not usually problematic in the early years[7,8]. The evolving strategies for anti-viral therapy in this group of patients are likely to have a significant impact on survival in this group of patients[9-11].

Alcoholic liver disease (ALD) is the commonest cause of cirrhosis in many parts of the western world although during the evolution of liver transplantation very few cases were accepted. Many transplant physicians were initially reluctant to consider liver replacement in these patients because of the risks of returning to alcohol and public attitudes[12]. The alcoholic who can prove abstinence prior to grafting has an equivalent survival to those transplanted for other chronic liver disease and recidivism is surprisingly uncommon[13]. There has therefore been an increasing pressure to acceptre formed alcoholics and an increasing proportion are now being grafted[14].

The commonest cause of chronic liver failure in children is biliaryatresia. If diagnosed early and treated surgically with a portoenterostomy (Kasai operation), the progression of liver disease is delayed and up to 40% of children will survive long term[15]. Many children however, will develop end stage liver disease and die within the first few years of life if not transplanted. Failure to thrive is a common sequelae of chronic liver disease in children and should be considered an indication for grafting.

The development of hepatic encephalopathy within eight weeks of onset of symptom s in a patient without previous liver disease is defined as acute fulminant hepa tic failure (AFHF)[16]. Sub-acute or late onset hepatic failure has al so been recognised with encephalopathy developing between eight weeks and six months of onset of symptoms. The commonest causes of AFHF in the UK include drugs and toxins[17-20], viral hepatitis (Hepatitis A, B, and non-A non-B)[21,22] and miscellaneous causes including Wilson's disease, fatty liver of pregnancy and Budd-Chiari syndrome[23-26]. Specific prognostic factors for spontaneous recovery from AFHF have been published and are helpful in decision making about transplantation[27].

An increasing number of inborn errors of metabolism with a deficiency of a single hepatic enzyme are being treated by liver transplantation, even though the liver is otherwise structurally and functionally normal (Table 1). Timing is important and transplantation should be performed before irretrievable damage is don e to other organs e.g. renal failure in primary oxalosis or cerebral damage in Crigler-Najjar syndrome[28,29].

Hepatocellular carcinoma (HCC) is the commonest primary liver malignancy and although it is rare in the UK it is one of the commonest cancers worldwide[30]. The majority of cases occur in the background of liver cirrhosis with the presence of HCV and HBV being additional risk factors (Figure 2). It has been recommended that transplantation be restricted to patients with HCC who have lesions up to 3 cm and up to three in number[31-33]. There are other rare unresectable hepatic tumours that are occasionally conside red for transplantation. These include epithelioid haemangioendothelioma, sarcomata, cholangiocarcinoma and secondary neuroendocrine tumours[34-36]. Hilar cholangiocarcinomas almost invariably recur early after grafting and are no longer considered appropriate candidates[37].

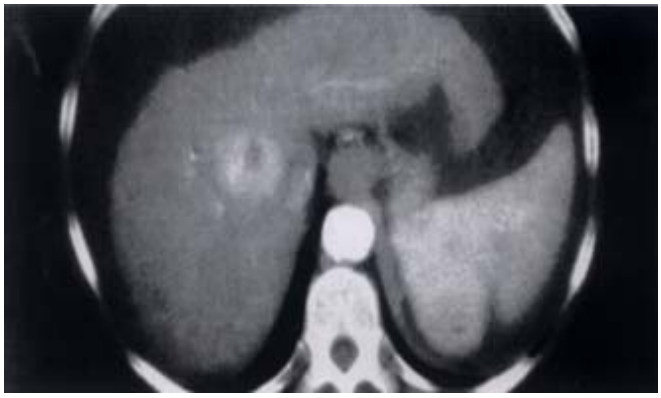

Figure 2 CT Scan showing HCC in cirrhotic liver with ascites and splenomegaly.

Table 1 Common indications for liver transplantation

		Birmingham series	
		n	%
Chronic liver failure	Primary biliary cirrhosis	434	24.7
	Primary sclerosing cholangitis	158	9
	Autoimmune chronic active hepatitis	96	5.5
	Alcoholic cirrhosis	122	6.9
	Cryptogenic cirrhosis	92	5.2
	HBV or HBC cirrhosis	165	9.4
	Biliary atresia	142	8
	Alpha-1 anti-trypsin deficiency	65	3.7
	Budd-Chiari syndrome	8	0.1
Acute liver failure	Viral hepatitis (non-A, non B, HBV, HAV)	10	8
	Drugs (Paracetamol, anti-tuberculosis therapy, halothane)	66	3.8
	Toxins and solvents	0	
Primary hepatic malignancy	Unresectable HCC	20	0.4
	Small HCC in cirrhotic liver	78	10.2
Inborn errors of metabolism	Crigler-Najjar type 1	1	0.05
	Proprionic acidaemia	6	0.08
	Primary oxalosis	8	0.1
	Urea cycle defects	0	

REFERRAL AND ASSESSMENT

In patients with acute or chronic liver failure timely referral is necessary if a successful outcome is to be achieved[38-40]. Many patients with chronic liver disease can remain stable for long periods and decompensation may occur secondary to a complication such as variceal bleeding, portal vein thrombosis, development of hepatic malignancy or spontaneous bacterial peritonitis. The ability to intervene before any major deterioration is dependent on the recognitio n of early indicators of disease progression; in cholestatic conditions (PBC and PSC) the level of bilirubin is an obvious indicator of the underlying disease severity and is likely to lead to an early referral for specialist opinion but for many liver conditions the appearance of jaundice is a late feature and other signs of a deterioration in liver synthetic function, such as a falling albumin or rising prothrombin time are a better indicator of the need for referral.

A multi-disciplinary team including hepatologists and transplant surgeons usually assess patients in the UK. A careful review is required to determine the diagnosis of the liver disease and this will include a specialist pathologist at the transplant centre reporting on the liver histology. Often patients who drink moderate amounts of alcohol are labelled as having alcoholic liver disease but an open mind for these cases is encouraged because modest alcohol intake may unmask an underlying liver condition such as alpha-1 anti-trypsin deficiency or haemochromatosis[41,42].

Assessment for transplantation includes both physical fitness for major surgery as well as psychological evaluation and counselling. A detailed evaluation of the cardio-respiratory system is often indicated and this may require ECG, echocardiogram, exercise ECG, coronary angiography and pulmonary artery catheter isation in those with evidence of ischaemic heart disease, pulmonary hypertension or suspected major pulmonary shunts as seen in the hepatopulmonary syndrome[43].

Technical considerations such as patency of the portal vein are also required an d this can be determined by Doppler ultrasound, angiography, spiral computerised tomography (CT) or magnetic resonance imaging (MRI). An absent portal vein is not a contraindication to transplantation if a patent superior mesenteric vein or large coronary vein can be identified which would be suitable for anastomosis to the donor portal vein[44]. Patients with primary HCC require detail ed investigation for evidence of disease outside the liver and this should include laparoscopy to detect peritoneal disease or transcapsular spread[45], isotope bone scanning and computerised tomographic studies of the abdomen and chest. Difficulty may occur in patients with PSC in trying to differentiate between malignant and benign hilar strictures (Figure 3). In our series malignancy was present in 25% of the patients with significant biliary dilatation but that pre-transplant diagnosis was difficult (unpublished data).

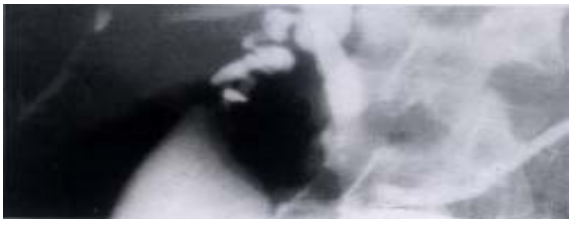

Figure 3 Cholangiogram showing dominant hilar stricture in a patient with PSC.

DONOR ORGANS

The number of liver transplants performed annually in the UK has remained largely stable over the last five years and this has been despite a slight fall in the number of cadaveric organ donors. The total number of liver transplants has been maintained in the UK by an increase in the number of split liver grafts perfor med and a wider use of more marginal liver donors. Over the last ten years the introduction of seatbelt laws and stricter drink driving legislation has reduced the number of cadaveric donors being derived from road traffic accident victims. Donor numbers have been largely maintained by utilising older donors who have usually died from cerebro-vascular disease and have concomitant co-morbidity. The use of such marginal donors does not seem to have been at the expense of worse outcomes. Successful outcome from liver transplantation is possible even in haemodynamically unstable donors and in those with abnormal liver function tests[46].

Assessment of the liver by an experienced transplant surgeon at time of retrieval is a useful guide to subsequent function but if there is evidence of fatty change, a frozen section histological assessment prior to implantation can be helpful[47-49]. A fit recipient can often cope with a marginal graft but a poor recipient will need a graft which functions well immediately for the best chance for survival.

Size matching of donor and recipient is attempted when selecting a patient for a particular liver. Attempting to place a large graft in a small recipient can cause major technical problems. Patients with cholestatic diseases such as PSC and particularly PBC often have large livers and will accept grafts from significantly larger donors, as can patients with marked ascites.

Approximately 60% of potentially suitable organ donors (approximately 1000 per year) are missed each year in the UK[50]. UK organ donation rates remain some of the lowest in Europe but a more aggressive approach to the identification and confirmation of brainstem death and improved family requesting could achieve significant improvements in organ donation in the UK[51]. A number of initiatives such as presumed donor consent and elective ventilation are currently being considered[50].

OPERATIVE TECHNIQUE

Many factors can be identified which have contributed to the improved early outcome after liver replacement. Semi-elective daytime operating ensures that the surgical and anaesthetic team produce the best technical results. The ability to store livers long enough to allow this came from the development of University of Wisconsin preservation fluid which allows satisfactory immediate graft function for storage periods of eighteen hours or more[52].

Meticulous attention to haemostasis has been aided by developments in surgical techniques and instruments (conventional diathermy, argon beam coagulator, fibrin glue, etc.). The monitoring of coagulation parameters in the operating room with the help of the thromboelastogram (TEG) means that blood coagulation is optimised and that predictable deteriorations in clotting which often occur on reperfusion can be anticipated and minimised[53]. The role of anti-fibrinolytic agents such as aprotinin (Trasyslol) and human recombinant factors (Novoseven) remains unclear but are the subject of clinical study[54,55].

Technical innovations

The introduction of venovenous bypass for the anhepatic phase

produced a significant stabilisation of haemodynamic parameters during portal vein and caval clamping with a clear reduction in transfusion requirements and an improvement in renal function[56]. The alternative to venovenous bypass is to preserve the vena cava at the time of hepatectomy and anastomose the back of the donor vena cava to the front of the recipient cava (piggyback technique)[57]. Several techniques have been described but the piggyback technique is not without its complications[58-62]. Most units currently utilise a combination of techniques and a minority of units still perform liver replacement without either bypass or the piggyback technique.

Early techniques of biliary reconstruction involved utilising the donor gall bladder as a conduit between the donor and recipient duct. This technique has been abandoned because of the almost universal development of stones in the conduit. An end-to-end duct anastomosis is now the routine but this has been followed by stricture formation in up to 13% of cases[63-65] and techniques of anastomosing the ducts obliquely with the ends spatulated or by utilising a side-to-side anastomosis are gaining wider acceptance[66,67]. The use of a T-tube has been abandoned by most units[63-65,68].

Liver reduction and splitting

The shortfall in size matched grafts for small children led to the development o f reduced grafts in the mid 1980's[69-71]. The most commonly used technique is to transplant the left lateral lobe segments II and III with venous outflow based on the left hepatic vein which is anastomosed to the retained recipient vena cava[72]. Weight ratios as high as ten-to-one between donor and recipient have been reported[73] the ideal weight ratio however, is four, five or six to one. Reduced liver grafts are not without their comp lications[74] but there appears to be a low incidence of hepatic artery thrombosis[75,76]. The introduction of this technique has led to a significant reduction in mortality from liver disease in children[77]. The techniques of graft reduction have led to the development of splitting livers where the left lobe (or left lateral segments) is transplanted into a paediatric recipient and the right lobe is grafted usually into an adult recipient (split-liver)[78-80]. This technique was developed on the backbench following removal of the cadaveric donor organ. The pro cedure can take approximately two hours and during this time the donor organ is subject to some re-warming that might be detrimental to its initial function. Recently the technique of *in situ* splitting of cadaveric donor organs has been developed as an extension of the development of living related liver transplantation. The advant age of this technique is that the splitting of the liver is performed during the warm phase dissection prior to organ perfusion and cooling and the organ is then not subject to re-warming during a subsequent splitting procedure. The results of this technique appear to result in better initial graft function[81-83].

Living related liver transplantation

In countries that do not have legal recognition of brainstem death and therefore have no access to cadaveric organs, solid organ transplantation has been limited to living related organ donation and this has led to the development of living related liver transplantation[84]. The increasing donor organ shortfall with the increasing number of potential recipients; despite the option of organ splitting, has meant that even in countries that do recognise brainstem death living related liver transplantation has had to be undertaken[85-88]. The organ shortfall in the UK for patients with liver disease is less than in other countries and the number of units performing this procedure is small with only 12 being performed in 1999[89,90]. The greatest experience with this technique has been with adult-to-child left lateral lobe because of the obvious size discrepancy and donor to recipient weight ratios[91] but increasing experience of the technique has led to the expansion of the technique to include adult-to-adult donation[92-96]. The increasing demand for liver transplantation in the UK and the reduction in cadaveric donor organs[90] suggest that this technique is likely to become established practice but careful preoperative evaluation of the donor is needed[97-100].

Complications

The one-year survival following liver transplantation has improved from approx imately 30% in the 1960s and 1970s to more than 80% in the 1990s[14,101,102].

The immediate complications following liver transplantation include primary non -function, haemorrhage and acute renal failure. The incidence of these is sign ificantly influenced by the quality of the donor liver and technical aspects of the transplant operation itself. Over the last 10 years in the UK there has been an increase in the use of marginal organs[46] but this has been offset by improvements in technical aspects of the surgical procedure, per-operative anaesthetic management and post-operative intensive care management. In our own unit the incidence of these complications between 1985 to 1989 and 1995 to 1999 was; primary non-function 1.9% and 1.7%, return to theatre for pack removal or haemorrhage 8.4% and 2. 4% and post-operative renal failure 18.6% and 16.4% respectively (unpublished data). Despite the use of an increasingly marginal donor pool the incidence of these complications hastherefore reduced.

Primary non-function may be due to pre-existing but occult problems in the donor, poor retrieval or preservation, or injury caused by reperfusion (post-reperfusion syndrome). The clinical picture mimics acute fulminant hepatic failure and death rapidly follow unless urgent regrafting can be undertaken. Fortunately primary non-function is rare although primary dysfunction occurs in 5% to 10% of cases and is associated with a worse long-term outcome[103,104].

The majority of routine liver transplants require minimal or no transfused blood . In our own series 47% of liver transplants required four units or less of blood per-operatively (unpublished data). Patients with severe portal hypertension and previous major upper abdominal operations can pose a major surgical challenge, meticulous haemostasis, venovenous bypass, warming of blood and blood products and strict control of coagulation parameters will usually be effective.

A significant number of transplant candidates already have impaired renal function and a combination of factors lead to a rise in the serum creatinine after surgery[105-107]. This will usually respond to optimisation of hydration and pharmacological manipulation but a proportion of patients will develop anuria and require renal replacement therapy at least in the short term[108].

Histological evidence of acute rejection can be documented in approximately 80% of liver grafts at the end of the first week but many of these do not require ad ditional immunosuppression if other parameters of graft function are improving[109]. Histological evidence of severe cellular rejection and less severe histological forms associated with significant biochemical abnormalities (approximately 30% of liver grafts) are usually

treated with high dose steroids[110,111]. Steroid resistant rejection may respond to other agents including monoclonal (OKT3) and polyclonal antibodies (ATG) or by switching immunosuppression regimes[112,113]. Chronic or irreversible rejection in the liver is a biliary rather than a vascular phenomenon in which the small bile radicals are destroyed[114,115]. This can occur very early on after grafting and if progressive leads to loss of the graft although predicting which patients might require regrafting can be difficult[116,117]. Chronic rejection accounts for approximately 5% of graft loss within the first three to five years following transplantation[118]. Lower rates of chronic rejection and graft salvage in early chronic rejection may occur with newer immunosuppressive regimes[119-121]. Histological examination of the transplanted liver in stable long-term patients often shows evidence of chronic post-transplant hepatitis[122]. The causes of the histological changes are unknown although unrecognised viral infections may be responsible for some cases and the steroid sparing immunosuppression regimes may also be partly responsible.

Serious cytomegalovirus (CMV) infections tend to be primary (transmitted by the donor liver) rather than reactivation infections and should be avoidable if CMV-matched donors are used. Clinical infection usually presents between four and eight weeks with fever and leucopenia but asymptomatic sero-conversion does not require treatment. This will respond well to a combination of reduction in base line immunosuppression and ganciclovir therapy[123]. The traditional se rological tests vary between centres, take time and are less sensitive than PCR tests[124]. In patients with symptoms specific to an organ histologi cal analysis should be used in conjunction with PCR tests[125,126].Significant CMV infection is associated with acute rejection and may result in a worse long-term outcome[127]. The routine use of prophylactic ganciclovir reduces the incidence of clinical CMV infection although a high index of suspicion and prompt treatment will also result in negligible mortality[128-132].

Biliary complications are a significant problem in most units undertaking liver transplantation and these include bile leaks, anastomotic strictures, non-anast omotic strictures of the donor bile duct and sludge formation. The overall incidence in adults is approximately 10% but is higher in children[74,133]. In our own series the overall incidence of biliary complications requiring inte rvention is 12%, this rises to 27% in those patients undergoing re-transplantation (unpublished data). The ability to image the biliary tree effectively using ultrasound, MRI cholangiography, endoscopic retrograde cholangiography (ERCP) or percutaneous transhepatic cholangiography (PTC) has led to most biliary complications being managed without reoperation[134]. The presence of a major biliary disruption or an associated biliary obstruction is an indication for urgent biliary reconstruction[135]. Biliary obstruction without leakage will usually be evident from simple ultrasound, can be confirmed by ERCP or PTC and can usually be managed without recourse to open surgery[136-138]. Non-anastomotic biliary strictures involving the confluence or intra -hepatic bile ducts are a rare but serious complication that were once attributed to prolonged preservation times[139]. These strictures are complicated but a proportion can be resolved using a PTC approach by a skilled radiologist although a number of cases will require regrafting. In any patient with a biliary complication patency of the hepatic artery should be confirmed, as hepatic artery thrombosis will cause ischaemia and necrosis of the biliary tree[140]. The late biliary complications seen after transplantation are usually obstruction with possible secondary sepsis and cholangitis. The commonest cause is an anastomotic stricture, with or without stone or sludge formation in the proximal dilated biliary tree. An ERCP may enable duct clearance, dilatation of any stricture and stent insertion. Most strictures will recur and therefore formal biliary reconstruction is usually required.

Hepatic arterial thrombosis (HAT) after liver transplantation occurs most frequently in the first postoperative month and leads to graft necrosis, intra-hepatic abscess or biliary necrosis and bile leakage. In all suspected cases patency of the artery should be checked with Doppler ultrasound and confirmed with spiral CT or angiography[141-143]. Per-cutaneous attempts at revascularizati on of stenosed or thrombosed hepatic arteries can be attempted and urgent thromb ectomy has been successful in some cases but the majority of cases of early HAT will need regrafting[144-148]. Late arterial thrombosis may be occult an d if asymptomatic can probably be ignored. In our own series HAT has occurred in 4.6% of adult grafts and 9.1% of paediatric grafts (unpublished data). Technical problems account for the majority of cases but over transfusion at the time of surgery, producing a high a haematocrit, has been reported as a risk factor[149,150].

Malignancy is well recognised as a potential complication of long term immunosup pression. Longer survival is seen with the liver compared to other solid organ transplants and therefore the time exposed to the risk of malignancy is greater. The most common malignancies seen secondary to prolonged immunosuppression are the lymphoproliferative diseases and lymphoma and skin malignancy[151,1 52]. Reduction in the level of immunosuppression is often enough to treat lymphoproliferative disease[153]. A proportion of liver transplants are performed for primary hepatic malignancy and paradoxically the donor liver (free from malignancy at the time of transplant) is the commonest site of recurrence. The predilection for circulating malignant cells to return and then grow in the liver is well recognised.

IMMUNOSUPPRESSION

The widespread introduction of cylosporine A in the early 1980s was responsible for the improvement in liver graft survival from 35% to 70% survival at one-year[154]. Immunosuppression with cylosporine, azathioprine and steroids remained the main immunosuppressive regimen until the development of tacrolimus in 1989[155]. Tacrolimus was initially used to salvage grafts failing from rejection on cylosporine based regimens[156] but has subsequently been increasingly used as first line immunosuppression by many units. Although structurally different to cylosporine, it also acts by inhibiting calcineurin and subsequent interleukin (IL) 2 production and therefore prevents T cell proli feration[157]. Three prospective randomised trials have compared the ef ficacy of tacrolimus and cylosporine in liver transplant recipients[158-160]. The incidence of rejection was significantly lower with tacrolimus in all studies but there was no difference in one-year patient and graft survival. Long-term follow up has shown a trend towards en hanced survival in patients treated with tacrolimus[161]. The toxicity profile of tacrolimus is similar to that of cylosporine (nephrotoxicity, neurotoxicity, hypertension and diabetogenic potential) but without the gingival hyperplasia and hirsutism commonly seen with cylosporine[162].

Mycophenolate mofetil (MMF) is another new agent that

blocks purine metabolism by inhibiting inosine monophosphate dehydrogenase in T and B lymphocytes[163]. The role of MMF in liver transplant recipients remains to be fully defined but initial reports suggest that when combined with tacrolimus the incidence of acute rejection is reduced[164,165]. MMF has haematological and gastrointestinal side effects but is not nephrotoxic and may be useful in patients with compromised renal function so that the dose of tacrolimus can be reduced[166]. New immunosuppressants continue to be developed and some are currently under eva luation including sirolimus (inhibits action of IL2), basiliximab (chimeric IL2 receptor monoclonal antibody) and daclizumab (humanised IL2 receptor monclonal antibody)[167,168]. Polyclonal antibody therapy that has previously b een used to treat steroid resistant rejection has however, been rendered almost obsolete by current immunosuppressant protocols. In our own centre the current immunosuppression regimen is tacrolimus combined with azathioprine and prednisolone, with steroid taper and withdrawal over three months. MMF is used in place of azathioprine to allow low dose tacrolimus regime ns in those patients with renal impairment prior to transplantation and is also used in place of azathioprine in those patients undergoing retransplantation for chronic rejection.

The available immunosuppressive options will continue to increase and with it the permutations of immunosuppressive regimens. This may make it difficult to effectively evaluate individual regimens. Immunosuppression will however, continue to be a balancing act, with over immunosuppression culminating in toxicity, life threatening infections and malignancy and under immunosuppression leading to rejection and graft loss.

RETRANSPLANTATION

In our own series 10% of nearly 2000 liver transplants were regrafts, although the proportion of patients requiring a regraft is decreasing[169]. HAT accounts for 30% of regrafts, primary non-function for 16%, chronic rejection for 31% and recurrent disease for 6%, although the incidence of HAT and primary non-function is decreasing and the incidence of recurrent disease (PSC and HCV) is increasing[169]. Early re-transplantation is technically straightforward and usually performed for HAT or primary non-function. In an era of donor shortage and donor/ recipient number mismatch the role of re-transplantation has been questioned but the outcome of re- transplantation is good with survival rates only slightly worse than those achieved for the first graft[170].

SURVIVAL

One-year survival rates for elective liver transplant in patients with benign disease now exceed 90% in many centres, with predicted 10 year survival rates expected to exceed 70%[102,171]. Patients transplanted for AFHF have a worse one-year survival with higher post-operative death rates usually related to cerebral complications and multi-organ failure. Experienced centres have however, obtained one-year survival rates of approximately 70%[172-174]. The long-term outcome for patients undergoing liver transplant for AFHF is as good as those transplanted for chronic disease. The increasing interest in living related transplantation offers a new opportunity for those patients with AFHF who cannot wait for a cadaveric organ[175]. The outcome in children undergoing liver transplantation is equally good, even in high-risk groups such as children age under 1 year in whom donor organ shortage might prevent grafting at the optimal time[75].

Survival rates for patients grafted for primary liver cancer (HCC) are less good however, patients transplanted for asymptomatic lesions up to 3 cm in diameter have survival rates close to those seen in patients grafted for benign disease[32]. In our own unit overall survival (including fulminant hepatic failure) at one-year is 81% for adults and 86% for children with different long-term survival depending on disease type (Figure 4).

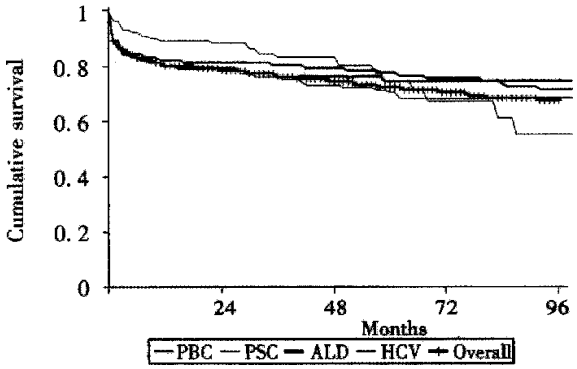

Figure 4 Survival following liver transplantation by disease type: birmingham series 1988-2000.

THE FUTURE

The most serious issue currently facing liver transplant physicians is the short fall in donor organs needed to meet demand. This deficit is greater in the US than in the UK. If the UK could increase its rates of organ donation to levels seen in other European centres and split all livers that meet appropriate criteria (approximately 25% of UK cadaveric organs) then current organ demand could be met. Patient demand will however, mean that increasingly transplant physicians will be asked to justify why certain categories of patient are not considered suitable for transplantation. The limited supply of cadaveric organs allows these physicians to justify transplantation criteria on the basis of the scarcity of this resource. The continued success of living related liver transplant programmes around the world is likely to lead to increasing pressure to relax the criteria for liver transplantation for those patients able to provide their 'own' source of suitable transplant organs. This will require strict control and the application of new National guidelines if the UK is to avoid an expensive and potentially dangerous situation in the application of universal standards of care. A successful UK living related programme would certainly help to ease the deficit in urgent organs for those with AFHF and could address the deficit that currently exists for liver transplantation in chronic liver disease but we believe that this should only occur after the UK has exhausted the potential that is currently un tapped in potential cadaveric organs.

The use of genetically modified xenografts could be potential major breakthrough for organ recipients but is not easily applicable to liver failure patients and there remain many biological and ethical obstacles before these organs become a sustainable source[176].

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

The impact of new technology on surgery for colorectal cancer

Gregory B Makin[1], David J Breen[2] and John RT Monson[1]

University of Hull Academic Surgical Unit[1] and Department of Radiology[2], Castle Hill Hospital, Castle Road, Cottingham HU16 5JQ, United Kingdom

Correspondence to: Professor John RT Monson, University of Hull Academic Surgical Unit, Castle Hill Hospital, Castle Road, Cottingham HU16 5JQ, United Kingdom.j.r.monson@hull.ac.uk
Telephone: 0044-1482-623-225, Fax: 0044-1482-623-274
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Abstract

Advances in technology continue at a rapid pace and affect all aspects of life, including surgery. We have reviewed some of these advances and the impact they are having on the investigation and management of colorectal cancer. Modern endoscopes, with magnifying, variable stiffness and localisation capabilities are making the primary investigation of colonic cancer easier and more acceptable for patients. Imaging investigations looking at primary, metastatic and recurrent disease are shifting to digital data sets, which can be stored, reviewed remotely, potentially fused with other modalities and reconstructed as 3 dimensional (3D) images for the purposes of advanced diagnostic interpretation and computer assisted surgery. They include virtual colonoscopy, trans-rectal ultrasound, magnetic resonance imaging, positron emission tomography and radioimmunoscintigraphy. Once a colorectal carcinoma is diagnosed, the treatment options available are expanding. Colonic stents are being used to relieve large bowel obstruction, either as a palliative measure or to improve the patient's overall condition before definitive surgery. Transanal endoscopic microsurgery and minimally invasive techniques are being used with similar outcomes and a lower mortality, morbidity and hospital stay than open trans-abdominal surgery. Transanal endoscopic microsurgery allows precise excision of both benign and early malignant lesions in the mid and upper rectum. Survival of patients with inoperable hepatic metastases following radiofrequency ablation is encouraging. Robotics and telemedicine are taking surgery well into the 21st century. Artificial neural networks are being developed to enable us to predict the outcome for individual patients. New technology has a major impact on the way we practice surgery for colorectal cancer.

Subject headings colorectal neoplasms; technology; surgery; radiology

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INTRODUCTION

Advances in technology continue at a rapid pace and affect all aspects of life. Medicine is no exception. Colorectal carcinoma (CRC) is the third most commonly diagnosed cancer in the western world[1]. There have been considerable steps forward in the survival and outcome of CRC in recent years. New technology has in no small way contributed to this improvement. One of the major advances in surgery in the 20th century was the development of circular stapling devices in Russia in 1967[2]. These instruments have revolutionized the colorectal anastomosis, particularly deep in the pelvis and almost certainly contributed to the increase in sphincter preserving operations. Another was the introduction of minimally invasive techniques, which has altered the approach to many surgical procedures. Imaging investigations are shifting from analogue film storage to digital data sets. Once acquired this imaging data can be stored, reviewed remotely, potentially fused with other modalities and reconstructed as 3 dimensional images for the purposes of advanced diagnostic interpretation and computer assisted surgery. There will be an increased reliance on 3D-imaging in all aspects of patient care[3]. Now that we have entered the 21st century, how will these and other new technologies affect the practice of coloproctology The AIM of these advances is to continue to improve the outcome of patients with CRC, by altering the way physicians diagnose and treat their diseases. We discuss a number of new technological advances and their impact on surgery for CRC (Table 1).

Table 1 New technology available for colorectal surgeons

INVESTIGATION
Modern endoscopes
Virtual colonoscopy
Trans-rectal ultrasound
Magnetic resonance imaging
Positron emission tomography
Radioimmunoscintigraphy
INTERVENTION
Colonic stents
Transanal endoscopic microsurgery
Minimally invasive surgery
Radiofrequency ablation
Robotics
OUTCOME
Artificial neural networks

INVESTIGATION OF COLORECTAL CANCER

The management of CRC relies on its early detection and characterization. Improving the outcome of CRC depends not only on sensitive investigations but also the encouragement of early presentation by symptomatic patients and population screening. Currently, fibre optic endoscopy is the investigation of choice for the diagnosis of CRC. New colonoscopes are being developed with improved optics, magnifying and localisation capabilities and dual channels allowing endoscopic resection and endoscopic assisted surgery. Endoscopy though, is invasive and carries a small risk of perforation of around 0.2%[4]. Virtual colonoscopy is being

developed which will alleviate this risk and still examine the entire colon in 3-dimensions. There are many treatment modalities available once CRC has been diagnosed. The best option for an individual patient depends on the stage of the disease and the surgeon's expertise. Ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and radioimmunoscintigraphy can all be used to diagnose tumours, provide staging information pre operatively and detect recurrence. Improvements in the sensitivity of these investigations will result in a better outcome for patients.

Modern endoscopes

Fibre optic endoscopy and biopsy remains the investigation of choice for examining the colon and diagnosing CRC. Unfortunately in the United Kingdom a complete colonoscopic examination to the caecum is achieved in only 70% of cases[5]. This results in the patient having to make another visit to the hospital for either a repeat colonoscopy or a completion barium enema. Incomplete colonoscopy is usually due to a combination of looping of the scope, redundant or angulated sigmoid colon and patient intolerance. Several different configurations of looping are possible in the colon, including alpha, gamma, and N loops. Many of these require a different manoeuvre to un-loop the scope. Magnetic imaging localisation and variable stiffness endoscopes are being developed to reduce these loops forming and aid in their reduction, making the procedure more comfortable for the patient and improving the success rate of a complete examination of the colon. Magnifying endoscopes can help differentiate between neoplastic and non-neoplastic lesions without the need for histologic examination. As a result, the overall efficiency of colonoscopy should improve.

The position of the colonoscope within the colon and presence of any looping can be demonstrated using real time 3-dimensional magnetic imaging, which is built into the scope. Shah[6] has shown that even in the hands of expert endoscopists, correct identification of loop configuration occurred only 31% of the time. As the image generated is in real time, loops can be detected and straightened as soon as they form, resulting in shorter intubation time and higher completion rates[7]. Appropriate position change of the patient or a bdominal pressure can be used to correct to loop, if it's configuration is known. The magnetic imaging endoscope can also be used to indicate the extent of the examination. It has the advantage over fluoroscopic screening of having no radiation exposure to either the patient or endoscopy staff. However, identification of the ileocaecal valve, appendix orifice or ilealmucosa remains the gold standard of assuring complete examinati on of the colon. Not only are these new scopes good for patient care, but they are a useful tool for teaching as it shows the trainee exactly what is happening to the scope inside the colon. This feedback gives the operator a better understanding of how loops form, how this changes the resistance of scope insertion and how to prevent and straighten these loops. Shah[7] has shown that trainees using the MR imaging colonoscope have a higher completion rate and spend less time looping the instrument. As a result we should have more proficient endoscopists in the future.

Another new development is a variable stiffness colonoscope. The endoscopist can increase the stiffness of the scope, which reduces looping. Brooker[8] in a randomised control trial has found significantly quicker caecum intubation time (7 *vs* 11 minutes) and less patient discomfort (median pain scores 7 *vs* 24) when using this scope compared with a conventional colonoscope. As a result the rate of incomplete examinations should decrease and patient satisfaction increase.

Currently colonic polyps are biopsied or removed for histological diagnosis to determine if they are neoplastic. Patients with neoplastic lesions require ongoing surveillance, while those with non-neoplastic lesions can be reassured of their low risk of CRC and discharged. The complication rate with colonic biopsy is around 0.5% and includes bleeding, perforation and trans-mural thermal injury[9]. The crypt pattern of polyps has been shown to predict their underlying histology[10]. Magnifying endoscopes are being used to examine t he crypt patterns of polyps. Togashi[11] used a 100 times magnifying scope to examine the crypt pattern of 923 polyps following dye spray. The crypt or ifices were classified into 6 categories: medium round, asteroid, elliptical, small round, cerebriform and no apparent pattern. The first 2 were considered to be a non-neoplastic lesion, while the remainder was neoplastic. Neoplastic and non-neoplastic lesions could be distinguished by the crypt pattern in 88%, when compared to their histological diagnosis. With the use of the magnifying scope in the future, non-neoplastic lesions may not need to be biopsied. Advances in endoscopes will enable more complete and less painful examination of the colonic mucosa, with more accurate evaluation of polyps and a lower overall morbidity, making colonoscopy an even more valuable investigation.

Virtual colonoscopy

Virtual colonoscopy, CT colography CT colonography and CT pneumocolography are all terms which have been used to describe essentially similar investigations. It was first reported by Vining in 1994. Advances in CT software and hardware, particularly the advent of helical data acquisition, have enabled rapid high resolution 2 and 3-dimensional images of the colon to be created. The majority of colonic lesions can be detected on the standard 2-dimensional data set, while the 3-dimensional data is used for problem solving by simulation of an endolu minal image. However, Hara[12] reported that the accuracy of this test is superior when both the 2-D and 3-D images are reviewed, compared to the 2-D images alone. After the patient has taken mechanical bowel preparation, the clean colon is distended with 1.5 L-2 L of air or carbon dioxide, via a rectal catheter. This technique has several advantages. Firstly it is a rapid examination, with the volumetric CT data acquisition taking only a few minutes. It is non-invasive (apart from the introduction of a rectal catheter) and is performed without the need for sedation or abdominal compression. Virtual colonoscopy can examine other abdominal organs, avoids the 1 in 1000 risk of perforation by colonoscopy and is well tolerated by patients. Hara[12] reported that patients were more comfortable during virtual colonoscopy than with barium enema or colonoscopy. The imaging data can then be viewed and manipulated at a remote works station at a convenient time. The disadvantages of virtual colonoscopy are that histological specimens cannot be taken and at present, mucosal detail is poor, relying mostly on polypoidal morphology for lesion detection. Data interpretation can be time consuming, particularly if 3-D reconstruction is required.

Initially the main limitation of this investigation was a difficulty in distinguishing small polyps from faecal residue and examining collapsed segments of colon. The addition of prone imaging to the routine supine imaging has largely overc ome this problem, with improvement in the detection of polyps 5 mm in size or greater, from 75% to 88%[13]. Recent years have seen developments in spatial resolution and image manipulation such as colon mapping. The colon is "digitally

straightened" and opened for purposes of analysis of the colonic mucosal surface. In addition bowel preparation which permits the tagging of faecal residue and subsequent digital subtraction of these artifacts should lead to enhanced polyp detection. Currently the accuracy of polyp detection by virtual colonoscopy is superior to barium enema and is approaching conventional colonoscopy. Results are dependent on polyp size. A large randomised control trial of 180 patients, with 420 colonoscopically proven polyps, showed virtual colonoscopy to have a sensitivity and specificity of 85% and 93% respectively of detecting polyps 1 cm or more in size and 88% and 72% of detecting polyp s 5 mm or greater[13]. Fenlon reported that both virtual and conventional colonoscopy had a similar rate of complete examination of the colon (87% and 89% respectively). The former detected 89% of polyps greater than 6 mm[14].

The problem with conventional endoscopy remains incomplete examination of the pr oximal colon, either due to an occlusive lesion or patient intolerance. Virtual colonoscopy has the ability to examine the proximal colon above an obstructing o r impassable lesion. Morrin[15] compared virtual colonoscopy with bariu m enema in 40 patients who had a failed endoscopy. Virtual colonoscopy had significantly better views of all colonic segments and was better tolerated by the patient. The right colon is easier to evaluate than the sigmoid colon because a greater degree of distension is achievable and it lacks muscle spasm and hypertr ophy. Endoscopic blind spots behind mucosal folds are eliminated by virtual colonoscopy as the 3-D "fly through" can be done in both directions.

Virtual colonoscopy has the advantage of being able to examine the other organs in the abdomen and pelvis at the same time as the colon. Cross sectional views of the colon can show the wall thickness. Evidence of enlarged lymph nodes and liver involvement can be obtained. Morrin[16] correctly staged 13 out of 16 CRC's. Imaging of the liver makes it an efficient use of resources as many patients with a carcinoma have hepatic imaging to stage their disease. Much of the necessary pre operative data can be obtained in one visit with virtual colonoscopy. The uncovering of unrelated asymptomatic pathology has evoked some controversy. Hara[12] found highly clinically important secondary pathology in 11% of patients, including asymptomatic aortic aneurysms. The detection of other potentially life threatening diseases, which are common in this age group, can only be beneficial.

Virtual colonoscopy may revolutionise population screening for CRC as it is edging towards most of the factors needed for a screening tool. It is quick, non invasive, has a high patient acceptance, minimal morbidity, increasing sensitivity and can screen for several diseases at once. At present though, there are no reports in the literature on its use as a screening modality.

Trans-rectal ultrasound and magnetic resonance imaging

The importance of accurate pre operative staging of rectal cancer has grown with the increasing number of treatment options available. These include, local resection, trans abdominal resection, pre operative down staging and palliative radiotherapy. Benign or early malignant (pT1) rectal lesions can be excised locally. Patients with stage pT3 and pT4 tumours benefit from pre operative radiotherapy[17]. Asymptomatic or elderly patients with distant metastases may be given palliative chemoradiotherapy. Accurate pre operative staging of rectal cancer is thus essential in the planning of optimal therapy. Clinical examination, trans abdominal and trans-rectal ultrasound (TRUS), CT, phased-array pelvic body coil and endo-rectal MRI scanning are all used in some combination to assess the depth of rectal wall tumour invasion, and the presence of lymph no de and distant metastases. Despite these modern investigations, the "educated" digital rectal examination remains an important part of the assessment of recta l lesions, particularly in assessing tumour fixity and the need for down staging pre operative radiotherapy[18].

TRUS and MRI are currently both being used clinically in the pre operative assessment of rectal lesions. Both modalities can assess the depth of rectal wall invasion by the primary tumour and detect enlarged lymph nodes, suggesting tumour involvement. MRI has an advantage that it can simultaneously examine the liver for distant metastases. Unlike CT, there is no radiation risk to the patient with either modality. Many studies have shown that TRUS and MRI are more sensitive than CT in staging rectal lesions[19,20]. However, there are conflicting reports as to which of these 2 former investigations is the most accurate in determining the depth of invasion. This in part depends on the MRI technique, whether a surface (phased-array) or an endo-rectal coil is used and is operator dependent. Satoh[21] found TRUS more accurate than MRI, while Thaler[22] and Waizer[23] found no difference between the two. In two recent prospective comparative studies there was no significant difference between TRUS and endo-rectal MRI in both T and N[24] (Tables 2 and 3). Drew[25] highlighted the problem of inter-observer variation with only 31% accuracy in assessing depth of invasion. The accuracy of pre operative staging continues to improve as refinement and understanding of the MRI technique improves. Brown[26] has achieved a 100% T-staging accuracy, predicting depth of extramural tumour invasion to within 5 mm, with the use of high resolution phased-array pelvic coil MRI.

Results of lymph node staging are also conflicting,with neither investigation consistently showing a high level of accuracy. Thaler[22] found TRUS mo re accurate than MRI (80% versus 60%). Using MRI, McNicolas[27] reported a 95% accuracy, where as Drew[25] reported only a 58% positive predictive value. While TRUS and MRI are both reasonably sensitive at staging the depth of tumour, MRI is better at assessing tumour extension into adjacent organs. Both MRI and TRUS rely on highly skilled interpretation of the images, with the former being more expensive and the latter user dependent.

Hepatic metastases can be detected either by ultrasound, CT or MRI. Although ultrasound is quick and easy, MRI is the most sensitive by virtue of enhanced contrast resolution, with an accuracy of 81% in detecting liver[28]. The time has passed when the only pre operative staging of a rectal lesion was with the educated digital rectal examination. Pre-operative staging continues to improve with Brown[26] achieving a 100% T-staging accuracy with the use of high resolution phased-array pelvic coil MRI. Such technological developments will continue to slowly enhance patient outcome, as accurate pre operative staging will triage patients to the appropriate treatment.

The usefulness of intense follow up programs after curative CRC resection remain s controversial. Survival benefits have not been shown in a randomised control trials comparing intensive follow up to no follow[29]. Tumour recurrence is often diagnosed late, with less than 30% amenable to further curative surgery[30]. This recurrence is usually a marker of advanced disease. Radiologic pelvic surveillance is hindered by its inability to distinguish tumour recurrence from post operative fibrosis. Dynamic contrast enhanced MRI is still clAIMed to be the most accurate means of detecting early recurrent disease in the pelvis[21,23].

Table 2 Rectal cancer staging by TRUS and MRI[24]

	TRUS (%)	MRI (%)
T stage accuracy	80	85
N stage sensitivity	72	81
N stage specificity	80	66

Table 3 Rectal cancer staging by TRUS, MRI and CT[20]

Accuracy of detection	TRUS (%)	MRI (%)	CT (%)
Depth of invasion	81	81	65
Lymph node metastases	63	63	56

Positron emission tomography

Positron emission tomography (PET) was first applied to CRC in 1982. PET detects abnormal cellular metabolic activity. PET utilises a number of radiolabelled an alogues, including F-18-FDG, which are preferentially concentrated in malignant cells due to their accelerated glycolysis. F-18-FDG decays by positron emiss ion, which releases 2 protons, which are detected by the imager. Ultrasound, CT and MRI rely largely upon morphologic changes to detect tumour recurrence. The a dvantage of PET is that these cellular changes precede any structural abnormality. There have been 2 large studies showing PET to be more sensitive in detecting recurrent CRC than conventional imaging. Valk[31]reported a sensitivity of 93% *vs* 69% and a specificity of 98% *vs* 96% of PET versus CT respec tively. Whiteford[32] found similar results when comparing PET with CT and colonoscopy combined (sensitivity 90% *vs* 71% and specificity 92% *vs* 85% respectively). PET has detected unsuspected metastases in up to 32% of patients with normal[33,34]. The sensitivity and specificity of PET versus CT in detecting extrahepatic metastases is 94% *vs* 67% and 98% *vs* 96% respectively. As a result there should be a reduction in the number of unnecessary laparotomies, which should help to offset the high purchase cost of the PET hardware. In the future PET may also have a role in evaluating tumour response to chemotherapy and radiotherapy. Findlay[35] reported that the rate of uptake of F-18-FDG in CRC liver metastases compared to normal liver could discriminate tumour response from non-response to fluorouracil.

The main disadvantage of PET is its inability to distinguish between tumour and inflammation. PET has increased uptake in the setting of acute inflammation. This may be the reason for the high false positive rate reported by Schiepers[36], who had 11 false positive results in the chest out of 25 positive tests. Likewise, assessing the response of CRC to radiotherapy is limited by the inflammatory response it produces. This may decrease as experience with this modality increases.

The current role of PET in the management of CRC remains unclear. Early benefits have been shown in detecting recurrent and metastatic disease. Fusing the information it yields with CT and MRI images is enhancing its diagnostic interpretation. There are few reports of its use in screening and detection of primary lesions. Other disadvantages of PET include its limited availability and high cost (around 1.5 million capital cost) and tracer production and distribution.

Radioimmunoscintigraphy

Radioactive labelled antibody scans are being used in many aspects of medicine today. Like PET it detects cellular abnormalities prior to any structural change s in the tissues. There have been many monoclonal antibodies developed to detect CRC antigens, with sensitivity ranging from 74% to 92%. These antigens include B72.3, carcinoembryonic antigen (CEA), BW431/21 and PR1A3[37-40]. CEA molecules break off the colonic columnar cell membrane and circulate in the blood stream. This shed antigen significantly reduces the accuracy of CEA scanning. In contrast, PR1A3 is an anti-CEA monoclonal antibody that binds preferentially to columnar cell surface bound CEA rather than soluble CEA found in circulating[41]. Technetium labelled PR1A3 has been shown to detect 100% of primary CRC's. At present however, colonoscopy remains the investigation of choice for detecting primary lesions, although 99m-Tc PR1A3 is being used clinically to detect recurrent CRC, with a sensitivity and specificity of 96% and 50% respectively[42]. Despite the realization that it is difficult to detect recurrence early enough to be curable, in this study 25% had a beneficial alteration in their management plan.

Despite modern radiology and endoscopy, the aetiology of a recto-sigmoid mass or stricture may remain undiagnosed. Every surgeon has been in the dilemma of whether the lesion is malignant or diverticular in origin. The timing and extent of the resection is determined by the underlying aetiology. Malignant lesions require early surgery with radical en-bloc resection, while diverticular disease should have an initial conservative approach, followed by a limited resection when the inflammation has resolved. Ongoing efforts are concentrating on whether PR1A3 can differentiate between malignancy and inflammatory conditions and early results are encouraging[43]. Overall therefore, the role of radio immunoscintigraphy in the management of CRC seems likely to continue to expand as new, more specific antibodies are developed.

MANAGEMENT OF COLORECTAL CANCER

Colonic stents

The management of a malignant large bowel obstruction in the acute setting, in t he elderly with co-morbidity and those with unresectable disease remains problematic. A new treatment option, the expanding metallic stent, was first reported by Itabashi[44] in 1993. They can be used to relieve obstruction prior to surgery or as palliation and are inserted either with fluoroscopic or endosc opic guidance. Preoperative stenting of large bowel obstructions relieves sympto ms in 87%-100% within 96 hours[45-47]. Stenting AIMs to allow time for the patient's overall medical condition to improve. This reduces the complexity and number of stages of the surgery, with 90% of patients able to undergo an elective single stage procedure[47,48]. The short-term benefits of stenting are obvious, but we do not know of any long-term risks, such as tumour fracture and dissemination. If the procedure can be shown to be safe, then all patients should have a stent placed to improve their nutritional status, electrolyte balance and permit bowel preparation prior to surgery. All these factors will reduce mortality and morbidity and the number of defunctioning stomas required. The main risk of the procedure is perforation of the colon, but this is usually recognised early and patients can proceed to surgery, reverting to a conventional treatment plan. Law[46] reported a series of 24 malignant large bowel obstructions, with 1 perforation requiring a Hartmann's procedure and 3 patients later requiring a stoma. Turegano-Fuentes[49] reports severe tenesmus in 2 patients where the stent was placed low in the rectum.

Palliative stenting of advanced colorectal carcinoma is a quick, effective and non-invasive way of relieving symptoms. It avoids both a laparotomy and a stoma in a patient with a life expectancy of less than 12 months. At present the role of

stents in the management of resectable colon cancer remains uncertain. Stent design and technology and appropriate patient selection is continually evolving, making this a rapidly changing field. Overall results in the literatur e are variable and have mostly been confined to palliation. A prospective randomized trial comparing stenting with primary surgery in resectable disease is clearly required. Further studies need to be done into patient selection for stenting and timing of subsequent resection.

Minimally invasive surgery

Minimally invasive techniques have gained popularity over the past two decades, especially for upper gastrointestinal and biliary pathology. Laparoscopic cholec ystectomy has revolutionised biliary surgery and it is regarded as the procedure of choice for gallstones. Schlinkert[50] performed the first laparosco pically a ssisted hemicolectomy in 1991. Colorectal surgeons have been more cautious in ad opting laparoscopic techniques, particularly for malignant disease. Concern over the ability to perform safe dissection with adequate oncologic tumour and lymph node resection, intracorporeal anastomosis and port site recurrences has led regulating bodies in both the United Kingdom (National Institute for Clinical Exce llence) and United States of America (National Cancer Institute) to recommend that laparoscopic resections for CRC be restricted to clinical trials only. The advantages of minimally invasive surgery are well documented and include statistically significant less post operative pain, a reduced post operative ileus, a shorter hospital stay, better cosmesis and an earlier return to normal activity (Table 4)[51-53]. There is no difference in the cancer related outcome in experienced hands. In randomized control trials comparing laparoscopic and open colectomy, there was no difference in the length of colon, margin distance, length of mesentery and number of lymph nodes excised[54]. There is also no difference in 2 year recurrence free and crude survival rates[55]. Concern over the initially high rate of port site recurrences of up to 26% has not been sustained[56]. Since 1993, fifteen papers on port site recurrences have been published. Rates range from 0% to 1.7%, with a mean of only 0.65%. The threelargest series have a rate between 0.65% and 1.1%[57-59]. This is similar to the reported wound recurrence rates of 0.6% and 0.8% after open resection[60,61]. The cause of port site recurrence is still unknown, although Whelan[62] has suggested that they may be related to the "learning curve" phenomenon. Many patients, in both the laparoscopic and open groups however, had signs of disseminated intra abdominal disease. This fact is believed to be a more signifi cant risk factor for wound recurrence rather than the method of access. Over the years a number of preventative measures have been put forward to reduce recurrence, including the use of wound protectors, gasless laparoscopy, wound excision and peritoneal irrigation.

The advantages of laparoscopic resection for CRC is more pronounced in procedure s which do not require an incision to remove the specimen and perform the anasto mosis. An abdomino-perineal resection is an ideal laparoscopic case as the specimen is removed through the perineal wound and an end colostomy is created through a port hole. Darzi[63] suggests that better views of the mesorectal plane can be obtained using the laparoscope, allowing more precise dissection.

There is no doubt that minimally invasive surgery for CRC is safe, feasible and beneficial for the patient. It should however be limited to experienced laparoscopic and colorectal surgeons, with a low threshold for conversion to an open procedure. Patience is required as it usually takes longer to perform the procedure laparoscopically, especially in the beginning. Surgical principles of oncologic resection must not be compromised by the laparoscopic approach.

Table 4 Advantages of laparoscopic colectomy compared to open colectomy

Mean	Laparoscopic colectomy	Open colectomy
Narcotic use (days)	2.7	4.8
Ileus (days)	3.9	5.9
Hospital stay (days)	6.5	10.2

Transanal endoscopic microsurgery

Transanal endoscopic microsurgery (TEMS) was first performed by Buess[64] in 1983 in Germany. It has revolutionised local resection of rectal lesions, particularly malignant and those in the upper rectum. Traditionally, trans anal resection was limited to benign disease in the low and mid rectum. Many surgeons find this surgery cumbersome, with difficult access and poor views of the operating field. This usually limits its use to small lesions, usually less than 4cm diameter, within 6 cm-8 cm from the anus[65]. Surgery, with a curative intent, for malignant or upper rectal lesions, previously required trans-abdominal resection if endoscopic resection was not feasible. Mortality following trans-abdominal resection is around 5%-8%, but increases to 20% in patients over the age of 80[66]. Operative morbidity is around 25%[67].

There are two main advantages of TEMS in benign rectal disease. Firstly it allow s access to lesions in the mid and upper rectum. In two series of TEMS resection s most of the lesions would have been too high for a trans anal approach without TEMS, as the mean distance of the lesions from the dentate line was greater than 7 cm (Table 5)[68,69]. Secondly the local recurrence rate after TEMS (5%-9%), is much lower than traditional trans-anal resection (12%-25%)[70]. We feel that this low recurrence rate is due to the technique, as the rectum is constantly dilated by insufflation of CO_2 gas, enabling more precise surgery.

Table 5 TEMS excision of benign lesions[68,69]

	Lev-Chelouche	Neary
Number	46	21
Distance from dentate line (cm)	3-18 (mean 7)	5-17 (mean 10)
Size (cm)	1- 7 (mean 2.5)	2-12 (mean 3.9)
Recurrence	4 (9%)	1 (5%)

The use of local excision for malignant rectal lesions remains controversial. Willett[71] reported no difference in the outcome of pT1 and pT2 carcinomas, having favourable histologic features, resected trans-anally or trans-abdominally. The 5 year recurrence free rates were 87% and 91% respectively. Mellgren[72], on the other hand, reports a worse outcome with local resection when compared with trans abdominal resection. Overall 5 year survival rates were 69% and 82% respectively and local recurrence rates were 28% and 4% respectively. Mellgren however, did not exclude poorly differentiated lesions from trans anal excision, nor analyse the results of histological grade subgroup s. Also, these resections were performed using the less precise traditional trans-anal approach. The disadvantage of TEMS in malignant disease is the lack of lymph node sampling and clearance. The incidence of nodal involvement in pT1 tumours with a favourable

histological grade is only 3%, compared to 12% in pT1 lesions with poor prognostic grade[73,74] advocated TEMS for pT1 lesions due to lower morbidity, similar local recurrence rates and similar survival benefit to that of a major resection . We feel that TEMS is a suitable alternative treatment option for pT1 and pT2 lesions with favourable histology. It can also be used in more advanced lesions and those with unfavorable histology in the elderly or those unfit for major surgery[69]. Results of TEMS for rectal malignancy are shown in Table 6. By carefully selecting the patients who undergo local excision for a malignant rectal lesion, acceptable results in overall survival and local control can be achieved. Neary[69] reports no recurrence after a mean follow up of 20 months, which is better than 3% local recurrence after total mesorectal excision[75].

Table 6 TEMS excision of malignant lesions[68,69]

	Lev-Chelouche	Neary
Number	29	19
Distance from dentate line (cm)	3-15 (mean 8)	4-14 (mean 10)
Size (cm)	2-5 (mean 3.2)	1-6 (mean 3.2)
Recurrence	4 (18%)	0 (0%)

Mortality and morbidity rates of TEMS are less than 1.3% and 20% respectively. This extremely low mortality makes it a very valuable alternative to a trans-abdominal approach, provided the cancer related outcomes remain equal. Most of the morbidity is minor and include urinary retention and haemorrhage managed conservatively. Intra-peritoneal perforation is the major complication of this procedure, which can be sutured locally or treated by reverting to the original operative choice of an anterior resection. The hospital stay is only 3 days on average, which is much shorter than a trans abdominal resection[69]. The saving in bed costs more than offsets the initial outlay to purchase the reusable equipment. The advantages of TEMS are listed in Table 7.

Table 7 TEMS excision of benign and malignant lesions[68,69]

	Lev-Chelouche	Neary
Number	75	40
Mortality	1 (1.3%)	0 (0%)
Morbidity	10 (13%)	8 (20%)
Hospital stay (days)	2-13 (mean 5.5)	1-6 (mean 3.2)

TEMS will continue to play an increasing role in the management of rectal lesion s. It has the advantage of allowing precise surgery in the mid and upper rectum on both benign and early malignant lesions. Advanced cancers are still better de alt with by trans-abdominal resection, unless the patient is elderly or unfit for major surgery. In these two groups the risk of major surgery out weighs the benefits.

Radiofrequency ablation

Liver metastases ultimately develop in 50% of patients with colorectal cancer[76]. Surgical resection offers the only potential for cure and long term survival, with 5 year survival rates up to 46% after "curative surgery"[76,77]. However, less than 25% of patients with colorectal liver metastases are considered suitable for hepatic resection[78]. In those patients where their tumour load, tumour distribution or co-morbidity prevents attempte d curative resection or they have recurrent disease, there are other therapeutic options. Palliation can be achieved with systemic or hepatic arterial chemotherapy, cryotherapy, and radioactive yttrium-90 microspheres, although cure is ext remely unlikely[79]. The mean survival of patients undergoing the different treatments for CRC liver metastases is listed in Table 8.

Table 8 Median Survival following treatment for CRC liver metastases

Treatment	Median survival
Supportive care[91]	7-11 months
IV Fluoruoracil chemotherapy[91]	11-14 months
Hepatic artery chemotherapy[91]	15-17 months
Radiofrequency ablation[84]	27 months

Radiofrequency ablation (RFA) by applying an alternating current at 300-500 kHz causes frictional heating of tissues to between 50-85 degrees Celsius. This thermal injury results in coagulative necrosis of spheres of tissue usually between 3 cm-5 cm in diameter[80,81]. It is safe and effective, with up to 98% of lesions showing persistent complete necrosis at 15 months[82-84]. Gillams[84] has shown RFA improves survival of patients with CRC liver metastases unsuitable for resection. RFA was performed in 69 patients, with an average number of metastases of 2.9 (range 1-16) and a mean maximum diameter of 3.9 cm (range 1-8). 26% had undergone previous hepatic resection and 93% received chemotherapy. The 3 year survival rate and mean survival time from diagnosis was 34% and 27 months respectively. 58% developed new hepatic metastases and 33% new extrahepatic disease. A subgroup of 24 patients, with less than 4 metastases, with a maximum diameter of 5cm, performed better, with a median survival of 33 months. Bilchik[85] recommends RFA for un-resectable primary or secondary malignant liver lesions up to 3 cm in size. Mortality and major morbidity occurred in 1.4% and 3.2% respectively, the latter being needle tract seeding. Minor morbidity, including pain and sepsis, occurred in 12%. RFA has the advantages of being safe, well tolerated and can be applied percutaneously, laparoscopically or at laparotomy. Lesions close to the diaphragm and colon are best accessed under direct vision by either of the latter 2 approaches. Percutaneous application has the added advantage of a shorter hospital stay of less than 24 hours. The role of RFA should continue to expand, as it is beginning to be applied to small lesions in the remaining liver segments during resection. In turn, this will broaden the horizon of hepatic resection.

Robotics

Robotics is at the forefront of the technological advances in medicine. Many innovations are being developed to aid laparoscopic surgery. Robotic arms has enabled solo laparoscopic surgery, which abolishes the need for an assistant, has greater stability of views and does not fatigue[86]. Voice activated robotic arms are being produced, which further enhances their ease of use. One of the drawbacks of laparoscopic surgery is the lack of depth perception. Robots (Davinci from Intuitive Surgical Ltd) and stereoscopic glasses (Optimize Interna tional) are being developed with 3-dimensional tactile and visual capabilities. Computer assisted colonoscopy, using a miniature self propelled robotic probe, is being developed to reduce the discomfort and increase patient acceptance of colonoscopy[87]. Tele-medicine will have an increasing role as we shift to digital data based investigations. Expert opinions can be given over the telephone, thus improving patient care.

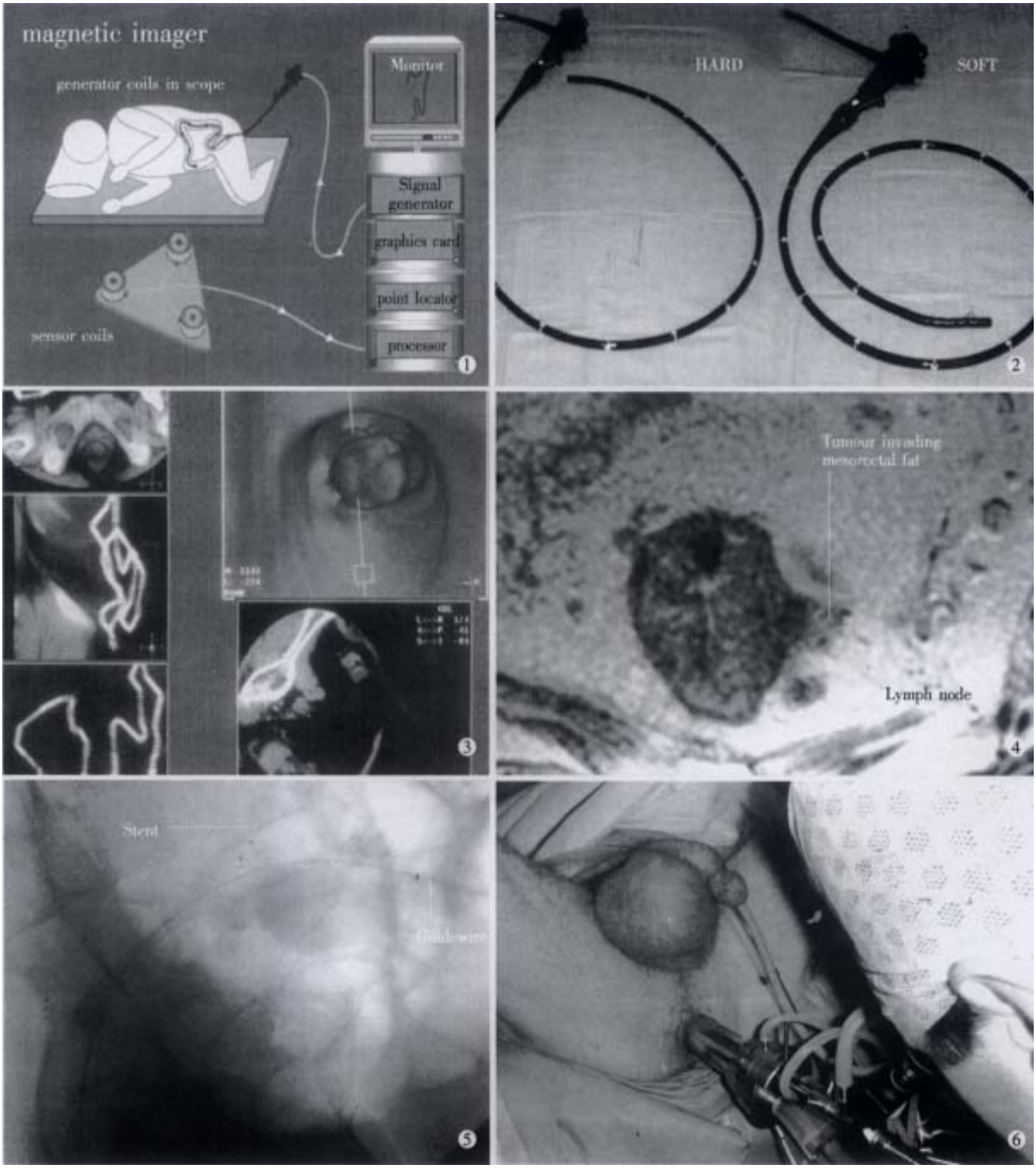

Figure 1 Magnetic imager colonoscope: magnetic generator coils built into the endoscope enable its position and presence of looping in the colon to be identified on the monitor.
Figure 2 Variable stiffness colonoscope: illustrating the stiff (hard) and flexible (soft) modes of the endoscope.
Figure 3 Virtual colonoscopy workstation interface (Marconi). T he operator is able to assess the 3-D data set simultaneously in 3 orthogonal planes. The upper right hand image demonstrates the endoluminal viewpoint and shows a 3cm polypoidal malignancy at the splenic flexure.
Figure 4 Thin cut imaging of a rectal carcinoma perpendicular to the long axis of the tumour displays the tumour breaching the muscularis propr ia and an enlarged lymph node within the mesorectum. Suggested staging T3N1.
Figure 5 Lateral view demonstrating a metallic stent (Boston Sc ientific, Watertown, MA) being deployed across a rectosigmoid carcinoma with the aid of a guidewire. The patient achieved prompt and effective decompression.
Figure 6 Trans-anal endoscopic microsurgery: 50 mm rectos cope is inserted through the anus. An attached stereoscopic binocular viewing eyepiece allows six-fold magnification of the operative field. Constant flow insufflation with carbon dioxide keeps the rectum dilated.

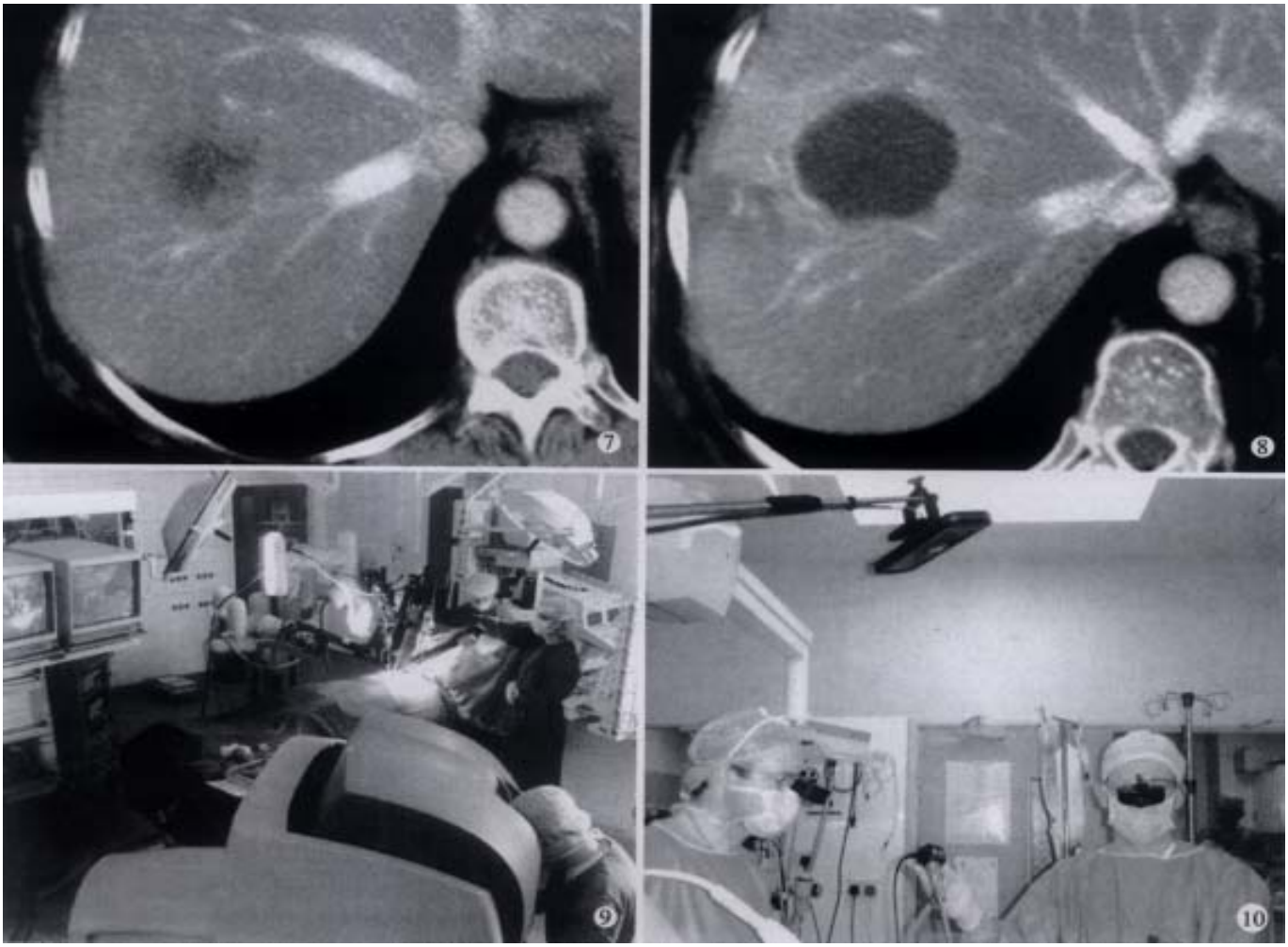

Figure 7 Radiofrequency ablation: CT scan demonstrates A 3.5 cm solitary colorectal metastasis in segment 8 of the right lobe of the liver.
Figure 8 Radiofrequency ablation: the post treatment CT scan sh ows a 4.5cm necrotic RFA lesion at the site of the previous tumour (Figur e 7). There is typical marginal enhancement and some perfusional anomalies in the subtended liver.
Figure 9 "Davinci": Operating robot (Intuitive Surgical Ltd).
Figure 10 Stereoscopic 3-D projection laparoscopic glasses (Optimize International).

OUTCOME OF COLORECTAL CANCER

Artificial neural networks

Analysis of the treatments available to patients with CRC traditionally relied on population statistics. These predictions have little meaning for individual patients. Artificial neural networks (ANN) are particularly suited for the analysis of complex databases and the relations within these data sets[88]. They allow recognition of patterns in complex biological data sets, which cannot be detected in conventional linear statistical analysis. The advant age of ANN's is that once the network is established, it can be used to predict outcome for individual patients. Imputation of data is the first step in setting up and training a network, and the more data entered the better. Outcome is entered at the same time. Once the network is validated using a second set of data and outcome, data from an individual can be entered and a prediction of outcome for that individual calculated.

Neural networks have been applied to several aspects of CRC. Bottaci[89] analysed pre operative, operative and follow up data on 334 patients treated for CRC. ANN's were used to predict the death of an individual patient and compared to a surgeon's opinion. In the first institution where the initial data to train the network was collected, the network was marginally more accurate (80% *vs* 75%). When this ANN was used on data from a second institution, its accuracy increased to 90%, compared to the surgeon's prediction of death of 79%. Neural networks have also been used to accurately predict the lymph node status and tumour stage of a resected colonic malignancy, based on the patient's age and tumour biopsy grade and immunohistochemistry[90]. Data from 75 patients were used to train the network. The ANN correctly predicted the lymph node status of 20 of 24 test cases (sensitivity 85%, specificity 80%) and the tumour stage of 21 of 24 test cases (sensitivity 92%, specificity 82%). The application of neural networks in coloproctology is endless. Once a network is established from a data set, the outcome for each individual patient on any aspect of CRC can be predicted.

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Edited by Rampton DS and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Recent advances in the surgical treatment of pancreatic cancer

A Shankar and RCG Russell

Department of Surgery, The Middlesex Hospital, Mortimer Street, London, W1N 8AA, UK
Correspondence to: A Shankar, Department of Surgery, The Middlesex Hospital, Mortimer Street, London, W1N 8AA, UK
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INTRODUCTION

Pancreatic cancer remains the fourth commonest cause of cancer related death in the western world[1]. The prognosis remains dismal due partly to late presentation, with associated low resectability rates, and the aggressive biological nature of these tumors. The median survival time from diagnosis in unresectable tumors remains only 4-6 months.

For those patients amenable to surgical resection over the last 20 years have seen marked improvements in postoperative mortality and morbidity, especially in specialist pancreatic centres[2,3]. Despite these changes long-term survival remains low, with a total 5-year survival rate remaining less than 5%. Patients with ampullary cancer have a better 5-year survival of 40%-60%.

Resection, however, remains the only chance of long term survival with adjuvant therapies providing disappointing results. Operability remains low due to the local and distant extent of the disease. Assessment of this extent has been greatly advanced by modern radiological techniques.

PREOPERATIVE ASSESSMENT

Once distant disease has been excluded, selection of patients for resection is crucial if the rate of irresectability discovered at operation is to be kept to a minimum.

Angiography

Once considered crucial in the assessment of operability, angiography is now virtually unnecessary. It was argued that the venous phase of the arteriogram was fundamental if invasion of the superior mesenteric and portal vein were to be excluded. This has now been superseded by improvements in helical CT scanning, which is able to accurately determine venous involvement. The issue of preoperative detection of vascular anomalies should not be an indication for routine angiography in the hands of experienced pancreatic surgeons.

Computerised tomography (CT)

CT is still the traditional imaging modality for staging pancreatic cancer, although it lacks specificity and sensitivity. Spiral CT with intravenous contrast offers higher resolution than conventional CT and improves diagnostic and staging accuracy[4]. Unfortunately CT is still limited by its ability to differentiate between benign and malignant lesions and also may miss subcentimetre hepatic deposits and peritoneal seedlings. The impact of helical CT scanning using defined pancreatic protocols with multiplanar reconstruction has yet to be assessed, but the extent of local disease, involvement of arteries and veins and lymphatic spread can be assessed with improved accuracy (as illustrated in the following CT images).

Magnetic resonance imaging (MRI/MRCP)

Although a relatively new technique for assessing pancreatic lesions, MRI is particularly useful at differentiating inflammatory from neoplastic pancreatic lesions. Pancreatic adenocarcinomas are usually low signal on T1 and T2 weighted images[5], although it has as yet not been shown to be superior to CT in assessing operability. The use of magnetic resonance endoscopy may in the future improve the accuracy of MRI. At present, both MRI and CT scanning give additional information such that both techniques are of value in assessment.

Laparoscopy and laparoscopic ultrasound

Laparoscopy has the advantage of being able to detect small (<10 mm) hepatic deposits (with and without laparoscopic ultrasound), assess degree of lymph node involvement and identify peritoneal disease over that detected by conventional imaging. The position of the pancreas in the retroperitoneal area does, however, limit its ease of application.

When combined with laparoscopic ultrasound, a sensitivity up to 90% for predicting operability have been suggested[6,7] which may be further improved with the addition of peritoneal lavage cytology[8]. Positive lavage cytology for tumor cells is associated with lower resectability and lower survival rates[9].

Determination of irresectability by laparoscopy in patients deemed operable by CT is usually due to the detection of occult liver metastases and peritoneal seedlings. Laparoscopic ultrasound allows direct placement of the probe onto the pancreas providing views of the tumor in relation to the superior mesenteric and portal veins. Such reports have led to the routine use of staging laparoscopy in some units prior to resection, although laparoscopic ultrasound is still relatively uncommon.

The issue of port site metastases is a risk in this technique, although the mechanism is not completely understood.

Endoscopic ultrasound (EUS)

This technique involves real time ultrasound scanning from within the lumen of the stomach and duodenum. Initial reports suggested highly reproducible results in expert hands in relation to staging of pancreatic cancer, especially in regard to vascular invasion, providing more accurate results than conventional CT[10]. It is less useful at demonstrating malignant lymph node involvement due to confounding factors such as nodal reactivity and inability to differentiate nodal micrometastases[11]. EUS is also used to obtain fine needle

aspiration cytology of pancreatic masses and has yielded almost 80% diagnostic sensitivity[12]. One of the main drawbacks to this technique is that it is highly user dependent.

Positron emission tomography (PET)

PET involves the injection of radiolabelled glucose which is preferentially picked up by malignant cells and metabolised. PET scanning has found increasing applications throughout oncological assessment and when combined with CT scanning provides both anatomical and functional information. The most commonly used agent is 2-Fluoro-2-deoxy-D-glucose (FDG), which when taken up by cells and metabolised emits positrons that collide with electrons creating gamma rays, which are then picked up by a ring detector.

PET aids diagnosis of adenocarcinoma of the pancreas and also improves the sensitivity of conventional imaging in the detection of peritoneal and hepatic disease, enabling correlation between CT detected abnormalities and PET findings. Initial studies suggest that when PET is combined with EUS it may be more accurate than CT in detecting metastatic disease[13].

In addition to aiding diagnosis and staging, the extent of uptake of the radiola belled substrate may correlate with the aggressiveness of the tumor, with a higher uptake predicting shorter survival[14,15]. This extent of expression may also help differentiate benign from malignant lesions[16] .

SURGICAL APPROACH TO RESECTABLE PANCREATIC CANCER

Ninety percent of all malignant pancreatic exocrine tumors are accounted for by pancreatic duct cell cancer, with about two thirds occurring in the head of the gland. Convention states that tumors arising from the left side of the gland have a worse prognosis due to later presentation. Whilst it is true that tumors arising in the head may be detected at an earlier stage due to the associated jaundice, more recent data suggests that resectable tumors in the head, uncinate process and neck have a similar survival to those in the body and tail[3].

A number of issues arise in relation to the surgical approach to pancreatic cancer and include: preoperative biliary drainage, extent of lymphadenectomy, vascular resection and pylorus-preserving versus standard Whipple.

Preoperative biliary drainage

The issue of preoperative biliary drainage either endoscopically or transhep atically remains controversial. Trede and Schwall, in a large retrospective study, demonstrated that patients who underwent preoperative endoscopic stenting had a reduced incidence of postoperative infective complications[17]. Other studies failed to show any benefit and suggested an increased postoperative risk associated with stenting in relation to sepsis introduced with instrumentation. Povoski et al demonstrated that the primary predictor of postoperative complications was the preoperative placement of biliary stents[18].

For patients in whom the date for resection is to be delayed, or in those undergoing palliation of jaundice, biliary stenting is usually recommended.

Extent of lymphadenectomy

A wide variety of opinion exists regarding the extent of lymphatic dissection for both tumors on the right and left side of the pancreas. Results vary depending upon the institution / country of origin and is partly due to the disparity that exists in the definitions used. Recent standardisation of terminology in this regard may clarify future studies, with precise definitions of 'standard', 'radical' and 'extended radical' lymphadenectomy.

Although extended surgical procedures popularised in Japan may increase the resectability rate up to 50%, this does not necessarily translate into improved survival. The retrospective nature of much of this data renders interpretation difficult and requires validation. Node positivity in many series is a strong negative predictor for survival with a 5-year survival stated to be less than 5%[19,20] whilst others using radical lymphadenectomy demonstrate a 5-y ear survival of up to 70% for node negative patients[21], results not reproduced elsewhere. These results are supported by the finding of metastatic disease in lymph nodes removed during extended lymphadenectomies for early disease[22].

Extended lymphatic resection when performed by exponents of this technique report equivalent rates for morbidity and mortality to those found in standard resections[23]. Hirata et al reported a retrospective series of 1001 resected pancreatic cancers from 77 centres in Japan[24]. The majority of these were stage IV according to the standard of Japanese Pancreatic Society (JPS). One hundred and thirty-one patients had a D0 resection (no lymph nodes), 365 a D1, and a D2 in 505 (422 of whom also underwent a further resectional procedure such as IV C reconstruction). The mortality rate was 2.5%. Improved survival was only found in those patients with N1 nodes positive who had a D1 resection.

In a retrospective series, Naganuma *et al* demonstrated a significant improment in survival for patients who underwent extended resections compared to earlier more 'standard' procedures[25]. This study has several failings, one of which is its retrospective nature, and another is the highly selective process of determining which patients underwent a extended resection in the second group.

A relatively small (n = 81) randomised, multicentre, prospective study of standard versus extended lymphadenectomy was reported by Pedrazzoli *et al*[23]. There was a significant improvement in survival for the node positive patients in the extended lymphadenectomy group, although when the whole group was analysed there was no difference. The survival curve of node positive patients undergoing extended lymphadenectomy could be superimposed on that for node negative patients. There was no difference in postoperative morbidity and mortality between the two groups. Multivariate analysis of all the patients demonstrated that long-term survival was significantly affected by tumor differentiation, tumor size, nodal involvement and more than a four unit blood transfusion.

Vascular resection

Whilst technically feasible in some patients *en bloc* resection of infiltrated peripancreatic vessels does not appear to improve survival, with vessel involvement a strong negative predictor of outcome[26,27]. There are few groups reporting worthwhile survival figures for patients undergoing vascular resection and again in a retrospective format[28]. Morbidity is high due to the extensive autonomic neurectomy causing diarrhoea.

Some groups have advocated a more widespread use of venous resection in view of the high rate of vessel involvement by tumor in some patients thought to be macroscopically clear, although the relevance of such findings to survival is hard to quantify[29].

Pylorus-preserving versus standard Whipple

Traditional teaching had been that proximal pancreatic resection should be combined with distal gastrectomy to reduce

gastric acid production and hence minimise the risk of postoperative stress ulceration and also to improve oncological clearance. This approach has now been modified with preservation of the en tire stomach and proximal duodenum. This reduces the complications associated with gastrectomy and does not appear to increase the risk of marginal ulceration. Apart from dorsally placed tumors abutting the duodenum oncological clearance does not appear to be affected although there is a higher incidence of postoperative delayed gastric emptying[30].

COMPLICATIONS OF PANCREATIC RESECTION

In specialist centres perioperative mortality should be less than 5% and in some are less than 1%, although the incidence of postoperative complications is still 30%-40%[2,3,34]. The complications most commonly found are leakage of the pancreatic anastomosis, haemorrhage, abdominal abscess and delayed gastric emptying.

Pancreatic fistulae occur with a reported frequency of 4%-24%[3,35] and may progress to a full leak with associated sepsis and haemorrhage. Such serious leaks occur in less than 5% in specialist units and may carry a mortality of up to 40%. Clearly, access to experienced interventional radiology is crucial in these cases to deal with associated intra-abdominal collections. There is a correlation between the frequency of leaks and the pancreatic consistency, duct diameter and extent of residual exocrine function.

Although some groups routinely stent the pancreatic duct there is currently no randomised data to substantiate this practice, which also applies to the practice of creating separate Roux loops for high risk cases. The method of reconstruction is also debatable, *i.e.* end-to-side or end-to-end, or invagination versus mucosa to mucosa, but again lacks randomised data. The experience of the surgeon is probably the most crucial factor in determining leak rates[36]. Inhibition of pancreatic exocrine function using the somatostatin analogue octreotide prophylactically in the perioperative period has been shown to reduce postoperative morbidity associated with pancreatic fistulae in a number of randomised studies in Europe[37,38].

SURVIVAL POST RESECTION

According to the review on the resection of pancreatic cancer published in 1996 by Sperti *et al*[39], the rate of resection was approximately 20% with a mean operative mortality of 9% and a 5-year survival rate of 12%.

Tumor biology appears to be the most important predictor of survival, with multivariate analysis demonstrating that aneuploidy, tumor size and nodal status are all independent predictors of outcome[40].

Clear resection margins is a crucial measure of long-term survival, especially in relation to the posterior aspect of the tumor[41]. This mode of spread occurs predominantly along the perineural sheath and lymphatics.

Extended resections, whilst improving resectability rates, do not appear to improve survival but await results of randomised studies.

The largest single institution series to date reported by Sohn *et al* has an overall 5-year survival rate of 17% for the 208 patients followed for at least five years (of a total of 616 patients included in the series)[42]. Improved survival was found in those with less than 750 mL intra-operative blood loss, no transfusions, negative nodes, clear margins and tumors less than 3 cm. In addition, there was a demonstrable survival advantage for those patients receiving adjuvant chemoradiotherapy. For those patients with tumors less than 3 cm, node negative with clear margins, the 5-year survival rate was 31% in this series. The issue of blood loss has been noted previously to be a predictor of survival[40] and should be minimised in all cases.

Two other large retrospective series from France[43] and USA[44] of 555 and 327 resections demonstrated 5-year survival rates of 15% and 12% respectively for the groups as a whole.

The use of postoperative chemoradiotherapy has been the subject of much debate. After an apparently curative resection a large number of patients will subsequen tly develop liver and/or peritoneal metastases. The North American Gastrointestinal Tumor Study Group (GITSG) randomised patients to receive radiotherapy and 5-fluorouracil or supportive therapy only, with treatment continuing for two years. Patients receiving adjuvant therapy had a significant survival advantage[45].

These results were contradicted by the results of two European studies-ESPAC-1[46] and The EORTC gastrointestinal study group[47]. The EORTC study randomised 218 patients with T1-2 N0-1a M0 pancreatic head or T1-3 N0-1a M0 periampullary cancer to receive post resection either 5FU and external beam radiotherapy or observation alone between 1987 and 1995. There was no significant improvement in either survival or local recurrence rates in all groups studied. The only difference in treatment protocol between this study and the GITSG trial[45] was a reduced dose of 5FU, 5FU was only given in the first week of chemotherapy. Currently adjuvant therapy cannot be recommended in the context of a randomized control trial.

Some success has been reported in the use of neoadjuvant therapy to down-stage tumors to an operable state, although these are all small, non-randomised studies. Snady *et al* looked at two groups of patients over an 8-year period[48]. Those with initially diagnosed locally invasive but regional tumors were given a combination of 5FU, streptozocin, cisplatin and radiotherapy. The others were patients who underwent resection without preoperative treatment (the majority of whom underwent postoperative adjuvant chemotherapy with or without radiotherapy). Those patients who became down-staged and underwent subsequent resection had significantly better survival than those in the non-pr e treated group.

QUALITY OF LIFE (QOL) AFTER PANCREATICODUODENECTOMY

Few studies have addressed the issues about quality of life after pancreaticoduo denectomy, concentrating rather on morbidity, mortality and survival. McLeod et al compared 25 age- and sex-matched controls undergoing cholecys tectomy with an equivalent number undergoing pancreaticoduodenectomy[31], measuring 6 quality of life assessments. This study demonstrated no difference between the two groups in terms of the quality of life measures and also showed a return to normal preoperative body weight and nutritional status in the pancreaticoduodenectomy patients. Melvin et al compared the quality of life in patients undergoing a standard Whipple with those undergoing a pylorus-preserving procedure[32]. Mental health was the same but physical health quality of life was lower in the Whipple group, with no association noted with the pathology found, *i.e.* benign or malignant. Huang et al assessed 192 patients undergoing pancreaticoduodenect omy using the three domains of quality of life, physical, psychological and social and compared these to healthy patients and those undergoing laparoscopic cholecystectomy[33]. The pancreatectomy patients manifested

similar quality of life profiles to the control groups, but did experience problems related to weight loss, abdominal pain, steatorrhoea and diabetes. When a subgroup analysis was performed, the patients undergoing surgery for chronic pancreatitis scored significantly lower in all three parameters when compared to the control group, presumably secondary to the chronicity of their disease.

SURGICAL PALLIATION OF ADVANCED PANCREATIC CANCER

Given the low rate of resectability for patients with pancreatic cancer, the issue of palliation of symptoms becomes of paramount importance. Surgery may play a part in the management of jaundice, duodenal obstruction and pain.Relief of jaundice plays a key role in improving quality of life and survival in patients with advanced pancreatic and periampullary cancer. The two options are endoscopic/percutaneous stenting (either metal or plastic) and open/laparoscopic surgical drainage. Endoscopic drainage has a success rate of 85%-90% with a relatively low complication rate. Stent occlusion may be a problem and in an effort to overcome this metal stents are now commonly utilize d for proven inoperable malignant tumors. Their greater patency rates compared to plastic stents have been demonstrated in randomized controlled trials. Even if occlusion does occur, tumor ingrowth can be ablated using laser/thermal techniques, otherwise further stents can be placed through the middle, beyond the site of occlusion. For those patients in whom endoscopic access has failed, percutaneous transhepatic stents can be used, although with a higher risk of complications[49].

For those patients who at the time of surgery are found to be inoperable and who have biliary obstruction, surgical biliary drainage is necessary. A variety of techniques are available, although the original cholecystjejunostomy has now been superseded due to the low long-term patency rates. The preferred method of bypass in this institution is choledochoduodenostomy, as this is relatively simple to perform with minimal morbidity and long-term patency. Other groups used choledochojejunostomy, which is suggested by some to have longer patency rates[50].

Operative drainage has a higher morbidity and mortality than stenting[51] and should be reserved for those patients found to be inoperable at surgery and the rare instance in which stenting by either route has failed, bearing in mind the mortality rates of open drainage of 19%-24%.

The issue of duodenal obstruction is controversial, with debate over whether or not routine gastric bypass should be performed at the same time as surgical biliary bypass. Approximately 5% of patients with pancreatic cancer will have actual mechanical obstruction and 17% of patients undergoing biliary bypass alone will develop subsequent duodenal obstruction. A proportion of patients undergoing gastric bypass procedures will develop delayed gastric emptying, with significant morbidity attached to this procedure. Therefore, it seems sensible to reserve gastric bypass for those with definite obstruction or evidence of impending problems, especially given the availability of endoscopically placed metal duodenal stents.

Pain is a major problem in the management of patients with pancreatic cancer and aside from standard analgesic regimens a variety of nerve ablative technique s are available. Coeliac plexus blockade, either at the time of surgery or percu taneously, has been reported as having significant success rates[52]. More recently thoracoscopic division of the splanchnic nerves has reported variable success rates and the results of large randomised studies are underway. Initial enthusiasm was tempered by high complication rates which have now drastically reduced. Unilateral versus bilateral approaches have been assessed and the left side appears to be the most important for its analgesic effect in pancreatic cancer.

CONCLUSION

Pancreatic cancer remains a disease with a dismal prognosis despite improvements in surgical technique. Accurate staging of disease is crucial if inappropriate surgery is avoided, with the choice of technique depending upon available facilities and expertise.

Modifications in surgical approach are unlikely to modify the outcome, and only improvements in adjuvant treatments, coupled with a greater understanding of the biological nature of the disease process, will the survival be improved. Meanwhile, patients with pancreatic cancer should be managed by multidisciplinary teams in specialist centres, if morbidity and mortality associated with the management of this disease are to be minimized.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Pharmacology of tetrandrine and its therapeutic use in digestive diseases

Ding-Guo Li, Zhi-Rong Wang and Han-Ming Lu

Department of Gastroenterology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Correspondence to: Dr. Ding Guo Li, Department of Gastroenterology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
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INTRODUCTION

Tetrandrine (Tet) is a dibenzylisoquinoline alkaloid isolated from Stephania tetrandra S. Moore, a Chinese herbal medicine. In the past decade, lots of studies demonstrated that Tet has multiple bioactivities. It is promising to use Tet as an antifibrogenetic in liver or lung fibrosis with or without portal or pulmonary hypertension, as well as an immunomodulating and anticarcinoma drug.

PHARMACOLOGY

Ca^{2+} channel blocking activity

Abnormal Ca^{2+} signaling and elevated concentration of intracellular free Ca^{2+} are the basic pathophysiological events involved in various diseases. As a Ca^{2+} antagonist, Tet can inhibit extracellular Ca^{2+} entry, int ervene in the distribution of intracellular Ca^{2+}, maintain intracellular Ca^{2+} homeostasis, and then disrupt the pathological processes. As shown in whole cell patch-clamp recordings, Tet blocked bovine chromaffin cells voltage-operated Ca^{2+} channel current in a time- and concentration-dependently manner. In rat phaeochromocytoma PC 12 cells, 100 μmol·L^{-1} Tet abolished high K^+ (30 mmol·L^{-1}) - induced sustained increase in cytoplasmic Ca^{2+} concentration, inhibit bombesin-induced inositol triphosphate accumulation in NIH/3T3 fibroblast and abolish Ca^{2+} entry[1]. In rat glioma C6 cells, studied with fluorometric ratio method, Tet did not affect the resting cytoplasmic Ca^{2+} concentration, but it inhibited IP3 accumulation and the sustained and peak elevation of cytoplasmic Ca^{2+} concentration induced by bombesin and thapsigargin, a microsomal Ca^{2+}-ATPase inhibitor, in a dose-dependent manner. The dose of Tet needed to abolish the sustained and peak elevation of cytoplasmic Ca^{2+} concentration induced by bombesin and thapsigargin was 30 μmol·L^{-1}[2]. Bickmeyer *et al*[3] demonstrated that NG108-15 cells treated with 100 μM Tet for seven minutes could block voltage-dependent Ca2+ entry induced by depolarization with 50 mM KCl. Tet could block non-voltage-operated Ca^{2+} entry activated by intracellular Ca^{2+} store depletion induced by thapsigargin and could release intracellular Ca^{2+} in HL-60 cells, and could therefore increase concentration of intracellular free Ca^{2+}, elicit therapeutic effects. We have previ ously demonstrated that Tet could concentration-dependently block extracellular Ca^{2+} entry into hepatocytes, promote mitochondria Ca^{2+}-uptake, and inhibit Ca^{2+}-mobilizing from mitochondria. However, the blockade of Ca^{2+} channel played the most important role in maintaining Ca^{2+} homeostasis, but not intracellular Ca^{2+} distribution.

In the presence of extracellular Ca^{2+} (1.3 μmol·L^{-1}), glutamate, serotonin and histamine significantly increased the intracellular Ca^{2+} concentration in a dose-dependent manner. 30ìmol·L^{-1} Tet significantly inhibited the increase in intra cellular Ca^{2+} concentration induced by glutamate, serotonin and histamine by 28.0%, 46.8% and 29.0%. In Ca^{2+} free Hanks' solution, Tet did not produce a significant inhibitory effect on the increase in intracellular Ca^{2+} concentration caused by serotonin and histamine. These results indica ted that Tet conducted blocking of Ca^{2+} influx from the extracellular site via NMDA, 5-HT_2 and histamine type I receptor-operated Ca^{2+} channels and has no obvious effect on the Ca^{2+} release from intracellular Ca^{2+} stores[4]. In addition, Tet also inhibited extracellular Ca^{2+} entry and intracellular Ca^{2+} mobilization induced by norepinhprine and angiotonin II via corresponding receptor respectively[3,5]. Taking together, in different tissues and different kinds of cells, Tet may block the Ca^{2+} channel through different mechanisms. It can block the voltage- and/or receptor-operated Ca^{2+} channel. Nevertheless, in some kind of carcinoma cells, Tet does not affect Ca^{2+} channel, but promote Ca^{2+} release from intracellular stores and elevate the cytosolic free Ca^{2+} concentration.

Immunomodulating activity

Clinically, Stephania tetrandra S. Moore has been thought to be effective in treating autoimmune diseases such as rheumatoid arthritis and systemic lupus erythe matosus. Tet, the active ingredient isolated from *Stephania tetrandra* S. Moore, has potential immunomodul ating and anti-inflammatory effects. T-lymphocytesplay a critical role as autoactive and pathogenic population in autoimmune and inflammatory diseases. Some experimental data showed that, through down-regulating the protein kinase C (PKC) signaling, interleukin-2 secretion and the expression of the T cell activation antigen (CD71), Tet inhibited phobol 12-myristate 13-acetate (PMA)+ionomycin-induced T cell proliferation dependent on interleukin-2 receptor alpha chain and CD69, such an action was unrelated to Ca^{2+} channel blockade[6]. Tet (0.1-10 μmol·L^{-1}) significantly inhibited neutrophil-monocyte chemotactic factor-1 upregulation and adhesion to fibrinogen induced by N-formyl-methionyl-leucyl-phenylalanimne and PMA. Tet at 0.1-100 μmol·L^{-1} caused dose- and time-dependent loss of cell viability of mouse peritoneal macrophages, guinea-pig alveolar macrophages and mouse macrophage-like J774 cells, reduced production of oxygen free radical oxygen, down-regulated synthesis and

release of some pro-inflammatory cytokines[7,8].

Nuclear transcription factor kappa B (NF-kappa B) is a multiprotein complex which regulates a variety of genes concerned with immunity and inflammation. For the alveolar macrophages, Tet could inhibit the activation of their NF-kappa B and NF-kappa B-dependent reporter gene expression induced by endotoxin , PMA, and silica in a dose-dependent manner. Western blot analysis suggested that the inhibitory effects of tetrandrine on NF-kappa B activation could be attributed to its ability to suppress signal-induced degradation of I kappa B alpha, a cytoplasmic inhibitor of the NF-kappa B transcription factor[7,9].

Conducting tumor cell apoptosis

To induce tumor cell apoptosis is one of the important chemotherapeutic strategies for malignent tumors. Tet inhibited both proliferation and clonogenicity of human leukemic U937 cells at an optimal concentration of 2.5 $mg \cdot L^{-1}$. The characteristic morphological changes of apoptosis were observed under light microscopy and DNA fragmentation was noted by gel electrophoresis in these cells. Moreover, flow-cytometric detection of surface phosphatidyl serine expression of cells after treatment with Tet confirmed the induction of apoptosis in these cells[10]. Tet concentration-dependently inhibited the proli feration of human leukemic HL-60 cells. Morphological observation and DNA analysis revealed that Tet caused cell shrinkage with the formation of apoptotic bodies, and showed clear evidence of DNA fragmentation[11]. Tet was found to induce pronounced morphological changes characteristic of apoptosis and extensive DNA fragmentation in the human BM13674 cell line 8 h after treatment[12]. The induction of apoptosis by Tet was much more rapid in CEM-C7 cells (4 h) than in the same cells treated with glucocort icoids (40 h), and did not require de novo protein synthesis[13]. These results indicate that Tet may have value as an anti-neoplastic agent.

Reversing multidrug resistance (MDR)

The occurrence of MDR to chemotherapeutic drugs is a major problem for successful cancer treatment. The overexpression of cell membrane P-glycoprotein (P-gp) is one of the major mechanisms of MDR. P-gp pumps antitumor drugs out of tumor cells, causing drug resistance.

Tet (3 $\mu mol \cdot L^{-1}$) reduced the paclitaxel concentration required to achieve 50% inhibition of cell growth (EC50) of HCT15 (P-gp-positive) cells about 3100-fold, and also reduced the EC50 value of actinomycin D about 36.0-fold in the cells. Meanwhile, Tet had no effect on the cytotoxicity of the drugs to SK-OV-3 (P-gp-negative) cells[14]. The non-cytotoxic concentrations of Tet potentiated the growth-inhibitory actions of doxorubicin (Dox) in the Har-resistant HL60 cells. The colony formation efficencies were reduced from 60% by Dox to 0.2% by Tet + Dox. Retardation of the G2M phase cells was increased. But Tet did not potentiate Dox cytotoxity in the sensitive HL60 cells. Dox accumulation in the harringtonine-resistant HL60 cells treated by with was increased. These results indicated that Tet enhanced the cytotoxicity of MDR-related drugs via modulation of P-gp[15]. In addition, Tet could also inhibit platelet- activating factor-induced human platelet aggregation and decrease thromboxane B2 production and thrombus formation[16].

THERAPEUTIC USE IN DIGESTIVE SYSTEM DISEASES

Protective effects on hepatocyte injury

Hepatocyte lesions are common and very important clinically[A17-A21]. Chen *et al*[22] observed the effects of Tet on hepatocytic injury ind uced by CCl_4. The result showed that, compared with control group, Tet (1-1000 $nmol \cdot L^{-1}$) increased viability of liver cell (from 71% to 72%-89%), reduced lactate dehydrogenase release, and malondialdehyde (MDA) formation. Tet prevented the increase of the intracellular Ca^{2+} concentration and the attenuation of the membrane microflow of liver cells. Tet (30 $mg \cdot kg^{-1} \cdot d^{-1}$ via gavage for two wk) could markedly reduce the elevation of serum alanine aminotransferase, alkaline phosphatatase and MDA induced by azathioprine. The level of reductive glutathione and SOD were not different from the normal control group. Histological changes in the Tet-treated group were slight[23]. The protective effect on CCl_4- or azathioprine-injured hepatocytes may be elicited by inhibiting the lipid peroxidation, improving the membrane microflow, and lessening the Ca^{2+} concentration. With flow-cytometric technique, we demonstrated that 10-60 $mg \cdot L^{-1}$ Tet could concentration-dependently accelerate the G1 phase cells transforming to S phase cells, and increase the level of DNA in the S phase and protein in the G1, G2 phase cells significantly. Further stu dies indicated that the effect of Tet in promoting hepatocytes proliferation was not related to blockade of Ca^{2+} influx[24].

Anti-hepatofibrogenetic activity

Tet could significantly reduce the degree of experimental hepatic fibrogenesis induced by CCl_4 in rats; the levels of serum hyaluronic acid and procollagen peptide were decreased, and the liver dysfunction was ameliorated, Tet could also obviously inhibit extracellular matrix formation and collagen deposition. In the liver tissue of rats treated with Tet, hepatic stellate cell (HSC) activation, proliferation, and transformation were down-regulated; the number of desmin-positive cells were reduced significantly. The anti-fibrotic effect of Tet had no significant difference from that of colchicine[25]. HSC activation, proliferation, and transforming into fibroblast are the putative events in hepatic fibrogenesis. Tet could significantly inhibit conventional cultured HSC activation and type I and type III collagen mRNA expressions and protein synthesis were down-regulated. Tet could block HSC proliferation colla gen synthesis induced by platelet-derived growth factor (PDGF), reduce the level of PDGF, PDGF receptor (PDGF-R beta1), transforming growth factor beta1 (TGF beta1) and alpha-smooth muscle actin mRNA, and also down-regulate the au tocrine of PDGF, PDGF-R beta1, TGF beta1. These data suggest that Tet may block hepatic fibrogenesis directly and/or through inhibiting cytokine express ions[26,27]. After taking Tet orally for three months, liver functions of the patients with cirrhosis were obviously improved. Administrat ion Tet for six to eighteen months, serum levels of PIIIP and HA of the patients were markedly reduced. Histological examination showed that, compared with pretreatment or placebo, inflammatory cell infiltration was reduced, and even abolished, and that the deposition of ECM, type I and type III collagen were decreased significantly[28].

Anti-portal hypertension

Portal hypertension is one of important manifestations of the patients with cirrhosis. Upper gastrointestinal hemorrhage caused by portal hypertension commonly led to the patient's death. After injecting Tet intravenously (2.0, 6.0 and 20.0 $mg \cdot kg^{-1}$), portal venous pressure and mean arterial pressure were assessed in cirrhotic rats induced by CCl_4. The results

demonstrated that Tet induced dose-dependent decreases in portal venous pressure and mean arterial pressure. The maximum percentage reductions of portal venous pressure after Tet in the three different dosages were 5.4%±1.0%, 9.2%±0.8%, and 23.7%±1.2% of baseline, respectively. Total peripheral resistance was also reduced by Tet[29,30]. In portal hypertensive rats induced by partial portal vein ligation, Tet (4, 8, 16 and 24 mg·kg^{-1}) induced dose-dependent decreases of portal venous pressure and mean arterial pressure after intravenous infusion. Tet (16 mg·kg^{-1}) caused the portal venous pressure decreasing from a baseline of 12.5 mmHg to 10.0 mmHg, and the mean arterial pressure from a baseline of 90 mmHg to 80 mmHg. At 24 mg·kg^{-1}, Tet reduced portal venous pressure and mean arterial pressure to 20.3%±2.4% and 28.4%±1.4% of baseline, respectively[31]. The effects of Tet on portal hypotension may be attributed to its actions of blocking voltage- and receptor-operated Ca^{2+} channels in vascular smooth muscle cells, inhibiting intracellular Ca^{2+} mobilization and dilating peripheral blood vessels. We had previously observed its clinical therapeutic effects on portal hypertension. Taking Tet orally for 2 consecutive years, the esophageal variceal pressure and the portal blood flow in cirrhotic patients with portal hypertension were significantly reduced. The proportion of patients with no recurrent gastrointestinal bleeding during 2 years' medication of tetrandrine was 87.9%. It is suggested that Tet would be effective for cirrhotic patients with portal hypertension in preventing recur rent variceal bleeding[32].

Therapeutic effect on portal hypertensive gastropathy

Portal hypertensive gastropathy is caused by dysfunction of submucosal circulation and gastric mucosal barrier damage. Recent studies found that Tet increased prostgalandin E2, GMBE and GAM secretion, reduced the degree of gastric mucosa injury, and lowered the portal pressure. This result indicates that Tet may be useful in portal hypertensive gastropathy.

Preventing pancreatic islet beta cells from toxic injury

Pancreatic islet beta cells could be damaged by alloxan (50 mg·k g^{-1} i.v.)[aa] in rats, and diabetic animal models were thus prepared. Pancreatic islet beta cells density in experimental groups pretreated with Tet (100 mg·kg^{-1} via gavage) at 1.5 hours and 5 hours prior to alloxan injection increased from the control value of 13±4 to 62±9 and 65±7 (P<0.001). When the doses of Tet decreased from 100 mg·kg^{-1} to 50 mg·kg^{-1} and 25 mg·kg^{-1}, the pancreatic islet beta cell density were 45±5 and 38±4 (P<0.01 and P<0.001)[33].

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97

Yan Li, Zhao-You Tang, Sheng-Long Ye, Yin-Kun Liu, Jie Chen, Qiong Xue, Jun Chen, Dong-Mei Gao and Wei-Hua Bao

Liver Cancer Institute and Zhongshan Hospital of Fudan University (Former Liver Cancer Institute of Shanghai Medical University), Shanghai 200032, China
Supported by the State Key Basic Research Program Grant G1998051211, and the Fund for Leading Specialty of Shanghai Metropolitan Bureau of Public Health.
Correspondence to: Professor Zhao-You Tang, M.D., Liver Cancer Institute & Zhongshan Hospital of Fudan University, 136 Yixueyuan Road, Shanghai 200032, China.zytang@scrap.stc.sh.cn
Telephone: +86-21-64037181, Fax: +86-21-64037181
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Abstract

AIM **To establish clone cells with different metastatic potential for the study of metastasis-related mechanisms.**

METHODS **Cloning procedure was performed on parental hepatocellular carcinoma (HCC) cell line MHCC97, and biological characteristics of the target clones selected by *in vivo* screening were studied.**

RESULTS **Two clones with high (MHCC97-H) and low (MHCC97-L) metastatic potential were isolated from the parent cell line. Compared with MHCC97-L, MHCC97-H had smaller cell size (average cell diameter 43 μm *vs* 50 μm) and faster *in vitro* and *in vivo* growth rate (tumor cell doubling time was 34.2 h *vs* 60.0 h). The main range s of chromosomes were 55-58 in MHCC97-H and 57-62 in MHCC97-L. Boyden chamber *in vitro* invasion assay demonstrated that the number of penetrating cells through the artificial basement membrane was (37.5±11.0) cells/field for MHCC97-H *vs* (17.7±6.3)/field for MHCC97-L. The proportions of cells in G0-G1 phase, S phase, and G2-M phase for MHCC97-H/MHCC97-L were 0.56/0.65, 0.28/0.25 and 0.16/0.10, respectively, as measured by flow cytometry. The serum AFP levels in nude mice 5wk after orthotopic implanta tion of tumor tissue were (246±66) μg·L^{-1} for MHCC97-H and (91±66) μg·L^{-1} for MHCC97-L. The pulmonary metastatic rate was 100% (10/10) *vs* 40% (4/10).**

CONCLUSION **Two clones of the same genetic background but with different biological behaviors were established, which could be valuable models for investigation on HCC metastasis.**

Subject headings hepatocellular carcinoma; clone cells; metastasis

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INTRODUCTION

Cancer cell population, either as a solid tumor mass *in vivo* or as a continuous cell line *in vitro*, is an ever-changing entity due to their geneticins tability and selective environmental pressure. A tumor mass consists of different cell clones, a phenomenon known as tumor heterogeneity[1,2]. Based on this phenomenon, tumor cell clones of different biological properties have been isolated from a number of human and animal tumor cell lines. These differences include a variety of biological characteristics such as tumor cell morphology, karyotypes, *in vitro* and *in vivo* growth patterns[3-7], DNA ploidy[8,9] tumorigenicity and drug sensitivity[5], metastatic patterns[4] and metastatic potentials[10-12], albumin secretion[13] and hyaluronan production[14].

Liver cancer is the 4th most common cause of death from cancer and China alone accounts for 53% of all liver cancer death worldwide[15], and the incidence is on slow but steady rise in both developing and the developed countries[16-20] Primary liver cancer in China, of which more than 90% is hepatocellular carcinoma (HCC), remains the second leading cancer killer that mainly affects middle-aged people-those in the prime of their most productive years[21]. Although gratifying progress has been achieved in clinical treatment at some centers, the overall survival for the whole HCC population at large is still very poor. Another dismal problem is that HCC is more prone to recurrence and metastasis even after curative resection[16,22-24]. Therefore researches in the mechanism and intervention of liver cancer recurrence and metastasis have special priority in China's anti-cancer campaign. For a better insight into the mechanisms of HCC metastasis and for the development of new treatment strategies, an ideal HCC model system is essential. To serve this purpose, animal model of metastatic human HCC LCI-D20[25] and metastatic human HCC cell line MHCC97[26] have been established at the authors' institute.

Although several human and animal liver tumor cell clones[13,27-37] have been established, few of these were suitable for the study of human HCC metastasis-the most fundamental characteristics of cancer and the ultimate cause of most cancer mortality. Recently, we isolated two human HCC clones with different metastatic potential from the parent cell line MHCC97, and explored some of their differences.

MATERIALS AND METHODS

Animals

Male athymic BALB/c nu/nu mice, 4 - 6 wk old, were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Science, and housed in laminar- flow cabinets under specific pathogen-free (SPF) condition. All studies on mice were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". The study protocol was approved by Shanghai Medical Experimental Animal Care Committee.

Parent Cell Line

The parent cell line MHCC97[26] is a human HCC cell line established from the animal model of human HCC LCI-D20[25].

Cloning Procedure

Cells of the 25th passage of the parent cell line were used for the current work. The cells were cultured at 37 ℃ in a humidified atmosphere of 50 mL·L^{-1} CO_2 and 950 mL·L^{-1} air. The culture medium was high glucose Dulbecco's modified Eagle medium (DMEM) (GibcoBRL, Grand Island, NY, USA) suppl emented with 100 mL·L^{-1} fetal bovine serum (Hyclone, Utah, USA). Two days after cell passage, the medium was transferred into a sterile tube (Corning Incorporated, Corning, NY, USA), centrifuged at 2000 r·min^{-1} for 10 min, and the supernatant was stored at -20 ℃ as conditioned medium. When the cells grew to approximately 80% confluence, the culture flask was placed in 4 ℃ refrigerator for 4 h to synchronize the cells, followed by incubation overnight at 37 ℃ as usual. In the following morning, single cell suspension was prepared after trypsinization (2.5 g·L^{-1} trypsin, Difco, prepared in Ca^{2+} and Mg^{2+} free Hanks solution d-Hanks), cell viability was confirmed by trypan blue exclusion, and cloning procedure was performed using the limited dilution method[6]. The cloned cells were preserved in culture medium containing 100 g·L^{-1} dimethyl sulfoxide (DMSO, Sigma Chemical Co, St Louis, MO, USA), and stored frozen in liquid nitrogen till used for *in vivo* screening.

In vivo Screening

In vivo screening was conducted in nude mice, when the cloning process was complete. The stored cells were thawed and propagated. Approximately 1×10^7 cells in 0.2 mL culture medium were injected sc into the right flank of the mice, which were then observed daily for signs of tumor development. Once the subcutaneous tumor reached 1-1.5 cm in diameter, it was removed and cut into pieces about 2 mm×2 mm×2 mm which were implanted into the liver of each of 6 nude mice, using the method as described previously[25]. Five weeks later, the animals were sacrificed and autopsied. Lungs and other organs suspected of tumor involvement were sampled for histopathological studies. This was the first round of *in vivo* selection. In order to identify clones with maximal and minimal metastatic potential, all the recovered clones still viable after thawing were tested for initial screening. The parent cell line was used as controls.

Confirmation Test

Once the clones with maximal and minimal metastatic potential were targeted. They were subjected to the second round of confirmation test, which was performe d essentially in the same way as the first round of selection, but more animals were used. Target clone cells were propagated and 5×10^6 cells in 0.2 mL culture medium were injected into the left lower flank region of each of 5 nude mice (4 wk-old, 13 g-17 g). The animals were observed for latency period, defined in this study as the time interval from the day of injection till the day of definite tumor mass about 5 mm in diameter at the injection site. The growth of subcutaneous tumor was recorded for 30 d, then fresh tumor tissues were implanted into the liver exactly the same manner as the first round of selection, 10 mice for each clone. Animal care and pathological studies were the same.

The confirmed clones were subjected to the following studies.

Morphological Observations

The cells were cultured on culture chambers (Lab-Tek) Chamber Slide, Nunc Inc. Naperville, III, USA) for 2 d and stained in Giemsa solution. Cell morphology was viewed under light microscope and representative pictures were taken. Transmission electron microscopy was conducted as described previously[11]. For scanning electron microscopy, cells grown on cover slips were fixed with 25 g·L^{-1} glutaraldehyde fixative (pH 7.2), and observed directly under scanning electron microscope (HITACHI S-520, Japan).

Chromosome analysis

Chromosome preparation was performed with the method described by Seabright[38], with slight modification. Briefly, cells after 60 h of subculture were used. Colchicine (Shanghai Chemical Reagents Co. Shanghai, China) was added to the culture flasks to yield the final concentration of 0.04mg·L^{-1}, and the flasks were incubated for 4h before the cells were harvested. The cells were treated in hypotonic solution consisting of 1:1 mixture (in volume) of 4 g·L^{-1} potassium chloride and of 4 g·L^{-1} sodium citrate, and then fixed in ice-cold methanol: glacial a cetic acid (2:1, volume ratio). The slides stained in Giemsa solution (1:10 dilution in pH 6.8 PBS). Metaphase chromosome spreads were analyzed with Cytovision Chromosome analysis system (CytoVision™ Image Analysis Workstations, USA).

Cell Growth Curves

Cells in exponential growth phase were trypsinized to give single-cell suspensi on. 4×10^4 viable cells in 1mL of medium were added to every well of the 24-well tissue culture plates, which were incubated at 37 ℃ with 50 mL·L^{-1} CO_2. Cell numbers in two wells were counted in a hemocytometer every 24 h for 7 consecutive days, and cell growth curves were plotted based on these results. The tumor cell doubling time was calculated according to the following formula: TD = Tlg2/lg (N/N_0) (TD: doubling time, T: time interval, N_0: initial cell number, N: end-point cell number)[39].

Plate Efficiency (PE)

1×10^7 cells·L^{-1} of single-cell suspension were made from cells of exponential growth phase. 0.2 mL of cell suspension (containing 2000 viable cells) and 4 mL of culture medium were added to each well (3.5 cm in diameter) of 6-well culture plate, which was incubated at 37 ℃ with 50 mL ·L^{-1} CO_2 for 12 d, washed twice with warm PBS, and stained with Giemsa solution. The number of colonies was counted under microscope (40×). PE was calculated using the following formula: PE = (number of colonies/ number of cells inoculated)×100%.

In vitro invasion assay

Matrigel invasion assay was performed using the method by Albini *et al*[40], with modification. Boyden chamber inserts (Nunclon™, Denmark) with filter membrane pore size of 8 μm were used in the assay. 50 μg matrigel (from the Department of Biology, Medical Center of Beijing University, Beijing, China) was coated to each filter and the chamber was incubated at 37 ℃ for 2 h to produce the artificial basement membrane. Tumor cells in serum-free DMEM (200 μL containing 1×10^5 cells) were added to the upper compartment of the chamber, and 800 μL of conditioned medium was added to the lower compartment. After 24 h incubation, the matrigel was removed, the filter was washed, fixed and

stained in Giemsa solution. Cells that had migrated to the under side of the filter were counted under a light microscope (200×). The results were expressed as the number of migrated cells per field and presented as the [AKx-D]±s of three assays.

Flow cytometry

Cells at exponential growth phase were harvested and single-cell suspensions containing 1×10^6 cells were made. The cells were treated following the standa rdized protocol and cell cycle analyses were performed by flow cytometry as described previously[41].

Immunocytochemistry

Cells directly cultured on slides were washed two times with PBS and then fixed in acetone for 5 min at room temperature. Albumin, alpha-fetoprotein (AFP), cytokeratin 8 and hepatitis B surface antigen (HBsAg) were detected by immunocyt ochemistry using a two-step labeled avidin-biotin immunoperoxidase method, as recommended by the supplier. Primary detection was by either rabbit polyclonal or murine monoclonal antibodies. Biotinylated secondary envision antibodies were goat anti-rabbit IgG and rabbit anti-mouse IgG (Dako, Denmark). Negative controls consisted of omission of the primary antibody, and all cells were counterstained with hematoxylin. The slides were viewed under microscope and the degree of staining was recorded.

Detection of hepatitis B virus DNA

Cells were harvested by trypsinization when they were at 90% confluence, and was hed twice in PBS. Total cellular genomic DNA was extracted using the Qiangen DNA extraction kit (Qiagen GmbH, Germany). Hepatitis B virus (HBV) DNA was exami ned by fluorescent primer polymerase chain reaction (PCR) (LightCycler, Roche, USA) using 2 μg cellular DNA, according to the instruction of the HBV DNA detection kit (Shenzhen Piji Biotechnology Development Co. Shenzhen, China). Positive and negative standards were tested at thesame time.

Alpha-fetoprotein determination

At the end of the second test, when the mice were sacrificed, blood was taken from each animal, and the serum AFP levels were determined automatically (ACS: 180 Automated Chemiluminescence System, Bayer Corporation, USA).

Statistical analysis

Fisher's exact test and student's *t* test were used, respectively, for comp arisons of enumeration data (number of mice with lung metastases) and measuremen t data. The statistical analysis software package Stata 5.0 was used for the tests, and $P<0.05$ was considered as statistically significant.

RESULTS

Identification of Clones with Different Metastatic Potential

A total of 28 clones were isolated from the single-cell culturing of four 96-well plates. Among them, clones 2, 3, 12, 14 and 15 were discarded because of suspected microbial contamination; clones 5, 9, 16, 17, 18, 19, 20, 21, 22, 23, 26, 27, 28 were not viable when the cells in the first few passages were thawed from liquid nitrogen. The remaining ten clones (clones 1, 4, 6, 7, 8, 10, 11, 13, 24, and 25) were propagated for *in vivo* screening. The cells of each clone were first injected into the subcutis of nude mice. Then the subcutaneous tumors were implanted into nude mice liver, for evaluation of spontaneous pulmonary metastasis after 5 wk. The results were summarized in Table 1. It was found that most of these clones had similar metastatic potential. Lung metastases were 6/6 for clones 1, 7, 8, 10, 11 and 13, 5/6 for clone 4, 4/6 for clones 6 and 24, and 2/6 for clone 25.

Table 1 Lung metastases of 10 clones

Clone number	*n* Tumor implanted	*n* Lung metastases after 5 wk
1, 7, 8, 10, 11, 13	6	6
4	6	5
6, 24	6	4
25	6	2
MHCC97	6	6

NOTE. Each clone was first sc inoculated into nude mice. Then small pieces of subcutaneous tumor were implanted into the liver of 6 nude mice for every clone. The number of mice with lung metastases was determined by histopathology 5 wk after orthotopic tumor implantation (see the *in vivo* screening for detail.).

Among the clones with greatest metastatic potential, clone 8 produced the most numerous lung metastases (median number of lung colonies 12/mouse). Therefore, clone 8 and clone 25 were selected as target clones and used for the second round *in vivo* confirmation test. For evaluation of subcutaneous tumor development, 5 nude mice for each clone were used, and every mouse wasinjected with 0.2 mL of cell suspensions containing 5×10^6 cells. For evaluation of spontaneous metastasis after orthotopic implantation, 10 animals were used for each clone. The results were shown in Table 2 and Figures 1, 2.

Table 2 Abdominal events and pulmonary metastases after liver implantation of subcutaneous tumor tissue

	Clone number	
	No. 8 (MHCC97-H)	No. 25 (HMCC97-L)
No of mice with tumor implantation	10	10
Tumor size by d 35/cm	1.42±0.11	0.90 ±0.26[a]
Abdominal events		
Abdominal wall invasion	40% (4/10)	20% (2/10)
Bloody ascites	10% (1/10)	0% (0/10)
Intrahepatic metastases	80% (8/10)	0% (0/10)
Diaphragm invasion	10% (1/10)	0% (0/10)
Hepato-splenic/hepato-gastric ligament invasion	10% (1/10)	0% (0/10)
Loco-regional lymph node enlargement	0% (0/10)	0% (0/10)
Pulmonary metastases	100% (10/10)	40% (4/10)[b]

NOTE. [a]$P<0.01$, *t* test. The length (L), width (W) and height (H) of liver tumor was measured at autopsy using a caliper, and the tumor size was expressed as the geometric mean diameter (GMD) GMD = (L×W×H) 1/3; [b]$P<0.05$ Fisher's exact test.

Although both clones were tumorigenic, the latency period differed considerably between them, being (5-10) (6.4±2.2) d for clone 8 and (20-25) (21.3±2.5) d for clone 25. By d 30, the subcutaneous tumor sizes were (1.94±0.36) cm for clone 8 and (0.84±0.47) cm for clone 25 ($P<0.01$, *t* test). Both clones mainly exhibited expansive growth pattern, although clone 8 showed some tumor invasion in 2 of the 5 animals tested, one invading the hipbone and another invading the lumbar spine. The most obvious difference between the two clones regarding the subcutaneous tumor development was the growth rate.

Their differences became even more apparent after small

bits of subcutaneous tumor tissues were implanted into the liver of nude mice for 5 wk, when it was found that clone 8 produced notable intrahepatic metastases in 8 of the 10 recipients examined, whereas clone 25 did not produce any observable nodules. Apart from intrahepatic metastases, clone 8 also caused abdominal wall invasion in 4/10 (40%), diaphragm invasion in 1/10 (10%), hepato-splenic and hepato-gastric ligaments invasion in 1/10 (10%), and bloody ascites in 1/10 (10%). These changes were not observed for clone 25, except 2/10 (20%) animals showed abdominal wall invasion. No enlargement of lymph nodes was observed for either clone.

Pathological studies of liver tumors from the two clones showed similar histology. The tumor cells were polygonal epithelial-like cells forming large tumor nests surrounded by thin fibroconnective tissues. Tumor necroses were prominent at the center of large tumor nests. Anaplastic tumor spindles aggressively infil trating the adjacent tissues and tumor cells invading blood vessels were observe d in clone 8, but not in clone 25. Moreover, the lung metastatic lesions formed by these two clones were also different. The lungmetastases by clone 25 were usually small and located near the surface of the lung, while those formed by clone 8 were large and usually located in the lung parenchyma, pressing blood vessels and the bronchioles (Figures 3A, B).

Thus it was established from these selections and observations that clone 8 was the most metastatic and clone 25 the least metastatic. They were designated as MHCC97-H and MHCC97-L, respectively.

Cell Morphology

Both clones showed polygonal epithelial-like morphology, with firm attachment to the culture flask. However, they were different in cell size (average cell diameter (50±5) μm in MHCC97-L *vs* 43±2 μm in MHCC97-H) and cell morphology (multiform in MHCC97-L *vs* uniform in MHCC97-H). Both cells had large conspicuous nucleus, with 1-3 big nucleoli scattered in the nucleus of MHCC97-L and 3-7 smaller nucleoli in MHCC97-H (Figure 4A, B). Electron microscopy revealed abundant microvilli and projections on the cell surface. Some of the projections on MHCC97-H extended far and formed bulges at the end, while those on MHCC97-L were short and compact. Both cells had many lysosomes in the cytoplasm, which were usually concentrated on one side of the cell in MHCC97-H and scattered around the cytoplasm in MHCC97-L. No obvious desmosomes, tight conjunction or other cell junction structures were observed, nor were virus particles or other particular particles.

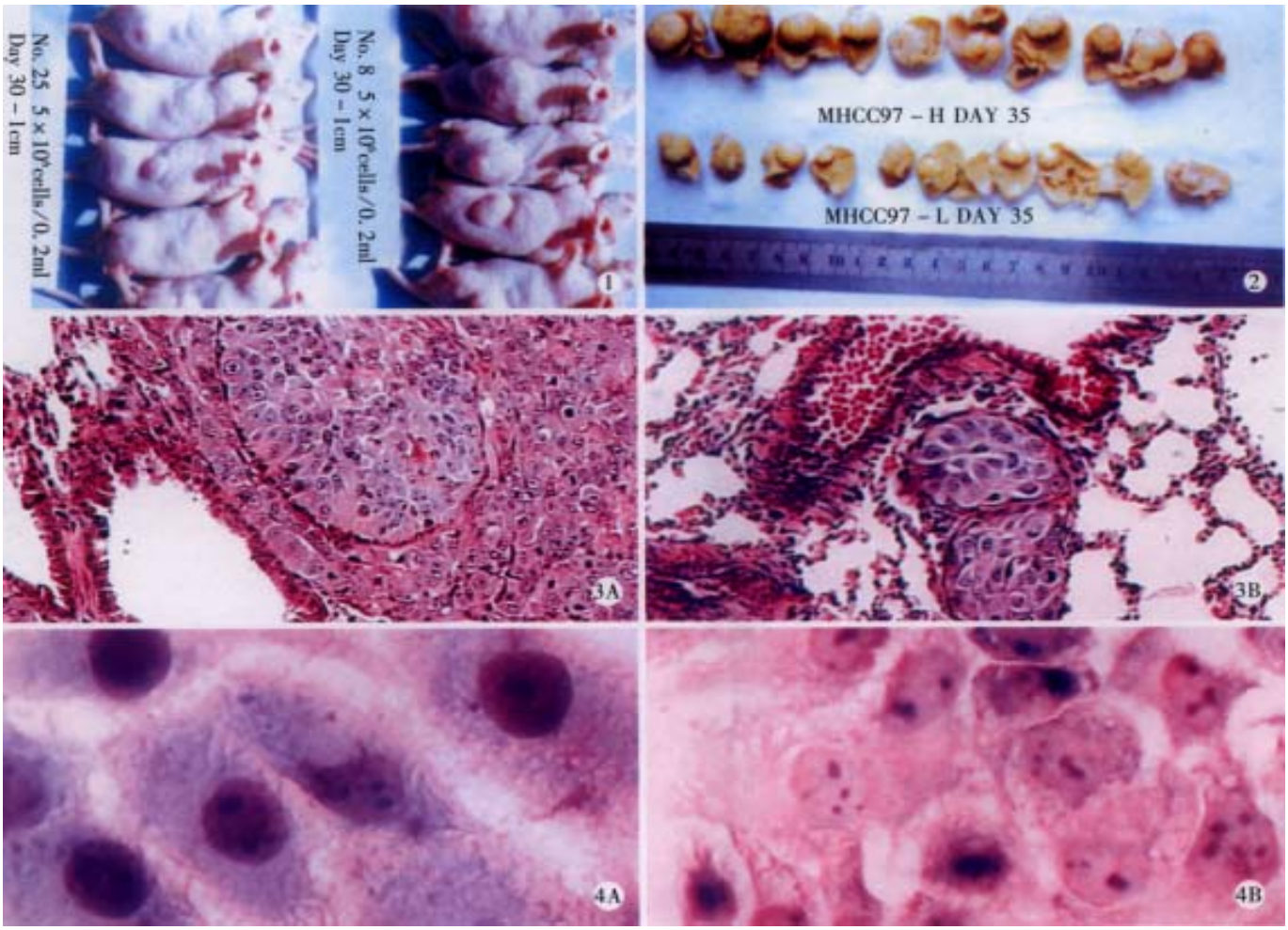

Figure 1 Subcutaneous tumor formation of the two clones. 30d after sc injection of 5×10^6 tumor cells for each nude mouse, the average s.c tumor diameter was (1.94±0.36) cm for MHCC97-H as against (0.84±0.47) cm for MHCC97-L.
Figure 2 The liver tumor size of the two clones 5wk after orthotopic inoculation. The tumor geometric mean diameter (GMD) for MHCC97 -H was (1.42±0.11) cm as against (0.90±0.26) cm for MHCC97-L.
Figure 3 Photomicroscopy of lung metastases of the two clones. MHCC97-H (3A) produced large metastatic lesion pressing the bronchioles while MHCC97-L (3B) only formed small metastasis. HE ×100.
Figure 4 Photomicroscopy of MHCC97-L (4A) and MHCC97-H (4B) illustrating the clear differences in the number of nucleoli. Giemsa×400.

Chromosome analysis

Both clones were heteroploid. The chromosome number in MHCC97-H ranged from 37 to 68, with 68% of cells in the main range of 55-58 chromosomes. The range of ch romosomes for MHCC97-L was from 44 to 105, and 58% in the main range of 57-62 c hromosomes.

Cell growth curve

As shown in Figure 5, MHCC97-H grew much faster than MHCC97-L, their populatio n doubling time being 34.2 h and 60.0 h, respectively.

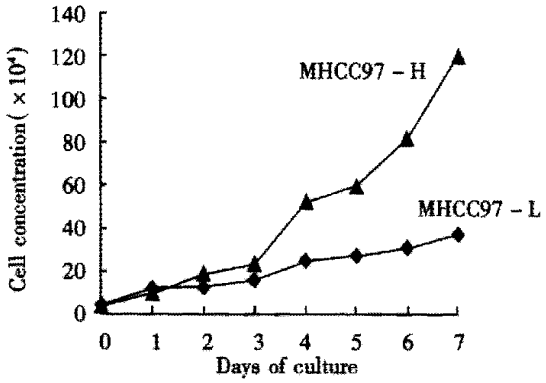

Figure 5 Cell growth curve of two clones. 4×10^4 viable cell s were cultured in each well of the 24 well culture plate. Cell numbers were determined for 7 consecutive days. Each time point represents the mean of duplicate cell counts.

Plate efficiency

Colony formation rates were (22.2±3.7)% in MHCC97-H and (18.6±4.7)% in MHCC97-L, the difference being of no statistical significance ($P>0.05$, *t* test). However, the number of cells in each colony did differ between the two cell clones, with 8-15 cells in each colony of MHCC97-H and 3-5 cells in each colony of MHCC97-L.

In vitro invasion assay

The numbers of cells that penetrated the artificial basement membrane were (37.5±11.0) cells per high power field in MHCC97-H and (17.7±6.3)/ HP field in MHCC 97-L ($P<0.05$, *t* test).

Flow cytometry

Cell cycle analysis by flow cytometry revealed that the proportion of cells in G0-G1 phase, S phase, and G2-M phase for MHCC97-H/MHCC97-L were 0.56/0.65, 0.28%/0.25 and 0.16/0.10, respectively. MHCC97-H had more cells in S phase, and G2-M phase than MHCC97-L.

Immunocytochemistry

Both clones were positive for albumin, AFP and cytokeratin 8, attesting that they were indeed liver cancer cells. However, neither of the two had any positive stain for HBsAg.

Detection of HBV DNA Integration

Both clones were positive for HBV DNA.

AFP Production

Five weeks after orthotopic implantation of the tumor from MHCC97-H and MHCC97-L, AFP levels in nude mice serum were (246±66) $\mu g\cdot L^{-1}$ and 91±66 $\mu g\cdot L^{-1}$, respectively ($P<0.01$, *t* test).

DISCUSSION

Heterogeneous nature of tumor in terms of its *in vitro* and *in vivo* characteristics has been well recognized. Populations of human and animal tumors frequently demonstrate a great variation in a number of cellular and functional properties. Our study reported here confirmed that the metastatic human HCC cell line MHCC97 was also a heterogeneous population, consisting of subpopulations with different metastatic potentials. Immunocytochemical studies demonstrated that these two clones were positive for albumin, AFP and cytokeratin 8, demonstrating they were still HCC cells. HBV DNA integration into the cell genome also confirmed that they were related to their parent cell line MHCC97, which was HBV positive[26].

Systemic comparisons of these two clones revealed many differences between them. Heterogeneity of cell morphology is the most easily observed feature among different clones of the same origin, and it is usually the beginning of the study of tumor heterogeneity. This phenomenon has been well documented in various tumors , including lung cancer[10], colon cancer[42,43], breast cancer[6,44], squamous cell carcinoma of the skin[3] and the tongue[7,11]. In our study, the two clones identified here also showed differences in cell morphology. The highly metastatic variant MHCC97-H was small (average diameter 43 μm) and more uniform than the clone with low metastatic potential MHCC97-L (average diameter 50 μm). Cell size itself may be a mechanical factor influencing metastasis. The small size may facilitate cells to traverse through the blood vessels, evade the immune attacks in the circulation during tumor cell transport, and come up with less mechanical resistance during tumor cell penetration in the target tissue. Since our study was a spontaneous metastasis model, this difference in cell size between the two clones may have some impact on the ability to overcome host barriers. This is in keeping with a earlier finding by Suzuki *et al*[45], who used a mouse fibrosarcoma system to study the experimental metastasic ability (via tumor cell injection into the tail vein to observe the lung colony formation abilities) of various clones, and found that the clone with nearly 10-fold higher lung colony-forming ability was much smaller in cell volume than the low metastatic clone.

Another prominent difference in cell morphology is that MHCC97-H had more nucleoli (3-7 per cell) than MHCC97-L (1-3 per cell). More nucleoli are a marker of active cell proliferation. Derenzini *et al*[46] suggested that nucleoli perse could tell the metatatic potential of tumor cells. Their study indicated that larger nucleoli predicted more rapid tumor cell proliferation. However, our results seem to suggest that nucleolar number rather than the size is an indicator of fast tumor proliferation. Indeed, in our model system, the highly meta static variant with more nucleoli grew much faster than the one with few nucleol i and low metastasis. This could be reflected in cell growth rate curve (tumor c ell doubling time was 34.2 h in MHCC97-H and 60.0 h in MHCC97-L), fraction of cells in S phase and G2-M phase of the cell cycle, latency period in subcutaneous tumor development and liver tumor size at the endpoint of the study. Whether or not tumor cell growth rate is directly related to metastasis is not clear yet. Yasoshima *et al*[47] using metastatic gastric cancer cell line, and Samiei *et al*[48] using metastatic mammary clones found, that metastasis was independent of tumor cell growth; while other works[49,50] showed close association between tumor cell growth rate and metastasis. Our results suggest fast growing tumor is more prone to metastasis.

Cytogenetic studies also revealed the differences in the chromosome number between the two clones. This is in

keeping with a recent findings by Takeuchi *et al*[11], who used similar method to have isolated cloned cancer cells with different metastatic potentials, and found marked difference in modal chromosome number between the highly metastatic clone and the non-metastatic clone.

As HCC is a special health issue in China, basic and clinical researches in this field have been intensive. As early as in the 1960s, Chen[51] establish ed the first human HCC cell line in the world. Later on several HCC cell lines were established[52-58]. Although most of these showed tumorigenicity when inoculated into experimental animals, rarely did they demonstrate the full potential for loco-regional and distant metastases, as seen so frequently in clinical patients. The metastatic HCC cell line MHCC97 was established in order to meet the urgent need for suitable models to study the mechanisms of and interventions on HCC metastasis. And now we took one step further to have isolated clones of different metastatic potential from the same cell line. These new models could be valuable for the study of HCC metastasis.

Cancer metastasis is the ultimate display of complex interactions between the ma lignant cells and the host defense mechanism. The process of metastasis consists of selection and sequential steps that include angiogenesis, detachment, motility, invasion of the extracellular matrix, intravasation, circulation, adhesion, extravasation into the organ parenchyma and growth[2]. The ability of cancer cells to form metastasis depends on a set of unique biological properties that enable the malignant cells to complete all those steps of metastatic cascade. For HCC invasion and metastasis, extensive studies have unveiled many molecular mechanisms involved in these processes, including P53[59,60]/CDKN$_2$ mutation, overexpression of H-ras/EGFR, nm23/TIMP[60], over-expression of CD44v6 and under-expression of nm23-H1[61], over-expression of metalloproteinase-9 and CD34[62], high level of laminin in the blood and tumor[63], intercellular adhesion molecule-1 (ICAM-1)[64], N-Actylglucosaminyltransferase V (GnTV) activity[65], high expression of urokinase-type plasminogen activator (uPA), its receptor (uPAR) and inhibitor (PAI-1)[66], chromosome 8p deletion[67,68]. But it is likely that other genes or gene locus in addition to these genes are also involved in the process of meta stasis in HCC, since most of these studies were focused only on a few genes or their products. It is reasonable to assume that there could be a group of relevant genes rather than a single or a few genes to account for tumor metastasis. The identification of those unknown genes related to metastasis is important in order to gain a complete picture of the molecular biology of HCC metastasis. For this end, a dependable model system that consists clones with high and low metastatic potential from the same origin should be the ideal study material. It is based on this rationale that some recent works seeking metastasis-related genes were conducted on the cloned cells from the same biological background. Reichner *et al*[35] isolated two clonal cell lines with different metastatic potentials from a rat hepatocellular carcinoma model induced by chemical carcinogens, and studied the differences in metastasis related mechanisms. They found no differences in the expression of several antigens noted to correlate with metastatic potential, including CD44 variant glycoprotein, p53, transferrin receptor, and E-cadherin. The only notable difference in the parameters studied was the level of IL-6. The highly metastatic clone released much more IL-6 than the low metastatic clone. Other studies use metastatic and non-metastatic human mammary carcinoma clone lines as comparing materials to seek metastasis-associated genes, and provided a vast amount of information on gene expression and metastatic phenotype[69].

In summary, our study confirmed that the metastatic HCC cell line MHCC97 is a he terogeneous population, consisting of cells with divergent biological properties. The two clones isolated from the parent cell line differed not only in *in vitro* characteristics like cell morphology and growth kinetics, but also in the most fundamental biological behavior-tumor metastasis. Since these two clones are from the same parent cell line, thus having the same genetic background, the differences in their phenotypes must have some underlying molecular mechanisms. In-depth study on their differences might help us gain new insight into the mechanisms of liver cancer metastasis.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

High frequencies of HGV and TTV infections in blood donors in Hangzhou

Jie Yan[1], Li-Li Chen[2], Yi-Hui Luo[1], Ya-Fei Mao[1] and Meng He[1]

[1]Department of Pathogenic Biology, Medical School of Zhejiang University, Hangzhou 310006, Zhejiang Province, China
[2]Department of Stomatology, The Second Affiliated Hospital, Medical School of Zhejiang University, Hangzhou 310009, Zhejiang Province, China
Correspondence to: Jie Yan, Department of Pathogenic Biology, Medical School of Zhejiang University, 353 Yan An Road, Hangzhou 310006, Zhejiang Province, China.
Telephone: +86-571-7217385, Fax: +86-571-7217044
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Abstract

AIM **To determine the frequencies of HGV and TTV infections in blood donors in Hangzhou.**

METHODS **RT-nested PCR for HGV RNA detection and semi-nested PCR for TTV DNA detection in the sera from 203 blood donors, and nucleotide sequence analysis were performed.**

RESULTS **Thirty-two (15.8%) and 30 (14.8%) of the 203 serum samples were positive for HGV RNA and TTV DNA, respectively. And 5 (2.5%) of the 203 serum samples were detectable for both HGV RNA and TTV DNA. Homology of the nucleotide sequences of HGV RT-nested PCR products and TTV semi-nested PCR products from 3 serum samples compared with the reported HGV and TTV sequences was 89.36%, 87.94%, 88.65% and 63.51%, 65.77% and 67.12%, respectively.**

CONCLUSION **The infection rates of HGV and/or TTV in blood donors are relatively high, and to establish HGV and TTV examinations to screen blood donors is needed for transfusion security. The genomic heterogeneity of TTV or HGV is present in the isolates from different areas.**

Subject headings China; DNA virus infections/epidemiology; hepatitis, virus, human/ epidemiology; blood transfusion/ adverse effect; blood donors; hepatitis agents, GB/ isolation & purification

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INTRODUCTION

Viral hepatitis is relatively common in China[1-12]. Among them hepatit is G virus (HGV) and hepatitis GB virus (GBV-C) were recently identified as the two isolates of a novel positive-stranded RNA virus belonging to *Flaviviridae* family associated with human non-A-E hepatitis[13,14]. In 1997, a negatively stranded DNA virus, named transfusion transmitted virus (TTV), was isolated from a patient suffering from non-A-G hepatitis. At present TTV was proposed to be a member of a new virus family temporarily named *Circinoviridae*[15,16]. HGV RNA could be detected in patients with non-A-E hepatitis or fulminant hepatic failure (FHF) at relatively high percentages[17-22] and the coinfection of HGV and HCV may accelerate the progression of chronic liver disease[23-25]. However, many investigation data revealed that HGV was able to establish a long-term asymptomatic viremia in non A-E hepatitis patients and only a few of the patients had biochemical evidence of liver damage[26-30]. TTV DNA was frequently found in n on-A-G hepatitis patients with a single elevation of alanine aminotransferase (ALT) and the persistence of TTV infection might be a causative factor of human FHF[31-33]. A high frequency of TTV virus infection among patients with non-B, non-C hepatocellular carcinoma was also reported by Nakagawa *et al*[34]. However, some literatures reported that clinical implication of TTV infection was insignificant because of minimal role of liver injury in non-A-G hepatitis patients[35-37]. The clinical importance of HGV and TTV infections in human hepatic diseases is still present even though the real pathogenic potentials of the two viruses have remained unanswered. Therefore, the prevalence of HGV and TTV infections in blood donors is a significant subject for investigation.

In the present study, HGV RNA and TTV DNA in the serum samples of 203 blood donors in Hangzhou, eastern China, were detected using RT-nested PCR and semi-nested PCR, respectively. The HGV RNA or TTV DNA positive amplification products from part of the serum samples were cloned and sequenced. The results of this study may help determine the frequencies of HGV and TTV infections in blood donors in the local area and provide the basis for screening blood donors to control transmission of the two viruses.

MATERIALS AND METHODS

Materials

A total of 203 serum samples of healthy blood donors were obtained from four hospitals in Hongzhou, Zhejiang Province of China. The blood donors were confirm ed to have neither clinical signals of hepatitis and nor elevation of ALT by conventional hepatic examinations, and the serum samples were negative for hepatitis A-C viruses by EIA and PCR. The reagents used in reverse transcription (RT) and PCR were purchased from Sangon and the other reagents used in this study from Sigma.

Methods

Isolation of serum RNA and DNA Total RNA in 200 μL of each serum samples was prepared by Trizol method according to the manufacturer's instruction and then dissolved in 50 μL

of DEPC treated water. Total DNA in 200 μL of each serum samples was extracted by phenol-chloroform method[38], and then dissolved in 50 μL TE buffer (pH 8.0).

RT-nested PCR for HGV RNA detection Ten microliters of total RNA preparation was mixed with 10 μL RT master mixture containing 1 μmol·L^{-1} of random hexanucleotide as primer, 2 mol·L^{-1} each of dNTP, 20U M-MuLV-reverse transcrip tase, 20U RNase inhibitor and 4 μL of 5× RT buffer (pH 8.3). The steps of RT were described as follows: at 70 ℃×5 min for denaturation, at 42 ℃×60 min for cDNA synthesis, and at 70 ℃×10 min to stop the reaction.

Primers derived from HGV 5'-NCR were used in the RT-nested PCR[39]. External primers: 5'-ATGACAGGGTTG-GTAGGTCGT AAATC-3' (sense), 5'-CCCCACTGGTC-CTTGTCAACTCGCCG-3' (antisense). Internal primers: 5'-TGGTAGCCACTATAGGTGG GTCTTAA-3' (sense), 5'-ACATTGAAGGGCGACGTGGACCGTAC-3' (antisense). For the first round PCR, 10 μL of RT product was mixed with 90 μL PCR master mixture containing 250 nmol·L^{-1} each of the primers, 2 mol·L^{-1} each of dNTP, 25 mol·L^{-1} $MgCl_2$, 2.5 U of Taq DNA polymerase and 10 μL of 10×PCR buffer (pH 9.1). For the second round PCR, 5 μL product from the first round PCR was used as template, and the other reaction reagents were the same as that in the first round PCR except for the primers. The parameters for each of the PCR rounds were: 94 ℃ 5 min (×1); 94 ℃ 1 min, 56 ℃ 1 min, 72 ℃ 1.5 min (×35) and 72 ℃ 7 min (×1). The expected size of target fragments amplified from HGV RNA was 193 bp.

Semi-nested PCR for TTV DNA detection The primers used in the semi-nested PCR for TTV DNA detection were the same des cribed by Okamoto *et al*[40]. External primers: 5'-ACAGACAGAGGAGA AGGCAACATG-3' (sense), 5'-CTGGCATTTTACCATTTCCAAAGTT-3' (antisense). Inte rnal primers: 5'-GGCAACATGTTATGATAGACTGG-3' (sense), 5'-CTGGCATTTTACCATT TCCAAAGTT-3' (antisense). Except for specific primers, $MgCl_2$ concentration (15mol·L-1), total reaction volume (50 μL) and annealing temperature (60 ℃), the compositions and concentrations of other reaction agents and the parameters for semi-nested PCR was the same as that of the RT-nested PCR for HGV detection. The expected size of target fragment s amplified from TTV DNA was 271 bp.

Examination of amplification products The results of amplificat ion reactions were observed on UV light after 20 g·L^{-1} ethidium bromide stained agarose electrophoresis, and 100 bp DNA ladder was used as a size marker to estimate the length of products.

Analysis of nucleotide sequences of amplification products The target DNA fragments from HGV or TTV amplification products by PCR were cloned into plasmid pUCm-T-vector using T-A cloning kit according to the manufa cturer's instruction. The recombinant plasmid was amplified in *E.coli* strain DH 5α and then recovered by Sambrook's method[38]. The nucleotide sequence of inserted fragment was analyzed by Sangon. Homology of the nucleotide sequences was compared with those of reported[13,40].

RESULTS

Positive rates of HGV RNA and TTV DNA in the serum samples The respective target fagments respectively amplified from HGV RNA and TTV DNA are shown in Figure 1. Thirty-two (15.8%) 30 (14.8%) and 5 (2.5%) of the 203 serum samples were positive for HGV RNA, TTV DNA and both of the two respectively (Table 1).

Table 1 Positive rates of HGV RNA and TTV DNA in the 203 blood donors

Virus	Tested (*n*)	Positive (*n*)	%
HGV	203	32	15.8
TTV	203	30	14.8
HGV and TTV	203	5	2.5

Nucleotide sequence analysis and homology comparison Homology of the nucleotide sequences of HGV RT-nested PCR products from 3 serum samples compared with the reported HGV sequence[13] was 89.36%, 87.94% and 88.65% respectively (Figure 2). Homology of the nucleotide sequences of TTV semi-nested PCR products from 3 serum samples compared with the reported TTV TA278 genotype-1a sequence[40] was 63.51%, 65.77% and 67.12% (Figure 3). The homology in comparison of these sequences mentioned above did not contain the primer sequences.

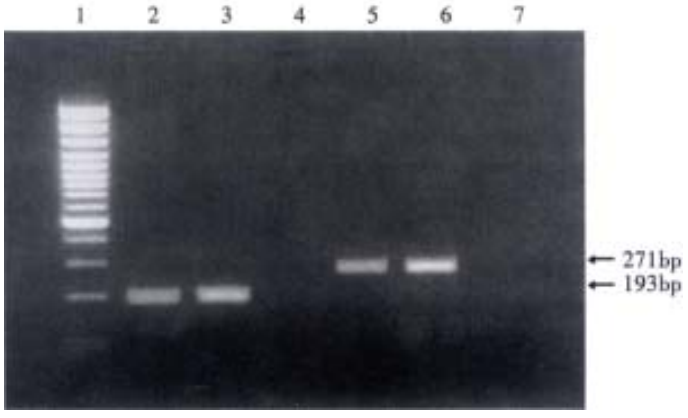

Figure 1 Target amplification fragments from HGV RNA and TTV DNA. (1: marker; 2 and 3: HGV positive serum samples; 4: blank for HGV detection; 5 and 6: TTV positive serum samples; 7: blank for TTV detection)

Figure 2 Homology of the nucleotide sequences from HGV RT-nested PCR products from 3 serum samples as compared with the reported sequence. (1. The reported HGV sequence[13]; 2-4. The sequences of HG V RT-nested PCR products from 3 serum samples. Underlined areas indicate the primer positions.)

Figure 3 Homology of the nucleotide sequences from T TV semi-nested PCR products from 3 serum samples as compared with the reported sequence.
1. The reported TTV sequence[40]; 2-4. The sequences of TTV semi-nested PCR products from 3 serum samples. Underlined areas indicate the primer positions.

DISCUSSION

Viral hepatitis is a common infectious disease in the world and causes a serious healthy problem. Although hepatitis viruses A-E have been demonstrated to be responsible for human hepatitis A-E, approximately 20% of acute and 15% of chronic hepatitis were associated with unknown aetiology[41]. HGV and TTV were recently identified as the transfusion-transmitted viruses and the causative agents of human non-A-E hepatitis[13-15]. However, many investigation data revealed that the patients infected with HGV or TTV were usually asymp tomatic and only a few of them showed mild liver injury[42-44]. Therefore, a wide variety of questions about the potential pathogenicity of HGV and TTV infection remain unanswered[31,45-47].

Since HGV and TTV are generally transmitted by transfusion, high infection frequencies of the two viruses in blood recipients and in hemodialyzed patients have been demonstrated[31,43,48-50]. Many investigations demonstrated that HGV RNA was detectable in 1.3%-10.6% of blood donors in different areas abroad[51-55]. Blood donors were also frequently infected with TTV but the infection rates abroad were usually lower than 5%[56,57]. In China, HGV and TTV infection rates in blood donors were reported to be approximately 8% and 15%, respectively[46,58]. In the present study, HGV infection rat e in the 203 blood donors was as high as 15.8%, which seems obviously higher than the reported HGV infection rates in the blood donors from other areas of China and abroad. Such high HGV infection rate in the blood donors in Hangzhou is probably due to the geographic difference. In this study, TTV viremia was found in 14.8% of the same 203 blood donors, which is higher than that of the reported abroad but similar to the reported in Chinese blood donors from other areas. In addition, 5 (2.5%) serum samples from the 203 blood donors were positive for both HGV RNA and TTV DNA, indicating the existence of coinfection of the two viruses. However, we can not exactly evaluate the significance of the co-infection because of being unable to get the detailed information of the five co-infection blood donors.

None of the blood donors tested in this study showed clinical symptoms and laboratory markers for hepatitis, which suggested that most of blood donors infected with HGV and/or TTV are usually latent. These asymptomatic blood donors carrying HGV and/or TTV may be more risky and important for the source of infection. Since HGV and TTV at least cause mild hepatitis in human[15,41] and high frequency of HGV and/or TTV infections in blood donors, it is necessary to establish HGV and TTV examination items to screenblood donors for transfusion security.

The nucleotide sequences of HGV RT-nested PCR products from 3 serum samples¡¡are highly homologous (87.94%-89.36%) to the HGV sequence reported by Linnen *et al*[13]. This result of sequence analysis indicates that the RT-nested PCR used in this study is reliable for HGV RNA detection. To analyze the details of the nucleotide sequencing data, it seems to show an HGV genotype different from the literature[59-62]. Lower homology (63.51%-67.12%) of the nucleotide sequences of TTV semi-nested PCR products from 3 serum samples compared with the reported TTV sequence[40] reveals the genomic heterogeneity of TTV in the isolates from different areas, and this founding accords with the conclusions of previously published reports[37,40].

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Prokaryotical expression of structural and non-structural proteins of hepatitis G virus

Ning-Shao Xia[1], Hai-Jie Yang[1], Jun Zhang[1], Chang-Qing Lin[1], Ying-Bin Wang[1], Juan Wang[1], Mei-Yun Zhan[2] and MH Ng[3]

[1]Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province, China
[2]Institute of Virology, Chinese Academy of Preventive Medicine Beijing 100052, China
[3]Department of Microbiology, Hong Kong University, Hongkong, China
Supported by National 863 Project, No. 102-07-02-07, and the 9th Five-Year Sci-Tech Plan, No. 96-906A-03-08
Correspondence to: Prof. Ning-Shao Xia, Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province,
Telephone: +86-592-2184110, Fax: +86-592-2184110
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Abstract

AIM **To study the epitope distribution of hepatitis G virus (HGV) and to seek for the potential recombinant antigens for the development of HGV diagnositic reagents.**

METHODS **Fourteen clones encompassing HGV gene fragments from core to NS3 and NS5 were constructed using prokaryotic expression vector pRSET and (or) pGEX, and expressed in E.coli. Western blotting and ELISA were used to detect the immunoreactivity of these recombinant proteins.**

RESULTS **One clone with HGV fragment from core to E1 (G1), one from E2 (G31), three from NS3 (G6, G61, G7), one from NS5B (G821) and one chimeric fragment from NS3 and NS5B (G61-821) could be expressed well and showed obvious immunoreactivity by Western blotting. One clone with HGV framment from NS5B (G82) was also well expressed, but could not show immunoreactivity by Western blotting. No obvious expression was found in the other six clones. All the expressed recombinant proteins were in inclusion body form, except the protein G61 which could be expressed in soluble form. Further purified recombinant proteins G1, G31, G61, G821 and G61-821 were detected in indirected ELISA as coating antigen respectively. Only recombinant G1 could still show immunoreactivity, and the other four recombinant proteins failed to react to the HGV antibody positive sera. Western blotting results indicated that the immunoactivity of these four recombinant proteins were lost during purification.**

CONCLUSION **Core to E1, E2, NS3 and NS5 fragment of HGV contain antigenic epitopes, which could be produced in prokaryotically expressed recombinant proteins. A high-yield recombinant protein (G1) located in HGV core to E1 could remain its epitope after purification, which showed the potenti al that G1 could be used as a coating antigen to develop an ELISA kit for HGV specific antibody diagnosis.**

Subject headings hepatitis agents; GB/genetics; genes, viral; viral proteins/biosynthesis

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INTRODUCTION

Hepatitis G virus (HGV), also known as GBV-C, is a novel human virus, which can cause acute and persistent infection in humans[1-4]. The clinical significance of HGV infection is still controversial[5-10]. Some studies have reported the seroprevalence of HGV RNA in general population as well as voluntary blood donors between 0.3% and 6%, but the frequency is always significantly higher in high risk groups such as intravenous drug users (IVDU), patients with acute and chronic hepatitis B and C, patients with blood transfusion and hemodialysis, and patients with cryptogenic hepatitis[11-22]. HGV is a member of flaviviridae[3,4,19]. The genome organization of HGV resembles that of hepatitis C virus (HCV). Its positive- stranded RNA genome is about 9.4 kb in length that contains a single open reading frame (ORF), which encodes a polyprotein of about 2900 amino acids. The polyprotein is cleaved by viral and host proteases into structural proteins (Core, E1 and E2) and non-structural proteins (NS2, NS3, NS4, NS5a and NS5b)[1,2,23,24].

Up to now, RT-PCR is the most commonly used method for the diagnosis of HGV infection. It is necessary to develop a convenient antibody detection assay. In this study, we had serial fragments selected from core to NS3 and NS5 region of HGV Chinese strain expressed in *E.coli*, and detected their immunoreactivity by Western blotting and ELISA.

MATERIALS AND METHODS

Bacterial strains and plasmids

E.coli strain DH5α and BL21(DE3) were stored in our laboratory. Serial expression vectors pRSET and pGEX were purchased respectively from Invitrogen Co. and Pharmacia Co.. Clones include gene fragments of Chinese HGV strain (HGVch, Genbank Accession Number U94695) constructed in our laboratory before[25].

Enzymes and other biochemical reagents

Restriction endoenzymes, T4 DNA ligase and DNA polymerase Taq were purchased from Promega. Sepharose 4B GST matrix, His Trap™ and isopropyl- β-D-thiogalactopyranoside (IPTG) were purchased from Pharmacia. Goat anti-human IgG-alkaline phosphatase conjugate and substrates BCIP, NBT were purchased from Boehinger Mannheim.

Serum samples

Three HGV positive serum samples were kindly provided by Dr. Qiu (Beijing Wantai Biological Medicine Co.). Two of them

were HGV RNA positive by RT-PCR, the other one was positive in the synthetic peptides based HGV ELISA kits and with high titer anti-HGV antibodies. These three serum samples mixed in equal ratio and were used in Western blotting.

Construction of recombinant plasmids

By the regular molecule biological METHODS, pRSET and pGEX were digested with single endoenzyme or two endoenzymes, gene fragments of HGV in pGEM T-Easy were digested with the same restriction enzyme (s), and they were ligated by T4 DNA ligase, and the expression clones of HGV gene fragments were constructed.

Expression of HGV gene fragments in E.coli

Two mL fresh overnight cultured BL21(DE3) carrying HGV gene fragment expression plasmids were diluted with 200 mL fresh LB medium in the presence of 100 mg·L^{-1} ampicilin and grew to A_{600} = 0.8 at 37 ℃ at a shaki ng speed of 210 r·min^{-1}. The culture was induced by adding IPTG to a final concentration of 0.2 mmol·L^{-1} at 37 ℃ for 3 h. One mL culture was harvested by centrifugation, cell pellets were resuspended in 400 μL SDS/PAGE loading buffer, and aliquots were run on 120 g·L^{-1} SDS-PAGE gels. In order to obtain soluble recombinant proteins, induction expression was carried out at 20 ℃ for 3 h.

Western blot analysis of recombinant proteins

Total cell lysates were run on SDS-PAGE gels and transferred electrophoretically to nitrocellulose membrane for 1 h under the voltage of 100 V. The membrane was then incubated in blocking solution (50 g·L^{-1} nonfat milk in Tris-buffered saline, TBS) for 1 hour at room temperature followed by incubation at room temperature for 2 h in the sera that prediluted to 1:200 with blocking solution. The membrane was washed three times with TTBS (0.5 g·L^{-1} Tween-20 in TBS) for 10 min, and alkaline phosphatase-labeled goat anti-human IgG antibodies diluted in TTBS (1:2000) were exposed to the membrane at room temperature for 1h. The membrane was visualized with a substrate solution of BCIP and NBT after another washing for 3 times for 10 minutes with TTBS.

Purification of recombinant proteins of HGV

For soluble recombinant proteins, the harvested bacterial pellets were resuspend ed in PBS, after ultrasonification and high speed centrifugation, supernatants were collected and used for purification. Purification of expressed proteins in pRSET was carried out according to the manual of His Trap™ (Pharmacia Co.), and that in pGEX was done following the manual of GST purification modules (Pharmacia Co.).

For insoluble recombinant proteins, the harvested bacterial pellets were resuspended in PBS, and collected after ultrasonification and high speed centrifugation, and then resuspended in washing buffer (50 mmol·L^{-1} Tris-Cl pH 7.2, 5 mmol·L^{-1} EDTA, 150 mmol·L^{-1} NaCl, 5 g·L^{-1} Triton-x 100). Following washing with 2 mol·L^{-1} and 4 mol·L^{-1} urea, the recombinant proteins in pellets were dissolved in 8 mol·L^{-1} urea, which were run on SDS-PAGE gels and stained with 0.3 mol·L^{-1} $CuCl_2$ solution for 5 minutes. The bands of the recombinant proteins could be seen clearly (background was light blue, and the bands of proteins were blank fielded), they were cut carefully and sealed into dialyzer in elution buffer (250 mmol·L^{-1} EDTA, 250 mmol ·L-1 Tris-Cl pH 9.5) to elute target proteins with electrophoresis.

Enzyme linked immunoadsorbent assay (ELISA)

Purified recombinant antigens were coated to microplate in a amount of 100 ng each well in 0.05 mol·L^{-1} CB (pH 9.6) buffer for 2 h at 37 ℃ and overnight at 4 ℃. Plates were washed with PBS containing 0.5 g·L^{-1} Tween 20 and blocked with blocking buffer (0.5 g·L^{-1} Tween 20 and 10 g·L^{-1} bovine serum albumin in PBS)for 2 hours at 37 ℃. Sera (1:1000) were applied for 30 min at 37 ℃. A peroxidase-conjugated goat anti-human IgG used as secondary antibody was incubated for 30 min at 37 ℃ and visualized with o-phenyl-diamine-2HCl (10 g·L^{-1} in 5 mmol·L^{-1} Tris-HCl, pH 7.0). Wells were washed five times with PBST (0.5 g·L^{-1} Tween 20 in PBS) between each step. The reaction was stopped with 50 μL of 2 mol·L^{-1} H_2SO_4. Absorption was measured at A_{495}.

RESULTS

Construction and identification of recombinant plasmids

The recombinant plasmids were digested with proper restriction endoenzymes. Agarose gel electrophoresis showed that all HGV gene fragments were cloned into the vectors with correct orientation and size. The recombinant protein expressed by pGEX vector had a GST fusion protein in N-terminal, while that by pRSET vector had a hexahistidine in N-terminal. The locations of corresponding fragments in HGV genome of these plasmids are listed in Table 1.

Table 1 Amino acid location of constructed expression vector in HGV CH strain, yields in E.coli and immunoreactivity in Western blotting

Clone	Vector	Target fragments	Amino acid location in ORF	Molecular mass of recombinant protein (ku)	Yield in *E.coli*	Immuno-reactivity
G1	pGEX	Core to E1	1- 144	42	High	+
G2	pRSET	E1 to E2	101- 284	23	No	
G3	pGEX	E2	247- 578	62	No	
G31	pGEX	E2	491- 578	36	High	+
G4	pRSET	E2 to NS2	542- 876	40	No	
G5	pRSET	NS2 to NS3	854-1078	28	No	
G6	pGEX	NS3	1073-1345	56	High	+
G61	pRSET	NS3	1160-1345	24	High	+
G7	pGEX	NS3	1267-1427	44	High	+
G8	pRSET	NS5	2151-2524	45	No	
G81	pRSET	NS5	2151-2412	32	No	
G82	pGEX	NS5	2408-2524	40	High	-
G821	pRSET	NS5	2357-2524	23	High	+
G61-821	pRSET	NS3+NS5	1160-1345+2357-2524	44	High	+

Expression of recombinant proteins in E.coli

Fourteen clones were constructed, covering the core, E1, E2, NS2, NS3 and NS5 region of HGV (Figure 1). The virus fragment in G1 covered a region from the beginning of the core to the aa144, which was located in the middle of E1. The yield of G1 was about 20% in total bacterial proteins (Figure 2, lane B). No visible expression was found in G2 (covering a region from E1 to E2) and G3 (including almost entire E2). But when the C-terminal 88 residues of G3, named G31, were expressed, a yield of about 30% was obtained (Figure 2, lane C). Both of the clones included NS2 fragment, G4 and G5, and could not produce obvious recombinant proteins. G6 and G7, both located in NS3, were expressed well (Figure 2, lane D and G). To obtain a soluble form of NS3 antigen, a fragment from aa1160 to aa1345 was subcloned from G6 to vector pRSET, the result ed clone G61 was expressed much better than G6 and G7, and the soluble form recombinant protein could be found in the supernatant after centrifugating the ultrasonificated bacteria (Figure 2, lane E and F). The NS5 fragment G8 was not expressed, so did the N-terminal two-three (G81) when G8 was spliced into two parts and subcloned, but the C-terminal one-three (G82) was expressed quite well (Figure 2, lane H). Better expression was found in the yield of G821, resulting from the 78 residues extended from G82 to N-terminal, (Figure 2, lane I), so did the chimerical clone, G61-821 (Figure 2, lane J).

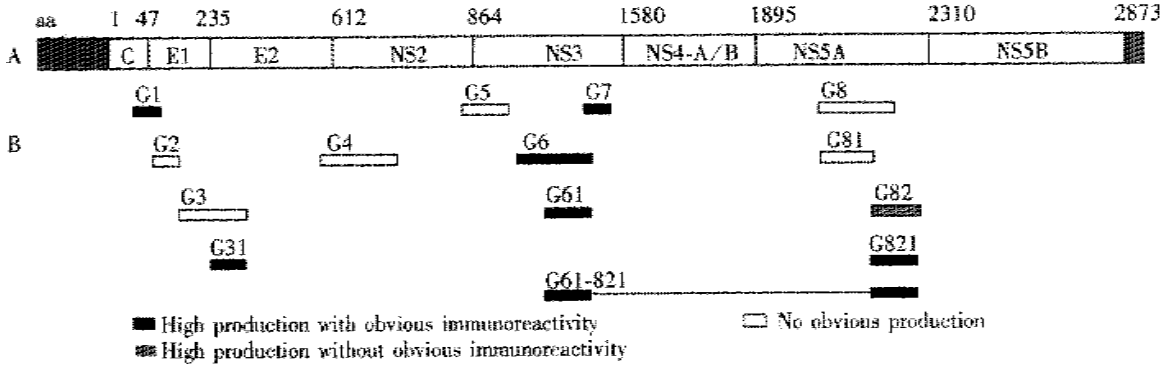

Figure 1 Schematic representation of HGV genome and ex pressed proteins. A: Putative genomic organization of HGV CH strain. B: Fragments expressed from the HGV CH strain polyprotein.

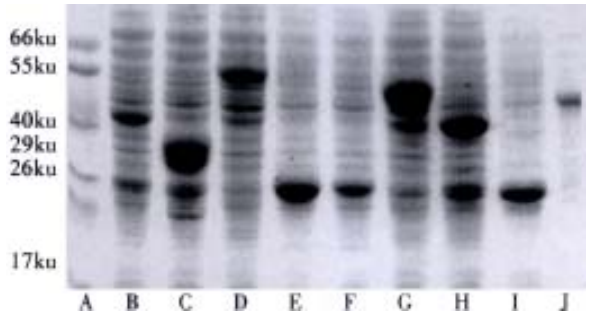

Figure 2 Expression of the recombinant HGV proteins in *E.coli* analyzed by SDS-PAGE.A. Molecular mass standard; B.G1; C. G31; D.G6; E.G61; F.G61 in soluble form; G.G7; H.G82; I.G821; J. G61-821

Western blotting analysis

Eight well-expressed recombinant proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose for immumoblotting. As shown in Figure 3, except for fragment G82, all the other seven proteins showed immunoreactivity. G1 and G82 had strong reactivity with HGV positive sera, and reactivity of G31 was relatively weaker.

Purification and ELISA assay of recombinant proteins of HGV

Four immunoreactive antigens, G1, G31, G61 and G821, which were located in the core, E2, NS3 and NS5 respectively, and a chimerical antigen G61-821 was selected for further purification. After electrophoretical elution, the purity of these five proteins was higher than 90% (Figure 4). These antigens were coated respectively to microplate in a amount of 100ng each well. Three HGV antibody positive sera and five negative sera were used in ELISA. The results showed that only G1 could detected all positive sera effectively (Figure 5).

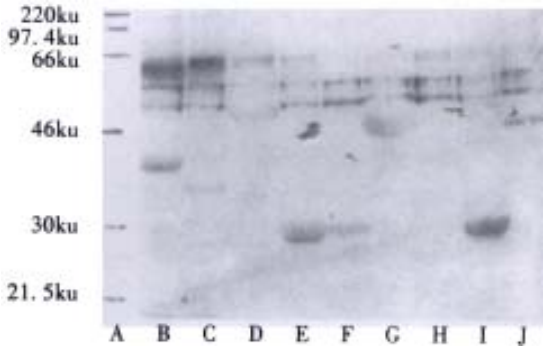

Figure 3 Western blot analysis of expressed recombinan t HGV proteins. A. Molecular mass standard; B.G1; C.G31; D.G6; E.G61 F. G61 in soluble form; G.G7; H.G82; I.G821; J.G61-821

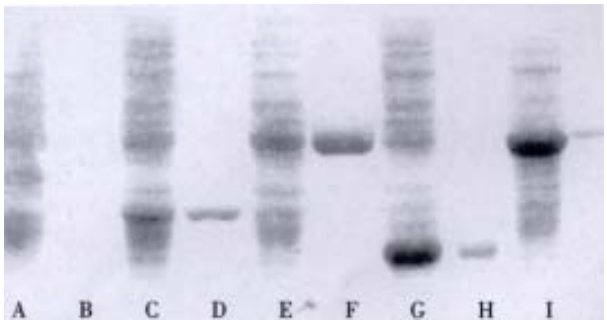

Figure 4 SDS-PAGE Analysis of purified recombinant HGV proteins. Lane designations refer to purified HGV proteins or corresponding

E.coli lysates: A.G1; B.purified G1; C.G31; D.purified G31; E.G61; F.purified G61; G.G821; H.purified G821; I.G61:821; J.purified G61-821

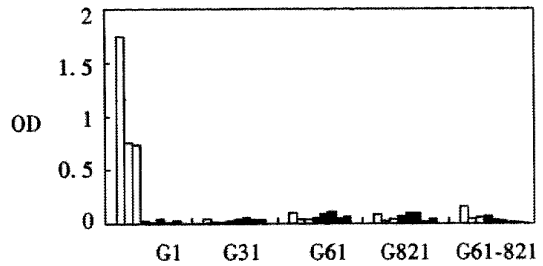

Figure 5 ELISA results of purified recombinant antigens. □ Positive sera ■ Negative sera

DISCUSSION

We have obtained full-length sequence of a Chinese HGV strain (HGVch) through overlapping RT-PCR[25]. Seven overlapping clones covering from the beginning of core to the end of NS3, were named G1 to G7, and one clone (G8) was located in NS5. They were subcloned to prokaryotic expression vectors pRSET or pGEX and expressed in E.coli in this study. The SDS-PAGE results showed that only fragments within C-E1 (G1) and NS3 (G6 and G7) could be expressed efficiently, but the clones located in E2 (G2 and G3), NS2 (G4 and G5) and NS5 (G8) could not. To improve the yield, one subclone (G31) for E2 and two subclone s (G81 and G82) for NS5 were constructed. The G31 was located in the C-part of G3, the G81 located in the N-terminal two-three of G8, and the G82 in the C-terminal one-three of G8. The results showed that G31 and G82 expressed well, but not G81.

In order to detect the immunoreactivity of different proteins at the same time, it is important to choose serum containing multiclonal antibodies against different epitopes. Due to the lack of reliable METHODS to detect anti-HGV, in this study, we chose a mixed serum as first antibodies in the immunoblotting assay. The Western blotting showed that G1, G6 and G7 had strong immunoreactivit y, the immunoreactivity of G3 could be identified too, but no reactivity could be found with G82. Then we constructed another clone G821, which resulted from the 78 residues, and extended to N-terminal from G82, its immunoreactivity in Western blotting was quite strong, indicating that this 78 residues played an important role in the epitopes of NS5. Both G6 and G7 showed a strong immunoreactivity, suggesting that the epitopic might cluster in NS3 region. We had a truncated fragment of G6 named G61 expressed as an endeavor to obtain the soluble NS3 antigen, because a soluble antigen usually displays more natural epitopic conformation. But we could not see a stronger reactivity in the soluble form of G61 than the insoluble form of G61 in Western blotting. Chimerical gene G61-821 was expressed as an endeavor for making a better diagnostic antigen, and it showed a strong immunoreactivity in Western blotting.

Among the four immunoreactive antigens, G1, G31, G61 and G821, which were locate d in the core, E2, NS3 and NS5 respectively, the chimerical antigen G61-821 was selected for further purification and coating respectively to microplate to make an ELISA kit. The results showed that only G1 could detect HGV positive sera effectively, the other four recombinant proteins could react with none of the three tested HGV positive sera, which may suggest that the immunodominant epitopes in these four antigens were conformationally depended, and were destructed during our purifying procedure, as sustained by the Western blotting results for these five purified proteins, which showed only the reactivity of G1.

Epidemiological studies of HGV have been hampered by the lack of convenience serologic assays. The virus has not been successfully grown in culture, thus no native antigen is available for serologic detections. Many authors had reported the expression of HGV proteins in prokaryotic systems or eukaryotic systems[26-28]. The recombinant proteins of the core, NS3, NS4 and NS5 gene of HGV expressed in E.coli showed immunoreactivity in Western blot assays, but none of them could be used in ELISA. It may be due to the loss of antigenicity during the purification procedure as shown in this study. Dille *et al*[29] successfully established an ELISA for anti-HGV E2 using CHO expressed E2 antigen, however, the presence of anti-E2 and HGV RNA was almost mutually exclusive: few were positive for both markers at the same time, and the utility of this ELISA in epidemiological studies was very limited[30,31]. Two HGV RNA positive sera were positive in ELISA based on the core-E1 antigen G1 obtained in this study. Although the serum samples in this study are very limited, they suggested the potential utility of this ELISA in epidemiological studies.

Although the prevalence of HGV infection is higher than that of HCV infection in the general population, there is absence of an obvious relationship between elevated level of alanine aminotransferase (ALT) and presence of HGV infection. Besides, whether the liver is the replication site of HGV has remained unclear. Saito *et al*[32] had detected both positive and negative stranded HGV RNA by HGV RT-PCR in the liver tissues of all the six tested HGV infected patients, which indicated that liver might be the primary site of HGV replicatio n, but many other scientists could not repeat these findings in their HGV infected patients by the same METHODS[33-36]. Recently, Reshetnyak et al reported that patients with HGV monoinfection had demonstrated the increase of the DNA single-stranded breaks peripheral blood lymphocytes (PBL) quantity[37]. Whether HGV is pathogenic to the liver or not, we should remain open to the possibility that its major pathological consequences, if any, may lie outside of the liver. A convenient serologic assay is undoubtedly crucial for the clarification of these unclear points.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Effects of hypoxia, hyperoxia on the regulation of expression and activity of matrix metalloproteinase-2 in hepatic stellate cells

Ping-Sheng Chen[1,2], Wei-Rong Zhai1, Xiao-Mei Zhou[3], Jin-Sheng Zhang[1], Yue-E Zhang[1], Yu-Qin Ling[1] and Ying-Hong Gu[1]

[1]Department of Pathology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China
[2]Ping-Sheng Chen now works in the Department of Pathology, School of Basic Medical Sciences the (former Nanjing Railway Medical College), Southeast University, Nanjing 210009, China
[3]Institute of Cancer Research, Shanghai 200032, China
Supported by the Scientific Research Fund for Doctorate Education, State Educational Commission, No.9837
Correspondence to: Dr. Wei-Rong Zhai, Department of Pathology, School of Basic Medical Sciences, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China.wrzhai@online.sh.cn
Telephone: +86-21-64041900 Ext.2536(O)
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Abstract

AIM **To study the effects of hypoxia, hyperoxia on the regulation of expression and activity of matrix metalloproteinase-2 (MMP-2) in hepatic stellate cells (HSC).**

METHODS **The expressions of MMP-2, tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and membrane type matrix metalloproteinase-1 (MT1-MMP) in cultured rat HSC were detected by immunocytochemistry (ICC) and *in situ* hybridization (ISH). The contents of MMP-2 and TIMP-2 in culture supernatant were detected with ELISA and the activity of MMP-2 in supernatant was revealed by zymography.**

RESULTS **In the situation of hypoxia for 12 h, the expression of MMP-2 protein was enhanced (hypoxia group positive indexes: 5.7±2.0, n = 10; control: 3.2±1.0, n = 7; P<0.05), while TIMP-2 protein was decreased in HSC (hypoxia group positive indexes: 2.5±0.7, n = 10; control: 3.6±1.0, n = 7; P<0.05), and the activity (total A) of MMP-2 in supernatant declined obviously (hypoxia group: 7.334±1.922, n = 9; control: 17.277±7.424, n = 11; P<0.01). Compared the varied duration of hypoxia, the changes of expressions including mRNA and protein level as well as activity of MMP-2 were most notable in 6 h group. The highest value (A hypoxia-Acontrol) of the protein and the most intense signal of mRNA were in the period of hypoxia for 6 h, along with the lowest activity of MMP-2. In the situation of hyperoxia for 12 h, the contents (A_{450}) of MMP-2 and TIMP-2 in supernatant were both higher than those in the control, especially the TIMP-2 (hyperoxia group: 0.0499±0.0144, n = 16; control: 0.0219±0.0098, n = 14; P<0.01), and so was the activity of MMP-2 (hyperoxia group: 5.252±0.771, n = 14; control: 4.304±1.083, n = 12; P<0.05), and the expression of MT1-MMP was increased.**

CONCLUSION **HSC is sensitive to the oxygen, hypoxia enhances the expression of MMP-2 and the effect is more marked at the early stage; hyperoxia mainly raises the activity of MMP-2.**

Subject headings liver/pathology; liver/metabolism; metalloproteinases/biosynthesis; metalloproteinases/metabolism; anoxia/metabolism; oxygen/pharmacology

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INTRODUCTION

It is well known that the key event in the hepatic fibrogenesis is the activation of hepatic stellate cells (HSC) due to the altered circumstances and the activated cells are the main source of MMP-2 which may promote the activation of HSC owing to degradation of the basement membrane matrix rich in collagen type IV around the cells[1-23]. We also know that liver fibrosis may be induced or worsened by hypoxia and reperfusion[24-27]. However, it has not been reported that the effects of oxygen on the expression and the activity regulation of MMP-2 in HSC. In this paper, the regulation of the expression and the activity of MMP-2 in rat HSC was investigated *in vitro* under the conditions of hypoxia or hyperoxia.

MATERIALS AND METHODS

Isolation and culture of HSC

HSC were isolated from adult Sprague Dawley rats weighing 380 g to 420 g (bought from the Experimental Animals Center of Shanghai Medical University, China) according to the method of Di Sario *et al*[28,29]. The cells ($10^5 \cdot mL^{-1}$) were inoculated in culture flasks and dishes with cover-glasses, and then cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. The medium (DMEM, Sigma Co.) was changed 24 h later and thereafter every 2 d to 3 d. After 7 d culturing, the medium was replaced with serum-free medium (DMEM/F12, V/V = 1:1). Meanwhile, some of the dishes were cultured under the condition of hypoxia or hyperoxia, as previously described[30]. Briefly, the dishes were put in a sealed container with two holes (for the gas in and out), through which 100% N_2 or O_2 (Shanghai Biouxi Gas Co. Ltd, China) was inflated for 30 min, and then with the holes shut the dishes were incubated in hypoxia or hyperoxia continually for 12 h. The culture supernatant was collected and centrifuged, and preserved at -20 ℃. The cells on the cover-glasses were

rinsed in phosphate-buffered saline (PBS) for three times, fixed in 40 g·L^{-1} paraformaldehyde/PBS, and preserved in 700 mL·L^{-1} ethanol at 4 ℃[31]. In another experiment for observing the differences among the varied durations of hypoxia, the dishes were cultured with hypoxia for 6 h, 12 h and 24 h, 12 dishes for each group, along with three dishes as parallel controls for each group.

ELISA

Sandwich technique was used to detect the relative contents of MMP-2, TIMP-2 in the culture supernatant with polyclonal antibody against human MMP-2 (present of Dr. Stetler-Stevenson; 1:20 00), polyclonal antibody against human TIMP-2 (1:800), goat anti rabbit IgG-HRP (Huamei Co. Shanghai, China. 1:1 000) and the colorific tetramethyl benzidine (TMB) (Huamei Co. Shanghai, China). Fresh serum-free medium served as negative control. The O.D.values(A_{450} values) measured with the Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA) at 450 nm represented the relative contents of the protein.

Detection of the MMP-2 activity with zymography[10]

The activity of MMP-2 was detected by gelatin zymography using 80 g·L^{-1} polyacrylamide gels co-polymerized with 1 g·L^{-1} gelatin which served as the substrate of MMP-2. Culture supernatant (15 μL) was mixed with 2×sample buffer (1:1) and electrophor ised (80V-150V) for 4 h-5 h. Subsequently, SDS was extracted with Triton X-100 from the gels, which were then incubated for 48 h at 37 ℃ in 50 mmol·L^{-1} Tris/HCl, pH 7.4, containing 5 mmol·L^{-1}$CaCl_2$ and 5 mmol·L^{-1} $ZnCl_2$. Gels were stained in 300 mL·L^{-1} methanol/100 mL·L^{-1} acetic acid containing 5 g·L^{-1} Coomassie brilliant blue G250 and decolorized. The clear band against a blue background representing the activity of MMP-2 was measured by using Gel Image System (Image master 1D analysis software, Pharmacia)and recorded with the total *A* (area of clear band times mean *A*).

Immunocytochemistry (ICC)

Labeled streptavidin biotin method with HRP/DAB (Dako Co.) was used in ICC for detecting the expression of MMP-2, TIMP-2, MT1-MMP and desmin in the cells on the cover-glasses. The specific antibodies were: monoclonal antibody against the human MMP-2 (CalBiochem; 1:100), polyclonal antibody against human TIMP -2 (present of Dr. Stetler-Stevenson; 1:300), monoclonal antibody against the human MT1-MMP (CalBiochem; 1:10), polyclonal antibody against chicken desmin (made by the Department of Pathology, Shanghai Medical University; 1:200). According to the stain intensity, the positive result was recorded as the value of 1 (mild), 2 (middle), 3 (intense), and the positive indexes were given by the average values of the positive cells in 9 HP (×400) fields per slide.

In situ hybridization (ISH)

Recombinant plasmids of human MMP-2, TIMP-2 cDNA were gifts from professor Marmer (Medical Center of Washington University, USA). And that of MT1-MMP was presented by Dr. Xiao. Expansion, extraction and purification of the recombinant plasmids were performed as routine. Three cRNA probes were transcripted in vitro according to the protocol of the kit (Boehringer Mannheim Co., Germany). ISH was performed as previuosly described[32,33] with immuno histochemical detection using an alkaline phosphatase (AKP)-conjugated anti-digoxigenin monoclonal antibody (Boehringer Mannheim Co., Germany). Hybridization signal was visualized through the substrate of AKP (NBT and BCIP).

Statistical methods

t or *t'*test was used for statistical analysis.

RESULTS

Morphology and growing state of HSC

The HSC isolated freshly appeared round in shape and rich in cytoplasmic lipid droplets. The cells stretched the cytoplasmic processes and numerous lipid droplets were still seen around nuclei on the third day after seeding. Thereafter the cells became stellate with several long processes, grew in clumps, and took about 7 d to 10 d to cover all over the flasks or dishes. More than 95% of the cells showed expression of desmin. It was noticed that the morp hology and the number of HSC did not change obviously after treated with hypoxia or hyperoxia, as compared with control.

Relative content of MMP-2, TIMP-2 proteins in supernatant

Relative contents of MMP-2 protein in each of three hypoxia periods were higher than those in the control. The highest increase in the value ($A_{hypoxia}$- $A_{control}$) of the protein was in the period of hypoxia for 6 h, and the lowest was in 12 h. The relative content of TIMP-2 was higher in 12 h, while it lowered in both 6 h and 24 h, compared with the control; but there were no significant differences among them (Table 1). The contents of both MMP-2 and TIMP-2 in supernatants of the hyperoxia group (12 h) were higher than those of the control, especially the TIMP-2 (P<0.01, Table 2).

Activity of MMP-2 in supernatant

Activity of MMP-2 in hypoxia group (7.334±1.922, n = 9) was lower than that in control (17.277±7.424, n = 11; P<0.01, Figure 1). Compared the varied periods of hypoxia, the activity came down obviously in 6 h and 24 h, but slightly in 12 h (Table 1). On the contrary, the activity of MMP-2 in supernatant rose remarkably as the cells were cultured in hyperoxia for 12 h (P<0.05,Figure 1, Table 2).

Table 1 Increased values of MMP-2, TIMP-2 proteins and activity of MMP-2 in supernatants in three hypoxia periods

t / h	MMP-2		TIMP-2		MMP-2 activity	
	n	$\bar{x}\pm s$	*n*	$\bar{x}\pm s$	*n*	$\bar{x}\pm s$
6	12	0.0264±0.0168[b]	12	-0.0042±0.0100	9	-1.110±2.612
12	12	0.0026±0.0111	12	0.0023±0.0181	11	-0.392±1.543
24	10	0.0100±0.0136[a]	10	-0.0012±0.0140	10	-1.323±2.194

[a]P<0.05 *vs* 6 h; [b]P<0.01 *vs* 12 h.

Table 2 Changes of MMP-2, TIMP-2 contents and activity in supernatant in hyperoxia group

Group	MMP-2		TIMP-2		MMP-2 activity	
	n	$\bar{x}\pm s$	*n*	$\bar{x}\pm s$	*n*	$\bar{x}\pm s$
Hyperoxia	16	0.1958±0.0448	16	0.0499±0.0144[b]	14	5.252±0.771[a]
Control	15	0.1729±0.0409	14	0.0219±0.0098	12	4.304±1.083

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

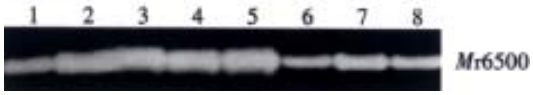

Figure 1 Zymographic analyses.
Lane1: hypoxia group; Lane 2: control; Lane 3-5: hyperoxia group; Lane 6-8: control.

Expression of MMP-2, TIMP-2, MT1-MMP proteins In cell situ

The positive stain of MMP-2, TIMP-2 and MT1-MMP appeared brown and yellow and located in cytoplasm. The expression of MMP-2 in hypoxia was more intense (positive indexes: 5.7±2.0, n = 10) than that in control (3.2±1.0, n = 7, P<0.05, Figures 2,3). The expression of TIMP-2 in hypoxia(positive indexes: 2.5±0.7, n = 10) was weaker (control, 3.6±1.0, n = 7, P<0.05, Figures 4,5). No significant statistical difference was found between hyperoxia and control for the expression of MMP-2. The expression of MT1-MMP was slightly more intense in hyperoxia than that in control (Figures 6,7).

Expression of MMP-2, TIMP-2 and MT1-MMP mRNA

The hybridization signal of MMP-2 in HSC was found in each of three periods of hypoxia. It was most intense in the 6 h group, milder in the 24 h group (Figures 8-10). The signal of TIMP-2 was weak and there was no difference among three periods. The expression of MT1-MMP mRNA was up regulated (Figure 11), TIMP-2 and MMP-2 were slightly elevated in HSC cultured in hyperoxia for 12 h.

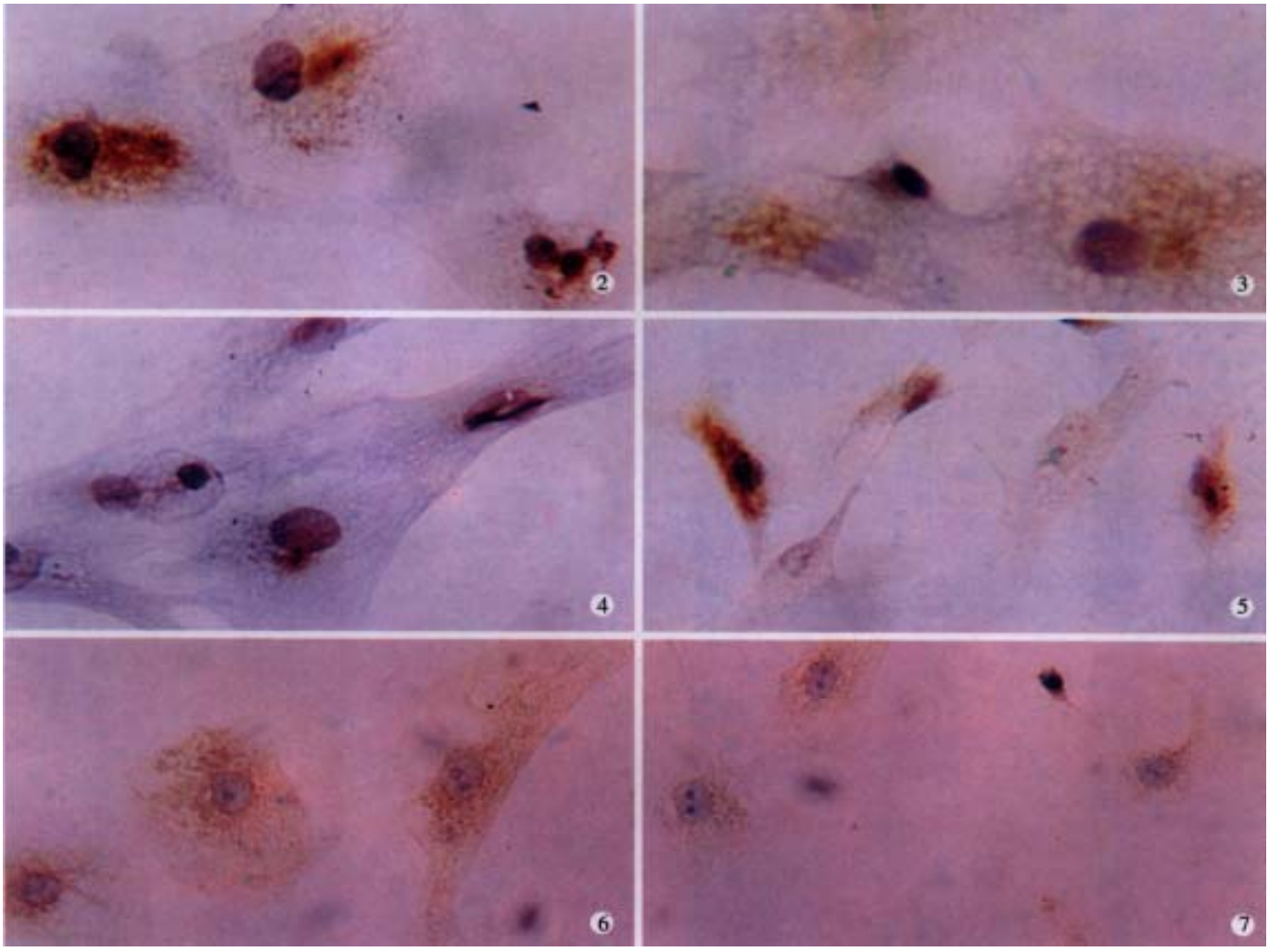

Figure 2 MMP-2 protein in HSC presented intense positive in hypoxia group. LSAB (DAB),×200
Figure 3 MMP-2 protein in HSC presented positive in hypoxia control. LSAB(DAB),×200
Figure 4 TIMP-2 protein in HSC presented weak positive in hypoxia group. LSAB(DAB),×200
Figure 5 TIMP-2 protein in HSC presented intense positive in hypoxia control. LSAB(DAB), ×200
Figure 6 MT1-MMP protein in HSC presented positive in hyperoxia group. LSAB(DAB), ×200
Figure 7 MT1-MMP protein in HSC presented weak positive in hyperoxia control. LSAB(DAB), ×200

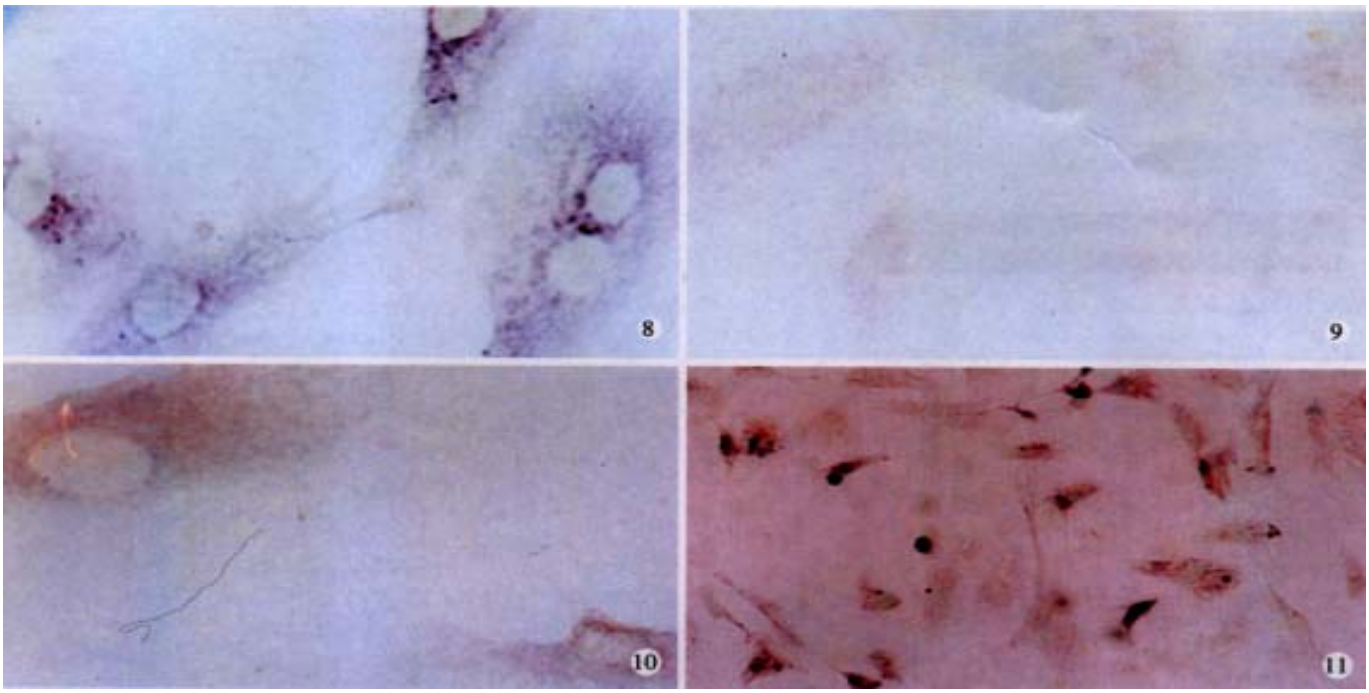

Figure 8 Signal of MMP-2 mRNA in HSC was intense in hypoxia for 6h. ISH(NBT/BCIP), ×400
Figure 9 Signal of MMP-2 mRNA in HSC was weak in hypoxia for 12h. ISH(NBT/BCIP), ×400
Figure 10 Signal of MMP-2 mRNA in HSC was intense in hypoxia for 24h. ISH(NBT/BCIP), ×400
Figure 11 Signal of MT1-MMP mRNA in HSC was intense in hyperoxia group. ISH(NBT/BCIP), ×100

DISCUSSION

It is known to all that oxygen is essential for cell living, and hypoxia will lead to cell dysfunction, or even death. Unfortunately, hypoxia always happens during the liver damage or inflammation, in which swelling of the hepatocytes, constriction of vessels, capillarization of sinusoids, increasing of ECMs in Space of Disse, construction of hepatocytes regenerating nodules and fibrotic septa, abnormal vessels network, increasing endothelin promoted by enterogenous endotoxin, and others may result in liver cell hypoxia[34-37]. In other words, tissue hypoxia occurs in the whole course of the liver fibrogenesis including the initiation and development. Meanwhile, ischemia-reperfusion is not rare in liver injury, and counterpulsation and hyperbaric oxygen therapy are suggested to treat some chronic liver diseases recently[38]. However, there are only a few reports about the effects of hypoxia or hyperoxia in liver fibrogenesis. Avila *et al* reported that methionine adenosyltransferase (MAT) mRNA level was down-regulated by hypoxia, the synthes is of glutathione (GSH) decreased because of a drop of MAT, the free radicals could not be eliminated timely, and the antioxidation ability of the liver came down[39]. Blanc et al found that hypoxia-reoxygenation had direct toxic effects on sinusoidal endothelial cells with an increase in xanthine oxidase activity and lipid peroxidation[40]. Up to now, apart from our work[11,14], we have not found any report about the effects of hypoxia or hyperoxia on the expression and the activity regulation of MMP-2 in HSC.

In our *in vitro* experiments, the expression of MMP-2 in HSC in hypoxia was increased, among the three different hypoxia periods, the changes of expression and activity of MMP-2 were most remarkable in the 6h group, suggesting that the harmful effects of the hypoxia were more serious at the early stage of the liver damage. In the meanwhile, the activity of the enzyme went down, this might be related to the regulation of the enzyme activity[41-46]. As we know, the activation of MMP-2 from HSC not only requires interactions with hepatocytes[47], but also is regulated by MT1-MMP and TIMP-2 which directly mediates the binding of pro-MMP-2 to the cell surface. The cell surface-localized complex is then promoted by MT1-MMP to generate a cell surface-bound active MMP-2: TIMP-2 complex. Upon dissociation of the complex, the active site is exposed, and TIMP-2 binds MMP-2, thereby suppressing its activity[48-54]. Apparently a lack of cooperation with hepatocytes and low expression of TIMP-2 in this experiment may contribute to a drop in the enzyme activity. The change of MMP-2 was opposite to that of TIMP-2 in the different hypoxia periods, suggesting that the former might induce the expression of the latter in the negative direction. It is clear that cell function in vitro is not all the same as it is *in vivo*, so the activity of MMP-2 in the liver tissue under hypoxia is worthy of further study. The mechanism in detail of the MMP-2 and TIMP-2 expression variations induced by hypoxia remains to be clarified. It may be the reason why HSC under hypoxia produced transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) which up-regulate the level of transforming growth factor beta 1 (TGF-β1) gene expression. TGF-β1 promotes the production of MMP-2 and inhibits that of TIMP-2[55].

The expression of MMP-2 and its regulation in HSC in the state of hyperoxia have not been reported. We found that the content of MMP-2 in supernatant was increased, and the activity of MMP-2 rose obviously in hyperoxia group. This suggests that the regulation of MMP-2 activation is stronger than that of the enzyme expression in hyperoxia. As hyperoxia promotes the expression of MT1-MMP in HSC and also that of TIMP-2, the elevated activity of MMP-2 in hyperoxia may be related to a combined action of MT1-MMP and TIMP-2.

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Intestinal expressions of eNOSmRNA and iNOSmRNA in rats with acute liver failure

Jian-Min Qin[1] and Yang-De Zhang[2]

[1]Department of Hepatobiliary & Enteric Surgery, Xiangya Hospital, Hunan Medical University, Changsha 410008, Hunan Province, China
[2]Department of Hepatobiliary & Enteric Surgery, Xiangya Hospital, Hunan Medical University, Changsha 410008, Hunan Province, China
Correspondence to: Jian-Min Qin, M.D., the Biological Signal Transduction Center, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, Shanghai 200438, China. Jianminqin@yhaoo.com
Telephone: +86-21-25070846
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Abstract

AIM **To observe the gene expression change of eNOSmRNA and iNOSmRNA in the small and large intestines with acute liver failure (ALF), and to reveal the biological function of NO on the pathogenesis of ALF and multiple organs dysfunction at the molecular level.**

METHODS **Sixty male Wistar rats were selected, weighing from 250 g to 350 g, and divided into 5 groups randomly: SO, ALF (6h, 12h), L-Arg, L-NAME, L-Arg and L-NAME, each group with 10 rats. The dose of L-Arg was 300 mg·kg^{-1}, and L-NAME was 30 mg·kg^{-1}, the reagents diluted by normal saline were injected through tail vein 30 minutes pre- and post-operation. The rats in the ALF group were respectively sacrificed postoperatively at 6 h, 12 h, and the rats in the other groups were sacrificed postoperatively at 6 h. The tissues of small and large intestines were harvested in 4% paraforaldehyde containing the reagent of DEPC and fixed at 6 h, embedded in paraffin, and 4 μm section was cut. The expression of eNOSmRNA and iNOSmRNA in these tissues was determined with in situ hybridization, and analyzed with the imaging analysis system of CMM-3 and SPSS statistical software.**

RESULTS **The expression of eNOSmRNA in the large intestine and iNOSmRNA in the small and large intestines increased significantly at 6 h after ALF, but the expression of iNOSmRNA in the small and large intestines reduced notably at 12 h after ALF ($P<0.05$); the expression of eNOSmRNA in the large intestine and iNOSmRNA in the small and large intestines decreased significantly with the reagents of L-Arg at 6 h ALF, but the expression of eNOSmRNA and iNOSmRNA in the small and large intestines decreased totally with the reagents of L-NAME or association with L-Arg 6 h ALF.**

CONCLUSION **The expression of eNOSmRNA in the large intestine increased notably at the early stage of ALF, NO induced by the enzyme of eNOS from the transplantation of eNOSmRNA can protect the function of the large intestine, the high expression of iNOSmRNA is involved in the damaged function of the small and large intestines. NO precursor can reduce the expression of iNOSmRNA in the small and large intestines and the damage to intestines; NOS inhibitor or association with NO precursor can totally lower the expression of eNOSmRNA and iNOSmRNA in the small and large intestines, it cannot notably influence the NOS inhibitor in the gene expression of eNOSmRNA and iNOSmRNA to supply the additional NO precursor.**

Subject headings liver failure, acute/pathology; liver failure, acute/metabolism; nitric oxide synthase/biosynthesis; nitric oxide synthase/analysis; nitric oxide donors/pharmacology; nitric oxide/pharmacology

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INTRODUCTION

Acute liver failure (ALF) can severely influence the metabolism, secretion, synthesis and biological transformation, resulting in multiple organs dysfunction (MOD). The effect of gastrointestinal ischemiaon the whole body is extensive and profound, which not only causes the increase of intestinal permeability and the movement of bacteria and toxin in the intestinal cavity, but also release a large quantity of inflammatory media; neuroendocrine element after ALF is closely related to intestinal damage. nitric oxide (NO) is a gaseous molecule with multiple biological function and "two sword" characters, and nitric oxide synthase (NOS) is a key enzyme that can synthesize NO and there are three subtypes including endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neural nitric oxide synthase (nNOS). Abundant nitric NOS is distributed in gastrointestinal tract, NO induced to produce by NOS is a nonadrenergic and noncholinergic (NANC) active media, it participates extensively in various physiological and pathological functions in the intestinal tract. eNOS is Ca^{2+} type and mainly distributed in endothelium, regulated and con trolled by Ca^{2+}/CaM, activated by bradykinin, histamine, PAF, P substance and blood flow shear energy, and catalyzes to produce a little amount of NO that regulates vascular dilation and protects adhesive to vascular wall from platelets and neutrophils. iNOS is a non-Ca^{2+} type and mainly distri buted in macrophages and endothelium, not regulated and controlled by Ca^{2+} /CaM, activated by endotoxin as well as several cytokines (such as IFN-γ, TNF-β, IL-1β), and catalyzes continuously to produce a large amount of NO that has cytotoxic effect. NO induced to produce by iNOS down-regulates the gene expression of iNOS. The negative feedback is conducted by mainly inhibiting DNA binding activity of NF-êB, it may restrict excessive production of NO under the pathophysiological conditions. The physiological and pathological effect of NO to the body depends chiefly on the quality and strength of stimulative elements, the dosage and reactive sites[1-4]. Few reports about the effects on intestinal eNOS and iNOS after ALF are available, and the gene expression of eNOS and iNOS in the intestinal tract. The

study intends to disclose the biological function of NO in ALF and MOD by establishing an ALF rat model and determining the expression of eNOSmRNA and iNOSmRNA in small and large intestines so as to provide a theoretical basis for clinical treatment in ALF.

MATERIALS AND METHODS

Experimental animal

Sixty male wistar rats were obtained from the Experimental Animal Center of Hunan Medical University, weighing from 250 g to 350 g, and divided into 5 groups randomly: sham operation, ALF (6 h, 12 h), L-Arg, L-NAME, L-Arg and L-NAME group. Each group had 10 rats.

Drug and reagents

L-arginine (L-Arg) NO precusor, 300 mg·kg^{-1} , obtained from Shanghai Chemical Reagent Company.

N-nitric -L-arginine methyl ester (L-NAME) NOS inhibitor, 30 mg·kg^{-1}, obtained from Sigma Chemical Company.

eNOSmRNA and iNOSmRNA *in situ* hybridization kit

Obtained from Wuhan Boster Biological Company. Oligoneucleotide probe sequences deriving from human gene: AI925269 GI5661233--① 121---155 (probe sequence --GAGGA CTTTC TCCGT TCTCC TTAGA TTGTA AGCTG---); ② 661---695(probe sequence---ACGTG CCAGA CGTCC TGCAA CAACC GTACC ATTTA---), rat gene AJ011116 GI3676237 -- and 216---250 (probe sequence---CCTAA GACCG TTCTG GCTAA TGTGC TGTAA CTCTA---).

METHODS

The ALF rat model was established by 90% hepatectomy according to Lu *et al*'s method[5]. L-Arg or L-NAME diluted with normal saline was preoperatively and postoperatively injected into the tail vein 30 min after operation in the L-Arg group or L-NAME group; L-Arg was injected preoperatively, L-NAME was preoperatively injected at a 30 min interval, normal saline was injected 30 min preoperatively and postoperatively to SO group and ALF group. The animals were sacrificed at 6 h and 12 h in ALF group after operation, the animals of the other groups were sacrificed at 6 h after operation. The tissues of small and large intestines were fixed in 4% paraformaldehyde containing the reagent of DEPC for 6 hours, dehydra ted gradually, embedded in paraffin, and cut into 4 μm sections.

Determining the expression of eNOSmRNA and iNOSmRNA

The detailed manipulations were conducted according to INTRODUCTION of reagent kits, phosphate buffered saline was used as the negative control in stead of oligoneucleotide probe, and the tissue of breast cancer as positive control. Five views were randomly selected in each tissue sections, measured under the 10×20 fold statistics microscope and analyzed with the imaging analysis system of CMM-3. The mean absorbance optical density (OD) was measured, *P* value less than 0.05 was regarded as having significant difference.

RESULTS

The expressions of eNOSmRNA in large intestine and iNOSmRNA in small and large intestines in ALF increased significantly at 6h (Figures 1-3), and the expressions of iNOSmRNA in small and large intestines in ALF decreased significantly at 12 h ($P < 0.05$). The expressions of eNOSmRNA in large intestine and iNOSmRNA in small and large intestines in ALF decreased significantly at 6 h using L-Arg, but the expressions of eNOSmRNA and iNOSmRNA in small and large intestines in ALF decreased significantly at 6 h using L-NAME or combined with L-Arg (Figure 4, $P<0.05$). The expression of eNOSmRNA and iNOSmRNA in bowel tissues are compared among all groups in Table 1.

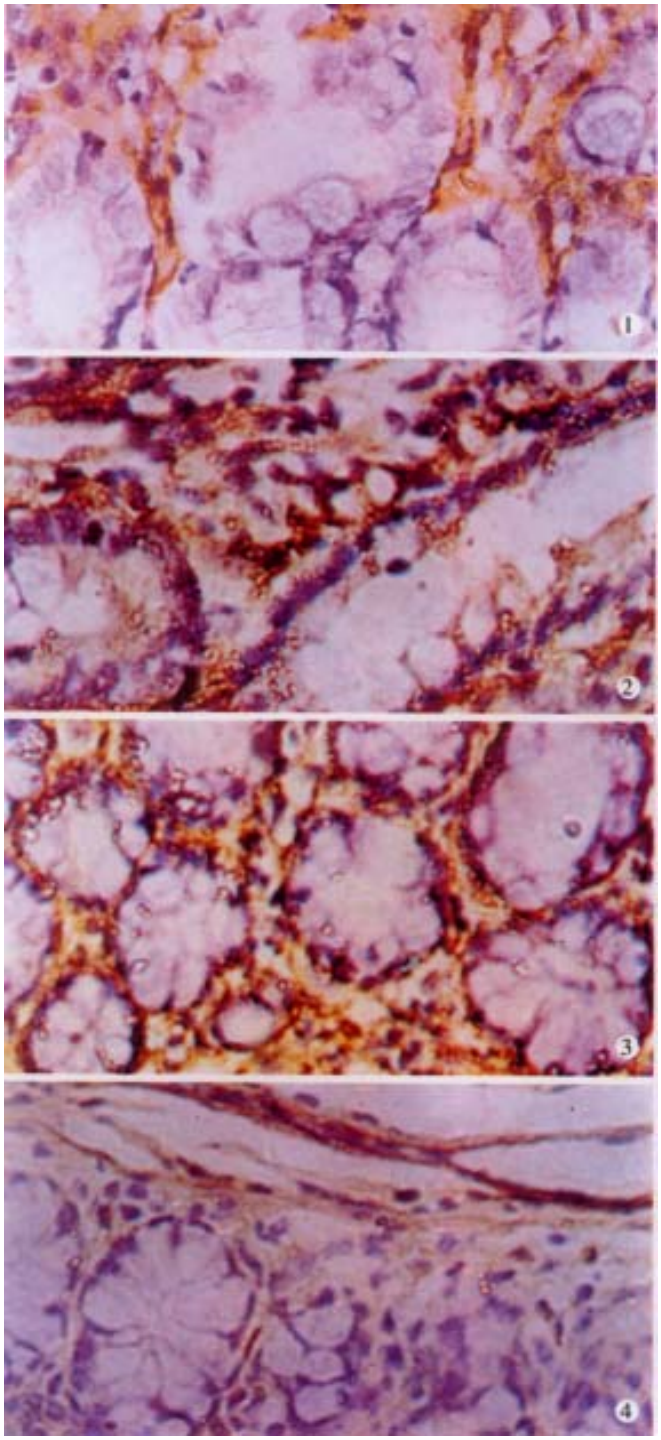

Figure 1 Significant increase of expressions of eNOSmRNA in large intestine with ALF at 6 h. ISH×400
Figure 2 Significant increase of expressions of iNOSmRNA in small intestine with ALF at 6 h. ISH×400
Figure 3 Significant increase of expressions of iNOSmRNA in large intestine with ALF at 6 h. ISH×400
Figure 4 Significant decrease of expressions of iNOSmRNA in large intestine with ALF with the reagents of L-Arg and L-NAME at 6 h. ISH×400

Table 1 intestinal expressions of eNOSmRNA and iNOSmRNA in rats with acute liver failure ($\bar{x}$±s, n=10)

Group	t/p	Gene expression	Small intestine	Large intestine
SO	6	eNOSmRNA	0.05±0.02	0.05±0.02[a]
		iNOSmRNA	0.06±0.02[c]	0.06±0.02[c]
ALF	6	eNOSmRNA	0.10±0.06[e]	0.18±0.06[e]
		iNOSmRNA	0.19±0.05	0.33±0.08
ALF	12	eNOSmRNA	0.05±0.02	0.10±0.04[e]
		iNOSmRNA	0.05±0.01[c]	0.17±0.03[c]
L-Arg	6	eNOSmRNA	0.06±0.01	0.06±0.01
		iNOSmRNA	0.06±0.02[c]	0.07±0.02[c]
L-NAME	6	eNOSmRNA	0.06±0.01	0.09±0.01
		iNOSmRNA	0.07±0.02[c]	0.16±0.03[c]
L-Arg+	6	eNOSmRNA	0.06±0.01	0.07±0.02
L-NAME		iNOSmRNA	0.08±0.03[c]	0.14±0.04[c]

[a]P<0.05 *vs* ALF 6 h eNOSmRNA; [c]P<0.05 *vs* ALF 6 h iNOSmRNA; [e]P<0.05 *vs* bith enzymes in every group.

DISCUSSION

ALF is a cooperative consequence of endotoxicemia, microcirculation dysfunction as well as inflammatory cells (such as macrophage, lymphocyte, etc) that release inflammatory mediators and cytokines (such as TNF, IFN, IL-1, etc) when stimulated, but definite mechanism remains unclear. The damage and decrease of phagocytosis in the liver result in enteral endotoxicemia and decrease in Na^+/K^+-ATP enzyme activity of liver cellular membrane, it causes intrahe patic cholestatic jaundice, dramatical decrease in glomerular filterable rate (GFR), increase in serum Cr, BUN and decrease in urine narium. Collection of glutamine in cerebral cells results in hepatic coma because of tyrosine, tryptophane and phenylalanine in brain, it suggests that enteral endotoxicemia is common substantial basis causing liver function failure syndrome, important pathophysiological stage of liver function failure, and mutual cause and result with liver function failure. It produces poison to hepatocytes, results in microcirculation dysfunction, aggravates liver damage and inhibits liver regeneration. Irreversible liver damage is also an initiate factor of subsequent multiple organs function failure because of liver function failure, it causes not only jaundice, haemorrhage but also renal function failure and hepatic coma[6,7]. Deficient L-arginine inhibits intrahepatic L-Arg/NO pathway and influences systematic blood flow when liver ischemia-reperfusion, L-arginine injected by portal vein inhibits arginase release in liver, reverses L-Arg/NO pathway, and mitigates liver injury[8,9]. Administration of arginine into intestinal tract can mitigate liver function impairment by acute liver injury,activate immune system of intestinal mucosa and regulate and decrease bacterial translocation[10]. GSH can participate in the regulation of iNOS activity, up-regulate the expression of iNOSmRNA, aggravate liver injury under stress condition[11]. Deficient TNF-α and IL-6 caused hepatic steatosis and high mortality, replenishment of TNF-α and IL-6 restored the hepatocyte regeneration[12]. NO can prevent cell apoptosis induced by TNF, and activate extra endothelial regulating kinase 1 and 2, inhibit NF-κB activation in cell[2,13-16]. Furthermore, NO inhibits Caspase-3-like proteinase and cell apoptosis, iNOS gene transfection mediated by adenovirus into hepatocytes can produce NO, which efficiently inhibits Caspase- 3-like proteinase activity, and local apoptosis in hepatocyte[17-20]. IL-1β can induce iNOS expression in hepatocyte by activating iNOS primer by IFN-γ, and produce a large amount of extra cellular matrix (ECM) such as hyaluronan, which induced iNOS to produce NO and form $ONOO^-$, and aggravate tissue injury by NF-κB signal transduction when liver is damaged, moreover, stabilize microcirculation and protect against liver injury. The effect of protection or impairment on the liver mainly depends on the NO concentration and cell derivation[21-23]. NO induced by iNOS in endotoxemia is closely related to liver function impairment, but NO induced early by hepatocyte cNOS can protect liver tissue[24,25]. The dual stimulation of endotoxemia and ischemia-reperfusion influenced microvascular blood flow and impaired liver tissue by selectively inhibiting eNOS or/and iNOS[26,27]. Enterogenous endotoxin permeated blood circulation and stimulated the expression of iNOS in intestinal tissues by portal-cavity lateral circulation. NO participates in hyperdynamic circulation of portal hypertension and general hyperdynamic circulation in cirrhosis[28,29]. Selectively inhibiting eNOS can cause vascular constriction and thrombosis and lower liver perfusion pressure, but had no influence on portal pressure; the difference may be related to different time interval of endotoxin and NOS inhibitor[30]. The expression of NOSmRNA decreased remarkably in the kidney of endotoxemia of cirrhosis, deficient NO or low sensitivity to NO in renal vessels can aggravate renal damage, and promote hepato-renal syndrome (HRS)[31]. Endogenous NO has anti-inflammatory function and inhibits neutrophiles to adhere to vascular endothelial cells and translocate from vessels. NO deriving from aminoformylp enicillamine can alleviate small intestine impairment induced by endotoxin and plasma leakage, suggesting that endogenous NO can maintain intestinal mucosal microvascular integrity, dilate intestinal vessel and improve gut microvasculation, react with superoxide (O_2^-) to be antioxidation, contribute to recovery of impaired enteric function. L-arginine and solution nitric prusside caused absorption of water and ions, but NOS inhibitor (L-NAMA) caused secretion of water and ions in the ileum, synchronous infusion of L-arginine and L-NAME cause absorption of water and ions in the ileum, indicating that endogenous NO can mediate absorption of water and ions in the ileum, maintain homeostasis of water and electrolytes. Neutrophil activation in circulation has been implicated in the capillary leakage during ischemia-reperfusion. It suggests that endogenous NO can maintain the capillary integrity and prevent macromolecular leakage. Capillary permeability and macromolecular leakage are critical factors causing ARDS during intestinal ischemia-reperfusion, and increased endogenous NO can maintain normal capillary function. It is very important to prevent systemic response such as ARDS caused by intestinal ischemia-reperfusion[32-36]. Endotoxemia results in increased cons titutive NOS and inducible NOS enzyme activities in the jejunum and ilem. LPS can upregulate constitutive NOS and inducible NOS in jejunal and ileal smooth muscle oxyhemoglobin increased intestinal transit in nonendotoxemia. NO may play a major role in mediating the rapid intestinal transit induced by endotoxemia. Hemoglobin attenuated the effect of endotoxin on intestinal motor function. Synthetic hemoglobins currently being a test as blood substitutes may be of therapeutic value in treating intestinal dysfunction complicated with sepsis [37-41]. However, the gene expression increase of iNOAmRNA in the intest inal tract produced a large amount of NO, slowed intestinal transit, promoted intestinal bacteria overgrowth during endotoxemia, and reacted with superoide (O_2^-) to form peroxynitrite anion ($ONOO^-$), causing inactivation of mitochondrial aconitase in entercytes and activation of poly-adenosine diphosphate ribosyl polymerase, leads to depletion of intracellular ATP stores by interfering with mitochondrial

respiration and intestinal mucosal barrier dysfunction, prolonged the exposure of cells to large amounts of NO may inhibit cell respiration, cause maldistribution of regional blood flow and cell damage, increase gut permeability; but inhibition of cNOS can decrease dramatically the blood flow of intestine, increase the damage of enteric hemorrhage by induction of LPS, even small dosage of LPS could cause enteric hemorrhage. LPS can inhibit cNOS,decreasing NO and increasing active oxidizing free radicals . L-Arg can protected the enteric hemorrhage from LPS or LPS associated with L-NAME. Inhibiting NO release can increase active oxygen production, and contribute to acute enteric damage by LPS[42-46]. Mishima *et al*[47] found that the iNOS-deficient mice could resist enterocyte apoptosis induced by LPS, and attenuate intestinal mucosal injury. Bacterial translocation induced by LPS was not found, suggesting that LPS could induce iNOS activation and increase NO production, resulting in intestinal mucosal injury and bacterial translocation, inhibiting iNOS can decrease NO production and prevent intestinal mucosal injury and bacterial translocation. Fruchterman *et al* observed that decrease of activity of cNOS and NO production in the small intestinal endothelial cell resulted in intestinal microvascular dysfunction such as low perfusion, platlets and neutrophils converged into postcapillary venule when hemorrhage shock and resuscitation occurred. $ONOO^-$ deriving from NO connection with O_2^- could decrease NO production, inhibit the synthesis of prostaglandin I_2 (PGI_2), impaie endothelial cell mitochondrial and lead to endothelial cell dysfunction and intestinal nucosal impairment[1,48-50]. NOSmRNA expression in colonic mucosa increased remarkably, especially iNOSmRNA, suggesting that vasodilation and blood flow increase because of increased NO production in colonic tissues, mucosal congestion was active not passive, inhibtion of iNOS and NO production may protect or mitigate the pathogenesis of portal hypertensive enteropathy (PHE). NO plays an important role in maintaining gastrointestinal vascular integrity and gastrointestinal motility under physiological and pathophysiological circumstances, it can relax vascular and gastrointestinal tract smooth muscle and contributes to LPS-induced intussusception pathogenesis by activating soluble guanylate cyclase (sGC) to increase intracellular cGMP, inhibiting NOS worsens the intestinal injury induced by ischemia-reperfusion and endotoxin, decreases the incidence of intussusception, but L-Arg can increase the incidence of intussusception, suggesting that endogenous NO plays an important in regulating normal and pathop hysiological circumstance by activating sGC and altering intracellular level of cGMP[51,52]. our study indicated that the expression of eNOSmRNA increased dramatically at early stage of ALF, NO produced by eNOS can keep the integrity of enteral microvascular mucosa, expand blood vessel in enteral mucosa, improve microcirculation, and has antioxidant function by connecting with superoxide anion and protects enteral function from damage. but the high expression of iNOSmRNA in large intestine can induce iNOS synthesis constantly and produce a large amount of NO, slow enteric peristalsis, promote bacterial outgrowth, increase calcium ion concentration, result in protective barrier dysfunction in enteral epithelium, penetration and bacterial translocation increase. The low expression of eNOSmRNA in small and large intestines might be related to consumable increase of eNOS, furthermore, the low expression of iNOSmRNA in small and large intestines might be related to producing excessive NO locally can and feedbackly inhibiting the expression of iNOSmRNA after replenishing exogenous larginine. L-Arg contributes to water and electrolyte absorption in small intestine and plays an important role in keeping microcirculatory stability, suggesting that replenishing exogenous NO precursor can reduce the enteral expression of iNOSmRNA and beneficial to organism, NOS inhibitor can significantly influence the gene expression of both enzymes in large intestine, especially reducing the eNOSmRNA expression more significantly. NO produced by high expression of iNOSmRNA may influence local microcirculation and integrity in enteral mucosa, and contribute to enteral bacterial transl ocation in ALF. It is similar to influence to eNOSmRNA and iNOSmRNA expression using NO precusor connecting with NOS inhibitor or only using NOS inhibitor, and indicating that replenishing exogenous NO precusor can not significantly influence the effect of NOS inhibitor on both gene expression of eNOS and iNOS. It is necessary to further on the mechanism of mutual interaction with them.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Relationship between ABO blood groups and carcinoma of esophagus and cardia in Chaoshan inhabitants of China

Min Su[1], Shan-Ming Lu[1], Dong-Ping Tian[1], Hu Zhao[1], Xiao-YunLi[2], De-Rui Li[3] and Zhi-Chao Zheng[4]

[1]Department of Pathology, [2]Tumor Hospital, [3,4]Second Teaching Hospital, Shantou University Medical College, Shantou 515031, China
Supported by the Key Teacher Fund, Ministry of Education of China and Sir Li Ka-Ching Foundation.
Correspondence to: Prof. Min Su, Department of Pathology, Shantou University Medical College, Shantou 515031, China. minsu@ncrc.stu.edu.cn or minsusm@21cn.com
Telephone: +86-754-8900429, Fax: +86-754-8525667
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Abstract

AIM **To study the relationship between ABO blood groups and carcinoma of esophagus and cardia in Chaoshan inhabitants of China, which is a unique Littoral high-risk area of esophageal carcinoma in China. The poor communication and transportation in the past has made Chaoshan a relatively closed area and kept its culture and custure of old China thousand years ago.**

METHODS **Data on age, sex, ABO blood type and X-ray or patholog ical diagnose of the patients with carcinoma of esophagus or cardia were collected from the Tumor Hospital. First Affiliated Hospital, Second Affiliated Hospital of Shantou University Medical College; and the Central Hospital of Shantou and the Central Hospital of Jieyang. A total of 6685 patients with esophageal carcinoma (EC) and 2955 patients with cardiac cancer (CC) in Chaoshan district were retrospectively assessed for their association with ABO blood groups.**

RESULTS **The distribution of ABO blood groups in patients with EC or CC was similar to the normal local population in Chaoshan. However, blood group B in male patients with CC and in the patients with carcinoma in the upper third esophagus was 2.3% and 4.7% higher than the corresponding controls. The relative risk B:O was 1.1415 ($P<0.05$) and 1.2696 ($P<0.05$), respectively. No relationship was found between ABO blood groups and tumor differentiation.**

CONCLUSION **ABO blood group B is associated with the incidence of CC in male individuals and carcinoma in the upper third esophagus. The distri bution of ABO blood groups varies in the different geographical and ethnic groups. As a result, proper controls are very important for such studies.**

Subject headings esophageal neoplasms; stomach neoplasms; ABO blood-group system; Guangdong; human

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INTRODUCTION

Chaoshan is an enclosed littoral region surrounded by mountains located at the boundary between Guangdong Province and Fujian Province of China. Chaoshan residents mostly came from Henan, Shanxi, and Changan thousands years ago, and are a relatively isolated population with a high risk of esophageal carcinoma (EC) and cardiac carcinoma (CC). Within Chaoshan, the Nanao Island has the highest risk[1,2]. According to the report from the Department of Public Health. Guangdong Province in 1993, the mortalities of esophageal carcinoma in the Nana o Island were: 108.68±7.88/100 000 standardized population of China and 145.44±10.49/100 000 standardized world population and 261.16±25.01/100 000 standardized world population aged 35-64 years. The annual average incidence rates of male and female were 132.19/100 000 and 69.20/100 000 in the Nanao Island from 1987 to 1992[3,4]. It has been noticed that EC is a race-related disease. People of Chinese origin living in foreign countries have higher EC frequency than those of non-Chinese origin, and in comparison with the frequency in the people with Chaoshan origin is even higher. The mortality of EC within immigrants of the Nanao Island origin in Meixian County, Guangdong Province is 1.7 times that of the local residents[5]. The poor communication and transportation in the past has made Chaoshan a relatively closed society and formed its unique culture, customs and habits. A preliminary study showed that Chaoshan residents were migrants from central China, and their traditional culture, custom and habits were well kept. This unique society provides us an unparalleled base to study cultural and genetic factors and their interaction in the study of EC.

Since the association between blood group A and gastric cancer was reported in 1953, the relationship with blood groups and incidence[6-16], clinicopathologic parameter[17,18] and prognosis[19-21] had been studied in many cancer and other disease[22,23]. However, there is no consistent result[24-27]. Additionally, ABO genes are distributed differently among socioeconomic groups[28] and we know that socioeconomi c status is one of the risk factors for diseases. Thus, a retrospective assessment of the relation between blood groups and EC, CC was performed with a view to start the study of the genetics of these cancers in Chaoshan district.

MATERIAL AND METHODS

Clinical data

Data about age, sex, ABO blood type and X-ray or pathological diagnosis of the patients with EC and CC were collected from the Tumor Hospital (1978-1998). The First Affiliated Hospital (1989-1998), the Second Affiliated Hospital (1983 - 1998) of Shantou University Medical

College; the Central Hospital of Shantou and the Central Hospital of Jieyang. A total of 6685 patients with EC and 2955 patients with CC in Chaoshan district were retrospectively assessed for the association with ABO blood groups. In these series, histopathological diagnoses were available in 4719 patients with EC and 2120 patients with CC. The differe ntiation of the carcinomas was categorized as grade 1 (well-differentiated tumors) to grade 3 (poorly-differentiated tumors). The remaining cancers were diagnosed by X-ray. In 5638 patients with EC, there is definite information on tumor sites within the esophagus. All patients' medical records, including operative notes and histopathological reports, were reviewed.

Normal control

The ABO blood group distribution of the blood donors from the Central Blood Bank in Shantou city was used as control groups 7276 donors were sampled, of the same ethnic group and from the same native place as the patients.

Statistical analysis

Data were stored in a computer data base (FoxPro, version 2.5b) and analyzed using a computer spread sheet (Microsoft Excel 97) and professional statistical computer software (SPSS, version 6.12 and SAS, version 6.08). The differences of ABO blood group distribution in patient groups and controls were assessed using Chi-square test. The gene frequencies of blood group antigen were calculated by the method of Bernstein and the relative risks were estimated using Woolf's methods. P values <0.05 were regarded as statistically significant.

RESULTS

The distribution of ABO blood groups in the EC group was similar to that of a normal population (Table 1) and so was the gene frequencies (Table 2). Although blood group B in total patients with CC and female patients with CC were 2.3% and 1.5% higher than the corresponding controls, the difference was not significant. In male patients with CC, there was a 1.6% deficiency of blood group A and a 2.3% excess of blood group B as compared with the male controls, and the two groups differ significantly in their ABO distribution ($\chi^2 = 8.518$, $P<0.05$). The gene frequencies of blood group B were 0.196 in male patients with CC, which was higher than that of the male controls (0.175), and the relative risk B:O was 1.14 (95% confidenc e interval 1.01-1.29) with statistical significance (Table 2). This suggests that male individuals with blood group B are more susceptible to CC. With respect to the site of EC, carcinoma in the middle third esophagus is most common (73%), but the prevalence of blood groups in this group and the lower third group were identical to the controls. The blood group B patients with carcinoma in the upper third esophagus was 4.7% higher than the controls, with a higher gene frequency (0.2072), and the difference of the blood group distribution between the two groups was of statistical significance. When blood group B was compared with blood group O, the relative risk was 1.27 (95% confidence interval 1.05-1.54), which was statistically significant by Chi-square test. Although the prevalence of blood group B was a little lower in the poorly differentiated squamous cell carcinoma of the esophagus (ESCC) than expected, the deficiency did not reach significance. No relationship between the tumor differentiation and ABO blood group distribution was found.

Table 1 ABO blood group distribution in EC patients (n, %)

Groups	Total	Blood groups				P value
		A(%)	AB(%)	B(%)	O(%)	
Control						
Male	5447	1468(27.0)	345(6.3)	1398(25.7)	2236(41.0)	
Female	1829	480(26.2)	112(6.1)	477(26.1)	760(41.6)	
Total	7276	1948(26.8)	457(6.3)	1875(25.8)	2996(41.1)	
EC						
Male	4987	1336(26.8)	271(6.0)	1362(27.3)	1990(39.9)	0.260
Female	1698	443(26.1)	102(6.0)	442(26.0)	711(41.9)	0.997
Total	6685	1779(26.6)	401(6.0)	1804(27.0)	2701(40.4)	0.399
CC						
Male	2437	620(25.4)	177(7.3)	683(28.0)	957(39.3)	0.036[a]
Female	518	138(26.7)	27(5.2)	143(27.6)	210(40.5)	0.790
Total	2955	758(25.7)	204(6.9)	826(27.9)	1167(39.5)	0.057
Site of EC						
Upper third	673	164(24.4)	46(6.8)	205(30.5)	258(38.3)	0.045[a]
Middle third	4121	1126(27.3)	243(5.9)	1079(26.2)	1673(40.6)	0.725
Lower third	844	206(24.4)	51(6.1)	245(29.0)	342(40.5)	0.186
Diff. (ESCC)						
Well Diff.	378	106(28.0)	25(6.6)	107(28.3)	140(37.0)	0.444
Moderately Diff.	1290	340(26.4)	75(5.8)	334(25.9)	541(41.9)	0.893
Poorly Diff.	572	160(28.0)	20(3.5)	152(26.6)	240(41.9)	0.064
Diff. (AC)						
Well Diff.	22	6(27.3)	2(9.1)	6(27.3)	8(36.4)	0.936
Moderately Diff.	161	45(28.0)	9(5.6)	43(26.7)	64(39.8)	0.954
Poorly Diff.	311	82(26.4)	24(7.7)	86(27.7)	119(38.3)	0.569

[a]$P<0.05$. EC: Esophagus cancer; CC: Cardiac cancer; AC: Adenocarcinoma; ESCC: Squamous cell carcinoma in esophagus; Diff: Differentiation

Table 2 Gene frequency of abo blood type in EC and CC patients

Group	Gene frequency			A/O			B/O		
	p	*q*	*r*	RR	CI	*P*	RR	CI	*P*
Control	0.1816	0.1754	0.6424						
Total (EC)	0.1758	0.1784	0.6405	1.01	0.93－1.10	NS	1.07	0.98－1.16	NS
Upper (EC)	0.1696	0.2072	0.6210	0.98	0.80－1.20	NS	1.27	1.05－1.54	<0.02
Total (CC)	0.1790	0.1925	0.6285	1.00	0.90－1.11	NS	1.13	1.02－1.26	NS
Male (CC)	0.1794	0.1954	0.6252	0.99	0.88－1.11	NS	1.14	1.01－1.29	<0.03

NS: Not significant; CI: Confidence interval; EC: Esophageal cancer; CC: Cardiac cancer

Table 3 Comparison of ABO blood group distribution in different districts in China

Districts	Blood groups				Characteristics
	A (%)	AB (%)	B (%)	O (%)	
Linxian	2936(24.5)	1254(10.5)	4279(35.6)	3536(29.5)	B>O>A>AB
Shanxi	2908(23.3)	987(7.9)	4120(33.0)	4469(35.8)	O>B>A>AB
Dezhou	994(28.4)	318(9.1)	1064(30.4)	1124(32.1)	O>A>B>AB
Wuhan	10799(33.2)	2970(9.2)	7180(21.9)	11641(35.7)	O>A>B>AB
Hubei	4461(32.5)	1125(8.2)	3376(24.6)	4762(34.7)	O>A>B>AB
Lanzhou	597(33.0)	171(9.4)	512(28.3)	530(29.3)	A>O>B>AB
Yancheng	947(30.3)	299(9.6)	845(27.0)	1037(33.2)	O>A>B>AB
Chaoshan	1948(26.8)	457(6.3)	1875(25.8)	2996(41.2)	O>A>B>AB

DISCUSSION

There seemed to be no association between ABO blood groups and the incidence of EC or CC when the studied patients were not grouped by sex and tumor site. This result is consistent with previous reports. However, in the present study, male individuals with blood group B were more susceptible to CC than those with other blood types, and blood group B was associated with carcinoma of the upper third of the esophagus. It suggested that the susceptibility of male to certain cancer was not the same as female. Thus, further classification by sex may be needed when studying the relation between ABO blood groups and cancer. No relation was found in the current study between blood groups and the histological differentiation of EC or CC. The relationship between ABO blood groups and carcinoma of esophagus and cardia has been controversial[29,30]. Mourant and his colleagues reported that blood groups A and B were both associated with EC and no relation was found between blood groups and CC, based on the data from 31 districts in 13 countries and 7 districts of 6 countries, respectively[31]. But some authors have reported different associations according to geographical locations. Thus, blood group A was associated with EC in the British population of Belfast and blood group B individuals were susceptible to EC in British in Bristol, Bantu in South Africa and Iowa in America. But ABO blood groups have shown no correlation with EC in other districts in these countries[29]. These inconsistent results reflect two fundamental methodological problems with such association studies, the statistical methods and the selection of controls. Since the association between ABO blood groups and different cancers is not the same, associations may be observed if several kinds of carcinoma are analysed together. Small sample size is another possible inconsistency.

The importance of careful selection of controls is illustrated by the comparison of populations from different districts whthin China (Table 3). The distribution of ABO blood groups in different districts differs significantly (χ^2 = 0.141, P<0.01). For instance, there was a 10% excess of blood group A individuals in the population of Wuhan, compared with that of Shanxi, a 13.7% excess of blood group B individuals in Linxian compared with Wuhan, and a difference in the frequency of blood group O in Chaoshan compared to Shanxi, even though Chaoshan residents came from Henan, Shanxi, a thousand years or more ago. This suggests that the frequency of different alleles in immigrants to Chaoshan from Shanxi has drifted, compared with their originating population. The best control is, therefore, the ABO blood group distribution of the healthy population of the same ethnic group and sex and from the same native place as the patients[32]. It is possible that blood group B is just a marker of the ancestral population from Shanxi which is now mixed into the Chaoshan region, Shanxi people are also the population at high risk of EC/CC.

The explanation for the association between ABO blood groups and some special diseases was still unclear. Many reports have shown that blood group antigen expression in tumor is correlated with metastasis[33,34] and prognosis[35,36]. The loss or presence of blood group antigens can increase cellular motility or facilitate the interaction between tumor cells and the endothelium of distant organs[37,38]. ABO(H) blood group genes are map at 9q in which the genetic alteration is common in many cancers[39-44]. Thus, ABO(H) blood group antigen expression may be affected by the genetic change of tumors. On the other hand, it is possible that the observe d associations are not due to the blood group antigens themselves, but to the effects of genes closely associated with them. Additionally, it might have nothing to do with molecular mechanisms or genetics. It is merely the result of population history[45-47], environment[45,48,49], diet[50] and customs[51-55].

In conclusion, the current study shows no association of ABO blood groups with EC and CC, when the patients are not separated by sex and tumor site. If they are so grouped, male individuals with blood group B are found to have increased risk for the development of CC, and blood group B individuals are more likely to get carcinoma in the upper third of the esophagus. These findings may contribute to the genetic or custom study of EC and CC.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

The effect pathway of retinoic acid through regulation of retinoic acid receptor α in gastric cancer cells

Su Liu, Qiao Wu, Zheng-Ming Chen and Wen-Jin Su

The Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005, Fujian Province, China
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Corresponding to: Dr. Qiao Wu, the Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005, Fujian Province, China. xgwu@xmu.edu.cn
Telephone: +86-592-2182542, Fax: +86-592-2086630
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Abstract

AIM **To evaluate the role of RARα gene in mediating the growth inhibitory effect of all-trans retinoic acid (ATRA) on gastric cancer cells.**

METHODS **The expression levels of retinoic acid receptors (RARs) in gastric cancer cells were detected by Northern blot. Transient transfection and chlorophenicol acetyl transferase (CAT) assay were used to show the transcr iptional activity of β retinoic acid response element (βRARE) and AP-1 activity. Cell growth inhibition was determined by MTT assay and anchorage-independent growth assay, respectively. Stable transfection was performed by the method of Lipofectamine, and the cells were screened by G418.**

RESULTS **ATRA could induce expression level of RARα in MGC80-3, BGC-823 and SGC-7901 cells obviously, resulting in growth inhibition of these cell lines. After sense RARα gene was transfected into MKN-45 cells that expressed rather low level of RARα and could not be induced by ATRA, the cell growth was in hibited by ATRA markedly. In contrast, when antisense RARá gene was transfected into BGC-823 cells, a little inhibitory effect by ATRA was seen, compared with the parallel BGC-823 cells. In transient transfection assay, ATRA effectively induced transcriptional activity of βRARE in MGC80-3, BGC-823, SGC-7902 and MKN/RARα cell lines, but not in MKN-45 and BGC/aRARα cell lines. Similar results were observed in measuring anti-AP-1 activity by ATRA in these cancer cell lines.**

CONCLUSION **ATRA inhibits the growth of gastric cancer cells by up-regulating the level of RARα; RARα is the major mediator of ATRA action in gastric cancer cells; and adequate level of RARα is required for ATRA effect on gastric cancer cells.**

Subject headings receptor; retinoic acid/pharmacology; stomach neoplasm/drug therapy; stomach neoplasm/pathology

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INTRODUCTION

Retinoic acid (RA) exerts profound effects on the growth, differentiation and apoptosis of normal, premalignant and malignant epithelial cells *in vivo* and *in vitro*[1-7]. The effects of retinoic acid are mainly mediated by two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs)[8-13], which belong to steroid/thyroid receptor superfamily, and are encoded by three distinct genes, α, β and γ. RXRs form homodimers (RXR/RXR) and heterodimers (RAR/RXR) with RARs receptively, then bind to specific RA response elements (RARE), and regulate positively and negatively their transcriptional activities of target genes[9,13-17]. These receptors, thus, display distinct patterns and exert specific functions on anti-cancer effects in various cancer cell lines.

There have been sufficient evidences showing a link between the alteration of RARs activity and some diseases[18-22]. t (15;17) chromosomal translocation leads to the forming of PML-RARα fusion and abnormal RARα transcription in acute promyelocytic leukemia[23-26] . High frequency of the deletion next to RARα gene in chromosome 3P is observed in human lung cancer. Lack of RARα expression is responsible for the resistance of RA in breast cancer cells[1,20,21,27- 29]. Investigation the functions of retinoic acid receptors, therefore, is essential to elucidate their anticancer effects of RA. In the present study, we evaluate the role of RARα gene in mediating the effect of all-trans retinoic acid (ATRA) in gastric cancer cells. The results indicated that RARá is required for ATRA to exert its growth inhibition on gastric cancer cells.

MATERIALS AND METHODS

Cell lines and culture conditions

The human gastric cancer cell lines, BGC-823, SGC-7901 and MKN-45, were purchased from Institute of Cell Biology, Shanghai, China. MGC80-3 cell line was established by Cancer Research Center in Xiamen University. All of four cell lines were maintained in RPMI1640 medium, supplemented with 100 mL·L^{-1} FCS, 1 mmol·L^{-1} glutamine, and 100×10^3 U·L^{-1} penicillin.

RNA preparation and Northern blot

Total RNA was prepared by guanidine hydrochloride/ultracentrifugation method. About 30 μg total RNA was fractionated on 10 g·L^{-1} agarose, then transferred to nylon, and probed with ^{32}P-labeled probe as previously described[30]. The probes of RARα, RARβ, RARγ and RXRα were provided

by Dr. Zhang (The Burnham Institute, CA, USA). 28S and 18S were shown in quantitation of RNA.

Transient transfection and CAT assay

Cells were seeded in six-well plates with approximately 70% confluent at the time of transfection. Cells were transient transfected by Lipofectamine™ (Gibco/BRL). Transient transfection was performed utilizing βRARE-tk-CAT reporter gene plasmid, containing the βRARE linked with tk-CAT promoter[29], or -73col-tk-CAT receptor gene plasmid, containing an AP-1 binding site located between residues -73 and -63 in collagenase promoter[31,32]. Transfection condition was as follows: 6 μL Lipofectamine™ in 1.0 mL standard medium was added to each well, togelher with 1.0 mL of standard medium containing 400 ng reporter gene plasmid, 400 ng β-galactosidase expression vector (pCH110, Pharmacia), and carrier DNA (pBluescript) added up to 1000 ng total DNA. CAT activity was normalized for transfection efficiency to the corresponding β-galactosidase activity as described elsewhere[1,30-32].

Stable transfection

Sense RARα- and antisense RARα expression vectors (provided by Dr. Zhang) were stably transfected into gastric cancer cells, MKN-45 and BGC-823, respectively, by Lipofectamine™ (Gibco/BRL) as described above, and then screened with 600 μg of G418. Expression of endogenous RARá was determined by Northern blot.

MTT assay

Cells were seeded at 1000 cells per well in 96-well plates, and treated with ATRA (Sigma) at various concentrations. Medium was changed and ATRA was added every other day. After treatment for one week, cells were stained with 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma) for 3 h-4 h. Cell viability was determined by the MTT assay[1,30,32]. An underlayer of 5 $g \cdot L^{-1}$ agar in medium supplemented with 100 mL $\cdot L^{-1}$ FCS was first prepared and hardened in 6-well plate. Cells $1\times10^{8} \cdot L^{-1}$, in culture medium containing 100 $mL \cdot L^{-1}$ FCS, 5 $g \cdot L^{-1}$ agar, and 10^{-6} $mol \cdot L^{-1}$ ATRA (only for experimental groups), were seeded onto the underlayer. The plate was incubated for three weeks in CO_2 incubator. Number of colonies with diameter >80 μm was counted under microscope[5].

RESULTS

Expressions of RARα, RARβ, RARγ and RXRα in gastric cancer cells

Northern blot analysis showed that the level of RARα expression was high in MGC80-3, BGC-823 and SGC-7901 cells, while rather low level in MKN-45 cells. After treated with ATRA, MGC80-3, BGC-823, and SGC-7901, cells exhibited a marked increase in RARα expression, whereas MKN-45 cells had no change in RARα expression. RARβ expressed in MGC80-3, BGC-823, and SGC7901 cells, but not in MKN-45 cells. As for RARγ, none of the four cell lines expressed RARγ (data not shown). All cell lines showed a relatively low-level expression of RXRα. However, the expressions of RARβ, RARγ and RXRα could not be induced by ATRA in these four cell lines (Figure 1).

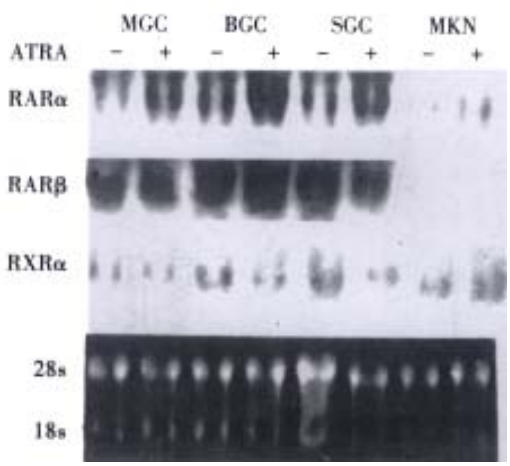


Figure 1 Expressions of RARα, RARβ and RXRα in gastric cancer cell lines detected by Northern blot. Cells were treated with 10^{-6} $mol \cdot L^{-1}$ ATRA.

Transfection and expression of RARα gene in gastric cancer cells

Based on these results mentioned above, we transfected antisense RARα gene and sense RARα gene into BGC-823 and MKN-45 cells, respectively. It was demonstrated by Northern blot that when antisense RARα gene was transfected into BGC-823 cells, RARα expression was repressed, and could not be induced by ATRA, compared with parallel cells BGC-823 (Figure 2A). On the contrary, MKN/RARα cells that transfected with sense RARα gene had a higher expression of RARα than parallel MKN-45 cells, and the expression of RARα could be induced by ATRA (Figure 2B).

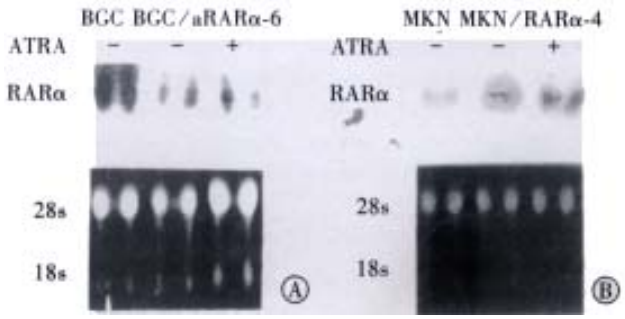


Figure 2 A. Expression of RARα mRNA in BGC-823 cells transfected with antisense RARα gene. B. Expression of RARα mRNA in MKN-45 cells transfected with sense RARα gene.

Effect of ATRA on the growth inhibition of gastric cancer cells

ATRA could effectively inhibit the growth of MGC80-3, BGC-823 and SGC-7901 cells, but had a rather weak effect on MKN-45 cells (Figure 3A). As for the transfected cells, BGC/aRARα, the inhibition rate by ATRA dropped obviously from 61.0% to 18.4%. The opposite result was seen in another transfected cell, MKN/RARα, in which ATRA could effectively suppress the growth of MKN/RARα cells, with an enhanced inhibition rate from 3.9% to 31.7% (Figure 3B).

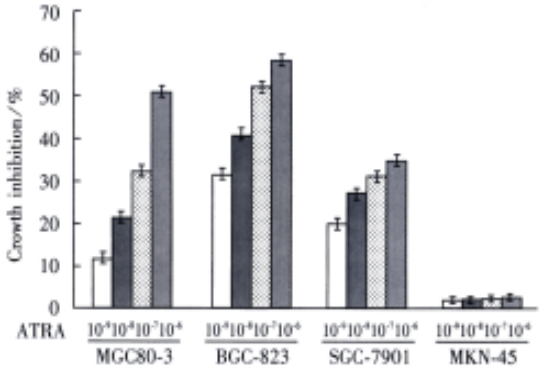


Figure 3A Growth inhibitory effect of ATRA on gastric cancer cell lines measured by the method of MTT. Cells were treated with various concentrations of ATRA indicated.

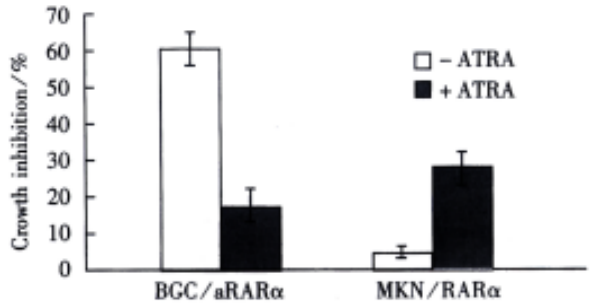


Figure 3B Growth inhibitory effect of ATRA on BGC-823 cells transfected with antisense RARα gene and on MKN-45 cells transfected with sense RARα gene, respectively.

Effect of ATRA on cell clone formation in soft agar

ATRA could inhibit the ability of clone formation in four cell lines and the inhibition for MKN-45 cells was lowest among four cell lines (Table 1). In contrast, in the transfected cells, the highest inhibition on MKN/RARα cells transfected with sense RARα gene was observed, compared with BGC/aRARα cells transfected with antisense RARα gene (Table 1).

Table 1 Inhibitory rate of clone formation of cells treated with 10^{-6} mol·L^{-1} ATRA in soft agar

Cell lines	MGC	BGC	SGC	MKN RARα	MKN / aRARα	BGC / aRARα
Inhibitory rate (%)	48.8[b]	45.2[b]	65.3[b]	14.3[b]	56.1[b]	15.2[b]

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

Regulation of ATRA on αRARE transcriptional activity

When transient transfection was performed with reporter gene, βRARE-tk-CAT, MGC80-3, BGC-823 and SGC-7901 cells exhibited a stronger induction of CAT activity by ATRA than MKN-45 cells, with an increased induction (CAT activity induced by ATRA deletes CAT activity in control) by 3.67, 3.44 and 2.25 fold, respectively, compared with that of MKN-45 cells by 1.04 (Figure 4A). However, ATRA could not significantly induce CAT activity in BGC/aRARα cells, and the induction was 1.76 fold, compared with 3.40 fold in MKN/RARα cells whose CAT activity was induced by ATRA obviously (Figure 4B).

Inhibitory effect of ATRA on AP-1 activity

AP-1 (activator protein-1) activity is associated with proliferation and trans fomation of tumor cells, and can be induced by some agents for mitogen, such as TPA (12-O-tetradecanoylphorbol-13-acetate)[31-33]. Detection of AP-1 activity by transient transfection and CAT assay was carried out in gastric cancer cells. As shown in Figure 5, the AP-1 activity (CAT activity) induced by TPA was suppressed by ATRA in MGC80-3, BGC-823 and SGC-7901 cells, with an ATRA-dose dependent manner. However, the suppressive effect of ATRA could not be observed in MKN-45 cells (Figure 5A). In the transfected cells, ATRA treatment resulted in a decrease of AP-1 activity induced by TPA in MKN/RARα cells transfected with sense RARα gene, but with a little effect in BGC/aRARα cells transfected with antisense RARα gene (Figure 5B).

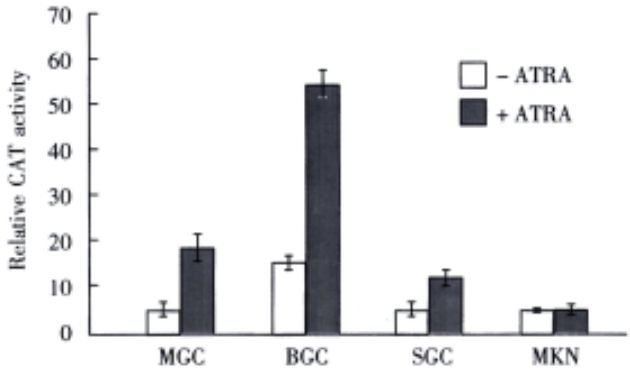


Figure 4A Regulation of ATRA on βRARE transcriptional activity in gastric cancer cell lines detected by CAT assay.

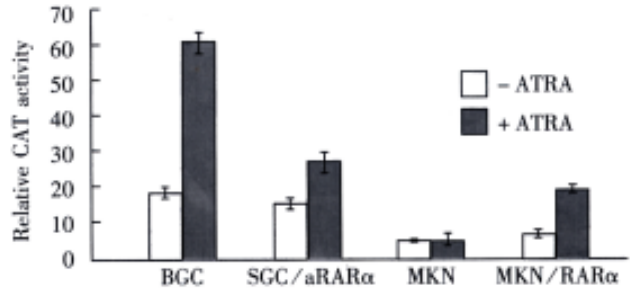
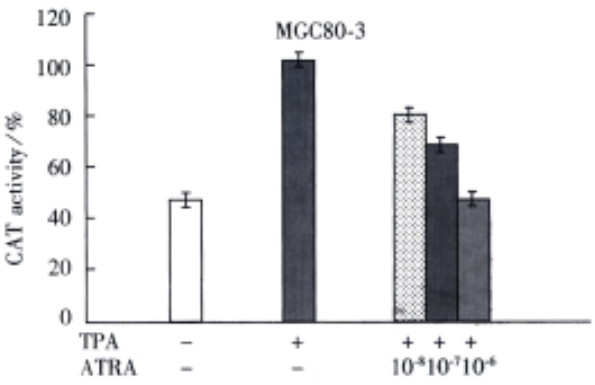


Figure 4B Regulation of ATRA on βRARE transcriptional activity in BGC-823 cells transfected with antisense RARα gene and in MKN-45 cells transfected with sense RARα gene, respectively.



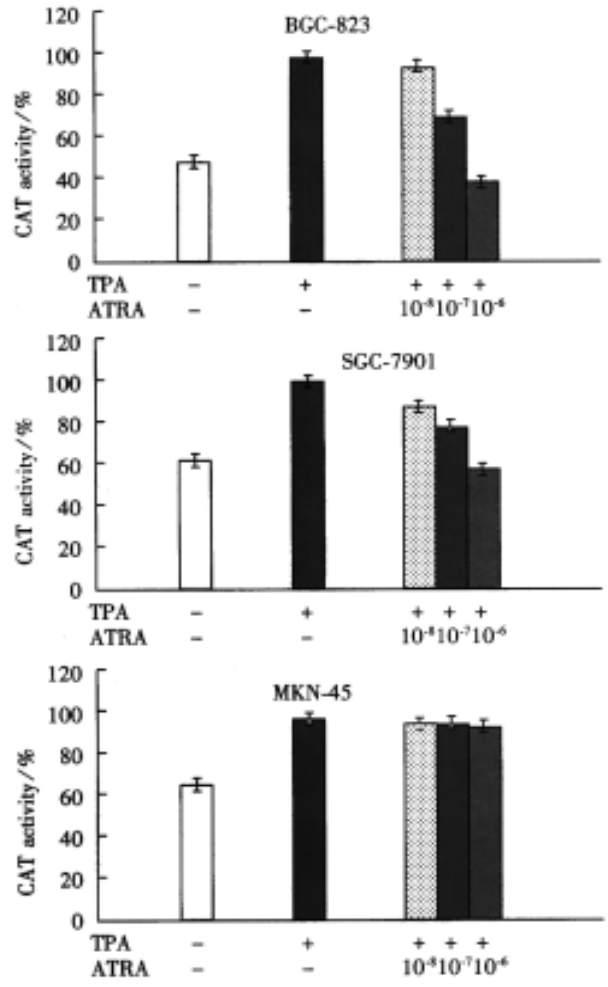


Figure 5A Inhibitory effect of ATRA on AP-1 activity in gastric cancer cell lines at various concentrations of ATRA shown by CAT assay.

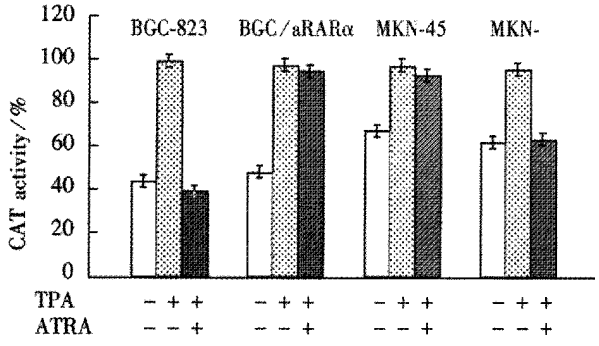


Figure 5B Effect of ATRA on AP-1 activity in BGC-823 cells transfected with antisense RARα gene and in MKN-45 cells transfected with sense RARα gene, respectively.

DISCUSSION

Retinoicacid (RA) is known to inhibit the growth of cancer cells *in vitro*, including cells of breast cancer, lung cancer, gastric cancer and liver cancer[1,15,30,34-36]. Effects of retinoic acid are mediate d by its receptors RARs and RXRs[8-13]. In the present study, we demons trated that the molecular mechanism by which RA inhibited the growth of gastric cancer cells was involved in RARα-mediated signal transduction pathway. Although ATRA did not show any inhibitory effects on MKN-45 cells (Figure 3A, Table 1), the expression of exogenously transfected sense RARα gene at elevated level in MKN-45 cells resulted in acquisition of sensitivity to growth inhibition by ATRA (Figures 2B, 3B, Table 1). In contrast, exogenous transfection of antisense RARα gene into BGC-823 cells, which expressed RARα, and RARα could be induced by ATRA (Figure 1, 2A), failed in growth inhibition by ATRA (Figure 3B, Table 1). These data suggested that the growth inhibitory effect of ATRA is due to the presence of RARα. In addition, we noted that although RARα mRNA was detected in MKN-45 cells, its mRNA level was rather low, compared with that in MGC80-3, BGC-823 and SGC-7901 cells (Figure 1). This may be the reason why ATRA could not exert its anti-proliferation effect on MKN-45 cells. RARα, thus, plays a major role in mediating growth inhibition of ATRA on gastric cancer cells, and adequate level of RARα is required for such action.

AP-1 is a transcriptional factor mainly composed of the products of cJun and cFos[31,37,38], which relate with proliferation and transformation of tumor cells. Our observation that ATRA could effectively inhibit AP-1 activity induced by TPA in MGC80-3, BGC-823 and SGC-7901 cells, but not in MKN-45 cells (Figure 5A) indicated that the suppression of AP-1 activity might contribute to cell growth inhibition by ATRA in gastric cancer cells. The anti-AP-1 effect of ATRA was mediated by the activation of RARα. When transfecting sense RARα gene into MKN-45 cells, a clear inhibition of AP-1 activity was seen (Figure 5B), thus leading to growth inhibition of MKN-45 cells (Figure 3B, Table 1). However, a little effect by ATRA in BGC/ aRARα cells observed in this study (Figure 5B) was associated with a weakened inhibition in BGC/aRARα cell proliferation (Figure 3B, Table 1). Thus, anti-AP-1 activity is one of the mechanisms for ATRA to inhibit growth of gastric cancer cells, and RARα does play a critical role.

RARα, once activated by RA, forms a heterodimer with RXR, then bind to retinoic acid response element (such as βRARE), and regulates transcription and expression of target genes[13-17]. In acute promyelocytic leukemia cells and RA-resistant breast cancer cells, RA could up-regulate the expression of RARα via modulation of RARE motif located in RARα promoter[39-41]. The fact that when the reporter gene βRARE-tk-CAT was transfected into MGC80-3, BGC-823 and SGC-7901 cells, a marked increase in βRARE transcriptional activity induced by ATRA was observed (Figure 4A) suggested that RARs are functional in these cell lines, i.e., to activate βRARE transcriptional activity in the presence of ATRA, and then to stimulate cell growth inhibitory signals to repress the growth of cancer cells. However, when the same reporter gene was transfected into MKN-54 cells, the âRARE transcriptional activity induced by ATRA was relatively low (Figure 4A), indicating the abnormality of βRARE transcriptional regulation or functional loss of RARα in MKN-45 cells, which caused the failure of growth inhibition of MKN-45 cells by ATRA. The similar results were further confirmed by transient transfection assay in transfected gene cell lines, BGC/aRARα and MKN/ RARα, respectively (Figure 4B). All these data are consistent with those observed in breast cancer cells and lung cancer cells[1,42], and imply that low-level expression of retinoic acid receptors in cancer cells is closely associated with the development of malignant tumor. RARα might serve as a candidate marker to determine which gastric cancer patient would respond to and benefit from the retinoid therapy, and this is also useful for the synthesis of RARα-selective retinoids. Of course, some further experiments to verify this issue are needed.

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Effect of cholecystokinin on cytokines during endotoxic shock in rats

Yi-Ling Ling[1], Ai-Hong Meng[1], Xiao-Yun Zhao[1], Bao-En Shan[2], Jun-Lan Zhang[1] and Xiao-Peng Zhang[3]

[1]Department of Pathophysiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China
[2]Research Center of Fourth Hospital, Hebei Medical University, Shijiazhuang 050000, Hebei Province, China
[3]Department of Chest Surgery of Hebei Provincial People's Hospital, Shijiazhuang 050000, Hebei Province, China
Correspondence to: Professor Yi-Ling Ling, Department of Pathophysiology, Hebei Medical University, Shijiazhuang 050017, Hebei Province, China.LingYL20@sina.com.cn
Telephone: +86-311-6052263
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Abstract

AIM **To study the effect of cholecystokinin-octapeptide (CCK-8) on systemic hypotension and cytokine production in lipopolysaccharide (LPS)-induced endotoxic shock (ES) rats.**

METHODS **The changes of blood pressure were observed using physiological record instrument in four groups of rats: LPS (8 mg·kg^{-1}, iv) induced ES; CCK-8 (40 μg·kg^{-1}, iv) pretreatment 10 min before LPS (8 mg·kg^{-1}); CCK-8 (40 μg·kg^{-1}, iv) or normal saline (control) groups. Differences in tissue and circulating specificity of the proinflammatory cytokines (TNF-α, IL-1β and IL-6) were assayed with ELISA kits.**

RESULTS **CCK-8 reversed LPS-induced decrease of mean artery blood pressure (MABP) in rats. Compared with control, LPS elevated the serum level of IL-6 significantly (3567±687) ng·L^{-1} *vs* 128±22 ng·L^{-1}, *P*<0.01), while contents of TNF-α and IL-1β elevated significantly (277±86 ng·L^{-1} *vs* not detectable and 43±9 ng·L^{-1} *vs* not detectable, *P*<0.01) but less extent than IL-6. CCK-8 significantly inhibited the LPS-induced increase in serum TNF-α, IL-1β and IL-6. LPS elevated spleen and lung content of IL-1β significantly (5184±85 ng·L^{-1} *vs* 1047±21 ng·L^{-1} and 4050±614 ng·L^{-1} *vs* not detectable, *P*<0.01), while levels of TNF-α and IL-6 also rose significantly but in less extent than IL-1β. CCK-8 inhibited the LPS-induced increase of the cytokines in spleen and lung. In the heart, CCK-8 significantly inhibited LPS-induced increase of TNF-α (864±123 ng·L^{-1} in CCK-8+LPS group *vs* 1599±227 ng·L^{-1} in LPS group, *P*<0.01), and IL-1β (282±93 ng·L^{-1} in CCK-8+LPS group *vs* 621±145 ng·L^{-1} in LPS group, *P*<0.01).**

CONCLUSION **CCK-8 reverses ES, which may be related to its inhibitory effect on the overproduction of cytokines.**

Subject headings sincalide/ pharmacology; lipopolysaccharides; shock, septic/drug therapy; shock, septic/ blood; tumor necrosis factor /analysis; interleukins/blood; hypotension/drug therapy; hypotension/etiology

Ling YL, Meng AH, Zhao XY, Shan BE, Zhang JL, Zhang XP. Effect of cholecystokinin on cytokines during endotoxic shock in rats. *World J Gastroenterol*, 2001;7(5):667-671

INTRODUCTION

Lipopolysaccharide (LPS), a main component of gram-negative bacterial endotoxin[1], is the leading cause of sepsis or endotoxic shock (ES), and when administered experimentally to animals, mimics the same inflammatory response. The pathophysiological changes seen in sepsis are often not due to the infectious organism itself but to the uncontrolled production of proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Accumulation of these mediators leads to tissue damage, ultimately producing the lethality of sepsis[2]. Studies suggest that alterations in their circulating levels do not always reflect changes at the tissue level and this has led to the reassessment of tissue cytokine content as a more accurate reflection of the host response to stress[3]. Cholecystokinin (CCK), a component of the gastrin-CCK family and first isolate d from hog intestine, shows a widespread distribution in different organs and tissues. The sulfated carboxy-terminal octapeptide (CCK-8), which has been isolated from the central nervous system and digestive tract, is the predominant active form. CCK-8 possessed both excitatory and inhibitory action on contractile activity of different regions of stomach in guinea pigs[4]. CCK-8 could antagonize the elimination of morphine on the potentiations of ACh to duodenal activities, and these effects were mediated by CCK-A receptor[5]. Besides the effects of CCK-8 on the digestive tract, other biological actions of this peptide have been observed, such as appetite inhibition[6,7]. In the spleen, CCK-8 is formed in high abundance in the white pulp where it appears to surround cell clusters. It seems that CCK -8 increases in vivo the secretion of immunoglobulins[8,9], while it causes *in vitro* an inhibition of Molt-4 lymphoblast proliferation[10] and modulates mitogen-induced lymphoproliferation and intracellular calcium mobilization[11-13]. CCK-8 is a chemoattractant for human monocytes and rat macrophages[14], enhances human eosinophil chemotaxis induced by PAF and LTB4 in allergic patients[15] and is a negative modulator of several murine macrophage and human neutrophil functions[16-18]. It was reported that CCK-8 reversed hemorrhagic shock[19,20]. Our previous studies demonstrated for the first time that CCK-8 could protect animals from LPS-induced ES[21]. However, whether this protecting effect of CCK-8 is related to its modulation of cytokines is still not clear. The AIM of this work is to study the effect of CCK-8 pretreatment on systemic hypotension and on production of cytokines such as TNF-α, IL- 1β and IL-6 in spleen, lung, heart and serum of ES rats.

MATERIALS AND METHODS

Materials

CCK-8 (sulfated), LPS (E.coli LPS, serotype 0111:B4), leupeptin, pepstatin A and Triton X-100 were all purchased from Sigma and aprotinin from Boehringer. ELISA kits were purchased from Endogen (USA) and Medsystem (Austria). All other reagents used were of analytic grade.

Methods

Animal preparation Specific pathogen-free male Sprague-Daw ley rats (n = 48, weighing 150 g-200 g, obtained from Experimental Animal Center of Hebei) were housed in a controlled environment, exposed to 12 h:12 h light-dark cycle and fed standard rat diet. On the day of experiment, animals were randomly assigned to four groups injected different agents via tail vein. For group receiving LPS, a bolus dose (8 mg·kg^{-1}, 5 g·L^{-1}) of LPS was injected into the tail vein. For group of CCK-8+LPS, a bolus dose (40 μg·kg^{-1}, 0.05 g·L^{-1}) of CCK-8 was administered 10 min before injection of LPS. Negative control animals received saline, CCK-8 (40 μg·kg^{-1}) was also administe red alone in the other group.

Mean arterial blood pressure (MABP) detection Catheter was inserted into arteriae femoralis before agents administration and MABP was detected using physiology record instrument (RM-6000, Japan). ES model was made by LPS administration, the effect of CCK-8 on MABP of ES rats was observed.

Enzyme linked immunoabsorbant assay (ELISA) Animals were sacrificed at 2 h or 6 h after treated with LPS, spleen, lung and hear t were rapidly excised, rinsed of blood, and the blood was centrifuged for collection of serum. The samples were stored at -80 ℃. The samples collected at 2 h were for the assay of TNF-α and at 6 h for the assay of IL-1β and IL-6. Serum TNF-α was measured using an ELISA kit (Bender MedSystem, Austria) specific for rat TNF-α with an inter-assay coefficient of variation to be <10% and intra-assay coefficient of variation to be <5%; the lowest limit of detection was 17 ng·L-1. Serum IL-1β was determined with a rat ELISA (Endogen Inc, USA). Intra- and inter-assay coefficients of variation were 5.3%-6.1% and 6.8%-8.8%, respectively. The limit of detection for this assay was <12 ng·L^{-1}. Serum IL-6 was determined with a rat ELISA (Endogen Inc, USA). The intra and inter-assay coefficients of variation were 8.3%-9.2% and 6.9%-7.8%, respectively. The lowest limit of detection was 16 ng·L^{-1}.

Forzen tissue samples were weighed and placed in homogenization buffer (4 ℃) at a ratio of 100 mg per milliliter of buffer. Buffer contained a protease-inhibitor cocktail including 1 mmol·L^{-1} phenylmethylsulfon glfluoride (PMSF), 1 mg·L^{-1} pepstatin A, 1 mg·L^{-1} aprotinin, and 1 mg·L^{-1} leupeptin in phosphate-buffered saline solution, pH 7.2, containing 5 g·L^{-1} Triton X-100. Samples were homogenized and centrifuged at 18000 r·min^{-1}. Tissue supernatants were analyzed for TNF-α, IL-1β and IL-6 using the above described ELISAs.

Statistical analysis

Data were reported as x±s. Statistical differences between values from different groups were determined by one way ANOVA. Significance was set at P<0.05.

RESULTS

Changes of MABP

There was no significant difference among groups before treatment. LPS administr ation resulted in a significant sustained decrease in MABP during the period of 2 h, decreased to 7.82±0.43 kPa 30 min after LPS administration and restored to 9.33±0.63 kPa by pretreatment with CCK-8 (Figure 1).

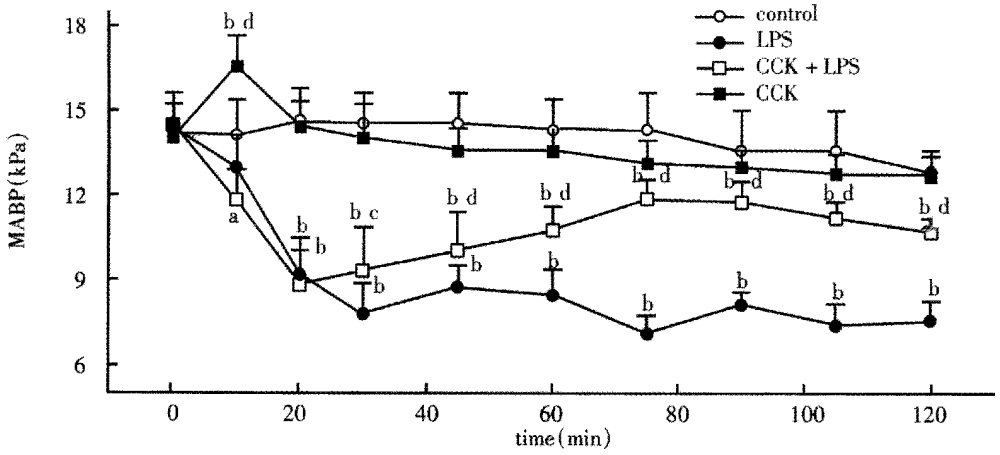

Figure 1 Mean arterial blood pressure (MABP) of animals injected normal saline, LPS, CCK+LPS and CCK. n = 6, ^aP<0.05, ^bP<0.01, *vs* Control; ^cP<0.05, ^dP<0.01, *vs* LPS.

Serum cytokine levels

TNF-α and IL-1β were not detectable in the serum of saline or CCK-8 treated rats, while IL-6 concentration in serum of saline or CCK-8 treated rats were 128±22 ng·L^{-1} and 84±78 ng·L^{-1}, respectively. Significant increase in serum level of TNF-α was observed in LPS group at 2 h to an average of 277±86 ng·L^{-1}, which was inhibited by CCK-8 to 12±21 ng·L^{-1}. Serum levels of IL-1β and IL-6 increased to 43±9 ng·L^{-1} and 3567±687 ng·L^{-1}, respectively, 6 h after administration of LPS, while administration of CCK-8 prior to LPS significantly inhibited this LPS induced increase in IL-1β and IL-6 concentration (not detectable and to 1797±69 ng·L^{-1}, respectively. LPS elevated the serum level of IL-6 significantly, while serum levels of TNF-α and IL-1β rose significantly but less than IL-6 (Figure 2).

Tissue cytokine contents

Spleen TNF-α content was significantly higher 2 h after administration of LPS than in control animals 941±149 ng·L^{-1} *vs* 282±30 ng·L^{-1}, P<0.01, while CCK-8 significantly inhibited the LPS induced increase of TNF-α 462±87 ng·L^{-1}, P<0.01. Similar results of TNF-α were noted in the lung and heart following 2 h post-LPS administration. CCK-8 pretreatment attenuated LPS-

induced increases of IL-1β and IL-6 contents in spleen and lung 6 h after LPS administration. LPS elevated content of IL-1β in the spleen and lung significantly 5184±85 ng·L^{-1} *vs* 1047±21 ng·L^{-1} and 4050±614 ng·L^{-1} *vs* not detectable, respectively (Figure 2), while the levels of TNF-α and IL-6 rose but in less extent than IL-1β. CCK-8 pretreatment attenuated LPS-induced increases of IL-1β content in the heart 6 h after LPS administration from 621±145 ng·L^{-1} to 282±93 ng·L^{-1}, P<0.01. No significant changes were noted in contents of cytokines following CCK-8 administration compared with normal saline administration.

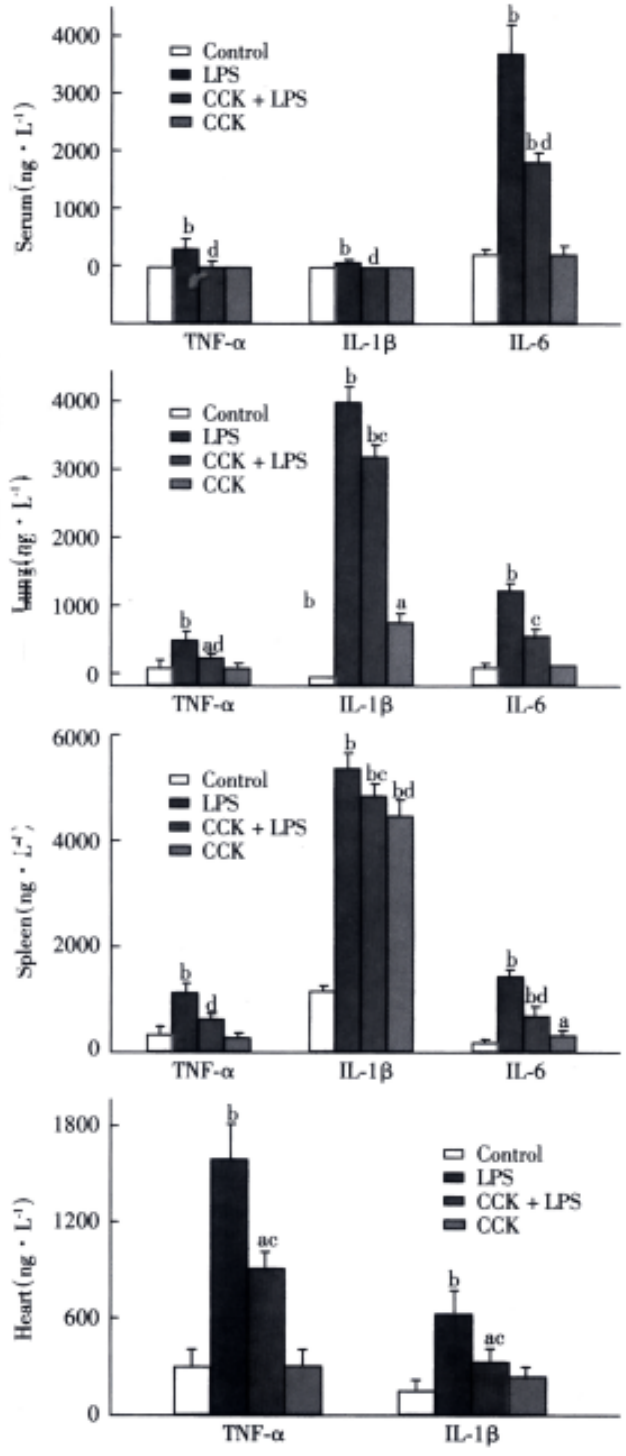

Figure 2 Effects of CCK on TNF-α, IL-1β and IL-6 2 h (TNF-α) or 6 h (IL-1β and IL-6) following LPS administration. n = 6. [a]P<0.05, [b]P<0.01, *vs* Control; [c]P<0.05, [d]P<0.01, *vs* LPS.

DISCUSSION

Interaction of the central nervous system (CNS) with immune system can occur via several endocrine pathways, the presence of various neuropeptide receptors on cells of the immune system suggests more direct communication pathways. Moreover, immune cells release cytokines, which can influence neuronal activity. Thus direct communication between the CNS and the immune system may well be bi-directional[22]. The present study show that administration of brain- gut peptide, CCK-8, prevents the LPS-induced decrease of MABP and attenuates LPS-induced increase of proinflammatory cytokines (TNF-α, IL-1β and IL-6) *in vivo*. Inhibition of these cytokines by CCK-8 may have contributed to the prevention of ES, such as restoring the blood pressure. The macrophages are the sources of proinflammatory cytokines (TNF-α, IL-1 and IL-6)[23]. These three important proinflammatory cytokines respond to the initial stimulation[24] and play predominant roles in the normal inflammatory response. Exaggerated endogenous production is likely responsible for the complications associated with sepsis such as tissue injury and ultimate organ failure[25-28], induce a wide range of pathophysiologic changes in LPS-induced ES. TNF is known to have cytotoxic and cytostatic effects on certain tumor cells, and with a pivotal role in inflammatory reactions and regulation of immunologic al response[29,30]. TNF can enhance the adhesion of PMN to endothelium[31]. Although the migration of leukocytes to and accumulation at the site of inflammation are important for killing micro-organisms, activation of recruited neutrophils coupled with excessive release of oxygen metabolites and proinflammatory mediators may induce tissue injury which can lead to organ dysfunction[32]. TNF-α was mainly produced in the early stage of endotoxemia, and decreased obviously from 6 h to 9 h after challenge[33]. The effect of TNF-α is influenced by other cytokines[34]. IL-1β and IL-6, produced mainly by activated phagocytes and lymphocytes, show a wide variety of biological functions. They can influence secretion of other cytokines and inflammatory mediators in an autocrine or paracrine fashion, induce expression of surface immune molecules of antigen-presenting cells to serve as an activation factor and differentiation factor on T cells and B cells, mediate immunoglobulin secretion, activate the complements, killer cells and phagocytes, and enhance tissue injury mediated by cellular and humoral immune reactions[35]. Most of our knowledge concerning the induction of these cytokines in response to LPS or gram-negative bacteria comes from studies performed either in vitro or in fluids obtained from animal models or patients with sepsis, whereas the major site of action of the cytokines are probably at the tissue and cell level[36]. Our study show that the increase in spleen and lung content of IL-6 following LPS administration was lesser than in serum. In contrast, the increase in spleen and lung content of IL-1β following LPS administration was more than in serum, while the content of IL-1β in heart was more than in serum and less than in spleen and lung. The results demonstrated a dissociation or poor correlation between intra-splenic, intra-pulmonic or intra-cardiac abundance of TNF-α, IL-1β and IL-6 and the magnitude of the increase in circulating cytokine levels. The production of cytokines varies from organ to organ in response to systemic administration of LPS. Activated macrophages in spleen and lung are known to be active producers of pro-inflammatory cytokines. This has led to the suggestion that spleen, lung and heart are important organs in the production of TNF-α

and IL-1β following LPS administration, releasing the cytokines produced into the circulation, thereby contributing to the elevated serum levels of the cytokines. We found that CCK-8 significantly inhibits LPS-induced increase of pro-inflammatory cytokines in vivo. While Cunningham *et al*[37] reported that CCK-8 stimulated production of TNF-α, IL-1β and IL-6 by monocytes, but was considerably less than LPS response. Later studies[38]suggest that the increase of cytokines induced by CCK-8 may be due to the detection of endotoxin/LPS in medium.

Despite convincing data indicating the protective function of CCK-8 to organism in ES, the precise mechanism remains elusive. Our previous study demons trated that exogenous or endogenous CCK reversed pulmonary arterial hypertension (PAH) during endotoxic shock[39]. In vitro studies showed CCK can protect pulmonary artery endothelium against detrimental effects by LPS or TNF-α[40,41]. The anti-inflammatory effect of CCK- showed in this study may mediate the cell protective function of CCK-8 in ES. CCK-8 dose dependently attenuated gastric lesions induced by 75% ethanol, blockade of CCKA receptor with loxiglumide abolished the protective effects of CCK[42]. The effect of CCK may depend on its modulation of immunocyte function, which could occur through CCK receptors. There exists CCKB receptor in human lymphoid cells[43]. It was demonstrated recently that wild-type transcripts of both CCK receptor subtypes and splice variants of the CCK-B/gastrin receptor are expressed in nontransformed human mononuclear cells[44]. Endogenous opioids, such as β-endorphin, are produced within the immune system and are active regulators of the immune response. There were elevations in the circulating levels of β-end orphin in LPS-treated animals, which indicated the potential contribution to modulating the tissue cytokine response to LPS[45]. The hypotensive response was related to release of endorphin, while the mechanism of CCK-8 anti-ES may be related to its anti-endorphin effect[3]. CCK-8 decreased heart rate via CCK-A receptors located in the atrium of the rats[46] . Intravenous administration of CCK-8 to guinea pigs inhibited the motility of the left ventricle of heart[47]. CCK had hypertensive effect on rats and increased the cardiac contraction amplitude, decreased coronary outflow, while it had no effect on heart rate[48]. CCK-B agonist pentagastrin induced significant and very rapid, dose-dependent elevations in adrenocorticotropin and cortisol levels, and significant elevations in heart rate and blood pressure were seen[49]. The different effect of CCK-8 on cardiovascular system may due to the different doses and animals. However, additional mechanisms may be present in mediating the cytokine responses to LPS and CCK. Our study shows for the first time a previously unknown physiological function for CCK- 8 in a model of LPS-induced ES. CCK-8 may have modulatory effects on the immune functions of ES rats. CCK-8, therefore, might be used therapeutically to treat septic shock syndrome and other inflammatory disease states.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Effects of extract F of red-rooted Salvia on mucosal lesions of gastric corpus and antrum induced by hemorrhagic shock-reperfusion in rats

Li-Hong Zhang[1], Chang-Bai Yao[2] and He-Quan Li[3]

[1]Department of Anesthesiology, Second Clinical College, China Medical University, Shenyang 110003, Liaoning Province, China
[2]Department of General Surgery, Second Clinical College, China Medical University
[3]Department of Pathophysiology, China medical University
Supported by the National Natural Science Foundation of China, No. 3870890
Correspondence to: Li-Hong Zhang, Department of Anesthesiology, Second Clinical College, China Medical University, 36 Sanhao Street, Shenyang 110003, Liaoning Province, China. cumzhanglihong@163.net
Telephone: +86-24-24133628
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Abstract

AIM **To compare the effects of extract F of red-rooted Salvia (EFRRS) on mucosal lesions of gastric corpus and antrum induced by hemorrhagic shock and reperfusion in rats.**

METHODS **The rats were subject to hemorrhagic shock and followed by reperfusion, and were divided randomly into two groups. Group 1 received saline, and group 2 received EFRRS intravenously. The index of gastric mucosal lesions (IGML) was expressed as the percentage of lesional area in the corpus or antrum. The degree of gastric mucosal lesions (DGML) was catalogued grade 0, 1, 2 and 3. The concentrations of prostaglandins (PGs) were measured by radioimmunoassay. The concentration of MDA was measured according to the procedures of Asakawa. The activity of SOD was measured by the biochemical way. The growth rates or inhibitory rates of above-mentioned parametes were calculated.**

RESULTS **As compared with IGML (%), grade 3 damage (%) and MDA content (nmol/g tissue) of gastric antrum which were respectively 7.96±0.59 , 34.86±4.96 and 156.98±16.12, those of gastric corpus which were respectively 23.18±6.82, 58.44±9.07 and 230.56±19.37 increased markedly ($P<0.01$), whereas the grade 0 damage, grade 1 damage, the concentrat ions of PGE_2 and PGI_2 (pg/mg tissue), the ratio of PGI_2/TXA_2 and the activity of SOD (U/g tissue) of corpus which were respectively 3.01±1.01, 8.35±1.95, 540.48±182.78, 714.38±123.74, 17.38±5.93 and 134.29±13.35 were markedly lower than those of antrum which were respectively 13.92±2.25, 26.78±6.06, 2218.56±433.12, 2531.76±492.35, 43.46±8.51 and 187.45±17.67 ($P<0.01$) after hemorrhagic shock and reperfusion. After intravenous EFRRS, the growth rates (%) of grade 0 damage, grade 1 damage, the concentrations of PGE_2 and PGI_2, the ratio of PGI_2/TXA_2 and the activity of SOD of corpus which were respectively 632.56, 308.62, 40.75, 74.75, 92.29 and 122.25 were higher than those in antrum which were respectively 104.89, 58.40, 11.12, 56.58, 30.65 and 82.64, whereas the inhibitory rates (%) of IGML, grade 3 damage and MDA content of gastric corpus were 82.93, 65.32 and 59.09, being higher than those of gastric antrum which were 76.64, 53.18 and 42.37.**

CONCLUSION **After hemorrhagic shock-reperfusion, the gastric mucosal lesions in the corpus were more severe than those in the antrum, which were related not only to the different distribution of endogenous PGs in the mucosa, but also to the different ability of anti-oxidation of the mucosa. The protective effect of EFRRS on the gastric mucosa in the corpus was more evident than that in the antrum, which was related to higher growth degree of PGs contents and anti-oxitative ability in gastric corpus after administratio n of EFRRS.**

Subject headings plant extracts/pharmacology; gastric mucosa/pathology; shock hemorrhagic; reperfusion; hydroxyl radical

Zhang LH, Yao CB, Li HQ. Effects of extract F of red-rooted Salvia on mucosal lesions of gastric corpus and antrum induced by hemorrhagic shock-reperfusion in rats. *World J Gastroenterol*, 2001; 7(4):672-677

INTRODUCTION

More and more stress has been put on gastric mucosal reperfusional injury. Though scholars both at home and abroad have performed plenty of researches on it, there has been no satisfying method or drug yet[1-5]. Red-rooted Salvia is the traditional Chinese medicine for accelerating blood circulation and ameliorating congestion, and its pharmacological effect is very extensive. Resources of red-rooted Salvia in China is sufficient. It has been evidenced that the proportion of red-rooted Salvia dissolving in water can treat acute or chronic gastric mucosal lesions, and has protective effect on gastric mucosa. Extract F of red-rooted Salvia (EFRRS) is extracted from the proportion of red-rooted Salvia dissolving in water[6-11]. Because Prostaglandins (PGs) and oxygen free radical (OFR) play important roles in reper fusional injury[12-18], the present experiment was AIMed at studying the endogen ous PGs and the anti-oxidation of the gastric corpus and the antrum, and comparing their ability to resist the lesions induced by hemorrhagic shock-reperfusi on in rats and the protective effect of EFRRS on the gastric corpus and the antrum.

METERIALS AND METHODS

Drug

EFRRS was extracted from red-rooted Salvia solution provided by Chemical Assay Center of China Medical University using column chromatography.

Animal models

Male Wistar rats, weighing 260 g-300 g, were fasted overnight. The rats were anesthetized intraperitoneally with 5 mg·100g^{-1} of 20% Urethane. Tracheostomy was performed and PE-250 tubing was inserted into the trachea to maintain an open airway. Then open the abdomen and lavage the gastric lumen gently with warm saline. The right carotid artery was cannulated using a polyethylene tube to monitor the blood pressure. The femoral artery was canulated for withdrawing the blood and reinfused the shed blood. After the blood pressure was stabilized, normal saline or EFRRS (1 g·100gw·t^{-1}) was administered for 25 min via a tail vein (0.03 mL·min^{-1}). 1 mL of 0.1 mol/L HCl per 100 g body wt was then instilled into the stomach via the gastric tube, five min after the intragastric instillation of HCL, blood was withdrawn from the femoral artery. The mean arterial blood pressure fell to 2.67 kPa-4.0 kPa and was maintained at that level for 20 min. The shed blood was then reinfused, and 20 min later the rats were sacrificed[19]. Rats were allocated into two groups. Group 1 (n = 9) received NS via the tail vein, and group 2 (n = 7) received EFRRS (1 g·100gwt^{-1}) via the tail vein.

Index of gastric mucosal lesions (IGML) and inhibitory rate (IR) of IGML

The corpus and antrum lesional areas were measured in square millimeters. IGML was expressed as the percentage of lesional area in the corpus or antrum[20]. IR was calculated by the following formula.

$$IR=\frac{\text{Difference of mean value of lesional areas between the two groups}}{\text{Mean value of lesional area in group 1}}\times 100\%$$

Depth of gastric mucosal lesions (DGML) , growth rate(GR) and IR of DGML

After meaursing the lesional areas, the samples for light microscopy (LM) and scanning electron microscopy (SEM) were taken from the proximal anterior wall of the corpus or the middle of the antrum. The samples analyzed by LM were evaluated as follows[21]. The damage was graded as 0, 1, 2 and 3. Grade 0 was defined as normal intact surface mucous cells with intact gastric pits and glands. Grade 1: Surface mucous cells were vacuolated with pyknotic nuclei. Some exfoliation was present. Grade 2: In addition to the above changes, the cells lining the gastric pits were also disrupted and exfoliated. Grade 3: Cell destruction extended into the gastric glands (Figures 1-6). Samples analyzed by SEM were evaluated as follows[22]. Grade 0: The mucosa showed closely packed, polygonal surface mucous cells and narrow openings to the gastric pits. Grade 1: Surface cells were flattened with irregular shape, and gaps between individual cells. Grade 2: The basal lamina was exposed and was largely devoid of surface mucous cells, but still showed continuity, wide openings to the gastric pits were visible. The picture resembled that of a honeycomb. Grade 3: Most of the basal lamina were disrupted, and only a portion being still intact. Regular surface cells were no longer present (Figures 7-12). A close correlation between LM and SEM grading was found (r = 0.846, P<0.01). The percentage of damage of each grade was calculated in each group. The METHODS to calculate the GR or IR of DGML were the same as that of IR of the lesional area.

PGs contents and GRs of PGs

Prostaglandin E_2 (PGE_2), 6-keto-$PGF_{1\alpha}$ (6-keto-$PGF_{1\alpha}$: 6-keto is the metabolite of PGI_2), and TXB_2 (metabolite of TXA_2) boxes were provided by Biochemistry Laboratory of Liberal Army General Hospital, and their concentrations were assayed by using radiommunoassay. GRs of PGs were calculated in the same way as that of IR of the lesional area.

MDA content and IR of MDA

Malondialdehyde (MDA) is the final metabolism product of OFR. It can be measured by the way of Asakawa[23]. IR of MDA was calculated in the same way as that of IR of lesional area.

SOD activity and GR of SOD

Superoxide dimutase (SOD) was measured according to the biochemical method[24]. GR of SOD was calculated in the same way as that of IR of lesional area.

In all experiments, the date was represented by the mean value ± standard error, and analyzed by paired t test. P value of <0.05 was considered significant.

RESULTS

Comparison of IGML and its IRs between the gastric corpus and antrum

The results are shown in Table 1. After hemorrhagic shock-reperfusion, IGML in the corpus was much higher than that in the antrum (P<0.01). As compared with that in the corpus, IR in the antrum was lower after administratio n of EFRRS.

Table 1 IGML and its IR in gastric corpus and antrum (%, $\bar{x}\pm s$)

	IGML	IR
Group 1 (n = 9)		
Corpus	23.18±6.82	
Antrum	7.96±0.59[b]	
Group 2 (n = 7)		
Corpus	4.42±1.39[a]	82.93
Antrum	1.62±0.37[a]	76.64

[b]P<0.01, *vs* corpus; [a]P<0.01, *vs* group 1.

Comparison of DGML, and its IRs and GRs between the gastric corpus and antrum

The results are shown in Table 2. As compared with those in the corpus, grade 0 and 1 damages in the antrum were much increased (P<0.01), and grade 3 damage markedly decreased (P<0.01) after hemorrhagic shock-reperfusion. After administration of EFRRS, the GR of grade 0 and 1 damage and the IR of grade 3 damage in the antrum were much less than those in the corpus.

Comparison of the concentrations of PGE_2, 6-keto and TXB_2, the ratio of 6-keto/TXB_2 and their GRs between the gastric corpus and antrum

In Table 3, higher PGE_2 and 6-keto levels and 6-keto/TXB_2 ratio were found in the antrum compared with those in the corpus after hemorrhagic shock-reperfusion (P<0.01), and the GRs of PGE_2, 6-keto and 6-keto/TXB_2 in the corpus were higher than those in the antrum after administration of EFRRS.

Table 2 DGML and its IR or GR in the gastric corpus and antrum (%, $\bar{x}\pm s$)

	DGML							
	0	GR	1	GR	2	IR	3	IR
Group 1 (n = 7)								
Corpus	3.01±1.01		8.35±1.95		31.32±4.49		58.44±9.07	
Antrum	13.92±2.25[b]		26.78±6.06[b]		25.98±8.32		34.86±4.96[b]	
Group 2 (n = 6)								
Corpus	22.05±5.96[a]	632.56	34.12±8.12[a]	308.62	25.96±10.04	17.11	20.32±6.95[a]	65.32
Antrum	28.52±8.12[a]	104.89	42.42±8.58[a]	58.40	14.03±3.13[a]	45.98	16.32±4.05[a]	53.18

[b]P<0.01, *vs* corpus; [a]P<0.01, *vs* group 1.

Table 3 PGs contents (pg/mg tissue), and their GRs in the gastric corpus and antrum (%, $\bar{x}\pm s$)

Group	PGE_2	GR	6-keto	GR	TXB_2	6-Keto/TXB_2	GR
Group 1 (n = 6)							
Corpus	540.48±182.78		714.38±123.74		58.28±6.74	17.38±5.93	
Antrum	2218.56±433.12[b]		2531.76±492.35[b]		62.49±9.51	43.46±8.51[b]	
Group 2 (n = 6)							
Corpus	759.77±192.00[aa]	40.75	1248.37±158.54[a]	74.75	45.37±7.54[aa]	33.42±9.24[a]	92.29
Antrum	2465.17±480.36	11.12	2698.31±526.71	56.58	50.02±7.50[a]	56.78±5.45[a]	30.65

[b]P<0.01, *vs* corpus; [a]P<0.01, *vs* group 1; [aa]P<0.05, *vs* group 1.

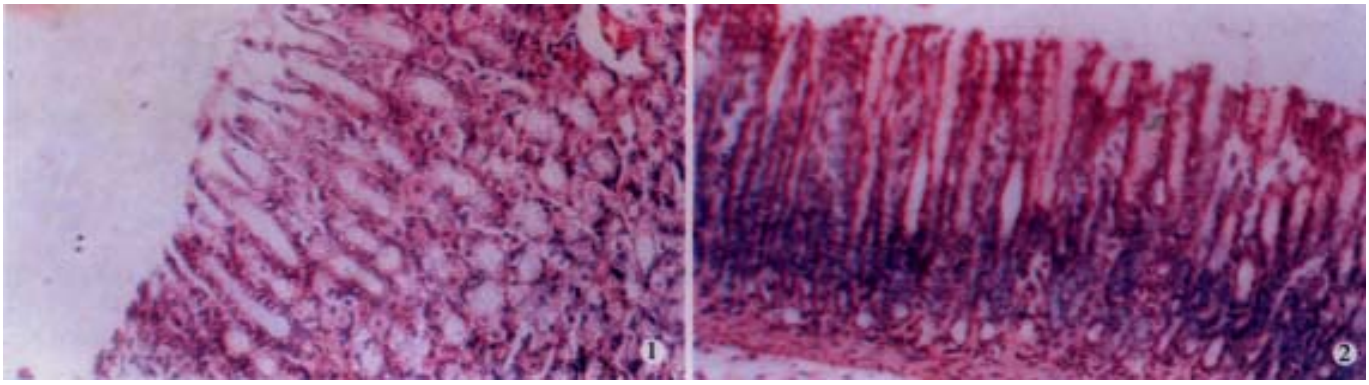
Comparison of MDA content, IR of MDA, SOD activity and GR of SOD between the gastric corpus and antrum

In Table 4, higher SOD activity and lower MDA level were found in the antrum com pared with those in the corpus after hemorrhagic shock-reperfusion (P<0 .01), and the GR of SOD and IR of MDA were higher in corpus than those in antrum after administration of EFRRS.

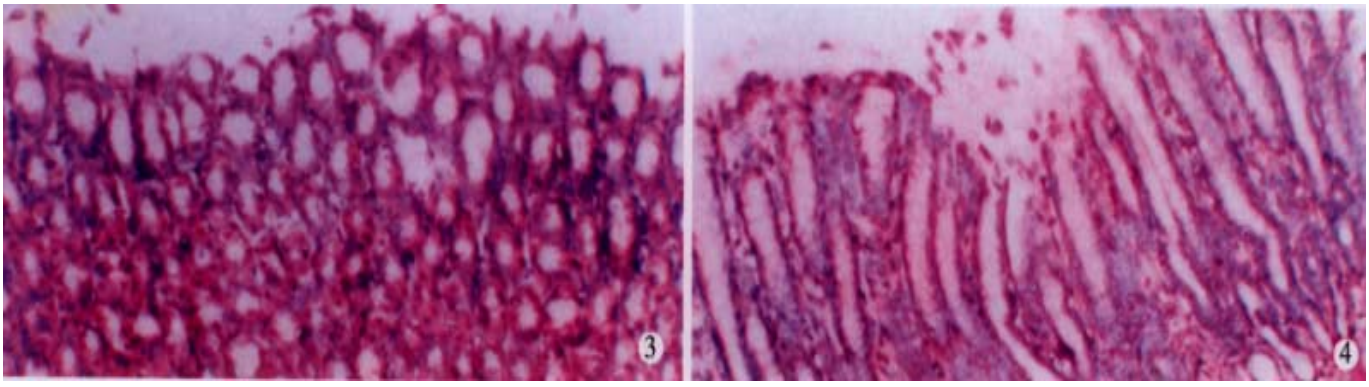
Table 4 MDA content (nmol/g tissue), SOD activity (U/g tissue), IR of MDA and GR of SOD in the gastric corpus and antrum (%, $\bar{x}\pm s$)

Group	MDA	IR	SOD	GR
Group 1 (n = 6)				
Corpus	230.56±19.37		134.29±13.35	
Antrum	156.98±16.12[b]		187.45±17.67[b]	
Group 2 (n = 6)				
Corpus	94.32±11.32[a]	59.09	298.47±20.12[a]	122.25
Antrum	90.46±12.45[a]	42.37	342.35±26.58[a]	82.64

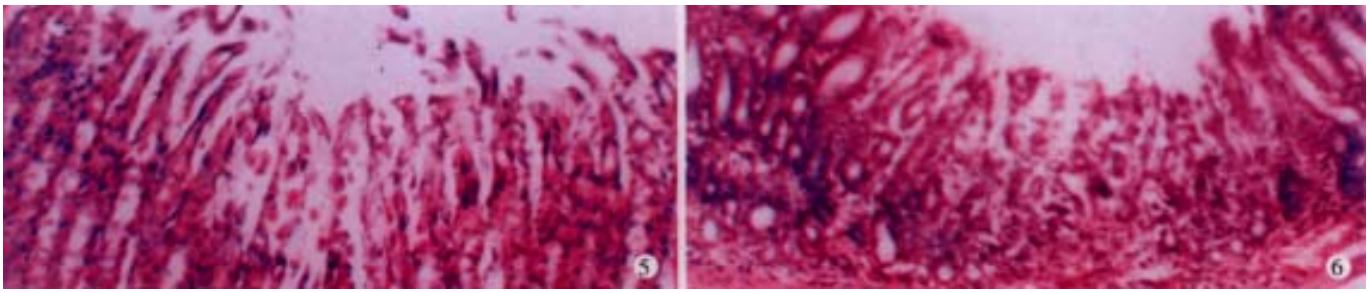
[b]P<0.01, *vs* corpus; [a]P<0.01, *vs* group 1.



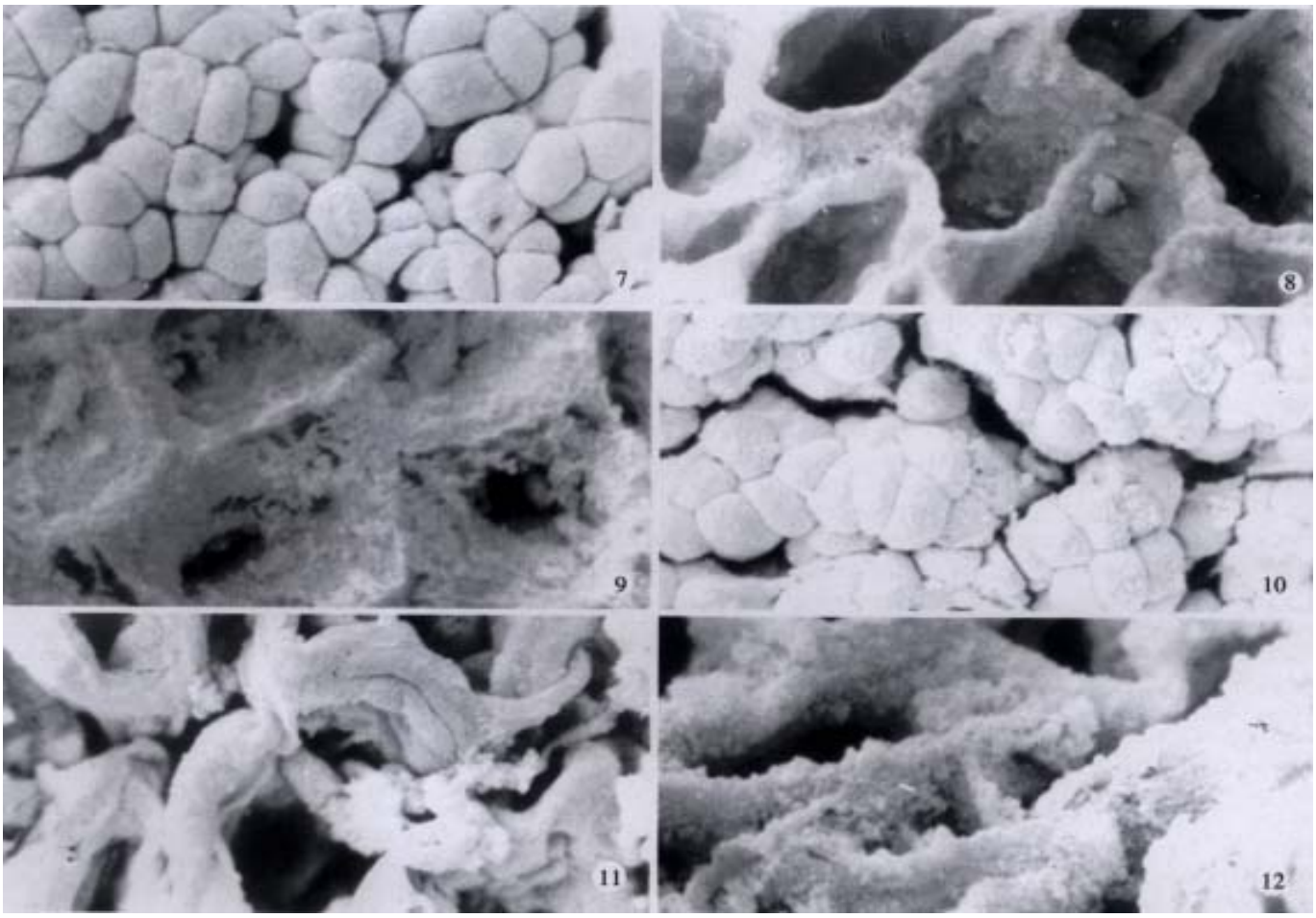
Figures 1, 2 Grade 1 damage in gastric corpus and antr um. LM×330.
In grade 1 damage, surface mucous cells were damaged.



Figures 3, 4 Grade 2 damage in gastric corpus and antrum. LM×330
In grade 2 damage, the cells kining the gastric pits were also disrupted.



Figures 5, 6 Grade 3 damage in gastric corpus and antrum. LM×330
In grade 3 damage, cell destruction extended into the gastric glands.



Figures 7, 8, 9 Grade 1, 2 and 3 damage in gastric corpus. SEM×1500
Figures 10, 11, 12 Grade 1, 2 and 3 damage in gastric antrum. SEM×1500
In grade 1 damage, surface cells were of irregular shape, and gaps between individual cells were present. In grade 2 damage, the basal lamina was exposed, but still showed continuity. Wide openings to the gastric pits were visible. In grade 3 damage, most of the basal lamina was disrupted, Regular surface cells were no longer present.

DISCUSSION

Reperfusion after hemorrhagic shock can lead to multiple organ damage, *i.e.* reperfusional injury. The gastric lesions include stress ulcer, hemorrhage, necrosis, or perforation[25-28]. The present study showed that the area and depth of gastric mucosal lesions caused by hemorrhagic shock-reperfusion in the gastric corpus of rats were more severe than those in the antrum. This indicated that there were differences in resistance in gastric mucosa of the corpus and the antrum, which was probably related to differences in gastric mucosal blood flow, energy metabolism and the capacity to dispose the influxing hydrogen ion, but most propably was related with the different distribution of endogenous PGs and the different ability of anti-oxidation[29-33]. The present study also showed that the protective role of EFRRS was different in the gastric corpus and the antrum, EFRRS possessed more powerful capability to reduce the area of lesions and to lighten the extent of lesions in corpus than those in the antrum, indicating EFRRS had potential protective effect on the corpus mucosa, which was related to the higher changes of PGs and OFR in the corpus caused by EFRRS.

It was generally thought that gastric mucosa was affected by both injury factors as gastric acid and pepsin and protective factors as PGs and gastric mucus. Large quantities of PGs were

found in the gastric mucosa. Numerous studies have doc umented that PGs possessed potent cytoprotective action. PGE_2 could obviously inhibit the secretion of basal gastric acid and acid stimulated by histamine, p entagastrin and food in dogs and humans. In addition, PGE_2 could increase the gastric mucus layer. PGE_2 and PGI_2 could dilate the blood vessel, increase the blood flow and carbohydrate secretion, and enhance the resistibility of gastric mucosa to injury. PGs could also lengthen the life span of epithelia and thick en the mucosa layer[34-37]. In many physiological and pathophysiological conditions, PGI_2 has protective effect on gastric mucosa. On the contrary, TXA_2 may aggravate the gastric mucosal injury[38,39]. The present findings showed the PGE_2 and PGI_2 contents and PGI_2/TXA_2 ratio in the antrum were markedly higher than those in the corpus after hemorrhagic shock-reperfusion, showing gastric antrum was more resistant than gastric corpus. Arakawa[40] found PGE_2 levels in the gastric corpus were significantly lower than that in the antrum, and drug like indomethacin could easily damage the mucosa of gastric corpus. He thought that the concentration of endogenous PGE_2 decided the defensive ability of gastric mucosa. The present study also showed that PGE_2 and 6-keto levels and 6-keto/TXB_2 ratio in the antrum and the corpus both increased after administration of EFRRS, but the GRs of PGE_2, 6-keto and 6-keto/TXB_2 in corpus were higher than t hose in antrum, demonstrating the reinforcement of defensive ability of gastric corpus was more powerful after administration of EFRRS.

OFR played an important role in reperfusional injury. OFR caused lipid peroxid ation (LPO) of polyunsaturated fatty acid of biomembrane, which resulted in the impairment of metabolism and function of cells, even the death of cells. Plenty of OFRs could lead to irreversible damage of gastric mucosa, because they could cause intracellular calcium overload besides of extensive LPO of tissues and cells[41-47]. OFR was produced by the system of enzyme and no-enzyme. Malondialdehyde (MDA) is the metabolite of LPO of OFR, and may reflect the degree of cells attacked by OFR, therefore MDA is usually an marker to monitor OFR. Superoxide dismutase (SOD) can clear superoxide anion,and may reflect the ability of scavenging system of free radical. Under normal conditions, OFR could be promptly cleared by the body. Only when the production of OFR markedly increased, or the ability of scavenging OFR much decreased, tissues were injured[48,49]. The study showed that there were higher SOD activity and lower MDA level in the antrum compared with those in corpus after hemorrhagic shock-reperfusion, so that the ability of anti-oxidation was more powerful in gastric antrum, and the reperfusional injury was easier in gastric corpus. This study also showed that the activity of SOD and the concentration of MDA decreased in the antrum and the corpus after administration of EFRRS, but the GR of SOD and IR of MDA in corpus were higher than those in antrum, demonstrating reinforcement of anti-oxidation of gastric corpus was more powerful after administration of EFRRS. The increase of SOD could accelerate the clearance of OFR to protect cell from the attact of OFR, while the decrease of OFR made cells produce more SOD, and clear more OFRs to form good cycle possessing the role of protective gastric mucosa.

It had been demonstrated in our previous studies[50-54], that EFRRS could increase the PGs contents, decrease the production of OFRs, and had calcium block effect, which resulted in some effects against gastric mucosal lesions induced by hemorrhagic shock-reperfusion. The present study discussed the mechanisms that the gastric injury in the corpus was easier after hemorrhagic shock-reperfusion, and the causes that the protective effects of EFRRS against gastric mucosal injury was moer powerful in the corpus through both PGs contents and OFR system. This made the researches of reperfusional injury of gastric mucosa and the protective effects of EFRRS perform more profoundly and detailedly.

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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Pathogenic and pathological characteristic of new type gosling viral enteritis first observed in China

An-Chun Cheng, Ming-Shu Wang, Xiao-Yue Chen, Yu-Fei Guo, Zhao-Yu Liu and Peng-Fei Fang

College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Xinkang Road, Yaan City 625014, Sichuan Province, China
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Correspondence to: An-Chun Cheng, College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Yaan 625014, Sichuan Province, China.Chenganchun@Hotmail.com; Anchun@sicau.edu.cn
Telephone: +86-835-2226327/2242544/2242326, Fax: +86-835-2242754
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Abstract

AIM **To study the purifying method and characteristics of new gosling viral enteritis virus (NGVEV), the etiological agent of new gosling viral enteritis (NGVE) which was first recognized in China, as well as the pathomorphological development in goslings infected artificially with NGVEV.**

METHODS **① NGVEV virions were purified by the procedure of treatment with chloroform and ammonium sulfate precipitation, dialysis to remove the sulfate radical and ammonium ion and separation by gel filtration chromatog raphy, and SDS-PAGE. ② Forty 2-day-old White Sichuan goslings were orally administered with NGVEV and 24 hr later 2 birds were randomly selected and killed at 24 hr intervals until death occurred. Specimens(duodenum, ileum, liver, heart, kidney, spleen, lung, proventriculus, pancreas, esophagus, and the intestinal embolus) were taken until all birds in this group died and were sectioned and stained with hemotoxylin and eosin and studied by light microscope.**

RESULTS **NGVEV shared the typical characteristics of Adenovirus and which structural proteins consisted of 15 polypeptides. Necrosis and sloughing of the epithelial cells covering the villus tips of the duodenum were first observed in goslings 2 days postinfection artificially with NGVEV. With the progress of infection, this lesion rapidly occurred in the epithelium at the base of the villus and with infiltration of the inflammatory cells, the jejunum tended to be involved. With the intensification of mucosa necrosis and inflammatory exudation of the small intestine, fibrinonecrotic enteritis was further developed and embolus composed of either intestinal contents wrapped by pseudomembrane or of the mixture of fibrous exudate and necrotic intestinal mucosa were observed in the middle-lower part of the small intestine. This structure occluded the intestinal tract and made the intestine dilated in appearance. The intestinal glandular cells underwent degeneration, necrosis and might be found sloughed into the lumen. Hemorrhage and hyperemia could be observed on the lung and kidney. Epithelial cells of the renal tubular underwent degeneration. In some cases, granular degeneration and fatty degeneration could be found in the liver and in some cases at a later stage of this disease the epithelial cells of trachea and proventriculus might be found sloughed. In some cases at an early stage of this disease, cardiac hyperemia and hemorrhage could be observed. Esophagus, pancreas and brain were found normal. Analyses and comparisons between the pathologic lesions of NGVE and Gosling Plague (GP) were available in this paper as well.**

CONCLUSION **① NGVEV is adenovirus. ② Pathological characteristic could be as the data for NGVE diagnosis.**

Subject headings enteritis/virology; enteritis/pathology; adenoviridae/isolation & purification; gosling/virology; gosling/new infectious disease

Cheng AC, Wang MS, Chen XY, Guo YF, Liu ZY, Fang PF. Pathogenic and Pathological characteristic of new type gosling viral enteritis first observed in China. *World J Gastroenterol*, 2001;7(5):678-684

INTRODUCTION

Cheng *et al*[1] reported that a disease which extremely resembled Gosling Plague (GP)[2-5] in aspects of epizootiology, clinical signs and pathologic lesions was observed in goslings less than 30 days of age in a variety of areas in Sichuan province and that the sausagelike lesion found in birds which died at a later stage of the acute case was almost identical to that observed in the case of GP in terms of gross and histopathological changes. This disease with the name of NGVE was regarded as a new one caused by Adenovirus through preliminary epizootiological investigation, clinical signs, histopathological examination, causal agent isolation and the experiment of artificial infection[1,7-9]. The isolated virus of NGVEV was capable of reproducing the disease identical to natural infection in aspects of clinical signs and histopathological lesions by a variety of routes and the oral route was considered to be the best one. Goslings infected with duck plague virus (DPV) or gosling plague virus (GPV) could be observed enteritis[2,3,5,10-16], but the 36 strains of NGVEV isolated from a variety of areas were antigenically identical and no antigenic relationships with DPV and GPV was demonstrated[1,2,6-9]. The characteristics, purifying method, structural proteins of the representative causal agent of NGVEV-CN as well as the histopathologic developments observed in goslings infected with NGVEV are reported as follows:

MATERIALS AND METHOD

Virus strain

The strain of NGVEV with its minimum lethal dose being 10^{-6}/0.5 mL to 1-day-old goslings by oral administration was isolated from the natural cases of NGVE[1].

Characteristics of NGVEV-CN

According to directions presented in referencer[17], experiments were performed to identify the following properties of NGVEV-CN: hemagglutination, buoyant density, sensitivity to temperature, pH, chloroform and trypsin, type of the nucleic acid and type of the nucleic acid strand.

Virus purification and viral structural polypeptide analysis by SDS-PAGE

Experiments were performed according to directions presented in referencer[18-21].

Virus purification The virus strain of NGVEV-CN inoculated on the primary duck fibroblasts was harvested when 75% of the cells showed cytopathic effect and were centrifugated at 4000 rpm for 10 min after treated with chloroform for 5 times. The supernatant was collected and with stir was slowly added the same volume of saturated ammonium sulfate. After placed overnight at 4 ℃ statically, it was subjected to centrifugation at 10 000 rpm for 1 hr. The precipitate was dissolved in small volume of sterile distilled water and then underwent dialysis to remove sulfate radical and ammoniumion. Sephadex G200 chromatography was performed to elute the solution with phosphate buffered saline solution (PBS) of 0.15 M and pH 7.2 as buffer. Nucleic acid-protein detector was employed to determine the absorbancy at 280 nm of the separately collected eluates. Cellulose acetate electrophoresis and disc polyacrylamide gel electrophoresis (PAGE) were performed to examine the purification of the isolated virus after the virus containing eluates sharing the same absorption peak were mixed. Transmission electronic microscope type H-600 was employed to examine the negatively stained virions.

Viral structural polypeptide analysis by SDS-PAGE 20 μL of the purified virus was boiled for 5 min after mixed with the same volume of buffer whose concentration was 2 times of that used for electroph oreses and the virus lysate underwent PAGE, with the discontinuous gradient gel containing 0.4% SDS. The gel was 1.5 mm, 11cm, 12 cm in depth, width, length respectively and the concentration of the stacking gel and the separating gel was 3% and 12.5% respectively. A solution of Tris-Gly with a pH of 6.8 was used as buffer and electric current was raised from 160 v to 200 v when the sample moved from the stacking gel to the concentrating gel. Electrophoresis was performed at 4 ℃ for 4 hr and six proteins were employed as molecular mass references. These proteins and their molecular masses (Daltons) were: chicken albumen lysozyme (14 400), trypsin inhibitor (20 100), bovine carbonic anhydrase (31 000), rabbit actin (43 000), bovine serum album in (66 200) and rabbit phosphorylase (97 400). The gel was then removed and stained with 0.25% Coomassie brilliant blue for 12 hr. After that, it was decolored with a solution containing methanol and glacial acetic acid until the proteins were visible as discrete blue bands and their background was hyaline, which took about 48 hr. The gel was then photoed by camera and scanned by automa tic gel image-forming and analysis system. After analyzed and processed by the software of Gel, molecular masses and relative percentages of each structural polypeptides were obtained.

The pathomorphological development observed in goslings artificially infected with NGVEV

Eighty 2-day-old White Sichuan geese whose mother birds were all vaccinated twice with attenuated GPV vaccine just before egg production were employed and each one was inoculated subcutaneously with 1mL anti-GPV hyperimmune serum and after observed for one day these birds were divided randomly into 2 groups with each containing 40. Each of the first group was administered orally with 0.5 mL NGVEV which was diluted 1:10 and 24 hr later 2 birds were randomly selected and killed at 24 hr intervals until death occurred in this group. Specimens were taken until all birds in this group died. Each of the second group was administered orally with 0.5 mL sterile physiological saline solution and was kept as control. One bird was killed each day and specimens were taken until all the birds in the first group died. The specimens included the duodenum, ileum, liver, heart, kidney, spleen, lung, proventriculus, pancreas, esophagus, and the intestinal embolus. These specimens were sectioned and stained with hemotoxylin and eosin and studied by light microscope. Special staining method of Maun methylene blue and eosin was also employed for detecting inclusion bodys.

RESULTS AND DISCUSSION

Characteristics of NGVEV-CN

Hemagglutination Under the temperature of 4℃, 25℃, 30℃, 37℃, 42℃, or with the pH of 6.6, 6.8, 7.0, 7.2, 7.4, or with either physio logical saline solution or phosphate buffered saline employed, the isolated virus of NGVEV did not agglutinate the newly prepared erythrocytes of chicken, duck, goose, pigeon, yellow cattle, buffalo or pig. This indicated that NGVEV did not have the property of hemagglutination and this agreed with the conclusion that "The majority of fowl adenovirus serotypes (group 1) do not hemagglutinate"[17].

Buoyant density The buoyant density of NGVEV in cesium chlorid e was 1.32 g/mL.

Sensitivity to temperature The ability of NGVEV to cause primary duck embryo fibroblasts CPE and to cause goslings mortality was unaffected by storage for 36 months at -15℃, for 20 months at 0℃, or for 45 days at 37℃. Ability to cause primary duck embryo fibroblasts CPE and infectivity to goslings were not destroyed by heating at 45℃ for 48 hr, at 56℃ for 5 hr, or at 60℃ for 1 hr. Heating for 5 min at 80℃ or for 10 sec at 96℃ (boiled water) could make the virus lose infectivity to goslings and make the virus lose the ability to cause primary duck embryo fibroblasts CPE, which was revealed by the phenomenon that no CPE was observed when even seven blind passages were performed. All these indicated that the virus of NGVEV was very resistant to heating.

Sensitivity to pH The pathogenicity and infectivity of NGVEV was stable at pH ranging from 3.0 to 8.0 and the titre of NGVEV dropped to certain extent at pH 2.0 or 9.0, which was indicated by the fact that more time was needed for CPE to appear. NGVEV was inactivated at pH 3 or at pH 10. All these suggested that the isolated virus of NGVEV was adaptable to a comparatively wide range of pH.

Sensitivity to Chloroform Typical CPE could be observed in primary duck embryo fibroblasts inoculated with NGVEV that had been treated with chloroform for either 1 or 2 or 3 times and no differences was observed between the experiment group and the control group. This revealed that NGVEV was not sensitive to chloroform and this corresponded with the fact observed by electronic microscopy that the virions had no envelope.

Sensitivity to trypsin Whether NGVEV was treated with trypsin or not, the same CPE occurred. This suggested that NGVEV was insensitive to chloroform and was able to resist the gastroenteric proteases and gastric acid, therefore could penetrate the intestine easily[1]. NGVEV could result in the intestinal exudative or necrotic inflammation and could cause necrosis and sloughing of the intestinal epithelial cells.

Type of the nucleic acid

Drug inhibition It is well known that 5-iodedeoxyuridine (5-IudR) is similar to thymidine (T) in structure and can supersede T in DNA replication. Therefore DNA with no normal function is synthesized and virus propagation is prevented therefore make

DNA virus that is capable of resulting in CPE lose this ability. Five dilutions of NGVEV, 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, were prepared and 40 bottles of primary duck embryo fibroblasts were randomly divided into 2 groups with each containing 20. Each dilution of NGVEV was inoculated to 4 bottles of primary duck embryo fibroblasts of each group and cultivated at 37℃ for 1 hr for attachment of NGVEV to the cells before the virus containing media was removed. Then maintenance media containing 50 μg/mL 5-IudR was added to the experiment group and that containing no 5-IudR was added to the control group and observation was maintained for 14 days. It suggested that NGVEV was a DNA virus by the experimental result that no CPE was observed in the experiment group.

Enzymatic digestion The purified virus containing suspension was adjusted to a final concentration of 0.5% by adding 10% SDS and then under went extraction by saturated phenol which was dissolved in the solvent of Tris-HCl. The liquid phase was extracted by ether for 4 times and after the ether removed was precipitated by sodium acetate of pH 5.2 and dehydrated alcohol. Then it was placed overnight at -20℃. Then centrifugation at 16 500 rpm for 30 min was employed. After dried, the precipitate was dissolved in the buffer of Tris-EDTA with a pH of 7.3 and then was divided into 3 parts. The second part and the third part were added DNase and RNase respectively and the first part was added with no enzyme. λ DNA as well as DNA of Egg drop Syndrome Virus (EDSV-DNA) were employed as controls. It proved that the nucleic acid of NGVEV could be degenerated by DNase but could not by Rnase and the result suggested that NGVEV was a DNA virus with a nucleic acid of approximately 32 kb.

Nucleic acid strand type of NGVEV

Egg drop syndrome viruses(EDSV) DNA[20] and λ DNA were used as controls of double-stranded nucleic acid and Gosling Plague Virus (GPV)[17] was used as control of single-stranded nucleic acid.

Acridine orange staining It is known that the mechanism of acridine orange staining is based on the stain concentration and the nucleic acid space configuration, not on the type of nucleic acid (DNA or RNA). Under ultraviolet light the single-stranded nucleic acid of GPV was brilliant flame red and the nucleic acid of NGVEV and EDSV, along with ë DNA, was apple green. This suggested that the NGVEV nucleic acid was double stranded.

Nuclease digestion It is known that nuclease S1 usually degrade s the single-stranded nucleic acid, and is capable of, only at a higher concent ration, degrading double-stranded nucleic acid. The result that under the same condition NGVEV-DNA,¡¡along with the controls of EDSV-DNA and ë DNA, could not but GPV-DNA could be digested by nuclease S1, confirmed the double stranded nucleic acid type of NGVEV[21-25].

Virus purification and viral structural polypeptide analysis by SDS-PAGE

Virus purification It proved, by cellulose acetate electr ophoresis and electronic microscopy examination, that purified virions were obtained and could serve as a qualified material for the viral fine structure observation, the nucleic acid extraction and the viral structural protein analysis. Electronic microscopy of the virus revealed NGVEV virion, spherical or slightly elliptical in shape, was an unenveloped icosahedral structure 60-80 nm in diameter, with an average of 70-90 nm. And by morphological analysis, the virus appeared to fulfill the criteria of adenovirus.

Viral structural polypeptide analysis by SDS-PAGE SDS-PAGE of the disrupted purified virus particles revealed 15 discrete bands (Figure 1). After scanned and analyzed by automatic gel image-forming and analyzing system, the molecular mass and relative percentage of each structural polypept ide were obtained (Table 1). The fact that VP_4, VP_7, VP_8, VP_9 and VP_{14} were the main structural polypeptides and made up about 90.2622% of the gross protein revealed that NGVEV fulfilled the criteria of adenovirus to great extent[17,26-28]. From the relationship between DNA base number and the molecular mass of the encoded protein, it is known that NGVEV structural protein with an overall molecular weight of 71 300 D needed DNA of approximately 19.3 kb to code for. But the viral genome of DNA was about 32 kb. This suggested that NGVEV DNA codes not only for the viral structural protein. This property is shared by other adenoviruses as well[17].

From data above we have got, we know that NGVEV is adenovirus[29-45].

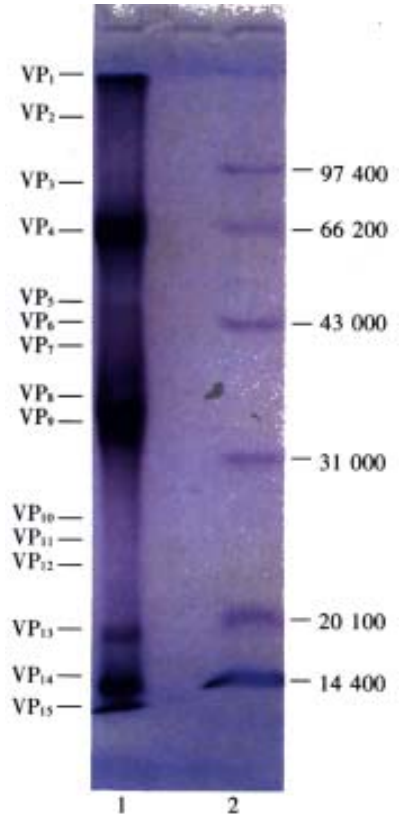

Figure 1 Protein polypetide map of NGVEV-CN virus.
1. Polypetide of NGVEV-CN virus;
2. Low MW. standard protein

Table 1 Molecular weights and relative percentages of NGVEV structural polypeptides

Polypeptides	VP1	VP2	VP3	VP4	VP5	VP6	VP7	VP8	VP9	VP10	VP11	VP12	VP13	VP14	VP15
Molecular weights(Daltons)	116 000	104 000	88 000	68 000	60 700	54 000	40 100	36 000	34 500	24 200	22 000	20 000	18 000	14 200	13 400
Relative percentage(%)	3.0215	0.0172	0.0014	24.595	0.5581	0.6225	12.6938	25.361	18.1807	0.1483	0.2275	0.3826	0.9072	10.0714	3.2118

The pathomorphological development observed in goslings artificia lly infected with NGVEV-CN

Signs

The incubation period of the experimental infection mainly varied from 2 to 3 days (36/40 or 85%) and only a few varied from 4 to 5 days; The early signs were: inactivity of the brood, reduced appetite, listlessness, somnolence, loose drooping, ruffled feathers accompanied by drooping of wings. Besides, the voice of the birds was not sonorous as before; At a later stage of this disease, wet vent feathers, soiled vents, inappetence, watery drooping with yellow or whitish yellow mucoid contained could be seen. Brown drooping might be observed in some individuals and the affected birds could not keep balance in walking and standi ng. Spasmodic prostration and convulsions, kicking spasmodically upwards with both legs might be observed in some affected goslings and opisthotonus might be found in most of the birds which died of this disease. The affected birds usually died of emaciation, extreme weakness, and somnolence. Retarded growth was observed in the affected birds and the body weight might be reduced by one fold compared with the control group. In the experiment group, mortality occurred 4 days postinfection and peaked 10-18 days postinfection and all the birds died at the 25th day postinfection. In the experiment group, altogether 8 birds were killed and 32 died.

Pathological changes

Gross changes

Small intestine In each small intestinal part, no gross lesions was demonstrated one day postinfection and only mild hyperemia could be seen 2 days postinfection; in each part of the small intestine, pronounced hyperemia, mild hemorrhage, obvious mucosa swelling as well as excess mucus production might be observed 3 days postinfection. In addition to pronounced hyperemia as well as excess mucus production, mucosa swelling and serious hemorrhage could be found in each small intestinal part of birds killed 4 days postinfection; severe hemorrhage might be found in each small intestinal part of birds that died of this disease. Besides, swollen intestinal mucosa which appeared bright and contained plenty of mucous secretory products might be observed as well; in addition to the severe hemorrhage which might be demonstrated in each part of the small intestine, a little whitish yellow coagulated fibrous exudate as well as a few pieces of necrotic intestinal epithelia might be found on the intestinal mucosa of birds that died 7-12 days postinfection; coagulative embolus wrapped by yellowish pseudomembrane was first found in birds that died 14 days postinfectio n and was about 0.2 cm in diameter and over 10 cm in length when first observed. This structure found in birds that died at a later time, which was between 0.5 and 0.7 cm in diameter and over 10 cm in length and whose length might reach 30 cm or more, was bigger than that observed previously and the small intestinal part containing it was 1-2 times distended than the control in appearance (Figure 2). The small intestinal part containing this embolus was much thin in its wall and that without this structure underwent severe hemorrhage with, its mucosa reddened. This structure of embolus was mainly observed in the middle-lower part of the small intestine before the bifurcation of the ceca and most of which was in the form of one section. There were emboluses in the form of two sections, but the number was greatly reduced. The intestinal coagulative embolus observed at necropsy could roughly be classified into 2 types. The first with a diameter of over 0.5 cm and a length of approximately 20 cm was big in size and dense in texture. And this embolus occupying the whole intestinal lumen, whether sectioned transversely or long itudinally, was found to be a two-layed structure. The outer layer, which was a pseudomembrane 0.5 mm-1 mm in depth was dry and brownish in appearance and was composed of the mixture of necrotic tissue and fibrous exudate. The inner layer was the intestinal contents which was dry and dense. The second kind of embolus with the appearance of thin rod and with a diameter of 30cm or more was obviously thinner but longer than the first and was composed of the coagulative mixture of necrotic intestinal tissue and fibrous exudate. Both kinds of emboluses did not adhere to, and were easily to be separated from the intestinal wall. The wall of the embolus containing part of the intestine was very thin and its transparency was greatly enhanced. The intestinal lesions observed in this disease, especially the coagulative embolus in the middle-lower part of the intestine that occluded the intestinal tract, resembled that observed in the case of gosling plague (GP)[46] to a great extent.

Rectum and the cecum Swelling, hyperemia and mild hemorrhage might be observed in birds that died at an early stage of this disease; much mucus was observed and the cloaca was filled with yellowish loose contents in cases that died at a later stage.

Other tissues or organs No gross lesions was found in killed birds. Birds that died at an early stage, usually less than 10 days postinfection, of this disease might show subcutaneous hyperemia or hemorrhage; epcardial mural hyperemia or small punctuate hemorrhages might be observed in a few of the m. The pectoral muscle and the leg muscle underwent hemorrhage and presented themselves dark red in appearance; the liver on which petechial or ecchymotic hemorrhages might be presented underwent venous congestion and was dark red in appearance. The swollen gall bladder which was dilated in appearance and 3-5 times larger than the control was dark greenish red and was full of bile; the kidney underwent hyperemia and mild hemorrhage and presented itself dark red in appearance; no obvious lesions was found in other tissues or organs. In birds that died at a later stage (more than 11 days postinfection), no obvious lesion was found except the liver which appeared dark red and the kidney which underwent mild hyperemia and hemorrhage.

Histopathological lesions

Duodenum In birds killed 1 day postinfection, no lesion was found different from the controls. In birds killed 2-4 days postinfection, some of the epithelial cells covering the villustips were found sloughed and some of the lamina propria cells underwent coagulative necrosis and presented themselves to be a sheet of red-stained granules among which fibrinoid necrotic interstitial tissue might be detected. In addition, hyperemia of the villi and necrosis of some of the intes tinal villus tips were observed (Figure 3). In birds that died 4 days postinfect ion, epithelium of the mucosa was found denuded and some of the villi might be completely denuded, with the lamina propria exposed. The lamina propria underwent edema. The intestinal glandular cells underwent vacuolar degeneration and were loosely packed. In birds that died 5-10 days postinfection, intestinal epithelial cells were found completely sloughed, parts of the mucosal axletree of lamina propria were found remained, in which large number of erythrocytes were detected . As the infection advanced, a large number of lymphocytes were observed in the swollen lamina propria and in some parts of it much fibrin was found. Most of the intestinal glandular cells underwent vacuolar degeneration, necrosis and were loosely packed; during the course of this disease, the duodenal fibrinonecrotic enteritis was developed and most of the intestinal villi were completely denuded , with the neat separation surface formed (Figure 4). Abundant fibrin, blood cells and bacteria filled the lumen; in birds that died 11 days postinfection, epithelial cells was completely sloughed from the mucosa and the mucosal axletree of lamina propria was exposed. As for the lamina propria, some was infiltrated with abundant lymphocytes and some underwent necrosis and sloughing. Some intestinal glandular cells

were contracted and were detached from their surrounding connective tissue, with a space around them formed. The intestinal glandular underwent necrosis, sloughing and a small number of intestinal glandular cells underwent vacuolar degeneration and were loosely packed. The blood vessels in the muscle lays were congested and abundant fibrin as well as sloughed or necrotic cells filled the lumen.

Ileum No differences was found between the birds killed 1-4 days postinfection and the controls; in birds that died 4 days postinfection , epithelial cells covering the villus tips underwent necrosis and sloughing. The glandular cells underwent swelling and vacuolar degeneration and were loosely packed, some of which were represented by their outlines, another part of which might be replaced by abundant proliferated connective tissues and lost their outlines. Some of the intestinal villi were observed completely sloughed from the mucosa and with the prolonged course of this disease, the intestinal villi were observed completely sloughed and the typical fibrinonecrotic enteritis was eventually developed. The pseudomembrane covering the embolus in the intestinal tract was mainly composed of necrotic mucosa, droppings, inflammatory cells, bac terium and the exudate of fibrin (Figure 5).

Liver No differences was found between the birds killed 1-4 days postinfection and the controls; in some cases that died of this disease , local congestion, mild granular degeneration and fatty degeneration (7/32) (Figure 6) was observed. As for the pathological changes in the case of gosling plague (GP)[46], pronounced inflammatory lesion and focal necrosis of the liver were successively observed 24-48 hours postinfection and granular degeneration as well as vacuolar degeneration were observed successively. With the prolonged course of GP, the hepatic cells underwent severe vacuolar degeneration and progressive necrosis, which were most evident in birds that died of this disease. The hepatic cells were swollen and their shape changed from polygonal to round. Many vesicles appeared in the hepatic cytoplasm and thus made it appeared loosely foamy. Cytoplasm dissolution, ballooning degeneration, together with the marked vacuolation of the hepatocytes were observed in more serious cases and the Sudan III staining for fat was negative. With the further development of this disease, hepatocytes underwent rupture, necrosis and dissolution and the necrosis-dissolution foci were developed, in which were light red -stained oedematous fluid as well as a small number of monocytes and lymphocytes. Dehydration, condensation and enhanced cytoplasmic acidophilia occurred in a few hepatocyte cytoplasm, in which round eosinophilic droplets were formed and shared great similarity to that structure observed in the case of human viral hepatitis.The hepatic cords were disorganized in structure and some of the hepatic sinusoid,with the necrosis and dissolution of the hepatocyte,were disrupted and erythrocytes were released.The hepatic interstitial blood vessels and the hepatic sinusoids were congested and loose and lost their normal structure and underwent fibrinoid necrosis. The interlobular monocytes and lymphocytes underwent diffuse proliferation and showed diffuse infiltration.Nodular proliferative foci were formed in some parts of the interlobular tissue.

Kidney In birds killed 1-4 days postinfection, the main lesions were: the renal tubular tract was not clear in structure, mild grandular degeneration could be seen in the epithelial cells of the renal tubular; in birds that died of this disease 4 days postinfection, hemorrhage focus and granular degeneration of the epithelium of the renal tubular might be found in some cases (19/32); vacuolar degeneration (Figure 7) and abundant sloughed epithelial cells in the ureter tract might be observed in some cases (9/32), Some cases (5/32) were found normal.

Spleen Mild hyperemia might be observed in some (4/8) of the birds killed 1-4 days postinfection; Mild hyperemia, hemorrhage might be observed in a few (5/32) of the cases that died of this disease and the lymphoid follide of these cases were found equivocal in structure. As for the case of gosling plague[46], no obvious lesions was found during the early stage of this disease and during the later stage, splenic sinusoid might be found congested and contained a large number of monocytes and a small number of heterophiles. Besides, the splenic corpuscles underwent atrophy and was equivocal in structure; lymphocytes underwent necrosis and karyorrhexis; small necrotic foci might be found in the lymphoid nodule; proliferation of monocytes or reticular cells might be observed in the parenchyma of the spleen.

Lung In cases 1 day postinfection, the blood vessels were congested; in cases 2 days postinfection, tertiary bronchuses underwent hyperemia and hemorrhage; in cases 3-4 days postinfection, hyperemia, hemorrhage was found and the vein was congested. The tertiary bronchus and the atrium contained a lot of erythrocytes and the secondary bronchus contained quantities of blood; most of the cases that died 4 days postinfection showed pulmonary congestion and hemorrhage, caseation necrosis foci could only be observed in a few cases (2/32).

Proventriculus No lesions were observed in birds killed 1-4 days postinfection or birds that died less than 5 days postinfection; sloughing of the epithelium from the mucosa was observed in most (30/32) of the birds that died more than 5 days postinfection and there were abundant sloughed cells in the cavity. Besides, sloughed glandular cells might be found in parts of the glandlobular cavities. As for the cases of GP[46], 48 hour s until death, degeneration, necrosis and sloughing of the epithelial cells of the mucosa might be observed. The lamina propria underwent hyperemia and was infiltrated with inflammatory cells. Compound tubular glandular cells might be observed sloughed; many inflammatory cells and sloughed glandular cells filled the collecting sinusoid.

Heart Most (27/32) of the killed birds were found to be normal and only a few (5/32) that died at an early stage of this disease showed mild hyperemia and hemorrhage in their hearts. But in the case of gosling plague[46], the following might occur 12-36 days postinfection: granular degeneration of the myocardium, Cross striations of the myocardium got dim or disappeared; the myocardium underwent karyorrhexis and karyolysis; small necrotic foci and inflammatory cell infiltration might be observed; Focal pericardit is might developed.

Pancreas, brain and esophagus These organs were found normal. But in the case of GP[46] 72 hr postinfection, the following was observed: Interstitial blood vessels were congested, the glandular cells underwent degeneration and was separated from the basal membrane. In birds that died of GP, the acinuses were found disorganized in structure and the glandular cells were necrosed, sloughed, dissolved and finally the necrosis-dissolution focus was formed; the interstitial tissues were infiltrated with inflammatory cells. In the case of GP[46], basically the same lesion, which was not obvious at an early stage, was shown in the cerebrum and the cerebellum; at a later stage, the following might occur: nenix underwent swelling and hyperemia, blood vessels in the cerebral parenchyma were dilated and congested with the perivascular space enlarged, foci formed by the perivacular proliferation of a small number of lymphocyte and monocytes could be seen, matrix of the cerebrum was swollen, the neuron underwent degeneration and vesicles might be observed in its cytoplasm, pykosis was observed and the nucleus was equivocal in structure, the neurogliocytes underwent diffuse proliferation and glial nodules might be formed in some areas within it.

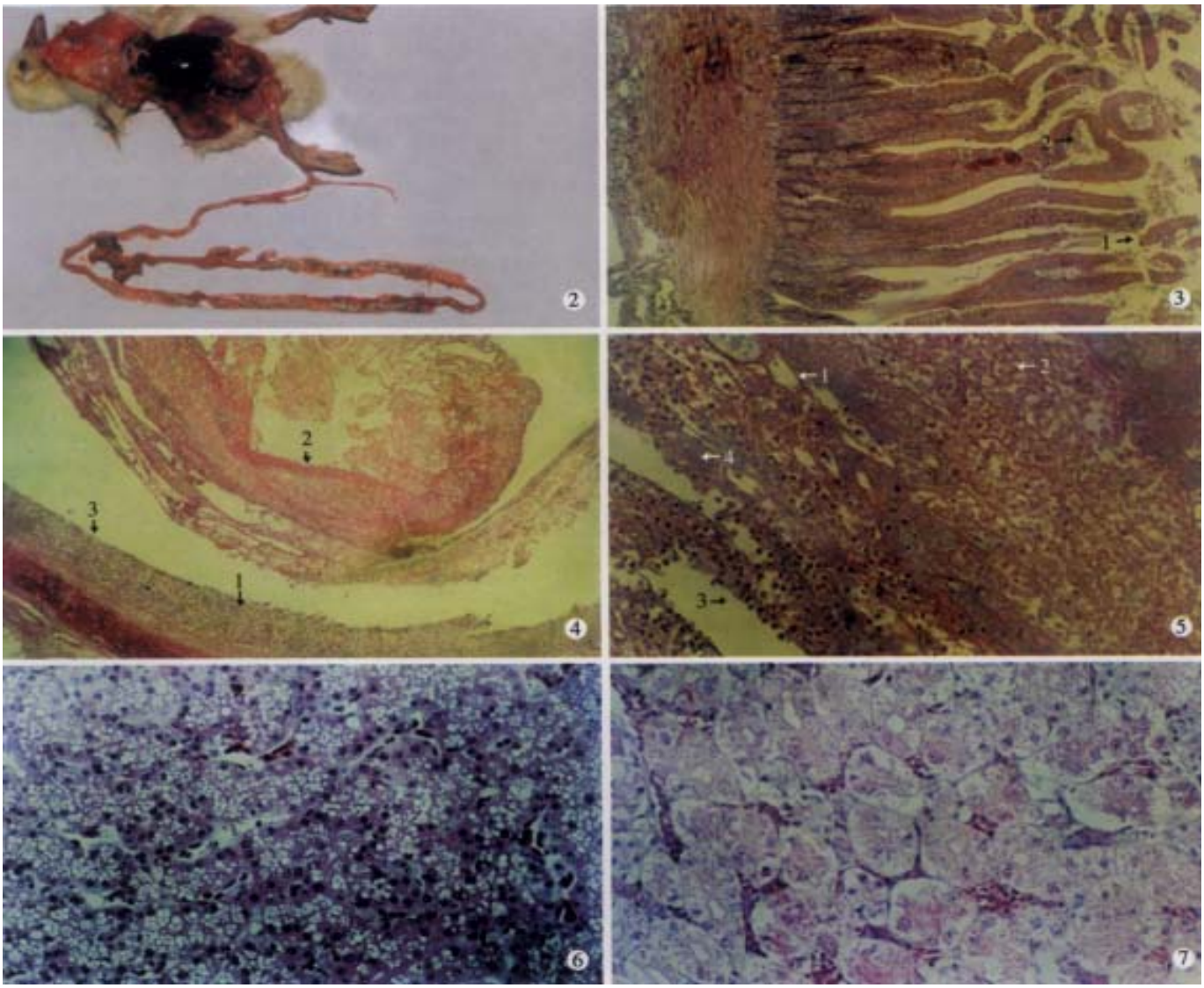

Figure 2 Particular coagulative embolus was formed in small in testine of death gosling (13 days postinfection), the length is over 40 cm. Haemorrhage was occured in small intestine wall and be dyed red.
Figure 3 Many pieces of falled epidermal cells in duodenum cavity (arrow 1), coagulative necroses was occured in some villus top (arrow 2). (150×, H.E)
Figure 4 Particular fibrinous and necrosed enteritis of ileum: necrosed mucosae (arrow 1) and fibrinous edudate coagulated into artificial me mbrane and dropped into the cavity (arrow 2), and surface of separation boundry was smooth (arrow 3). (100×,H.E)
Figure 5 Particular fibrinous and necrosed enteritis of ileum: the embo lus consisted of the necrosed mucosal tissue coagulated and dropped materials, it include fibrinous edudate which like thread and inflammatory cells. Fibrin (arrow 1), necrosed cells (arrow2), inflammatory cells (arrow 3) and bacteria (arrow 4). (400×,H.E)
Figure 6 Fatty degeneration were occured in liver cells. (500×,H.E)
Figure 7 Kidney: Hyperaemia. Granular degeneration was occured seriously in kidney small vessles (arrow 1), or even vacuolar degeneration (arrow 2). (500×,H.E)

About the structure of inclusion body According to the related reports[17,18,46], intranuclear or intracytoplasmic inclusions might be observed in myocardium, hepatocytes, and epithelial cells of the intestine in the case of GP. Wang[46] reported that eosinophilic granules were found in the hepatocytes. In many cases of poultry infected with adenovirus, inclusion body could be observed[47-52]. But none of these mentioned above was observed in the case of NGVE.

Histopathological and ultrastructure analysis is the key method to understand mechanism of human ang animal disease[53-64]. The histopathological change of Goslings be infected with NGVEV can help us to understand mechanism of NGVE, Pathological characteristic could be provide helpful materials for the NGVE diagnosis and differential diagnosis from GP.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Hepatitis C virus infection of human hepatoma cell line 7721 *in vitro*

Zhi-Qiang Song[1], Fei Hao[1], Feng Min[2], Qiao-Yu Ma[2] and Guo-Dong Liu[2]

Department of Dermatology[1], Department of Infectious Diseases[2], Southwest Hospital, Third Military Medical University, Chongqing 400038, China
Supported by the National Natural Science Foundation of China, No. 39670672.
Correspondence to: Dr. Zhi-Qiang Song, Department of Dermatolo gy, Southwest Hospital, Third Military Medical University, Chongqing 400038, China.songzq@yeah.net.
Telephone: +86-23-68753298
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Abstract

AIM **To establish a cell culture system with long-term replication of hepatitis C virus *in vitro*.**

METHODS **Human hepatoma cell line 7721 was tested for its susceptibility to HCV by incubating with a serum from a patient with chronic hepatitis C. Cells and supernatant were harvested at various phases during the culturing periods. The presence of HCV RNA, the expression of HCV antigens in cells and/or supernatant were examined by RT-PCR, in situ hybridization and immunohisto-chemistry respectively.**

RESULTS **The intracellular HCV RNA was first detected on d2 after infection and then could be intermittently detected in both cells and supernatant over a period of at least three months. The expression of HCV NS_3, CP_{10} antigens could be observed in cells. The fresh cells could be infected by supernatant from cultured infected cells and the transmission of viral genome from HCV-infected 7721 cells to PBMCs was also observed.**

CONCLUSION **The hepatoma line 7721 is not only susceptible to HCV but also supports its long-term replication *in vitro*.**

Subject headings hepatitis C virus; cell culture; cell model; carcinoma, hepatocellular/pathology; tumor cells, cultured; hepatitis B virus; virus replication

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INTRODUCTION

Hepatitis C virus (HCV) is the major cause of transfusion-associated non-A, non-B hepatitis[1]. Acute HCV infection leads, in more than 70% of the patients, to the development of chronic hepatitis[2], and then cirrhosis and hepatocellular carcinoma[3,4]. HCV is an enveloped positive-stranded RNA virus which contains a genome of about 9500 nts encoding a polyprotein by host and viral proteases results in the production of structural and nonstructual (NS) protein[5-8]. Despite increasing knowledge of genome structure and individual viral proteins, studies on virus replication and pathogenesis have been hampered by the lack of reliable and efficient cell culture systems. To identify critical epitopes that elicit a neutralizing antibody response and to understand the pathogenesis of persistent infection, efficient infection cell models for HCV are needed[8-10]. Many cell lines has been sought to support HCV infection and replication *in vitro*. Human T-lymphocyte cell lines[11-18], human fibroblast cells[19] (VH3), peripheral blood mononuclear cells[20,21] (PBMCs) and hepatocytes[22-28] have been shown to support HCV replication *in vitro*. These cell models have been proved to be useful for the study of HCV. To be mentioned, hepatocytes are the main target cells for HCV replication *in vivo*. Whether the replication of HCV in non-hepatocytes entirely mimics the behavior of human hepatocytes and whether the molecular characterization of growing HCV in these cell models has changed are still poorly understood[28].

In this study, we employed a human hepatoma cell line 7721 to test its susceptibility to HCV and established an infection cell model that can support HCV long-term replication *in vitro*. The presence of both plus- and minus- strand of HCV RNA and the expression of viral antigens in infected cells were demonstrated.

MATERIALS AND METHODS

Inoculum

The principal inoculum was a serum obtained from a 45 year old female chronic patient, who was positive for anti-HCV antibody (Abbott) and HCV-RNA (1b genotype). Sera from patients without any evidence of HCV infection or chronic liver diseases were also used as controls.

Cell line

The hepatoma cell line 7721 was obtained from Lab. of Gastroenterology in Third Military Medical University. Cells were maintained in PRMI1640 medium supplement ed with non-essential amino acids and 100 mL·L^{-1} fetal bovine serum . The cells were fed fresh medium every 3 d to 5 d, and were subcultured at the ratio of 1:2 to 1:4.

Viral inoculation and sample collection

The HCV-positive serum (inoculum) was diluted at the ratio of 1:5. 7721 cells maintained in 25 cm 2 polystyrene flasks and 6-well dishes were incubated with the inoculum at 37 ℃ for 8 hr. Then all medium was removed and cells were washed 6 times with phosphate buffered saline (PBS). Cultures were refed with medium with 100 mL·L^{-1} fetal bovine serum and the media were changed every 4 d-5 d.

Cells and supernatants were harvested according to the method introduced by Iacovacci *et al*[27], with slight modification. Briefly, cells (approximately 2×10^6 cells) and 1 mL aliquots of cell supernatants from infected and uninfected cell cultures were harvested at different periods after infection for up to 70 d, and slices grown with cells were also collected accordingly (for study of the localization of different HCV antigens and minus- strand of HCV RNA),

replacing the medium removed at each collection. Supernatants were centrifuged at 10 000×g to remove cellular debris, and the ultracentrifuged pellets were stored at -20℃ until detection of HCV RNA. Cells were harvested for RNA extraction after 6 washes with phosphate buffered saline (PBS).

Detection of HCV RNA by RT-PCR

For detection of HCV RNA, total RNA was extracted from 1mL of the ultracen trifuged supernatants and approximately 2×10^6 cells by using a nucleotide and protein extraction kit (Tripure, Roche). 10 μL of the RNA solution was denatured at 72℃ for 4 min and incubated at 37℃ for 1 h with 200 U of murine moloney leukaemia virus reverse transcriptase (MMLV-RT, Promega) and 50 pmol of the outer antisense oligonucleotide primer (antisense, CACTCGCAAGCACCCTATCA-; nucleotides 302-285). Synthesis of cDNA was stopped by heating the samples at 95℃ for 10 min. Amplification of the cDNA was performed by using 15 μL cDNA solution and 50 pmol of one of the outer primers (sense,-GGCGACACTCCACCATA GAT-: nucleotides 9-28). Thirty cycles of DNA amplification were carried out followed by and extension step for 10 min at 72℃. Each cycle of PCR consisted of 94℃ for 60 s, 55℃ for 90 s and 72℃ for 120 s. The second PCR was carried out in the same way with 10 μL of the first PCR mixture and 50pmol of each inner prime (sense, -CTGTGAGCAACTACTGTCT-; nucleotides36-55 and antisense, -CGGTGTACTCACCGGT TCC-; nucleotides 161-143). The amplified DNA was visualized by 20 $g\cdot L^{-1}$ agarose gel electrphoresis and ethidium bromide staining.

Detection of HCV antigens by immunohistochemistry

A modification of immunohistochemical staining of viral antigens by streptavidin peroxidase (SP) method was performed. Monolayers of infected and uninfected cells grown on slides were fixed for 15 min in 40 $g\cdot L^{-1}$ formaldehyde pH (7.3) in PBS solution. After washing, endogenous peroxidase was blocked by a 10-minute incubation with 3 $mL\cdot L^{-1}$ H_2O_2 in methanol. Sections were washed in PBS, 15 min at 37℃. Sections were washed in PBS (pH 7.4); then 200 $mL\cdot L^{-1}$ normal goat serum was used for 15 minutes.

The sections were incubated with 1:200 dilution of the primary antibody (anti- NS3) or with 1:60 dilution of primary antibody (anti-CP_{10}) for 16 h at 4℃ in a moisture chamber. The immunostaining was also done without the primary antibodies. These reagents and all subsequent reagents were diluted in PBS with 1 $g\cdot L^{-1}$ bovine serum albumin (BSA) and used at a volume of 100 mL per slide. After a PBS rinse, biotinylated second antibody was applied for 30 min at room temperature. After another PBS rinse, streptavidin peroxidase was applied for 30 min. After a final PBS rinse, the cell sections were incubated with 0.6 $g\cdot L^{-1}$ diaminobenzidine (Sigma) and 0.1 $mL\cdot L^{-1}$ H_2O_2. The sections were then conterstained with hematoxylin, dehydrated with graded alcohols and xylene, and observed under coveslips.

In situ hybridization

The localization of intracellular minus-strand of HCV RNA by in situ hybridization was done according to the METHODS by Cribier *et al*[20] and Nouri-Aria *et al*[29] with modifications. The slides were rehydrated through an ethanol series (1000-500 $mL\cdot L^{-1}$) and rinsed in PBS for 5 min, and then the cells were digested with 1mg $\cdot L^{-1}$ proteinase K (Roche) for 30 min at 37℃. The slides were rinsed twice in PBS containing 2 $g\cdot L^{-1}$ glycine (10 min each), refixed in 40 $g\cdot L^{-1}$ PFA for 5 min at room temperature, then rinsed three times for 10 min in 2×SSC buffer, and finally 10 $mmol\cdot L^{-1}$ DDT was then added to the slides. Dehydration in an ethanol series (500-1000 $mL\cdot L^{-1}$) was followed. The probe was denatured for 15 min at 85℃ and added to hyridization solution (500 g $\cdot L^{-1}$ formamide, 100 $g\cdot L^{-1}$ dextran sulphate, 2×SSC, 2×Denhardt's solution, 1 $g\cdot L^{-1}$ Triton X-100, 0.01 $mol\cdot L^{-1}$ DDT, 200 $mg\cdot L^{-1}$ herring sperm DNA). The final concentration of the probe was 1 $mg\cdot L^{-1}$. The slides were denatured for 2 min at 95℃, and various amounts of probe, depending on the area, were added on the cell section. Samples were covered with siliconized coverslips, sealed with paraffin, and incubated in a humidified chamber at 37℃ for 10 h-20 h. The coverslips were removed and the slides were rinsed three times in 1×SSC buffer and washed twice in 500 $g\cdot L^{-1}$ formamide, 1×SSC for 15 min at 42℃. After two washes at 42℃ in 1×SSC and another wash in 0.1×SSC (10 min each), the slides were dehydrated through an ethanol series and dried under vacuum. The sections were incubated with a streptavidin-biotin alkaline phosphatase complex and successively developed with nitroblue-tetrazolium and bromo-chloro-indolyl phosphate (NBT/BCIP), according to the manufacturer's instructions. After being rinsed in water, the slides usually were stained briefly with eosin. Uninfected cells were hybridized with the HCV probes and served as a first negative control. The Dig-labeled genomic strand RNA transcript was 41mer and its sequences is 5'-CTGCTAGCCGACT AGTGTTGGGTCCGCGAAACC-GCTTGTGG-3'.

RESULTS

Detection of HCV RNA in the infected cells

The intracellular RNA was determined by RT-PCR in infected cells at various times during culture, ranging from d 2 to d 95, as shown in Table 1. Plus- strand of HCV RNA was intermittently detectable after infection, but not detectable on d 25, d 30, d 52 and d 75 after infection. Minus-strand RNA was detectable from d 3 after infection, and still detect able at d 95 after infection.

Table 1 Detection of HCV RNA in 7721 cells and supernatant

Day p.i.	Cells		Supernatant	
	Plus-strand RNA	Minus-strand RNA	Plus-strand RNA	Minus-strand RNA
2	+	-	+	-
3	++	++	+	-
10	+	+	+	ND
20	++	+	-	-
25	-	-	-	ND
30	-	-	-	-
40	+	-	-	-
52	-	-	+	-
60	+	-	-	-
75	-	+	-	-
82	++	++	++	-
90	++	-	+	-
95	+	+	-	-

*+, Weakly positive; ++,Positive; -,Negative; ND, Not detection.

Detection of HCV RNA in the culture medium

The presence of HCV RNA in culture supernatant was also determined by RT-PCR. Plus-strand of HCV RNA in

supernatant was less often than that in cells. The HCV negative-strand could not be detected in the supernatants and inoculum, as shown in Table 1. Immediately after incubation and six washes of the uninfected 7721 cells, the RT-PCR were negative on the buffer used in the final wash of the cells.

These results were confirmed by three other repeated experiments.

The expression of HCV antigens in infected cells

7721 cells were tested for the expression of virus-encoded proteins by SP method using mouse monoclonal antibodies directed against NS_3 and CP_{10}. Figure 1A, B show that infected 7721 cells were positive for different viral antigens. The positive cells were scattered and displayed diffuse cytoplasmic yellow staining similar to that reported in other cell culture systems and naturally infected liver cells[30-34]. Moreover, uninfected cells and cells incubated with normal serum were tested negative using these same antibodies (Figure 1C).

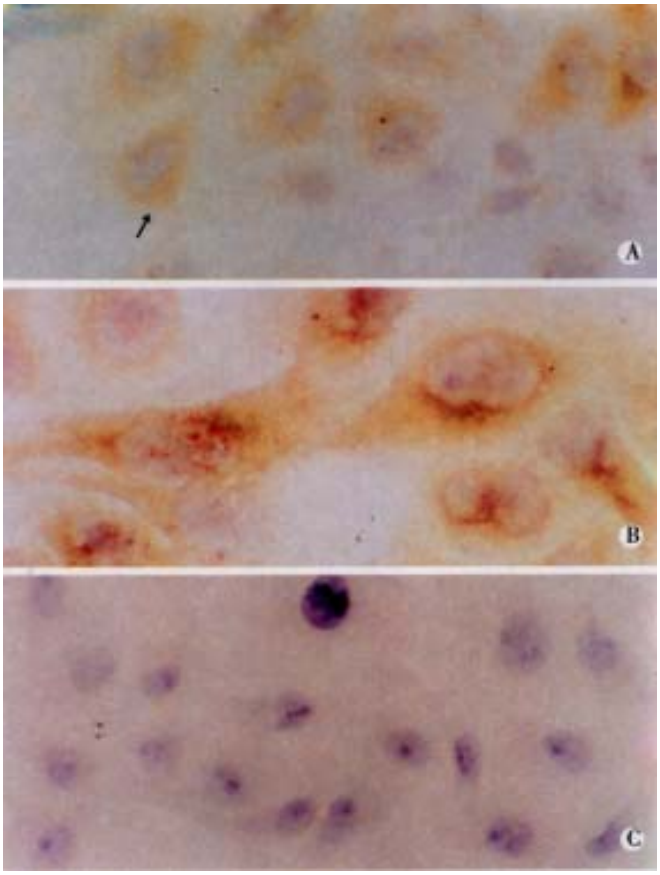

Figure 1 Immunohistochemical detection of HCV-encoded protein in infected 7721 cells. Positive reaction (yellow) with anti-NS_3 (A) and anti-CP_{10} (B) in 7721 cells, were scattered in the lobules (SP method, original magnification, A×200, B×400). Lack of staining with the same antibody in uninfected 7721 cells processed at the same times and 7721 incubated with oormal serum (C).

In situ hybridization

Using the HCV positive-strand probe, positively labeled cells could be observed at various time after infection. The positive signals (blue) also mainly localized in cytoplasm, and were similar to that reported in other cell culture systems and naturally infected liver cells[35-40], as shown in Figure 2A, B. Uninfected 7721 as controls showed no labeling with the same probe (Figure 2C).

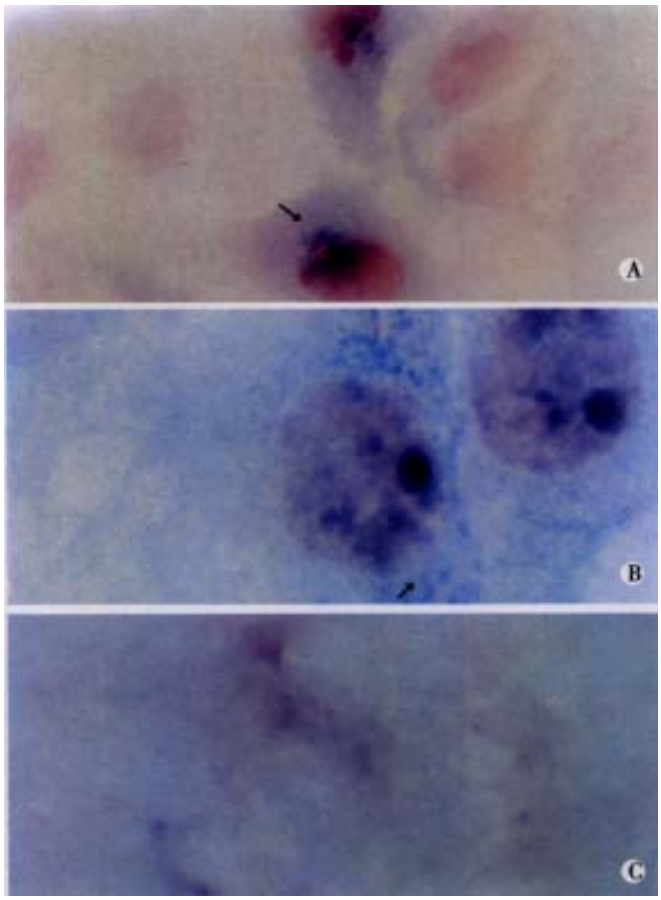

Figure 2 Detection of minus-strand of HCV RNA in cells using in situ hybridization. Positive reaction (blue) was mainly present with cytoplasm (A, original magnification, ×400). Higher magnification showed the positive cells looked morhologically normal (B, original magnification, ×1000). No signal in uninfected cells (C).

Infectivity of the released HCV in the culture supernatant

To determine the infectivity of HCV particles released into the culture supernatant, we harvested cell-free culture supernatant from primary infected-7721 culture on d6 after infection, as described by Mizutani *et al*[14-16]. Fresh (uninfected) 7721 cells were incubated with the culture supernatant containing HCV for 8 h at 37℃ and then the medium was washed.On d 4 and d 8 after initiating the culture, we harvested the medium and cells for the presence of HCV RNA and HCV antigens as above described. Figure 3A shows that minus- strand RNA could be detected in fresh 7721 cells on d 4 and d 8. HCV NS_3 antigen could also be detected in cells on day 8 (Figure 3B).

Transmission of HCV in cell culture

In order to demonstrate whether this system is abortive or productive of infectious HCV, the transmission of the viral genome in HCV-infected 7721 cells to new cells was tested by coculture. For this purpose, we separated the PBMCs from healthy man as described[20]. PBMCs (2×10^5) were mixed with an equal number of HCV-infected 7721 cells. The presence of HCV NS_3 antigen and minus-strand RNA in PBMCs were tested on day 4 and 10 after coculture. The result showed that minus-strand RNA and NS_3 antigen of HCV could be detected by *in situ* hybridization and immunohistochemistry, indicating that the genome was successfully transmitted to PBMCs.

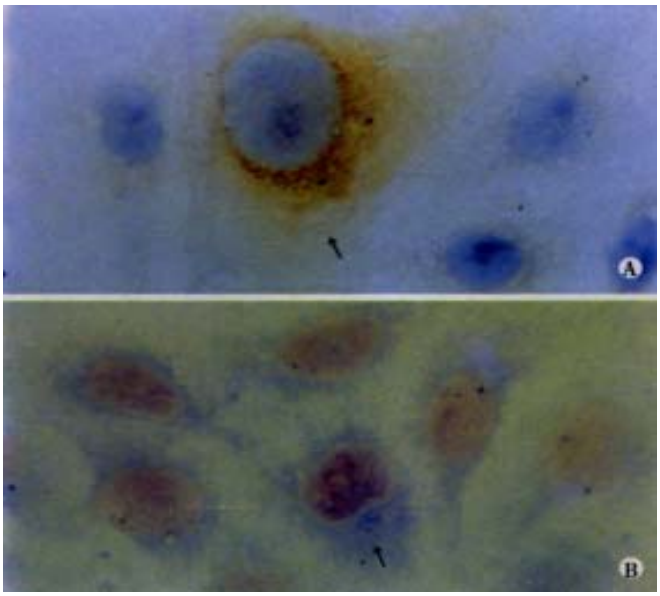

Figure 3 Cell-free transmission of HCV in cell culture. HCV transmission to fresh/uninfected 77 21 was carried out by using the supernatant of culture medium derived from HCV-infected cell culture. The presence of minus-strand of HCV RNA and NS 3 antigen in cells was detected by in situ hybridization (A, original magnification, ×400) and immunohistochemistry (B, original magnification, ×400), respectively.

Cell growth and phenotyic change

7721 cells presented a spindle or round shape in culture and grew in a monolayer. Untreated cells and cells incubated with negative or positive serum, prolifer ated at the same rate with no visible phenotypic changes. Cell growth was not affected by incubation with the HCV-positive serum.

DISCUSSION

Although the knowledge of the molecular biology of HCV has progressed rapidly, our understanding of viral replication and pathogenicity is still hampered by the lack of reliable and efficient cell culture systems. Development of an efficient *in vitro* culture system for HCV will faeilitate the study of HCV. We have demonstrated the susceptibility of a hepatoma cell line 7721 to HCV and established a stable cell model that may support HCV long-term replication *in vitro*. Because HCV RNA in both cells and supernatant after infection may be the residue of the inoculated virus attached to the cell surface, it is necessary to identify that HCV sequences detected in the infected cultures are not from inoculum but newly produced.

We tested both plus-and minus-strands of HCV RNA, the expression of HCV antigens, the localization of minus-strand of HCV RNA, separately, and could show that HCV replication in the cultured cells was not the residue of inoculum. The data presented here indicate that the 7721 cells support HCV infection for at least 3 months. The infected 7721 cultures released detectable levels of HCV into the medium as early as d 2 after infection. Reduction of adsorption time (from 8 h to 2 h) did not significantly reduce the efficiency o f viral replication (not shown), suggesting that binding to cell receptors occurs in a relatively short time[14,28].

We found that both plus- and minus- strands were not often but intermittently detectable in cells and supernatant. Such intermittent detection of the HCV RNA was also reported in other culture systems[20,27,28] and the infection *in vivo*[41-45]. Interestingly, in our study, we showed that the fresh 7721 cells could be infected by the supernatant from cultured infected cells, and that the HCV-infected cells were able to transmit the virus to new cells (PBMCs) after coculture. These results suggested that HCV-infected 7721 cells could deliver infectious and mature virus as others cell models[11,28].

Our findings are consistent with a productive *in vitro* infection of 7721 cells by HCV and are in agreement with the results of Tagawa *et al*[26-28]. However, these authors did not study the expression of viral antigens in hepatoma cell lines after infection. Detection of the HCV protein in cultured 7721 cells by immunohistochemistry indicated that HCV RNA was translated and HCV proteins were produced in cultured cells. The frequency of antigen-positive was less than 30%. The expression of different viral antigens and the localization of minus-strand of HCV RNA were similar to the naturally infected liver cells[30-34]. The results of this study correlates well with experiments as shown in naturally infected hepatocyes, suggesting that the HCV infection and replication occurring in 7721 cultures closely mimic the infection occurring *in vivo*[46-50]. Blocking of viral attachment by a hyperimmune rabbit serum against hypervariable region 1 has been demonstrated on the basis of this model[51]. The mechanisms of HCV infection, replication, assembly, and neutralization can now be studied in detail *in vitro*. This system should also be useful for analysis of antigen epitopes and the evaluation of vaccine candidates, which is especially important because of the limited availability of chimpanzees for vaccine studies and the high degree of variability in the HCV glycoprotein domains.

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Effect of a cancer vaccine prepared by fusions of hepatocarcinoma cells with dendritic cells

Juan Zhang[1], Jin-Kun Zhang[2], Shao-Hong Zhuo[3] and Hai-Bin Chen[2]

[1]Clinical Laboratory, The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, Guangdong Province, China
[2]Cancer Pathology Laboratory, Shantou University Medical College, Shantou 515031, Guangdong Province, China
[3]Department of Gastroenterology, Third Municipal Hospital of Shantou, Shantou 515073, Guangdong Province, China
Supported by the Natural Science Foundation of Guangdong Province, China, No.980180
Correspondence to: Juan Zhang, Clinical Laboratory, The First Affiliated Hospital of Shantou University Medical College, Shantou 515041, Guangdong Province, China. Juanzh005@163.net
Telephone: +86-754-8950794, Fax: +86-754-8557562
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Abstract

AIM **To prepare a cancer vaccine (H_{22}-DC) expressing high levels of costimulatory molecules based on fusions of hepatocarcinoma cells (H_{22}) with dendritic cells (DC) of mice and to analyze the biological characteristics and induction of specific CTL activity of H_{22}-DC.**

METHODS **DCs were isolated from murine spleen by metrizamide density gradient centrifugation, purified based on its characteristics of semi-adhesion to culture plates and FcR^-, and were cultured in the medium containing GM-CSF and IL-4. A large number of DC were harvested. DCs were then fused with H_{22} cells by PEG and the fusion cells were marked with CD11c MicroBeads. The H_{22}-DC was sorted with Mimi MACS sorter. The techniques of cell culture, immunocytochemistry and light microscopy were also used to test the characteristics of growth and morphology of H_{22}-DC *in vitro*. As the immunogen, H_{22}-DC was inoculated subcutaneously into the right armpit of BALB/C mice, and their tumorigenicity *in vivo* was observed. MTT was used to test the CTL activity of murine spleen *in vitro*.**

RESULTS **DC cells isolated and generated were CD11c+ cells with irregular shape, and highly expressed CD80, CD86 and CD54 molecules. H_{22} cells were $CD11c^-$ cells with spherical shape and bigger volume, and did not express CD80, CD86 and CD54 molecules. H_{22}-DC was $CD11c^+$ cells with bigger volume, being spherical, flat or irregular in shape, and highly expressed CD80, CD86 and CD54 molecules, too. H_{22}-DC was able to divide and proliferate in vitro, but its activity of proliferation was significantly decreased as compared with H_{22} cells and its growth curve was flatter than H_{22} cells. After subcutaneous inoculation over 60 days, H_{22}-DC showed no tumorigenecity in mice, which was significantly different from control groups (P<0.01). The spleen CTL activity against H_{22} cells in mice implanted with fresh H_{22}-DC was significantly higher than control groups (P<0.01).**

CONCLUSION **H_{22}-DC could significantly stimulate the specific CTL activity of murine spleen, which suggests that the fusion cells have already obtained the function of antigen presenting of parental DC and could present H_{22} specific antigen which has not been identified yet, and H_{22}-DC could induce antitumor immune response; although simply mixed H_{22} cells with DC could stimulate the specific CTL activity which could inhibit the growth of tumor in some degree, it could not prevent the generation of tumor. It shows that the DC vaccine is likely to become a helpful approach in immunotherapy of hepatocarcinoma.**

Subject headings cancer vaccine; dendritic cells; hepatocarcinoma cells; cell fusion; spleen; mouse

Zhang J, Zhang JK, Zhuo SH, Chen HB. Effect of a cancer vaccine prepared by fusions of hepatocarcinoma cells with dendritic cells. *World J Gastroenterol*, 2001;7(5):690-694

INTRODUCTION

T lymphocyte-mediated immunoresponse plays an important role in the antitumor immune response. The sensibilization, activation and proliferation of T lymph ocytes depend on antigen presenting cells (APC) which was able to present corres ponding antigen peptides and to provide costimulatory signals[1]. However, many tumor cells have weak immunogenicity which expressed low levels or no MHC and costimulatory molecules, so that tumor antigen can not be effectively presented. Therefore, they cannot induce effective antitumor immune response in host, and can not effectively activate specific killing mechanism. APC cancer vaccines are expected to enhance the immunogenicity of tumor cells and increase the presenting ability of antigen presenting cells as well as induce effective specific T lymphocyte mediated antitumor immune response. Dendritic cells (DC) are a kind of the most potential and professional antigen presenting cells *in vivo*[2-8]. DC can catch, process and present antigens, and enhance the killing activity of lymphokine[9-11], especially it can powerfully stimulate the primary immune response[12-16]. So more and more attention has been paid to the function of tumor immunotherapy of DC[17-19]. In this experiment, in order to effectively strengthen the function of antigen presenting of DC, to enhance the immunogenicity of tumor cells and to stimulate the specific CTL activity of host, dendritic cells derived from murine spleen were fused with hepatocarcinoma cells.

MATERIALS AND METHODS

Materials

Male BALB/C mice, 6-8 weeks old, weighing 15 g-20 g, purchased from Shanghai SIPPR/BK Experimental Animal Limited, were randomly divided into test group and control group. Mouse monoclonal antibody CD80, CD86, CD54 were purchased from Coulter Co. rmGM-CSF and rmIL-4 were obtained from R&D Co. Mini MACS (magnetic cell seperation) and CD11c (N418) microBeads were bought from Miltenyi GmbH Biotec. Metrizamide was obtained from

Amresco Co. and PEG was from Sigma Co. Mouse hepatocarcinoma cell line (H_{22}) was obtained from the Cancer Research Institute of Dalian Medical University.

Methods

Isolation of DC According to the previous METHODS[20-24] with minor modefications, DCs were isolated from murine spleen by metrizam ide (145 g·L^{-1}) density gradient centrifugation, purified based on its characteristics of semi-adhesion to culture plates and FcR$^-$, and cultured in the medium containing GM-CSF and IL-4 (500 ng·L^{-1}), and a large number of DC were harvested.

Cell fusion and selecting[25,26] DCs were fused with H_{22} cells by PEG and the fusion cells were marked with CD11c MicroBeads. The H_{22}-DCs were sorted with Mimi MACS sorter. Fused cells were cultured in RPMI 1640 medium containing 20 mL·L^{-1} fetal bovine serum, rmGM-CSF and rmIL-4 (500 ng·L^{-1}) for 2-3 wks.

Cellular morphological analysis Light microscopy and phase contrast microscopy were used to identify the morphological characteristics of H_{22}-DC, H_{22} and DC.

Immunocytochemical staining for CD80, CD86 and CD54 Cells were incubated with antibodies against CD80, CD86 and CD54. Membrane proteins were detected by ABC reagen and DAB staining, and photomicrographs were taken with an Olympus microphoto-microscope.

Cell proliferation analysis *in vitro* DCs were added into 24-well plates at 1.25×10^4 cells per well and three wells were randomly seclected to be counted every 24 h. Then, growth curve of H_{22}-DC was drawn accoding to their average value, using H_{22} as control group at the same time.

Tumorigenecity assays This experiment was conducted in 3 groups. Each group included four experimental subgroups (H_{22}-DC, H_{22}+DC, H_{22} and PBS). Immunogen (0.1 mL 1×10^{10}-2×10^{10} ·L^{-1}) of H_{22}-DC was inoculated subcutaneously into the right armpit of H_{22}-DC subgroup mice of each group and the same amount of H_{22}, H_{22}+DC and PBS were inoculated into the mice in each corresponding subgrou p in the same way. The growth of tumors was observed every day and the survival time of mice was calculated. Meanwhile, mice in the second group were killed on the 14th day after implantation, and tumors were isolated and tumor weight was compared.

CTL activity assays The third group mice were killed for examination at the 10th day after implantation, and the spleen was separated to prepare cell suspension, then the cells were cultured in 100 mL·L^{-1} FCS-RPMI1640 medium containing the final concentration of 100 KU·L^{-1} rhIL-2 by genetic recombination at 37℃ in a saturated humidified 50 m L·L^{-1} CO_2 atmosphere for 3 days. The anti-tumor experiment was conducted in four subgroups. Two ratios of effect (CTL) to target (H_{22}) (5:1 and 10:1) were used in all groups. ① Group A: CTL (H_{22}-DC subgroup) + H_{22}; ② group B: CTL (H_{22}+DC subgroup) + H_{22}; ③ group C: CTL (H_{22} subgroup) + H_{22}; ④ group D: CTL (PBS subgroup) + H_{22}. In addition, T groups were only consisted of CTL as the corresponding control groups and group E was H_{22} control group. Culture medium of the control group only contained 100 mL·L^{-1} FCS-RPMI1640. All of these groups were cultured in 96-well culture plates and each group had 3 wells at 37℃ in a saturated humidified 50 mL·L^{-1} CO_2 atmosphere for 48 h. Cytotoxity activity was determined by MTT assay as previously described[27,28]. Briefly, freshly prepared and filtered 20 μL MTT (5 g·L^{-1} in PBS) were added to each well, and the cells were continuously cultured for 4 h. Then the supernatant was removed and 150 μL DMSO was added to each well and agitated for 10 min to fully liquefy crystals, followed by reading on BIO-RAD 3550-UV type automatic ELSIA reader at 570 nm wavelength.

Statistical analysis

Statistical analysis was made using analysis of variance, if $P<0.05$, the result was considered statistically significant.

RESULTS

DC morphology

DCs are irregular shaped cells with many surface membrane processes, including spiky or spherical pseudopod-like processes. They have oval or irregular-shaped nucleus with wavy movement. The cytoplasm contains rich spherical mitocho ndrias. Determined by immunocytochemical staining, DCs were CD80 and CD86 and CD54 positive cells with irregular shape and brown-yellow fine granules in cytoplasm (Figures 5-7).

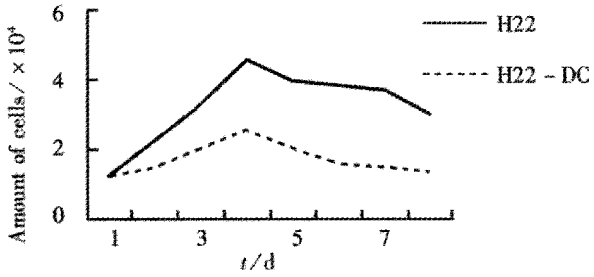


Figure 1 H_{22}-DC growth curve.

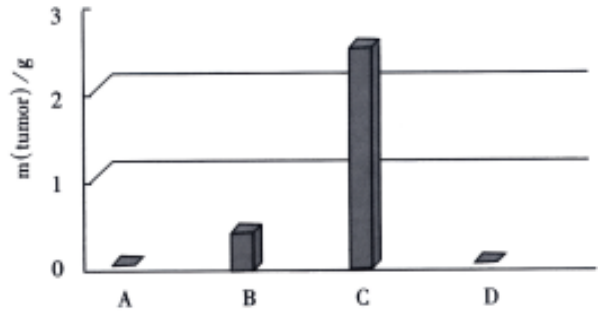


Figure 2 Tumor mass of BALB/c mice on d14 after inoculation.

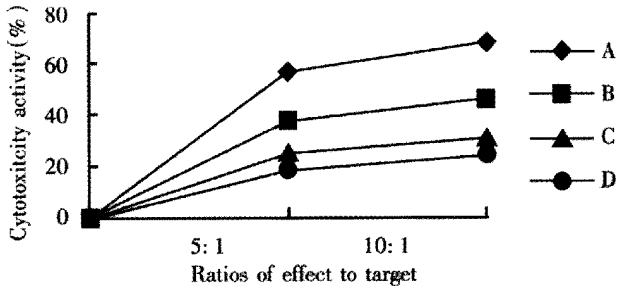


Figure 3 Influence on CTL cells of kill activity in vitro on d10 after inoculation.

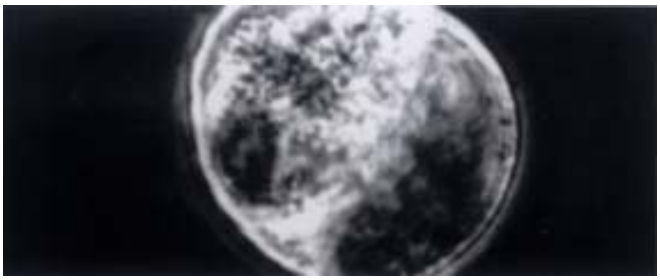


Figure 4 H_{22}-DC fusion cells under phase microscope. 5×40

Characteristics of H_{22}-DC and H_{22} and sorting of fusion cells

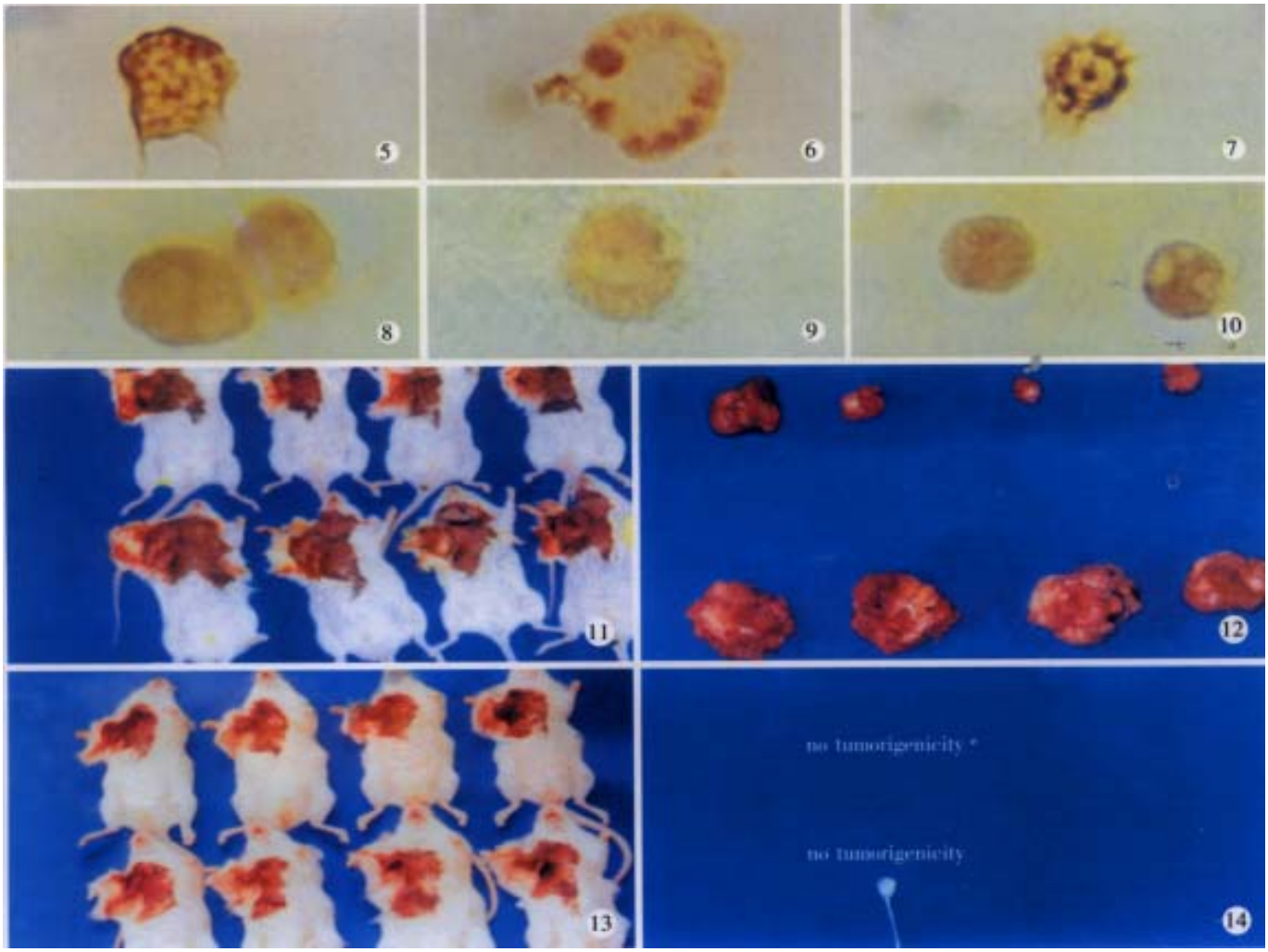
Marked with CD11c MicroBeads and sorted with Mini MACs, H_{22}-DCs were $CD11c^+$ cells (Figures 8-10), but H_{22} was $CD11c^-$ cells. By immunocytochemical staining, H_{22}-DCs were CD80, CD86 and CD54 positive cells and H_{22} was negative cells. Cytokine of rmGM-CSF and rmIL-4 was able to induce proliferation of fusion cells and prolong their survival time. The fusion cells which were marked with CD11c MicroBeads and sorted with Mini MACs were mixed with unfused DC. If there were no rmGM-CSF and rmIL-4 in the medium, natural apoptosis would occur in DC after 10-14 days, but H_{22}-DC would be still alive.

Identification and analysis of characteristics of fusion cells

H_{22}-DCs have some characteristics of both of their parental cells such as suspended growth, oval, flat and irregular in shape, they also have irregular shape nucleus and rich mitochondrias (Figure 4). Proliferation of H_{22}-DC in incubation without rmGM-CSF and rmIL-4 showed slow growth and low activity. However, H_{22}-DC incubation with rmGM-CSF and rmIL-4 was able to divide and proliferate, but compared with H_{22}, its activity of division and proliferation was significantly decreased and their growth curve was flatter. After subcutaneous implantation over 60 days, no tumorigenesis was induced in H_{22}-DC of mice, but induced tumorigenesis (100%) was observed in control subgroups (iH_{22}+DC and H_{22} subgroup) (Figure 1). The tumor weight of H_{22}+DC subgroup mice implanted on day 14 was significantly different from that of H_{22} subgroup ($P<0.01$, Figures 2, 11-13).

CTL activity assays

MTT assays showed that CTL activity of spleen in H_{22}-DC group was significantly higher than that in H_{22}+DC, H_{22} or PBS group ($P<0.01$, Figure 3).



Figures 5-7 The expression of CD80, CD86, CD54 in DC, being brown yellow, 3.3×100
Figures 8-10 The expression of CD80, CD86, CD54 in DC-H_{22}, being brown yellow, 3.3×100
Figures 11-14 Tumorigenecity assays in BALB/c mice on d 14 following DC-H_{22} inoculation.

DISCUSSION

Steinman and Cohn first isolated DC from the spleen of mice in 1973[20]. Since then, scholars have successfully isolated DC from thymus, aggregated lymphoid follicle, tracheas of mice, livers of rats and human peripheral blood. In recent years, mature DC was considered able to effectively present tumor-peptide epitopes and induce cytotoxic T lymphocytes (CTL) to produce strong specific antitumor immune response[29-32]. Wu and Kufe *et al* prepared DC vaccine using activated B cells and DC fused with tumor cells by traditional fusion METHODS in 1994 and 1997, respectively. This experiment was based on the established METHODS of isolation and generation of DC[20-24] by chemical fusion with PEG and techniques of immunomagnetic beads, it not only apparently simplifies the complicated sorting process of traditional fusion methods, but also effectively increase the purity of cell sorting. It is simple and feasible. The CD11c monoclonal antibody N418 is specific for the integrin αx subunit of αxb2 which was the leukocytic integrin expressed on mouse splenic DC[33,34]. The principle of MACS CD11c+ cell sorting is that the cells are labelled by MicroBeads coupled with CD11c antibodies and passed through a sorting column which is placed in the magnetic field of a MACS sorter. The magnetically labelled $CD11c^+$ DC are retained in the column while the unlabeled $CD11c^-$ cells passed away. After getting the column from the magnetic field, the magnetically retained $CD11c^+$ DC can be eluted as the fraction of positively sorting cells. The effect of MACS sorter is confirmed by fluoroimmunoassay, PCR, FISH and FACS. The advantages of MACS are: it can process numerous cells, its sorting purity is very high, and it can be operated easily.

Much data in recent years show that DC played a very important role in the tumor immune response[35-39], especially with the develpment of gene therapy against tumor specific antigen. But at present, T cells epitopes of tumor specific antigens in most of the human cancers besides melanoma, breast cancer and ovarian cancer are not very clear[40]. Thus, cancer vaccine directly fused DC with tumor cells has become an important way in active immunotherapy of tumors[41-45]. It is simple and reliable and of practical value. At the same time, a tumor immunotherapy approach of specificly distinguishing and killing tumor cells but normal cells of host *in vivo* has developed[45-50].

By sorting with Mini MACS marked with mouse CD11c MicroBeads, H_{22}-DCs have some characteristics of two parental cells, being irregular in shape. Apoptosis occurred in DC-DC and DC mixed with H_{22}-DC respectively after 7-10 d and 10-14 d in the medium with no rmGM-CSF and rmIL-4, but H_{22}-DCs were still alive. H_{22}-DC could divide and proliferate quickly in the initial stage but soon their growth slowed down and their activity of dividing and proliferating reduced. We failed to establish the cell line in vitro, possibly due to the growth nature of the parental cells in vitro and loss of chromosome with the time of incubation.

After subcutaneous implantation over 60 days, H_{22}-DC showed no induced tumorigenesis in BALB/C mice, but did it in H_{22} control group (100%). It suggested that H_{22}-DC has lost its tumorigenecity in vivo. The tumor weight in H_{22}+DC control group was significantly different from that of H_{22} control group when it had been implanted for 14 days ($P<0.01$). It shows that DC simply mixed with tumor cells could obviously inhibit the development of tumor in the early stage, but could not prevent the generation of tumor, which means that DC played a positive role in the course of presenting tumor antigen and inducing sepecific antitumor immune response in the early stage of tumorigenicity. By selecting the spleen of mice in H_{22}-DC, H_{22}+DC and H_{22} group on d10 after implantation spleen CTL activity in vitro was induced in our experiment, and the results showed that the spleen CTL activity of H_{22}-DC inoculated group was significantly higher than H_{22} inoculated group ($P<0.01$), which suggests that the active immunity of cancer vaccine can produce specific antitumorimmune protection in mice. DC and H_{22}-DC could induce specific antitumorimmune response and stimulate production of effective T lymphocytes in mice, and H_{22}-DC induced no tumorigenesis. It indicates that DC directly fused with hepatocarcinomar cells is likely to become a helpful appreach in immunotherapy for hepatocarcinoma.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Gastroesophageal manometry and 24-hour double pH monitoring in neonates with birth asphyxia

Mei Sun[1], Wei-Lin Wang[2], Wei Wang[2], De-Liang Wen[1], Hui Zhang[1] and Yu-Kun Han[1]

[1]Pediatric Department, 2Pediatric Surgery Department, Second Clinical College, China Medical University, Shenyang 110003, China
Project supported by the Research Fund of the Ministry of Health of China, No.96-2-170 (1996)
Correspondence to: Dr. Mei Sun, Pediatric Department, Second Clinical College, China Medical University, Shenyang 110003, China. meis@ihw.com.cn
Telephone: +86-24-23893501 Ext.6503, Fax: +86-24-23844734
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INTRODUCTION

Birth asphyxia may lead to disturbances of gastroenteric motility of newborn infants[1,2]. The change of gut pressure and reflux are the major manife stations of the motor disturbance[3-9]. To evaluate the effects of perinatal asphyxia on the gastroenteric motility, gastric and esophageal pressure and double pH were measured in a group of asphyxiated newborns. And, their pathophysiological and anatomical effects on gastroenteric function were discussed.

MATERIALS AND METHODS

Subjects

The neonates admitted to our neonatal intension care unit of the Second Affiliated Hospital, China Medical University from August 1995 to March 1996 were studied. The criterion for asphyxia: Apgar score at birth (1 min) $\leqslant$7, accompanied with hypoxia, acidosis and other organ damage caused by asphyxia[10]. The asphyxia group consisted of 35 asphyxia neonates with a mean age of 3.3 d, gestational age 40 wk, and mean body weight of 3189 g. In the asphyxia group, there were 7 infants with severe asphyxia (1 min Apgar score$\leqslant$3). The control group included 17 normal infants, who were admitted for intensive care. These infants showed no symptoms of gastroenteric, neurological and respiratory diseases, with a mean age of 5.2 d, mean gestation age of 39.6 wk, and mean body weight of 3371 g. Two groups were fed with same formula. No prokinetic agent was administrated.

Gastric and esophageal manometry

Multiple channel physiological recorder (RMP-6018, Japan), TP-400 pressure transmitter and Au-601G automatic drawing device were used in this study. The manometry was performed after 3h fasting. By the fluid transformation and synchronous device, the pressure within the stomach, lower esophageal sphincter (LESP), body of esophagus and upper esophageal sphincter were measured respectively and the difference between LESP and gastric pressure was calculated. The distance between the nasal cavity and the LES was calculated as well.

Double pH monitoring technique

Crystal antimony double pH microelectrode (diameter 2.1 mm, type 90-0011, Synectics Medical, Sweden) was used in this study. Before each monitoring, pH calibration was performed. The esophageal electrode was introduced to 2 cm above LES and the distal electrode was advanced 15 cm apart. The infants' diet and activity were not limited during the test. The beginning and ending of each feeding, occurrence of vomiting and crying were written down. The pH signals were recorded during 24 h in a pocket pH meter. After that, the meter was connected with computer and the results were analyzed with a pH analysis software system (Esophogram 5.550 B3 improved edition, Gastrosoft Inc.).

Monitoring index

The indexes of acid gastroesophageal reflux (GER) (esophageal pH<4): acid reflux index (RI) (percentage of total time pH<4); number of reflux episodes; number of reflux episode >5 min; duration of longest episode pH<4 in minutes; total time of pH <4 in minutes; the area under pH<4 curve (pH×min); and esophageal clearance time (min/reflux). The indexes of alkaline duodenogastric reflux (gastric pH>4): gastric alkaline index (AI): percentage of total time pH>4; number of gastric alkaline episodes; number of gastric alkaline episode >5 min; duration of longest episode pH>4 in minutes; total time of pH>4 in minutes; and the area under pH>4 curve (pH×min).

Statistics

The data were expressed as $\bar{x}\pm S_{\bar{x}}$. Inter-group comparison was performed with *t* test. The results were considered statistically significant if *P* value <0.05.

RESULTS

Esophageal and gastric pressure

The gastric pressure of the study group was higher than that of control group (Table 1). The LESP, difference between LESP and gastric pressure, esophageal body pressure and UESP were lower than those of control group, but the differences were not significant (P>0.05). The gastric pressure of severe asphyxia group was significantly lower than that of mild asphyxia group (P<0.01).

24-h esophageal pH monitoring

The GER parameters of asphyxia group were all higher than those of control group (Table 2). The parameters were also higher than the consulting pathological GER diagnosis standard for the age group of <12 mo, i.e. reflux index > 5%, number of reflux episodes >132, number of episodes over 5 min >1, duration of longest episode of pH<4 >13 minutes, the area under pH<4 curve >51. This pH analysis

system recommended a Boix-Ochoa value reflecting infant acid gastroesophageal reflux and a value less than 11.99 was considered normal statistically[11]. In our current study, the asphyxia group had an average value of 37.1 whereas 6.4 in the control group.

24-h gastric pH monitoring

The difference in the parameters between the asphyxia and control groups was not statistically significant ($P>0.05$, Table 3) and with no sensible tendency.

Double pH monitoring in infants with asphyxia of different degree

The acid GER in mild asphyxia group was more severe than that in severe asphyxia group (Tables 2,3). The gastric alkaline parameter was not significantly different between the asphyxia groups of different degree.

Table 1 Esophageal and gastric pressure in asphyxia infants ($\bar{x}\pm s_{\bar{x}}$, kPa)

Groups	*n*	Gastric pressure	LESP	LESP-gastric pressure	Esophageal pressure	UESP
Asphyxia	35	0.89±0.07	3.22±0.14	2.33±0.13	-0.33±0.05	2.38±0.13
Mild	28	0.99±0.06	3.32±0.15	2.31±0.14	-0.34±0.05	2.34±0.15
Severe	7	0.49±0.20[b]	2.90±0.33	2.42±0.36	-0.30±0.09	2.55±0.31
Control	17	0.86±0.07	3.37±0.29	2.47±0.29	-0.38±0.06	2.45±0.22

[b]$P<0.01$, *vs* mild asphyxia group.

Table 2 24-h esophageal pH monitoring in asphyxia ($\bar{x}\pm s_{\bar{x}}$)

Groups	*n*	Acid reflux index	Reflux episodes	Episode >5 min	Duration of longest episode pH<4	Total time pH<4	Area under pH<4curve	Esoph. clear time
Asphyxia	35	7.1±1.3[b]	137±24[b]	5.9±1.1[b]	24.9±8.0[a]	157±32[b]	187±62[a]	0.95±0.12[b]
Mild	28	8.7±1.5	167±28	7.3±1.3	30.3±9.8	192±37	230±76	1.00±0.12
Severe	7	0.8±0.4[d]	19±9[d]	0.9±0.7[d]	3.4±1.3[c]	19±10[d]	17±9[d]	0.76±0.36
Control	17	1.3±0.5	29±11	0.7±0.5	4.2±1.7	20±10	20±9	0.39±0.09

[a]$P<0.05$, [b]$P<0.01$, *vs* control; [c]$P<0.05$, [d]$P<0.01$, *vs* mild asphyxia.

Table 3 24-h pH monitoring of stomach in asphyxia ($\bar{x}\pm s_{\bar{x}}$)

Group	*n*	Gastric index	Alkaline episodes	Episode >5 min	Duration of longest episode pH>4	Total time pH>4	Area under pH>4 curve
Asphyxia	35	21±6	111±17	10.1±1.7	243±59	554±79	1132±202
Mild	28	23±7	124±20	10.3±1.8	234±58	558±83	1102±188
Severe	7	15±13	56±24	9.3±5.3	281±196	539±230	1252±723
Control	17	20±7	80±17	8.7±2.1	388±96	802±124	1769±352

DISCUSSION

The pressure measurement of inside gut is a major index reflecting gastroenterol motility. LESP and UESP are important elements to resist GER[12]. Our results showed that gastric pressure in asphyxia group was higher than that of control, LESP, esophageal body pressure and UESP were all lower than those of control group. Though the differences were not significant, it does partly contribute the pathophysiological and anatomical bases for GER[13-16].

GER is a disorder of gastroenterol motility malfunction. Among many inspecting METHODS, continuous 24 h esophageal pH monitoring is regarded as "golden standard" to diagnose and manage GER[17-22]. This study introduced this technique to the digestive tract motility research of asphyxia newborn babies. The acid GER parameters derived from the normal control group were similar with a large sample study (92 cases of same age) done by Vandenplas *et al*[23] with the same technique, which suggests that this method has good stability, reliability and repetition[24].

In children with impaired central nervous system, there is a tendency of more occurrence of GER[25-29]. This is associated with the long-term supine position, uncoordinated or lack swallow movement, impaired function of esophageal movement, abnormal motility of gastric sinus or pylorus duodenum, increased abdominal pressure caused by swallowing too much air, convulsion and effects of some medicine. It has been observed in animal that slight increase of intracranial pressure can cause obvious decrease of LESP[26,27]. Prenatal asphyxia can easily cause temperate or permanent injury of central nervous system, it should be noted that prenatal asphyxia may play an important role in the mechanism of GER.

GER is not all pathological, so it has no feature of all-or-no. There are large laps between physiological and pathological GER[30]. Normal physiological GER occurs when a baby is in a standing position, or in a state of wakening and post meal, whereas the pathological GER occurs when the baby is in supine position, or during sleep and before meal[31]. In our study,the GER in asphyxia patients obviously belong to the pathological GER no matter using Boix-Ochoa Index or other diagnostic standards. So these patients can easily suffer from reflux, vomiting even without feeding, and complications[32-35] such as problems of nutrition and respiratory system.

There is few research of alkaline GER in neonates[36-39]. It is generally regarded that esophageal pH>7 can be called alkaline GER. Because this value is near to the normal esophageal pH (5.0-6.8), the single esophageal pH monitoring can not give an accurate judgment about alkaline GER. But the gastroesophageal continuous double pH monitoring has resolved this problem[39]. It can help analyze the relevance of the changes of gastroesophageal pH and in the meantime it helps understand the pathological bases of the alkaline duodenal GER. However, the gastric alkaline parameter showed

nosignificant difference between the asphyxia group and normal control, mild and severe asphyxia infants. Furthermore, there was no relevance with the esophageal alkaline reflux.

To our surprise, severe asphyxiated infants showed obviously decreased gastric pressure, slighter acid GER and lower gastric alkaline than that of their mild asphyxia cohort. Combining with the clinical symptoms of feeding difficulty, gastric retention, abdominal distension, and constipation in severe asphyxiated infants, this result suggested that after severe asphyxia, a phenomenon similar with "gastropa resis" in adults, developed in newborn infants due to the multiple factors such as inhibition of central nervous system[40,41] and ischemia-refilling injury.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Influence of L-methionine-deprived total parenteral nutrition with 5-fluorouracil on gastric cancer and host metabolism

Hong-Bing Xiao[1], Wei-Xin Cao[2], Hao-Ran Yin[2], Yan-Zhen Lin[2] and Shi-Hui Ye[1]

[1]Department of Surgery, Affiliated Railway Hospital, Tongji University, Shanghai 200072, China
[2]Department of Surgery, Affiliated Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
Supported by the National Natural Science Foundation of China, No.39370780.
Correspondence to: Dr. Hong-Bing Xiao, Department of Surgery, Affiliated Railway Hospital, Tongji University, Shanghai 200072, China. xhawbin@online.sh.cn
Telephone: +86-21-56533693, Fax: +86-21-56773983
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Abstract

AIM **To investigate the influence of L-methionine-deprived total parenteral nutrition with 5-FU on gastric cancer and host metabolism.**

METHODS **N-methyl-N'-nitro-nitrosoguanidine (MNNG) induced gastric cancer rats were randomly divided into four groups: Met-containing TPN group (*n* = 11), Met-deprived TPN group (*n* = 12), Met-containing TPN+5-FU group (*n* = 11) and Met-deprived TPN+5-FU group (*n* = 12). Five rats in each group were sacrificed after 7 days of treatment and the samples were taken for examination. The remaining rats in each group were then fed separately with normal diet after the treatment until death, the life span was noted.**

RESULTS **The tumors were enlarged in Met-containing group and shrank in Met-deprived group markedly after the treatment. The DNA index (DI) of tumor cells and the body weight (BW) of rats had no significant change in the two groups, however, the ratio of tumor cells' S phase was increased. The ratio of G2M phase went up in Met-containing group, but down in Met-deprived group. In the other two groups that 5-FU was added, the BW of rats, and the diameter of tumors, the DI of tumor cells, the S and G2M phase ratio of tumor cells were all decreased, particularly in Met-deprived plus 5-FU group. Pathological examination revealed that the necrotic foci of the tumor tissue increased after Met-deprived TPN treatment, and the nucleoli of tumor cells enlarged. In -MetTPN+5-FU group, severe nuclear damage was also found by karyopyknosis and karyorrhexis, meanwhile there was slight degeneration in some liver and kidney cells. The serum free Met and Cysteine decreased markedly (*P*<0.001), while other amino acids, such as serum free serine and glutamine increased significantly (*P*<0.005). All the rats died of multiple organ failure caused by cancer metastasis. The average survival time was 18.6 days in Met-containing TPN group, 31 days in Met-deprived TPN group, 27.5 days in Met-containing TPN+5-FU group, and 43 days in Met-deprived TPN+5-FU group (*P*<0.05).**

CONCLUSION **Met-deprived TPN causes methionine starvation of tumor cells, and can enhance the anti-tumor effect of 5-FU and prolong the life span of gastric cancer-bearing rats.**

Subject headings stomach neoplasms/therapy; stomach neoplasms/pathology; parenteral nutrition; methionine/therapy use; fluorouracil/therapy use

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INTRODUCTION

Many tumor cells can not grow in the medium replaced L-methionine (Met) with its direct precursor L-homocyateine. However, normal cells can grow well in that medium. It means that the growth of tumor cell is Met-dependent[1-9]. In this study, we prepared a Met-free amino acid solution and used it as the sole nitrogen source of total parental nutrition (TPN), to investigate the influence of Met-deprived TPN on the gastric cancer and host metabolism.

MATERIALS AND METHODS

Animal grouping and treatment

Male Wistar rats, with N-methyl-N'-nitro-nitrosoguanidine (MNNG) induced gastric cancer (*n* = 46), were verified to have tumor diameter larger than 8 mm in laparotomy and confirmed pathologically. The 46 rats were randomly divided into four groups: Met-containing TPN group (*n* = 11), Met-deprived TPN group (*n* = 12), Met-containing TPN+5-FU group (*n* = 11) and Met-deprived TPN+5-FU group (*n* = 12). Five rats in each group were sacrificed after 7 d of treatment and the specimens were taken for examination and the other rats were then fed with normal diet separately after the treatment until death for noting the life span. After 24 h fasting, the rats were cannulated to the vena cava immediately after peritoneal cavity anesthesia with 25 $g \cdot L^{-1}$ barbital and placed in metabolic cage respectively and infused with different TPN solution after fixing the TPN tube at the back of rats with a plate and tension spring.

Composition and infusion of TPN solution

The rats were infused with 50 $g \cdot L^{-1}$ glucose normal saline 200 mL $\cdot kg^{-1}$ on the cannulation day. Different TPN solutions were infused continously 24 h a day on the next 7 d. During the experiment, the rats were housed individually in metabolic cages. The composition and usage of TPN solutions are shown in Table 1.

Specimen sampling and detection

Pre- and post- TPN, the rats' body mass (BM) and the tumor

diameter were measured, the tumor tissues were sampled for flow cytometry (FCM) tumor cell cycle analysis and DNA index calculation. Blood samples were taken for serum amino acid profile test. Tissues of the rats' gastric cancer, heart, lung, liver and kidney were sampled and fixed with 100 mL·L^{-1} formalin, enbedded with paraffin, sliced in 5 μm, and HE stained for pathohistologic examination.

Table 1 Composition and dosage of TPN solution (kg^{-1}·d^{-1})

Composition	+MetTPN	-MetTPN
250 g·L^{-1} Glucose / mL	100	100
200 g·L^{-1} Intralipid / mL	35	35
+Met amino acid solution / mL	100	0
-Met amino acid solution / mL	0	100
Non-protein energy / J	710	710
Total nitrogen / g	1.30	1.03
Total volume / mL	235	235

Usage and dose of 5-FU

5-FU was added to the TPN solution and infused for 6 d (from d 2 to d 7, 15 mg·kg^{-1}·d^{-1}).

Observation of survival time

The other rats in each group not sacrificed were moved to common cage after withdrawing TPN tube and fed with normal diet until death. The survival time was noted.

Statistical analysis

Student t test was used to examine the data. Survival time was examined with time sequence examination. The difference was considered significant when the *P* value was less than 0.05.

RESULTS

Alteration of the rats' BM, tumor size and DI

The BM and DI had no significant change in both +MetTPN and -MetTPN groups after treatment. The tumor size was markedly enlarged in +MetTPN group and shrank in -MetTPN group. All of the BM and the DI as well as the tumor size were decreased in both groups of +MetTPN+5-FU and -MetTPN+5-FU, and the change was more significant in the -MetTPN+5-FU group (Table 2).

Change of tumor cell cycles

The S phase percentage of tumor cells was increased in both +MetTPN and -MetTPN groups, and it is higher in the other groups. However, the change of G2M phase ratio was different. It was increased in +MetTPN group and decreased in -MetTPN group. The S and G2M phases were lowered in the other groups with 5-FU. The change was more significant in -MetTPN+5-FU group (Table 3).

Table 2 Body mass, tumor diameter and DNA index of tumor cells after TPN ($\bar{x}$±s)

TPN solution	mb / g		dt / mm		DNA index	
	Pre-TPN	Post-TPN	Pre-TPN	Post-TPN	Pre-TPN	Post-TPN
+MetTPN	242±20	243±20	11.2±1.0	14.7±0.7^{b}	1.13±0.24	1.21±0.26
-MetTPN	248±20	245±18	11.5±1.2	6.9±0.8^{ad}	1.15±0.21	1.12±0.23
+MetTPN+5-FU	245±13	233±12^{b}	11.1±1.1	9.3±0.8^{a}	1.15±0.03	1.08±0.02^{a}
-MetTPN+5-FU	245±14	225±13^{b}	11.4±0.9	5.5±0.5^{bc}	1.15±0.03	1.02±0.02^{bc}

^{d}P<0.05, ^{b}P<0.01, *vs* preTPN; ^{c}P<0.05, ^{d}P<0.01, *vs* +MetTPN.

Table 3 Change of tumor cell cycles after TPN ($\bar{x}$±s, %)

TPN solution	S		G2M		G0 / G1	
	Pre-TPN	Post-TPN	Pre-TPN	Post-TPN	Pre-TPN	Post-TPN
+MetTPN	6.2±1.2	10.3±1.4^{b}	10.5±1.1	16.6±1.4^{a}	83.4±1.9	73.1±1.8^{b}
-MetTPN	6.4±1.0	28.5±1.0^{ac}	11.2±1.1	8.3±1.5^{ad}	82.4±0.9	63.2±5.5^{a}
+MetTPN+5-FU	6.6±0.3	4.5±0.5^{a}	12.6±0.5	9.7±1.2^{a}	80.8±0.3	85.8±1.2^{b}
-MetTPN+5-FU	6.5±0.7	4.5±0.3^{a}	12.4±0.9	6.2±0.5^{bc}	80.1±0.5	89.3±0.7^{b}

^{a}P<0.05, ^{b}P<0.01, *vs* preTPN; ^{c}P<0.05, ^{d}P<0.01, *vs* +MetTPN.

Serum amino acid profile

The serum L-methionine and L-systein were markedly decreased in the -MetTPN rats. However, the other amino acids such as asprine and glutamine as well as serine were significantly increased after treatment (Table 4).

Pathohistological findings

The number of tumor necrotic foci were increased after -MetTPN and -MetTPN+5-FU treatment. Nuclei was enlarged in tumor cells and liver cells.

Survival time

All the gastric cancer bearing rats died of cancer metastasis and cachexia. The mean survival time was 18.6 d in +MetTPN group, 31 d in -Me tTPN, 27.5 d in +MetTPN+5-FU and 43 d in -MetTPN+5-FU (P<0.05).

Table 4 Serum FAA value ($\bar{x}$±$s_{\bar{x}}$, μmol·L^{-1})

	+MetTPN	-MetTPN
Asp	36.1±1.2	88.9±10.3^{a}
Glu	27.3±4.2	193.2±17.4^{a}
Ser	124.8±21.5	231.5±32.3^{a}
Gly	116.9±18.3	286.7±21.9^{a}
Gln	103.4±14.4	90.1±10.3
His	11.2±1.5	25.0±3.6
Thr	53.5±3.8	93.2±7.1^{a}
Ala	138.7±30.1	218.8±31.4^{a}
Arg	83.6±9.4	173.4±25.1^{a}
Pro	33.9±8.2	79.7±13.4
Tyr	20.8±5.1	44.8±6.3
Val	88.4±14.1	31.6±1.6
Met	76.1±1.3	10.9±3.1^{b}
Cys	87.3±3.2	43.2±5.4^{b}
Ile	5.4±1.1	10.1±1.2
Leu	34.2±1.3	55.2±4.3
Phe	22.1±2.2	43.1±2.9
Trp	42.5±3.6	50.7±4.4
Lys	212.4±43.1	387.8±58.3^{a}

^{a}P<0.05, ^{b}P<0.001, *vs* +MetTPN.

DISCUSSION

Patients with malignant tumors often show severe protein-amino acid metabolism disorder and uncorrectable negative nitrogen balance as well as low immune function caused by malnutrition[10-21]. TPN support is considered beneficial to improve the patients' nutritional status and immune function, and to lower the surgical complications, and to improve the quality of life[22-28]. But, TPN can also stimulate proliferation of the tumor cells[29,30]. So, there is much concern to study the special feature of tumor cells' metabolism and to find a regimen of TPN that is beneficial to the host, but pernicious to the tumor cells, particularly through the regulation of tumor cells' metabolism[31-52].

In this study, a special regimen of TPN deprived of Met was used in MNNG induced gastric cancer rats. The results showed that the TPN, containing Met or not, has regulative effects on the tumor cells' dynamics. The effects were different between Met-containing TPN and Met-deprived one. Met-containing TPN stimulated tumor cells' proliferation and promoted the tumor cells from G0/G1 phase into S and G2M phase, and made the ratio of S phase to G2M phase increased simultaneously. However, the Met-deprived TPN disturbed the metaboli sm of DNA, especially DNA methylation through Met starvation and inhibited the tumor proliferation by blunting S phase into G2M phase, resulting in increase of S phase ratio and decrease of G2M phase ratio. The inhibitory effect of tumor growth of the Met-deprived TPN was enhanced by simultaneous use of 5-FU, and it was manifested by a longer life span in the rats treated. Pathohistological examination found that the necrotic foci were increased in the tumor tissue and the nuclei of the tumor cells were enlarged. The pathohistologic findings were concordant with FCM analysis of cell cycles, meaning that the metabolism of DNA was blunted. One must be careful when using -MetTPN, particularly -MetTPN+5-FU, as some adverse effects may occur. In this practice, we noticed that the host liver and kidney cells had light degeneration. Hoffman *et al*[53] studied the growth of SV-40 fibroblast cells in the medium deprived of Met and found that the cell proliferation was blunted in S/G2 phase reversibly. Further studies[54] found that in the Met-free environment, the intracellular free Met of tumor was extremely decreased and it lowered greatly the S-Adeosylmethionine (S-AdoMet) which had decreased because of the tumor cell's over active transmethylation, and resulted in a low ratio of S-AdoMet to S-AdoHcy. This directly inhibited the activity of transmethylase and suppressed the transmethylation reaction, including the DNA methylation. When the tumor cells were returned to the Met-containing environme nt, DNA methylation recovered and the cell cycle circulated. This reversible block of tumor cell cycle at S/G2 phase enhanced the anti-cancer effect of 5-FU and hinted the combined and sequenced use of other cycle specific chemotherapy agents with Met-deprived TPN.

By autopsy, we found that the liver and peritoneum metastasis of gastric cancer were much less in the group using Met-deprived TPN and the group using Met-deprived TPN plus 5-FU. It suggests that not only the primary tumor proliferation was inhibited, but also the invasive ability for metastasis was suppressed. Breillout *et al*[55] reported that Met-deprived TPN suppressed the metastasis potential of Lewis lung cancer and rhabdomyosarcoma in experimental animals. They considered that it was Met-starvation which disturbed the methylation of DNA and membrane lipids of the tumor cells, inhibiting their metastastic ability. Some authors even suggested that the inhibitory effect of -MetTPN against metastasis is more powerful than in proliferation of primary tumors. However, this still needs further studies.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Experimental study on antitumor effect of arsenic trioxide in combination with cisplatin or doxorubicin on hepatocellular carcinoma

Wei Wang[1], Shu-Kui Qin[1], Bao-An Chen[2] and Hui-Ying Chen[1]

[1]Chinese PLA Cancer Center, Chinese PLA 81 Hospital, Nanjing 210002, Jiangshu Province, China
[2]Affliliated Zhongda Hospital of Southeast University Medical College, Nanjing 210087, Jiangsu Province, China
Supported by the Youth Science Grant of Jiangshu Province, No. BQ98048.
Correspondence to: Prof. Shu-Kui Qin, Chinese PLA Cancer Center, Chinese PLA 81 Hospital, Nanjing 210002, Jiangshu Province, China. Qinsk@jlonline.com
Telephone: +86-25-6648090 Ext.529
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INTRODUCTION

The main component of a traditional Chinese drug "Pishuang", arsenic trioxide (As_2O_3), has obviously selective anti-tumor effect on human hepatocellular carcinoma (HCC) in both *in vitro* and *in vivo* studies[1-5]. Due to limited effectiveness when any anti-carcinogen is used alone and obviously increased toxicity when the dose is raised, there is no exception for As_2O_3. Furthermore, combined chemotherapy contributes to improve therapeutic effectiveness, disperse toxicity and surmount drug-resistance, in which the combination of traditional Chinese and modern medicine has more advantages and characteristics. As a result, we made an experimental study on anti-tumor effect of As_2O_3 in combination with cisplantin (PDD) or doxorubicin (ADM) on HCC, to investigate the possibility of As_2O_3 in combination with PDD or ADM and nature of interaction between them, and to provide experimental basis for clinical application.

MATERIALS AND METHODS

Materials

Drugs and reagents As_2O_3 for injection (5 mg per am poul, Lot No. 998068, provided by Professor Ma Jun of Harbin Hamaeotoloy and Oncolony Institute), PDD for injection (20 mg per vial, Lot No. 990618, Shandong Qilu Pharmaceutical Factory) or ADM hydrochloride for injection (10 mg per vial, Lot 990406, Shanxi Mlarmaceutical Co. LTD).

Cell lines Human hepatoma Bel-7402 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in our laboratory. Bel-7402 cells were routinely cultured in RPMI1640 medium (Gibco) containing 100 mL·L^{-1} fetal calf (FCS) serum at 37℃ in humidified incubator with 50 mL·L^{-1} CO_2/95 mL·L^{-1} air.

Animals Mice with hepatoma HepA were obtained from the Shanghai Institute of Materia Medica of Chinese Academy of Sciences and Kunming mice (famale and male weighing, 18 g-22 g) from the Experimental Animal Center of Southerneast University Medical College.

methods

Measurement of anticancer activity *in vitro* The exponent growing Bel-7402 cells in culture flasks were harvested by 2.5 g· L^{-1} EDTA, suspended in RPMI1640 medium with 100 mL·L^{-1} FCS, adjusted to the concentration of 3×10^4 cells·L^{-1}, plated into 40-well plates (100 μL cells·well-1) and incubated at 37℃ in 50 mL·L^{-1} CO_2/95 mL·L^{-1} air until the cells were stuck with the plates. The cells were then exposed to 100 μL of various concentrations of a drug alone or combination for 48 h, and the controls to 100 μL of RPMI1640 medium with no FCS. After that, the absorption was detected by adding 20 μL tetrazolium (MTT) to each well, incubating for 4 h, sucking out the media, adding 150 μL dimethylsul foxide (DMSO) to dissolve the violet-crystal and measuring at 570 nm. Double wells were used for each drug concentration. Experiments were triplicated. The inhibitory rate was calculated as follows:

IR(%)=(1-mean absorption in experiments/mean absorption in controls)×100%

Measurement of anticancer activity *in vivo* The mice with hepatocarcinoma HepA were killed and their ascites Abstracted, adjusted to 2×10^7·mL^{-1} and implanted by subcutaneous injection 200 μL to each mouse. Sixty mice with implanted HepA tumor were randomly divided into control group (saline), and groups of As_2O_3 alone (2 mg·kg^{-1}·d^{1}), PDD alone (1 mg·kg^{-1}·d^{1}), As_2O_3 combin ed with PDD (As_2O_3 2 mg·kg^{-1}·d^{1} + PDD 1 mg·kg^{-1}·d^{1}), ADM alone (1 mg·kg^{-1}·d^{1}), and As_2O_3 combined with ADM (As_2O_3 2 mg·kg^{-1}·d^{1} +ADM 1 mg·kg^{-1} ·d^{-1}). Each group was injected intraveously 24 h after transplatation once a day for 7 days continuously. The mice were killed on the 8th day after the treatment and the tumors isolated and weighed. The inhibitory rate of tumor was calculated as follows:

Inhibitory rate of tumor (%)=(1-mean tumor weight in experiments/mean tumor weight in controls)×100%

Statistical method Analysis of variance of two-factor factoria l experiment was applied to evaluate anti-cancer activity *in vitro* and analysis of variance of random experiment was used to evaluate anti-cancer activity *in vivo*.

Evaluation of interaction of drug combination *In vitro* experiment: the interaction between As_2O_3 and PDD or ADM was evaluted by coefficient of drug in interaction (CDI), which was calculated as follows: CDI = AB/(A×B). AB is the absorption ratio between a drug combination group and controls and A or B is that between a drug alone and controls. When CDI value was equal to 1.0, or more than 1.0 or less than 1.0, the nature of the interaction between A and B was considered to be additive or antagonistic or synergistic[6]. *In vivo* experiment: the interaction between As_2O_3 and PDD or ADM was evaluated by Q value, which was calculated as follows: Q=E(AB)/[EA+(1-EA)×EB]. E(AB) is the inhibiting tumor rate and EA or EB is that of a drug alone. When Q value was equal to 0.85-1.15, or less than 0.85 or more than 1.15, additive or antagonistic or synergistic interaction was thought to occur[7].

RESULTS

The effect of As_2O_3 and/or PDD on HCC

The inhibition rates of As_2O_3 *in vitro*, in combination with PDD at various concentrations were more than that of As_2O_3 or PDD alone ($^aP<0.01$, F = 58.96), in which the inhibition rates increased more evidently at low concentrations (Figure 1). CDI values of As_2O_3 and PDD in combination at low concentration were less than 1.0 (Table 1).

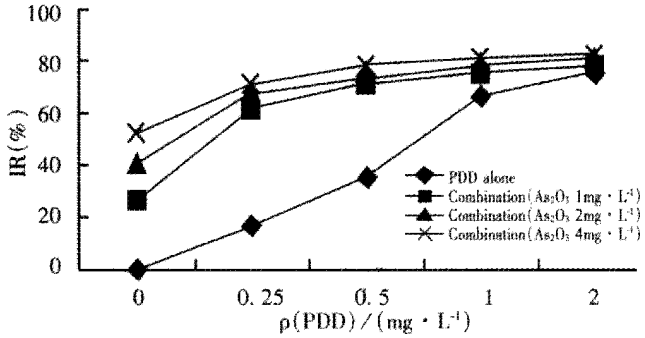

Figure 1 The effect of As_2O_3 and/or PDD on the growth of Bel-7402 cells *in vitro.*

Table 1 CDI value of As_2O_3 in combination with PDD against Bel-7402 cells

Cell line	As_2O_3 (mg·L-1)	PDD (mg·L-1)			
		0.25	0.5	1	2
Bel-7	1	0.60	0.60	1.00	1.22
	2	0.62	0.70	1.03	1.38
	4	0.60	0.65	1.13	1.50

The effect of As_2O_3 and/or ADM on HCC

In vitro the inhibition rates of As_2O_3 in combination with ADM in various concentrations were more than those of As_2O_3 or ADM alone ($^aP<0.01$, F = 64.77), in which the inhibition rates increased more evidently in low concentrations (Figure 2). CDI values of As_2O_3 and ADM in combination in low concentrations were almost equal to 1.0 (Table 2).

The effect of As_2O_3 and/or PDD on HepA implanted tumor

The inhibiting tumor rate of As_2O_3 in combination with PDD was more than that of As_2O_3 or PDD alone and *Q* value was more than 1.15 (Table 3).

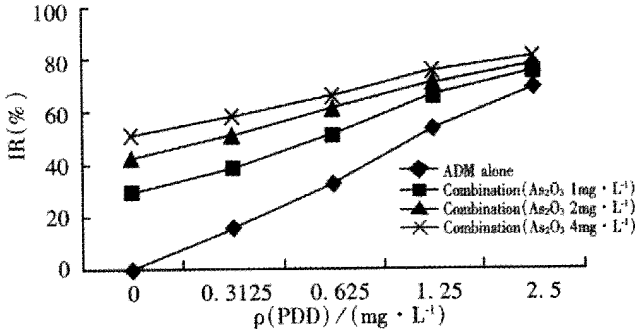

Figure 2 The effect of As_2O_3 and/or ADM on the growth of Bel-7402 cells *in vitro.*

Table 2 CDI value of As_2O_3 in combination with ADM against Bel-7402 cells

Cell line	As_2O_3(mg·L-1)	ADM (mg·L-1)			
		0.3125	0.625	1.25	2.5
Bel-7	1	1.03	1.02	1.05	1.12
	2	1.03	0.98	1.06	1.31
	4	1.00	1.05	1.08	1.28

Table 3 The effect of As_2O_3 and/or PDD on HepA implanted tumor in mice (n = 10)

Groups	Dose ($mg·kg^{-1}·d^{-1}$)	Tumor mass ($\bar{x}$±s,g)	Inhibition (%)	Q value
Control	NS	1.53±0.35		
As_2O_3	2	1.07±0.21	30.1	
PDD	1	0.82±0.11	46.2	
As_2O_3+PDD	2+1	0.40±0.05	73.9[a]	1.18

[a]$P<0.01$, F = 54.05, *vs* As_2O_3 or PDD alone.

The effect of As_2O_3 and/or ADM on HepA implanted tumor

The inhibiting tumor rate of As_2O_3 in combination with ADM was higher than that of As_2O_3 or ADM alone and *Q* value was less than 1.15 but more than 0.85 (Table 4).

Table 4 The effect of As_2O_3 and/or ADM on HepA implanted tumor in mice (n = 10)

Groups	Dose ($mg·kg^{-1}·d^{-1}$)	Tumor mass ($\bar{x}$±s,g)	Inhibition (%)	Q value
Control	NS	1.53±0.35		
As_2O_3	2	1.07±0.21		
ADM	1	0.91±0.12	40.5	
As_2O_3+ADM	2+1	0.61±0.11	60.1[a]	1.03

[a]$P<0.05$, F = 24.40, *vs* As_2O_3 or ADM alone.

DISCUSSION

As_2O_3, the main component of traditional Chinese drug "Pishuang", has been applied to treat acute promyelocytic leukemia and yielded notable results. Complete remission rate and long-term survival rate are high and the relapse rate is low in APL patients treated with As_2O_3[8-10]. The main mechanism of As_2O_3 is to induce apoptosis of leukemia cells, which is different from all-trans retinoic acid (ATRA)[11-23]. Based on the achievements, the experimental studies on anti-tumor effect of As_2O_3 in such hematopathy as malignant lymphoma[24] and myeloma[25,26] and solide tumors such as cancers of lung[27],

esophagus[28], stomach[29-32], colone[33-35] pancreas[36], mamma[37], cervix[38] and neuroblastoma[39] are in the ascendant.

The morbidity and mortality of hepatocarcinoma is high in China, which is the first cause of death among all kinds of cancers in Jiangshu Province. Due to the hidden onset, low rates of early diagnosis and rapid progression, most patients with hepatocarcinoma cannot be operated on and have to depend on chemotherapy, but the therapeutic effect of the present agents is unsatisfactory. So it is urgent and necessary to go on seeking new drugs and the improving therapeutic METHODS. Our group has taken the lead in conducting the study of As_2O_3 against liver cancer and found that As_2O_3 had obviously selective anti-tumor effect on hepatocarcinoma both *in vitro* and *in vivo*[1-5]: *in vitro* As_2O_3 inhibited the proliferation of several hepatocarcinoma cell lines but not normal human liver cells and *in vivo* inhibited implanted hepatocarcinoma in mice and prolonged the survival phase of mice with hepatocarcinoma but produced no obvious toxicity. The main mechanism is to induce apoptosis of hepatocarcinoma cells, which also has been proved by other reports[40-46].

To further investigate the best therapeutic way of As_2O_3 and raise the effect on hepatocarcinoma, we studied As_2O_3 and PDD or ADM in combination. The experiments *in vitro* showed that As_2O_3 in combination with PDD or ADM can increase the effect on HCC Bel-7402 and the increase extent varies at different concentrations, which was greater at lower concentrations. The possibl e reason is that the anti-tumor activity of an individual drug is saturated at high concentrations and difficult to increase after combination or there was antagonistic action to some extent between two drugs in combination and counteracted part of anti-tumor activity of a drug. CDI values showed that *in vitro* the nature of interaction is markedly synergistic between As_2O_3 and PDD and addictive between As_2O_3 and ADM in low concentrations. On the basis of the experiments *in vitro*, low-dose PDD or ADM combined with As_2O_3 was applied to treat HepA tumor implanted in mice, and inhibitory rate of tumor evidently increased as compared with that of a drug alone. Q value showed that *in vivo* synergistic interaction between As_2O_3 and PDD and addictive between As_2O_3 and ADM were thought to occur, which agreed with the results *in vitro*. These results suggested that low-dose PDD or ADM and As_2O_3 in combination could increase evidently anti-hepatocarcinoma effect. PDD and ADM are the main anti-hepatocarcinoma agents, but their toxicities in kidney, liver or heart restrict their clinical application, as a result patients cannot tolerate the high-dose agents whereas low dose is difficult to achieve satisfactory results. Considering selectively inhibitory effect of As_2O_3 on HCC *in vitro* and unobvious toxicity *in vivo*, the effect may be improved evidently without increased toxicities or keep satisfactory in poorly-tolerated patients with low dose of PDD or ADM when As_2O_3 and PDD or ADM in combination are applied to treat hepatocarcinoma.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Experimental research on TECA-I bioartificial liver support system to treat canines with acute liver failure

Xiao-Ping Chen[2], Yi-Long Xue[1], Xin-Jian Li[1], Zuo-Yun Zhang[1], Yan-Ling Li[1] and Zhi-Qiang Huang[2]

[1]Institute of Basic Medical Sciences, 2Department of Hepatobiliary Surgery, PLA General Hospital, Beijing 100853, China
Correspondence to: Dr. Yi-Long Xue, Institute of Basic Medical Sciences, PLA General Hospital, Beijing 100853, China. xueyl@plagh.com.cn
Telephone: +86-10-66937914
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Abstract

AIM **To evaluate the efficacy and safety of the TECA-I bioartificial liver support system (BALSS) in treating canines with acute liver failure (ALF).**

METHODS **Ten canines with ALF induced by 80% liver resection received BALSS treatment (BALSS group). Blood was perfused through a hollow fiber tube containing 1×10^{10} porcine hepatocytes. Four canines with ALF were treated with BALSS without porcine hepatocytes (control group), and five canines with ALF received drug treatment (drug group). Each treatment lasted 6 hours.**

RESULTS **BALSS treatment yielded beneficial effects for partial liver resection-induced ALF canines with survival and decreased plasma ammonia, ALT, AST and BIL. There was an obvious decrease in PT level and increase in PA level, and there were no changes in the count of lymphocytes, immunoglobulins (IgA, IgG and IgM) and complement (C3 and C4) levels after BALSS treatment. In contrast, for the canines with ALF in non-hepatocyte BALSS group (control group) and drug group, there were no significant changes in ammonia, ALT, AST, BIL, PT and PA levels. ALF canines in BALSS group, control group and drug group lived respectively an average time of 108.0 h±12.0 h, 24.0 h ±6.0 h and 20.4 h ±6.4 h, and three canines with ALF survived in BALSS group.**

CONCLUSION **TECA-I BALSS is efficacious and safe for ALF canines induced by parcial liver resection.**

Subject headings liver, artificial; liver failure, acute/surgery; liver failure, acute/physiopathology; liver failure, acute/immunology

Chen XP, Xue YL, Li XJ, Zhang ZY, Li YL, Huang ZQ. Experimental research on TECA-I bioartificial liver support system to treat canines with acute liver failure. *World J Gastroenterol*, 2001;7(5):706-709

INTRODUCTION

The incidence of acute liver failure (ALF) is high, and the survival rate of ALF patients is only 10%-15%[1,2].The current comprehensive treatment for ALF is not satisfactory. Orthotopic liver transplantation (OLT) is an effective therapy for patients with ALF. However, because of the shortage of donor organs, many patients died before they could receive OLT. The recently developed bioartificial liver support system can temporarily replace the complex functions of liver such as synthesis, detoxication and "bridge" the patients with ALF to liver transplantation. To what extent can TECA-I BALSS replace normal liver functions In order to answer the question, we have introduced the basic techniques of a hollow fiber bioartificial liver system, used Chinese experimental miniature swines as the donor of xenogeneic hepatocytes, improved the culturing conditions of porcine hepatocytes in the BALSS, and identified the efficacy and safety of TECA-I BALSS in treating drug-induced ALF canines[3,4]. In the present study, we developed a partial liver resection-induced ALF canine model to assess the efficacy and feasibility of the TECA-I BALSS in treating ALF canines.

MATERIALS AND METHODS

Animal

Nineteen healthy adult hybrid canines weighing 18 kg-22 kg were obtained from the Experimental Center of General Hospital of PLA, and fourteen adult Chinese experimental miniature swines weighing 8 kg-10 kg from the Breed Center of Chinese Agricultural University.

Reagents

All chemicals were provided by Hong Kong TECA Ltd Co. and Sigma Chemical Co.

Development of a canine model of ALF

The canines received eighty percent liver resection. Left lateral, left anterior , right lateral and caudate lobe were removed.

Isolation and culturing of porcine hepatocytes

Hepatocytes were isolated from Chinese experimental miniature swines by collagen ase digestion. Viability of the cells was assayed by trypan blue exclusion and AO/PI fluorescence staining.

BALSS treatment

Freshly isolated hepatocytes were cultured in the TECA-I BALSS provided by Hong Kong Ltd Co. The porcine hepatocytes circulated through the exterior space of capillary hollow fiber in the BALSS. Blood from femoral artery and vein on one side of the ALF canine circulated through the inner space of capillary hollow fiber and the substances exchanged through the membrane of hollow fibers between porcine hepatocytes and ALF blood.

Experimental groups

Nineteen ALF canines were randomly divided into three groups: ① BALSS group (n = 10): perfused with hollow fiber

tube of BALSS containing 1×10^{10} porcine hepatocytes. ② non-hepatocyte BALSS group (control group, $n = 4$): perfused with hollow fiber tube without porcine hepatocytes. ③ drug group ($n = 5$): intravenous injection with arginine, sodium glutamate and branch amino acid. Each treatment lasted six hours.

Biochemical assays

The plasma levels of ammonia, ALT, AST, bilirubin, PT and PA were measured before and 2, 4, 6 hours, 1, 3, 5, 7, 14 and 30 days after treatment.

Immunological assays

The count of lymphocytes, immunoglobulins (IgA, IgG, IgM) and complement (C3, C4)levels were measured before and 7, 14 and 30 days after BALSS treatment.

Morphological observation

The volume of the remnant liver was observed, and the remnant liver, kidneys, heart and lungs were taken out when the canines died or survived for 30 days for paraffin section, and HE stained and examined under light microscope.

Statistical analyses

The values of blood biochemical and immunological parameters were expressed as means±SD. Data of pre- and post-treatment among the three groups were compared using the Chi square test. The differences were considered significant at $P<0.05$.

RESULTS

Development of ALF canine models induced by eighty percent liver resection (Figures 1-6)

The canines demonstrated a significant increase in plasma ammonia, ALT, AST, bilirubin and PT levels, and decrease in PA level 24 hours after oper ation: ammonia (64.52±10.07 μmol/L-128.33±17.32 μmol/L, Figure 1), ALT (14.33±5.12 IU/L-277.04±53 .35 IU/L, Figure 2), AST (16.56±8.91 IU/L-236.06±75.68 IU/L, Figure 3), BIL (5.07±1.56 mmol/L-21.20±3.22 mmol/L, Figure 4), PT (9.68±0.76 s-19.91±1.87 s, Figure 5) and PA (79.16±4.31%-54.66±4.84%, Figure 6). ICG excretion rate of post-operation was lower than that of pre-operation respectively at 5, 10 and 15 minutes (15%, 23% and 30% *vs* 20%, 55% and 76%).

Porcine hepatocytes

Using the modified enzymatic digestive method, each liver yielded approximately $1\text{-}3\times10^{10}$ hepatocytes. Viability of the final suspensions averaged 75%-90% by typan blue exclusion and AO/PI fluorescence staining.

Liver and clotting function recovery of ALF canines after treatment with BALSS

BALSS treatment yielded beneficial effects with decreased plasma ammonia, bilirubin at 2 hours of the treatment, and then returned to normal from 4-6 hours of the treatment. There was an obvious decrease in PT, ALT and AST level and increase in PA level at 7 days after BALSS treatment. In contrast, there were no changes in ammonia, ALT, AST, bilirubin, PT and PA levels for the ALF canines in the control group and drug group. Among seven of ten canines in BALSS group which received one BALSS treatment, only one survived, and six lived an average time of 108.0 h±12.0 h (96 h-120 h). Three often canines received two BALSS treatments, two canines survived, and one died of lung infarction. There was no evidence that BALSS treatment had adverse effects on the morphology of kidneys, lungs and heart. In contrast, four ALF canines in the control group lived an average time of 24.0 h±6.0 h (18 h-50 h). Five ALF canines in the drug group lived an average time of 20.4 h±6.4 h (12 h-28 h).

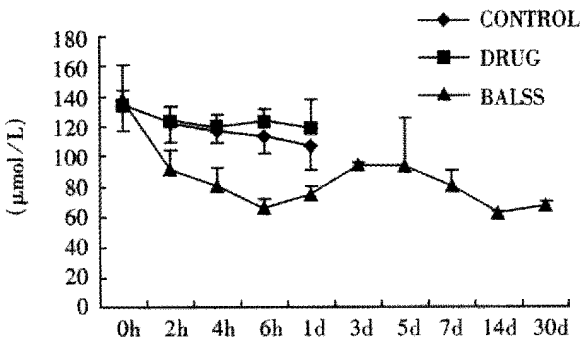

Figure 1 Effects of the BALSS on ammonia of subtotal hepatectomy induced ALF canines.

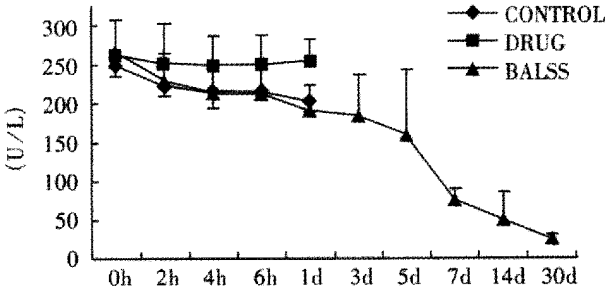

Figure 2 Effects of the BALSS on GPT of subtotal hepatectomy induced ALF canines.

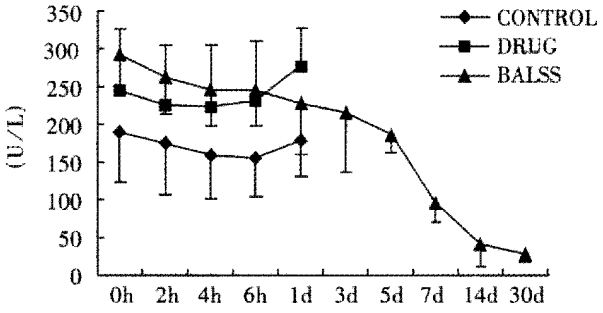

Figure 3 Effects of the BALSS on GOT of subtotal hepatectomy induced ALF canines.

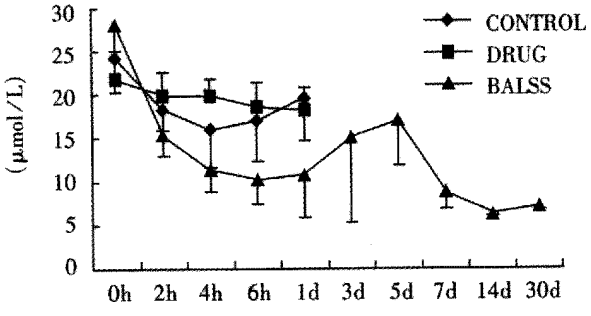

Figure 4 Effects of the BALSS on bilirubin of subtotal hepatectomy induced ALF canines.

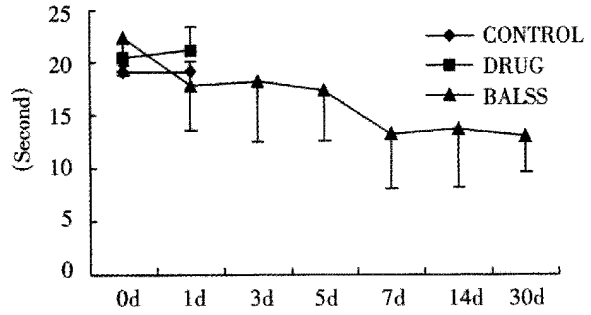

Figure 5 Effects of the BALSS on PT of subtotal hepatectomy induced ALF canines.

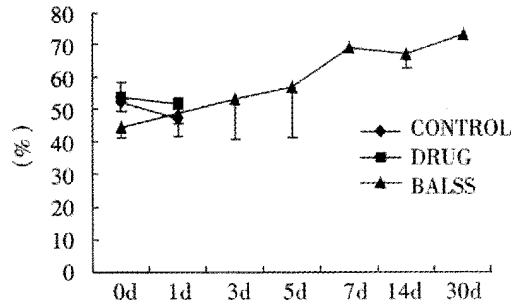

Figure 6 Effects of the BALSS on PA of subtotal hepatectomy induced ALF canines.

Immunological changes of ALF canines after BALSS treatment

There were no changes in count of lymphocytes, immunoglobulins (IgA, IgG, IgM) and complement (C3, C4) levels for 3 survived canines.

Morphological changes of remnant liver, kidneys, lungs and heart of ALF canines after treatment with BALSS

The volume of the remnant liver at 30 days after treatment became larger than that of ALF canines before treatment. BALSS treatment had no adverse effects on the morphology of kidneys, lungs and heart.

DISCUSSION

Acute liver failure is a kind of severe disease, and no effective clinical therapy is available currently. Since the liver has many complex physiological functions, such as synthesis, detoxication and biotransformation. The current non-bioartificial liver, for example, activated charcoal hemoperfusion and plasma exchange can not replace the liver function completely. It only can clear some middle and small molecule weight substance in circulation. Fortunately, encouraging success of currently developed bioartificial liver support system has been made in the experimental and clinical research. In BALSS, xenogeneic hepatocytes were used to exchange the substance with ALF blood so that it can temporarily replace the complex liver functions.

BALSS is made of biological and synthetic materials. The main biological material is hepatocytes which include human liver tumor cell lines[5,6], human embryo[7] and porcine hepatocytes[8]. There are possible dangers of the tumor cells to receptors and the scarce of the embryonic liver cells which hinders the application of BALSS, we therefore selected the pure strain Chinese experimental miniature swine as the source of hepatocytes in our experiment. Using modified enzymatic digesting and culturing method, we acquired a large quantity of porcine hepatocytes to meet the needs of BALSS. Porcine hepatocytes 1-3×10^{10} could be acquired from one swine liver, and the viability of hepatocytes reached 75%-90%. Hollow fiber tube is the main synthetic material in which hepatocytes are cultured and exchange substance with ALF blood through semipermeable membrane. A surgical model of acute liver failure is preferred to a toxic model, because it is more reprodu cible and induces only liver damage. In our study, we developed a subtotal hepatectomy induced ALF canine model. Twenty-four hours after operation, the blood hepatic biochemiscal parameters had obvious changes. The ALF canines died within 36-52 hours without treatment. The ALF canine model had a good reproductivity and could suit the studies of BALSS.

One of the most striking observations from the present work is that the ammonia l levels of the ALF canines in BALSS group were lowered dramatically at 2 hours of the treatment, and then returned to normal from 4-6 hours of the treatment. In contrast, there were no changes in the ammonial level for the ALF canines in the control group and drug group. The result indicated that ammonia could move through the semipermeable membrane of hollow fiber tube and was metabolized by porcine hepatocytes through urea cycle enzymes and glutamine synthetase. Significant decreases in plasma bilirubin levels after BALSS treatment were observed and demonstrated that porcine hepatocytes cultured in BALSS could accelerate metabolism of bilirubin in ALF blood. It was reported that hyperbilir ubinemia in animals had been corrected by microencapsulated hepatocyte transplantation.

Results of a phase I clinical trial of BALSS treatment showed that more than two BALSS treatments could clear the toxins in ALF blood completely and provide sufficient metabolic, liver functional support[9,10]. Our experiment indicated that the survival rate of ALF canines that received two BALSS treatments was higher than that of ALF canines that received only one BALSS treatment. Certainly the ultimate goal of a liver support treatment is to treat patients early enough and repeatedly to allow them to regenerate their livers and recover normal liver function.

Although in the development of a bioartificial liver, the use of hepatocytes from xenogeneic origin is the most promising alternative for human hepatocytes due to the shortage of human donor material, potential immunological problems may arise. The central unit of TECA-I BALSS is the so-called bioreactor (hollow fiber tube) which is divided into two sets of compartment by semipermeable membrane: extracapillary and intracapillary compartment. The semipermeable membrane is set such that those substances which are metabolized, detoxified, or synthesized can readily cross the membrane. However, blood cells and hepatocytes should not cross the reactor membrane. In our experiment, in the 3 survived canines, there was neither change in the count of lymphocytes, immune proteins (IgA, IgG, IgM) and supplementary (C3, C4) levels and nor pathological change in kidneys, remnant liver, lungs and heart after BALSS treatment.

On one hand, porcine hepatocytes cultured in BALSS can provide directly liver functional support, and on the other hand, hepatocytes can produce some proteins such as growth factors, and stimulate liver regeneration. Hepatocytes transpla nted into the spleen of rats could reverse the neurologic disorders associated with hepatic encephalopathy[11]. Thus, it is likely that both mechanisms direct action of hepatocytes cultured in BALSS and indirection stimulation of native liver are

involved[12].

It is suggested that the TECA-I BALSS could temporarily replace the hepatic function of ALF canines effectively and safely, and it may provide a new approach for the treatment of ALF.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Overexpression of hepatic plasminogen activator inhibitor type 1 mRNA in rabbits with fatty liver

Jian-Gao Fan[1], Liang-Hua Chen[2], Zheng-Jie Xu[1] and Min-De Zeng[3]

[1]Department of Gastroenterology, Shanghai First People's Hospital, Shanghai 200085, China
[2]Department of Cardiology, Shandong Provincial Hospital, Jinan 250021, China
[3]Shanghai Institute of Digestive Diseases, Shanghai 200080, China
Correspondence to: Dr. Jian-Gao Fan, Department of Gastroenterology, Shanghai First People's Hospital, Shanghai 200085, China. fanjg@online.sh.cn
Telephone: +86-21-63240090, Fax: +86-21-63240825
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Subject headings hyperlipidemia; fatty liver; plasminogen activator inhibitor type 1(PAI-1)

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INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1), an approximately M_r50000 glycoprotein, is the major physiological inhibitor of plasminogen activators. It is not only the priming factor for atherosclerosis and coronary thrombosis[1-3], but also participates in the genesis of chronic hepatitis and liver fibrosis[4-11]. However, there has been no available report yet about the research of hepatic PAI-1 gene expression in hyperlipidemia and fatty liver. The present study AIMed to explore the change of hepatic PAI-1 mRNA and its plasma activity by means of animal model.

MATERIALS AND METHODS

Animal model

Seventeen male New Zealand white rabbits weighing 2 200 g-2 500 g were randomly divided into two groups. The seven rabbits of control group were fed with normal rabbit diet. The ten rabbits of model group were fed with a fat-rich diet, which was prepared by addition of 10 g·kg^{-1} cholesterol and 100 g·kg^{1} lard oil to the normal diet. Vein blood samples of rabbits were collected before the beginning of study and at the 6th week and 12th week of the study respectively. Then the plasma samples were obtained and maintained at -70℃ until assayed. When the rabbits were sacrificed at the 12th week, their body mass and wet liver mass were measured. And two small samples of liver tissue were obtained from the right hepatic lobe. One sample was fixed in formalin, embedded in paraffin and stained with H&E. The other sample was stored in liquid nitrogen for detection of PAI-1 mRNA.

Measurement of plasma biochemistry

Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG) and total cholesterol (TCH) were determined by automatic biochemical analysis instrument (Olympus AU 1000, made in Japan). Plasma PAI-1 activity was determined by a chromogenic activity kit purchased from Biotechnology Co., Ltd. of the former Shanghai Medical University.

Detection of hepatic PAI-1 Mrna

The primer for rabbit PAI-1 was designed based on its complement DNA (cDNA) sequence published previously[6,12,13] and synthesized by DNA synthesis instrument. The sequence is 5' ATG GAA TTC CCG TGG AAC AAG AAT GAG ATC AG 3'and 5' TGA GCC ATC ATG GGC ACA GAG. The primer for β-actin was given by Shanghai Institute of Cell Biology of the Chinese Academy of Sciences, and the sequence is 5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTGC 3' and 5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3'. The samples of liver tissues were cut into pieces quickly and the total RNAs were extracted with Trizol Reagent (Gibco BRL Co., Ltd.). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to amplificate the mRNA of PAI-1 and β-actin, and followed by electrophoresis of the products, photography and computer image scan. And the results were validated by the OD value of β-actin because of its equal expression in various tissues.

Statistical analysis

All results were expressed as $\bar{x}\pm s$. Statistical differences between means were determined by Student *t* test. $P<0.05$ was selected to reflect significance.

RESULTS

General condition

During the experiment, no animal death occurred in both groups. The body mass of rabbits in the model group increased more rapidly than in the control group. The model group had significantly greater body mass and liver index (wet liver mass per body mass) than the control group ($P<0.05$ respectively, Table 1). Compared with the control group, the plasma TCh and TG levels in the model group already increased significantly at the 6th week ($P<0.05$ *vs* controls) and increased more significantly at the 12th week ($P<0.01$ *vs* controls). However, the plasma levels of ALT and AST in the model group only had a tendency to increase as compared with those of control group (Table 2).

Table 1 The changes of body mass and liver index

Groups	*n*	m (body)/(kg)		Liver index/(g·kg^{-1})
		Start	End	End
Control	7	2.4±0.3	3.1±0.2	26±3
Model	10	2.3±0.6	3.4±0.1[a]	31±2[a]

[a]$P<0.05$ *vs* control.

Table 2 Plasma biochemical test at the 12th week

Groups	*n*	c(TG)/(mmol·L^{-1})	c(TCh)/(mmol·L^{-1})	b(ALT)/(nkat·L^{-1})	b(AST)/(nkat·L^{-1})
Control	7	0.34±0.11	0.76±0.17	372±130	215±45
Model	10	0.54±0.17[b]	26.40±3.89[b]	505±215	263±127

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

Changes of plasma PAI-1 activity

In the model group, the plasma PAI-1 activity of rabbits already increased significantly at the 6th week ($P<0.05$) and increased more significantly at the 12th week ($P<0.01$, Table 3) compared with those at the beginning of the experiment. Moreover, the difference in plasma PAI-1 activity between the two groups at the same period also had highly statistical significance ($P<0.05$ and $P<0.01$, respectively).

Table 3 The changes of plasma PAI-1 activity ($\times 10^3 AU \cdot L^{-1}$)

Groups	*n*	0 wk	6 wk	12 wk
Control	7	5.6±2.6	6.8±1.4	4.8±2.2
Model	10	4.9±3.1	7.7±1.1[a]	14.0±2.5[b]

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

Changes of liver pathology

In the gross, the livers of the model group enlarged markedly as compared with those of control group. Under the light microscope, all the liver tissue sections stained with H&E of the model group showed diffuse and severe foamy steatosis of hepatocyte with mild necrosis and inflammatory cell infiltration, among which monocytes were dominant. And these lesions were located mainly in portal areas.

Expression of hepatic PAI-1 mRNA

The mRNA fragments of PAI-1 and β-actin were 360 bp and 828 bp respectively (Figures 1, 2). The lanes (No.3,4,5) of hepatic PAI-1 mRNA of the model group were markedly more intense than those of control group (No.1,2). The ratio of hepatic PAI-1 mRNA to β-actin mRNA of model group was significantly greater than that of the control group (3.474±0.051 *vs* 1.210±0.031, $P<0.01$).

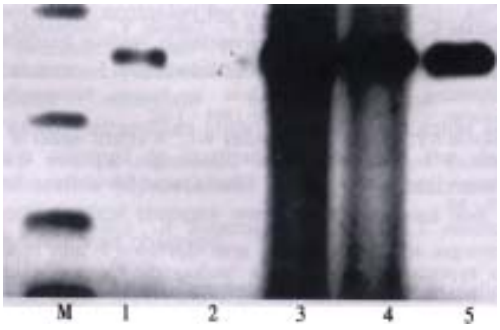

Figure 1 The expression of hepatic PAI-1 mRNA. 1,2: control group; 3,4,5: model group; M: marker

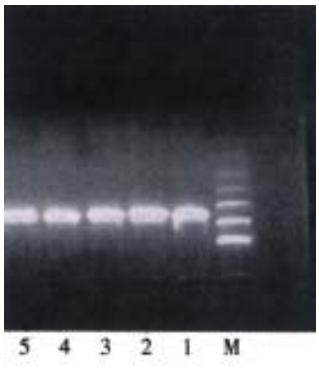

Figure 2 The expression of hepatic β-action mRNA. 1,2: control group; 3,4,5: model group; M: marker

DISCUSSION

Fibrinolysis system has an inactive zymogen that is called plasminogen. It can be activated by plasminogen activators and be converted to plasmin[11]. Plasmin can not only degrade the fibrin and remove it from the blood circulation, but also degrade extracellular matrix (ECM) directly and protect the tissues from fibrosis[1-11]. Among all fibrinolysis components, PAI-1 plays a central role in the pathophysiology of cardioangiological diseases. It is the major physiological inhibitor of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). The plasma PAI-1 regulates the plasmin cascade by its interaction with the tPA[1,2]. The increased activity of plasma PAI-1 could inactive the tPA in circulation, and the PAI-1 depositing in ECM could accelerate the clearance of tPA in tissues. PAI-1 exists in all of human tissues. Analysis of human tissues for PAI-1 antigen and activity has shown that the greatest quantity and highest specific activity were found in the liver. In plasma and ECM, the free PAI-1 produced by hepatocytes, endothelial cells and smooth muscle cells binds vitronectin (VN) to maintain the stable activity. The binding PAI-1 which increases the anti-fibrinolysis activity of blood vessels and accelerates the deposition of ECM can induce the genesis of atherosclerosis, coronary heart disease, pulmonary fibrosis and kidney fibrosis[1-3]. Recent studies have shown that the activity of PAI-1 is also associated with the occurrence and progression of chronic viral hepatitis and liver fibrosis. With the progression of chronic liver disease, the plasma PAI-1 antigen level and expression of PAI-1 in the liver increased gradually and culminated in the stage of liver cirrhosis, then decreased with the deterioration of liver function (Child-Pugh classification A to C)[4-8]. In vitro, hepatic stellate cells (HSC), also known as hepatic lipocytes, fat-storing cells, or Ito cells, could express PAI-1 when activated[12-15]. And previous studies have already demonstrated that HSC played a critical role in the genesis and development of liver fibrosis[16,17]. However, there has been no report yet about the effect of PAI-1 on the pathogenesis of fatty liver.

The present study established a rabbit model with overweight, hyperlipidemia and fatty liver via a fat-and-cholesterol-rich diet. The rabbits of this model had significantly greater body weight and higher plasma lipid levels than the normal control rabbits and their serum levels of ALT and AST had a tendency to increase. In hepatic histopathology, this model showed severe hepatocyte steatosis with mild hepatocyte necrosis and inflammatory cell infiltration. These characteristics resemble the presentation of human fatty liver associated with obesity and hyperlipidemia[18]. So this model can be used to explore the pathogenesis of nonalcoholic fatty liver. In the present study, while hyperlipidemia occurred in the model rabbits after 6 weeks of the fatty-rich diets, the plasma PAI-1 activity also increased. Then the plasma lipid and the plasma PAI-1 activity increased more significantly at the 12th week. Moreover, it was shown in the RT-PCR that PAI-1 mRNA was markedly intense in the liver with steatosis.

It is known that various factors could influence the expression and translation of hepatic PAI-1 gene. In the non-alcoholic fatty liver with obesity and hyperlipidemia, many accompanying factors such as lipoprotein (VLDL, LDL), hyperinsulinemia or gut-derived endotoxemia can stimulate hepatic PAI-1 production[19-22]. When the cytokines such as IL-1, TNF, PDGF, TGF-β or IGF-1 exist simultaneously, hepatic PAI-1 production will be further increased[23-28]. Besides, other factors such as dexamethasone, prothrombin, or angiotensin II (Ang II) can also accelerate the secretion of

PAI-1[29-32]. Subsequently, the increase in PAI-1 caused the imbalance of tPA and PAI-1, which leads to the decrease in fibrinolysis activity of hepatic microcirculation and degrading of hepatic ECM. Then the dysfunction of hepatic microcirculation, even the microthrombosis could be induced and the occurrence and progression of liver fibrosis promoted[5-11]. However, some studies have indicated that PAI-1 can inhibit the conversion of inactive TGF-β (transforming growth factor-β) into active TGF-β, so that PAI-1 might inhibit liver fibrogenesis since TGF-β can promote the production of ECM and inhibit its degrading[4]. It is therefore, necessary to reinforce the study of the relationship between PAI-1 and fibrosis in fatty liver in order to find an effective method for the prevention and treatment of liver fibrosis.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Safe upper limit of intermittent hepatic inflow occlusion for liver resection in cirrhotic rats

Dao-Xiong Lei[1,2], Cheng-Hong Peng[1], Shu-You Peng[1], Xian-Chuan Jiang[1], Yu-Lian Wu[1] and Hong-Wei Shen[1]

[1]Department of Surgery, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China
[2]Department of Surgery, Zhongnan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China
This work was supported by the grant from the Science and Technology Committee of Zhejiang Province, No. 971103132
Correspondence to: Dr. Dao-Xiong Lei, Department of Surgery, Zhongnan Hospital, Wuhan University School of Medicine, Wuhan 430071, Hubei Province, China. Nicholaslei@21cn.com
Telephone: +86-27-87317906, 87308376, Fax: +86-27-87307966
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Abstract

AIM **To evaluate the effects of varying ischemic durations on cirrhotic liver and to determine the safe upper limit of repeated intermitten t hepatic inflow occlusion.**

METHODS **Hepatic ischemia in cirrhotic rats was induced by clamping the common pedicle of left and median lobes after non-ischemic lobes resection. The cirrhotic rats were divided into six groups according to the duration and form of vascular clamping: sham occlusion (SO), intermittent occlus ion for 10 (IO-10), 15 (IO-15), 20 (IO-20) and 30 (IO-30) minutes with 5 minutes of reflow and continuous occlusion for 60 minutes (CO-60). All animals received a total duration of 60 minutes of hepatic inflow occlusion. Liver viability was investigated in relation of hepatic adenylate energy charge (EC). Triphenyltetrazollum chloride (TTC) reduction activities were assayed to qualitatively evaluate the degree of irreversible hepatocellular injury. The biochemical and morphological changes were also assessed and a 7-day mortality was observed.**

RESULTS **At 60 minutes after reperfusion following a total of 60 minutes of hepatic inflow occlusion, EC values in IO-10 (0.749±0.012) and IO-15 (0.699±0.002) groups were rapidly restored to that in SO group (0.748±0.016), TTC reduction activities remained in high levels (0.144±0.002 mg/mg protein, 0.139±0.003 mg/mg protein and 0.121±0.003 mg/mg protein in SO, IO-10 and IO-15 groups, respectively). But in IO-20 and IO-30 groups, EC levels were partly restored (0.457±0.023 and 0.534±0.027) accompanying with a significantly decreased TTC reduction activities (0. 070±0.005 mg/mg protein and 0.061±0.003 mg/mg protein). No recovery in EC values (0.228±0.004) and a progressive decrease in TTC reduction activities (0.033±0.002 mg/mg protein) were shown in CO-60 group. Although not significantly different, the activities of the serum aspartate aminotransferase (AST) on the third postoperative day (POD_3) and P OD_7 and of the serum alanine aminotransferase (ALT) on POD_3 in CO-60 group remained higher than that in intermittent occlusion groups. Moreover, a 60% animal mortality rate and more severe morphological alterations were also shown in CO-60 group.**

CONCLUSION **Hepatic inflow occlusion during 60 minutes for liver resection in cirrhotic rats resulted in less hepatocellular injury when occlusion was intermittent rather than continuous. Each period of 15 minutes was the safe upper limit of repeated intermittent vascular occlusion that the cirrhotic liver could tolerate without undergoing irreversible hepatocellular injury.**

Subject headings hepatic inflow occlusion/intermittent/continuous; liver resection; cirrhosis; rat; energy charge; triphennyltetrazollum chloride

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INTRODUCTION

The effective control of intraoperative bleeding is one of the most important measures for successful hepatectomy[1]. Up to now, temporary portal triad clamping (Pringle maneuver) has been used widely as a means of reducing blood loss. In China, hepatocellular carcinoma (HCC) is commonly associated with cirrhosis[2-6], bleeding during hepatic resection is a major factor in determining the severity of postoperative liver damage and the prognosis[7-9]. The safe time limit of hepatic vascular occlusion during the cirrhoti c liver resection remains the major concern of liver surgeons[10,11]. Intermittent occlusion may not only reduce intraoperative bleeding but also increase the total duration of ischemia for hepatectomy[7,12,13]. Thus it is important for the improvement of the safety of the cirrhotic liver resection that how long each time is suitable for the intermittent occlusion[14,15]. The purpose of this experiment is to study the effects of various ischemic durations on cirrhotic liver and determine the safe upper limit of intermittent hepatic inflow occlusion without irreversible damage in rats with cirrhotic liver.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200 g±30 g were allocated randomly into six experimental groups of 20 animals, depending on the duration and form of ischemia: Sham occlusion (SO group), intermittent occlusion for 10 min (IO-10 group), 15 min (IO-15 group), 20 min (IO-20 group) and 30 min (IO-30 group) with 5 min of reperfusion between each period, continuous occlusion for 60 min (CO-60 group). The rat cirrhosis was induced by injecting 600 $g \cdot L^{-1}$ CCl_4 oil subcutaneously twice weekly at a dose of 4 $mL \cdot kg^{-1}$ bw and drinking 50 $mL \cdot L^{-1}$ ethanol. A validated model[16] used by clamping the common pedicle of left and median lobes after

non-ischemia lobes resection did not require splanchnic decompressing. All animals received a total duration of 60 min of hepatic inflow occlusion.

Operative procedure

Rats were anesthetized with intraperitoneal pentobarbital 0.2 mg·kg^{1}. A midline laparotomy was performed. Ligamentous attachments around the liver were divided and the total liver hilum of right and left portal vein, hepatic artery, bile duct as well as the right hepatic vein were also dissected. Ischemia was induced in the median and left hepatic lobes by clamping the corresponding arterial and portal venous branches en masse. This allowed an uninterrupted blood supply to the right lobe and caudate lobe, avoiding congestion of alimentary tract and consequent haemodynamic instability. When the assigned total ischemic duration of 60 min was completed, the clamp was removed and the non-ischemic right lobe and caudate lobe were resected (30% hepatectomy) after ligation of the corresponding pedicles en masse. This technique abrogates the effect of splanchnic congestion caused by total occlusion of the portal hilum during hepatic ischemia.

Assessment of liver viability[17]

A small portion of excised liver (about 30 mg) was immediately frozen by freeze -clamp precooled with liquid nitrogen, lyophilized overnight, and further kept at -80℃ until analysis. The dry tissue was then weighed and homogenized in 1 mL ice-cold 0.6 mol·L^{-1} $HClO_4$. Thirty minutes later, the homogenate was centrifuged for 20 min at -4℃, 20 000×g and the supernatant with 0.3 mL was neutralized with 1 mol·L^{-1} potassium carbonate 0.2 mL and centrifuged in the same manner. The final supernatant was filtered through a filter of 0.45 μm and used as a sample with 20 μL for determining the liver tissue concentration of ATP, ADP and AMP by high-performance liquid chromatography (Shim-pacr CLC-ODS analytical column, 150 mm×6.0 mm i.d, λ=254 nm) at a flow rate of 0.1 mL·min^{-1}. The results were calculated as micromoles of nucleotide per gram of dry tissue. Energy charge was calculated as follows: EC = (ATP+1/2ADP)/(ATP+ADP+AMP).

Quantitation of the extent of irreversible hepatocellular injury

The degree of irreversible cellular injury was assessed by a modified technique described by Rodriguez *et al*[18]. A portion of each liver tissue specimen was rinsed in ice-cold Ringer's lactate solution, and then placed on ice, weighed, and homogenized in 0.25 mol·L^{-1} sucrose to make an 8% homogenate by weight. The homogenate was filtered through a fine stainless steel mesh to remove the remaining fragments. Protein content of the homogenate was determined by the method of Lowry. A 1 mL aliquot of homogenate was then mixed with 1 mL of 10 g·L^{-1} TTC (Sigma) in 0.033 mol·L^{-1} phosphate buffer (pH 7.4). The reaction was made in triplicate. The reactive mixture was incubated at 37℃ in an agitating water bath for 30 min and added with an equal volume of acetone to each reaction tube and then vortexes. The extracted mixture was centrifuged for 15 min at 2 000 r·min^{-1} and the absorbance of the clear red supernatants was measured at 485 nm in a spectrophotometer. The absorbances of the reaction triplicates were averaged and unreacted reagent blanks were subtracted to arrive at a final absor ption. Comparison of these absorption values to a standard curve of known reduce d TTC concentrations to determine the micrograms of TTC reduced per 30 min per milligram of the liver tissue protein.

Serum AST, ALT, lactic dehydrogenase (LDH), alkaline phosphatase (AKP) and total bile acid (TBA) contents were measured at 4℃ using the available kits. Animals of each group were treated as scheduled. The excised median lobe liver was fixed in 100 mL·L-1 formalin and embedded in paraffin. Sections 3 μm thick were stained with hematoxylin and eosin for light microscopy. A segment of the liver tissue of the median lobe, 2 mm×2 mm×2 mm in size, was taken and fixed in 40 g·$^{-1}$ glutaraldehyde-phosphate buffer (0.1 mol·L^{-1}, pH: 7.3) for 24 h and then post-fixed in 10 ·L^{-1} osmian tetroxide for two hours, dehydrated with a descending series of alcohol, and embedded with epoxied resin. The specimens were cut into ultrathin slices pigmented double with uranium acetate and lead citrate before being observed under the HITACHI-600 transmission electron microscope. Ten animals in each group were considered to have survived the procedure if they remained alive on the seventh day after operation.

Statistical analyses

Values were expressed as x±s. Differences among groups were analyzed using one-way analysis of variance (ANOVA). A post hoc analysis using Fisher's PLSD test was used to account for multiple comparisons. Differences in survival were determined using the Kaplan-Meier test. *P* values less than 0.05 were considered significant. All the statistical analyses were made using SPSS software package.

RESULTS

At a total of 60 min of hepatic inflow occlusion, the liver tissue levels of ATP in each ischemia group were significantly decreased, the extent of ATP restoration at 60 min after reperfusion was markedly related with the METHODS of hepatic inflow occlusion. The ATP levels in IO-10 and IO-15 groups were rapidly restored to that in SO group, but there was a progressive decrease in IO-20 and IO-30 groups, the recovery was significantly suppressed in CO-60 group, to only 5.8% of ATP levels in SO group (Table 1). There was significant difference among IO-10, IO-15, IO-20, IO-30 and CO-60 groups.

Table 1 Adenine nucleotide concentrations in the dry cirrhotic livers (x±s, μmol·g^{-1}, n = 6)

Groups	Ischemia 60 min			Reperfusion 60 min		
	ATP	ADP	AMP	ATP	ADP	AMP
SO	5.00±0.13	3.09±0.38	0.97±0.39	5.07±0.15	3.19±0.67	0.71±0.14
IO-10	0.53±0.06^{b}	1.85±0.28^{b}	3.40±1.53^{a}	4.99±0.14	3.41±0.29	0.53±0.25
IO-15	0.45±0.05^{b}	1.39±0.55^{bc}	3.81±2.07^{b}	4.99±0.16	3.35±0.16	1.20±0.09^{bd}
IO-20	0.43±0.03^{b}	1.13±0.43^{bd}	3.21±1.37^{a}	1.01±0.13^{bdf}	2.22±0.24^{bdf}	1.41±0.15^{bd}
IO-30	0.45±0.12^{b}	0.79±0.05^{bde}	2.29±0.81	0.90±0.19^{bdf}	2.41±0.35^{bdf}	0.69±0.24^{f}
CO-60	0.41±0.08^{b}	1.00±0.34^{bd}	3.10±0.45^{a}	0.30±0.01^{bdfgh}	0.85±0.15^{bdfgh}	2.02±0.19^{bdfgh}

^{a}P<0.05, ^{b}P<0.01 *vs* SO; ^{c}P<0.05, ^{d}P<0.01 *vs* IO-10; ^{e}P<0.05, ^{f}P<0.01 *vs* IO-15; ^{g}P<0.01 *vs* IO-20; ^{h}P<0.01 *vs* IO-30.

The EC levels at a total ischemic duration of 60 min significantly and immediately decreased in each ischemia group. At 60 min after reperfusion, there was a rapid restoration in IO-10 and IO-15 groups (0.748±0.016 in SO group, 0.749±0.012 in IO-10 group, 0.699±0.002 in IO-15 group). The EC levels were restored partly in IO-20 and IO-30 groups (0.457±0.023 and 0.534±0.027) and no recovery in CO-60 group (0.228±0.004) (Figure 1). A significant difference was shown among IO-10, IO-15, IO-20, IO-30 and CO-60 groups.

At a total ischemic period of 60 min, TTC reduction activities in IO-20, IO-30 and CO-60 groups (0.098±0.007 mg/mg protein, 0.099±0.005 mg/mg protein and 0.068±0.007mg/mg protein, respectively) markedly declined to 66.7%, 67.3% and 46.3% of SO group (0.147±0.004 mg/mg protein), respectively. At 60 min after reperfusion, TTC reduction activities remained high in IO-10 and IO-15 groups (0.139±0.003 mg/mg protein and 0.121±0.003 mg/mg protein) and significantly decreased in IO-20 and IO-30 groups (0.070±0.005 mg/mg protein and 0.061±0.003 mg/mg protein), there was a progressive decrease in CO-60 group (0.033±0.002 mg/mg protein) in comparison with that in each intermittent occlusion group (P<0.01) (Figure 2).

Although serum AST and ALT activities on POD_1 markedly increased in IO-10 (986±49 μkat·L^{-1} and 1356±221μkat·L^{-1}), IO-15 (1431±116μkat·L^{-1} and 1611±149μkat·L^{-1}), IO-20 (1558±78 μkat·L^{-1} and 1186±187 μkat·L^{-1}), IO-30 (1743±96 μkat·L^{-1} and 2466±489 μkat·L^{-1}) and CO-60 (1773±181 μkat·L^{-1} and 2190±397 μkat·L^{-1}) groups as compared with SO group (294±16 μkat·L^{-1} and 669±26 μkat·L^{-1}), there was no difference among five ischemia groups. However, serum AST activities in CO-60 group on POD_3 and POD_7 (203±14 μkat·L-1, n = 5 and 183±6 μkat·L-1, n = 4) and ALT activities on POD_3 (484±38 μkat·L^{-1}, n = 5) remained to be higher than that in intermittent ischemia groups (P<0.05). In addition, no significant difference was found in postoperative serum LDH, AKP and TBA levels among ischemia groups. All animals in SO and IO-10 groups survived during the 7 d period. The mortality rates in IO-15, IO-20 and IO-30 groups were 10%, 30% and 40%, respectively. In contrast, the mortality rate in CO-60 group increased to 60%, with a significant difference between IO-10 and CO-60 groups (P<0.05).

The morphologic findings revealed that hepatic cells slightly swollen in IO-10 and IO-15 groups, but the dilatation of the rough-surfaced endoplasmic reticulum (RER) and the degeneration changes in mitochondria within cytoplasm were mild, the shape of nucleus was regular. In IO-20 and IO-30 groups, hepatic cells had a moderate swelling with cytoplasmic microvacuolisation, predominantly in the midzonal areas. Ultrastructural changes were shown with a gross dilatati on of RER and mitochondria, an irregular nucleus and an aggregation of the heterochromatins within the nucleus. In CO-60 group, the hepatocytes had vacuolar degeneration accompanied with infiltration of polymorphonuclear leucocytes in the sinusoids. RER and mitochondria were severely dilated, the heterochromati ns within the nucleus markedly increased and nucleus concentration occurred (Figure 3).

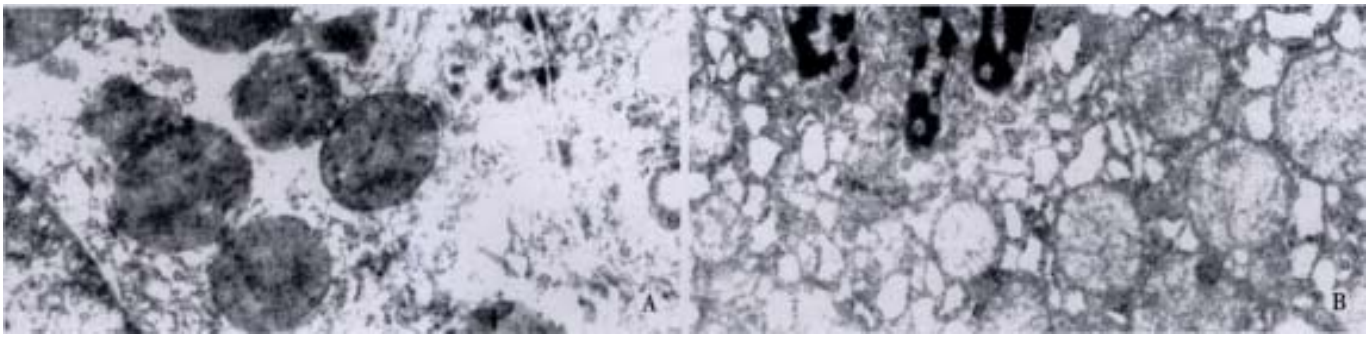

Figure 1 Changes of energy charge in hepatic tissues.
Figure 2 Changes of TTC reduction activities in hepatic tissues.

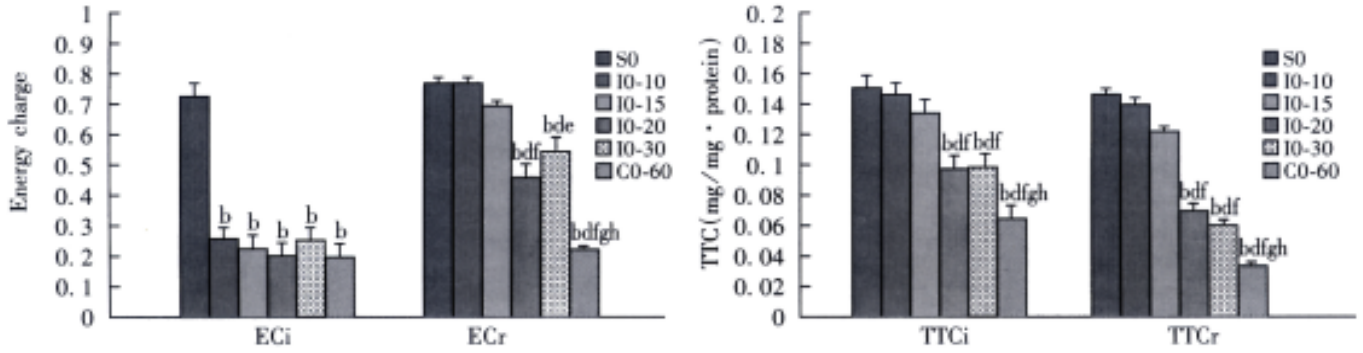

Figure 3 Electron micrograph of hepatocyte at a total ischemic duration of 60 min in cirrhotic rats. A: The mitochondria were regular, chroma tin in nucleus was well-distributed, nuclear membrane was clear in SO group, TEM×17 000, B: Mitochondria and RER were severely dilated, heterochromtins within the nucleus markedly increased, nucleus concentration occurred.TEM×15 000

DISCUSSION

In China,the incidence of HCC is high and almost 85% of HCC were accompanied with liver cirrhosis[4,19].Bleeding during liver resection is known to be an important prognostic indicator of both morbidity and mortality, particularly for the cirrhotic patients[14,20,21]. Generally, blood loss has been controlled by the Pringle maneuver, which is one of the simplest and most commonly used METHODS and attention has focused on the

length of time for which such ischemia to the liver can be safely tolerated[20,22-26]. Although the healthy human liver could tolerate a major hepatectomy with ischemia of more than 60 min under normothermic condition[24,25], in their studies, there were complications in 18 of 34 patients and a thirteen-fold increase in the activities of plasma transaminase on the first postoperative day. Moreover, the cirrhotic liver would be much more vulnerable to ischemia, Therefore, intermittent portal triad clamping for the control of bleeding during cirrhotic liver resection has been advocated that this would be less detrimental to the liver than continuous clamping[7,12,13,20,21] . How long the cirrhotic liver could tolerate intermittent hepatic inflow occlusion each time remains to be determined.

EC, which expressed the balance between ATP-consuming and -producing reactions and was of central importance in the regulation of metabolic sequences, had bee n used to assess liver viability[17]. These studies had suggested that enhanced ATP synthesis and prompt recovery of EC after revascularization were prerequisites for maintaining the liver viability. In the present study, althoug h ATP content and EC at a total ischemic duration of 60 min were retarded during the ischemic period, a significant difference was shown at 60 min after reperfusion. Because the ability of the liver to restore EC after reperfusion played a decisive role in determining hepatic viability[19], the prompt restoration of the EC levels in IO-10 and IO-15 groups reflected the maintenance of liver viability. However, EC had only partial recovery in IO-20 and IO-30 groups, could not be restored after reperfusion in CO-60 group, indicating the irreversible mitochondrial dysfunction, subsequent ATP depletion and loss of liver viability in IO-20 and IO-30 groups, which were more severe in CO-60 group. These findings were further supported by the morphological results.

Of the variety of cell types in the liver, the hepatocytes were the most sensitive to normotheric ischemia. The liver could tolerate a temporary ischemia of short duration but prolonged ischemia caused irreversible hepatocellular injury and led to liver function failure even if the liver was reperfused. The TTC reduction activity assay reflected the ability of the intact mitochondrial reduction-oxidation enzyme systems to convert the colorless TTC to a red formazan dye. This indicator had been validated to quantitate the extent of irreversible cell damage in the study of the hepatic and cardiac ischemic injury[18].The inability to reduce TTC indicated the irreversible loss of mitochondrial function. In the present study, the TTC reduction activities had no changes during ischemia of 60 min and remained at higher levels at 60 min after reperfusion in IO-10 and IO-15 groups, but had an aggressive decrease in IO-20, IO-30 and CO-60 groups at 60 min of ischemia or reperfusion. These results demonstrated that the irreversible hepatocellular injury firstly occurred in IO-20 group, significantly greater injury occurred in CO-60 group.

As mentioned above, when changes in EC levels were examined in conjunction with changes in TTC reduction activities and morphological features, it was possible to identify a significant injury that was completely reversible when the intermittent occlusion duration was limited to 15 min. However, the intermittent occlus ion of prolonged 20 or 30 min would produce irreversible hepatocellular damage, the extent of damage became greater in continuous occlusion group.

In addition, it has been emphasized in the previous literature that uncontrollab le massive hemorrhage during hepatic resection would lead to a deterioration of liver function and increased postoperative morbidity and mortality, particularly in the patients with liver cirrhosis[7-9,14,20,21]. These patients could tolerate liver ischemia within certain limits better than they do the consequ ences of massive bleeding and blood transfusion. Wu *et al*[20] showed that a cirrhotic liver could tolerate intermittent ischemia for up to 200 minutes without increased postoperative complications and mortality. In another report, immediate postoperative liver function was better preserved in the intermittent occlusion group than in those patients who were operated on without using the intermittent occlusion[15]. This may be due to less hemodynamic disturbance induced by the bleeding as well as hepatovenous retrograde perfusion during the liver transection to maintain the liver viability[21]. However, the preoperative status of liver function should still be taken into account. An improved prognosis appeared to be related to a suitable selection of cirrhotic patients with well-compensated liver function and an increased proportion of limited resections of cirrhotic livers[14,27-29].

In the clinical practice, an intermittent rather than a continuous hepatic vascular occlusion was advocated during limited resection of the cirrhotic live r because it could increase the ability of the liver to tolerate the consequences of prolonged ischemia and splanchnic venous stasis[20,21]. However, the relation between the selected duration of intermittent vascular occlusion and the risk of bleeding from esophageal varices in the cirrhotic patients during the liver resection remains to be determined.

It is concluded from this experiment that intermittent hepatic inflow occlusion to the liver resulted in less hepatocellular damage when compared with continuous occlusion and suggested that intermittent occlusion of the hepatic pedicle was the preferred method to control the intraoperative hemorrhage during cirrhotic liver resections. Furthermore, each period of 15 min was the safe upper limit of repeated intermittent hepatic inflow occlusion that the cirrhotic liver could tolerate without irreversible liver damage.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Relationship between autoimmune hepatitis and HLA-DR4 and DRβ allelic sequences in the third hypervariable region in Chinese

Xiong Ma and De-Kai Qiu

Shanghai Institute of Digestive Disease, Shanghai Renji Hospital, Shanghai Second Medical University, Shanghai 200001, China
Correspondence to: Dr. Xiong Ma, Shanghai Institute of Digestive Disease, Shanghai Second Medical University, Affiliated Renji Hospital, 145 Shandong Zhonglu, Shanghai 200001, China. Xiongma@netease.com
Telephone: +86-21-63200874
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Abstract

AIM **To analyze the association of HLA-DRB1 with autoimmune hepatitis (AIH) in patients from China.**

METHODS **In 32 patients and 48 healthy controls, polymerase chain reaction amplification with sequence-specific primers (PCR-SSP) was performed to examine the association of certain alleles or polymorphic sequences of HLA-DRB1 with AIH.**

RESULTS **HLA-DRB1 typing by PCR-SSP showed that DR4 had a significantly increased frequency among patients with AIH versus healthy control (46.9% versus 20.8%; relative risk = 3.35, *P* = 0.014). In subtypes of DR4, there was a trend of increase in the gene frequency of DRB1 *0405 in patients with AIH versus healthy controls (21.9% *vs* 6.3%, *P* = 0.04, but P_c = 0.08). In addition, a significant increase was found in the alleles frequency encoding QRRAA from the third hyperpolymorphic region of DR4 in the patients with AIH (86.7% of DR4 positive patients *vs* 40.0% in DR4 positive controls, *P* = 0.016, P_c = 0.028, RR = 9.75).**

CONCLUSION **AIH in Chinese is associated with HLA-DR4. There is a relationship between QRRAA sequence within the third hyperpolymorphic region of the DRB allele and AIH in Chinese.**

Subject headings hepatitis, autoimmune/immunology; HLA-DR antigen/genetics; alleles; sequence analysis; polymerase chain reaction

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INTRODUCTION

Autoimmune hepatitis (AIH) is an inflammation of the liver characterized by the presence of periportal hepatitis in microscopic examination (piecemeal necrosis or interface hepatitis), hypergammaglobulinemia, and serum autoantibodies[1-5]. Infiltration of the liver by T lymphocytes and a peripheral defect in T-suppressor cell function suggest an immune basis for the pathogenesis and liver injury in AIH[6-9]. Among northern European and North American white populations, various studies have shown that the serologically defined HLA-DR3 and DR4 antigen are the primary determinants of HLA-encoded susceptibility to AIH[10]. The principal susceptibility allele for type 1 autoimmune hepatitis among white northern Europeans and Americans is HLA-DRB1 *0301, and a second, independent risk factor is HLA-DRB1*0401, which encodes DR3 and DR4 respectively[11-13]. In Japan, where DR3 is very rare in the normal population, the primary HLA associated with DR4 and almost all patients are in the older age groups, with a peak onset at 50-60 years of age[14-16]. Patients from Argentina[17], Brazil[18], and Mexico[19] have susceptibility alleles for type 1 autoimmune hepatitis that are different from those in white northern European and American patients. These discrepant observations emphasize the importance of studying ethnically homogeneous populations[20]. In present study, we performed HLA genotyping with respect to DRB1 in Chinese patients with AIH using PCR amplification by the sequence-specific primers (PCR-SSP) method to investigate the relationship between the distribution of the HLA-DRB1 allele and the susceptibility to autoimmune hepatitis.

MATERIALS AND METHODS

Subjects

A total of 32 patients, all female, who satisfied the diagnostic criteria for AIH[1,2,21-23], were enrolled in this study. They were followed up at Shanghai Institute of Digestive Disease, Shanghai Renji Hospital from 1996 to December 2000. Each patient denied illicit drug use, contact with jaundiced individuals, family history of liver disease, alcohol abuse, and exposure to hepatotoxic medication or chemicals. Each lacked evidence of infection with hepatitis B and C virus. All patients were seropositive at a titer of at least 1:40 by indirect immunofluorescence for antinuclear antibodies or smooth muscle antibodies. The control population comprised 48 healthy female Chinese who were unrelated to the patients.

Methods

DNA extraction High molecular weight DNA was isolated from peripheral blood leukocytes by phenol/chloroform extractions.

Amplification primers Primer pairs used for HLA-DRB1 and subtypes of HLA-DR4 were synthesized by the Shanghai Branch, Canadian Sangon Bioengineer Company, according to REFERENCES[24,25]. In each PCR reaction a primer pair was included that amplified the third intron of DRB1 genes. These two primers matched non-allelic sequences and thus functioned as an internal positive amplification control. 5'-prime C5 $^{5'}$-TGCCAAGTGGAGCACCCAA$^{3'}$ (Tm 60℃, complementary to codons 173-179 in the 3'end of exon 3) and 3'-primer

C3 5' GCATCTTGCTCTGTGCAGAT3' (Tm 60℃, complementary to codons 193-200 in the 5'end of exon 4) gave rise to a 796 base pair (bp) fragment.

PCR-SSP[24,25] Each PCR reaction mixture contained 2-4 allele- or group-specific DRB1 primers and the internal positive control primer pair in a 5-fold lower concentration. The PCR reaction mixtures (13 μL) consisted of 100 ng genomic DNA in 2 μL, PCR buffer [50 mmol·L^{-1} KCl/1.5 mmol·L-1 $MgCl_2$/10 mmol·L^{-1} Tris-Cl, pH 8.3/0.001% (w/v) gelatin], 200 μmol·L-1 of each dATP, dCTP, dGTP and dTTP, 1 μmol·L^{-1} of the allele- and group-specific DRB primers, 0.2 μmol·L^{-1} of Ampli Taq (diluted 1 to 10 in 1×PCR buffer, Shanghai Branch, Canadian Sangon Bioengineer Co.). PCR amplifications were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Instruments). DNAs were amplified by 30 three-temperature cycles in 1 h and 20 min. Each cycle consisted of denaturation at 94℃ for 20 s, annealing at 65℃ for 50 s and extension at 72℃ for 20 s.

Visualization of amplification Absence or presence of PCR products was visualized by agarose gel electrophoresis. After addition of 2.5μL loading buffer [300 mL·L^{-1}% (v/v) gllycerol stained with bromophenol blue and xylene cyanol], the PCR reaction mixtures were loaded in 3 mm wide slost in 10 g·L^{-1} ME agarose gels pre-stained with ethidium bromide (0.5 mg·L^{-1} gel). Gels were run for 25-30 min at 7-8 V·cm^{-1}, then were examined under UV illumination and documented by photography.

Statistical analysis

Data were analyzed by the standard statistical procedure of χ^2 contingency table analysis with Yates' correction. Corrected *P* values were calculated using the Bonferroni inequality method. Relative risk was obtained by the cross-product ratio of the entries in the 2×2 table. A level of $P<0.05$ was considered as statistically significant.

RESULTS

Allelic frequencies of HLA-DRB1 are shown in Table 1. HLA-DRB1 typing by PCR-SSP showed that DRB1 *04, which encodes DR4 antigen, had a significantly increased frequency among patients with AIH versus healthy control (46.9% *vs* 20.8%; relative risk = 3.35, $P = 0.014$). No other allele was significantly associated with autoimmune hepatiti s. DRB1 *04 has 11 subtypes officially assigned as DRB1 *0401-0411. As shown in Table 2, there was a trend of an increase in gene frequency of DRB1 *0405 in patients with AIH versus healthy controls (21.9% *vs* 6.3%, $P = 0.04$, $P_c = 0.08$).

The "shared motif hypothesis" suggests that many susceptibility-associated alleles encode a conserved epitope spanning the third allelic hypervariable region of the DRâ molecule, which may form the molecular basis of susceptibility to autoimmune diseases such as rheumatoid arthritis. The sequence over this third allelic hypervariable region, which spans amino acid 70-74 of the DRβ chain, are QKRAA and QRRAA. QKRAA is encoded by allele DRB1 *0401, QRRAA by DRB1 *0404, DRB1 *0405, DRB1 *0408 and DRB1 *0410. Our analysis indicates a significant increase in the alleles encoding QRRAA from the third hyperpolymorphic region of DR4 in the patients with AIH (13 cases or 86.7% of 15 DRB1 *04 positive patients *vs* 4 cases or 40.0% of 10 DRB1 *04 positive controls, $P = 0.016$, $P_c = 0.028$, RR = 9.75).

Table 1 Allelic frequencies of DRB1 in autoimmune hepatitis

DRB1 allele	DR Ag	Autoimmune hepatitis % (n = 32)	Healthy control % (n = 48)	RR	P value	Corrected P value
DRB1*01	DR1	0.0	4.2			
DRB1*15,*16	DR2	37.5	35.4			
DRB1*03	DR3	15.6	14.6			
DRB1*04	DR4	46.9	20.8	3.35	0.014	0.026
DRB1*11,*12	DR5	12.5	29.2			
DRB1*13,*14	DR6	9.4	12.5			
DRB1*07	DR7	3.1	4.2			
DRB1*08	DR8	9.4	10.4			
DRB1*09	DR9	34.4	37.5			
DRB1*10	DR10	0	2.1			

P values are given only where significant at <0.05 level.

Table 2 Allelic frequencies of DRB1*04 subtypes in autoimmune hepatitis

Allele	Autoimmune hepatitis % (n = 32)	Healthy control % (n = 48)	RR	P value	Corrected P value
DR4	46.9 (15)	20.8 (10)	3.4	0.014	0.026
DRB1*0401	3.1 (1)	4.2 (2)			
DRB1*0402	0	0			
DRB1*0403	3.1 (1)	4.2 (2)			
DRB1*0404	12.5 (4)	2.1 (1)			
DRB1*0405	21.9 (7)	6.3 (3)	4.2	0.04	0.08
DRB1*0406	3.1 (1)	4.2 (2)			
DRB1*0407	0	0			
DRB1*0408	0	0			
DRB1*0409	0	0			
DRB1*0410	6.2 (2)	0			
DRB1*0411	0	0			

P values are given only where significant at <0.05 level.

DISCUSSION

Autoimmune hepatitis (AIH) is a disease of unknown etiology but is presumed to have a basis in aberrant autoreactivity[26,27]. It is characterized by hypergammaglobulinaemia due mainly to selective elevation of serum IgG, a wide range of circulating tissue autoant ibodies, a picture of periportal (interface) hepatitis on liver biopsy with a predominantly lymphoplasmacytic necroinflammatory infiltrate without cholangiolitic or other changes normally associated with liver diseases of other aetiologies, and usually a notable response to immunosuppressive therapy[1,23,28,29]. Diagnosis is based on the combination of these features together with careful exclusion of all other possible causes of liver disease. Corticosteriods remain as the standard therapy for patients with severe autoimmune hepatitis but the treatment is not universally effective[30]. Novel drugs with potent immunosuppressive and cytoprotetive functions must be evaluated[31,32]. Liver transplanta tion has been an extremely successful treatment option for the decompensated patients[33], but better drug regimens are needed to conserve this limited resource.

The association of AIH with inheritance of the HLA-DR3 allotype in Caucasians has long been recognized. The secondary association with DR4 (in DR3 negative patients) has been more recently defined[34-39]. DR3 tends to occur more frequently in younger patients with severe disease whereas DR4 is mostly associated with an older age at presentation and with generally milder disease that is easier to control[11-15].Using HLA-DR typing by PCR-SSP, we found that DRB1*04 (encoding DR4) was presented in 46.9% of the AIH group versus 20.8% of healthy control individuals,

giving a relative risk of 3.35. No other allele was significantly associated with autoimmune hepatitis in Chinese. In white patients, a dual association of HLA-DR3 and -DR4 has been found in patients with autoimmune hepatitis. The discrepancy in HLA association between Chinese and white patients with AIH is probably caused by the racial or disease differences in the distribution of HLA alleles. Other genetic predispositions or environmental factor responsible for the pathogenesis of these autoimmune diseases may vary among ethnic groups. Our results are similar to the reports from Japan, where relationship between the susceptibility of AIH and the presence of HLA-DR4 had been firmly demonstrated[14-16]. In our study, there was a trend of an increase in gene frequency of DRB1*0405 increased in patients with AIH versus healthy controls, but the difference between patients and controls was not statistically significant, probably due to the small number of patients. "The shared epitope hypothesis" suggests that many alleles encode a similar amino acid sequence in the critical portion of the antigen-binding groove of the HLA DR molecule. The presence of shared third allelic hypervariable region epitopes QKRAA and QRRAA at position 70-74 of the HLA-DRâ chain was analyzed in our study. Unlike in the Caucasian population, the DRB1*0401 allele, which possesses the QKRAA sequence, was very uncommon in the Chinese subjects. There was a significant increase in the percentage of patients who possess the QRRAA sequence. This was accounted for mainly by the DRB1*0405 allele, with the rest being DRB1*0404 allele.

Autoimmune hepatitis is thought to be triggered by various environmental factors in individuals rendered susceptibility by a distinct and genetic background[40]. The immune response is initiated in human when the T-cell receptor recognizes foreign or self-peptide fragments that can be bound to self-HLA molecules encoded by the major histocompatibility complex genes on the short arm of the sixth chromosome. Differences in immune responsiveness among individuals are considered to be caused by allelic variations in HLA antigens with autoimmune diseases associated with specific HLA antigens. DR4 may regulate immune responsiveness to some autoimmune hepatitis, but not all. Other genetic polymorphism of HLA, such as HLA-DR6, -DQ, -C4[41-45], may also play an important role in the pathogenesis of AIH in the white patients, but there has been no data in Chinese yet. Autoimmune promoter genes outside the HLA, such as CTLA-4 (cytotoxic T-lymphocyte antigen-4)[46], T cell repertoire[47,48], cytokine gene[49,50], were investigated in white patients with AIH. So the genetic background of AIH may be very complex, but the clarity of this genetic predisposition and its clinical consequences will render defining susceptible populations and seeking novel therapeutic interventions.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

*p*16 gene methylation in colorectal cancers associated with Duke's staging

Jing Yi[1], Zhi-Wei Wang[1], Hui Cang[1], Yu-Ying Chen[1], Ren Zhao[2], Bao-Ming Yu[2] and Xue-Ming Tang[1]

[1]Department of Cell Biology, 2Department of Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
Supported by the grants from Ministry of Public Health of China, No. 98-1-303 and The Educational Committee of Shanghai, No.2000B02.
Correspondence to: Dr. Jing Yi, Department of Cell Biology, Shanghai Second Medical University, 280 Chongqing South Road, Shanghai 200025, China. yijing@shsmu.edu.cn
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Abstract

AIM **To explore the association of methylation of the CpG island in the promotor of the P16 tumor suppressor gene with the clinicopath ological characteristics of the colorectal cancers.**

METHODS **Methylation-specific PCR (MSP) was used to detect P16 methylation of 62 sporadic colorectal cancer specimens.**

RESULTS **P16 methylation was detected in 42% of the tumors. Dukes' staging was associated with P16 methylation status. p16 methylation occurred more frequently in Dukes' C and D patients (75.9%) than in Dukes' A and B patients (12.1%).**

CONCLUSION **P16 methylation plays a role in the carcinogenes is of a subset of colorectal cancer, and it might be linked to poor prognosis.**

Subject headings colorectal neoplasma/pathology; genes, P16; polymerase chain reaction/METHODS; CpG region; methylation; MSP

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INTRODUCTION

The alterations of p16 gene, i.e. homozygous deletion, point mutation and promoter methylation, and their consequence, P16 gene inactivation, were frequently observed in many human tumors. In colorectal cancers (CRC), since the first two mechanisms have never been demonstrated[1], the role of P16 gene methylation in carcinogenesis has attracted considerable attention recently[2-4]. Whether P16 methylation is associated with clinicopat hological characteristics has not been clear[4,5]. In the present study, we detected the occurrence of p16 methylation in primary CRC using a recently developed methylation specific PCR method (MSP) and explored the relationship between methylated p16 and Dukes' staging and other clinicopathological characteristics of the patients.

MATERIALS AND METHODS

Tumor specimens

Fresh specimens (n = 62) of surgically resected CRC were collected from Rui Jin Hospital and were immediately frozen at -80℃ after rinsed with phosphate buffered saline.

DNA isolation

Tumor DNA was isolated and purified with Trizol reagent (GIBCO Life Technologies) according to the manufacturer's instruction.

Bisulfite conversion of DNA samples

Bisulfite conversion of DNA samples for methylation detection was carried out based on the principle that treatment of DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil, while methylated cytosine residues remain unchanged. Thus, the DNA sequences of methylated and unmethylated genomic regions following bisulfite conversion would be different and distinguishable by sequence specific PCR primers. CpGenome DNA modification kit (Intergen, New York, NY) was used. One microgram of DNA was treated with sodium bisulfite and the bisulfite-converted DNA was resuspended in a total volume of 30 μL of TE.

Methylation-specific PCR (MSP)

MSP was performed using CpG p16 WIZ amplification kit (Intergen). With a complete chemical modification reaction, U primers amplified only unmethylated DNA, and M primers amplified only methylated DNA in the region of P16 gene promoter. W primers amplified unmodified DNA no matter they were methylated or not. Each chemically modified experimental DNA sample was amplified with primers U, M and W respectively. The PCR mixture contained 1×PCR buffer (500 mmol·L^{-1} KCl, 100 mmol·L^{-1} Tris-HCl, 10 g·L^{-1} Triton-100, 15 mmol·L^{-1} $MgCl_2$), dNTPs (each at 0.25 mmol·L^{-1}), U or M primers 1.0 μL, Ampli Taq Gold polymerase (Perkin-Elmer, Foster city, CA) 1 U, bisulfite-modified DNA 0.1 μg in a final volume of 30 μL. Reactions were started at 95℃ for 12 min. Amplification was carried out in a thermal cycler (Hybaid, Teddington, England) for 35 cycles (45 s at 95℃, 45 s at 60℃, and 60 s at 72℃), followed by a final 10 min extension at 72℃. U, M and W controls provided by the kit served as validation of the reagents and PCR conditions. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

The expected PCR products visualized on the gel should be an 154 bp nucleotide for unmethylated P16 and 145 bp nucleotide for methylated p16. If the sample contains unmethylated DNA, Uprimer will produce an 154 bp products (band U), and if it contains methylated DNA, M primer will produce 145 bp products (band M). The appearance of band W from the sample indicated an incomplete bisulfite conversion, and was considered a sign of unqualified chemical modification of the sample.

Clinicopathological data

Clinicopathological data were collected after MSP for p16 methylation was accomp lished. The clinicopathological data

included age, gender of the patients, anatomical location[2] and Dukes stage of CRC.

Statistical analysis

The χ^2 test was performed to analyze the relationship between p16 methylation status and each of the clinicopathologic parameters.

RESULTS

MSP analysis of p16 methylation

Of 62 CRC specimens, 26 displayed bands M, 154 bp products, signifying that 42% of the tumors had detectable p16 methylation. Band U, 154 bp product, was visible in all specimens with varied intensity, indicating the existence of unmethylated DNA in these specimens. No band W appeared in all specimens (Figure 1).

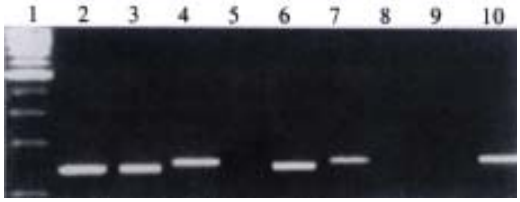

Figure 1 Electrophoresis of MSP products of p16 CpG region. 1. 100 bp marker, 2. W control (142 bp), 3. M control (145 bp), 4. U control (154 bp), 5,6,7. The same sample (from patient No.9) reactive with W, M,U primers respec tively, 6. 145 bp product, 7. 154 bp product, indicating p16 was methylated in this sample, 8,9,10. Same sample (from patient No.34) reactive with W, M, U primers respectively, 10. 154 bp product, indicating that p16 was unmethylated in this sample.

Clinicopathological correlations with p16 methylation

No correlation was found between p16 methylation and some of clinical factors, including age, gender, tumor location, etc. However, P16 methylation was signif icantly associated with Dukes' staging. Dukes' C, D patients were more likely to contain methylated p16 compared with Dukes' A, B patients (Table 1).

Table 1 Clinicopathological correlations with p16 methylation

	n	Methylated	Unmethylated
Male	35	18 (51.4)	17 (48.6)
Female	27	8 (29.6)	19 (70.4)
Age (yrs)			
<50	15	7 (46.7)	8 (53.3)
>50	47	19 (40.4)	28 (59.6)
Tumor location[2]			
Proximal	21	12 (57.1)	9 (42.9)
Distal	41	14 (34.1)	27 (65.9)
Dukes' staging			
A, B	33	4 (12.1)	29 (87.9)
C, D	29	22 (75.9)	7 (24.1)[b]

[b]$P<0.01$, *vs* A, B.

DISCUSSION

DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferase in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide[6,7]. The potential contribution of DNA methylation to oncogenesis is mediated by one or more of mechanisms that include DNA hypomethylation, hyper methylation of tumor suppressor gene and chromosomal instability in cancers[8-13]. The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation[6]. P16 tumor suppressor gene plays a monitor role in the passage of cells through the G1 to S phase of the cell cycle by binding to cyclin-dependent kinase 4 and inhibiting its effect on cyclin D1[14-16]. Methylation of cytosine residues at CpG sites in p16 gene promotor, resulting in a silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas in varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma and so on[17-30].

In the previous studies on p16 methylation, most researchers used Southern analysis and digestion of methylation-sensitive enzymes to detect methylation[15,31]. We used MSP, in the present study, for analysis of the methylat ion status of p16. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. In summary, MSP is a simple, sensitive and specific method for determining the methylation status of CpG rich region[32]. This might account for that the occurrence of p16 methylation detected by MSP (20%-50%)[4,5,33] was usually higher than those by Southern analysis and other METHODS (10%-20%)[15,31]. In our study, methylated p16 in primary CRC was found in a frequency as high as 42%, probably revealing a number more precise than those detected not by MSP.

Though p16 methylation is common in CRC cell lines[4,34,35] and has been suggested to involve also in the primary CRC[2,4,5,33,36-38], the association of p16 methylation with the clinicopathological characteristics of primary CRC has rarely been investigated and concluded with conflicts[2,5,37-39]. The role of p16 methylation in the development and progress of the primary CRC therefore awaits to be elucidated. Wiencke *et al* found that women were much more likely than men to have p16-methylated tumors and that proximal tumors were more likely to contain methylated p16. They reported that p16 methylation was also associated with poorly differentiated tumors[2]. Liang *et al* reported that the presence of p16 methylation predicted shorter survivals in colorectal cancer patients[5]. Zhang et al found that p16 methylation in CRC was not associated with clinicopathologic data[37,38]. In our study, no correlation was found between p16 methylation and various clinicopathologic factors including age, gender or tumor location. However, p16 methylation was significantly associated with Dukes' staging. Dukes C, D patients were more likely (75.9%) to contain methylated p16 as compared with Dukes' A, B patients (12.1%). Dukes' staging is a clinical classification for colorectal cancers based on the tumor size, local extent, metastatic status, i.e. lymph node involvement. Hence, Dukes' staging has been considered a most important prognostic determinant. In the present study, p16 methylation was more likely to occur in Dukes' C, D patients. This result suggested that p16 methylation might link to a more malignant outcome of CRC.

How does the existence of methylated p16 promoter affect the expression pattern of p16 protein in primary tumors Controversy has emerged over recent years on the

relationship of p16 promoter methylation and its gene expression in whole tumors. Although most of authors hold the traditional viewpoint that detectable p16 methylation necessarily link to the inactivation of p16 protein, or transcriptional silencing of p16 gene[3,10,19,31,40], coexistence of p16 methylation and p16 expression in one specimen has been frequently described[2,41-44]. It was noticeable that, in our study, band U, i.e. PCR products amplified by primer U, was visible in each specim en. Similar results were also reported by other investigators[2]. This might first be attributed to the contamination of non-neoplastic cells that naturally harbored unmethylated DNA. However, Guan *et al* demonstrated that band U appeared even in the samples acquired by microdissection which got rid of the non-neoplastic cells[4] . This indicated the possibility that tumor cells in one sample were virtually the mixture of those contained methylated and unmethylated DNA. In fact, the cell line in which p16 methylation was detected could still express p16 protein, due to a partial methylation[45]. In summary, coexistence of p16 methylation and p16 expression in one sample might reflect the cell heterogeneity, in which, as we speculated, a part of cells contained methylated p16 and loss of p16 expression whereas another part of cells kept expressing or even overexpressing p16 protein. We found that, in a previous immunohistochemical study on 71 archival specimens of CRC, p16 expression-positive tumor cells were seen in roughly 50% specimens, and these p16 expressive specimens were coincident with those with a poorer differentiation grade[46]. Other investigators have also proposed that activation but not inactivation of p16 gene was associated with primary CRC[47]. We will continue our effects to explore the correspondent relation of p16 methylation and p16 expression in the identical CRC specimen, so as to determine how the seemly paradoxical events might unite in one tumor. The elucidation of the relationship between p16 expression and p16 gene methylation in primary tumors will certainly help better understand the role of methylation of tumor suppressor genes in carcinogenesis.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Identification of differentially expressed genes in normal mucosa, adenoma and adenocarcinoma of colon by SSH

Min-Jie Luo and Mao-De Lai

Department of Pathology, School of Medicine, Zhejiang University, Hangzhou 310031, P.R.China
This study is supported by Science Foundation of the Education Department of Zhejiang Province.
Correspondence to: Mao-De Lai, M.D., Department of Pathology, School of Medicine, Zhejiang University, Hangzhou 310031, P.R. China. lmd@sun.zju.edu.cn
Telephone: +86-571-87217134, Fax: +86-571-87951358
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Abbreviation CRC colorectal cancer. SSH suppression subtractive hybridization. LD PCR long distance polymerase chain reaction. HLA human leukocyte antigen. IGFBP insulin-like growth factor binding protein. GN guanylin. EF1 elongation factor 1

Abstract

AIM **To construct subtracted cDNA libraries and further identify differentially expressed genes that are related to the development of colorectal carcinoma (CRC).**

METHODS **Suppression subtractive hybridization(SSH) was done on cDNAs of normal mucosa, adenoma and adenocarcinoma tissues from the same patient . Three subtracted cDNA libraries were constructed and then hybridized with forward and backward subtracted probes for differential screening. Positive clones from each subtracted cDNA library were selected for sequencing and BLAST analysis. Finally, virtual Northern Blot confirmed such differential expression.**

RESULTS **By this way, there were about 3-4×102 clones identified in each subtracted cDNA library, in which about 85% positive clones were differentially screened. Sequencing and BLAST homology search revealed some clones containing sequences of known gene fragments and several possibly novel genes showing few or no sequence homologies with any known sequences in the database.**

CONCLUSION **All results confirmed the effectiveness and sensitivity of SSH. The differentially expressed genes during the development of CRC can be used to shed light on the pathogenesis of CRC and be useful genetic markers for early diagnosis and therapy.**

Subject headings colonic neoplasms/genetics; adenoma/genetics; adenocarcinoma/genetics; intestinal mucosa/metabolism; nucleic acid hybridization

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common forms of malignancy and the major cause of mortality in the population. The adenoma-carcinoma sequence has been accepted as the main pathway involving a series of molecular changes, which include activation of oncogenes, inactivation of tumor suppressor genes, etc. in colorectal carcinogenesis for several years[1]. However, due to the complexity of carcinogenesis, there are still many problems unsolved for the model of CRC. Not only are all the known genetic alterations not enough to explain fully the progression toward malignancy, but also there is no one genetic marker which is entirely tissue and / or cancer specific. Hence, an attempt was made to find new genes that are related to the development of CRC. Our goal was to clone and identify new cancer-predisposing genes.

In almost any area of biology or medicine, questions arise from the differential gene expression. Transformations in the normal mucosa-adenoma-carcinoma sequence result from the expression changes of specific gene(s) too. The identification and characterization of human genes expressed exclusively or preferentially in tumor tissue will hopefully shed light on the mechanisms of CRC development and provide useful genetic markers for screening, diagnosis, prognosis, therapeutic monitoring and even follow-up development of therapeutic vaccines.

There are many techniques that AIM at producing an inventory of differential transcripts between two populations of mRNAs. Although all these methods have proven successfully isolating differentially expressed genes, they all had some intrinsic drawbacks: need for plenty of initiating materials, difficulty in obtaining rare transcripts, significant incidence of false positives, labor intensive, etc. Suppression subtractive hybridization (SSH), a PCR-based cDNA subtracted method[2], combines normalization and subtraction in a single procedure and generates subtracted cDNA libraries. This method overcomes many drawbacks mentioned above.

In this research, we conducted SSH to identify genes differentially expressed am ong cDNAs of normal mucosa, adenoma and adenocarcinoma tissues from the same patient. Three subtracted cDNA libraries were constructed and numerous sequences were isolated.

MATERIALS AND METHODS

Tissue sample preparation

All normal (N), adenoma (A) and adenocarcinoma (T) tissue samples obtained from the same patient were histologically confirmed by a pathologist. The tissues were frozen in liquid nitrogen and homogenized with mortar. Total RNA was prepared using Trizol reagent (Gibco BRL, USA). RNA integrity was checked on 1% formaldehyde agarose gel. Poly (A)+ RNA was then purified using PolyATract mRNA cDNA system kit (Promega, USA) as per manufacturer's instructions.

SMART cDNA synthesis

0.5 μg mRNAs from each sample were reversely transcribed using MuLV SuperScript II reverse transcriptase (Gibco BRL, USA), SMART II oligonucleotides and CDS primers from SMART cDNA synthesis kit (Clontech, USA). The first strand cDNA mixtures were amplified by LD PCR. Before amplification, the number of PCR cycles was optimized (19 for N, 17 for both A and T) to ensure that cDNA products remain in the exponential phase of amplification to reduce nonspecific amplification.

SSH

After cDNAs were produced, they were purified by passing through Chroma spin-400 columns (Clontech, USA). Each purified tester cDNA was digested with RsaI to give an average insert length approximately longer than that of 600 base pairs. Then they were phenol chloroform-extracted, ethanol-precipitated, and dissolved in 6 μL ddH_2O. The tester cDNAs were subdivided into two equal groups and then ligated to adaptor 1 and 2R in separate ligation reactions. Ligation efficiency was tested. Subtractive hybridi zation was performed by annealing an excess of driver cDNAs with each sample of adaptor-ligated tester cDNAs. The cDNAs were heat-denatured and incubated in 68℃ for 8 h. After the first hybridization, the two samples were mixed together and hybridized again with freshly heat-denatured driver cDNAs for 20 h at 68℃. Two rounds of hybridization would generate a normalized population of tester-specific cDNAs with different adaptors on each end.

After filling in the ends, two rounds of PCR amplification were performed to enrich desired cDNAs containing both adaptors by exponential amplification of these products. The optimized cycles for the first and second PCR were 26 and 12 respectively to increase representation and reduce redundancy of subtracted cDNA libraries.

Secondary PCR products were used as templates for PCR amplification of human G3PDH at 18, 23, 28 and 33 cycles to assure subtraction efficiency. PCR products were run on 1.8% agarose gel.

Both forward and backward SSH were performed. The difference between them was that the tester sample in forward SSH was used as driver sample in backward SSH.

Cloning and differentially screening of subtracted cDNA libraries

Products from the secondary PCR were T-A cloned into pGEM-T Easy Vector using pGEM-T Easy Vector System II kit (Promega, USA). The ligation products were transformed into DH5α competent cells. The transformed cells were plated on 2-YT agar plates containing ampicilin, X-Gal and IPTG allowed for color selection of colonies. Transformation efficiency was calculated and white clones were selected for further characterization.

Randomly selected individual clones were grown for 8 h in 96-well culture plates and then used for clone PCR amplification. The primers used were nested Primer1 and 2R of adaptors. After 27 cycles amplification, the PCR products were subjected to a differential screening process. Briefly, 3 μL of amplified products of each candidate gene was mixed with DNA dilution buffer (50 μg/mL herring sperm DNA, 10 mM Tris. Cl, pH 8.0, 1 mM EDTA), spotted onto duplicate Hybond N+ Membranes (Boeheringer Mannheim, Germany) and UV cross-linked for 1 min. The forward and backward subtracted cDNAs were used as probes. These cDNAs were DIG-labeled by PCR amplification using PCR DIG probe synthesis kit (Boeheringer Mannheim, Germany), digested with *Rsa*I, *Eag*I and *Sma*lI to remove the adaptors and purified with column. After prehybridization, the duplicate membranes were hybridized with forward and backward subtracted cDNA libraries probes respectively at 42℃ for 16 h-20 h in hybridization solution with 50% deionized formamide, 0.1% (w/v)-Lauroylsarcosinee, 2% blocking reagent, 0.02% (w/v) SDS, 2 μg/mL NP1, NP2R and CDS primer sequences. The membranes were then washed for 2×5 min with 2×SSC, 0.1% SDS at room temperature and for 2×15 min in 0.5×SSC, 0.1% SDS at 68℃. Then, they were blocked by gently agitating in blocking solution at 37℃ for 1 h, incubated in buffer I with Anti-DIG (1:4 000) at 37℃ for 30 min, washed in buffer I for 2×15 min and equilibrated in buffer III for 5 min. Finally, the membranes were incubated in mixture of NBT and BCIP for about 15 min. The clones giving at least 5-fold higher hybridization signals with the forward probes were selected for further analysis.

Sequencing and BLAST homology search

Candidate positive clones from each subtracted cDNA library were selected for sequencing. Cycle sequencing reactions were conducted with Thermo Sequenase cycle sequencing core kit (Amersham Life Science, USA). Each clone was sequenced on both 5' and 3' end. The BLASTN program was used to search for the DNA sequence homology of the isolated clones in the database of Genbank.

Virtual Northern blot

High yields of full-length cDNAs were generated from 1ìg total RNA of normal mucosa, adenoma and adenocarcinoma using SMART PCR cDNA Synthesis kit (Clontech, USA). 0.2 μg cDNAs were electrop hosed on a 1.0% agarosegel, transferred onto Hybond N+Membrane (Boeheringer Mannheim, Germany) and hybridized with DIG-labeled clone probes. These probes were generated by PCR amplification of the relevant inserts, adaptor-cutting and column-purification. Hybridization and washing conditions were the same as described above in screening of cDNA libraries. DIG-labeled G3PDH probes were used as control.

RESULTS

Subtracted cDNA library construction by SSH

In this research, SSH was done to identify differentially expressed genes among cDNAs of normal mucosa, adenoma and adenocarcinoma tissues from the same patient. Three subtracted cDNA libraries were constructed. The subtracted A-N cDNA library used normal mucosa as tester cDNA and adenoma tissue as driver cDNA; the subtracted T-N cDNA library used adenocarcinoma tissue as tester cDNA and normal mucosa as driver cDNA; the subtracted T-A cDNA library used adenocarcinoma tissue as tester cDNA and adenoma tissue as driver cDNA. Figure 1 shows subtractedcDNA libraries after secondary PCR amplification. They usually looked like smears with or without several discrete bands. However, the patterns among them were different.

Subtraction efficiency analysis showed the effectively reduced abundance of non -differentially expressed genes. In nonsubtracted cDNA libraries, housekeeping gene G3PDH PCR products were visible after 18 cycles of amplification and became saturated after 23-28 cycles. However, subtracted libraries required a higher number of amplification cycles for G3PDH to be detected. Depletion of G3PDH was more significant in the subtracted A-N and T-A cDNA libraries, suggesting that these two subtractions were more complete.

Differential screening of subtracted cDNA libraries

About 4×10^2,3×10^2 and 3.5×10^2 clones were respectively identified in subtracted A-N, T-N and T-A cDNA libraries with average insert size of 0.2-1 kb. These clones were

screened by hybridization with both forward and backward subtracted cDNA library probes. Clones corresponding to truly differentially expressed genes would mainly hybridize with the forward subtracted cDNA probe, but not, or only faintly, with the backward subtracted cDNA probe, asshown by signal comparison between two blots. By this way, more than 85% positive clones in three subtracted cDNA libraries were differentially screened.

Sequencing and homology search

Automated sequencing proved that most clones were isolated no more than two times. BLASTN homology search revealed that some clones contained substantial sequence homologies with known gene fragments and several possibly novel genes, showed few sequence homologies with any known sequences in the GenBank database. The results of homology search are shown in Table 1.

Virtual Northern Blot

The cDNAs of selected clones were mainly expressed in tester tissue but not, or to a lesser extent, in driver tissue as Figure 4 shows. Virtual Northern Blot showed such differential expression in most clones. These results were also confirmed by RNA Dot blot (data not shown).

Table 1 Characteristion of randomly selected differentially expressed clones from the three subtracted cDNA libraries screening of forwar subtraction. Clone name, insert size, sequence identity and redundancy are shown

Clone	Insert(bp)	Sequenceidentity	Redundancy
Subtracted A-N cDNA library			
a1	850	DNA sequence from clone RP5-991C6 on chromosome 6q14	1
a2	850	Mitochondrion genome	1
a3	850	DNA sequence from clone 75K24 on chromosome 6q13-1	1
a4	550	No match	1
a5	500	No match	1
a6	550	No match	1
a7	500	Guanylin mRNA, complete cds	1
a8	500	DNA sequence from clone GS1-279B7 on chromosome 1q25.1-31.1	1
a9	700	beta 2-syntrophin mRNA	1
a10	450	mRNA fragment coding for HLA-1	1
a11	500	mRNA for KIAA0212 gene	1
a12	650	No match	1
a13	800	PAC clone RP5-1131G17 from 7p15.1-p14	1
a14	600	BRX mRNA	1
Subtracted T-N cDNA library			
b1	480	mRNA fragment coding for HLA DR	2
b2	750	Insulin-like growth factor binding protein 7	1
b3	750	mRNA for KIAA0494 protein	1
b4	760	mRNA for immunoglobulin kappa light chain	1
b5	500	Complement component 3 gene, exons 1-30	1
b6	620	mRNA for anti-HbsAg immunoglobulin	1
b7	600	mRNA for fibronectin	2
b8	770	BAC clone RG158017 from 7q22-q31.1	1
b9	620	Ribosomal S25(RPS25) mRNA	1
b10	650	No match	1
b11	400	Mitochondrion, cytochrome oxidase subunit II	1
b12	450	Eukaryotic translation elongation factor1 gamma	1
b13	400	No match	1
b14	350	Ribosomal SII(RPSII)	1
Subtracted T-A cDNA librar			
r1	700	Ribosomal S12(RPS12) mRNA	2
r2	520	No match	1
r3	550	Clone P13 anti-phospholipid immunoglobulin 1	1
r4	500	mRNA for heat shock protein 90	2
r5	700	Chromosome 14 DNA sequence	1
r6	450	Chromosome 18, clone hRPK	1
r7	350	HLA-DR alpha-chain mRNA	2
r8	750	Novel human mRNA from chromosome1, which has similar to BAT2 genes	1
r9	520	mRNA for immunoglobulin kappa light chain	1

Species is human.

DISCUSSION

It was learned in the last two decades that genetic changes lie at the root of all cancers. In response, the Cancer Genome Anatomy Project (CGAP) will unite the newest technologies to identify all the genes responsible for the initiation and growth of cancer. In this research, we used SSH to identify candidate genes that were differentially expressed during CRC development.

The novel technique called SSH is an ideal subtractive system that combines high subtraction efficiency with normalized representation of differentially expressed genes, and is based on suppression PCR that permits exponential amplification of genes differing in abundance, at the same time that amplification of sequences of identical abundance genes in two populations is suppressed. Therefore, differentially expressed genes of low abundance can be cloned, while differentially expressed genes of high abundance are not

excessively isolated[3,4]. It was reported that SSH achieved more than 1000-fold enrichment of low-abundance genes but only required 2 μg mRNA instead of the 10-20 μg required in other methods. This method has been successfully used to isolate significant genes in many rese arches. Here we report the efficiency of SSH technique in identifying differenti ally expressed genes in normal mucosa, adenoma and adenocarcinoma from the same patient. All of the results confirmed the effectiveness and sensitivity of this method.

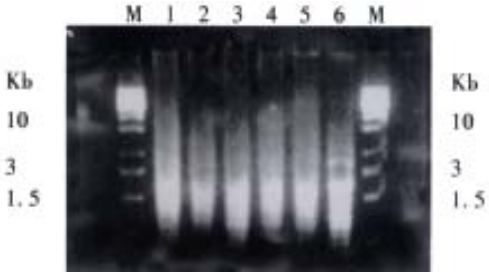


Figure 1 The results of secondary PCR amplification: Lane 1: Subtracted A-N cDNA library; Lane 2: Unsubtracted A cDNA library; Lane 3: Subtracted T-A cDNA library; Lane 4: Unsubtracted T cDNA library; Lane 5: Subtracted T-N cDNA library; Lane 6: Control

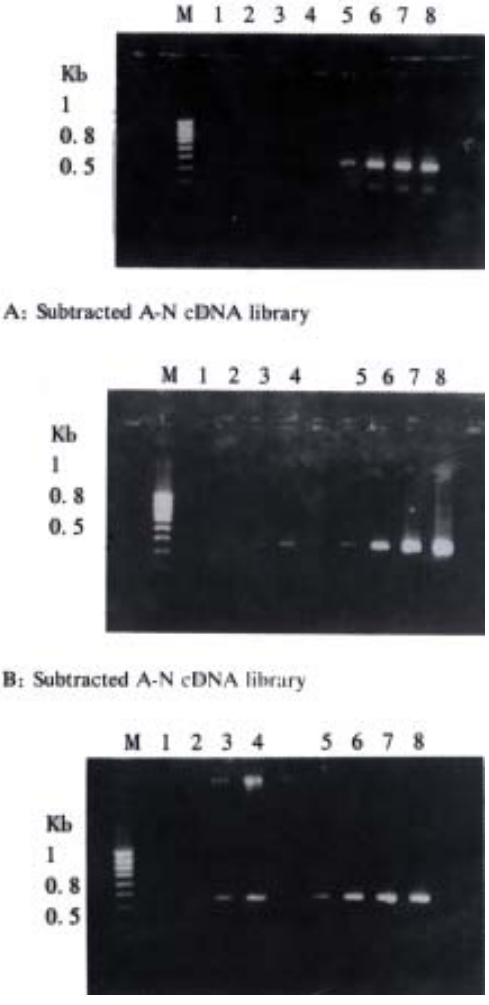


Figure 2 Analysis of subtraction efficiency: Lanes 1-4 were performed on subtracted cDNA libraries, Lanes 5-8 were performed on unsubtracted cDNA libraries. Cycles for 1&5, 2&6, 3&7 and 4&8 were 18, 23, 28 and 33 respectively.

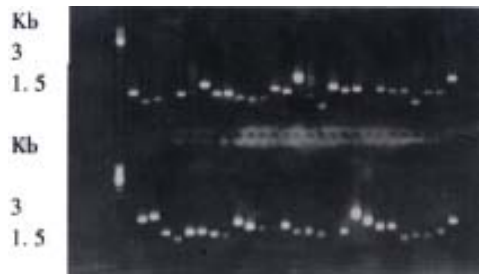
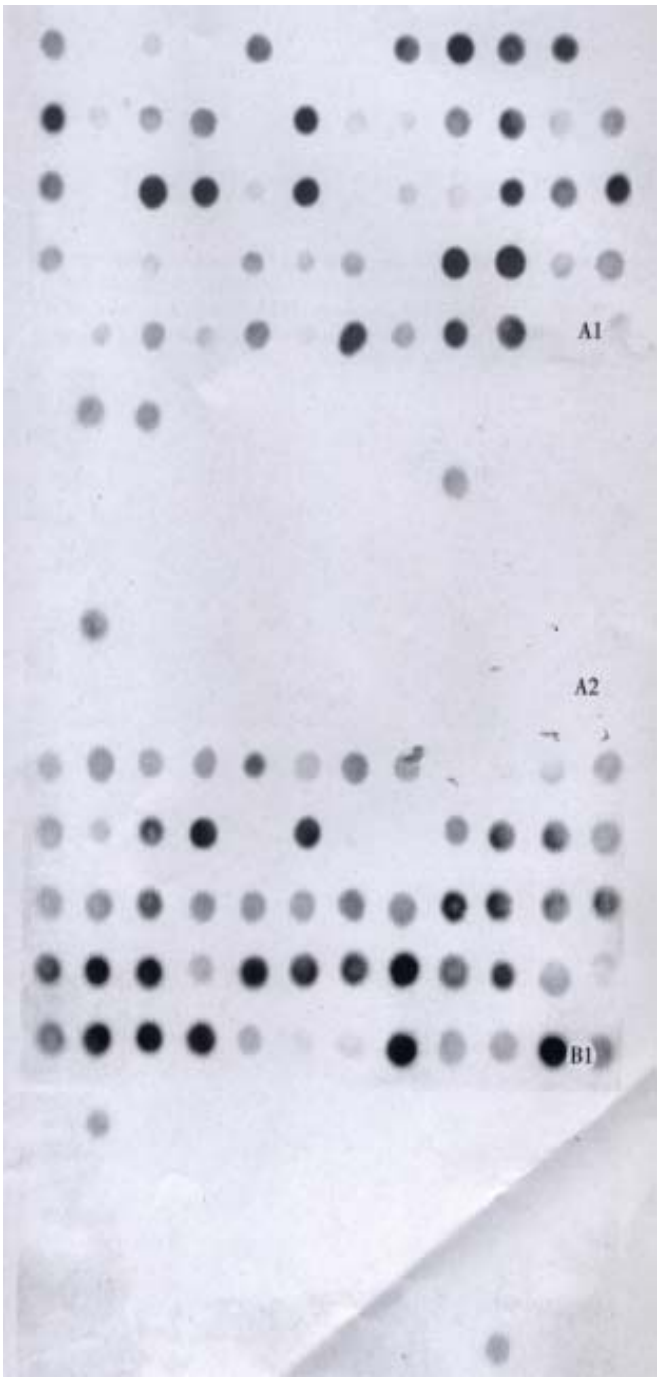


Figure 3 Clone PCR amplification with an average insert size of 0.2-1 kb.



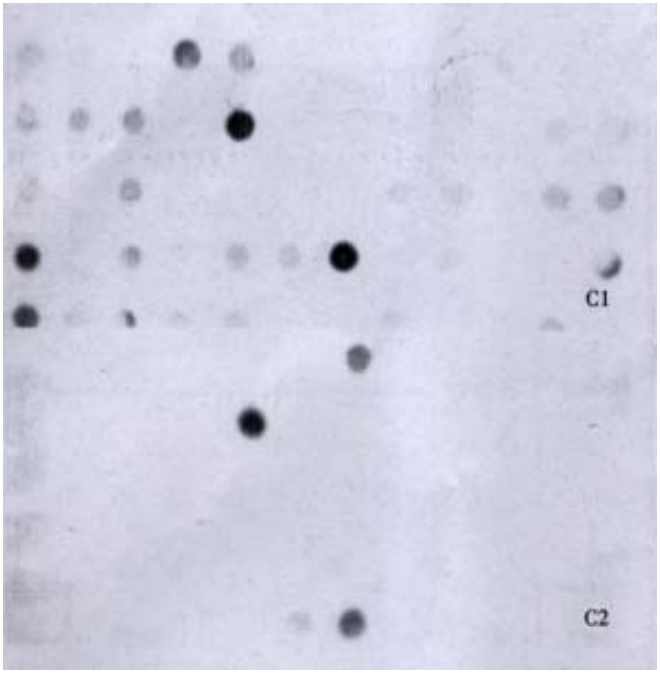


Figure 4 Differentially screening of clones using both forward (left) and backward (right) subtracted cDNA libraries. A1: Subtracted A-N cDNA library; A2: Unsubtracted A cDNA library; B1: Subtracted T-A cDNA library; B2: Unsubtracted T cDNA library; C1: Subtracted T-N cDNA library; C2: Unsubtr acted T cDNA library.

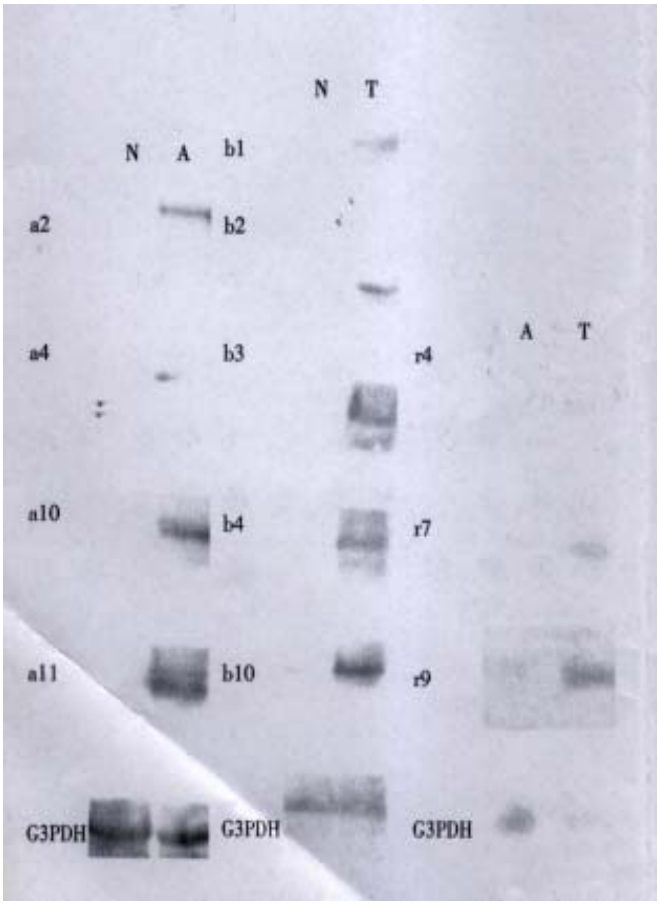


Figure 5 Virtual Northern Blot analysis of several differentially expressed cDNA: N: normal mucosa; A: adenoma; T: adenocarcinoma.

PCR analysis of the subtraction efficiency after subtractive hybridization was showed that the abundance of the non-differentially expressed gene G3PDH was effectively reduced. The difference of subtraction efficiency among the three subtracted libraries might be due to the diversity of starting materials for comparison. As we know, no subtraction technique is 100% effective. Although SSH greatly improved the subtraction efficiency, some false positive clones were still present in the studied material. Therefore, non-radioactive hybridization with forward and backward subtracted cDNA library probes was used to differentially screen these subtracted libraries before Virtual Northern blot confirmation[5]. About more than 85% positive clones in each subtracted cDNA library were differentially screened. von Stein OD *et al*[3] found about 94% positive rate in their research, so they considered confirmation of differential expression by Northern blot analysis for each clone obtained was probably unnecessary. In our research, Virtual Northern Blot and RNA Dot Blot were still performed to con firm such differential expression before further analysis of the clones.

By this way, we constructed three subtracted cDNA libraries, A-N, T-N and T-A , as mentioned above. About 3-4×10^2 clones were identified in each subtracted cDNA library through differential screening. After sequencing and BLASTN homology search, these clones could be divided into three groups: known genes preciously described as differentially expressed genes in cancer and closely related with carcinogenesis of CRC; known genes never before associated with carcinogenesis; and unknown genes that remain to be fully characterized. The identification of the first group of known genes attested to the validity of this method. In addition, no more than only two times isolation of most clones also proved the high efficiency of SSH.

HLA-DR, identified in both subtracted T-N and T-A cDNA libraries, was reported to be abundantly expressed in CRC but less so in adenoma and normal mucosa[6,7]. However, the relationship of HLA-DR expression with prognosis is still disputed[6,8]. Other molecules such as invariant chain may take part in this mechanism. So it is very important to conduct comprehensive research on the role of these molecules.

Heat shock protein (Hsps) is thought to play an important role in the cell cycle s and various processes of carcinogenesis. It can be divided into two types: Hsp90α and Hsp90β[9]. The former may play a role in cell proliferation; the later is inversely related to differentiation. Both of them are present in normal tissue but are more highly expressed in neoplasm. More important, Hsp90 was recently reported to interact with HLA-DR. In this study, Hsp90 was screened in the subtracted T-A cDNA library two times, which showed its high expression in carcinoma.

IGFBP-rp1 belongs to the IGFBP family with low affinity with IGFs. It had been shown to decrease expression with disease progression in breast carcinomas[10] and in the worsening from the benign to malignant prostate epithelial cells[11,12]. Methylation with down-regulated expression was found to be associated with liver tumorigenesis[13]. So it is inferred that IGFBP-rp1 may have a suppressive effect on cancer development. Surprisingly, in this study, IGFBP-rp1 was overexpressed in CRC in contrast to their down-regulation in carcinoma, which was also shown in prostate cancer tissue[14]. It remains to be further investigated what the role of IGFBP-rp1 is and whether such different expression is individual- specific, tissue- specific or even cell-specific.

Two cDNA clones were shown to have more than 90% homology with Ig κ gene, one screened in the subtracted T-N

cDNA library and the other in T-A. Hu *et al*[15] reported a new transforming gene Tx showed very high (99.5%) homology with the C region of Ig κ. A literature survey revealed Ig or Ig-like materials expression in carcinoma[16]. It is generally considered that Ig is mainly secreted by B-lympho cytes, so the relationship between Ig κ and carcinogenesis of CRC should be further confirmed.

Guanylin (GN) is the endogenous peptide ligand for guanylyl cyclase C (GCC) and can regulate intestinal Cl- secretion. It is robustly expressed in normal intestinal epithelium of colon but is absent in CRC[17]. So, it is possible that loss of GN activity leads to or is a result of adenocarcinoma formation. In this study, GN was expressed at higher levels in adenoma than in normal mucos a but not in CRC. It is uncertain if such increase of GN activity in adenoma means a kind of reaction to carcinogen stimulation.

Several genes shown to be dysregulated in neoplasm cells such as EF1-r[18], mitochondrion, RPS12, RPS 22/25, RPSII[19] were also identified. All of them were increased in cancer cells, as previously reported and some of them were related to the robust growth characteristics.

Besides the known genes, a very important finding in this study was the identification of several new genes that were previously undescribed in Genbank. Both Virtual Northern blot and RNA dot blot hybridization has showed such different expression. Among three subtracted cDNA libraries, A-N showed the highest rate of novel genes isolation. Although it remains to be explored the full-length sequence of these new genes by RACE or other methods and their function in carcinogenesis of CRC, these results really provide clues for us to understand newly proposed mechanisms of cancer formation.

In the past years, much of cancer research laid particular stress on analysis of single gene expressed differentially in tumor cells as compared with their normal counterparts, but little stress on comprehensive study of gene expression in cancer. It was interesting that in this study several genes including anti-HbsAg and IGFBP-rp1 were also differentially expressed in carcinoma. Based on available reports, anti-HbsAg gene was mainly related with the individual character, while IGFBP-rp1 was even supposed to be an anti-oncogene. So we propose that such diversity and specificity of gene expression may contribute to the heterogeneity in biological properties of cancer, which may be individual-specific, tumor-specific, process-specific or even cell-specific. Literature also indicated that there were differences of gene expression among cancers. Therefore, two novel concepts can be brought forward on carcinigenesis: one is the model formed by a total of individual gene expression in cancer, the other is the module formed by interaction among molecules[20]. Cellular functions, such as signal transduction, are carried out by modules. Connecting different levels of analysis from molecules, through modules, to models is essential for an understanding of carcinogenesis. Differentially expressed genes between tissues comprise the molecular basis for understanding model and module. So, despite the limitations of tissue from the same patient, this approach has provided interesting results for further research.

This is the first time that differentially expressed genes in colorectal adenoma and adenocarcinoma were isolated by the new technique SSH. Now, microarray detection of these differentially expressed genes as model and module to hybridize with other tumor samples is being investigated[21]. Comprehensive study of these genes will be helpful for us to create theoretical models of some systems and verify that such predictions match reality, which, in turn, will be useful for screening, detecting, classifying and therapy of CRC.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Diagnosis and treatment of congenital choledochal cyst: 20 years' experience in China

Liu-Bin Shi[1], Shu-You Peng[1], Xing-Kai Meng[1], Cheng-Hong Peng[1], Ying-Bin Liu[1], Xiao-Peng Chen[1],Zhen-Ling Ji[2], De-Tong Yang[2] and Huai-Ren Chen[2]

[1]Department of Surgery, The Second Affiliated hospital of Zhejiang University School of Medicine, Hangzhou 310009, China
[2]Department of Surgery, Affiliated Zhongda Hospital of Southeast University, Nanjing 210009, China
Correspondence to: Liu-Bin Shi, Department of Surgery, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China.slb5327694@sohu.com
Telephone: +86-571-87230541
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Abstract

AIM **To summarize the experience of diagnosis and treatment of congenital choledochal cyst in the past 20 years (1980-2000).**

METHODS **The clinical data of 108 patients admitted from 1980 to 2000 were analyzed retrospectively.**

RESULTS **Abdominal pain, jaundice and abdominal mass were presented in most child cases. Clinical symptoms in adult cases were non-specific, resulting in delayed diagnosis frequently. Fifty-seven patients (52.7%) had coexistent pancreatiobiliary disease. Carcinoma of the biliary duct occurred in 18 patients (16.6%). Ultrasonic examination was undertaken in 94 cases, ERCP performed in 46 cases and CT in 71 cases. All of the cases were correctly diagnosed before operation. Abnormal pancreatobiliary duct junction was found in 39 patients. Before 1985 the diagnosis and classification of congenital choledochal cyst were established by ultrasonography preoperatively and confirmed during operation, the main procedures were internal drainage by cyst enterostomy. After 1985, the diagnosis was established by ERCP and CT, and cystectomy with Roux-en-Y hepaticojejunostomy was the conventional procedures. In 1994, we reported a new and simplified operative procedure in order to reduce the risk of choledochal cyst malignancy. Postoperative complication was mainly retrograde infection of biliary tract, which could be controlled by the administration of antibiotics, there was no perioperative mortality.**

CONCLUSION **The concept in diagnosis and treatment of congenital choledochal cyst has obviously been changed greatly. CT and ERCP were of great help in the classification of the disease. Currently, cystectomy with Roux-en-Y hepaticojejunostomy is strongly recommended as the choice for patients with type I and type IV cysts. Piggyback orthotopic liver transplantation is indicated in type V cysts (Caroli's disease) with frequently recurrent cholangitis.**

Subject headings choledochal cyst/surgery; choledochal cyst/radiography; choledochal cyst/diagnosis; biliary tract/abnormalities; choledochal cyst/therapy; Caroli's disease/diagnosis; Caroli's/surgery

Hi LB, Peng SY, Meng XK,Peng CH, Liu YB, Chen XP, Ji ZL, Yang DT, Chen HR. Diagnosis and treatment of congenital choledochal cyst: 20 years' experience in China. *World J Gastroenterol*, 2001;7(5):732-734

INTRODUCTION

Choledochal cyst, or congenital cystic dilatation of the common bile duct, is a rare entity in western countries. Most of reported cases in the world come from Asia, about two-thirds of cases reported from Japan[1]. In recent years, cases of choledochal cyst are reported increasingly in China[2-5].We analyzed retrospectively the clinical data of 108 patients admitted from 1980 to 2000 in our two hospitals in order to summarize the Chinese experience of diagnosis and treatment of this congenital choledochal cyst.

MATERIAL AND METHODS

Patients

From October 1980 to February 2001, a total of 108 patients with choledochal cyst were treated In the Department of Surgery, the Second Affiliated Hospital of Zhejiang University School of Medicine and Zhongda Hospital of Southeast University. There were a total of 85 females and 23 males. The mean age was 27.8 years, with a range from 3 to 68 years. Among them, 91 cases were adults. According to Todani and colleagues' classification of congenital choledochal cyst[1]. Seventy-five patients belonged to type I (solitary extrahepatic cyst), 19 type IV A (extrahepatic and intrahepatic cysts), 5 type IV_B (multiple extrahepatic cysts), 1 type III (choledochocele) and 6 type V (only intrahepatic segmental ductal dilatation, also known as Caroli's disease); two cases were unclassifiable. In 23 patients their condition was associated with biliary tract stones. Abdominal pain, jaundice and abdominal mass were presented in most child cases, and clinical symptoms in adult cases were non-specific, resulting in delayed diagnosis frequently. Fifty-seven patients (52.7%) had coexistent pancreatiobiliary disease, 7 patients had synchronous and 11 had metachronous carcinoma lesions arising from the biliary duct cyst. Three patients appeared in pregnant period. Among complications at the time of admission for care there were jaundice in 77 patients, cholangitis in 61 patients. Ultrasonic examination was undertaken in 94 cases, ERCP performed in 46 cases and CT in 71 cases.

Surgical procedures

From 1980 to 1985, Roux-en-Y cystojejunostomy was performed in 19 patients, side to side cystoduodenostomy in 15 patients and exploratory laparotomy in 4 patients. From 1986 to 1994, 24 cases underwent cystectomy plus cholecystectomy with Roux-en-Y hepatoenterostomy, 6 underwent partial cystectomy plus cholecystectomy with Roux-en-Y hepatoenterostomy, 4 underwent cystectomy or cyst transection plus duodenocholedochotomy by jejunal

interposition, and 3 underwent left hepatectomy. After 1995, we also performed cyst excision plus cholecystectomy with Roux-en-Y hepatoenterostomy in 19 cases. Meanwhile, we reported a new and simplified operative procedure (cyst wall resection plus Roux-en-Y hepatoduodenostomy and doudenojejunuostomy) in order to reduce the risk of choledochal cyst malignancy. This procedure was performed in 9 patients. Two cases underwent piggyback orthotopic liver transplantation.

RESULTS

There was no operative death in this series. Twenty-eight patients developed early postoperative complications including cholangitis, bile leakage and wound infection. All of them recovered with conservative therapy. Late complications occurred in twenty-three patients, who suffered from ascending cholangitis, hepatolithiasis, pancreatitis and adhesive intestinal obstruction six months to five years after operation. Twenty-two cases underwent reoperation. Among them, 11 cases owing to bile duct malignant change resulting from previous internal drainage procedures (including cystoduodenostomy and cystojejunostomy); 6 cases owing to suppurative cholangitis or pancreatitis; 3 cases owing to postoperative biliary stricture accompanied stones; and 2 cases owing to calculus of bile duct. The reoperative procedures included resection of tumoror plastic repair of stricture. Sixty-two patients were under follow-up for an average period of ten years (6 months to 18 years).

DISCUSSION

Diagnosis

Choledochal cysts are recognized as a disease of childhood[1,6,7], but in our study, 91 (84.2%) of 108 patients were older than 14 years at the time of operation. Thereafter, the surgeon should be aware of choledochal cysts even in the adult patients. Abdominal pain, jaundice and abdominal mass were presente d in most child cases. In this series, 12 patients(70.5%) had these symptoms . Clinical symptoms in adult cases were non-specific, 57 patients (52.7%) had coexistent pancreatiobiliary disease. In addition, the increased likelihood of associated hepatobiliary disease, as well as previous surgery makes management in adults more complex[8]. Although the classical clinical triad of abdominal pain, jaundice and right hypochondriac mass has been reported in children, most patients (71 cases) described here had symptoms that were chronic and intermittent, often resulting in delayed diagnosis[9]. Furthermore, secondary hepatobiliary disease in adults may obscure the primary problem and compound the complexities of subsequent surgery[10-13]. Twenty-three patients with intra- or extra hepatic stones were found in this series, it deserved attention. In 1959, Alonso-Lej[1] and in 1977, Todani[1] classified choledochal cysts based on the location of the cyst. From that time, they were diagnosed more frequently with the aid of improved diagnostic technique, for example, Ultrasound, CT and direct cholangiography such as PTC and ERCP. US is the first imaging modality of choice in the evaluation of patients suspected to have extrahepatic bile duct dilatation[14]. With a new ultrasound technology, the normal common bile duct is easily identifi able, and recognition of localized or generalized dilatation is facilitated. In our case, the specific ultrasound diagnosis of a choledochal cyst was made by identifying two bile ducts entering into a large cystic mass which was separate from the gallbladder and extended deeply into the portahepatis. Biliary ductal obstruction or other cystic lesion, such as pancreatic pseudo-cyst, was excluded by detection of a normal-sized gallbladder and nondistended intrahepatic bile ducts[14]. We demonstrated the entrance of extrahepatic bile ducts into the choledochal cyst in 91 of 108 patients by US. After 1985, we considered that PTC and ERCP were the most useful and direct METHODS in establishing the diagnosis of choledochal cyst, but the former may result in some trauma and complication[15]. At present, we advocate performing ERCP at the time of US and CT examination in order to classify the type of cyst and to recognize the presence of an anomalous pancreaticobiliary duct junction (APBDJ)[16]. In our series, of 46 patiens examined with ERCP, 39 patients (93.4%) had APBDJ. Komi[1] also reported a 92.2% association between choledochal cysts and APBDJ in 645 cases, which is similar to our results. There were many theories about the etiology of the choledochal cysts[1]. Babbit suggested that in these patients there is an abnormal pancreaticobiliary duct junction that allows reflux of pancreatic secretions into the biliary system during a critical stage of its development. Consequent chemical and enzymatic destruction of the duct wall leads to cystic dilation[17,18]. Iwai[19] and other authors[16,18] found that the choledochal dilation has a close association with abnormal choledochopancreaticoductal junction, whether direct or indirect. CT can clearly visualize cyst location, number, scope, stone and relationships with surrounding structures. After 1994, 71 cases were routinely performed with CT examination, all of them were diagnose d definitely. In recent 5 years, CT cholangiography and MR cholangiography have been used in most advanced countries[20]. Our center also want to practice this new METHODS in the future.

Operative procedure

Choledochal cyst is a congenital abnormality that requires surgical intervention to prevent hepatobiliary and pancreatic complication[21-23]. A lot of reports have demonstrated that cystenterostomy, an internal drainage procedure without resection, carries a high morbidity rate and often requires subsequent reparative operation[24]. Todani[25] analyzed carcinoma arising from retained cysts with internal drainage procedures, and suggested that enteric drainage tend to creat a cul-de-sac in the choledochal cyst and to activate pancreatic juice when intestinal juice with enterokinase refluxes into the cyst through an anastomotic stoma. As a result, inflammation of the bile duct wall is accelerated, possibly resulting in carcinoma because of the long-standing irritation of the biliary epithelium. Kobayashi[26] points out that the incidence of bile duct carcinoma is still high, even after excision of extrahepatic bile ducts in APBDJ patients with choledochal dilatation. For these patients, careful long-term follow-up is necessary, especially after operations. In the present series, 11 of 108 patients who had previous cyst enterostomy developed carcinoma. However, all cases who developed carcinoma in our study were over 30 years of age, which supports the previous report that the risk of carcinoma increases with age[25,27-29]. After 1985, cyst internal drainage was abandoned and total excision of the cyst and Roux-en-Y hepatojejunostomy had been recommended as the first choice by us. In 1994, we reported a new and simplified operative procedure to separate the biliary and pancreatic flow so as to reduce the risk of malignancy of choledocha l cyst, which was clinically applied in 9 cases, and proved effective[30].

Total excision of type I and type IV choledochal cyst and Roux-en-Y hepatoj ejunostomy has been recommended by many investigators because of the lower incidence of postoperative complications, especially in Japan, and has been

increasingly popularized worldwide[24,31,32], we agree with their opinions. Treatment for type V cysts is still controversial. At present, hepatic resection is safe and effective for some of type V cysts (Caroli's disease)[33]. In our series, three left lobectomy and four nonanatomical hepatic resections were performed and symptomatic relief was obtained completely and permanently. About type V cysts with frequently recurrent cholangitis, resulting in biliary liver cirrhosis, liver resection is seldom feasible because of associated congenital hepatic fibrosis.

In this setting, liver transplantation may represent the only effective and durable form of treatment and offers the only hope for such patients. In this study, nearly two years ago we performed piggyback orthotopic liver transplantation for two cases of Caroli's disease and both patients recovered smoothly with satisfactory results after operation and are healthily surviving[34]. In conclusion, the surgical strategy should be selected based on the type of cyst. For those who had internal drainage operation before, careful long-term follow-up is required. We recommended total excision of type I and type IV choledochal cyst and Roux-en-Y hepaticojejunostomy. It is an important topic to actively explore new procedure in preventing malignancy of congenital cyst of common bile duct, and it deserves paying attention to. About type V cysts with frequently recurrent cholangitis resulting biliary liver cirrhosis, liver transplantation should be considered.

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Edited by Lu HM

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Treatment of rotaviral gastroenteritis with Qiwei Baizhu powder

Shuang-Teng He[1], Fei-Zhou He[2], Can-Rong Wu[3], Shun-Xiang Li[2], Wei-Xin Liu[4], Yong-Fang Yang[2], Shi-Sheng Jiang[2] and Gang He[1]

[1]Institute of Combined Traditional Chinese and Western Medicine, Xiangya Hospital, Hunan Medical University, Changsha, 410008, China
[2]Hunan Province Academy of Traditional Chinese Medicine, Changsha 410006, China
[3]Department of Microbiology, Hunan College of Traditional Chinese Medicine, Changsha 410007, China
[4]Department of Pediatrics, The Second Hospital of Shuangfeng County, Hunan Province, 411700, China
Supported by the National Natural Science Foundation of China, No. 39 270861.
Correspondence to: Dr. Shuang-Teng He, Institute of Combined Traditional Chinese and Western Medicine, Xiangya Hospital, Hunan Medical University, Changsha 410008, Hunan Province, China. shuangteng@yahoo.com.cn
Telephone: +86-731-8861841
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Abstract

AIM **To observe the effects of Qiwei Baizhu Powder (QWBZP) on rotaviral gastroenteritis in children and in animal models.**

METHODS **Enrolled patients were divided into two groups, and one group was treated with oral rehydration solution (ORS) and the other treat ed with oral liquid of QWBZP. Neonate mice were orally infected with 50 μL rotavirus suspension (4×10^8 PFU/mL) and treated with ORS or oral liquid of QWBZP, respectively.**

RESULTS **Eighty-three cases of rotaviral gastroenteritis treated with QWBZP revealed a better efficacy than that treated with ORS ($\chi^2 = 10.87$, $P<0.05$). The contents of sodium and glucose as well as number of patients with positive human rotavirus antigen in stool in QWBZP group were all less than that in ORS group. In a nimal models, QWBZP was found effective in treating rotavirus gastroenteritis in neonate NIH mice, as compared with control groups. In QWBZP group, the mortality of infected mice was decreased by 73.3%, the body weight of infected mice was increased, the contents of sodium and glucose as well as number of mice with positive rotavirus antigen in feces were significantly reduced, and the pathological changes such as damage of small intestinal mucosa and villi were also obviously alleviated.**

CONCLUSION **QWBZP has effects on improving the absorptive function of small intestine, shortening the duration of diarrhea and rotavirus shedding from stool and alleviating the pathological changes of small intestine induced by rotavirus.**

Subject headings gastroenteritis/drug therapy; rotavirus infections/drug therapy; Qiwei baizhu powder/therapeutic use; disease models, animal

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INTRODUCTION

Rotavirus is the most common cause of acute gastroenteritis in infants and young children all over the world[1-4]. Although rotaviral diarrhea is a self -limited disease, it caused 870 000 deaths per year in developing countries[5], even a small part of patients were involved in encephalopathy[6,7]. The primary treatment of this illness is oral or intravenous rehydration to modulate dehydration and electrolyte disturbances due to vomiting and wat ery diarrhea[8,9]. Based on the oral rehydration solution (ORS) recommen ded by World Health Organization (WHO)[10,11], reduced osmolarity ORS[8], addition of Lactobacillus GG to ORS[12,13], and rice-based solution[14] have been used to treat this disease. Also, oral immunoglobulins was used as antiviral therapy[15,16], and rotavirus candidate vaccines are developing[17], in which a oral tetravalant, rhesus rotavirus-based, human reassortant vaccine has been licensed in USA[18].

To seek more effective and cheaper drugs to treat rotaviral diarrhea from Chinese medicinal herbs, we found that a Chinese traditional medical prescription named Qiwei Baizhu Powder (QWBZP) had been employed to treat acute diarrhea in infants and children for nearly one thousand years in Traditional Chinese Medicine (TCM). QWBZP was written by a famous ancient Chinese pediatrician who named ZhongYang Qian and lived from 1035 to 1117. After Mr. Qian, many other ancient Chinese pediatricians demonstrated that QWBZP had good effects in shorting diarrhea and improving the symptom of thirst in their pediatric works. According to their description and our clinical experiences, we prepared oral liquid from QWBZP[19], and tested the effects of the oral liquid in treating rotaviral infection in clinical and experimental studies. This traditional Chinese prescription was shown to be effective in inhibiting replication of human rotavirus at monolayer of MA104 cells[20]. In the present study, we investigate the effects of QWBZP in treating rotaviral gastroenteritis in both clinic and animal models.

PATIENTS AND METHODS

Study population

All patients aged from 3 to 24 months (mean age 13.5±3.2 months) were observed at pediatric wards or out-patients service in both the hospital affiliated to Hunan Province Academy of Traditional Chinese Medicine and the Second Hospital of Shuangfeng County at Hunan Province in China from October 1993 to March 1996. Patients enrolled into the study were diagnosed based on clinical symptoms and laboratory tests in accordance with the diagnostic standard worked out by the Chinese Ministry of Health[21]. That was: the age of patients was less than 24 months, history of acute watery diarrhea less than 72 hours with or without upper respiratory symptoms, negative pathogenic bacteria in stool culture, positive of rotaviral antigen in stool samples. Any patient with negative rotaviral antigen or positive pathogenic

germs was excluded.

Preparation of oral liquid from QWBZP and oral rehydration solution

The oral liquid prepared from QWBZP was described as previously[19]. The prescription of Qiwei Baizhu Powder was composed of seven kinds plants or herbs as listed below: Panax ginseng C. A. May, 7.5 gm; Poriacocos (Schw.) Wolf, 15 gm; Atractylodes macrocephala Koidz, 15 gm; Glycyrrhiza uralensis Fisch, 3 gm; Pogostemon cablin (Blanco) Benth, 15 gm; Pueraria lobata (Willd.) Ohwi, 30 gm; and Viadimiria souliei (Franch.) Ling, 6 gm.Briefly, the seven medicinal materials were washed and dried. Among them, Glycyrrhiza was fried with a little honey. These medicinal materials were carefully weighed and grounded into powder. The seven kinds of powder were mixed together and decocted with eight-fold of water (w/w) until half the water was evaporated off. The volatile oil evaporated from these herbs was collected at the same time. The decoction was extracted and filtered through 50 μM hole of sieves, then clarified at 4℃ overnight. The supernatant was collected and mixed with the volatile oil, bottled in 200 mL per glass flask, sealed and sterilized. The bottled decoction were stored at 4℃ and used as oral liquid of QWBZP. Detected by high performance liquid chromatography (HPLC) or automatic analyzer (7170A type, Hitachi, Japan), the oral liquid contained 12.6mg/L gensenoside Rg^1, 95.3 mmol/L K^+, 50.6 mmol/L Cl^-, 37.4 mmol/L Na^+, 2.01 mmol/L Ca^{2+}, with pH 6.1. The oral rehydration solution was prepared according to the formulae recommended by WHO, and contained in mMol per liter, Na^+90, K^+20, HCO^3-30, Cl^-80 , glucose 110 with pH 7.5[2,10,11].

Treatment protocol

The enrolled patients were divided into two groups at random, one was ORS group, and the other was QWBZP group. There were 83 patients in QWBZP group, aged 3-24 months (mean age 13.6±3.5 months), with histories of watery diarrhea for 21 to 72 hours (mean history of diarrhea 53.5±12.4 hours), 50 boys and 33 girls, 65 moderate cases, and severe 18. And 72 cases in ORS group, aged 3-24 months (mean age 14.1±4.1 months), with histories of diarrhea for 19-72 hours (mean history 56.2±13.7 hours), 41 boys, 31 girls, 56 moderate cases, and 16 severe cases. Patients in QWBZP group were fed with the oral liquid of QWBZP, and ORS group were fed with oral rehydration solution for three days. Children aged less than 12 months were fed 5 mL of ORS or QWBZP each time, children aged 12-24 months were fed 10mL ORS or QWBZP each time. The total dose per day was 10-15 $mL \cdot kg^{-1} \cdot d^{-1}$. During the treatment, the children continued to have their mother's breast-milk or milk or their usual food, other treatment including antibiotics, and antimobility drugs were not used[2,21]. Their stool frequency was recorded. The children were observed until the cessation of diarrhea. If their symptoms such as diarrhea, vomiting, and dehydration became more severe after 24 hours of treatment in both groups, these patients would receive intravenous rehydration and other treatments, who were considered as inefficient cases statistically.

Laboratory tests

Before and during the observation, blood and stool samples of patients were collected for detection of glucose, electrolytes, hematocrit, antigen of rotavirus, and pathogenic bacteria including Salmonella, Shigella, Vibrio cholerae, and enteropathogenic Escherichia coli. Stool was placed in Eagle's medium complemented with 5% bovine serum, 5 U/mL penicillin and 5 μg/mL streptomycin, and frozen until examined for rotavirus antigen by enzyme-linked immunosorbbent assay (ELISA). The monoclonal antibody against human rotavirus was supplied by Dr. Zhu SH, Laboratory of Immunology at Medical College of Zhejiang University, Hangzhou)[22-24] or under direct electron microscopy[25]. Concentration of glucose in stool, and contents of electrolytes in serum and stool were measured by automatic analyzer. hematocrit was tested by microtube method.

Evaluation of curative effect

The curative effect was classified into three categories: ① cured: all of symptoms disappeared, normal appetite and stool, negative rotaviral antigen in stool, normal value of laboratory tests; ② efficient: stool output and frequency were obviously decreased, appetite improved, index of laboratory tests improved; and ③ inefficient: stool output and frequency as that before treatment, dehydration was not improved or even worse, rotavirus antigen was positive or negative.

METHODS IN ANIMAL MODELS

Cells and virus

MA_{104} cells, a cell line derived from fetal rhesus monkey kidney, were obtained from Prof. Hong T, Institute of Virology, Chinese Academy of Preventive Medicine. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM , Gibco Inc., USA) complemented with 10% fetal bovine serum, 5U/mL penicillin, 5ìg/mL streptomycin, and 10mmol/L HEPES. Human rotavirus (HRV) Wa strain was kindly granted by Dr. Qian Y, Laboratory of Virology at Capital Pediatric Institute of China through the authority of Dr. Kapikian AZ, National Institute of Allergy and Infectious Diseases, NIH of USA. The virus was replicated at the monolayer of MA_{104} cells[26-29]. When the viral titer was raised to 10^8 PFU/mL after several passages, the viral suspension was stored at -80℃ and employed to inoculate animals.

Animal infection

Pregnant dam mice (NIH strain, supplied by Experimental Animal Department at Hunan Institute of Medicinal Industry) were fed in sterile cages. The new born pups were maintained in germfree isolators at 20℃ room temperature and fed with sterilized milk in germfree conditions. The neonate mice were divided into 4 group at 2 days of age. The four groups were normal group, infected group, ORS group, and QWBZP group. Pups in the later three groups were orally inoculated with 50 μL of cold viral suspension of 4×10^8 PFU/mL human rotavirus according to the METHODS as described for mice[30-34]. Neonate mice in normal group were not inoculated. Pups in each group were maintained in isolated room after division.

Treatment

After 24 hours of inoculation, the pups in ORS group and in QWBZP group were fed with 0.1 mL of ORS or 0.1 mL oral liquid of QWBZP each time, respectively, 4 times per day for

three days. The total dose was 0.5 mL, or about 150 mL·kg^{-1}·d^{-1}. Pups in normal group and infected group were fed with sterilized distilled water at the same dose as other two groups. Aside the oraltherapy, all pups in the four groups were fed with 0.1 mL sterilized milk, three times per day.

Observation and laboratory tests

After inoculation, the intake of food, body weight, activity, and number of death of the pups in all four groups were carefully recorded daily until 10 days after inoculation. For the observation of the intake of food, when fed with 0.1 mL milk, there was a white round lump in the stomach of pup to be seen through the transparent skin. If the digestive function and absorptive function of neonate mouse were normal, the white lump in the stomach of pup gradually became smaller and smaller after feeding. If the digestive and absorptive functions were decreased, the white lump in stomach did not become smaller, in contrast, the a bdomen of the pup became distended. In this condition, the next feed of milk was not needed. For assessment of diarrhea, when the abdomen of pup was gently pressed[35,36], the watery stool or loose yellow stool was shedding fro m the anus of the pup. The blood samples and stool specimen were collected before the treatment and at day 4 and 7 after infection for the analysis of serous electrolytes, glucose, and hematocrit, and rotaviral antigen, electrolyte, and glucose in stool. The METHODS of analysis was described in clinical study.

METHODS of statistics

All quantitative data in the study were presented as $\bar{x}\pm s$, and compared by Student's *t* test between two group or by analysis of variance (ANOV) among three groups or more than three groups. The dichotomous data were compared with Chi-square test.

RESULTS

Effects of antidiarrhea

After treatment for 3 days, 71 cases in QWBZP group and 45 cases in ORS group had formed stool, and their symptoms of vomiting, diarrhea, dehydration, and fever disappeared. As seen in Table 1, the cured cases in QWBZP group we re more than that in ORS group ($\chi^2 = 10.87$, $P<0.01$) , while the inefficient cases in QWBZP group were less than that in ORS group ($\chi^2 = 5.85$, $P<0.05$). From these cured cases, the duration from beginning of treatment to stop of diarrhea was 25.9±13.5 hours in QWBZP group, and 41.8±19.3 hours in ORS group. The duration of cessation of diarrhea in QWBZP group was shortened as compared with ORS group (t = 5.86, $P<0.01$). The results showed that the curative effect of QWBZP in treating rotaviral diarrhea was better than that of ORS.

Table 1 The curative effect of QWBZP and ORS

Group	Total cases	Cured cases	Efficient cases	Inefficient cases
ORS	72	45	15	12
QWBZP	83	71	8	4

Changes of electrolytes in serum and sodium and glucose in stool

Hematocrit and serum electrolytes of patients were all improved in the two groups after treatment, but as seen in Table 2, more improvedment were shown in QWBZP group. Contents of sodium and in stool samples of patients were not changed in ORS group, but remarkably reduced in QWBZP group as seen in Table 3. The results showed that QWBZP had the effects in reducing the contents of sodium and glucose in stool and promoting the absorptive function of small intestine to intake sodium and glucose from intestinal cavity.

Table 2 Changes of electrolytes in serum and hematocrit after treatment ($\bar{x}\pm s$)

Treatment	Cases	Time point	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)	Hematocrit (%)
ORS	72	Before treatment	146.4±9.4	3.9±0.2	98.5±5.0	50.6±4.6
		After treatment	140.6±7.3[a]	4.1±0.3	102.4±6.7	45.7±3.8[a]
QWBZP	83	Before treatment	145.7±8.6	3.8±0.2	101.0±5.4	51.2±4.4
		After treatment	141.2±8.1[a]	3.9±0.3	103.5±4.8	46.1±5.8[a]

[a]$P<0.05$ *vs* the same group before treatment.

Table 3 Changes of sodium and glucose in stool after treatment (mmol/L, $\bar{x}\pm s$)

Treatment	Cases	Time point	Sodium	Potassium	Chloride	glucose
ORS	72	Before treatment	25.3±4.1	18.5±3.4	11.7±2.6	0.9±0.2
		After treatment	26.8±4.7	11.7±4.1	13.0±3.1	1.0±0.4
QWBZP	83	Before treatment	26.2±4.3	17.3±3.5	12.4±2.5	0.9±0.3
		After treatment	11.5±3.6[a,b]	10.4±2.8[a,b]	6.8±2.7[a,b]	0.2±0.1[a,b]

[a]$P<0.05$ *vs* the same group before treatment; [b]$P<0.05$ *vs* ORS group.

The clearance of rotaviral antigen in stool

There were positive rotaviral antigen or rotaviral particals in stool samples of all patients in two groups. After 3 days of treatment, 13 cases became positive for rotavirus in QWBZP group, 45 cases in ORS group. Significant difference was seen between the two groups ($\chi^2 = 30.47$, $P<0.01$). The result indicted that treatment with QWBZP can shorten the duration of rotavirus excretion from stool.

The taste of the oral liquid QWBZP

Compared with ORS, the oral liquid of QWBZP tastes slightly sweat and free from bitterness. So the oral liquid was easy to be drunk for infants and young children. In contrast, ORS was so bitter, which often induced vomiting maleings the patients refus to drink. Furthermore, the appetite, the intake of food, the activities, and nutrition of patients in QWBAZ group were considerably improved. No side-effect was presented during

the treatment with QWBZP.

The endpoint of diarrhea and mortality of mice

The neonate mice were ill with decreased digestive function (the white lump in stomach was not decreased after 4-8 hours of feeding with milk), abdominal distension, and watery diarrhea at 48 hours of rotaviral inoculation. Dehydration occurred after 3 days of rotavirus infection with the presence of dry, loose, and wrinkly skin, in activities. Symptoms of prostration, cyanosis, and weight loss appeared at day 4 after inoculation. All of the pups in infected group (15/15), 10 mice in ORS group (10/15), 4 mice in QWBZP group (4/15) died during the observation. But still 1 mouse died in the normal group. The surv ivals in QWBZP group were more than that in the infected group ($\chi^2 = 17.37$, $P<0.01$) and in ORS group ($\chi^2 = 4.82$, $P<0.05$). The diarrhea in survived mice stopped at day 6-12 after inoculation. The diarrhea in QWBZS group stopped at day 5-10 (7.4±1.3 days), and in ORS group at day 8-12 (9.9±1.8 days) after infection. There was significant difference between the two groups (t = 2.79, $P<0.05$).

Changes of body weight

The body weight of mice in all four groups are shown in Table 4. The body weight of mice in infected group was not increased, but that in QWBZP group was increased after three days of treatment. The result indicated that treatment with QWBZP could rectify dehydration and improve nutrition of mice infected with rotavirus.

Table 4 Changes of body weight of mice after inoculation (g, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	2.2±0.2	2.8±0.4	5.1±0.6
Infected	5	2.1±0.3	2.2±0.2	2.3±0.4
ORS	5	2.2±0.2	2.4±0.3	3.6±0.5[a]
QWBZP	6	2.1±0.3	2.7±0.4[a]	4.8±0.5[b]

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

Changes of laboratory value

As shown in Tables 5-9, contents of sodium and glucose in stool samples from the mice in QWBZP group were both decreased in comparison of infected group and ORS group at day 4 and 7 after inoculation. Hematocrit of mice in QWBZP and ORS groups were also improved. Content of sodium and glucose in blood were not changed in all four groups after treatment. These results suggested that QWBZP had effects in promoting the absorption of sodium and glucose after rotavirus infection.

Table 5 Changes of content of fecal sodium (mmol/L, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	9.5±0.6	10.2±0.8	9.3±1.2
Infected	5	10.1±1.1	22.9±2.2[a,b]	24.7±1.9[a,b]
ORS	5	9.7±0.9	26.5±1.3[a,b]	15.7±1.9[c,d]
QWBZP	6	10.4±1.1	11.6±1.4	11.2±1.1

[a]$P<0.001$, [b]$P<0.01$ *vs* QWBZP group. [c]$P<0.001$, [d]$P<0.01$ *vs* the same group before treatment.

Table 6 Changes of content of glucos in stool (mmol/L, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	0.1±0.2	0	0.1±0.1
Infected	5	0	1.1±0.3[a]	1.3±0.4[a]
ORS	5	0	1.2±0.4[a]	1.2±0.3[a]
QWBZP	6	0.1±0.1	0.2±0.2	0.1±0.1

[a]$P<0.01$ *vs* QWBZP group.

Table 7 Changes of content of glucos in serum (mmol/L, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	4.4±1.2	3.9±1.4	4.1±1.2
Infected	5	4.2±0.9	3.8±1.3	4.3±0.9
ORS	5	4.4±0.8	4.6±1.4	4.5±1.7
QWBZP	6	4.1±1.6	4.5±1.5	4.8±1.6

Table 8 Changes of content of serous sodium (mmol/L, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	140.6±7.8	142.1±8.6	141.7±8.4
Infected	5	141.8±9.6	143.5±9.9	142.3±8.7
ORS	5	142.5±8.8	145.8±7.6	144.7±9.2
QWBZP	6	140.6±7.5	141.6±8.6	142.4±8.5

Table 9 Changes of hematocrit after treatment (%, $\bar{x}$±s)

Group	*n*	Days post-inoculation		
		1 d	4 d	7 d
Normal	4	42.1±5.6	41.3±4.2	40.5±4.4
Infected	5	43.5±4.8	52.2±5.4[a]	54.2±5.1[a]
ORS	5	42.7±5.2	46.7±3.4	46.5±3.7
QWBZP	6	43.9±5.5	45.7±4.7	43.4±4.1

[a]$P<0.05$ *vs* normal group.

Reduction of rotavirus shedding from stool

There were 2 mice with positive rotaviral antigen in stool in QWBZP group (2/1 5), 9 mice with positive rotaviral antigen in infected group (9/15), and 10 mice with positive rotaviral antigen in ORS group (10/15) at day 4 after inoculation. No mouse with positive rotaviral antigen was seen in the normal group. The mice with rotaviral antigen positive in QWBZP group were less than that in infected group ($\chi^2 = 8.1$, $P<0.01$) and in ORS group ($\chi^2 = 13.3$, $P<0.01$).

Histological changes of intestine

Main morphological changes seen at day 4 after rotavirus infection were villous shorting and exfoliation, irregular construction of small intestinal mucosa with infiltration of lymphocytes, thinned intestinal wall. At day 7 after inoculation, the villi of small intestine became rarer and the intestinal wall became thinner. The pathological changes in ORS group were the same as that in infected group at day 4 after inoculation, and at day 7, the pathological changes were obviously moderated in comparison with that in infected group, but more severe than that in QWBZP group. Compared with control groups, the villi and mucosa construction of small intestine were alleviated at day 4 after in oculation, and were nearly normal at day 7 after rotaviral infection. These results suggested that QWBZP had the effect in protecting intestinal

mucosa against the damage induced by rotavirus infection.

DISCUSSION

The results of this study clearly indicated that treatment with the oral liquid of QWBZP significantly shortened the duration of diarrhea and rotavirus shedding from stool, reduced the contents of sodium and glucose in stool samples as compared with ORS treatment in clinic. In animal models, treatment with QWBZP remark ably reduced rotavirus antigen in feces, decreased the mortality of infected mice, reduced the contents of sodium and glucose in stool, and alleviated the damage of small intestinal mucosa and villi. These results suggested that QWBZP had the effects in improving in absorptive function, protecting intestinal mucosa against in jury induced by rotavirus infection, and enhancing the clearance of rotavirus from stool. Another noteworthy finding in this study during clinical observation w as that the less bitter taste of the liquid of QWBZP was easily accepted by children as compared with ORS.

According to the epidemiology, only 0% to 20 % of summer diarrhea cases were rot avirus associated, while 70% to 80% of winter diarrhea cases were due to rotavirus[11,37], and 55.9% of all hospitalized diarrheal children were associated with rotavirus and significantly clustered during winter months in China[38]. Resides vomiting, watery diarrhea, and dehydration, most patients with rotaviral diarrhea had upper respiratory symptoms such as fever, cough, and sneeze[39]. These clinical manifestations of rotavirus diarrhea were similar to one type of acute diarrhea named “cold diarrhea” in TCM. The “cold diarrhea” is thought to be caused by cold weather, cold wind, or cold food, which mostly occurred in winter or cold climates. The “cold diarrhea” was first described by Mr. Qian ZY. Qian said in his pediatric work: “If their diarrhea is caused by cold and wind , the children will have vomiting, fever, cough, sleepiness, thirst, and diarrhea with yellow watery stool. The patients need to drink decoction of Qiwei Baizhu Powder for quenching their thirst”. Following Mr. Qian, many other ancient Chinese pediatricians treated acute diarrhea in children with QWBZP. They recorded the effects of QWBZP in anti-diarrhea, quenching thirst, and improvement of appetite in detail. In our clinical practice, we observed that treatment wit h QWBZP not only shortened the duration of diarrhea and rotavirus shedding, but also improved the condition of nutrition and appetite of the ill patients. Therefore treating rotaviral diarrhea with QWBZP yielded better results than with ORS. The theory of TCM considered that the digestive and absorptive function in young children was immature, so children were susceptible to diarrhea. Treatment for acute diarrhea in childhood with QWBZP can improve the digestive and absorptive function and quench thirst induced by vomiting and diarrhea through the effect of “Bupi” of this prescription.

Following infection of rotavirus, the duodenal biopsies showed a patchy irrgulalarity of mucosal surface, shorting and bunting of the villi, distorting microvilli of absorptive cell[40]. As a consequence of virus replication in the cytoplasm and viral shedding, the infected cell at the surface of villilysed and the villious tip became atrophic[2,11,41], and the villious surface was replaced by immature cells migrating from the crypts[42]. The destruction of mature enterocytes and replacement of immature crypt cells result in reduced levels of disaccharidase and xylose absorption[43], and decrease in the absorptive surface of the small bowel. So diarrhea caused by rotavirus is mainly due to decreased absorption[11,44]. Clinical studies demonstrated that the stools of rotavirus diarrhea contained higher contents of sodium and glucose than normal[45]. In our observation in both clinic and animal models, we found that the contents of sodium and glucose in feces in ORS group or infected group were higher than that in QWBZP group. The result indicated that treatment with QWBZP could promote the absorption of sodium and glucose. The mechanism of ORS on rehydration was based on glucose-facilitated sodium transport[46], while the mechanism of QWBZP on treating rotavirus diarrhea would be more complex and involved in improving the digestive and absorptive function, protecting intestinal mucosa cells from damage induced by rotavirus, and clearance of rotavirus.

More recently, some studies identified that the non-structural protein 4 (NSP4) of rotavirus is a viral enterotoxin, which induced diarrhea by stimulating chloride secretion through a calcium-depedent signaling pathway after binding to the pulative receptor on the intestinal epithelium[34,44,47-50]. So the rotavirus diarrhea is caused by reduction of absorption as well as secretory hyperactivity[2,44,48]. The therapeutical method to improve the absorptive function to enhance the intake of sodium and glucose, therefore, plays an important role in cessation of diarrhea. That may be the reason why the efficacy of QWBZP is better than ORS. Indeed, a lot of studies in TCM in recent years demonstrated that some of medicinal herbs contained in QWBZP have effects of enhancing absorption of D-xylose.

As rotavirus diarrhea in children, rotavirus infection in animal models is also a self-limited disease[36,51]. But in our study, most of infected mice died of severe diarrhea and dehydration without treatment of QWBZP. This result might be due to different METHODS of feeding. In our study, we fed neonate mice with sterilized milk orally, Guerin-Danan, *et al* let the infected pups return to their dams and allowed to suckle[36]. Because breast milk contains IgA antibodies against rotavirus and trypsin inhibitors, which will provide protection to breast-fed infants[52]. Furthermore, it is well known that nutrition in breast milk was more easily absorbed than that in other milk. Treatment with QWBZP can promote the intake of nutrition through improvement of digestive and absorptive function. Therefore, the body weight was increased and most infected mice survived in the QWBZP group as compared with the infected group as well as ORS group.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Management of patients with a short bowel

Jeremy M D Nightingale

Consultant Gastroenterologist, Gastroenterology Centre, Leicester Royal Infirmary, United Kingdom
Correspondence to: Jeremy M D Nightingale MD, MRCP, Consultant Gastroenterologist, Gastroenterology Centre, Leicester Royal Infirmary, LEICESTER LE1 5WW, United Kingdom. jnightingale@uhl.trent.nhs.uk
Telephone :+44-116 258 6324, Fax :+44-116 258 6985
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Abstract

There are two common types of adult patient with a short bowel, those with jejunum in continuity with a functioning colon and those with a jejunostomy. Both groups have potential problems of undernutrition, but this is a greater problem in those without a colon, as they do not derive energy from anaerobic bacterial fermentation of carbohydrate to short chain fatty acids in the colon. Patients with a jejunostomy have major problems of dehydration, sodium and magnesium depletion all due to a large volume of stomal output. Both types of patient have lost at least 60 cm of terminal ileum and so will become deficient of vitamin B_{12}. Both groups have a high prevalence of gallstones (45%) resulting from periods of biliary stasis. Patients with a retained colon have a 25% chance of developing calcium oxalate renal stones and they may have problems with D (-) lactic acidosis. The survival of patients with a short bowel, even if they need long-term parenteral nutrition, is good.

Subject headings Short bowel syndrome; Patients/ statistics & numerical data

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INTRODUCTION

A patient has a short bowel when there is an insufficient length of functioning gut to allow adequate absorption, so macronutrient and/or water and electrolyte supplements are needed to maintain health and/or growth. If untreated or without compensatory mechanisms undernutrition and/or dehydration result[1]. This is likely to be the case if less than 200 cm small bowel remains. The problems experienced by patients with a short bowel depend upon the type and length of remaining small bowel and the presence or absence of a functioning colon. Patients traditionally described as having the short bowel syndrome were those with a retained colon who had intractable diarrhoea with malabsorption of fat, vitamin, and other nutrients so leading to undernutrition, with continuing weight loss and anaemia[2]. The normal adult human small intestinal length varies from about 275-850 cm as measured from the duodeno-jejunal flexure at autopsy or surgery and tends to be shorter in women[3-6]. Congenital cases of a short bowel have been reported and are usually associated with malrotation of the gut[7]. Patients who start with a small intestinal length at or below the lower end of the normal range may develop the problems associated with having a short bowel after relatively little small intestine has been removed[8]. It is important to refer to the remaining length of small intestine rather than to the amount resected.

REMAINING BOWEL

Ileum or jejunum

Ileal mucosa, in contrast to the jejunal mucosa, has "tight" intercellular junctions and thus can concentrate its contents. However, jejunal mucosa has "leaky" intercellular junctions and so the osmolality of the luminal contents is similar to that of plasma. Gastrointestinal transit is naturally slower in the ileum than jejunum so allowing more time for absorption[9,10]. The terminal ileum has the specific functions of absorbing vitamin B_{12}[11,12] and bile salts. Any ileum remaining, after a small bowel resection, can structurally and functionally adapt so increasing absorption[13-16], while remaining jejunum can only functionally adapt if some ileum or colon also remains[16-19]. Thus the outcome is more favourable after a jejunal than an ileal resection.

Ileocaecal valve

Preservation of the ileocaecal valve has traditionally been thought to slow transit and prevent the reflux of colonic contents into the small bowel. However, resection of the ileocaecal valve in adults does not affect these functions[9,20].

Colon

Conservation of the colon is advantageous as it absorbs water, sodium[21-25], magnesium[25,26], calcium[27], short and medium chain fatty acids[28-30], slows gastro-intestinal transit[31] and stimulates small intestinal hyperplasia[32]. Patients with a preserved colon after a small bowel resection may survive without parenteral support with a very short[33,34] or even no remaining jejunum[35].

TYPES AND CAUSES OF A SHORT BOWEL

There are three types of patient with a short bowel. Patients in whom the ileum, often with the ileocaecal valve has been removed leaving a jejuno-colic anastomosis (jejunum-colon); patients in whom some jejunum, the ileum and colon have been removed, so they have an end jejunostomy and patients who have had a predominantly jejunal resection, and have more than 10 cm of terminal ileum and the colon remaining (jejuno-ileal)[25,36,37]. Jejuno-ileal anastomosis patients are not common and as their management is broadly similar to those with a jejuno-colic anastomosis, they are not specifically discussed. Patients with a jejunostomy can be classified as net "absorbers" or net "secretors". The net "absorbers" absorb more water and sodium from their diet than they take orally (usual stomal output about 2 kg/24), thus they can be managed with oral sodium and water supplements and parenteral fluids are not needed. They usually have more than 100 cm residual jejunum. The net "secretors" usually have less than 100 cm residual jejunum and lose more water and sodium from their stoma than they take by mouth (usual stomal output may be 4-8 kg/24). The jejunostomy output from a net "secretor" increases markedly in the daytime in response to food and is minimal at night[38] (Figure 1). The three most common reasons for patients having less than 200 cm of small bowel are superior mesenteric artery thrombosis, Crohn's disease and irradiation damage[25,36,37,39] (Table 1). A short bowel more commonly arises in women (67%) thao men[25], this may be because women start with a shorter length of small intestine than men.

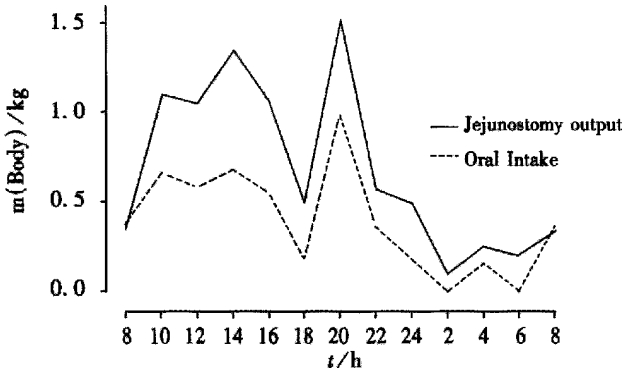

Figure 1 Oral intake (kg) and stomal output measured every 2 hours in a patient who has 30 cm of residual jejunum. There is a marked net "secretory" output in response to food and drink (38).

Table 1 Reasons for a short bowel (less than 200 cm small bowel) (25)

	Jejunum-colon	Jejunostomy
Total (sex)	38 (26F)*	46 (31F)
Age	46 (7-70)	42 (16-68)
Median jejunal length (cm)	90 (0-190)	115 (20-190)
Diagnosis		
Crohn's disease	16	33
Ischaemia	6	2
Irradiation	5	3
Ulcerative colitis	-	5
Volvulus	5	-
Adhesions	4	1
Diverticular disease	1	1
Desmoid tumour	1	1

*:7 had an ileocaecal valve and 31 a jejuno-colic anastomosis.

PHYSIOLOGICAL CHANGES

The problems that arise after a major intestinal resection reflect both normal and altered physiology. Most experimental work in animals involves a predominantly jejunal resection (jejuno-ileal anastomosis), which is not the common situation in man.

Gastro-intestinal motility

Gastric emptying and small bowel transit of liquid is fast in patients with a jejunostomy and normal in patients with a retained colon, probably due to circulating plasma levels of peptide YY being low and high respectively[40] (Figure 2). Peptide YY is an enteric hormone produced by the L cells in the terminal ileum and colon upon exposure to luminal nutrients, it slows gastric emptying and gastrointestinal transit[41].

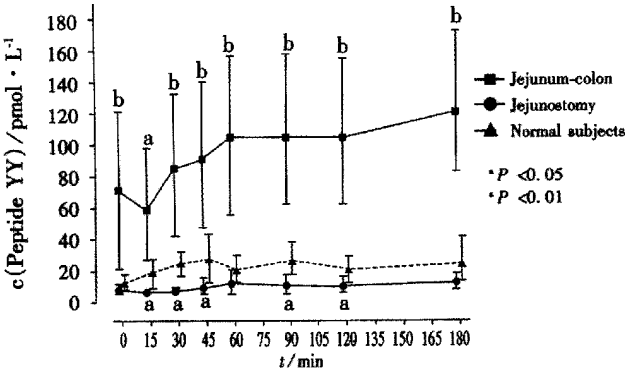

Figure 2 Median peptide YY levels with interquartile range for 6 jejunum-colon patients, 7 jejunostomy patients and 12 normal subjects after a pancake and orange juice meal (40). GLP-2 levels mirror this pattern (65,69).

Gastrointestinal secretions

Salivary secretion is reduced in patients with a jejunostomy[42]. Gastric acid secretion is increased in dogs with denervated gastric pouches. In man some cases of long-term survival in patients with a short bowel has been attributed to their previous gastric surgery[43-45]. High circulating gastrin levels are observed[40,46,47] and may be due to less small bowel being available to catabolizegastrin[48,49]. Gastric acid hypersecretion could increase the incidence of peptic ulceration[50]; impair nutrient absorption by causing bile salt precipitation[51]; reduced pancreatic enzyme function and increased jejunal motility. However although there may be an increase in gastric acid secretion in the first 2 wk after a small bowel resection[52], there is no good evidence for gastric acid hypersecretion in man in the long-term, especially in those with a jejunostomy[53]. Pancreatic function is reduced by undernutrition[54] and if there is no oral intake[55], but not after a small bowel resection leaving the colon[56].

Absorptive functions

The ileum absorbs vitamin B_{12} and bile salts. If more than 60 cm of terminal ileum is resected vitamin B_{12} deficiency is likely to occur[11,12,57]. Fat is absorbed over a longer length of the intestine than carbohydrate or protein and this distance increases as oral intake rises[58]. When more than 100 cm terminal ileum has been resected the increased hepatic synthesis of bile salts is not enough to keep up with the stool or stomal losses and a degree of fat malabsorption results[59].

Adaptation

Intestinal adaptation is the process that attempts to restore the total gut absorption of macronutrients, macrominerals and water, to that of before the intestinal resection[60]. This may occur by the patient eating more food than normal (hyperphagia)[61]. The remaining bowel may increase its absorptive area (structural adaptation) and/or gastrointestinal transit may slow (functional adaptation). After a jejunal resection in animals the ileal remnant undergoes structural changes, which include elongation of villi, deepening of the crypts and an increase in the number of enterocytes along a given length of villus. This structural adaptation may relate to circulating plasma levels of glucagon-like peptide-2 (GLP-2). GLP-2 is an enterocyte specific peptide growth hormone produced by the L cells in the terminal ileum and colon and it causes small and large bowel villus and crypt growth[62].

Jejunum-colon In patients with a jejuno-colic anastomosis, no structural adaptation has been demonstrated[17,63,64] even though high GLP-2 levels are observed[65]. Functional adaptation with slowing of gastric emptying and small bowel transit may occur[31] and may be due to high circulating peptide YY levels[40]. In these patients there is a small reduction in faecal weight in the 3 months following the small bowel resection[16]. There is increased jejunal absorption of macronutrients (e.g. glucose), water, sodium and calcium with time[15,18,19,66], and an increased chance of the patient being able to stop parenteral nutrition[25,36,37,67]. The intestinal calcium absorption may continue to increase for more than two years after a resection[66].

Jejunostomy Although adaptation occurs in the months after the creation of an ileostomy, there is no evidence for any structural[53] or functional[25,68,] adaptation at any time in patients with a jejunostomy. This may be because patients with a jejunostomy have low circulating plasma levels of GLP-2 and peptide YY[40,69,70].

PRESENTATION AND CLINICAL ASSESSMENT

Jejunum-colon patients often appear well after their resection except for diarrhoea/steatorrhoea, but in the following months may lose weight and become severely undernourished. Patients with a jejunostomy have problems of dehydration immediately after surgery due to large stomal water and sodium losses. This jejunal output is greatest after food and drink have been consumed.

The clinical assessment of a patient with a short bowel includes a measurement of residual small bowel length and an assessment of water, sodium, magnesium and nutritional status. The state of hydration and magnesium balance is of immediate importance to those with a jejunostomy.

Assessment of residual small intestine

From knowledge of residual bowel length predictions about the long-term need for fluid/nutrition can be made (Table 2).

Table 2 Guide to bowel length and long-term fluid/nutritional support needed by patients with a short bowel (5,37,38,74)

Jejunal length (cm)	Jejunum-colon	Jejunostomy
0-50	PN	PN+PS
51-100	ON	PN+PS*
101-150	None	ON+OGS
151-200	None	OGS

*:at 85-100 cm may need PS only
PN:Parenteral nutrition
PS:Parenteral saline (+/-magnesium)
ON:Oral (or enteral) nutritinon
OGS:Oral (or enteral) glucose/saline solution

Anatomical length The remaining small bowel length can be measured at surgery (10-30 cm segments are measured along the anti-mesenteric border with great care being taken not to over-stretch the bowel). If not measured at surgery, an opisometer, a device used for measuring distances on maps can be used to trace the long axis of the small bowel on a small bowel meal radiograph. This technique is relatively accurate if the total small intestinal length is less than 200 cm and if the entire small bowel is shown on one film[36,71].

Functional length Citrulline is a non-essential amino acid synthesised from glutamine and all the citrulline in the systemic circulation is derived from the enterocytes. Post absorptive plasma levels of citrulline relate to the length of remaining functional small bowel[72].

Hydration (water and sodium status)

Water and sodium deficiency (most common in jejunostomy patients) may result in hypotension and pre-renal failure. Daily body weight and accurate fluid balance (including stomal output) are the most important measurements. The serum creatinine, potassium and magnesium, and urinary sodium may be measured every 1-2 d initially, then once or twice a week and if long-term at home every 2-3 mo. Water and sodium deficiencies are detected by an abrupt fall in body weight, postural systolic hypotension (1.33 kPa), low urine volume and, if severe, by a rising serum creatinine and urea. The most helpful early measure of sodium depletion is the sodium concentration in a random urine sample. A concentration of 0-5 mmol·L^{-1} suggests sodium depletion. The aims are to maintain hydration/body mass and a daily urine volume of at least 801 ml with a sodium concentration greater than 20 mmol·L^{-1}.

Magnesium

Magnesium depletion is common especially in patients with a high stomal output. A serum value of less than 0.6 mmol·L^{-1} may give rise to symptoms.

Nutritional status

A patient may be assessed as undernourished if the body mass index is less than 18.5 kg·m^{-2}, if there is a recent weight loss of more than 10% or if the mid-arm muscle circumference is less than 19 cm in a woman or 22 cm in a man[73]. The degree of malabsorption and thus oral enteral nutrition requirements can be predicted from knowledge of the residual length of small bowel (Table 2). In terms of energy absorption parenteral nutrition is needed if a patient absorbs less than one-third of the oral energy intake[38,74]; though in people with high energy needs parenteral nutrition may be needed at a higher percentage energy absorption.

CLINICAL PROBLEMS AND TREATMENT

Salt and water depletion

Jejunum-colon The colon has a large capacity to absorb sodium and water; thus patients with a short bowel and a preserved colon are rarely in negative water and sodium balance and rarely need water or sodium supplements[23-25]. Although the colon secretes potassium, a low serum potassium level rarely occurs[25]. If sodium depleted, a glucose-saline drink can be sipped during the day as for patients with a jejunostomy. There is an exchange mechanism of chloride for bicarbonate in the colon; thus if much sodium chloride is consumed, bicarbonate may be lost in the stools, giving rise to a metabolic acidosis.

Jejunostomy Patients with a jejunostomy have a large volume of stomal output, which is greater after eating or drinking. Each litre of jejunostomy fluid contains about 100 mmol·L^{-1} of sodium[38]. This high volume output is mainly due to a loss of the normal daily secretions (0.5 L saliva, 2.0 L gastric juice and 1.5 L pancreatico-biliary) produced in responseto food and drink. Each day about 6 litres of chyme pass the duodeno-jejunal flexure, but by 100 cm distal to the duodeno-jejunal flexure the chyme volume is about the same as that of the oral intake[75,76]. If a jejunostomy is at a small bowel length of less than this more emerges from the stoma than is taken in by mouth ("secretors"). Even in the fasting state there is an obligatory loss of intestinal secretions produced with the migrating myoelectric complex (MMC)[77]. The effluent from a jejunostomy or ileostomy contains relatively little potassium (about 15 mmol·L^{-1})[23,24,38]. Potassium balance is not often a problem and net loss through the stoma occurs only when less than 50 cm jejunum remains[38]. A low serum potassium level is most commonly due to sodium depletion with secondary hyperaldosteronism and thus greater than normal urinary losses of potassium[23]. Hypokalaemia can also be due to hypomagnesaemia, which causes dysfunction of potassium transport systems and increases renal potassium excretion; this hypokalaemia is resistant to potassium treatment but responds to magnesium replacement[78,79]

Management of a high output stoma (jejunostomy/ileostomy) or enterocutaneous fistula

It is helpful both in predicting outcome and in choosing the type of nutritional support if the length of bowel above the stoma/fistula is known. Contrast studies (small bowel meal, fistulogram or stoma enema) may help with this. An examination of the output (colour/consistency/24 hr volume) gives an indication of the internal origin of the fistula.

Exclude other causes of a high output

A stoma may produce a high output if there is intra-abdominal sepsis, enteritis (e.g. clostridium or salmonella), partial/intermittent bowel obstruction, recurrent disease in the remaining bowel (e.g. Crohn's disease or irradiation) or sudden stopping of drugs (steroids or opiates).

Treatment of a high output jejunostomy

The concentration of sodium in the output remains constant (about 100 mmol·L^{-1}) whatever treatment is given. While there is a small stomal loss when fasting, the greatest increase in stomal output is after food or drink (Figure 2)[38]. Even in patients with a jejunostomy and receiving parenteral nutrition, the stomal output should be reduced, as this may reduce the amount or frequency of parenteral fluid required and the social problems of managing a high output stoma. If there is marked sodium and water depletion and severe thirst, it is often necessary to establish equilibrium by giving intravenous normal saline (2-4 L·d^{-1}) keeping the patient 'nil by mouth', which will also demonstrate that the output is driven by their oral intake. Over 2-3 d intravenous fluids are gradually withdrawn while food and restricted oral fluids are reintroduced. Great care must be taken not to administer too much fluid, which will readily cause oedema (partly due to the high circulating aldosterone levels[23,24,80,81]. To correct hypokalaemia in patients with a high output stoma, sodium/water depletion must be corrected and the serum magnesium brought into the

normal range. It is uncommon for potassium supplements to be needed.

Restrict oral fluids Jejunal mucosa is 'leaky' and rapid sodium fluxes occur across it. If water or any solution with a sodium concentration of less than 90 mmol/l is drunk there is a net efflux of sodium from the plasma into the bowel lumen[82], until a luminal sodium concentration of about 100 mmol/l is reached. In a patient with a jejunostomy this fluid is then lost in the stomal output. It is a common mistake for patients to be encouraged to drink oral hypotonic solutions to quench their thirst, but this causes large stomal sodium losses[80,82-85]. Treatment for the high output from a jejunostomy, ileostomy or high fistula begins with the patient restricting the total amount of oral hypotonic fluid (water, tea, coffee, fruit juices, alcohol or dilute salt solutions) to less than 500 $mL \cdot d^{-1}$. To make up the rest of the fluid requirement the patient is encouraged to drink a glucose-saline replacement solution. Many patients at home with marginally high stoma outputs (1.0-1.5 L) will be helped by a combination of oral fluid restriction (less than 1.0 $L \cdot d^{-1}$) and the addition of salt to their diet. Often patients are advised to take liquids and solids at different times (no liquid for half an hour before and after food) however there is no published evidence that this reduces stomal output or increases macro or micronutrient absorption[86].

Drink oral glucose-saline solution Patients with stomal losses of less than 1200 $mL \cdot d^{-1}$ can usually maintain sodium balance by adding extra salt to the limit of palatability at the table and when cooking. When stoma losses are in the range 1200 - 2000 mL, or sometimes more, it is possible for a patient to maintain sodium balance by taking a glucose-saline solution or salt capsules[84]. In hot weather, due to water and sodium loss in sweat, patients with a stoma are more likely to have problems of dehydration. As the sodium content of jejunostomy (or ileostomy) effluent is relatively constant at about 90 $mmol \cdot L^{-1}$ and as there is coupled absorption of sodium and glucose in the jejunum[87-89]; patients are advised to sip a glucose-saline solution with a sodium concentration of at least 90 $mmol \cdot L^{-1}$ throughout the day. The World Health Organization (WHO) cholera solution has a sodium concentration of 90 $mmol \cdot L^{-1}$[90] and is commonly used (without the potassium chloride) (Table 3). The concentration of sodium in this solution is much higher than many commercial preparations used to treat infective or traveller's diarrhoeas. There is no evidence that the sodium bicarbonate adds to the effectiveness of this solution[89] and it may be more palatable if the sodium bicarbonate is replaced by sodium citrate. If the sodium concentration is increased further (e.g. to 136 $mmol \cdot L^{-1}$) absorption of sodium and water is improved[91]. However even though taste perception is different in patients who are salt and water depleted, they may find a more concentrated salt solution too salty to drink. The patient should be encouraged to sip a total of one litre or more of one of these solutions in small quantities at intervals throughout the day. As compliance is often a major problem, patients need to understand the need for the solution. To improve palatability the solution may be chilled or flavoured with fruit juice.

Table 3 The modified WHO cholera rehydration solution

Sodium chloride	60 mmol (3.5 gm)
Sodium bicarbonate (or citrate)	30 mmol (2.5 gm) (2.9 gm)
Glucose	110 mmol (20 gm)
Tap water	one litre
Alternative rehydration solution	
Sodium chloride	120 mmol (7 gm)
Glucose	44 mmol (8 gm)
Tap water	one litre

A glucose-polymer (55 gm Maxijul) may be substituted for glucose to increase the energy intake by a mean of 648.8$kJ \cdot d^{-1}$ (115 kcal/day)[84]. A rice powder based oral rehydration solution can further increase the amount of energy absorbed, and if there is a functioning terminal ileum one study has shown that the sodium concentration in this solution can be reduced to 60 $mmol \cdot L^{-1}$[92]. The glucose-polymer/rice based solution can be especially useful in diabetic patients as it causes less extreme changes in blood glucose than glucose based solutions. Sodium chloride capsules (500 mg each) are effective when taken in large amounts (14/24 h), but can cause some patients to feel sick and even vomit[84]. If an enteral feed is given, sodium chloride needs to be added to make the total sodium concentration of the feed 100 $mmol \cdot L^{-1}$ while keeping the osmolality near to 300 $mmol \cdot L^{-1}$.

Drug therapy If restricting oral fluids and giving a glucose-saline solution to drink are not adequate, drugs may be needed. As the intestinal output, especially in net "secretors" rises after meals, it is important to give the drugs before food. Drugs used to reduce jejunostomy output actto reduce either intestinal motility or secretions. If any tablets/capsules emerge unchanged in stool/stomal output, they can be crushed, opened and/or mixed with water or put on food.

Antimotility (antidiarrhoeal) drugs Loperamide and codeine phosphate reduce intestinal motility and thus decrease water and sodium output from an ileostomy by about 20%-30%[93-95]. Loperamide is preferred to opiate drugs (e.g. codeine phosphate) as it is not sedative, addictive and does not cause fat malabsorption[94,96]. However loperamide does reduce postprandial pancreaticobiliary secretion of trypsin and bilirubin in patients with a short bowel and preserved colon[97]. They are usually taken half an hour before food. Oral loperamide 4 mg taken four times a day was more effective in reducing the weight and sodium content of ileostomy fluid than codeine phosphate 60 mg taken four times a day (94) but the effect of both together may be greater[98]. Loperamide circulates through the enterohepatic circulation, which is severely disrupted in patients with a short bowel and small bowel transit may be very rapid. Thus high doses of loperamide (e.g. 12-24 mg at a time) may be needed, as following a vagotomy and pyloroplasty[99]. These drugs are effective in most patients with a jejunostomy[38,100] particularly net "absorbers".

Antisecretory drugs Food and drink are diluted by digestive juices, thus the volume of stomal effluent can be reduced in 'secretors' by drugs that reduce the secretions from the stomach, liver and pancreas. Drugs that reduce gastric acid secretion (e.g. the H_2 antagonists or proton pump inhibitors or the somatostatin analogue octreotide) are most commonly used. There is one report of a patient with a jejunostomy for Crohn's disease having gastric irradiation to successfully reduce their stomal output[101].

H_2 antagonists/proton pump inhibitors Cimetidine (400 mg orally or intravenously four times a day)[102,103], ranitidine (300 mg orally twice daily)[38] and omeprazole (40 mg orally once a day or intravenously twice a day)[104,105] reduce jejunostomy output particularly in net secretors and generally in those with an output exceeding 2 litres daily. Omeprazole is readily absorbed in the duodenum and upper small bowel, but if less than 50 cm jejunum remains it may need to be given intravenously. The effect of these drugs is as good as octreotide (50 ìg subcutaneously twice daily) in terms of the reduction in stomal volume[38,104]. They do not change the absorption of energy, carbohydrate, lipid, nitrogen and divalent cations[102-105] and do not reduce jejunostomy output enough to prevent the need for parenteral fluid and electrolyte replacement.

Somatostatin/octreotide Somatostatin/octreotide reduce salivary, gastric and pancreatico-biliary secretions, slow small bowel transit and may delay gastric emptying. Somatostatin has a serum half-life of 3 min so is given by continuous infusion and does reduce stomal output[106]. Octreotide has a serum half-life of 90 minutes so is given as regular (two or three time's daily) subcutaneous injections before food. Several studies in adults have shown octreotide to reduce ileostomy diarrhoea and large volume jejunostomy outputs [100,107-114]. The greatest reductions in intestinal output have occurred in net

"secretors", and many patients have been able to reduce the volume of parenteral supplements needed[110,114]. All studies have shown a reduction in sodium output, which parallels that of the intestinal output[100,107-114]. Magnesium balance has not been changed by octreotide[107,112]. Octreotide does not significantly change total energy[110,113,114] or nitrogen absorption[107,109,112-114]. Fat absorption may be unchanged[112-114] or reduced[107] as might be predicted from a reduction in pancreaticobiliary secretion. The effect of octreotide is maintained in the long-term[107,109,110,112,114]. Somatostatin and octreotide both reduce the output from a high fistula and appear to accelerate the rate of spontaneous closure[115,116].

Steroids/fludrocortisone/desmopressin Mineralocorticoids (e.g. 2 mg oral fludrocortisone or 2 mg intravenous d-aldosterone)[117-119] or high dose hydrocortisone[120] can occasionally reduce the stomal output in patients with a retained ileum. Desmopressin (an analogue of antidiuretic hormone) has no effect upon ileal fluid or electrolyte loss in man[121]. Some patients cannot be maintained with any oral regimens (usually if jejunal length is less than 100 cm) and need regular parenteral saline supplements. Most such patients also need oral or parenteral nutritional supplements, but a few need only one or two litres of parenteral (or subcutaneous) saline daily, often with added magnesium sulphate (4-12 mmol).

Magnesium depletion

Magnesium deficiency occurs partly because its main absorptive sites have been removed (distal small bowel and colon)[122]. However the degree of malabsorption of magnesium does not correlate with the residual length of jejunum[42,123]. Fatty acids derived either from digestion of dietary fat or from bacterial fermentation of malabsorbed carbohydrate, combine with magnesium, calcium and zinc so preventing absorption and increasing faecal or stomal losses[124]. Another reason particularly in patients with a jejunostomy is that salt and water depletion causes secondary hyperaldosteronism, which increases renal magnesium excretion[125,126].

The clinical syndrome of magnesium deficiency in man includes fatigue, depression, jerky and weak muscles, ataxia, athetoid movements, cardiac arrhythmia's and, if severe, convulsions[127-130]. The occurrence of carpopedal spasm, positive Chvostek and Trousseau's signs generally occur if there is a concomitant hypocalcaemia[128,129,131]. Low serum magnesium levels are more common in patients with a jejunostomy (70%) than patients with a retained colon (40%)[25]. Hypomagnesaemia can perpetuate itself as it reduces both the secretion and function of parathormone[132]. Thus parathormone cannot promote magnesium absorption in the ascending limb of the loop of Henle or activate renal 1 alpha-hydroxylase, which catalyses the formation of 1,25 hydroxy-vitamin D. The failure to make 1,25 hydroxy-vitamin D results in reduced intestinal magnesium and calcium absorption[133].

Treatment of hypomagnesaemia

Dehydration and sodium depletion, which will cause secondary hyperaldosteronism (which leads to renal magnesium loss), must first be treated. A diet relatively low in fat reduces stool/stomal magnesium losses especially in patients with a retained colon[124,134]. Various magnesium salts have been given as a treatment orally: magnesium sulphate, chloride, hydroxide, acetate, carbonate, gluconate, lactate, citrate, aspartate, pyroglutamate, oxide (magnesia) and diglycinate. Most magnesium salts are poorly absorbed and may worsen diarrhoea/stomal output. Magnesium acetate has been shown to cause less diarrhoea than magnesium gluconate[135]. Magnesium oxide is commonly given and contains more elemental magnesium than the other salts, is insoluble in water and alcohol but soluble in dilute acid. In the stomach it is converted to magnesium chloride. It is given as gelatine capsules of 4 mmol magnesium oxide (160 mg of MgO) to a total of 12-24 mmol·d^{-1}. Magnesium oxide is usually given at night when intestinal transit is assumed to be slowest and hence there is more time for absorption. Magnesium diglycinate (chelate) is absorbed as well as magnesium oxide as an intact dipeptide in the proximal jejunum and it results in the passage of fewer stools, after an ileal resection, than magnesium oxide[136].

As hypomagnesaemia will have caused both a failure of parathormone release and a resistance to its action, 1-alpha hydroxy-cholecalciferol cannot be made in the kidney in adequate amounts. Thus if oral magnesium supplements do not bring the magnesium level into the normal range, oral 1-alpha hydroxy-cholecalciferol in a gradually increasing dose (every 2-4 wk) of 1-9 μg·d^{-1} will improve magnesium balance in patients, at least in those with a retained colon[137,138]. This action is by increasing both intestinal and renal magnesium absorption[138]. Occasionally magnesium is given as an intravenous or subcutaneous infusion.

Undernutrition - protein-energy malnutrition

Undernutrition only becomes apparent slowly and should be prevented from occurring by predicting its likelihood and from knowledge of the residual length of bowel remaining (Table 4). All patients who can be maintained on an oral diet need to consume more energy than normal subjects because 50% or more of the energy from the diet may be malabsorbed. Patients can achieve this by eating more high-energy food, having oral sip-feeds, or high energy enteral feeds given at night through a nasogastric or gastrostomy tube. There are rarely any problems inserting a percutaneous endoscopic gastrostomy (PEG) into patients with Crohn's disease providing that there is no distal obstruction[136]. Once weight is regained, a nocturnal feed can be reduced or stopped and sip-feeds during the day may be adequate. Only if these measures fail and the patient continues to lose weight, or fails to regain lost weight, is parenteral nutrition given. Parenteral nutrition may only be needed for a few weeks or months before oral supplements are adequate.

In the long-term parenteral nutrition is needed if a patient absorbs less than one-third of the oral energy intake[38,74], if there are high-energy requirements and absorption is 30-60% or if increasing the oral/enteral nutrient intake causes a socially unacceptably large volume of stomal output or diarrhoea. In addition to consuming a high energy diet the dietary advice given to the two types of patient is different.

Table 4 Summary of the problems of a short bowel

	Jejunum-colon	Jejunostomy
Presentation	gradual, diarrhoea and undernutrition	acute fluid losses
Water, sodium and magnesium depletion	uncommon in the long-term	common
Nutrient malabsorption	Common*	very common
D(-)lactic acidosis	occasionally	none
Renal stones (calcium oxalate)	25%	none
Gallstones (pigment)	45%	45%
Adaptation	functional adaptation	no evidence
Social problems	diarrhoea dehydration dependency on treatment	high stomal output

*:bacterial fermentation of carbohydrate salvages some energy, but D (-) lactic acidosis can occur if the diet is high in mono and oligosaccharides.

Jejunum-colon Unabsorbed long chain fatty acids in the colon reduce the transit time[139] and reduce water and sodium absorption[140] somaking diarrhoea worse. In addition they are toxic to bacteria so reduce carbohydrate fermentation[141]. They bind to calcium and magnesium increasing stool losses, and they increase oxalate absorption so predisposing to the formation of renal stones (see below). Theoretically, a low fat diet is ideal for patients after a small bowel resection[142], however in practice it is hard to implement. Fat yields twice as much energy as a comparable weight of carbohydrate or protein and makes food palatable. A high carbohydrate/low fat diet involves eating a large volume of food. A low fat diet may increase calcium, magnesium and zinc absorption[124] but makes essential fatty acid deficiency more likely[143]. Sunflower oil may be rubbed onto the

skin to ensure adequate amounts of essential fatty acids enter the body[144]. If a diet is high in monosaccharides D (-) lactic acidosis may occur (see below). Medium chain triglycerides are an alternative source of energy and are absorbed from the small and large bowel[145-148]. In order to increase the energy absorption, reduce the risk of renal stones and D (-) lactic acidosis, patients with a retained colon need a large totalenergy intake with a diet high in carbohydrate (polysaccharides)[29], but not increased in fat (long chain triglycerides) and low in oxalate.

Jejunostomy Patients with a jejunostomy absorb a constant proportion of the nitrogen, energy and fat from their diet[29,134,149]. Increasing fat in the diet, increases energy density, keeps the diet osmolality low, increases palatability and provides essential fatty acids[143]. It does raise fat excretion but does not usually increase stomal output, nor make the output offensive[29,134,149]. Two studies have shown fatty acids to increase the stomal output of divalent cations[124,150] and two others found no such effect [134,149]. There is no advantage in giving a diet of small molecules (e.g. an elemental diet), which causes a feed to be hyper-osmolar[149] and usually contains little sodium, so stomal losses of water and sodium from the stoma increase. A peptide diet has a relatively high osmolality and thus can increase stomal output[151]. Little advantage comes from taking a diet of water-soluble medium chain triglycerides in place of normal fat[148]. The addition of glutamine (15 gm) to a litre of rehydration solution in patients with jejunostomy resulted in no additional benefit in terms of water or sodium absorption[152]. The fibre content of the diet plays only a minor role in determining jejunal output[93]. As the stomal sodium losses are about 100 mmol·L^{-1} any diet will need added sodium chloride. These patients need a diet of high energy (carbohydrate or lipid) in which the osmolality is kept low by using large molecules (polysaccharides, protein and triglycerides)[149,150] and to which sodium chloride is added to give the meal/liquid feed a total sodium concentration of 90-120 mmol·L^{-1} and an osmolality of about 300 mmol·L^{-1}.

Conjugated bile acid treatment As fat malabsorption in both types of patient with a short bowel may partly be due to bile acid depletion, bile salts have been tried as a treatment. Cholylsarcosine, a synthetic bile acid resistant to bacterial deconjugation and dehydroxylation and which does not itself cause colonic secretion so does not cause diarrhoea. When 4 grams are taken three times a day, there is a variable improvement in fat and calcium absorption in patients with and without a retained functioning colon; however it may cause nausea[153-154].

Other vitamin and mineral deficiencies

Both groups of patient have had more than 60 cm of their terminal ileum removed, and need long-term hydroxycobalamin injections[11,12,57]. Patients receiving parenteral nutrition are commonly selenium deficient[155-157] and need larger amounts of selenium than are required in the diet of normal subjects[157] this suggests a loss of selenium from the gastrointestinal secretions. In patients with a jejunostomy there is a reduction in selenium absorption, which relates to the length of remaining jejunum[158]. The kidney can conserve selenium but this may not be adequate and selenium deficiency is common. This causes weak muscles and a dilated cardiomyopathy[159]. Zinc deficiency is not common unless stool volumes are large[160]. There may be impairment of absorption of the fat-soluble vitamins A D E K and essential fatty acids. Essential fatty acid deficiency can be treated by rubbing sunflower oil into the skin[144].

Diarrhoea (Jejunum-colon patients)

The oral intake determines the amount of stool passed. Diarrhoea, which may severely restrict a patient's life style, can be reduced by limiting food intake, but this may increase the problems of undernutrition. Rarely a patient needs parenteral nutrition to allow them to eat less and so reduce the diarrhoea.

Diarrhoea may be treated with loperamide 2-8 mg given half an hour before food in the same way as for patients with a jejunostomy and occasionally codeine phosphate is also added (30-60 mg half an hour before food). If less than 100 cm terminal ileum has been resected bile salt malabsorption may contribute to the diarrhoea and may be helped by cholestyramine, which has the additional advantage of reducing oxalate absorption, but may be detrimental by reducing fat absorption and by further reducing the bile salt pool[58]. Although a gastric anti-secretory drug may reduce diarrhoea shortly after surgery, they are not usually effective in the long-term.

Confusion

In addition to the many common general medical causes of confusion (e.g. hypoxia, hepatic, renal or cardiac failure, sepsis, hypoglycaemia, alcohol or other drugs) other specific causes should be sought in a patient with a short bowel. Hypomagnesaemia may cause mild confusion when the serum magnesium level is very low (less than 0.2 mmol·L^{-1}). Thiamine deficiency can cause a Wernicke/Korsikoff psychosis, which responds rapidly to large regular doses of thiamine. Two other specific causes in patients with a short bowel are D (-) lactic acidosis and hyperammonaemia. D (-) lactic acidosis is only seen in patients with a short bowel and a preserved colon. Lactic acid produced and utilized by mammalian cells is the L (+) isomer but colonic bacteria produce both the L (+) and the D (-) isomers. In rare circumstances the colonic flora may rapidly degrade a surplus of easily fermentable carbohydrate (mono and oligosaccharides and rapidly digestible starch[161] to D (-) lactate. A reduction in colonic gram-negative anaerobes and a predominance of gram-positive anaerobes, especially Lactobacillus, Eubacterium, and Bifidobacterium has been found[162]. D (-) lactate is absorbed and cannot be easily metabosised. It causes ataxia, blurred vision, ophthalmoplegia and nystagmus, slurred speech, aggressiveness, inappropriate behaviour, and stupor, which may progress to coma[163]. It is suspected when a patient is found to have a metabolic acidosis with a large anion gap, and confirmed by increased concentrations of D (-) lactate in blood and urine. The diet is changed so that simplecarbohydrates (mono and oligosaccharides)[164] are restricted and more slowly digestible polysaccharides (starch) encouraged and thiamine may be given. Broad-spectrum antibiotics (neomycin or vancomycin) can be given to reduce the bacterial production of lactate. Medium chain triglycerides may be omitted[165]. If very severe the patient may need to fast and be given parenteral nutrition.

Confusion may occur in both types of patients with a short bowel due to hyperammonaemia, this results because ammonia cannot be detoxified. The small amount of intestine remaining cannot manufacture adequate citrulline to detoxify ammonia in the urea cycle. The increase in blood ammonia is a problem if there is concomitant renal impairment, as the excess ammonia cannot be excreted. By giving arginine (an intermediary in the urea cycle) the hyperammonaemia can be corrected[166,167].

Drug absorption

Omeprazole can be absorbed in the duodenum/ upper jejunum and only if less than 50 cm jejunum remains are problems likely to occur. Many drugs will be incompletely absorbed by patients with a short bowel and may be needed in much higher amounts than usual (e.g. thyroxine, warfarin[168], and digoxin[169]) or may need to be given intravenously. As the enterohepatic circulation, round which loperamide circulates, is disrupted, higher doses than usual may need to be given.

Gallstones

Gallstones are common (45%) in both types of patient and are more common in men[25]. It was originally thought that gallstones in this circumstance were due to deposition of cholesterol because of a depleted bile-salt pool. However, the gallstones tend to contain calcium bilirubinate and these probably result from gallbladder stasis with the consequent formation of biliary sludge. These stones may

appear calcified on an abdominal radiograph[170]. Calcium bilirubinate crystals, within biliary sludge, are more commonly found in men than women[171]. The reasons for the formation of biliary sludge include, abdominal surgery, ileal resection, Crohn's disease, rapid weight loss, drug therapy (e.g. octreotide) and altered gastrointestinal transit and flora. Disturbed cholesterol or bilirubin metabolism, and changes in nucleation may also be important[172]. There are many therapies proposed to prevent the formation of gallstones. Cholecystokinin (CCK) injections have been successful[173]. Periodic pulsed infusions of amino acids or small amounts of enteral feed stimulate endogenous CCK release, cause gallbladder contraction and thus prevent gallbladder stasis[174]. Non-steroidal anti-inflammatory drugs may prevent gallstones by decreasing the mucin glycoprotein involved in nucleation[175], or by a prokinetic effect (via leukotrienes) on the gallbladder[176]. Ursodeoxycholic acid (UDCA) causes bile to become richer in glycine or taurine conjugates of UDCA and this results in a marked reduction in cholesterol crystallisation. This may be due to a reduction in some crystallisation promoting factors (e.g. aminopeptidase N, haptoglobin and some immunoglobulins)[177,178]. Reducing the amount of the more lithogenic secondary bile acids could be achieved by either increasing intestinal transit (e.g. cisapride[179]) or by inhibiting bowel bacteria (e.g. metronidazole)[180]). Some units advocate prophylactic cholecystectomy whenever a large intestinal resection is performed[181].

Renal stones

Patients with a retained colon have a 25% chance of developing symptomatic calcium oxalate renal stones[25]. Oxalate is a metabolic end product that cannot be metabolised in man. Under normal circumstances, most urinary oxalate derives from the metabolism of amino acids (mainly glycine) and of ascorbic acid. Less than 10% is derived from dietary oxalate[182]. However after an ileal resection there is increased colonic absorption of dietary oxalate especially by the distal colon[183].

There are many factors contributing to calcium oxalate renal stone formation[184].

Fat malabsorption Increasing the dietary intake of lipid results in increasing enteric hyperoxaluria[185]. Fat malabsorption leads to calcium soap formation and calcium malabsorption and to oxalate being unbound and thus easily absorbable. Colonic oxalate absorption is increased by low dietary calcium, hyperparathyroidism, and vitamin D administration, all of which reduce calcium concentrations in the colon and thus the extent to which oxalate is bound to calcium in the gut lumen.

Increased colonic permeability Unabsorbed bile acids increase colonic permeability to oxalate. Chenodeoxycholate, infused into the colon, increased oxalate absorption five-fold[186]. Cholestyramine taken orally decreased oxalate absorption[187,188].

Reduced bacterial breakdown of oxalate An anaerobic bacteria (Oxalobacter formigenes) within the colon degrades oxalate to carbon dioxide and formate[189]. A reduction in its amount could contribute to increased oxalate absorption. It is cultured less frequently from the stools of patients with Crohn's disease or steatorrhoea[190]. Its growth is inhibited by low bile acid concentrations as occur in patients with a short bowel and retained colon[191].

Vitamin deficiency Pyridoxine deficiency may increase the metabolic formation of oxalate and/or the ileal absorption[192,193]. Thiamine deficiency may increase oxalate production. In contrast ascorbic acid deficiency decreases oxalate absorption[194].

Hypocitraturia Citrate prevents nucleation, the first step in stone formation. Hypocitraturia is common in patients with malabsorption or jejunoileal bypass[195-197]. Citrate excretion and urine pH are reduced by systemic acidosis, including that caused by gastrointestinal bicarbonate wastage, and by hypomagnesaemia. Hypocitraturia in patients with malabsorption can be corrected by oral citrate supplementation and magnesium injections[196].

Type of oxalate ingested Sodium oxalate as found in tea is more readily absorbed that calcium oxalate in most foods.

To prevent calcium oxalate renal stone formation, patients should avoid dehydration and take a diet low in oxalate. A low oxalate diet means avoiding foods such as spinach, rhubarb, beetroot, nuts, chocolate, tea, wheat bran, and strawberries[184]. Fat restriction is theoretically good but may not be desirable for nutritional reasons. Substitution of medium chain triglycerides may be effective in reducing oxalate absorption[198]. Restriction of dietary oxalate and fat intake reduce urinary oxalate excretion[188,199-201]. Dietary calcium must not be restricted but must be increased. Calcium salts reduce absorption of dietary oxalate in patients with ileal resection or jejunoileal bypass[202-209]. Calcium-containing organic marine hydrocolloid decreases oxalate absorption without increasing calcium absorption[206]. Binding bile salts with cholestyramine has variable effects. It binds oxalate *in vitro*[210] and some studies showed it to decrease urinary oxalate excretion[187,210], however, other studies have shown no benefit[195,202] or even increased urinary oxalate excretion[211].

Aluminium salts can bind oxalate *in vitro*[205]. There was a halving of urinary oxalate excretion after oral aluminium hydroxide had been given to 4 patients with enteric hyperoxaluria[212], but no such reduction was observed in another study[211].

Social problems

Most long-term patients with a short bowel have a body mass index within the normal range and most are at full time work or looking after the home and family unaided[25]. Both groups may have diarrhoea and in those with a colon the diarrhoea is malodorous and bulky due to steatorrhoea.

The effluent from a small-bowel stoma, unlike from a colostomy, is not offensive but sometimes is a large volume (e. g. 3 or more litres/24 h). The bag then has to be emptied frequently and, if it becomes overfull, the adhesive flange may separate from the skin with embarrassing leakage of fluid, and with the likelihood of skin soreness. A patient with no remaining jejunum and a preserved colon can survive without parenteral nutrition but their quality of life is poor[35].

New and future treatments

New treatments aim to increase the absorptive function of the remaining gut. As plasma levels of GLP-2, which causes villus growth, and peptide YY, which slows upper gastrointestinal transit, are low in patients with a jejunostomy, treatments to correct these are being tried. GLP-2 gave a small increase in nutrient absorption[213]. Peptide YY agonists are available but have not yet been tried[214]. Other treatments that may increase structural adaptation include epidermal growth factor[215,216], colostrums and aminoguanidine[217]). Studies using growth hormone, glutamine and fibre have been disappointing[218,219]. Attempts are being made to replace colonic mucosa with small intestinal mucosa to increase absorption[220]. Other areas of importance are in the prevention of gallstones after an intestinal resection and randomised placebo controlled trials using prophylactic antibiotics, ursodeoxycholic acid and cholecystokinin are awaited. An oral nutrient solution containing 100 mmol/litre sodium and having an osmolality of 300 $mmol \cdot L^{-1}$ has yet to be commercially manufactured as an ideal nutrient solution to give to patients with a jejunostomy or high output ileostomy.

Small bowel transplantation is becoming a more successful operation, however few patients are suitable for this (no venous access or parenteral nutrition induced severe liver disease) and even when tacrolimus is used for immunosuppression only 40%-47% of patients will be alive 3 years later and 29%-38% will have a functioning

graft[221,222]. 78% of those who survive with the transplant are able to stop parenteral nutrition. These figures contrast to those for patients on home parenteral nutrition for non-neoplastic reasons who can expect to have a relatively good quality of life good and a survival rate of 70% at 3 years[223,224].

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Current gene therapy for stomach carcinoma

Chang-Tai Xu[1], Lian-Tian Huang[1], Bo-Rong Pan[2]

[1]Editorial Department, the Journal of Fourth Military Medical University, [2]Oncology Center, Xijing Hospital, Fourth Military Medical University, 169 Changle Xilu, Xi'an 710032, Shaanxi Province, China
Correspondence to: Dr. Chang-Tai Xu, Editorial Department, the Journal of Fourth Military Medical University, 169 Changle Xilu, Xi'an 710032, Shaanxi Province, China. xuct2000@263.net
Telephone: +86-29-3373456, Fax: +86-29-3224890
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INTRODUCTION

Gastric cancer is common in China[1-42], and its early diagnosis and treatment in advanced stage are difficult[31-50]. In recent years, gene study in cancer is a hotspot, and great progress has been achieved[41-80]. Cancer gene therapy has shifted from the imagination into the laboratory and clinical trials. The "logic of how genes function" coupled with the connections of cell cycle processes to specific gene actions is creating a promise of treating tumors by gene therapy. There have been significant advances against both local and metastatic growths. The potential role of gene intervention extends from diseases caused by single gene defects, through severe viral infections, to polygenic disorders, such as diabetes mellitus and arteriosclerosis. However, gene therapy can be defined as the introduction and expression of an exogenous gene into human cells for therapeutic benefit, and is conventionally restricted to human diseases associated with single gene defects. The rapid progress in our understanding of some of the molecular mechanisms involved in the pathogenesis of cancer and metabolic disorders, with the development of gene delivery vector technology, has urged us to consider novel gene approaches to digestive diseases. There is no shortage of ideas and applications for gene intervention in human diseases, but there are great limitations not only with the efficiency and targeting of the present generation of gene transfer vectors but also with our incomplete understanding of transcription control[1,2].

The graduation of gene therapy from unfulfilled dreams to conventional therapy for gene and acquired disorders will require a mastery of multiple disparate components including gene delivery vectors, regulated tissue-specific gene expression, control of immunity and manipulation of cell viability. Improvement in suicide genes has opened up a whole new treatment modality for treating hyperproliferative disorders and for designing animal models for disease[3]. Along with herpes simplex virus-1 thymidine kinase, a host of additional suicide gene has been developed. A critical comparison of these will follow along with progress in utilizing these reagents for therapeutic benefits[81-90].

The current delineation of the molecular basis of cancer provides a strong rationale to consider gene therapy approaches for cancer as a complement to other cancer therapies. Phase III trials focused on adenoviral vector-mediated delivery of wild-type *p*53 to compliment *p*53 mutations were recently initiated for head and neck cancer and ovarian cancer. Clinical testing of the tumor inhibitory gene E1A, delivered by synthetic vectors is ongoing. Positive clinical data from these clinical studies will establish the use of gene therapy as a component of the multimodal treatment for certain cancers[4-6].

Although the rapid technological advances continue to sustain the field of cancer gene therapy, few individual patients have benefited from the revolution so far. The plethora of clinical trials described confirms that each malignancy has its own ideal strategy based on the associated molecular defects, and there has been rapid progress in this viewpoint. At the same time, there has been a renewed appreciation for the limitations to gene therapy, which include low efficiency of gene transfer, poor specificity of response and methods to accurately evaluate responses, and lack of truly tumor-specific targets at which to aim. With all new therapies, we are climbing a steep learning curve in encountering treatment-related toxicities, as well as profound ethical and regulatory issues[5-9].

GENE THERAPY THEORY

Recent advances in understanding and manipulating genes—the biological units of heredity—have set the stage for scientists to alter patients' genetic material to fight or prevent diseases. One major goal of gene therapy is to supply cells with healthy copies of missing or flawed genes. This approach is revolutionary. Instead of giving a patient a drug to treat or control the symptoms of a gene disorder, physicians attempt to correct the basic problem by altering the gene makeup of some of the patient's cells(www.chgb.org.cn/spec-topics/therapy/text00.htm). Hundreds of major health problems are influenced by gene functions. In the future, gene therapy could be used to treat many of these conditions. Theoretically, it could also be used to alter germ cells (egg or sperm) in order to prevent a gene defect from being transmitted to future generations. However, difficult ethical and social questions as well as technical obstacles will be set in the possibility of germ-line gene therapy (Figure 1, www.chgb.org.cn/spec-topics/hgp/medicine/med03.htm). Gene therapy could also be used as a drug delivery system. To accomplish this, a gene that produces a useful product would be inserted into the DNA of the patient's cells[10-14]. For example, during blood vessel surgery, a gene that makes an ant-clotting factor could be inserted into the DNA of cells lining blood vessels to prevent dangerous blood clots from forming. Many other conditions might also lead themselves to treatment by using this general approach. As medicine operates increasingly at the molecular level, gene therapy for drug delivery could save much effort and expense. It could shortcut the lengthy and complicated process of collecting large amounts of a gene's protein product, purifying the product, formulating it as a drug, and administering it to the patient. However, gene therapy is still extremely new and highly experimental. The number of approved trials is small, and relatively few patients have been treated to date[8,11-13].

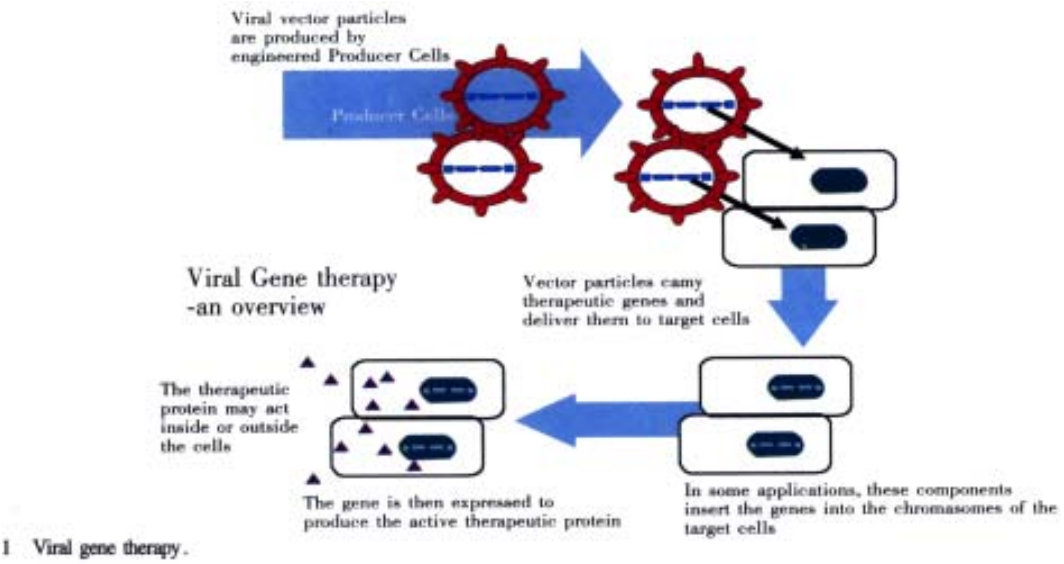

Figure 1 Viral gene therapy.

BASIC STEPS INVOLVED IN CURRENT GENE THERAPY EXPERIMENTS

In some current experiments, cells from the blood or bone marrow are removed from the patient and grown in the laboratory under conditions that encourage them to multiply. Then the desired gene is inserted into the cells with the help of a disabled virus, and the successfully altered cells are selected out, encouraged to multiply, and returned to the patient's body. In other cases, liposomes (lipid particles) or disabled viruses may be used to deliver the gene directly to cells within the patient's body. Basic requirements for gene therapy are as follows[91-110].

Potential of gene therapy

Gene therapy offers a new treatment paradigm for curing human disease. Rather than altering the disease phenotype by using agents that interact with gene products, or are themselves gene products, gene therapy can theoretically modify specific genes resulting in disease cure following a single administration. Initially gene therapy was envisioned for the treatment of gene disorders, but is currently being studied in a wide range of diseases, including cancer, peripheral vascular disease, arthritis, neurodegenerative disorders and other acquired diseases[15-20].

Gene identification and cloning

Even though the range of gene therapy strategies is quite diverse, certain key elements are required for a successful gene therapy strategy. The most elementary of these is that the relevant gene must be identified and cloned. Upon completion of the Human Genome Project, gene availability will be unlimited, but until then the starting point for any gene therapy strategy remains gene identification and cloning for relevant genes related to the disease[3,16].

Gene transfer and expression

Once the gene has been identified and cloned, the next consideration must be expression. Questions pertaining to the efficiency of gene transfer and gene expression remain at the forefront of gene therapy research. Currently much debate in the field of gene therapy revolves around the transfer of desired genes to appropriate cells, and then obtaining sufficient levels of expression for disease treatment. Hopefully, future research on gene transfer and tissue-specific gene expression will resolve these issues in the majority of gene therapy protocols[21-23]. Other important considerations for a gene therapy strategy include: a sufficient understanding of the pathogenesis of the targeted disorder, potential side effects of the gene therapy treatment, and understanding of the target cells to receive the gene therapy[22].

Terminology

Like most fields, gene therapy has unique terminology. The list provided below will clarify the meaning of some of the most common terms[1-3,6,9,22-26].

Ex vivo In gene transfer, transfer of gene material to cells located outside the host. Following transfer of the gene material, the cells are then implanted back into the host. This term has also been called the indirect method of gene transfer.

In vivo In gene transfer, transfer of gene material to cells located within the host. This has also been termed the direct method of gene transfer.

Gene therapy The transfer of selected genes into a host with the hope of ameliorating or curing a disease state.

Cell therapy (genome therapy) The transfer of entire cells, that have not been genetically modified, into a host with the hope that the transferred cells will engraft into and improve host function.

Somatic gene transfer Transfer of genes to non-germline tissues in the hope of correcting the disease state of a patient.

Germline gene Transfer of genes to germline (eggs or sperm) tissues in the hope of altering the genome of future generations.

Transgene The selected gene tested in a gene transfer experiment. For example, if you wished to treat a patient for phenylketonuria, you might plan to transfer a corrected version of the phenylalanine hydroxylase gene into the liver cells. In this example, the corrected version of the phenylalanine hydroxylase gene would be the transgene.

Reporter gene Genes that are used to test the efficiency of gene transfer. Examples include genes encoding luciferase, -galactosidase, and chloramphenicol acetyltransferase.

Gene transfer vector The mechanism by which the gene is transferred into a cell.

Transfer efficiency The percentage of cells that are expressing the desired transgene.

APPROACHES TO CANCER THERAPY

The idealized approach to gene therapy is the replacement of a mutated gene with a correct copy that restores normal functioning and therapeutically alters the malignant phenotype. One meaning of the

word "vector" is "carrier". In the field of infectious diseases, the term has been used to describe an agent, such as an insect, that carries an infectious organism from one individual to another. By analogy, the genetically disabled viruses used in gene therapy are referred to as vectors because they carry genes to cells. Most often, these vectors are derived from mouse retroviruses.

Scientists are working on ways to genetically alter immune cells that are naturally or deliberately targeted to cancers. They are interested in arming such cells with cancer-fighting genes and returning them to the body, where they could more forcefully attack the cancer. Clinical trials along these lines are in progress for the treatment of melanoma. Alternatively, cancer cells can be taken from the body and altered genetically so that they elicit a strong immune response. These cells can then be returned to the body in the hope that they will act as a cancer vaccine. A variety of clinical trials using this approach are now under way. It is also possible to inject a tumor with a gene that renders the tumor cells vulnerable to an antibiotic or other drug. Subsequent treatment with the drug should kill only the cells that contain the foreign gene. Since other cells would be spared, the treatment should have few side effects. Two trials using this approach are in progress for treatment of brain tumors[17-19,24].

Tumor-suppressor gene therapy

Goals of tumor suppressor gene therapy are: cell death and changes in growth of the cell, behavior of the cell, invasiveness of the cell, and metastatic ability of the cell.

Because *p*53 is the most common mutated in cancer and influences transcription, cell cycle movement, apoptosis, and angiogenesis, it is a prime target for gene replacement. In model systems, transduction of cancer cells with *p*53 has been demonstrated to inhibit growth, inhibit angiogenesis, and induce apoptosis.

Early clinical trials using a *p*53 retrovirus have also been encouraging. Current limitations of tumor-suppressor gene therapy are: limited number of target genes known to induce or maintain malignancy; and difficulty of transducing enough cancer cells to result in a cure.

A bystander effect—the death of more cells than are actually transduced—has been proposed although how it occurs has not been yet understood. This effect may result from cell-cell contact, immune mediated responses, *and/or* other local actions.

Other therapeutic possibilities are: combining *p*53 transduction with radiation or apoptosis-inducing chemotherapy, systemic delivery of *p*53 using liposomes, systemic delivery of *p*53 using hepatic artery infusion, use of an adenovirus that replicates only in *p*53 mutant cells and results in cell death through lysis of the cell.

Suicide gene therapy

Suicide gene therapy is the transduction of a gene that transforms a non-toxic "pro-drug" into a toxic substance. Systems under investigation are as follows[22-26]:

Escherichia coli cytosine deaminase (CD) gene + 5-fluorocytosine (5-FC). CD converts 5-FC to 5-fluorouracil (5-FU), a chemotherapeutic agent. This combination produces a bystander effect and has been demonstrated to have some success in animals with hepatic metastasis of gastrointestinal tumors. Delivery of CD to specific sites and the use of tissue specific promotors are a focus of work with this strategy.

Herpes simplex virus thymidine kinase gene (HSV-tk) + ganciclovir (GCV). HSV-tk phosphorylates GCV causing it to inhibit the synthesis of DNA. Like the first system, this also causes a bystander effect. This strategy has been looked at for treatment of localized brain tumors, liver metastases, peritoneal-based metastases, and mesotheliomas. Thus far, the unpredictability of the bystander effect and difficulties in transduction has kept cure rates low. The use of tissue-specific vectors to deliver the genes and the combination of the strategy with radiation may improve the efficacy of the approach in time.

Immunomodulatory gene therapy

Immunomodulatory gene therapy is a method to induce cellular immune responses to metastatic lesions. The strategy involves injecting into the skin of a patient a suspension of irradiated tumor cells that have been transduced with a cytokine gene to stimulate a systemic immune response against tumor-specific antigens[1-4,27-29]. In preclinical cancer models vaccination with tumor cells have been demonstrated to generate a cellular immunological response against "challenge" tumors. There are several problems that still need to be addressed to make this strategy successful[20,30-32]: ① there are only a few tumor-specific antigens that act as recognition targets; ② antitumor activity has been active against a relatively low tumor burden in several studies; and ¢Û the financial and labor costs are high and efficiencies will need to be developed.

Future modifications to the basic strategy may include: a combination of cytokine and costimulatory molecule vaccination to increase tumor vaccine efficiency; the use of *in situ* strategies to invoke immunologic responses, including direct injection into a tumor, as has been done with an adenoviral vector containing the IL-2 gene; combining a suicide gene therapy approach (eg, HSV-tk + GCV) with IL-2 transgene therapy or, more generally, using combinations of cytokines to induce antitumor activity both *in situ* within a tumor and systemically.

The future of cancer gene therapy appears to rest on increased competence with *in situ* transduction; further advances in techniques to induce expression of transduced genes; and further advances in inducing significant antitumor responses at the systemic level. These, in turn, will probably rest on advancing our understanding of tumor immunology, strategies to limit angiogenesis, and continued development of safe and effective vectors to carry genes to directed sites[27-31].

GENE TRANSFER TECHNOLOGY

The gene delivery technology is developing rapidly and there have been specific developments that could be translated into gene-based therapies for gastroenterological diseases. For example, *ex vivo* transfer methods are being studied extensively by using hepatocytes obtained through liver biopsy, partial hepatectomy, and from specimens harvested for liver transplantation. Adult liver cells transiently undergo active proliferation permitting *in vitro* gene transfer even with vectors that require active cell division for entry and expression. Gene transfer may then be facilitated by a number of methods, including viruses, liposome, calcium phosphate coprecipitation, particle bombardment, naked DNA injection, and electroporation. The transfected cells are reintroduced into the host by using, for example, a microcarrier system into the peritoneum, gel beads, hepatocyte coated cell support matrix implanted next to liver tissue, or into the spleen or portal circulation through direct injection[2-5,32-40].

The spectrum of delivery systems for *ex vivo* gene transfer is broadly applied also to the *in vivo* model. Although the transfer efficiency of liposomes is low, these lipids can be made comparatively easily to high chemical purity and have low immunogenicity, which may permit repeated administrations. They have been used successfully in an *in vivo* model, by topical administration to epithelial cells both in the airways and the intestinal tract and also by the intravascular route. A recent study showed high efficiency transfer of the APC tumor suppresser gene in liposome complexes delivered to normal mouse colonic epithelium by rectal catheter infusion. Almost 100% of epithelial cells expressed the gene for up to four days, which

is consistent with the known rate of turnover of this tissue[41]. Intravenous injection of a rat insulin gene expression vector in liposome complexes results in uptake primarily by the liver and spleen. Improvement in hepatocyte uptake can be achieved by incorporating lactosyl ceramide into the phospholipid bilayer; this galactosyl terminal asialoganglioside is specifically recognized by a receptor highly selective for hepatocytes. Many different lipid agents are now being explored for efficacy of DNA transfer and it seems likely that the composition of the complex will have to be optimized for different targets and different routes of administration.

Of the available methods of gene delivery, viruses have been proved the most efficient so far. Achieving viral gene transfer to specific organs for clinical application will be difficult, however, particularly as viral titres 10 to 1000 times higher than those usually attained (typically 106 infectious units per milliliter) will be necessary for *in vivo* strategies. There is now extensive experience with retroviruses whose main advantages include their small size and easy manipulation, and with stable colinear integration with host genome. They are comparatively non-toxic and are efficient for gene transfer. Retroviruses persist in up to 5% of hepatocytes three months after injection of an infected hepatocyte cell suspension into the portal vein after partial hepatectomy. The small intestinal epithelium is an attractive target for gene therapy because of its large surface area, easy accessibility, and the presence of stem cells with known locations. Although few studies have yet targeted the intestinal system *in vivo*, marker genes have been transferred to the epithelial surface with retroviral vectors in animal models. Clearly, unless the therapeutic or marker gene is transferred to the stem cells, the rapid turn-over of this specialized epithelium would seriously limit potential benefits of delivered genes. Retroviruses have a number of disadvantages, notably the requirement for cells that are actively dividing to permit viral DNA integration, the ability to carry only small DNA sequences, and a small but finite risk of causing insertional mutagenesis as a result of random integration[42-47].

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

GENE THERAPY FOR GASTRIC CANCER

The generation of retroviral vectors that infect specific cell types through recognition of cell surface antigens is a promising and effective approach to targeted gene therapy of cancer. Carcinoembryonic antigen (CEA), a highly characterized, cell surface glycoprotein overexpressed by various tumor cells, provides a specific tool for tumor tissue-specific targeting by retroviral vectors. The conventional suicidal gene delivery systems need additional drugs other than their gene products. The inducible nitric oxide synthase (iNOS) gene product yields nitric oxide (NO), which directly induces autocytotoxicity and cytolysis of bystander cells. In the present study, we have developed a novel bifunctional Moloney murine leukemia virus-based recombinant retroviral vector that displays a chimeric envelope protein containing a single-chain variable fragmented (scFv) antibody to CEA and carries the iNOS gene in the genome. The resultant bifunctional retroviral vector showed a specific delivery of the iNOS gene to human CEA-expressing carcinoma cells, resulting in the direct and efficient killing of CEA-expressing carcinoma cells by induction of apoptosis. This is the first report of successful killing of CEA-expressing cells by specific targeting of the iNOS gene. This approach may offer a one-step procedure for effective gene therapy of CEA-expressing tumors[44,48].

This study used a recombinant antisense c-myc adenovirus (Ad-ASc-myc) to evaluate how alterations of c-myc expression in the SGC7901 human gastric carcinoma cells could influence the proliferation, apoptosis and the growth of human gastric tumors in nude mice[42,49]. The human gastric carcinoma cell line, SGC7901 cells, treated with Ad-ASc-myc or adenovirus recombinants carrying LacZ gene (Ad-LacZ) were analyzed by using X-gal stain, MTT, DNA ladder, TUNEL assay, flow cytometric analysis, polymerase chain reaction and western blot *in vitro*. The tumorigenicity and experimental therapy in nude mice models were assessed *in vivo*. The Ad-ASc-myc could strongly inhibit cell growth and induce apoptosis in SGC7901 cells. The proliferation of the Ad-ASc-myc-infected SGC7901 cells was reduced by 44.1%. The mechanism of killing gastric carcinoma cells by Ad-ASc-myc was found to be apoptosis, which was detected by the use of a DNA ladder, TUNEL and flow cytometric analysis. Infection of Ad-ASc-myc in nude mice showed that all three mice failed to form tumors from a 7-30 day period, as compared with injection of Ad-LacZ and parent SGC7901 cells. Experimental therapy on the nude mice bearing subcutaneous tumors of SGC7901 cells showed that intratumor instillation of Ad-ASc-myc inhibited the growth of the tumors. Recombinant antisense c-myc adenovirus-treated tumors were inhibited by 68.9%, as compared with tumors injected with Ad-LacZ and control (LacZ and phosphate-buffered saline). The expression of Ad-ASc-myc can inhibit growth and induce apoptosis of gastric cancer cells *in vitro* and *in vivo*, thus it is a potential clinical utility in gene therapy for the treatment of gastric carcinoma.

The oncofoetal antigen 5T4 is a 72 ku glycoprotein expressed at the cell surface. It is defined by a monoclonal antibody, mAb5T4, which recognises a conformational extracellular epitope in the molecule. Overexpression of 5T4 antigen by tumors of several types has been linked with disease progression and poor clinical outcome. Its restricted expression in non-malignant tissues makes 5T4 antigen a suitable target for the development of antibody directed therapies. The use of murine monoclonal antibodies for targeted therapy allows the tumor specific delivery of therapeutic agents. However, their use has several drawbacks, including a strong human anti-mouse immune (HAMA) response and limited tumor penetration due to the size of the molecules. The use of antibody fragments leads to improved targeting, pharmacokinetics and a reduced HAMA. A single chain antibody (scFv) comprising the variable regions of the mAb5T4 heavy and light a chain has been expressed in *Escherichia coli*. The addition of a eukaryotic leader sequence allowed production in mammalian cells. The two 5T4 single chain antibodies, scFv5T4WT19 and LscFv5T4, described the same pattern of 5T4 antigen expression as mAb5T4 in normal human placenta and by FACS. Construction of a 5T4 extracellular domain-IgGFc fusion protein and its expression in COS-7 cells allowed the relative affinities of the antibodies to be compared by ELISA and measured in real time using a biosensor based assay. The small size of this 5T4 specific scFv should allow construction of fusion proteins with a range of biological response modifiers to be prepared whilst retaining the improved pharmacokinetic properties of scFvs[50].

The assessment of the angiogenic profile of tumors may become an important tool as a guide for the inclusion of novel drugs and molecular therapies into the standard chemoradiotherapy policy. Several studies have shown the prognostic importance of microvessel density (MVD) and of angiogenic factor expression in operable gastric cancer. In the present study we investigated the MVD, with immunohistochemistry the expression of vascular endothelial growth factor (VEGF) and of thymidine phosphorylase (TP) expression as

well as the nuclear expression of p53 protein, in a series of patients with locally advanced inoperable gastric cancer. A strong association of VEGF with TP expression was noted ($P<0.01$), and tumors coexpressing these factors had a statistically higher MVD ($P<0.01$). Nuclear *p*53 accumulation was also related to a high MVD ($P<0.01$), and this was independent of VEGF or TP expression. Microvessel density showed a bell-shaped association with prognosis; cases with an intermediate MVD exhibit a favorable outcome ($P<0.05$). A trend of nuclear TP expression to define a group of patients with poorer prognosis was noted ($P>0.05$), while none of the remaining variables showed any significant association. The immunostaining results allowed the grouping of the angiogenic profile in four major categories: ① highly vascularized tumors with VEGF *and/or* TP expression (about 36%); ② highly angiogenic tumors with p53 nuclear accumulation and low VEGF/TP expression (7%); ③ poorly vascularized tumor with low VEGF/TP and negative nuclear p53 staining (32%) and poorly vascularized tumors with TP expression (7%). Specific therapies targeting hypoxia, VEGF, or TP expression as well as p53 gene therapy have entered clinical experimentation or are already available for clinical use. Using the suggested markers, more than 80% of locally advanced gastric carcinomas can be grouped in different categories according to their angiogenic profile. Such a categorization may be useful for phase III trials on novel therapies targeting the major angiogenesis-related features studied here[51].

Mammalian degenerin (MDEG) is a member of the amiloride-sensitive sodium ion channel family, and its site-directed active mutant (MDEG-G430F) induces massive Na+ influx into cells, leading to cell ballooning and cell bursting. We attempted a novel therapeutic approach for gastric cancers by transferring MDEG-G430F into cancer cells using tumor-specific promoters[52]. In carcinoembryonic antigen (CEA)-producing gastric cancer cells, the level of cell death observed when MDEG-G430F was used with a CEA promoter was similar to that observed when using a potent nonspecific promoter such as the cytomegalovirus promoter. In an *in vivo* study, fusogenic liposome complexes containing MDEG-G430F driven by the CEA promoter were injected intraperitoneally into CEA-producing gastric cancer cells in a mouse peritoneal dissemination model. Although all 15 of the control mice were dead by 50 days postinoculation, 13 of the 15 mice treated with MDEG-G430F survived. These results indicate that transferring MDEG-G430F into cancer tissues using tumor-specific promoters can achieve striking and selective cancer cell death irrespective of the transcriptional efficiency of the promoters used *in vivo*, and suggest that this approach is a promising new strategy for cancer gene therapy[38,41].

Lymph node metastasis is one of the prognostic factors in gastric cancer. Sunami *et al*[63] have previously reported that decreased intercellular adhesion molecule-1 (ICAM-1) expression on cancer cells is associated with lymph node metastasis using a gastric cancer cell. In this study, ICAM-1 gene into a gastric cancer cell line was transected, 2MLN, and analyzed the effect on lymph node metastasis *in vitro* and *in vivo*. A significantly greater amount of peripheral blood mononuclear cells (PBMC) adhered to ICAM-1 transfected 2MLN cells, 2MLN/ICAM cells, than to 2MLN/vector cells. The lysis of 2MLN/ICAM cells by PBMC was significantly increased as compared with that of 2MLN/vector cells. The tumor growth rate of 2MLN/ICAM cells was significantly decreased *in vivo*. Lymph node metastases caused by 2MLN/ ICAM cells were found fewer in number and smaller in size, while many lymph node metastases were caused by 2MLN cells. Histologic findings showed that leukocytes were heavily infiltrated in both the 2MLN/ICAM tumors and metastasis lesions, while only a few leukocytes were observed in the lesions associated with 2MLN cells. The above findings indicate that ICAM-1 gene transduction could prove to be an effective gene therapy for lymph node metastasis of gastric cancer.

The elderly population has much to gain from the advances of molecular medicine, although at present genetic pharmacology remains mostly at the conceptual level. Cancer, in particular, is an increasing health burden and the majority (over 70%) of gene therapy trials aimed at tackling this problem. Available strategies employ both viral and synthetic vectors with the selective delivery and expression of therapeutic genes a pivotal requirement. Clinical trials are now in progress with a view to modulating disease at many different levels, including the direct replacement of abnormal genes. Suicide-gene formulations, and the delivery of "gain of function" genes, which seek to alter the malignant phenotype by indirect means, such as, immunopotentiation and stromal reorganisation. Early data from these studies is tantalising and we must remain optimistic that gene therapy will benefit the patients with cancer by both reducing morbidity and extending life[64].

The antitumoral effects of antisense RNA to K-ras were investigated in gastric cancer cell lines by examining the level of K-ras expression and the tumorigenicity *in vitro* and *in vivo*. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), DNA sequencing, and immunoblotting analysis revealed that YCC-1 gastric cancer cells overexpressed wild type K-ras, whereas YCC-2 cells had a homozygous mutation in codon 12 from GGT (glycine) to AGT (serine), while SNU-1 cells had a heterozygous mutation to GAT (asparagine) in the identical position. Both YCC-1 and YCC-2 cells were transduced by LNC-AS/K-ras containing the antisense 2.2 kb genomic K-ras DNA fragment covering exon 2 and exon 3 specific for K-ras. The application of antisense K-ras significantly downregulated the expression of K-ras and had no influence on the expression of either H-ras or N-ras. The antisense-transduced YCC-2 cells grew considerably slower than the control group transduced by LNCX, whereas the growth inhibition of antisense-transduced YCC-1 cells was less prominent than that of transduced YCC-2 cells. In addition, the tumorigenicity of YCC-2 cells transduced by LNC-AS/K-ras was totally lost. Therefore, our results imply that the specific inhibition of K-ras p21 protein can be accomplished by introducing the antisense covering the K-ras-specific region to gastric cancer cells with aberrant K-ras expression, resulting in a reduction of the growth rate and suppression of tumorigenicity[65]. Dysregulation of apoptosis may be closely related to the development of cancer and its chemoresistance. Overexpression of Bax, an inducer of apoptosis, has led to increased cell death in a variety of cancer cell lines. In this study, we investigated the effect of Bax overexpression in two gastric cancer cell lines, MKN-28 and MKN-45, using a Cre-loxP-mediated inducible expression system. After induction of bax, both cell lines showed decreased proliferation, partially due to increased cell death. Furthermore, Bax-expressing MKN-28 cells were more sensitive to cisplatin. These results indicate that up-regulation of the bax gene may provide a novel strategy for the treatment of gastric cancer[66].

In an attempt to obtain suitable *in vivo* models for optimizing new tumor therapy strategies for intestinal adenocarcinomas, carcinoembryonic antigen (CEA) promoter/SV40 T antigen gene constructs have been used to generate transgenic mice[67]. One transgenic line (L5496), which contains a 424-bp CEA promoter/SV40 T antigen transgene, exclusively developed multi-focal carcinomas in the pyloric region of the stomach in 100% of the offspring. Tumors were already observable in 37-day-old animals as dysplastic cell foci within the mucosal layer. In 50-day-old mice, the tumor mass was mainly restricted to the mucosa with invasive growth into the submucosal tissue. The animals became moribund at 100-130 days of age due to blockage of the pylorus. At this time, the tumor had penetrated into the duodenum and had invaded all tissue layers within the stomach. In contrast to most other stomach tumor

models, this one perfectly matches the development of the most common stomach cancers found in humans. Furthermore, after crossing these mice with mice that are transgenic for the human CEA gene, the double transgenic offspring revealed expression of CEA in the resulting tumors. Thus, being a model for studying gastric carcinoma development and prevention, this system should provide a useful preclinical model for CEA-targeted gastric tumor therapy[31,37].

PROSPECT IN THE FUTURE

Recent scientific breakthroughs in the genomic field and our understanding of the important role of genes in disease has made gene therapy one of the most rapidly advancing fields of biotechnology with great promise for treating inherited and acquired diseases. Many human diseases are caused by the absence or inappropriate presence of a protein. The first promise of biotechnology was to isolate and produce these natural proteins through genetic engineering and recombinant technology. The protein could then be administered to patients in order to compensate for its absence. Because proteins are not orally available, biotech companies focused on innovative methods of protein delivery and sustained drug delivery. Today, gene therapy is the ultimate method of protein delivery, in which the delivered gene enters the *body's* cells and turns them into small "factories" that produce a therapeutic protein for a specific disease over a prolonged period[82-93].

As gene therapy has shifted from the laboratory into the clinic, several issues have emerged as central to the development of this technology: gene identification, gene expression and gene delivery. Academic researchers supported by the *government's* Human Genome Project and more recently through genomics companies originally tackled gene identification. A number of disease-related genes with direct clinical have already been identified, and this number is growing as the field rapidly advances. Some of these genes are in the public domain and some are proprietary. Genes with broader clinical application are also being utilized to make cells express immune activating agents locally at the disease site or to become susceptible to further drug treatment or to immune response recognition[76-84,100-112].

The control of gene transcription is extremely complicated. For the most intensely investigated systems such as the globin genes, our understanding is still fragmental. While most protocols presently use strong viral promoters to drive expression of recombinant cDNA copies of therapeutic genes, future work must be directed to defining the genomic elements that enable temporal and spatial control of expression through a lifetime. The identification of locus control regions that can insulate gene clusters from interference by surrounding genetic influences has been an important step, and many investigators are now working to understand how the promoter and enhancer/silencer elements of a gene interact with structures within the nucleus. Advances in this area will require parallel developments in the sophistication of vector design before they can be transferred into practice[95-104].

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Oesophageal surgery

Erik J. Simchuk, Derek Alderson

University Department of Surgery, Bristol Royal Infirmary, Bristol BS2 8HW, UK
Correspondence to: Erik J. Simchuk, M.D., University Department of Surgery, Bristol Royal Infirmary, Bristol BS2 8HW, UK
Telephone: +44-117- 926 0601, Fax: +44-117- 925 2736
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INTRODUCTION

The origins of oesophageal surgery, like most surgical treatments, are based in the treatment of traumatic injury. The *Smith Surgical* Papyrus describes the examination, diagnosis and treatment of "a gaping wound of throat, penetrating the gullet"[1]. This article focuses on the important conditions facing the oesophageal surgeon today, with an emphasis on those where there have been significant changes in our understanding of disease or approaches to treatment.

BENIGN OESOPHAGEAL DISEASE: SURGICAL PRINCIPLES AND PRACTICE

Oesophageal motility disorders: Achalasia

Pathogenesis While the exact cause remains obscure, achalasia is clinically characterized by a triad of findings: oesophageal aperistalsis usually with atony, varying degrees of oesophageal dilatation, and failure of normal lower oesophageal sphin cter (LOS) relaxation in response to deglutition. It has been recognized recently that symptoms (particularly pain) can occur when the oesophagus remains undil ated and still exhibits contractions which are frequently of high amplitude, although it should be stressed, non-peristaltic. This is called vigorous achalasia. Most theories surround the concept of a neurogenic origin for achalasia, due to a loss of ganglion cells in Auerbach's plexus[2]. The cause remains obscure. Recent evidence suggests that achalasia may be part of a spectrum of oesophageal motility disorders with potentially common or similar aetiologies including diffuse oesophageal spasm (DOS) and nutcracker oesophagus[3].

Diagnosis The onset of symptoms is typically insidious and diagnosis is commonly made months to years later. Dysphagia is the most common presenting symptom, occurring in over 80% of patients. As the disease progresses, oesophageal dilatation occurs, which may lead to regurgitation and aspiration, resulting in pulmonary complications.

A clear diagnosis of achalasia can rarely be made on clinical grounds alone, and this usually relies on a combination of contrast radiography, motility studies, and endoscopy. Contrast oesophagography characteristically demonstrates a dila ted, possibly tortuous oesophagus, with smoothly tapered "birds beak" narrowing at the OGJ. Endoscopy may be indicated to ensure that no mucosal lesion is present and rule out the presence of a neoplasm in the lower oesophagus which can lead to a manometric pattern indistinguishable from achalasia (pseudoachalasia[4]). Manometry demonstrates absent relaxation of the LOS during wet and dry swallows, as well as usually simultaneous, low amplitude contractions in the body of the oesophagus. Although multiple medications have been tried for achalasia, including calcium channel blockers, nitrates, anticholinergics, prostaglandins, aminophylline and beta-agonists, none provide long-term symptomatic improvement. The intramuscular injection of botulinum toxin into the LOS was popularized in 1994. The toxin directly inhibits the release of acetylcholine from nerve terminals, causing the sphincter fibers to relax. Long term results have been disappointing. Of 28 patients enrolled in one study[5], only 20 experienced a remission lasting longer than 3 months, with most requiring additional treatments. Various strategies to improve results have not led to sustained relief of symptoms. Botulinum toxin injection is probably best regarded as an alternative to pneumatic dilatation in those who are not candidates for surgery.

The use of rigid mechanical dilators has given way to hydrostatic and pneumatic balloon dilators. Sanderson reported improved results in a cohort of 457 patients when successive dilatations were used[6]. Perforation occurs in 1%-5%, leading to a mortality rate of about 0.3%[7]. Balloon dilatation has compared favorably to oesophagomyotomy in some reviews[8], although in the only prospective, randomized trial to date, conducted by Csendes *et al*, oesophagomyotomy achieved 98% good to excellent results compared to just 68% good to excellent results after balloon dilatation[9]. Many authors have, however, considered that this was optimal surgery versus sub-optimal endoscopic therapy due to the nature of the "bag" used for dilatation. Since Heller described oesophagomyotomy, surgery has remained the standard against which all other methods are compared. The myotomy extends for approximately 5 cm to 6 cm above the oesophagogastric junction. It does not need to extend for any great length on to the stomach[10,11]. Intraoperative oesophagoscopy is helpful in assessing the proper proximal and distal extent of the myotomy, allowing the surgeon to accurately visualize the level of the squamocolumnar junction, and test the myotomy by air insufflation.

When performed through the abdomen, there seems to be a higher incidence of pathological gastroesophageal reflux than seen after a thoracic approach. DeMeester and colleagues reported an approximately 12% incidence of pathologic reflux after successful myotomy, reduced to 4% with the addition of fundoplication[12]. Surgeons who advocate the use of a Toupet fundoplication, argue that as it lies posterior and is sewn to the leading edges of the myotomy it allows the mucosa to bulge, keeping the myotomy pulled open so that it is less likely to scar. While reasonable arguments exist for the application of the Dor, Toupet, or Belsey partial fundoplications, none has been shown to have a clear advantage over the others. With the advent of minimally invasive surgery, thoracoscopic and laparoscopic approaches have become popular. Advocates of a thoracoscopic approach have propos ed that the chest offers a better view of the oesophagus in order to achieve a long myotomy[13], although difficulties extending the myotomy onto the anterior wall of the stomach may leave the muscular sling at the gastric cardia intact resulting in an ineffective myotomy and a higher incidence of post operative dysphagia[14]. Most early advocates of the thoracoscopic

approach have converted to laparoscopy.

In any event, the limited surgical morbidity afforded with a minimally invasive approach to oesophagomyotomy and the excellent results achieved have led to a change in the clinical decision making regarding the management of the patient with achalasia. Patients and physicians who once shunned the morbidity and time of recovery from open surgical treatment may consider a laparoscopic procedure to avoid the long term complications of achalasia.

Diffuse oesophageal spasm (DOS) The hallmarks of DOS are intermittent and unpredictable chest pain and non -progressive dysphagia. Dysphagia may be associated with chest pain, which is spontaneous, reproducible, retrosternal in location and may radiate to the left arm or neck. The dysphagia and chest pain may be triggered by certain hot or cold foods, carbonated beverages, or stress. Before the development of manometry, contrast oesophagography was the meth od of choice in making the diagnosis of DOS. A barium swallow may demonstrate s imultaneous, nonperistaltic contractions, with segmentation of the barium column, giving the characteristic "corkscrew" appearance to the oesophageal body, but is unreliable as these abnormalities exist in only a minority of patients[15]. While manometry remains the preferred method of establishing the diagnosis of DOS, disagreement exists over a precise definition. The most widely accepted manometric definition of DOS is two or more swallows resulting in simultaneous contractions in a series of ten wet swallows (the rest being peristaltic). Neither amplitudes, nor duration considered [16]. Recent authors have advocated the use of 24 hr ambulatory manometry, as DOS commonly produces intermittent symptoms that can be correlated with abnormal motility findings during a 24 hour study. An alternative and perhaps more acceptable definition of DOS is "the presence of multi-peaked contractions over at least 10cm of the oesophageal body lasting longer than 15 seconds and with maximum amplitudes of greater than 200 mmHg. Some of these spasm contractions should produce symptoms of ches t pain and/or dysphagia"[17]. Management of DOS is directed at symptom relief. Medical management has included the use of sublingual or oral long acting nitrates, calcium channel blo ckers, and hydralazine, all of which can provide some symptomatic and manometric improvement. Balloon dilatation has been shown to significantly relieve the severity of DOS symptoms in 70% of patients in whom initial medical management proved unsuccessful[18]. The surgical management of DOS is based on oesophagomyotomy, its length being de termined by the extent of manometric abnormalities. Most authors recommend carrying the myotomy through the LOS onto the gastric cardia. In carefully selected patients, the results have been excellent, with significant improvements (P<0.01) in symptoms, and a 93% effective palliation of dysphagia[19]. With the advent of minimally invasive surgery in the last several years, more surgeons are approaching DOS with a thoracoscopic long oesophageal myotomy, although there are no long-term results as yet.

Nutcracker oesophagus High amplitude peristaltic contractions were first described by Brand and collea gues in patients with noncardiac chest pain and dysphagia, and were confirmed by Benjamin and associates in 1979, who coined the term "nutcracker esophagus"[20], on the assumption that these contractions were the cause of symptoms. The manometric definition states that average peristaltic pressures are at least two standard deviations above the normal value, usually defined as 180mmHg[18]. The aetiology of nutcracker oesophagus is unclear. A psychological component has been implicated, and it has been reported to evolve eventually into diffuse oesophageal spasm or achalasia. It is the most common motor disorder diagnosed in patients with noncardiac chest pain.

Management focuses on symptom relief, and like DOS, many patients obtain relief with nitrates and calcium channel blockers, although dilatation seems ineffective. A few case reports have shown positive results after myotomy whose symptoms were not relieved with medical management, although there are no long-term results.

Gastroesophageal reflux disease and hiatus hernia

Gastroesophageal reflux disease (GORD) has become the most prevalent upper gastrointestinal disorder in the west. In 1946, Allison coined the term "ref lux oesophagitis" after identifying the fundamental pathophysiologic process resulting in the inflammation found at the gastroesophageal junction[21]. Since then, it has become clear that injury due to reflux may extend beyond the oesophagus to the larynx and lungs, and heartburn can occur without the changes in the oesophageal mucosa that define reflux oesophagitis. Thus, the broader term GORD was coined to describe any symptomatic condition or pathologic alterat ion that occurred as a result of reflux[22]. Although GORD is thought to account for approximately 75% of oesophageal disease in clinical practice, the epidemiology is not well defined, as no precise definition exists, nor is there a gold standard diagnostic procedure. Heartburn is experienced by approximately 20% to 40% of the western population[23], although the true prevalence is almost certainly higher than reported, as many sufferers treat themselves. Incidence increases with age in both sexes. Many foods exacerbate symptoms including coffee, chocolate, peppermint, and dairy products. Tobacco use and obesity are also related to increased incidence of GORD[24].

Symptoms are not reliable predictors of the presence of GORD. Only 60% of patients with "heartburn" have abnormal 24 hr pH and manometry testing, while chronic heartburn and regurgitation can be present without evidence of mucosal damage on upper endoscopy[25]. Complications develop in approximately 50% of patients with abnormal gastroesophageal reflux by pH testing including oesophagitis, oesophageal stricture, Barrett's metaplasia, and pulmonary disease[26].

Pathogenesis The anatomic and physiologic barriers to the development of GORD involve a lower oesophageal high pressure zone/sphincter (LOS), a gastroesophageal junction that is located intrabdominally, an anatomically intact gastroesophageal flap valve, an intact esophageal clearance mechanism that efficiently evacuates refluxed gastric contents from the oesophageal lumen, and a stomach that empties properly. The role of hiatus hernia in GORD has been controversial, although it clearly alters the anatomic relationship between the OGJ and diaphragmatic crura, and may contribute to LOS incompetence. Patients with large hiatus herni as have been demonstrated to have lower LOS pressures, be exposed to more reflux than patients with no or smaller hiatus hernias, and have prolonged acid exposure in t he oesophageal lumen, which may be secondary to impaired oesophageal clearance, rather than an increased number of reflux episodes[27]. Impaired oesophageal clearance has been demonstrated to correlate with inc reasing severity of inflammation[28]. As oesophageal clearance becomes increasingly impaired, even effective acid suppression may not be enough to reverse the mucosal injury in some patients, and for this reason it has been argued that surgical correction of a dysfunctional LOS should be considered before peristaltic function becomes impaired[29].

Diagnosis and treatment Oesophagoscopy provides the opportunity for the diagnosis of complications of GORD, including the identification of the presence of Barrett's metaplasia. Further

evaluation may involve 24 hour pH and manometry testing, to confirm the diagnosis of GORD and identify motility abnormalities.

The indications for surgery vary, but most recommend antireflux surgery in patients with symptoms refractory to medical management, and when complications exist such as stricture formation, bleeding, or pulmonary manifestations due to regurgitation and aspiration. It is important to recognize that most of the conditio ns which complicate gastroesophageal reflux disease are of a benign nature.

Many operations have been devised to treat GORD. While no single technique can guarantee excellent results under all circumstances, all involve reduction of any hiatus hernia and crural repair. The Nissen fundoplication is undoubtedly the most commonly employee and widely known antireflux procedure in the world. Multiple modifications to the original operation have been made since its original description by Nissen in 1956[30].

The Nissen-Rosetti operation incorporates a 360° fundic wrap without ligation of the short gastric vessels for simplicity and ease of dissection. The Rosett imodification proved quite effective, although postoperative morbidity with regard to dysphagia, gas bloat, and inability to belch are higher (8%) than the later "floppy" Nissen (3%), potentially reflecting the difference when a lower part of the fundus/ greater curve must be used in the wrap and the short gastric vessels not divided[31]. When performed correctly and for the right indications, long term outcomes with the "floppy" Nissen fundoplication have been excellent, with the alleviation of reflux and its symptoms in approximately 85%-90% of patients at 10 years, with morbidity including dysphagia, gas bloat, and inability to belch in the range of 3%-10%[32].

A variety of incomplete (<360°) wraps have been described (Toupet, Lind, Wats on). Historically, the most widely used was the 240° vertical fundoplication developed by Belsey and performed via a left thoracotomy. All produce excellent results in the hands of experienced surgeons with similar results to total fundiplication[33-35]. Hill championed the importance of the gastroesophageal flap valve as one of the chief natural barriers to reflux. He described a musculomucosal valve created by the angle of His, acting to prevent reflux by closing against the lesser curve with increased gastric pressure. The original Hill repair reconstructed the oe sophagogastric junction by posteriorly fixing it to the median arcuate ligament and accentuating the angle of His, thereby reestablishing the gastroesophageal flap valve apparatus[36].

The Hill operation has not enjoyed the popularity of the Nissen, probably as a result of the perceived difficulty in defining the median arcuate ligament. Vansant simplified the dissection in 1976[37], while further modifications have adopted the use of the preaortic fascia and condensation of the crus as the anchor for the repair. Long-term results with the modern Hill antireflux operation have been excellent, in one of the longest postoperative follow-up studies in antireflux surgery, after 15 to 20 years, 88% of patients had good to excellent outcomes[38].

With the development of minimally invasive techniques, it became possible to perform antireflux operations laparoscopically. Isolauri and associates demonstra ted that ten years worth of a proton pump inhibitor will cost 10 times more than a laparoscopic Nissen fundoplication, including the preoperative evaluation and postoperative care[39]. An initial report by Dallemagne[40] led to the rapid popularization of laparoscopic Nissen fundoplication throughout the world. Hinder reported on 198 patients who underwent laparoscopic Nissen fundoplication from 1991 to 1994, the main indication for surgery being symptom s refractory to medical management. The outcome for most patients was excellent , although nearly a third had problems with postoperative dysphagia or gas bloat[41]. Watson reported on 230 laparoscopic Nissen fundoplications, with a median follow-up of 16 months with symptom relief in 88%[42]. Whether the long term results of laparoscopic fundoplication will match those of open surgery remain to be seen, but initial results appear favourable when the surgery is performed at high volume centres.

Although laparoscopic antireflux surgery has proved successful for patients with small hiatus hernias, larger hiatus and paraesophageal hernias pose additional problems. The technical imperatives, as outlined by Horgan for open surgical technique, apply equally well to laparoscopic repair and include transhiatal oesophageal mobilization with reduction of the oesophagogastric junction into the abdomen, complete reduction of the hernia sac, effective crural closure, attention to the geometry of the fundoplication, and an anchored repair[43].

Although most series have demonstrated good to excellent results[44-46] they have been criticized for their short follow-up periods and lack of objectivity. Hashemi and associates demonstrated a concerningly high recurrence rate in patients who underwent laparoscopic repair of paraesophageal hernias. Routine postoperative contrast oesophagography demonstrated a 43% recurrence rate, compared to 15% following open surgery at 5 years[47], prompting many surgeons to discontinue the laparoscopic approach to large hiatus and paraesophageal hernias. While the presence of a large hiatus or paraesopha geal hernia is not a contraindication to laparoscopic repair, the high recurrence rate reported in the recent literat ure should prompt responsible surgeons to follow patients objectively while more data are obtained, preferably through prospective trials.

OESOPHAGEAL CANCER

The German-born surgeon, Dr. Franz Torek, working in New York in 1913, performe d the first successful transthoracic resection of the oesophagus for carcinoma[48]. In 1933 Grey Turner successfully resected an intra-thoracic oesophageal tumor by the trans-hiatal route, and in the same year Ohsa wa reported success in eight patients undergoing transthoracic oesophageal resec tion with intrathoracic oesophagogastrostomy for carcinoma of the lower oesophagus[49]. While refinements in techniques reduced morbidity and improved outcomes in the latter part of the 20th century, surgical treatment continues to be viewed unfavorably, mostly due to high operative mortality and poor survival statistics as documented by Earlam[50] and Muller[51]. Recent advances in perioperative care, more accurate staging techniques, better patient selection and the addition of chemo-radiotherapy have led to decreased perioperative mortality and improved survival rates, which has challenged the pessimistic attitudes toward oesophageal resection.

Pathogenesis Oesophageal carcinoma has a unique and changing epidemiologic pattern. Ge ographic variation is remarkable with 5 cases per 100 000 population in the United States, 7-10 cases per 100 000 in the United Kingdom, to over 500 cases per 100 000 population in parts of Iran, China, and Russia, leading most to believe that environmental causes play a large role in the development of oesophageal carcinoma[52]. Factors associated with an increased risk of oesophageal cancer include age, gender, race, excessive tobacco and alcohol use, diet and a variety of nutritional deficiencies including retinoic and ascorbic acids, riboflavin, and zinc. In areas where oesophageal carcinoma is endemic, diets appear to be low in fruit, vegetables, minerals, and vitamins A, C, and riboflavin[53].

Another remarkable feature of oesophageal cancer is the recent change in histologic pattern. While 20 years ago adenocarcinoma made up just 10% of oesophageal cancers, it now represents approximately 50% to 70% in the western world, virtually all due to a marked increase in the incidence of adenocarcinoma. While the exact reason for this is unknown, it has something to do with the rising incidence of Barrett's metaplasia, a condition which occurs as a response of the lower oesophageal epithelium to chronic

gastroesophageal reflux. The associ ation of specialized Barrett's oesophagus with an increased risk of developing oesophageal adenocarcinoma is well documented. Further understanding of the biology of oesophageal cancer, coupled with the identification of significant risk factors, such as Barrett's oesophagus, have permitted the surveillance of high-risk patients and the identification of early stage disease. The impact of this on future survival remains to be seen.

Diagnosis and staging The decision to consider a patient for curative therapy is essentially influence d by fitness to withstand treatment aimed at cure and accurate staging. At presentation, a careful history and physical exam may immediately reveal that the disease is beyond cure. Severe weight loss, spinal pain, recurrent laryngeal nerve palsy, or lymphadenopathy all indicate advanced disease. A chest x-ray may demonstrate mediastinal enlargement, tracheal deviation, a dilated fluid filled oesophagus, pleural effusions, or nodular lung involvement; all indicating advanced disease. Flexible endoscopic examination permits tumour visualization and biopsy. Contrast oesophagography can be used to confirm tumour location and length, and may be particularly useful in the detection of fistula formation or in clarifying anatomy in patients who have undergone previous gastric surgery.

Although computed tomography is not useful in staging early carcinomas of the oe sophagus[54], it can define the extent of more locally advanced disease as well as identify distant metastases. Patients with haematogenous metastases are incurable. With the advent of endoscopic ultrasound, the ability to more accurately define the locoregional extent of oesophageal carcinoma has improved significantly. Endoscopic ultrasound provides an accurate assessment of oesophageal wall p enetration (T-stage), as well as peri-tumoral and regional lymph node status (N-stage). Nodal positivity is the single factor which correlates most with the chance for cure[55], and the detection of contiguous organ invasion has made "open and close" or by-pass surgery virtually obsolete. Multiple reports have demonstrated an overall accuracy of approximately 90% for T-staging and approximately 80% to 85% accuracy for N-staging[56-58]. In addition, thoracoscopic and laparoscopic techniques are being more widely used to exclude pleural or peritoneal metastases again with accuracy approaching 90%[59].

Surgical treatment and results Exactly what constitutes an adequate resection margin or extent of lymph node dissection is widely debated, but it is evident that prolonged survival in oesopha geal carcinoma depends principally on the completeness of surgical resection as well as the pathologic stage of disease. Most surgeons recommend removing at least a 5 cm margin of normal oesophagus proximal to the lesion with surrounding pleura and fibro-areolar tissue along with the entire distal oesophagus and cardia. More proximal oesophageal tumors require a total thoracic oesophagecto my and cervical anastomosis for adequate resection margins, while more distal oesophagogastric junction tumors require removal of more stomach, especially along the lesser curve. A number of techniques have been developed for oesophageal resection, and while there does not appear to be a substantial difference in the overall mortality be tween procedures, the type and degree of morbidity does vary. The most popular approach in the west is the two-phase (abdomen, right chest) operation popularized by Lewis[60]. While devised principally for mid-oesophageal tumors, it is equally useful for distal tumors as well in providing adequate resection margins. A three-phase oesophagectomy combines a cervical incision for total thoracic oesophagectomy and cervical oesophagogastric anastomosis[61].

Transhiatal oesophagectomy involves the resection of the intrathoracic oesophagus through the oesophageal hiatus and the thoracic inlet with a cervical oesopha gogastric anastomosis. Most of the operation can be done under direct vision through the diaphragmatic hiatus, although the mid and upper thoracic dissection is done bluntly, with risk of tracheobronchial or great vessel injury. Many surgeons believe transhiatal oesophagectomy to be an appropriate choice only for distally located tumours due to the risk of radial margin positivity and the inability to perform an adequate intrathoracic lymph node dissection with mid-oesophageal tumours.

A left thoracoabdominal oesophagectomy is also widely used for lower oesophageal tumors. Anastomosis may be performed in the left chest or after further mobilization of the upper oesophagus a cervical oesophagogastric anastomosis can be fashioned. The incidence of symptomatic reflux is highest following 'low' anastomosis in the left chest and diminishes with higher anastomoses. There are relatively few series that have compared right and left thoracotomies for oesophageal resection, although Launois and associates demonstrated similar operative mortality and survival rates for both approaches[62]. The choice of conduit for reconstitution of gastrointestinal continuity is largely based on surgical preference, as there have been no controlled clinical trial s comparing techniques. Stomach, colon and jejunum have all been used successfully. The easily mobilized stomach has been found by many surgeons to be the best functional replacement for the resectedoesophagus. There is no standard for performance of the oesophagogastric anastomosis; the advent of circular stapling devices may have resulted in a decrease in anastomotic leak rates. Stricture rates of approximately 5% to 10% are seen with handsewn anastomotic techniques and medium sized stapling devices, although this can be as high as 30%-40% with smaller stapling anvils[63]. Stricturing occurs more commonly with cervical than intra-thoracic anastomoses irrespective of technique[64].

Emptying of the gastric conduit, occurs mainly as a gravity dependent mechanism with very little propulsive action in the gastric tube[65]. While there is continued debate regarding the need for a gastric emptying procedure, randomized studies do not support the use of pyloroplasty or pyloromyotomy to improve postoperative function. Gastric outlet obstruction after gastric pull-up may be due to axial rotation of the gastric tube and not pyloric function, thus, close attention should be paid intraoperatively to maintaining the correct anatomic orientation of the conduit. The extent of lymphadenectomy performed during oesophageal resection for carcinoma is highly variable. While no prospective trial has been performed in the was tern hemisphere demonstrating improved survival with more extended lymphadenecto my, the "three field" lymph node dissection has been claimed by Japanese autho rs to contribute to their improved survival statistics. Advocates of standard oesophagectomy argue that once an oesophageal carcinoma has penetrated the oesophageal wall and involved peritumoral lymph nodes, surgical cure is unlikely. Skinner, however, demonstrated that the removal of involved regional lymph nodes will improve survival rates[66], while Sasaki and colleagues showed that patients who underwent extended lymphadenectomy with oesophageal resection had significantly improved survival compared to patients who underwent more limited node dissection[67].

A standard lymphadenectomy in oesophageal cancer should include all paraesophageal, subcarinal, left gastric, common hepatic, proximal splenic, and crural nodes . A proper oncologic resection with adequate lymphadenectomy might improve survival, but it will certainly lead to more accurate staging and avoid the error of "improved" survival figures which reflect the stage migration inherent in a more accurate nodal assessment. Extended lymphadenectomy, however, does potenti ally increased morbidity, particularly when applied to nodes along the recurrent laryngeal nerve chains[68].

Postoperative mortality in oesophageal resection has decreased from approximatel y 25% to less than 8% in the past two decades. While complications have also decreased, a third of patients can be expected to have a major complication after surgery, including arrythmias, pulmonary embolus, pneumonia, or anastomotic leak . Most patients resume a normal diet within three to six months following surgery and maintain their weight at approximately 90% of normal. While surgery remains the mainstay of therapy for oesophageal carcinoma, there is considerable cur rent interest in combined modality approaches.

Loco-regional disease is a significant cause of morbidity and mortality, but even when this is controlled, most patients eventually die of metastatic disease. Combined modality therapy aims to improve local control, eliminate micrometas tatic disease and improve cure rates. Adjuvant systemic chemotherapy has not been shown to reduce the risk of metastases in randomized studies[69,70]. While some studies have shown a statistically significant reduction in loco -regional recurrence with the use of post-operative radiation, this also has not translated into a survival advantage[71].

Most enthusiasm now focuses on the use of neo-adjuvant multimodal therapy designed to reduce the size of the primary tumor to potentially improve the curative resection rate, and expose micrometastases to systemic chemotherapy at an early stage. Randomized trials comparing preoperative chemoradiation plus surgery to surgery alone vary considerably with regard to chemotherapy regimens, radiation doses, histologic type of cancer, and numbers of patients accrued making conclus ions difficult. Trials conducted by Walsh *et al*[72] and Forastiere *et al*[73] have demonstrated either a statistically significant survival advantage or trend toward improved survival with preoperative chemoradiation, while others have not[74]. While it is clear that some oesophageal carcinomas respond dramatically to preoperative chemoradiation, further prospective randomized trials are needed to see if this will translate into a clear survival advantage.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Liver resection for cancer

RW Parks, OJ Garden

Department of Clinical and Surgical Sciences (Surgery), University of Edinburgh, UK
Correspondence to: RW Parks, MD FRCS, Senior Lecturer in Surgery, Department of Clinical and Surgical Sciences (Surgery), University of Edinburgh, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh, EH3 9YW, UK. r.w.parks@ed.ac.uk
Telephone: +44-131-536 3817, Fax: +44-131-226 2881
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HISTORY OF HEPATIC RESECTION

The earliest hepatic surgery was almost exclusively performed for trauma with records from as far back as 1870[1,2]. Among the earliest liver resections performed for tumor were those of Langenbuch in 1888[3], Tiffany in 1890[4], and Lucke in 1891[5]. By 1899, 76 cases of liver resection had been reported with a mortality rate of 14.9%[6], a remarkably low figure for operations of this magnitude, all performed at the end of the 19th century. Wendel undertook the first anatomical resection for liver cancer in 1911 when he performed a right lobectomy. The patient who had a primary hepatocellular carcinoma survived for 9 years following the resection[7]. The earliest methods of achieving haemostasis were by electrocautery, elastic tourniquet, and suturing with flexible blunt needles. In 1902, Pringle described compression of the portal triad as a technique to reduce bleeding. A year later Anschutz described finger fracture although this was popularized much late r by Lin[8]. Over the past 50 years, the basic techniques of liver surgery have been refined and developed. Improved survival and reduced mortality rates associated with hepatic resection have resulted in a broader application of these operations, nonetheless, the majority of procedures are performed with a curative intent, although occasionally a palliative procedure may be considered.

BASIC HEPATIC ANATOMY

Precise knowledge of the surgical anatomy of the liver is essential before embar king on hepatic resection. The liver is supported beneath the diaphragm by the reflections of its visceral peritoneum, namely the right and left coronary ligaments, the left triangular ligament and the falciform ligament. There have been many descriptions of the internal architecture of the liver, but that reported by Couinard in 1957[9] is the most widely recognized and remain s the most clinically useful description for the hepatic surgeon.

The anatomical divide between the right and left liver is not at the falciform ligament but in a plane which runs from the gallbladder fossa to the inferior vena cava and is known as the midplane of the liver (the principal plane or Cantlie's line). Within this imaginary plane runs the middle hepatic vein which drains into the vena cava at a common confluence with the left hepatic vein. The right and left hemilivers are themselves further divided by the right and left hepatic vein and the right and left branches of the portal vein. Couinaud identified eight segments in the liver, each supplied by its own portal venous and hepatic arterial pedicle and each drained by a single bile duct. Terminology for various anatomical portions of the liver and the surgical removal of these portions continues to evolve. The Terminology Committee of the Intern ational Hepato-Pancreato-Biliary Association recently published a description of the newest terminology of hepatic anatomy and liver resections[10]. It had no responsibility to investigate anatomy per se and based its report on accepted internal liver anatomy. Right hepatectomy (or hemihepatectomy) and left hepatectomy (or hemihepatectomy) are the appropriate terms for removal of the right and left sides of the liver respectively. Any individual anatomical segment of the liver can be removed, the procedure being referred to as a segmentectomy. Groupings of segments can also be removed and these procedures may be referred to as sectionectomies (or sectorectomies) (Figure 1 a,b).

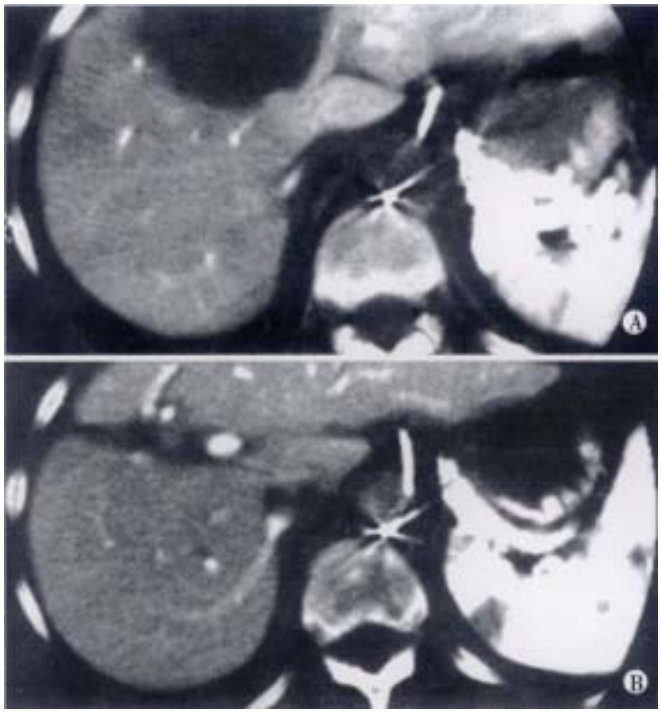

Figure 1 (a) Solitary hepatic metastasis occupying segment 4, 5 and 8, managed by trisegmental resection. (b) CT scan at one year showing hypertrophy of residual left lobe and segments 6/7.

PREOPERATIVE ASSESSMENT

The aim of preoperative investigations should be to determine the nature of the lesion and assess whether it is resectable. Furthermore, the relationship of the tumour to the hepatic vasculature will aid a decision as to the probable surgical procedure.

Liver function tests are frequently abnormal in patients with malignant liver disease and particular attention should be paid to the coagulation profile. Hepatitis B and C antigen screening should be undertaken in view of the associat ion between primary hepatocellular carcinoma and hepatitis. Elevation in tumour markers such as carcinoembryonic antigen (CEA) or alphafetoprotein (AFP) may point towards a diagnosis of colorectal liver metastasis or primary

hepatocellular carcinoma and will serve as a useful baseline for further follow-up.

Characterisation of hepatic lesions is provided by radiological imaging of the liver. Ultrasonography (US) and computed tomography (CT) are the cornerstone of diagnosis and often complement one another. However, magnetic resonance imaging (MRI) is becoming more widely available and may supersede these imaging techniques as the principal radiological investigation. A particular advantage of MRI is its ability to show major blood vessels accurately and demonstrate their relationship to tumour masses. Abdominal ultrasonography gives information regarding the number and size of hepatic lesions and will distinguish liver cysts from solid tumours. If ultrasonography shows multiple solid lesions suggesting incurable malignancy, a biopsy may be performed during the same session to obtain a pathological diagnosis. Duplex ultrasonography may provide additional information regarding involvement of majo r blood vessels and may be particularly useful in the preoperative evaluation of hilar cholangiocarcinoma[11].

Computed tomography may provide more detailed information on the number and size of liver lesions. Most metastases are hypovascular and appear as low attenuation lesions on contrast-enhanced CT scanning. Tumours that may be hypervascular i n relation to normal hepatic parenchyma (e.g. primary hepatocellular carcinoma and metastases from pancreatic islet cell tumour, carcinoid and renal cell carcinoma) may become isodense on a contrast-enhanced CT scan and therefore these patients should undergo both a non-contrast and dynamic contrast study. Dynamic CT scanning ensures enhancement of branches of the portal vein and the hepatic veins so that the relationship of the lesion can be assessed with regard to the hepatic vasculature. This enables determination of resectability and planning of surgical resection. CT arterioportography (CTAP) is a technique whereby the contrast medium is delivered into the portal venous system without prior systemic distribution and dilution. This is achieved by selective catheterisation of the superior mesenteric artery and results in greater hepatic parenchymal enhancement.

Technology now allows three-dimensional modelling of the liver based on spiral computed tomography images[12-14]. Such techniques not only permit detailed reconstruction of the vascular and biliary anatomy of the liver but also offer the potential to measure liver volume before surgery which could be useful in determining the extent and nature of hepatic resection. Accurate assessment of liver volume and an estimate of liver function may also allow prediction of postoperative liver failure in patients undergoing resection, assist in volume- enhancing embolisation procedures, help with planning of staged hepatic resection for bilobar disease and aid in selection of living-related liver donors. Wigmore *et al* have recently demonstrated that virtual hepatectomy of 3-D CTAP reconstructed images provides an accurate prediction of liver mass removed during subsequent hepatic resection[15].

Several techniques have been described for functional assessment of liver capaci ty. These measure drug excretion (e.g. lidocaine clearance[16] or its metabolite monoethylglycinexylidine[17]) or dye excretion (indocy anine green[18,19]). In the future, in patients with impaired hepatic function for whom liver resection is being contemplated, it may be advantageous to combine a functional assessment with an estimation of liver volume to be resected by virtual hepatectomy as described above.

Hepatic angiography is not employed routinely in modern clinical practice to pro vide a specific diagnosis or aid in the planning of surgical intervention, although it may facilitate infusion of lipiodol which may demonstrate “occult” hepatoma in cirrhotic patients being considered for curative resection of primary hepatic malignancy. Laparoscopy is increasingly used to allow direct visualisation of liver lesions and can be combined with laparoscopic ultrasonogr aphy to provide high resolution images[20].

Liver lesions amenable to resection in patients who are fit for surgical intervention should not be biopsied as this may be associated with haemorrhage, sampling error, misdiagnosis and needle-tract tumour seeding. Percutaneous biopsy should only be performed in those patients who are not considered candidates for surgical intervention and only where the results of biopsy might influence further management. In patients with primary hepatocellular carcinoma, it may be valuable to take a biopsy of the uninvolved liver to detect and determine the severity of parenchymal liver disease such as chronic hepatitis or cirrhosis.

Extrahepatic metastases should be sought by means of a chest CT scan before major resection is undertaken, although it is accepted that pulmonary nodules may not always represent metastatic deposits. Upper and lower gastrointestinal endoscopy or barium studies, intravenous urography and mammography (in female patients) may be valuable in patients with a solitary liver metastasis of unknown origin. More recently, positron emission tomography (PET) and isotope scanning using CEA antibodies have been investigated and have shown promising results in demonstration of the hepatic lesions and in determining the extent of extrahepatic disease[21].

Portal vein embolisation (PVE) of the hemiliver to be resected has been proposed to induce homolateral atrophy and contralateral compensatory hypertrophy of the remnant liver and thereby reduce the risk of postoperative liver failure[22]. The concept of PVE appears to be well accepted when performed on healthy liver when extensive resections are being considered[23,24], however, its use in injured liver is also becoming more widely accepted[23,25,26].

PREOPERATIVE PREPARATION

Anaemia and coagulation disturbances should be corrected preoperatively. Administration of vitamin K will improve coagulation disorders secondary to poor nutrition and absence of luminal bile salts in patients with biliary obstruction, but will not reverse coagulopathy secondary to hepatocellular dysfunction. Fresh frozen plasma should be administered to correct the prothrombin time to within 2 seconds of control before surgery if possible.

Patients with obstructive jaundice and portal hypertension have a higher risk of bleeding complications and hepatic decompensation in the postoperative period. Preoperative biliary drainage may improve some of the pathophysiological disturbances associated with obstructive jaundice[27], however placement of biliary endoprostheses may introduce infection and exacerbate subsequent complications[28,29]. The role of preoperative biliary decompression prior to definitive hepatic resection remains unclear.

Surgery in patients with active alcoholic hepatitis carries a substantial risk and abstinence for as little as 3 months will reduce this risk. Patients with active hepatitis who are on long-term steroid therapy may require an increase in steroid cover during the perioperative period. Ascites should be controlled preoperatively by salt restriction and diuretic therapy as ascites increases the risk of impaired wound healing.

Assessment of underlying liver disease is vitally important because, although ex tensive hepatic resection may be well tolerated when the remaining liver has nor mal function, even minor resections in cirrhotic patients may be poorly tolerate d. The use of clinical and biochemical parameters (using the modified Child-Pugh classification) in the assessment of surgical risk in cirrhotic patients is well established and correlates well with surgical risk.

OPERATIVE TECHNIQUES

Hepatic resection is performed under general anaesthetic with a controlled central venous pressure of less than 5 mmHg. For the majority of hepatic resections, the initial incision should be a bilateral

subcostal incision. Exposure may be further improved in some patients with a narrow costal margin by extending the incision in the midline upwards to the xiphoid process.

In patients with hepatic malignancy, a thorough search is made of the peritoneum and regional lymph nodes to exclude extrahepatic dissemination of malignancy. The liver is carefully palpated and intraoperative ultrasound is undertaken to confirm the position of the tumour and its relationship to the hepatic vasculature.

All major hepatic resections mandate control of the inflow vasculature and hepatic venous outflow to and from the portion of the liver to be resected with maint enance of good hepatic arterial and portal venous blood supply to the remnant. This may be done by dissection of the relevant portal pedicle at the hilus and outside the liver substance as is the authors' preference, or alternatively, the major branches may be secured within the liver following division of liver tissue. We have not found it necessary to consider the use of total vascular exclusion[30] in the last 10 years and have favoured "classical" hepatic resection in preference to segmental resections particularly for metastatic tumours[31]. Detailed descriptions of these various techniques are outwith the scope of this article but can be referred to in many major texts[32,33]. The liver parenchyma can be transected in a number of ways, but it is the authors' preference to employ a Cavitron ultrasonic surgical aspirator (CUSA) which skeletonises the vessels within the hepatic parenchyma, allowing their identification before they are damaged. Small vessels (<2 mm) can be secured by diathermy before division, although larger vessels and branches of the hepatic veins are best secured by ligation or application of clips. The relevant hepatic vein(s) may be divided using a vascular stapler or clamped, divided and oversewn with a continuous non-absorbable suture.

The exposed raw surface of the transected liver, vena cava and retroperitoneum are carefully inspected for any bleeding which should be controlled with diathermy or suture. An argon beam coagulator can be applied to the raw surface to ensure haemostasis. Thrombin glue can also be sprayed on these areas to minimize the risk of postoperative bile leakage. It is the authors' preference to place routinely a large tube drain connected to a closed drainage system before wound closure.

POST-OPERATIVE MANAGEMENT

High dependency nursing care is required to provide adequate observation of vital signs and conscious level in the postoperative period. Monitoring includes regular measurement of heart rate, blood pressure, central venous pressure, oxygen saturation, urine output and drain losses.

Patients undergoing major hepatic resection and those with poor preoperative liver function are at particular risk of developing postoperative hepatic decompensation. Maintenance of adequate liver function can be judged by regular assessment of conscious level, acid base status, blood glucose levels, blood lactate levels and prothrombin time.

COMPLICATIONS

Despite improvements in surgical technique and perioperative care, major complications and death may occur following major hepatic resection and the risks to the patient should not be underestimated. In a series of 133 hepatic resections in 129 patients published from our own unit[34], the overall operative mortality was 4.7%. Major early morbidity occurred in 20% of patients and resulted in unplanned radiological or repeat operative intervention, transfer to the intensive care unit in some patients and prolongation of hospital stay. Other major series report similar morbidity and mortality rates[35,36].

Postoperative liver failure from inadequate functional residual liver tissue is the leading cause of death after hepatectomy[37,38]. It has been previously reported that patients with a postoperative residual volume of 35% with good function are at low risk of developing liver failure[39]. However, in patients with impaired liver function, smaller resections may be hazardous.

INDICATIONS FOR HEPATIC RESECTION

The main indication for hepatic resection is primary or secondary hepatic malign ancy. (Figure 2 a,b). Primary malignant hepatic lesions include hepatocellular carcinoma, and less common tumours such as cholangiocellular carcinoma and haem angiosarcoma. Liver resection for metastatic disease is predominantly undertaken for patients with colorectal metastases, however, resection has been performed for non-colorectal liver metastases. Hepatic resection is also undertaken for contiguous tumours involving the liver, such as in selected patients with gallbladder carcinoma or cholangiocarcinoma involving the extrahepatic biliary tree. The indication for hepatic resection for malignancy in our own unit is shown in Table 1.

Hepatocellular carcinoma (HCC) is one of the world's most common malignancies and is particularly challenging because it usually develops on a background of chronic inflammatory liver disease[40]. The mean overall survival of patients with untreated HCC is generally reported to be 3 to 4 months after symptom s appear, however, in Japan and other parts of the world where HCC is being detected earlier, median survival times are nearly 6 months. Surgical resection is the treatment of choice for HCC if the resection can be performed safely and will not leave gross residual disease, however, unfortunately only a small proportion of HCC's are amenable to surgical removal. In non-cirrhotic patients, the tumour has often reached a substantial size by the time of presentation, whereas cirrhotic patients frequently have compromised liver function sufficient to preclude even segmental resection. The average survival time of patients who have undergone resection is about 3 years. Five year actuarial survival rates of 60%-70% have been reported in patients with Stage I/II disease compared with corresponding survival rates of 20%-30% in patients with more advanced disease (stage III/IV)[41,42]. Although the operative mortality rate from liver failure after hepatectomy for patients with HCC has decreased with experience[43], it still ranges from 0%[44,45] to 32%[46-48].

Table 1 Indication for hepatic resection for malignancy in Edinburgh (1988-2001)

Indication	Number
Primary liver tumor	
Hepatocellular carcinoma	30
Intrahepatic cholangiocarcinoma	8
Angiosarcoma	1
Clear cell carcinoma	1
Metastatic liver tumor	
Colorectal adenocarcinoma	131
Stromal tumor	8
Carcinoid tumor	7
Breast metastases	2
Appendiceal metastases	2
Metastatic melanoma	2
Contiguous tumor involving liver	
Hilar cholangiocarcinoma	29
Gallbladder carcinoma	7
Adrenocortical tumor	1
Total	230

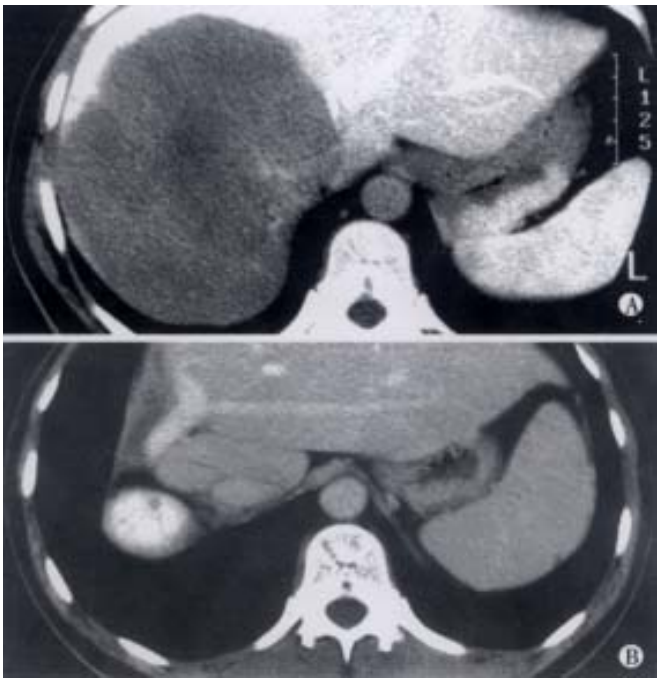

Figure 2 (a) Large solitary metastasis resected by extended right hepatectomy. (b) CT scan showing hypertrophied left and caudate lobes with no evidence of recurrence at one year.

Resection if possible is the treatment of choice for colorectal metastases and offers the only potential for cure[35,49]. Several studies have document ed the unfavourable prognosis of untreated hepatic metastases from colorectal cancer. Without treatment, 60%-75% of patients are dead at 1 year and the mortality rate at 3 years is almost 100%[50,51]. Five year survival rates in patients undergoing hepatic resection for colorectal metastases range between 25%-40% in most major centres[35,52,53]. Figures from the authors' unit demonstrate a 3-year survival rate of 65% in patients who underwent resection of colorectal liver metastases[34]. The number of metastases is no longer considered to be as important a predictor of long-term survival as previously[53,54]. Complete excision of all demonstrable tumour with clear resection margins has been shown to be of much greater importance[35]. Segmental-based resection allows excision of bilateral or multiple liver lesions that previously might have been deemed irresectable. Staged resection is another technique whereby large volumes of liver parenchyma may be resected without inducing hepatic insufficiency. There is increasing evidence that selected patients who develop recurrent hepatic tumour following previous resection of colorectal liver metastases will benefit from re-resection[55,56].

Surgical resection of hepatic metastases from neuroendocrine tumours is curative in some cases and is usually effective in relieving symptoms[57]. Palliative debulking or cytoreductive surgery is often worthwhile as it offers a chance of prolonged survival and may cause complete or partial relief of the incapacitating symptoms related to hormone production. McEntee *et al* reported the outcome of 37 patients (24 carcinoids and 13 islet cell tumours) who unde rwent hepatic resection for metastatic neuroendocrine tumours[58]. Seventeen resections were considered curative and in this group, results were encouraging in terms of survival and symptom relief, however in patients undergoing palliative resection the mean duration of symptom relief was only 6 months. Thompson *et al*, however, reported that half their patients with islet cell tumours had symptomatic improvement with a mean duration of 39 months after noncurative resection[59].

The role of hepatic resection for non-colorectal non-neuroendocrine metastases is less well defined. Most studies report small numbers of patients and must be regarded as anecdotal. Schwartz' review of the literature concluded that little improvement could be anticipated for resection of metastases from tumours of the oesophagus, stomach, small intestine or pancreas[60]. Similarly, there was little evidence to support routine resection of metastases from gynaecological or breast carcinomas, however, resection of metastases from primary renal cell carcinoma, Wilms' tumour, and adrenocortical carcinoma was indicated. Harrison *et al* reported a single centre experience of 96 patients who underwent liver resection for non-colorectal, non-neuroendocrine metastas es with no perioperative deaths[61]. The overall survival rate at 1, 3 and 5 years was 80%, 45% and 37% respectively (median survival, 32 months), with 12 actual 5 year survivors. Patients with genitourinary primary tumours exhibited the best outcome followed by patients with primary soft tissue tumours (breast, melanoma and sarcoma). Hepatic resection for non-colorectal gastrointestinal primary tumours was generally associated with a poor survival. Similar results have been reported from other centres where long-term survival was only seen in patients with non-GI-origin metastases[62].

Contiguous cancer arising from the extrahepatic biliary tree or gallbladder may also be amenable to hepatic resection. A more aggressive approach to the management of hilar cholangiocarcinoma in recent years has been associated with improve d long-term survival and quality of life[63-65]. In a recently reported series of 114 patients who presented with hilar cholangiocarcinoma, 98 patients had a radical resection, three underwent palliative resection and only 13 were not treated surgically[66]. The operative mortality rate was 4% and the 5-year survival rate was 28%. This report supports the widely held view that radical resection provides the best prognosis for selected patients with hilar cholangiocarcinoma. A number of recent studies have reported long-term survival after radical surgery for gallbladder cancer[67,68]. Radical regional lymphadenopathy may have survival benefits for patients with node-positive disease[69,70]. Aggressive reresection has been shown to be beneficial for patients with gallbladder cancer discovered during or after laparoscopic cholecystectomy for patients other than those with T1 tumours[71].

SUMMARY

Recent reports have highlighted consistently improved perioperative morbidity and mortality rates following hepatic resection. Operative mortality rates, even in cirrhotic patients, are less than 5% in most recent series. The commonest indication for hepatic resection is primary or secondary hepatic malignancy and therefore appropriate preoperative assessment of such tumours is vitally important. Accurate radiological imaging, including the use of three-dimensional reconstruction, will indicate if lesions are resectable and will aid the decision regarding the likely surgical procedure. Portal vein embolisati on is becoming a more widely accepted technique to induce contralateral hypertrophy reducing the risk of postoperative hepatic impairment and therefore increasing the indications for liver resection. The selection and subsequent management of patients with primary and secondary hepatic malignancy requires a multidisciplinary team approach invo lving hepatologists, radiologists, anaesthetists and surgeons and therefore the care of such patients should be undertaken in specialist hepatobiliary centres. Whilst accepting that hepatic resection offers the only prospect of cure for many patients, it is evident that future efforts will also be focused on determining the role of adjuvant treatments to reduce the inevitable recurrence of tumour which occurs in the majority of patients.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• REVIEW •

Pathogenesis of coeliac disease: implications for treatment

Jocelyn S Fraser, Paul J Ciclitira

Department of Gastroenterology, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK
Correspondence to: Professor PJ Ciclitira, Department of Gastroenterology (GKT), The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK. paul.ciclitira@kcl.ac.uk
Telephone: +44 20 7928 9292 Ext. 3063, Fax: +44 20 7620 2597
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INTRODUCTION

Coeliac disease (CD) is an enteropathy, characterised by villous atrophy, which occurs in genetically susceptible individuals. It affects mainly the proximal small intestine, and is caused by an intolerance to cereal storage proteins found in wheat, barley and rye. Due to earlier diagnosis, and the recognition of 'silent' or 'latent' forms of the disease, the very severe symptoms that were seen previously are not very common now[1]. Malabsorption, with steator rhoea and weight loss, occur less frequently. Anaemia, vitamin deficiencies, complications of pregnancy and associated autoimmune diseases, such as insulin dependent diabetes mellitus or thyroid disease are often the clues which lead to the diagnosis of coeliac disease. Coeliac disease affects people from all ethnic groups, though it is most common in people originating in Europe, including people in North America and Australia. It is rarely seen in people from an Afro-Caribbean background[2].

In the past, the prevalence of coeliac disease has been thought to be 1 in 1 500 of the population in Western countries, based on the number of identified cases. However, recent screening studies of blood donors has shown a far higher prevalence of 1 in 250 in Sweden[3] and the United States[4]. In Italy, a population screening study of 17 000 school children between the ages of 6 and 15 revealed a prevalence rate of 1 in 184[5]. This appears to be uniform throughout most of Europe, with some areas of higher incidence, such as the west coast of Ireland. It affects males and females equally.

The reason for this discrepancy between clinically apparent cases, and the number of individuals with positive screening results, lies in the concept of the 'coeliac iceberg'[2]. The majority of people with coeliac disease are symptom-free, or have only mild symptoms, and do not approach a health care professional for a diagnosis. These individuals have 'silent' coeliac disease, if they have abnormal screening antibodies and an abnormal small bowel biopsy, but no symptoms. Individuals with abnormal screening antibody tests, but a normal small bowel biopsy and no symptoms, have 'latent' coeliac disease[6]. Thus, there are large numbers of people who are undiagnosed, in the 'coeliac iceberg' analogy this would be the vast portion of the iceberg which is not visible, whereas the diagnosed individuals with symptoms form the tip of the iceberg (Figure 1).

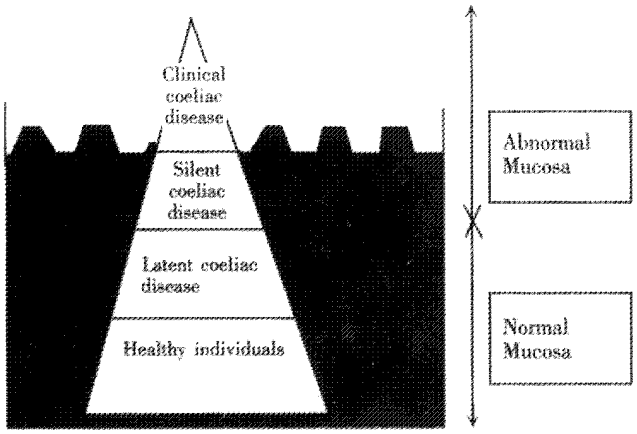

Figure 1 The coeliac iceberg. After A Ferguson[2]

HISTOPATHOLOGY

In the small intestine, the abnormalities are most marked proximally and decrease in severity with distal progression through the small intestine. In severe cases, the lesion may affect the ileum and even the stomach and rectum[7].

Flattening of the mucosa can vary from mild, through partial villous atrophy, to a total absence of villi. Classically, in untreated celiac disease, there is a flat mucosa with no villi (total villous atrophy), but more usually there is a reduction in the normal villous height, resulting in the villous height: crypt depth ratio being reduced from its normal value of between 3-5:1. The thickness of the mucosa is usually increased because of crypt hyperplasia.

The surface epithelial cells become pseudostratified compared to their normal tall columnar shape with resultant fall in enterocyte height. Crypt mitotic activity is no longer confined to the base and, although the histological appearance is usually normal, crypt abscesses have been described. Cell migration from the crypt base to the villous tip is reduced in untreated coeliac disease to 12-24 hours compared with the normal 3-5 days.

There is a chronic inflammatory cell infiltrate in the mucosa of the small intes tine in untreated coeliac disease with a rise in the number of plasma cells in the lamina propria. There is an increase in the ratio of intra-epithelial lymphocytes to surface enterocytes in active disease. Most of the intra-epithelial lymphocytes express the common leucocyte antigen CD3, 70% express the suppressor/ cytotoxic leucocyte antigen CD8, 5% express the helper/inducer CD4 phenocyte, whereas 20% of the cells are CD3+ve, CD4-ve and CD8-ve. Most of these cells express the more primitive γ/δ rather than the more usual α/β T-cell receptor. This results in a significant increase in the number of γ/δ T-cell receptor +ve lymphocytes in the surface epithelium of the small intestin e, both in treated and untreated coeliac disease[7].

CEREAL CHEMISTRY

Cereal storage proteins fall into four groups, the minor albumins, the globulins, the ethanol-insoluble glutenins, and the ethanol-soluble fraction termed prolamins.

Initial separation of wheat proteins depends on their relative solubility charac teristics. Gliadins are soluble in 40%-90% ethanol and high molecular weight glutenins are insoluble in neutral aqueous solution, saline or ethanol, although low molecular weight glutenins are ethanol soluble. The gliadins may be subdivided according to their relative electrophoretic mobility into α, β, γ and ω subfractions or according to their N-terminal amino acid sequence into α, β or ω subfractions, the previously described β-subfraction being reclassified as a type of α gliadin[8]. The molecular weight of these proteins rises from 32K to 58K daltons through α to ω gliadins.

Prolamins, the alcohol-soluble fraction of storage proteins are responsible for triggering the disease[9]. Wheat, barley and rye, being closely related, all contain prolamins (known respectively as gliadins, hordeins and secalins) with a high composition of glutamine and proline, whereas the prolamins of oats and more distantly related cereals, contain less glutamine and proline and more alanine and leucine[10]. The glutamine-rich peptide sequences appear to be responsible for the toxicity of wheat, barley and rye in coeliac disease.

Immune activation occurs after ingestion of these cereals, when these peptides a re presented, in conjunction with MHC class II molecules, to activate CD4+ T helper lymphocytes, causing release of Th_1 and Th_2 cytokines, which encourag e expansion of autoreactive B cell clones, and mucosal destruction. Much recent research has provided an insight into the mechanisms of pathogenesis by which this occurs.

Once the diagnosis of coeliac disease has been made, the patient needs to be established on a gluten free diet. All foods which contain wheat or wheat flour, as well as barley and rye must be avoided. There are a number of foods which contain hidden gluten, including soy sauce, mustard, mayonnaise and beer, which contains the barley prolamin, hordein. Gluten-free flour, bread, biscuits, pasta and snacks are available from a variety of companies.

An average Western diet contains 13 g of gluten, a "Codex-Alimentarius" gluten free diet contains <0.3% gluten from grains, and is sufficiently gluten free for the majority of coeliac patients, however, some patients continue to have symptoms, and improve only when gluten is completely removed from the diet. This was shown in a study by Faulkner-Hogg[11], where 15 out of 22 patients with symptoms on a Codex-Alimentarius gluten free diet improved after removal of all detectable gluten.

In the past, it was assumed that the small bowel mucosa returns to normal after introduction of a gluten-free diet, however, despite improvement of symptoms, persistent small bowel mucosal abnormalities may occur, and are not necessarily an indication of poor dietary compliance[12].

Oats are a member of the avena tribe of the gramineae, or grass family, of plant s, whereas wheat, barley and rye belong to the triticeae tribe, both tribes belonging to the pooideae sub-family. Thus avenin, the prolamin of oats, is genetically less like gliadin than secalin and hordein (Figure 2).

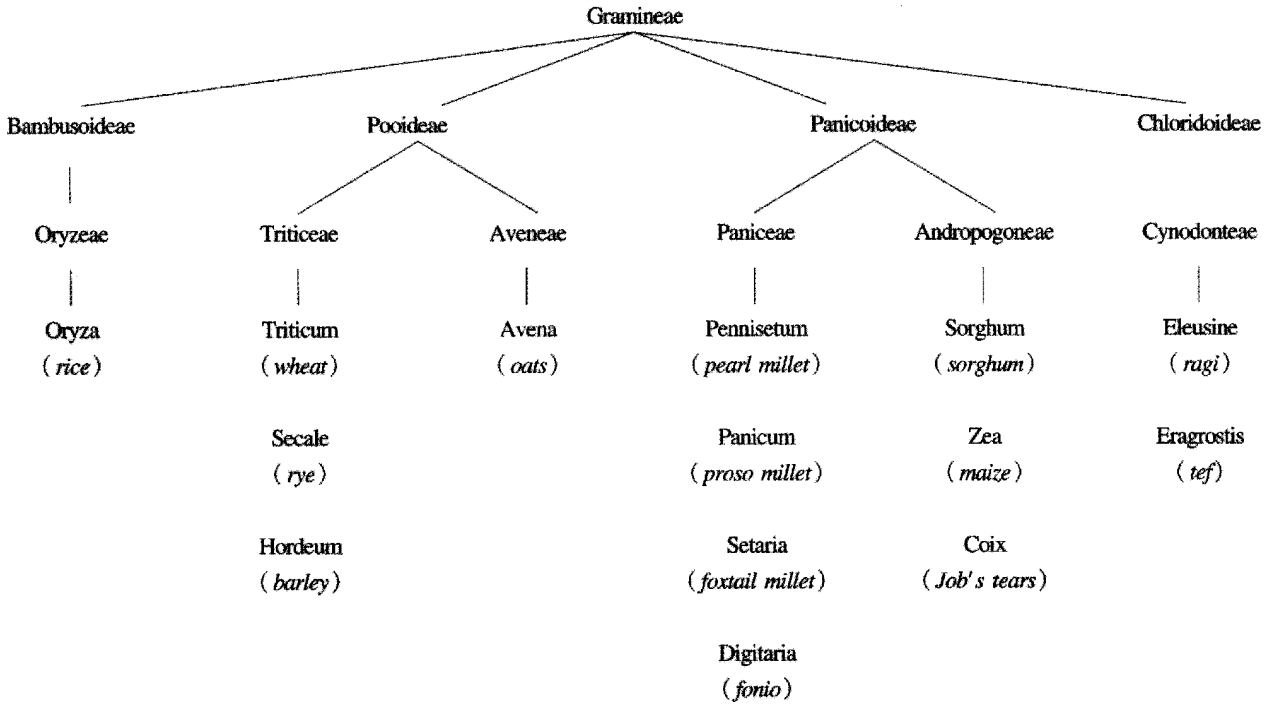

Figure 2 The taxonomic relationships of cereals. After P Shewr y, A Tatham and D Kasarda[10]

The toxicity of oats to patients with coeliac disease has been a controversial issue, as early studies have shown conflicting results. Harmful effects were observed by some workers[9,13], but not by others[14,15], and some investigators found variable results[16,17]. However, a recent Finnish study[18] on newly diagnosed patients, as well as coeliac patients in remission on a gluten-free diet, have shown that moderate amounts (up to 60 g/day) of oats are not detrimental, as witnessed by no significant differences in gliadin and reticulin antibodies, as well as numbers of intra-epithelial lymphocytes before and after introduction of oats into the diet.

Sequence homologies, and weak immunological cross reactivity, have been found between avenin and the prolamins of wheat, barley

and rye[10,19,20]. Additionally, only 5% to 15% of the total protein in oats is avenin, whereas 40% to 50% of the total protein in wheat, barley and rye are made up of their respective prolamins[21]. Thus, there is a smaller amount of avenin per gram if oat seed, and there are fewer toxic epitopes per gram of avenin. This suggests that a small amount of oats can be consumed by patients with coeliac disease, as long as the oats are not contaminated by wheat flour. In many mills however, the same equipment is used to grind wheat, as that used to grind oats, causing enough contamination to have a detrimental effect on the health of sensitive coeliac patients.

THE IMMUNODOMINANT PEPTIDE

The precise structure of the gluten proteins that exacerbate coeliac disease remains unknown, although there have been considerable recent advances in this area. Peptides from the fully sequenced α-gliadin, A-gliadin, have been used in a number of studies to try to identify the toxic epitope, which induces intestinal inflammation. A peptide corresponding to amino acids 31 to 49 of A gliadin was found to cause significant histological changes in small bowel biopsy specimens after *in vivo* challenge by intraduodenal infusion[22]. Anderson et al used 51 overlapping synthetic 15-amino acid peptides, spanning the complete sequence of the A-gliadin. They assessed optimal peripheral blood mononuclear cell (PBMC) secretion of the Th_1 cytokine gamma interferon (IFN-γ) in response to incubation with each of the peptides and demonstrated a transient, disease-specific, DQ2 restricted, CD4 T-cell response to a single dominant epitope. This peptide corresponded to amino acids 57-73 of A-gliadin, which had been partially deamidated by tissue transglutaminse at position 65 (Q65E)[23].

Arentz-Hansen *et al* produced eleven different recombinant antigens from α-gliadin, to demonstrate that the intestinal T cell response to α-gliadin in adult coeliac disease patients is focused on two immunodominant, DQ2 restricted peptides that overlap by a seven residue fragment. Gluten-specific T cell lines from small intestinal biopsies of 16 different patients all responded to one or both of the deamidated peptides, indicating that these epitopes are highly relevant to disease pathology. The peptides correspond to amino acids 62-75 (α2) and 57-68 (α9) with Q65E[24].

The identification of these peptide sequences, which act as potent T cell epitopes, may lead to the development of antigen specific therapy for coeliac disease. Once a target has been defined for immunomodulation, it may be possible to create non-toxic cereal based wheat, by removal or modification of the antigeni c sequence in gliadin proteins.

TISSUE TRANSGLUTAMINASE

Tissue transglutaminase (tTG) is a ubiquitous cytoplasmic enzyme, which is found mainly in respiratory and gut epithelial cells. It is important in prevention of tissue damage, by catalysing protein cross-linkage, causing formation of isopeptide bonds between glutamine and lysine residues. tTG also deaminates glutamine residues to glutamic acid.

Native gluten proteins have very few negatively charged residues, as they contai n approximately 40% glutamine and 20% proline, however, several of these glutamines are converted to glutamic acid in the presence of tTG. Deamidation of glutam ine residues to glutamic acid was found to strongly enhance T cell reactivity, due to the formation of negatively charged amino acids needed for efficient binding to DQ molecules, thus inducing maximal T cell proliferation[25].

Virtually all patients with CD have been found to express either HLA-DQ2 or -DQ8 class II molecules. HLA class II molecules are responsible for binding exogenous protein antigens and presenting them to CD^{4+} T cells. These molecules have a characteristic binding groove, which differ in size, shape and position between class II alleles, and which can be used to predict the sequence of pep tides needed to fit into it. Both DQ2 and DQ8 require negatively charged amino acids at certain positions for effective binding. Gluten specific HLA-DQ2 and -DQ8 restricted T cell clones can be isolated from small intestinal biopsy samples of patients with CD, and have been used to characterise gluten derived peptides capable of stimulating T cells[26].

Arentz-Hansen proposes that conditions may exist in the gut where T cell epitopes are both created and trapped locally by tTG, prohibiting their presentation by tolerogenic APCs in the gut. Alternatively, tTG may prevent these epitopes from spreading systemically as soluble antigen, a factor thought to be important in oral tolerance. Thus, it may be possible to administer soluble deamidated gliadin peptide to patients with coeliac disease, to induce tolerance to gliadin[24].

IMMUNOGENETICS

The precise mode of inheritance of coeliac disease is unknown, although l0%-15% of first degree relatives of probands are similarly affected[27,28]. There is 70%-100% concordance in affected monozygotic twins and 30%-50% concordance in human leukocyte antigen[29] (HLA)-identical siblings. Efforts to understand the mechanisms and genetics of polygenic human diseases have focused on the identification of DNA or protein products and protein molecules that segregate in both populations and families.

The most significant observation was the increased frequency of specific serologically defined lymphoid cell surface proteins, termed HLA class II molecules, in people with coeliac disease. These are glycosylated transmembrane heterodimer s comprising both α and β-chains, the genes for which are organised into three related subregions DR, DP and DQ. The genes are encoded within the HLA-class II region of the major histocompatibility complex on the short arm of chromosome 6. The association of particular HLA-DR and DQ types with coeliac disease is well established[30]. Associations with the HLA-DP region and the TNF-α genes have been reported but are thought to be secondary to linkage disequilibrium with HLA-DR and DQ haplotypes[31,32]. The genes most strongly associated with coeliac disease are DQAl *0501, DQB1 *0201[33,34]. 98% of northern Europeans with coeliac disease have these alleles in cis (DQ2) whereas in Southern Europe a third of the population with the condition express the same class II molecule from these alleles in trans (DR5,7)[30]. Italian and Argentinian coeliac disease populations are also reported to have a increased frequency of the DR5,7 haplotype[35]. In Israel there is also an association with the haplotype HLA-DR4, DQ8[36]. This genotype encodes a class II molecule with considerable similarity in the peptide binding groove configuration to that produced by the DQ2 genes, supporting a central role for the class II molecule in an immune mediated model of coeliac disease.

Twenty-five percent of people in the general population of Northern Europe have HLA-DQ2. It has been shown in epidemiological studies that only 30% of the genetic susceptibility to coeliac disease lies in the HLA region[37], the remaining 70% being elsewhere in the human genome. Two genome-wide linkage studies to identify these remaining susceptibility alleles have been undertaken using sibling pair analysis and have produced conflicting results. The first group from Ireland performed an autosomal screen using 40 affected sibling pairs, and identified five areas of interest at the following locations: 6p23, 7q31.3, 11p11, 15q26 and 22cen[38]. The second group from Italy using 110 sibling pairs, failed to confirm linkage in the areas identified in the previous study, but proposed to further areas of interest, at 5qter, and in a subgroup of patients at 11qter[39].

Our group, employing analysis of multi-generation families,

identified two new potential susceptibility loci at 10q23.1 and 16q23.3, and found evidence of linkage on chromosome 7, close to the γ T-cell receptor gene and on chromosome 2, near the CTLA4 gene[40].

The CTLA4/CD28 region, on chromosome 2q33, has been independently implicated in an association study from France[41] and a linkage study from Finland[42]. These genes control T cell proliferation, and play a part in other autoimmune dieases, such as type I insulin dependent diabetes mellitus and Graves' disease.

IMMUNE RESPONSE

It has been suggested that in coeliac disease there is a primary abnormal immune response of the small intestine to gluten proteins that produces an allergic phenomenon. There is considerable evidence to support this hypothesis, although the observed aberrant immune response may be secondary to an unrecognised alternative aetiopathology. There is a dense infiltration of the small intestinal lamina propria with lympho cytes and plasma cells in patients with untreated coeliac disease. There is also an increased ratio of intra-epithelial lymphocytes to surface enterocytes with the majority of these cells expressing the suppresser/cytotoxic phenotype and having the appearance of immunoblasts[43], and there is a strong assoc iation with the histocompatibility antigen HLA-DQ2 which is also associated with other auto-immune disorders.

High titres of antibodies to gliadin occur in coeliac disease as well as antibodies to reticulin and endomysium, which also implies an immune mediated mechanism[44]. The role of these antibodies in the disease pathogenesis is unknown. However, it has recently been shown that the antigen for anti-endomysial antibodies is the enzyme tissue transglutaminase, which is present in the small intestine and causes selective partial deamidation of gluten molecules.

There are an increased number of cells that express the cytokines IL-2, interferon-γ (IFN-γ) tumour necrosis factor-α (TNF-α) and tumour necrosis factor-β (TNF-β), IL-10, IL-1β and transforming growth factor-β (TGF-β). The expression of both Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) associated cytokine transcripts in the same biopsies indicate the activation of Th0 cells. The expression of IL-2 and IL-4 mRNA was not observed in the peripheral blood samples of patients with inactive coeliac disease, implying that they are associated with disease activity[45]. This also supports the immune-mediated hypothesis. The isolation of gluten-sensitive T-cells both from both the peripheral blood and small intestinal biopsies from coeliac patients[34,46], the HLA-DQ2 restriction of the majority of these cells and their production of pro-inflammatory cytokines when stimulated with wheat gliadin[47], provide further evidence that these represent an hyperimmune sensitivity to certain cereal peptides.

"Gliadin (or gluten) shock", is a rare condition which affects treated coeliac patients. In this condition a gluten challenge is followed by collapse with vomiting and tachycardia. The condition responds to treatment with corticosteroids.

In patients who do not respond to a strict gluten free diet, or who relapse on a gluten free diet, steroids can be used, such as prednisolone 20 mg/day, with a reducing course over the next 6 weeks. Azathioprine can also be used as a steroid-sparing agent[48]. Cyclosporine does not appear to be useful[49], indeed, some patients' symptoms increase, and budesonide, despite reducing the risk of osteoporosis, is not useful, as it is formulated to be slowly released in the terminal ileum, usually beyond the coeliac small bowel lesion, and is absorbed only in very small amounts.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• INVITED COMMENTARY •

Moving toward an understanding of the metastatic process in hepatocellular carcinoma

W. Michael Korn

Division of Gastroenterology and Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA 94143-0128, USA
Correspondence to UCSF Comprehensive Cancer Center, 2340 Sutter St. San Francisco, CA 94115, USA. mkorn@cc.ucsf.edu
Telephone: +1-415-502-2844, Fax: +1-415-502-3179
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INTRODUCTION

Clinical factors contributing to the therapeutic challenge of hepatocellular carcinoma (HCC) are manifold: tumors arise often in patients with compromised liver function, therefore limiting therapeutic options; symptoms develop only at later stages of tumor progression, and tumors tend to invade normal structures or occur in multiple locations simultaneously. Ninety percent of patients with tumors larger than 5 cm will have synchronous intrahepatic metastases at the time of presentation. In the majority of patients undergoing partial hepatectomy for HCC, intra-hepatic or distant metastases will occur[1]. Likewise, in about fifty percent of the patients who die within five years after liver transplantation for HCC, intra- and extrahepatic recurrences are the cause of death[2]. Tumor characteristics predicting recurrence of HCC following resection include portal vein invasion, intrahepatic metastasis, extratumoral spread, high mitotic index[3-5], and a sarcomatous phenotype[6]. Such observations demonstrate t hat tumor cells that are easily invading normal tissues and achieve access to the circulation harbor the greatest risk of tumor recurrence. The molecular events promoting invasiveness of HCC cells are still widely unknown. Further understanding of the se processes is urgently needed for the development of rational strategies for prevention and treatment of metastatic disease in HCC.

Investigations in different tumor types and murine models of cancer depict a characteristic cascade of events necessary for a tumor to achieve successful dissemination of met astases from a primary tumor. After malignant transformation and initial tumor growth, the first step towards metastasis formation is the initiation of vascularization of the tumor, a process known as the angiogenic switch. While the tumor progresses, new genetic changes occur leading to ① a reduced cell-cell adhesion, and, ② alterations in the interaction of the tumor cell with the extracellular matrix, allowing invasion into surrounding tissues including blood vessels (which are mediated by changes in integrin expression and activation of proteolytic enzymes). Tumor cells reaching the circulation must have developed mechanisms to suppress anoikis, which is programmed cell death caused by disruption of cell-substrate adhesion. At distant sites, tumor cells need to leave the circulation and migrate to sites with favorable conditions. Here, angiogenesis is again in itiated and cell progression of the metastasis continues. It is obvious that this sequence of events will be dependent on multiple molecular factors in malignant and normal (e.g. stroma and immune competent) cells. Some of these factors have been characterized in HCC. So far, and in agreement with data from other tumor types, there is evidence that a high proliferative index (as measured by PCNA or Ki-67 expression) is associated with reduced tumor differentiation and might b e predictive of recurrence after tumor resection[7-9]. In HCC, several signaling pathways are candidates for driving this proliferation, mainly those related to growth factor receptors, including Met, the receptor for the hepatocyte growth factor/scatter facter (HGF/SF) and epidermal growth factor receptor (EGFR)[10,11]. While enhanced proliferation is an important step during tumorigenesis, in creases in invasiveness and cell mobility are necessary for the generation of metastases. It has been demonstrated that signaling through the Ras/MAPK pathway (which confers signals from growth factor receptors, including EGFR and Met), could cooperate with the TGF-β signal transduction pathway in promoting the switch from a epithelial to mesenchymal phenotype in cancer cells, rendering them significantly more mobile and invasive[12]. *In vitro* data from HCC cell lines support this possibility, as it has been demonstrated that TGF-â signaling enhances prol if eration of HCC cell lines[13]. Disassembly of protein complexes involved in cell -cell adhesion has been demonstrated as a consequence of TGF-β and Ras/MAPK signaling[14]. Accordingly, loss of expression of the cell-adhesion molecul e E-cadherin and nuclear expression of its binding partner β-catenin, occurs frequently in high-grade HCC and correlates with a poor prognosis[15]. There is also strong evidence that HCC indeed activates angiogenesis. For example, vascular endothelial growth factor (VEGF), a promoter of angiogenesis, was found to be upregulated in HCC[16]. A recent prospective study in 100 patients undergoing resection of HCC demonstrated a strong correlation between serum concentration of vascular endothelial growth factor (VEGF) and microscopic vascular invasion[17]. In the same study, high serum levels were associated also with the presence of intrahepatic metastases and reduced disease-free survival. As indicated, successful invasion into surrounding tissues can also be facilitated by proteases that allow malignant cells to migrate through physiologic barriers, e.g. basement membranes. Indeed, experiments using HCC cell lines revealed that membrane-type 1 matrix metal loproteinase confer invasiveness and promote intrahepatic metastases[18]. In addition, expression of urokinase-type plasminogen activator (uPA), uPA receptor (u PAR) and plasminogen activator inhibitor type-1 (PAI-1) was found to be associated with tumor invasiveness in a mouse model of HCC and human HCC[19]. Overall, these data suggest that mechanisms of metastasis in HCC that are consistent with data in other types of solid tumors. However, most of

the evidence is circumstantial and correlative, reflecting a lack of appropriate model systems.

As Li *et al*. describe in this issue of the World Journal of Gastroenterology, new model systems might be available that could lend insight into the mechanisms underlying the metastatic process in HCC. The authors generated two clonally related cell line derivatives from human HCC cell line MHCC97 that show dramatic differences in their metastatic potential. Notably, the authors used an *in vivo* selecti on process that included the orthotopic transplantation of xenografts into the livers of mice, thus simulating the clinical situation as closely as possible. The differences in metastatic potential were mirrored by significant phenotypical differences. These included morphologic differences as well as faster proliferation, enhanced in vitro invasiveness, and increased alpha-fetoprotein (AFP) production of the highly malignant derivative compared to its less metastatic counter part. These findings are consistent with the expected metastatic phenotype of H CC and it will be most exciting to see results from molecular analyses of these cells. Particularly, analyses of differential gene expression, e.g. using cDNA expression arrays, will be revealing and could be compared to results from the an alysis expression profiles in human tumors[20].

Identification of differentially expressed genes represents an important step toward understanding metastasis in this disease. However, genetic evidence will be necessary to pinpoint the culprits in this process. So far, transgenic mouse models of HCC have bee n created in which liver-specific expression of potential oncogenes, including C yclin D1, c-myc, TGF-a, and Met leads consistently to the development of HCC- like tumors[21,11,22]. Interestingly, tumors occurring in these models lack a highly invasive or metastatic phenotype. A next step is to rebuild the full phenotype of HCC in mice using modified transgenic models that include combinations of growth and metastasis promoting genes. Based on these models, rational therapeutic approaches could be developed and tested.

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Edited by Pan BR

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Apoptosis, proliferation and *p*53 gene expression of *H. pylori* associated gastric epithelial lesions

Zhong Zhang[1], Yuan Yuan, Hua Gao, Ming Dong, Lan Wang, Yue-Hua Gong

[1]Department of Pathology, Shenyang Medical College, Shenyang 110031 Liaoning Province, China
Supported by National Ninth Five-Year Study Program for Tacking Key Scientific Problems,No. 96-906-01-04
Correspondence to: Yuan Yuan, Cancer Inst itute of First Hospital, China Medical University, Shenyang 110001, Liaoning Province,China. yyuan@mail.cmu.edu.cn
Telephone: +86-24-23256666-6153
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Abstract

AIM: **To study the relationship between *Helicobacter pylori* (*H.pylori*) and gastric carcinoma and its possible pathogenesis by *H.pylori*.**

METHODS: **DNEL technique and immunohistochemical technique were used to study the state of apoptosis, proliferation and *p*53 gene expression. A total of 100 gastric mucosal biopsy specimens, including 20 normal mucosa, 30 *H.pylori*-negative and 30 *H.pylori*-positive gastric precancerous lesions along with 20 gastric carcinomas were studied.**

RESULTS: **There were several apoptotic cells in the superficiale pithelium and a few proliferative cells within the neck of gastric glands, and n o *p*53 protein expression in normal mucosa. In gastric carcinoma, there were few apoptotic cells, while there were a large number of proliferative cells, and expression of *p*53 protein significantly was increased. In the phase of metaplasia, the apoptotic index (AI, 4.36%±1.95%), proliferative index (PI, 19.11%±6. 79%) and positivity of *p*53 expression (46.7%) in *H.pylori*-positive group were higher than those in normal mucosa (*P*<0.01). AI in *H.pylori*-positive group w as higher than that in *H.pylori*-negative group (3.81%±1.76%), PI in *H.pylori*-positive group was higher than that in *H.pylori*-negative group (12.25%±5.63%, *P*<0.01). In the phase of dysplasia, AI (2.31%±1.10%) in *H. pylori*-positive group was lower (3.05%±1.29%) than that in *H.pylori*-negative group, but PI (33.89%± 11.6 5%) was significantly higher (22.09±8018%, *P*<0.01). In phases of metaplasia, dysplasia and gastric cancer in the *H.pylori*-positive group, AIs had an evidently gradu all decreasing trend (*P*<0.01), while PIs had an evidently gradual increasing trend (*P*<0.05 or *P*<0.01), and there was also a trend of gradual increase in the expression of *p*53 gene.**

CONCLUSION: **In the course of the formation of gastric carcinoma, proliferation of gastric mucosa can be greatly increased by *H. pylori*, and *H. pylori* can induce apoptosis in the phase of metaplasia, but in the phase of dysplasia *H. pylori* can inhibit cellular apoptosis. And *H. pylori* infection can strengthen the expression of mutated *p*53 gene.**

Subject headings *Helicobacter pylori*; gastric precancerous lesion; apoptosis; proliferation; *p*53 gene

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INTRODUCTION

H. pylori infection is epidemiologically associated with the development of gastric cancer[1-7], but it is unknown how *H. pylori* does so . In this study, DNEL technique[8]and immunohistochemical staining were used to dynamically observe and compare the state of apoptosis, proliferation and *p*53 gene expression in *H.pylori*-negative or *H.pylori*-positive gastric precan cerous lesion as well as gastric carcinomas. The purpose is to probe into the effect of *H.pylori* on apoptosis, proliferation and *p*53 gene expression in gastric epithelium and to find out the relationship between *H.pylori* and gastric carcinog enesis, and the possible mechanism.

MATERIALS AND METHODS

Subjects

All samples were selected from people screened by endoscopy in a high risk area of gastric carcinoma in Zhuanghe, Liaoning Province. A total of 100 gastric mucosal biopsy specimens, including 20 normal mucosa, 30 metaplasia, 30 dysplasia and 20 gastric carcinoma cases. *H.pylori* infecti on was assessed by hematoxylin-eosin staining[9-11] and PCR[12]. If both results of the tests in a patient were positive, the patient was considered to be infected by *H.pylori*; if neither was positive, the patient was considered negative.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end la belling method

Using the kit (Oncor, San Diego, USA), the staining steps are as follows: ① the sections were deparaffinised through xylem and alcohol, and washed; ② diges ted for 15 min with proteinase K (20 mg·L^{-1}); ③ quenched with 30 ML·L^{-1} H_2O_2 for 20 min ; ④ applied with equilibration buffer for 15 s at room temperature ; ⑤ added with terminal deoxynucleotidyl transferase (TdT) and incubated at 37℃ for 60 min; ⑥ added with STOP/WASF buffer at 37℃ for 10 min ; ⑦ dropped with hydrogen peroxidase for 30 min at room temperature; and ⑧ visualized by immersi on in 3,3'-diaminobenzidine (DAB) solution, restained with methyl-green, and deh yd rated, transparency, mounted . PBS was substituted for TdT as negative control.

Immunohistochemical staining

SP kit was used (Zymed, USA).The primary a ntibodies were PCNA monoclonal antibody(diluted 1:50) and *p*53 monoclonal anti body (ready to use, Maixin, Fijian), respectively. Before staining, the sections were microwave heated in 0.05 mol·L^{-1} citric acid solution for antigen retrieval. PBS was substituted for primary antibodies as negative control.

Observed parameters

Two samples were stained by DNEL and immunohistochemical staining, then the DNEL-positive cells (apoptotic cell) and PCNA-positive cells (proliferative cell) were observed. Apoptotic index (AI) and proliferative index (PI) were obtained by calculating the percentage of positively stained cells evaluated for each tissue section after counting 1000 cells at more than 5 high power fields.

Statistical analysis

t test was used to compare the means. The positivity of *p*53 protein was analyzed by X^2 test

RESULTS

Charateristics of DNEL-positive, PCNA-positive and p53 protein-positive cells under microscopy

DNEL-positive cells (apoptotic cell) appeared brown corpuscular or diffuse in cell nuclei, and individual thickening nuclear membrane appeared brown. PCNA-positive cells (proliferative cell) appeared brown corpuscular in cell nuclei. Positive pro duct of *p*53 expression was restricted in cell nuclei. In normal mucosa, apoptotic cells sporadically scattered on the epithelium, a few proliferative cells scattered on the glandular neck and expression of *p*53 protein was not seen. But in the tissue of gastric carcinoma, apoptotic cells accounted for 1.62%; prolifer a tive cells for 41.99% and a cluster was formed all over the lesions; and there was a significant increase in the expression of p53 protein. Effect of *H. pylori* infection on apoptosis in gastric epithelium In the metaplasia mucosa, the apoptotic index in *H. pylori*-positive group was higher than that in normal mucosa ($P<0.01$), and it was also higher than that in *H. pylori*-negative group; while in the dysplasia mucosa, AI in *H. pylori*-positive group was lower than that in *H. pylori*-negative group. In the metaplasia, dysplasia mucosa and gastric carcinoma AI presented with an evidently gradual decrease trend ($P<0.05$ or $P<0.01$ Table 1) in *H. pylori*-positive group.

Table 1 Effect of *H. pylori* infection on AI in gastric epithelium (%, $\bar{x}\pm s$)

Group	*H.pylori* positive		*H.pylori* negative	
	n	AI	*n*	AI
Normal	-	-	20	2.08±1.07
Metaplasia	15	4.36±1.95[d]	15	3.81±1.76
Dysplasia	15	2.31±1.10[b]	15	3.05±1.29
Carcinoma	10	1.34±0.69[a]	10	1.89±1.03

[d]$P<0.01$, *vs* normal (*H.pylori* negative); [a] $P<0.05$, [b]$P<0.01$, *vs* upper adjacent group.

Effect of H.pylori infection on proliferation in gastric epithelium

In metaplasia and dysplasia mucosa the PI was significant higher than those in normal mucosa ($P<0.01$), and that in *H. pylori*-posi tive group was higher than that in *H. pylori*-negative group ($P<0.01$). From the normal mucosa to the gastric carcinoma, the PI has a gradual increase trend ($P<0.05$ or $P<0.01$, Table 2) in *H. pylori*-positive group.

Effect of H.pylori infection on the expression of p53 in gastric epithelium

In the metaplasia mucosa, the positivity of *p*53 protein expresion in *H.pylori*-positive group was higher than that in noraml mucosa ($P<0.01$). In the metaplasia,dysplasia mucosa and gastric carcinoma, there was a trend of gradual increase in positivity of *p*53 protein expression (Table 3) in *H. pylori*-positive group.

Table 2 Effect of *H.pylori* infection on PI in gastric epithelium (%, $\bar{x}\pm s$)

Group	*H.pylori* positive		*H.pylori* negative	
	n	PI	*n*	PI
Normal	-	-	20	9.78±3.65
Metaplasia	15	19.11±6.79[e]	15	12.25±5.63[d]
Dysplasia	15	33.89±11.65[eb]	15	22.09±8.18[d]
Carcinoma	10	48.27±15.67[ea]	10	34.70±12.74[c]

[e]$P<0.01$,*vs* normal (*H.pylori* negative); [a]$P<0.05$, [b]$P<0.01$,*vs* upper adjacent group; [c]$P<0.05$, [d]$P<0.01$, *vs* positive group.

Table 3 Effect of *H.pylori* infecion on expression of *p*53 protein in gastric epithelium

Group	*n*	*H.pylori*	*n*	*p*53 positive *n* (%)
Normal	20		20	0 (0.0)
Metaplasia	30	+	15	7 (46.7)[b]
		-	15	1 (6.7)
Dysplasia	30	+	15	8 (53.3)
		-	15	5 (33.3)
Carcinoma	20	+	10	8 (80.0)
		-	10	7 (70.0)

[b]$P<0.01$, *vs* normal (*H. pylori* negative).

DISCUSSION

Gastric mucosa consists of continuously renewed cells and cell proliferation an d apoptosis maintain their balance[13]. This study shows that the apoptotic cells were identified in gastric surface epithelium and formed “an apoptotic zone”; proliferative cells were seen in the neck region of the mucosal glands and formed “a prolife rating zone”. This distribution shows the proliferating zone gradually maturation, aging and death to the surface in the gastric mucosal epithelium. However in gastric carcinoma, apoptotic cells amount to 1.62%, proliferative cells amount t o 41.99%, which clustered all over the tumor tissue. This change obviously lost the distribution characteristics of apoptotic zone and proliferating zone which elucidates that the regulation of apoptosis and proliferation have already been beyond the normal mucosa and appear significantly disordered in gastric carcinoma.

Human gastric carcinogenesis is a multistep and multifactorial process[14-17]. In this process, the state of apoptosis and proliferation of gastric epithelium will change[18,19]. In this study, *H.pylori* infection was found to affect the cell apoptosis and proliferation. In the metaplasia mucosa, AI was higher than that in normal mucosa in *H.pylori*-positive group, and higher than that in *H.pylori*-negative one; however, in the dysphasia mucosa, AI in *H.pylori*-positive group is lower than that in *H.pylori*-negative group. In the process of gastric carcinogenesis[14,15], from the phase of metaplasia, dysplasia to gastric carcinoma, AI gradually decreased in *H.pylori*-positive group. This shows that from normal mucosa to gastric carcinoma, *H.pylori* may induce cell apoptosis in the phase of metaplasia; but it inhibits cell apoptosis in the phase of dysplasia, this is familiar with gastric carcinoma. Several reports suggest that *H.pylori* produces cytotixic protein (CagA and VacA)[20-28], and gastric mucosa can increasex some cytokines, nitrous oxide sythetase and oxygen radicals released after *H.pylori* infection[29-35]. At the same time, *H.pylori* infection can lower the gastric antioxidant ability. All those factors make DNA dam age or enhance the susceptibility of DNA-damage, and those DNA-damaged cells can be

cleared away by apoptosis[36],and this may be the mechanism that *H. pylori* in duces apoptosis. It has been proved that wild type *p*53 protein can induce cell apoptosis but the intracellular accumulation of mutant *p*53 protein can inhibit cell apoptosis and promote cell transformation and proliferation, resulting in carcinogenesis[37-41]. In this study, in the phae of metaplasia, positivity of *p*53 protein expression in *H.pylori*-positive group was higher than that in nor malmucosa. From the phase of metaplasia to gastric carcinoma, the positivity of *p*53 protein expression increased in *H. pylori*-positive group. As positive *p*53 protein confirmed by immunohistochemical staining was considered as mutation type[42]. It suggests that beginning with metaplasia, *H.pylori* infect ion can strengthen the expression of mutant *p*53 gene. With the accumulation of mutant and expression of *p*53, its inhibiting effect on apoptosis[43-45] will overpass the indution effect of *H. pylori*. Therefore in the *H.pylori*-positive dyspl asiamucosa, there was a decrease in apoptosis. In this study, in the *H. pylori*-positive gastric precancerous lesions-metaplasia and dysplasia, PI was significantly higher than that in normal mucosa, and higher than those in the c orresponding negative lesions. In the progress from normal mucosa to the malignant phenotype, PI gradually increased in *H.pylori*-positive group. T here sult shows that *H.pylori* apparently improves gastric epithelium proliferation which is very similar with the proliferating characteristics of gastric carcinoma. The mechanism may be that urease enzyme produced by *H. pylori* hydrolyses urea into carbon dioxide and ammonia, the latter can promote mitosis[46]; *H. pylori* infection will increase mucosal content and expression of EGF and TGF-α[47-52] and the effect of mutant *p*53 on proliferation, will result in rapid proliferation of epithelium.

In the phase of metaplasia, *H.pylori* can induce apoptosis, stimulate hyperproliferation and result in the regulation disorder of apoptosis and proliferation in the gastric epithelial cells, which increase the instability of gastric mucosa and carcinoma variability. Accompanied with the progress of lesions and the accumulation of *p*53 protein, *H. pylori* induce s gastric epithelial cell hyperproliferation and apoptosis reduction or even imb alance of the apoptotic and proliferative process, and accumul at ion of DNA-damaged cells, ultimately resulting in gastric carcinogenesis.This makes it clear that *H. pylori* infection may be an important factor of gastric car cinogenesis. Thus, it is significant to prevent *H. pylori* infection, eradicate *H. pylori* in early stage and study the relationship between *H. pylori* infection and gastric carcinoma in order to decrease the incidence rate and prevent gastric carcinoma.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Polymorphism of flagellin A gene in *Helicobacter pylori*

Wan-Sheng Ji[1], Jia-Lu Hu[1], Jun-Wen Qiu[1], Dao-Rong Peng[2], Bing-Long Shi[1], Shao-Juan Zhou, Kai-Chun Wu[1], Dai-Ming Fan[1]

[1]Institute of Digestive Diseases, [2] Department of Bacteriology, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032,China
Correspondence to: Jia-Lu Hu, Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. jiwsh@netease.com
Telephone: +86-029-3375229(lab), Fax: +86-29-7432505(home)
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Abstract

AIM: **To study the polymorphism of flagellin A genotype and its significance in *Helicobacter pylori* (*H. pylori*).**

METHODS: **As the template, genome DNA was purified from six clinical isolates of *H. pylori* from outpatients, and the corresponding flag ellin A fragments were amplified by polymerase chain reaction. All these products were sequenced. These sequences were compared with each other, and analyzed by software of FASTA program.**

RESULTS: **Specific PCR products were amplified from all of these *H. pylori* isolates and no length divergence was found a mong them. Compared with each other, the highest ungapped identity is 99.10%, while the lowest is 94.65%. Using FASTA program, the alignments between query and library sequences derived from different *H. pylori* strains were higher than 90%.**

CONCLUSION: **The nucleotide sequence of flagellin A in *H. pylori* is highly conservative with incident divergence. This information may be useful for gene diagnosis and further study on flagellar antigen phenotype.**

Subject headings *Hlicobacter pylori*; flagellin A; polymorphism; polymerase chain reaction

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is widely distributed among humans. It is the major pathogen for most of the gastric diseases. Several potential virulence factors have been suggested to play a role in Helicobacter pathogenesis, those studied in most details including the production of abundant amounts of urease, motility, the expression of cytotoxins and other extracellular enzyme, liposaccharides and adhesion to tissue-specific receptors[1-12].

Even in the viscous environment, a high degree of motility is is conferred by three to six flagellas that extends from one pole of the bacterium. The flagella consist of a basal body that contains the motor and the hook structure, the central filament and a membranous sheath enveloping each filament. Flagellin A is the major molecule of filament and responsible for bacterial motility to most degree in *H. pylori*[13-15].

According to the data reported previously, the sequence of flagellin A is highly conservative in both amino acid and nucleotide level; flagellin A is one of the major antigens to induce the production of antibody in serum[16-20]. Based on these points, flagellin A may be one of the best alternatives for gene diagnosis and designs of vaccine against *H. pylori*.

MATERIAL AND METHODS

H. pylori strains

Six *H. pylori* isolates were offered by the Department of Bacteriology, Xijing Hospital, the Fourth Military Medical University.

Amplification of flagellin A fragment in H. pylori

Genome DNA as template was purified from *H. pylori* isolates by using genome DNA purification kits (Shanghai Watson Biotech. LTD). Referring to the complete sequence of flagellin A gene in Genbank (X60746), primers were designed by primer3 program as follows[21]. Forward primer: 5'GCGGATAAGGCTATGGATGA 3'; reverse primer: 5'GATCGCTGCGACTAACCTTC 3'. Amplification was proceeded by two-step polymerase chain reaction; that is: 94℃ for 5 min, 94℃ for 45 s, 68℃ for 1 min, 30 cycles, and then 72℃ for 10 min.

Comparison of PCR products and alignment analysis by FASTA program

Sequences of PCR products amplified from six *H. pylori* isolates were compare d with each other. One of these sequences was presented and analyzed by FASTA program (version 3.3t07).

RESULTS

Any other sequences were compared with sequence A, the numbers of divergent nucleotides were eighteen, ten, twenty-one, thirteen, and twenty-eight between sequence A and B, A and C, A and D, A and E, and between sequence A and F, respectively (Figure 1).

PCR products including 561 nucleotides each were compared mutually and the identity was calculated as follows. Identity (%) =1-[100% × number of divergent nucleotides / 561nucleotides]. By comparison, it is found that sequence F has much more divergence from any other sequences, while sequence Ehas higher alignment with other sequences except sequence F (Table 1).

1	A	CCCCGGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTA
	B	CCCCCGGCTTCTGGGGATATTAGCTTGACTTTTAAGCAAGTGGATGGCGTGAATGATGTG
	C	CCCCGGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTA
	D	CCCCGGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTG
	E	CCCCGGGCTTCTGGGGATATTAGCTTGACTTTTAAGCAAGTGGATGGCGTGAATGATGTG
	F	CCCCGGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTA
61		ACTTTAGAGAGCGTGAAAATCTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
		ACTTTAGAGAGCGTGAAAATCTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
		ACTTTAGAGAGCGTAAAAATCTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
		ACTTTAGAGAGCGTGAAAGTTTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
		ACTTTAGAGAGCGTGAAAATCTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
		ACTTTAGAGAGCGTGAAAGTCTCTAGTTCAGCAGGCACAGGGATTGGCGTGTTAGCAGAA
121		GTGATTAACAAAAACTCTAACCGAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACG
		GTGATTAACAAAAACTCTAACCAAACAGGGGTTAAAGCTTATGCGAGCGTTATCACTACG
		GTGATTAACAAAAACTCTAACCAAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACG
		GTGATTAACAAAAACTCTAACCGAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACG
		GTGATTAACAAAAACTCTAACCAAACAGGGGTTAAAGCTCATGCGAGCGTTATCACCACG
		GTGATTAACAAAAACTCTAACCGAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACA
181		AGCGATGTGGCGGTCCAGTCAGGAAGTTTGAGTAATTTAACCTTAAACGGGATTCATTTG
		AGCGATGTGGCGGTTCAGTCAGGAAGTTTGAGTAATTTAACCTTAAATGGGATTCATTTG
		AGCGATGTGGCGGTCCAGTCAGGAAGTTTGAGTAATTTAACCTTAAATGGGATTCATTTG
		AGCGATGTGGCGGTCCAGTCAGGAAGTTTGAGTAATTTAACCTTAAATGGGATTCATTTG
		AGCGATGTGGCGGTCCAGTCAGGAAGTTTGAGTAATTTAACCTTAAATGGGATTCATTTG
		AGCGATGTGGCAGTCCAATCGGAAGATTTGAGTAATTTAACCTTAAATGGGATTCATTTG
241		GGCAATATCACAGACATTAAGAAAAACGACTCAGACGGAAGGTTAGTCGCAGCGATCAAT
		GGCAATATTGCAGATATTAAGAAAAACGACTCAGACGGAAGGTTAGTCGCAGCGATCAAT
		GGCAATATCGCAGACATTAAGAAAAACGACTCAGACGGAAGGTTAGTCGCAGCGATCAAT
		GGTAATATTGCAGATATTAAGAAAAACGACTCAGACGGAAGGTTAGTCGCAGCGATCAAT
		GGCAATATCGCAGATATTAAGAAAAACGACTCAGACGGAAGGTTAGTCGCAGCGATCAAT
		GGTAATATCGCAGATATTAAGAAAAATGACTCAGATGGGAGATTAGTCGCAGCGATCAAT
301		GCGGTTACTTCAGAAACCGGCGTGGAAGCTTACACGGATCAAAACGGGCGCTTGAATTTG
		GCGGTTACTTCAGAAACCGGCGTGGAAGCTTATACGGATCAAAATGGGCGCTTGAATTTG
		GCGGTTACTTCAGAAACCGGCGTGGAAGCTTATACGGATCAAAACGGACGCTTGAATTTG
		GCGGTTACTTCAGAAACCGGTGTGGAAGCTTATACGGATCAAAAAGGGCGCTTGAATTTG
		GCGGTTACTTCAGAAACCGGCGTGGAAGCTTATACGGATCAAAATGGGCGCTTGAATTTG
		GCGGTTACTTCAGAAACCGGTGTGGAAGCTTATACGGATCAAAAAGGGCGCTTGAATTTG
361		CGCAGTTTAGATGGCCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAATGGGCCTAGT
		CGCAGTTTAGATGGTCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAGTGGGCCTAGC
		CGCAGTTTAGATGGTCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAATGGGCCTAGC
		CGCAGTATAGATGGTCGTGGGATTGAAATCAAAACCGACAGCGTCAGTAATGGGCCTAGC
		CGCAGTTTAGATGGTCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAGTGGGCCTAGC
		CGCAGTATAGATGGTCGTGGGATTGAGATCAAAACCGATAGCACTAGTAATGGGCCTAGT
421		GCTTTAACGATGGTTAATGGCGGTCAGGATCTAACAAAAGGCTCTACTAACTACGGAAGG
		GCTTTAACGATGGTTAATGGCGGTCAGGATTTAACAAAAGGCTCTACTAACTACGGAAGG
		GCTTTAACGATGGTTAATGGCGGTCAGGATCTAACAAAAGGCTCTACTAACTACGGAAGG
		GCTTTAACGATGGTTCATGGCGGTCAGGATTTAACAAAAGGCTCTACTAACTACGGAAGG
		GCTTTAACGATGGTTAATGGCGGTCAGGATTTAACAAAAGGCTCTACTAACTACGGAAGG
		GCTTTAACGATGGTTAATGGCGGTCAGGATTTAACAAAAGGCTCTACTAACTATGGGAGG
481		CTTTCTCTCACACGATTAGACGCTAAGAGCATCAATGTCGTTTCGGCTTCTGACTCACAG
		CTTTCTCTCACACGATTAGACGCTAAGAGCATCAATGTCGTTTCGGCTTCTGACTCACAG
		CTTTCTCTCACACGATTAGACGCTAAGAGCATCAATGTCGTTTCGGCTTCTGACTCGCAG
		CTTTCTCTCACACGCTTAGACGCTAAGAGCATCAATGTCGTTTCGGCTTCTGACTCACAG
		CTTTCTCTCACACGATTAGACGCTAAGAGCATCAATGTCGTTTCGGCTTCTGACTCACAG
		CTTTCTCTCACACGATTAGACGCTAAGAGCATCAATGTGGTTTCGGCTTCTGACTCACAG
541		CATTTAGGTTTCACAGCGATTGGTTT
		CATTTAGGTTTCAGTGCGATTGGTTT
		CATTTAGGTTTTACAGCGATTGGTTT
		CATTTAGGCTTCACAGCGATTGGTTT
		CATTTAGGTTTCAGTGCGATTGGTTT
		CATTTAGGCTTCACAGCGATTGGTTT

Figure 1 Comparison of PCR products between sequence A and other sequences. Note: Divergent nucleotides were thickened and shadowed.

Table 1 Comparison of identity and divergent numbers among different PCR products

Identity (%)	Number of divergent nucleotides (base pairs)					
	A	B	C	D	E	F
A	-	18	10	21	13	28
B	96.79	-	16	18	4	29
C	98.21	97.15	-	18	13	30
D	96.25	96.79	96.79	-	17	22
E	97.68	99.29	97.68	96.97	-	30
F	95.01	94.83	94.65	96.08	94.65	-

Data of identity (%) were under dashed line.

Sequence A was forwarded to EMBL server, and fifty library sequences were searched and analyzed by FAS TA program. Ten library sequences with highest Z-score were shown here. All these sequences were derived from *H. pylori* strains and had identity with Sequence A higher than ninety-five percent (Table 2).

Table 2 Identity analysis of sequence A in DNA data base using FASTA program

Source of nucleotide sequence in Genbank	Z-score	Identity (%)	Length of compared sequences (base pairs)
X60746	2736.1	96.4	561
AE001487	2694.1	95.9	561
AE000574	2692.0	95.9	560
U63249	2139.5	98.1	424
U63250	2130.1	97.9	424
U63238	2120.6	97.6	424
AJ009373	2092.3	96.9	424
U63224	2082.9	96.7	424
U63236	2082.9	96.7	424
U63253	2082.9	96.7	424

DISCUSSION

Flagella are well-controlled organelle and essential for microbial motility in many bacterial genera[22-28]. Though much effort has been made to study the polymorphism of flagella in other genera like Salmonella, relatively little was known about that in *Helicobacter pylori*[29-34]. Recently, genome DNA of several isolates was sequenced, which offered the first structural information and made it possible to do further work. Flagellin A is unique in the known *H. pylori* database, which means that it has sequence quite different from analogs in other bacteria but high conservative within genus.

H. pylori are genetically highly diverse, the intraspecific free recombination of flagellin A is a frequent incident just as occurred in VacA and CagA[35-43]. Not like the diversity of serotypes in Salmonella or other bacteria, few of specific flagellar serotypes have been found in *H. pylori* so far[44-47]. The reported sequences of flagellin A were highly conservative and some difference may be caused by different methods. This fact was accordant to the results presented here. In this experiment, a fragment of about four hundred nucleotides in *H. pylori* flagellin A gene was found highly conservative between the oriental and the occidental.

As the unique pathogen, *H. pylori* are correlated with almost all of the gastric diseases[48-58]. Therefore, development of new methods to detect and eradicate *H. pylori* infection has become one of the central tasks to treat most of gastric diseases. The conservatism of flagellin A discovered in this experiment may be very helpful for PCR-based gene diagnosis.

Though the polymorphism of Flagellin in *H. pylori* was reported previously in some labs, it has not determined whether there are specific flagellar serotypes that may be relevant to the clinical symptoms[59-66]. In this experiment, one of the PCR products was found more divergent than that caused by geographical or ethnic fact ors. It is still not known whether the polymorphism shown here has influence on the flagellar serotype, motility, virulence or its colonization to gastric mucosa. To be one of the vaccine candidates against *H. pylori*, more work should be done on epitope mapping, analysis of molecular structure, and determination of antigen determinant region as well.

As one of the major known pathogenic factors, much research has been done on *H. pylori*. In one hand, much work was focused on its influence on gastric physiology, pathogenesis, carcinogenesis, and mutual reaction between host and pathogen; on the other hand, more attention was paid on its clinical aspect involving treatment, detection and prevention[67-84]. Though there have already been some methods to detect and treat *H. pylori* infection so far, much effort must be made to develop more effective w ay to deal with these worms.

In summary, the nucleotide sequence of flagellin A in *H. pylori* is highly conservative with incident divergence. This character is favorable for PCR-based diagnosis. The significance of the polymorphism in nucleo tide level has not well understood. Further study on the divergent isolate that discovered in this experiment might be helpful to interpret this phenomenon.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Human papillomavirus 16 E6 is associated with the nuclear matrix of esophageal carcinoma cells

Hai-Bin Chen[1,2], Ling Chen[2], Jin-Kun Zhang[2], Zhong-Ying Shen[2], Zhong-Jing Su[2], S.B. Cheng[3], E.C. Chew[4]

[1]Department of Histology and Embryology, [2]Tumor Pathology L aboratory, Shantou University Medical College, Shantou 515031, Guangdong Province, China
[3]Department of Physiology and 4Department of Anatomy, The Chinese University of Hong Kong, Shatin, NT. Hong Kong, China
Supported by Guangdong Provincial Natural Science Foundation of China, No. 990799 and Medical Science Foundation of Guangdong Province, No. B19 97083.
Correspondence to: Dr. Hai Bin Chen, Department of Histology and Embryology, Shantou University Medical College, 22 Xinlinglu, Shantou 515031, Guangdong Province, China. chenhb@stu.edu.cn
Telephone: +86-754-8900443, Fax: +86-754-8557562
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Abstract

AIM: **To explore the etiologic role of HPV infection in esophageal carcinoma, and the association of HPV-16 E6 with the nuclear matrix of carcinoma cells.**

METHODS: **Two esophageal carcinoma cell lines, EC/CUHK1 and EC/CUHK2, were tested for HPV-16 E6 subgenetic fragment by polymerase chain reaction amplification of virus DNA associated nuclear matrix. RT-PCR and immunocytochemistry were also used to visualize the expression of E6 subgene in the cells.**

RESULTS: **The HPV-16 E6 subgenetic fragment was found to be present in nuclear matrix-associated DNA, E6 oncoprotein localized in the nucleus where it is tightly associated with nuclear matrix after sequential extraction in EC/CUHK2 cells. It was not detected, however, in EC/CUHK1 cells.**

CONCLUSION: **The interaction between HPV-16 E6 and nuclear matrix may contribute to the virus induced carcinogenesis in esophageal carcinoma.**

Subject headings Esophageal neoplasms/virology; Esophaheal neoplasms/pathology; Tumor cells, cultured; Papillomavirus, human; Nuclear matrix

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INTRODUCTION

Esophageal carcinoma is the most common malignant epithelial neoplasm of the esophagus worldwide. The incidence of esophageal carcinoma reported by WHO has increased many folds in the past 20 years and is apparently still rising. However, the incidence of this disease varies widely among different geographic regions, being remarkably high in certain regions, including the northern parts of China, Iran and South Africa, whereas being remarkably low in Western Europe and North America. The wide geographic variation in incidence of esophageal carcinoma implies a causal role of environmental factors in the pathogenesis of esophageal carcinoma. Epidemio logical studies have shown that environmental factors, including tobacco, alcohol, certain dietary products, nutritional deficiency, and other chemical agents are associated with the development of esophageal carcinoma in both high and low risk regions[1,2].

Human papillomavirus (HPV) has recently been implicated as an important etiological factor in the development of esophageal carcinoma in high incidence areas[3-6]. HPV DNA sequences were detected in 24% to 60 % of esophageal squamous cell carcinoma in high risk regions. HPVs are small DNA viruses that can be classified as either high risk or low risk group. HPV-16 and 18 is most common in high risk group. In most HPV infected cells, E6E7 subgenetic fragments of viral genome are retained and integrated in host cell chromosomes[7,8]. The *in vivo* and *in vitro* transforming properties of the virus reside in these two genes. E6 interacts with a tumor suppressor protein P53 and has been shown to target the P53 protein for rapid degradation[9,10]. E6 expressing cells are the refore unable to express effective levels of P53 protein, resulting in the loss of normal P53 functions within these cells. The ability of the high risk HPV types to interfere with normal P53 function is particularly significant for oncogenetic activity since alterations of the p53 gene are the most common genetic event detected so far in almost all types of human cancers[11-14]. E6 also targets other proteins: the focal adhesion protein paxillin[15] and the interferon regulatory factor 3[16]. These data indicate the multifunctions of viral oncop roteins, modifying a multitude of cellular functions.

Nuclear matrix is operationally defined as the nonchromatin component of the nucleus after sequential extraction with non-ionic detergents, nucleases and high salt buffers. It consists of nuclear envelope and inner- fibrous web forming a scaffold for attachment of DNA[17]. Nuclear matrix has been shown to play a fundamental role in nuclei c acid m etabolism[18-21]. It has been found to harbor sites for DNA replication[22,23], transcription[24,25], RNA processing[26,27] and steroid hormone action[28-30]. In addition, proto-oncogene products and several viral oncoproteins such as the E B virus nuclear antigen leader protein[31],adenovirus E1A protein, the simian virus 40 large tumor antigen and HPV E7 protein[32,33] associate with the nuclear matrix. The involvement of nuclear matrix and its proteins in various nuclear a ctivities has also provided a potential target for anticancer agents[33-36] and toxicant[37].

In this study, the association of both HPV-16 E6 gene and E6 protein with nuclear matrix in HPV-16-containing esophageal carcinoma cell line was investigated by PCR, RT-PCR and immunocytochemistry.

MATERIALS AND METHODS

Cell culture

Two human esophageal carcinoma cell lines, EC/CUHK1 and EC/CUH K2, were used. EC/CUHK1[38] was established from a well-differentiated human esophageal carcinoma and it is HPV negative.

EC/CUHK2[39] was established from a Chinese male patient with a poorly differentiated squamous carcinoma of the esophagus, the cells contain HPV-16 DNA. The cell lines were cultured in DMEM (Gibco) supplemented with 50 mL/L fetal bovine serum (Gibco), 10^5 units/L penicillin an d 100 mg/L streptomycin (Sigma) at 37℃ in humidified 50 mL/L CO_2 incubator.

Nuclear matrix-associated DNA extraction

The method developed by Fey *et al* was modified[40]. Briefly, culture cells were trypsinized and resuspended in cytoske leton buffer (100 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose and 5 ml/L Triton X-100) for 15 min at 4℃. Chromatin was removed by incubation of the pellet with different concentrat ions (50 and 100 mg/L) of DNase I and RNase A (100 mg/L) in digestion buffer (same as CSK except with 50 mmol/L NaCl instead of KCl) for 30 min at room temperature. The digestion was terminated by addition of cold ammonium sulfate to a final concentration of 0.25 mol/L. After centrifugation, the resulting pellet containing nuclear matrix was digested overnight by proteinase K at 55℃ for DNA ext raction. The DNA was extracted by phenol-chloroform method and RNA was removed by DNase-free RNase treatment at 37℃ for 1 h. After re-extraction with phenol -chloroform, DNA was precipitated with ethanol in the presence of ammonium acetate and stored in Tris-EDTA buffer at 4℃.

Polymerase chain reaction

Purified primers specifying HPV-16 E6 sequences were designed, forming the viral sequence at position nucleotide 82-559 amplifying 477 bp fragments. The primer sequences are 5'-ATGCACCAAAAGAGAACTGC-3' and 5'-TTACAGCTGCGTTTCTCTAC-3'. PCR was performed in a 25 μl volume of PCR buffer with 1.0 mmol/L MgCl 2, 4×dNTPs, primers and sample DNA. The mixture was hot-started to reduce non-specificity. The amplificati on reaction was conducted with 30 cycles: 95℃ for 1 min; 55℃ for 1 min; and 72℃ for 1.5 min. HPV-16 plasmid DNA was employed as positive control. The PCR products were finally electrophoresed in 10 g/L agarose gel after ethidium bromide staining.

Total RNA extraction and RT-PCR

Total RNA was extracted by Trizol-chloroform method and precipitated with isopropanol. The RNA pellet was washed with ice-cold 750 ml/L ethanol, dried and resuspended in DEPC-treated water. The RNA quantity was determined with OD 260 and OD 280. Reverse transcription was performed with random hexamers, reverse transcriptase, RNase inhibitor, dNTPs and 1. 5 mmol/L $MgCl_2$ (Perkin Elmer) at 42℃ for 45 min. cDNA samples were then sub jected to PCR with HPV-16 E6 primer. The amplification condition was the same as the PCR.

Nuclear matrix preparations in situ and immunocytochemistry

To obtain whole mount nuclear matrix *in situ*, the method described by Staufenbiel *et al*[41] was followed with modifications. The monolayer of culture cells were grown on coverslips for 2 days. After three rinses with PBS at 4℃, the cells were incubated in cytoskeleton buffer, added 4 mmol/L ribonucleoside vanadyl complexes for 15 min at 4℃. Following the removal of supernatant, the cells were incubate d in reticulocyte standard buffer (42.5 mmol/L Tris-HCl pH 7.4, 8.5 mmol/L NaCl , 2.6 mmol/L $MgCl_2$, 1.2 mmol/L PMSF, 10 ml/L Tween 40, 12 mmol/L sodium deoxycholate, 2 mmol/L ribonucleoside vanadyl complexes) for 10 min at 4℃. Chromatin was removed by incubation of the cells in DNase I (100 mg/L) in digestion buffer f or 20 min at room temperature and terminated by addition of cold ammonium sulfate to a final concentration of 0.25 mol/L. The nuclear matrix on coverslips were fixed in PBS buffered 100 mL/L formalin for 10 min. After blocking the non-specificity, the cells were incubated with anti-HPV-16 E6 (Santa Cruz) antibody. The labeling was then detected by ABC method (Dako) and the result was visualized with DAB substrate. Negative controls were incubated in the absence of primary antibody.

RESULTS

PCR

A specific 477 bp PCR product was amplified by HPV-16 E6 primer in nuclear matrix associated DNA from EC/CUHK2 cell line, whereas DNA from nuclear matrix of EC/CUHK1 cell line showed negative reaction. Positive control using HPV-16 plasmid DNA confirmed the specificity and performance of PCR system. (Figure 1). Different concentrations (50 mg/L and 100 mg/L) of DNase I were employed to elute DNA during nuclear matrix preparation of EC/CUHK2. The residual nuclear matrix associated DNA was subjected to PCR. As shown in Figure 1, 477 bp PCR signal was detected in nuclear matrix associated DNA after 50 mg/L DNase and signal was found weakened in stronger DNase (100 mg/L) digestion groups.

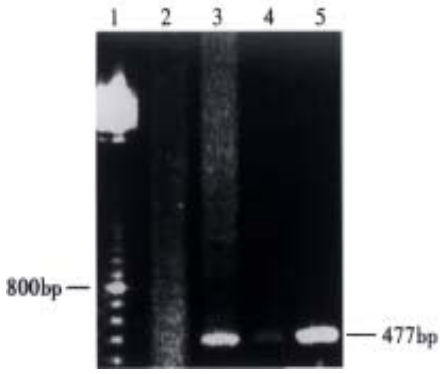

Figure 1 Results of PCR amplication with HPV-16 E6 primers. 1:100 bp DNA ladder marker. 2: nuclear matrix associated DNA of EC/CUHK1 cells, with 50 mg/L DNase digestion. 3: nuclear matrix associated DNA of EC/CUHK2 cells, with 50 mg/L DNase digestion. 4: nuclear matrix associated DNA of EC/CUHK2 cells, with 100 mg/L DNase digestion. 5: HPV-16 plasmid DNA.

RT-PCR

The expression of HPV-16 E6 in EC/CUHK1 and EC/CUHK2 cell lines were detected in the total RNA level. RT-PCR was conducted using specific HPV-16 E6 primer to detect HPV-16 E6 with 477 bp in length. The samples extracted from EC/C U HK2 showed specific amplification of 477 bp product, but negative results were observed in EC/CUHK1 (Figure 2).

Immunocytochemistry

HPV activity was assayed by anti-HPV-16 E6 oncoprotein antibody. EC/CUHK2 cells were immunolabelled wit h the antibody. The staining was found concentrated inside the nucleus. E6 oncoprotein distributed diffusely both in the nuclear filamentous structures and in the periphery of the nuclear lamin (Figure 3). In EC/CUHK1 cells, E6 protein immuno staining was negative (results not shown).

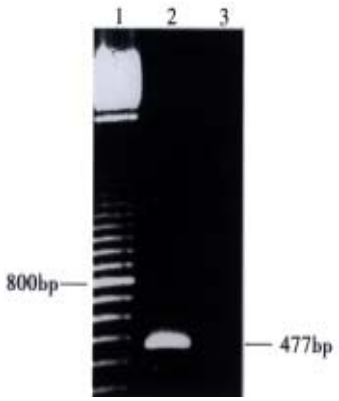

Figure 2 RT-PCR amplification with HPV-16 E6 primers. 1:100 bp DNA ladder marker. 2: EC/CUHK2 cells. 3: EC/CUHK1 cells.

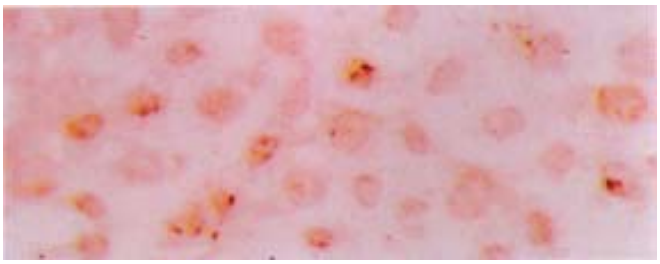

Figure 3 Immunocytochemical staining of anti-HPV-16 E6 protein in nuclear matrix of EC/CUHK2 cells.

DISCUSSION

HPV infections have been suspected to play a role in development of esophageal carcinoma. The prevalence of HPV varies among laboratories and different geographic regions from 0% to 67%. Recent reports found a relatively high rate (27.3% and 34.9%) of HPV positive biopsies in samples obtained from China and in 26.4% of samples obtained from cancer cases in South Africa[5,6]. In particular, HPV types of 16 and 18 are believed to play an important role in esophageal carcinogenesis in those areas with a high incidence of esophageal carcinoma[5,42]. Early observation and analysis of HPV genomes and the viral transcription pattern revealed frequent integration of viral DNA and the consistent expression of the early viral genes E6 and E 7. The same genes have subsequently been shown to be necessary for immortalizati on of various types of human cells[9,43,44]. The integration and retention of viral E6 and E7 fragments within the host cell genome is crucial in the initiation and promotion of virus induced carcinogenesis. The nuclear matrix has been repeatedly shown to be a site for DNA replication and transcription. The active genes are tightly bound to the nuclear matrix while the untranscribed genes are not[18,21]. Nuclear matrix is also an important site of viral host cell interactions[45-50]. The viral activities, such as viral replication and encapsulation were found highly associated with nuclear matrix of host cells. In our previous studies, we have demonstrated that the HPV-16 gene is associated with the nuclear matrix of cervical carcinoma cells[33,51-53]. The role of nuclear matrix in organizing HPV integration is also substantiated by our result. HPV-16 E6 DNA fragment was positively detected in the DNA pieces isolated from nuclear matrix after the treatment by a low or moderate concentration of DNase I. This suggests that so me of the nuclear matrix DNA or matrix attachment region may associate with HPV - 16 E6 DNA. It shows not only the integration of the E6 DNA fragment inside the genome of EC/CUHK2 cells but also a close relationship between the E6 fragment an d the site of active replication and transcription which is one of the proposed functions of nuclear matrix.

In this study, the results of RT-PCR and immunocy tochemistry after nuclear matrix preparation *in situ* show the expression of HPV-16 E6 gene and E6 oncoprotein in EC/CUHK2 cells to be localized in the cell nucleus where it is tightly associated with the nuclear matrix. The role of high risk HPV E6 oncoproteins in malignant transformation is supported by their immortalizing and transforming activities in cells in culture. Our findings provided evidence that the viral E6 protein is expressed inside EC/CUHK2 cells after persistent infection and helps efficient immortalization and survival of the infected cells. The interaction of E6 oncoprotein with P53 has been investigated extensively using *in vitro* systems and in cells[54-57]. The binding of E6 and P53 caused rapid ubiquitin-dependent proteolytic degradation of P53[9,10,58,59]. Cells expressing E6 therefore, have significantly depleted endogenous p53 expression, fail ed to show P53 accumulation following DNA damage and lost normal P53-mediatednegative cell growth control. The E6 targeting of P53 for degradation depends on the ability of the proteins to bind, but not the ability of P53 to form homo-oligomeric complexes. The subsequent degradation of P53 following the interaction with E6 is dependent on another cell protein, named E6-associated protein (E6-AP).

The E6/E6AP complex functions as a ubiquitin-protein ligase, equivalent to E3 in general ubiquitination pathways, allowing E2 catalysed conjugation of ubiquitin groups to lysine residues on P53[60-62]. The linkage of ubiquitin to P53 in this way serves as a recognition signal for specific protease targeting. In view o f the binding of E6 protein and nuclear matrix, we speculate that the E6-AP may be a nuclear matrix protein. It need to be further elucidated.

Since HPV-16 E6 subgenic fragments and oncoprotein are associated with nuclear matrix in esophageal carcinoma cell lines, further studies on the structure and function of these nuclear matrix proteins may provide more information on the relationship between HPV and esophageal carcinoma. It is of great importance to understand the car cinogenesis, and improve the diagnosis and treatment of esophageal carcinoma.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Cytochrome P450 2E1 genetic polymorphism and gastric cancer in Changle, Fujian Province

Lin Cai[1], Shun-Zhang Yu[2], Zuo-Feng Zhang[3]

[1]Department of Epidemiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China
[2]Department of Epidemiology, Shanghai Medical University, Shanghai 200032, China
[3]Department of Epidemiology, UCLA School of Public Health, Los Angeles California, USA
Supported by Natural Science Foundation of Fujian Province, China, No. C001009
Correspondence to: Lin Cai, Department of Epidemiology, Fujian Medical University, Fuzhou, 350004, Fujian Province, China.zjcailin@pub5.fz.fj.cn
Telephone: +86-591-3569264
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Abstract

AIM: **Genetic polymorphism in enzymes of carcinogen metabolism has been found to have the influence on the susceptibility to cancer. Cytochrome P450 2E1 (CYP2E1) is considered to play an important role in the metabolic a ctivation of procarcinogens such as N-nitrosoamines and low molecular weight organi c compounds. The purpose of this study is to determine whether CYP450 2E1 polymorphisms are associated with risks of gastric cancer.**

METHODS: **We conducted a population based case-control study in C hanglecounty, Fujian Province, a high-risk region of gastric cancer in China. Ninety-one incident gastric cancer patients and ninety-four healthy controls were included in our study. Datas including demographic characteristcs, diet intake, and alcohol and tobacco consumption of indivduals in our study were completed by a standardized questionnaire. PCR-R FLP revealed three genotypes:heterozygote (C1/C2) and two homozygotes (C1/C1 and C2/C2) in CYP2E1.**

RESULTS: **The frequency of variant genotypes (C1/C2 and C2/C2) in gastric cancer cases and controls was 36.3% and 24.5%, respectively. The rare homozygous C2/C2 genotype was found in 6 indivduals in gastric cancer group (6.6%), whereas there was only one in the control group (1.1%). However, the re was no statistically significan difference between the two groups (two-tailed Fisher's exact test, $P = 0.066$). Indivduals in gastric cancer group were m ore likely to carry genotype C1/C2 (odds ratio, OR = 1.50) and C2/C2 (OR = 7.34) than indivduals in control group ($\chi^2 = 4.597$, for trend $P = 0.032$). The frequencies of genotypes with the C2 allele (C1/C2 and C2/C2 genotypes) were compared with those of genotypes without C2 allele (C1/C1 genotype) among indivduals in gastric cancer group and control group according to the pattern of gastric cancer risk factors. The results show that indivduals who exposed to these gastric cancer risk factors and carry the C2 allele seemed to have a higher risk of developing gastric cancer.**

CONCLUSION: **Polymorphism of CYP2E1 gene may have some effct in the development of gastric cancer in Changle county, Fujian Province.**

Subject headings gastric neoplasm/ genetics; gastric neoplasm/ etiology; Cytochrome P-450 2E1; CYP2E1/ genetics; genotype; human; FUJIAN

Cai L, Yu SZ, Zhang ZF. Cytochrome P450 2E1 genetic polymorphism and gastric cancer in Changle, Fujian Province. *World J Gastroenterol*, 2001;7(6):792-795

INTRODUCTION

Fujian Province is one of the highest risk areas of gastric cancer in China[1,2]. Gastric cancer is the major cause of cancer mortality in Changle County,Fujian Province[3-5]. Epidemio logical studies have shown that an increased risk of developing gastric cancer is associated with diet and tobacco smoking[6-8]. One particular hypothesis, which has been paid more attention, is that N-nitroso compounds from dietary sources are involved in carcinogenesis of gastric cancer[9-10]. Recent advances in cancer research have revealed that the main etiology of human cancers are genetic changes in cancer related genes caused by carcinogens in the environment[11-14]. It is known that most of exogenous (xenobiotics) and endogenous chemical car cinogens require biotransformation to activated forms to be carcinogenic[15,16]. Most of the human metabolizing enzymes are genetically polymorphic, and those polymorphisms may affect the enzyme activity or inducibility[17-20]. The sensibitity to procarcinogen differs among individuals, which may have substantial importance in carcinogenesis[21-24]. Cytochromes P450 (CYPs) play an important role on metablism of several aspects of cancer. Cytochrome P450 2E1 (CYP2E1) is primarily responsible for the bioactivation of many low molecular weight carcinogens, and is involved in the metabolic oxidation of carcinogenic nitroso compounds, including N-nitrosoamines[25-26]. This study was designed to in vestigate the relationship between polymorphism of CYP 2E1 and gastric cancer.

MATERIALS AND METHODS

Subjects

All indivduals in our study are the residents of Changle county, Fujian Province of China. Ninety-one patients with pathologic diagnosis as primary gastric cancer between January 1996 to March 1998 and 94 age-and sex matched healthy controls were included in this study. Each indivdual was personally interviewed to obtain information on demographic characteristics, habits of cigarette smoking, alcohol drinking, and dietary consumption frequency. Blood specimens from them were also obtained.

DNA isolation and PCR-RFLP analysis

Genomic DNA was isolated from white blood cells by extraction u sing phenol/chloroform and precipitation using ethanol. PCR was performed using the primers 5'-CCAGTCGAGTCTACATTGTCA-3 (1370-1349) and 5'TTCATTCTGTCTTCTAACTGG-3 (999-978). The amplification reaction was conducted in a 50 (L solution

containing PCR buffer (1.5 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} KCl, 10 mmol· L^{-1} Tris-HCl, pH 8.3), 200 μmol·L^{-1} dNTP, 1ìmol·L^{-1} primer, 200 ng template DNA, and 2.5 μ Taq DNA polymerase (Promega Corp). Those reactions were performed about 35 cycle s at conditions following as denaturation for 1 min at 95℃, annealing for 1 min at 55℃, extension for 1 min at 72℃ and a final extension for 10 min at 72℃. T he PCR products were digested with PstI or RsaI (Fermantas-MBI. Vilnius, Lithuania) for 18 h at 37℃. The restriction sites was identified by 2.2% agarose gel electrophoresis. The genotypes of CYP2E1 were classified as following: a predominant homozygote (C1/C1), a heterozygote (C1/C2) and a rare homozygote (C2/C2). Figure 1, 2.

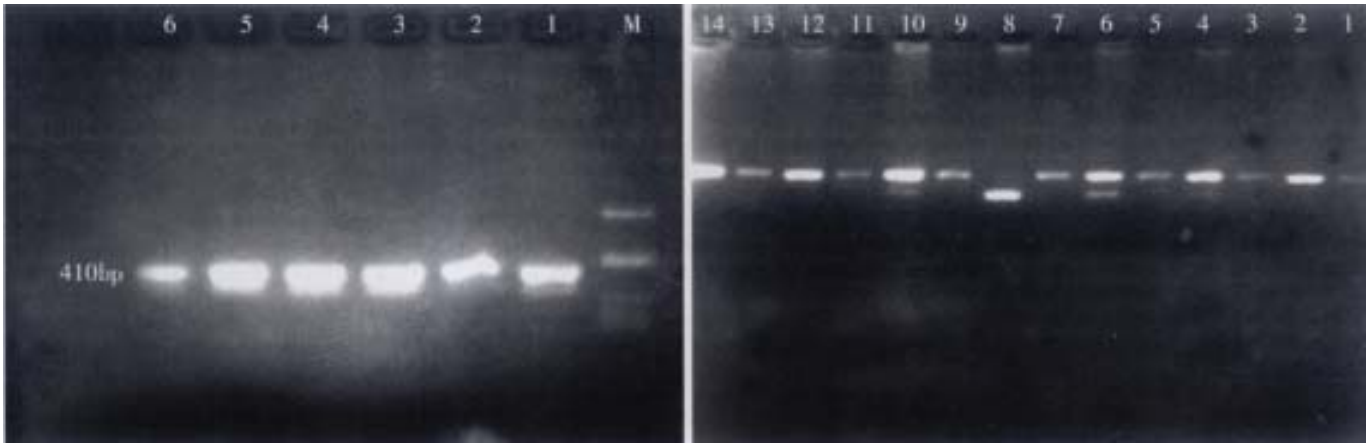

Figure 1 The PCR products of CYP2E1 gene. M: markers.
Figure 2 PCR-RFLP with Pst1.
Lanes 2, 12, 14: C1/C1; Lanes 4, 6, 10: C1/ C2; Lane 8: C2/C2; Lanes 1, 3, 5, 7, 9, 11, 13: Sample controls.

Statistical analysis

Fisher's exact tests were determined by standard methods. The relationships between CYP 2E1 genotypes and putative risk factors were measured using the odds ratios (ORs) and their 95% confidence intervals (95%CIs).

RESULTS

The age (x±s) of indivduals in gastric cancer group and control group were 58.4±11 years and 58.2±11 years, respectively. The distributions of age and sex were similar in cases and controls. The risks of gastric cancer were related to smoking, alcohol drinking, and fish sauce intake (a condiment commonly used by local residents), Table 1.

Table 1 Distribution of selected variables in gastric cancer group and group

	Gastric cancer (*n* = 91)	Control (*n* = 94)	*P* value
	n (%)	*n* (%)	
Age/y			
<50	21 (23.1)	22 (23.4)	
50-59	22 (24.2)	22 (23.4)	
60-69	32 (35.2)	34 (36.2)	
70+	16 (17.6)	16 (17.0)	0.9983
x±s	58.4±10.9	58.2±11.0	
Range	32-78	34-79	
Gender			
Male	77 (84.62)	82 (87.23)	
Female	14 (15.38)	12 (12.77)	0.6094
Education			
College	1 (1.1)	1 (1.1)	
High school	15 (16.5)	63 (67.0)	
Elementary school	57 (62.6)	22 (23.4)	
Illiterate	18 (19.8)	8 (8.5)	0.0000
Smoking/y			
0	31 (341)	60 (63.8)	
<10	2 (2.2)	16 (17.0)	
10-	16 (17.6)	7 (7.5)	
20-	22 (24.2)	4 (4.3)	
30-	20 (22.0)	7 (7.5)	0.0000
Alcohol drinking/y			
0	40 (44.0)	66 (70.2)	
<10	3 (3.3)	13 (13.8)	
10-	14 (15.4)	8 (8.5)	
20-	34 (37.4)	7 (7.5)	0.0000
Fish sauce intake			
Low (<3 times/w)	17 (18.68)	69 (73.40)	
High (≥ 3 times/w)	76 (81.32)	25 (26.60)	0.0000

The frequency of variant genotypes (C1/C2 and C2/C2) in gastric cancer group and control group was 36.3% and 24.5%, respectively. The rare homozygous C2/C2 genotype was found in 6 of gastric cancer group (6.6%), but in 1 of the controls (1.1 %). However, the result showed no statistical difference. Fisher's exact test $P = 0.066$ (Table 2). The allele frequencies of CYP2E1 fit with Hardy-Weinberg equilibrium ($\chi^2 = 0.242$, $P>0.05$).

Table 2 Cytochrome P450 2E1 genotype and risks of gastric cancer

CYP2E1	Gastric cancer		Control (n = 94)		OR (95%CI)
	n	(%)	n	(%)	
C1/C1	58	(63.7)	71	(75.5)	1.00
C1/C2	27	(29.7)	22	(23.4)	1 .50 (0.74-3.07)
C2/C2	6	(6.6)	1	(1.1)	7.34 (0. 84-166.60)

Fisher's test $P = 0.066$ *vs* controls χ^2 trend = 4.597 $P = 0.032$ *vs* controls

Gastric cancer indivduals were more likely to carry genotype C1/C2 and C2/C2 than indivduals in control group. Individuals carried at least one C2 allele (genotypes C1/C2 or C2/C2) seemed to have an increased risk of gastric cancer (OR = 1.86, 95%CI 1.07-3.25), Table 3.

Table 3 The allele frequencies of CYP2E1 in gastric cancer group and control group

	C1		C2	
	n	Frequencies	n	Frequencies
Gastric cancer	143	0.7857	39	0.2143
Controls	164	0.8723	24	0.1276

$\chi^2 = 4.91$ $P<0.05$ *vs* controls; OR = 1.86 95%CI 1.07-3.25.

The frequencies of genotypes with the C2 allele (C1/C2 and C2/C2) were compared with those of genotypes without C2 allele (C1/C1) among indivduals in gastric cancer and control group according to the pattern of gastric cancer risk factors of smoking, alcohol drinking and fish sauce intake. Indivduals who have been exposed to those risk factors of gastric cancer and carried the C2 allele seemed to have a higher risk of developing gastric cancer (Table 4).

Table 4 Relationships between CYP2E1 together with selected variables and risk of gastric cancer

CYP2E1	Variables	Gastric cancer	Controls	OR (95%CI)
	Smoking			
C1/C1	No	21	48	1.00
C1/C1	Yes	37	23	3.68 (1.67-8.19)
C1/C2 or C2/C2	No	10	12	1.90 (0.64-5.69)
C1/C2 or C2/ C2	Yes	23	11	4.78 (1.82-12.78)
	Alcohol drinking			
C1/C1	No	26	51	1.00
C1/C1	Yes	32	20	3.14 (1.42-6.99)
C1/C2 or C2/C2	No	14	15	1.83 (0.70- 4.77)
C1/ C2 or C2/C2	Yes	19	8	4.66 (1.65-13.53)
	Fish sauce intake[a]			
C1/C1	Low	11	53	1.00
C1/C1	High	47	18	12.58 (5.40-29.34)
C1/C2 or C2/C2	Low	6	16	1.81 (0.58-5.66)
C1/C2 or C2/C2	High	27	7	18.58 (6.47-53.37)

[a]High ⩾3 times/w; Low<3 times/w

DISCUSSION

Epidemiological studies have shown that up to 90% of all cancers are related to environmental factors. Most of the environmental carcinogens need to be metabolically activated to exert their carcinogenic effects[27-30]. Genetic polymorphisms in enzymes involved in carcinogen metabolism has shown to influence the susceptibility to cancer[31-33]. Cytochrome P4502E1 (CYP2E1) plays an important role in this process. It participates in the metabolic activation of carcinogenic nitrosamines. Several recent studies show that the genetic polymorphisms of metabolizing enzymes are associated with some cancers such as lung cancer[34-36], esophageal cancer[37-40] and colorectal cancer[41]. But the results of those studies of the relation between CYP2E1 and cancer susceptibility are inconsistent[42].

The possibility that N-nitrosated compounds are involved in gastric cancer has been an issue for many years. Nitrosamines occur in tobacco smoke and in some kinds of food, which are also formed endogenously in the stomach. In this study, fish sauce intake, cigarette smoke, and alcohol drinking were positively associated with gastric cancer. Fish sauce is a condiment that is particularly favored by the local residents in Fujian Province[43-45]. Many N-nitro compound precursors have been found in fish sauce[46,47]. Tobacco smoke contains many potential carcinogens, also including nitroso compounds[48]. CYP2E1 are known to vary and are induced by ethanol consumption. Several studies reported that the variant C2 was associated with enhanced enzyme activity. Hayashi *et al*[49] reported that enhanced activity of C2/C2 DNA was about 10 times than that of C1/C1 DNA. This difference in the transcriptional activities might associate with the susceptibility in human carcinogenesis. There is evidence suggesting that there may be a gene-environment interaction in the development of cancer so that cancer risk associated with a given exposure is modified by the genotype of the host[50]. In this study, a much higher risk was observed from those who exposed to the risk factors of gastric cancer and carried the C1/C2 or C2/C2 genotypes. The results suggest that polymorphic genes that code for tobacco carcinogen and alcohol metabolizing enzymes may play a role in susceptibility of gastric cancer. The intervention against cigarette smoking, alcohol drinking, bad eating habits may be important for the prevention of gastric cancer in high-incidence areas.

Because of the limited number of samples in this study, determination of precise dose-response relations with respect to a sufficient number of dose level for each of genotype groups may not be able to be conducted. Further studies with a larger number of samples are needed to confirm the role of genetic polymorphism of human CYP2E1 in gastric cacinogenesis.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Growth inhibition and apoptosis induction of Sulindac on Human gastric cancer cells

Yun-Lin Wu[1], Bo Sun[1], Xue-Jun Zhang[2], Sheng-Nian Wang[2], Heng-Yi He$_2$, Min-Min Qiao[1], Jie Zhong[1], Jia-Yu Xu[1]

[1]Department of Gastroenterology, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
[2]Institude of Biochemistry and Cell Biology, Shanghai Institues for Biological Sciences, Chinese Academy of Sciences. Shanghai 200025, China
Supported by Asahi Medical Foundation, No. 00-2000-03
Correspondence to: Yun-Lin Wu, Department of Gastroenterology, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China. Sunborjxh@yahoo.com.cn
Telephone: +86-21-64370045, Ext: 665246
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Abstract

AIM: **To evaluate the effects of sulindac in inducing growth inhibition and apoptosis of human gastric cancer cells in comparison with human hepatocellular carcinoma (HCC) cells.**

METHODS: **The human gastric cancer cell lines MKN45 and MKN28 and human hepatocellular carcinoma cell lines HepG$_2$ and SMMC7721 were used for the study. Anti-prolife rative effect was measured by MTT assay, and apoptosis was determined by Hoechst-33258 staining, electronography and DNA fragmentation. The protein of cyclooxygen ase-2 (COX-2) and Bcl-2 were detected by Western dot blotting.**

RESULTS: **Sulindac could initiate growth inhibition and apoptosis of MKN45, MKN28, HepG$_2$ and SMMC7721 cells in a dose-and time-depen dent manner. Growth inhibitory activity and apoptosis were more sensitive in HepG$_2$ cells than in SMMC7721 cells, MKN45 and MKN28 cells. After 24 hours incubation with sulindac at 2 mmol·L^{-1} and 4 mmol·L^{-1}, the level of COX-2 and Bcl-2 prote in were lowered in MKN45, SMMC7721 and HepG$_2$ cells but not in MKN28 cells.**

CONCLUSION: **Sulindac could inhibit the growth of gastric cancer cells and HCC cells effectively *in vitro* by apoptosis induction, which was associated with regression of COX-2 and Bcl-2 expression. The growth inhibition and apoptosis of HCC cells were greater than that of human gastric cancer cells. The different effects of apoptosis in gastric cancer cells may be related to the differentiation of the cells.**

Subject headings sulindac; apoptosis; gastric cancer; HCC;COX-2

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin were widely used for the treatment of inflammatory joint and muscle pain. The stuidies of past two decades indicated that NSAID could prevent colorectal cancer[1-3]. As a member of NSAID, sulindac has been shown to induce regression of adenomas in familial adenomatous polyposis (FAP) patients, prevent recurrence of adenomas[4-5] and reduce the risk of colon cancer[6]. Rahman *et al*[7] demonstrated that sulindac and its irreversible oxidized derivative, sulindac sulfone exhibited a growth inhibitory effect on human hepatocellular carcinoma (HCC) cell lines, indicating that sulindac has chemopreventive effect on colon cancer as well as on other types of cancer in GI tract. Gastric cancer is one of the most common causes of malignancy-related death in China[8-11]. It is imperative to find effective chemopreventive methods to reduce the morbidity and mortality of gastric cancer. Lots of chemical agents have been proved having the chara cter of inducing apoptosis in human gastric cancer cells[12-13]. Recent studies gave the concept that overexpression of cyclooxygenase-2 (COX-2) was the early event in carcinogenesis of gastric cancer[13] and made it the target of chem oprevention against gastric cancer[14]. The present study was undertaken to analyze the effect of sulindac in two gastric cancer cell lines as compared with two HCC cell lines.

MATERIALS AND METHODS

Cell lines and culture

The huamn gastric cancer cell lines MKN45 and MKN28 were obtained from the Japanese Cancer Res earch Resources Bank (Tokyo, Japan). HepG$_2$ and SMMC7721 cells were available commercially from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cells were grown in RP M I 1640 (Gibco) supplemented with 100 mL·L^{-1} fetal bovine serum (Sijiqing, HangZhou , China), penicilin (100 mg·L^{-1}) and streptomycin (100 mg·L^{-1}) in a humidified atmosphere of 50 mL·L^{-1} CO_2 at 37℃. Sulindac purchased from Sigma Chemical Co. (St Louis, MO, USA) was freshly prepared in DMSO (less than 5 g·L^{-1} in w hole medium) before use. Vehicle controls of DMSO was included in the studies.

MTT assay

Antiproliferative effects were measured by MTT assay. About 10 000 cells per well were plated in 96-well microtitre plates and incubated overnight in 100 μL of culture media. Then cells were treated with various concentrations of sulindac for 24 h and 48 h (0, 0.25, 0.5, 1, 2 and 4 mmol·L^{-1} for MKN45, MKN28 and SMMC77 21 cells, 0, 25, 50, 100, 200 and 400 μmol·L^{-1} for HepG$_2$ cells); 100 μL MTT (1 g·L^{-1}) was then added to each well, and the cells were further incubated for 4 h. The supernatant was removed and 100 μLDMSO was added to each well, and then incu bated for 30 min. The absorbance at wavelength of 570 nm was measured by a micro - ELISA reader. The negative control well has no cells and used as zero point of a bsorbance. Each assay was performed three times in triplicate.

Morphological measurement of apoptosis

The morphological change of apoptosis was assayed under fluorescence microscope following staining with Hoechst33258. The cells were fixed in 200 mL·L^{-1} ethanol/PBS, stained with 5 mg·L^{-1} Hoechst33258 (CNI) for 30 min at 37℃, then visualized under UV fluorescence microscope. Apoptotis cells were de fined as cells

showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptosis body. At least 300 cells were counted and the percentage of apoptotic cells (Apoptotic Index) was calculated.

DNA fragmentation analysis

Following sulindac treatment, cells were washed by PBS, and fixed in ice-c old 700 mL·L^{-1} ethanol for 24 h. After the ethanol was removed, cells were rinsed in 40 μL 0.2 mol·L^{-1} Na_2HPO_4/0.1 mol·L^{-1} citric acid (192:8, pH7.8) for 60 min at room temperature. After being centrifuged 5 min (1000G), the supernatant was c ollected in Eppendorf tubes, 4 μL 2.5 g ·L^{-1} NP-40 and 4 μL 10 g ·L^{-1} RNase A were added at 37℃ for 30 min, then 4 μL Protein K (1 mg/mL, Promega) were added at 50 ℃ for 30 min. Equal amount of DNA (15 μL) was electrophoresed in 10 g·L^{-1} a garose gels impregnated with ethidium bromide (5 mL·L^{-1}) for 2 h at 80 V. DNA fragments were visualized by ultraviolet transillumination.

Western Dot blot analysis of COX-2 and Bcl-2

Cells incubated with various concentrations of sulindac for 24 h (0, 2 and 4 mmol·L^{-1} for MKN28, MKN45 and SMMC7721 cells, 0, 400 and 800 μmol ·L^{-1} for $HepG_2$ cells). These cells were washed by PBS and dissolve d in 100iL extraction buffer (50 mmol·L^{-1} Tris-HCL (pH 8.0), 150 mmol·L^{-1} NaCl, 10 g·L^{-1} Triton-X-100 and 1mg·L^{-1} Aprotinine). Equal amounts of cell lysates (100 μg) were dotted on nitrocellulose membrane (Amersham). The membrane was incubated first with a primary antibody overnight at 4℃ and then with peroxidase- conjugated anti-rabbit or anti-mouse IgG antibody for 2 h. Protein was detected by chemilu minescence (ECL) system. The membranes were washed and added by equal amounts of luminal reagent A and B (Santa Cruz) for 2 min and exposed to film. Rabbit anti-h u man polyclonal antibody COX-2(H-62, sc-7951), goat anti-mouse IgG conjugate d with HRP(Cat#sc-2005) , Goat anti-rabbit IgG conjugated with HRP(Cat#sc-2004) we re purchased from Santa Cruz Co. Mouse anti-human bcl-2 monoclonal antibody (Cat.No.M-0025) was purchased from Antibody Diagnostica Inc(USA).

Statistical analysis

Student's *t* test was used for results comparison among different groups. The presented data were mean values of at least three different experiments and expressed as $\bar{x}\pm s$. A *P* value of less than 0.05 is considered statistically significant.

RESULTS

Effects of sulindac on cell growth

Various concentrations of sulindac were incubated with cells for 24 h and 48 h. Cell growth was determined by MTT assay. As shown in Figure 1, sulindac could inhibit the growth of gastric cancer cells and HCC cells in a dose-and time-dependent manner. Sulindac showed a more potent effect in reducing $HepG_2$ cells' growth as compared with SMM C77 21, MKN45 and MKN28 cells. The cell death rate was more obvious in MKN45 cells than in MKN28 cells (Figure 1).

Apoptosis of cells induced by sulindac

To evaluate the apoptosis of cells, Hoechst-33258 staining and agarouse gel electrophoresis of genomic DNA were used. The Hoechst-33258 staining showed apoptosis in all four types of cells, which was characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptosis body (Figure 2). The apoptosis was mo re evident in $HepG_2$ cells than in SMMC7721 and gastric cancer cells and the AI of MKN45 cells were higher than that of MKN28 cells (Figure 3). DNA fragmentation was shown as a ladder pattern on agarose gel.

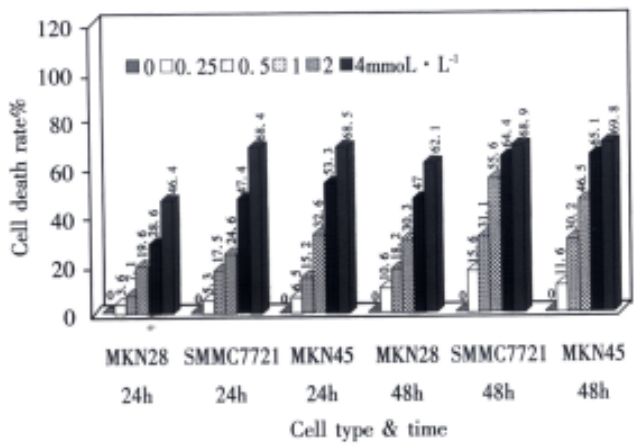

Figure 1A Dose-response of sulindac on growth of cell lines by MTT assay. (N = 3)

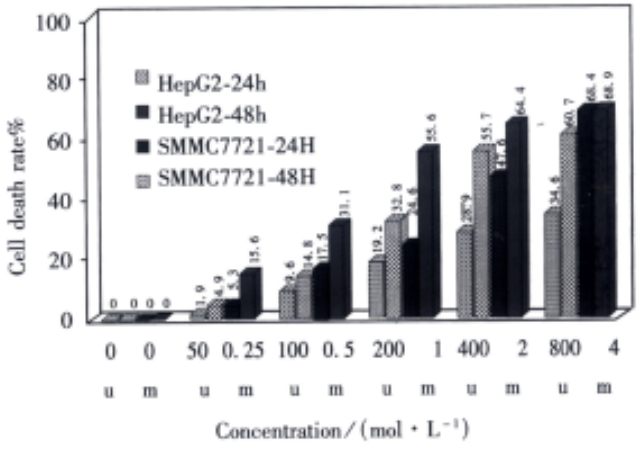

Figure 1B Dose-response of sulindac on growth of HCC cell line s by MTT assay. (N = 3)

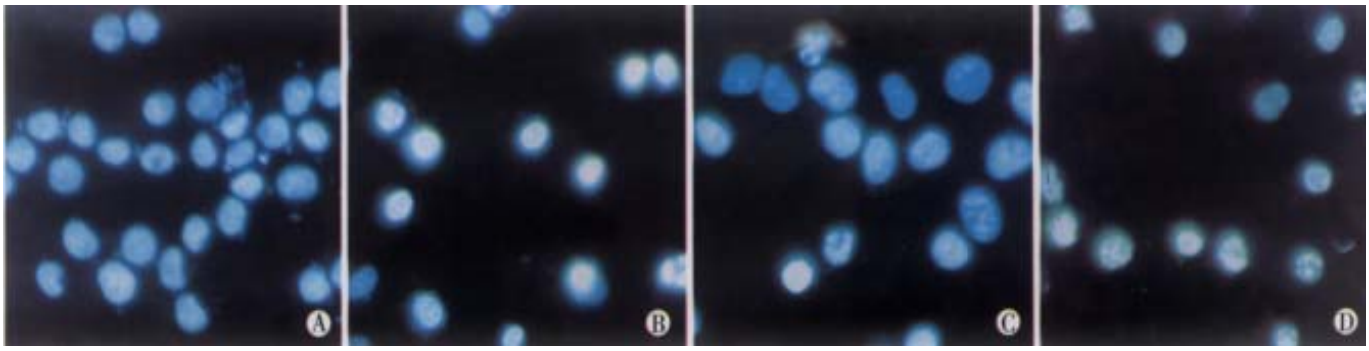

Figure 2 Morphological changes of MKN45 and $HepG_2$. Cells stained with Hoechst33258×400. A: MKN45 cells; B:MKN45 cells treated with 2 mmol·L^{-1} sulindac for 24 h; C: $HepG_2$ cells; D: $HepG_2$ cells treated with 400 μmol·L^{-1} sulindac for 24 h.

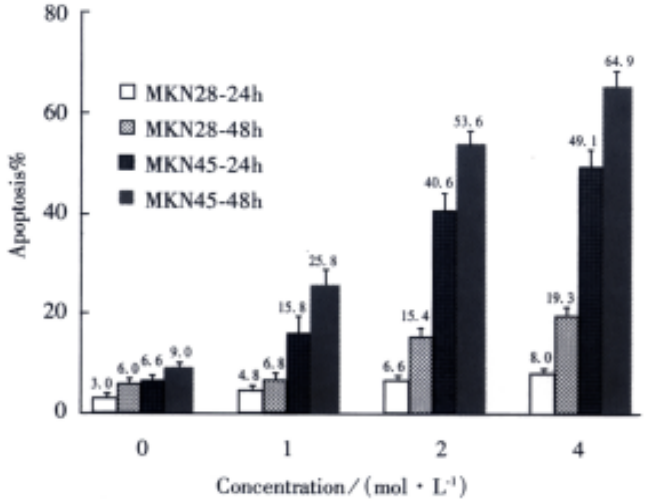

Figure 3A The apoptosis of gastric cancer cells induced by sulindac by Hoechst 33258 staining. (N = 3)

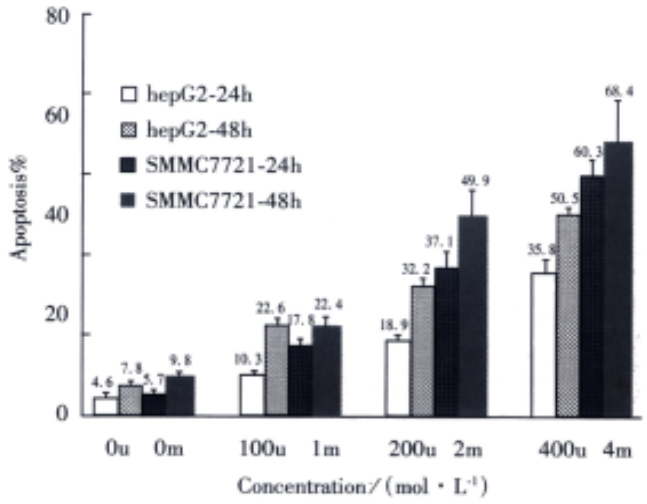

Figure 3B The apoptosis of 2 HCC cells induced by sulindac by Hoechst 33258 staining. (N = 3)

Differential expression of COX-2 and Bcl-2 protein in sulindac-treated cells

The protein levels of COX-2 and Bcl-2 were determined by Western dot blotting. After treatment with 2 mmoL ·L^{-1} and 4 mmoL·L^{-1} of sulindac for 24 h, the protein level of COX-2 and Bcl-2 showed marked decrease in MKN45, HepG$_2$ and SMMC7721 cells, whereas the protein level remained unchanged in MKN28 cells (Figure 4).

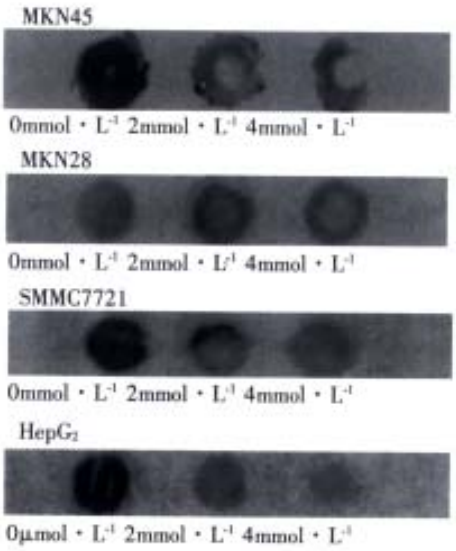

Figure 4A COX-2 protein levels in human gastric cancer and HCC cells with sulindac for 24 h.

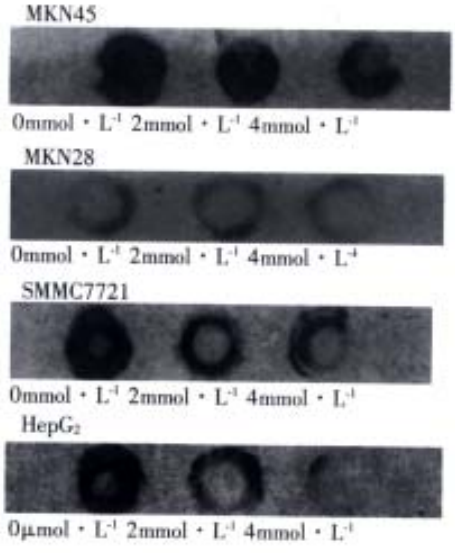

Figure 4B Bcl-2 protein levels in human gastric cancer cells and HCC cells with sulindac for 24 h.

DISCUSSION

Since Adolphie *et al* reported that certain NSAIDs were capable of inhibiting proliferation of Hela cells in 1972, the chemopreventive effect of NSAIDs has been widely studied *in vivo* and *in vitro* in recent years[15-16]. Most results indicated that the mechanism related to this capability was by the inhibition of cyclooxygenase-2 (COX-2) which was not found in most normal tissues and could be induced by cytokines and growth factors[17-19]. Elevated level of COX-2 suggested the existance of inflammation or carcinoma[20-25]. Lim *et al* found that all 104 gastric cancer tissues showed positive expression of COX-2 but not in normol gastric mucosa. Ratnasinghe *et al*[26] found that gastric cancer tissues could produce more prostagland in than normal gastric mucosa. COX-2 was also found to be related to tumor angiogenesis[27-28] and metastasis[29-31]. In this study, we confirmed the results of other studies that COX-2 was positive in MKN45, MKN28 and HepG$_2$ cell lines as well as in SMMC7721 cells and the level of COX-2 protein was much lower in MKN 2 8 cells. The results also confirmed that COX-2 was correlative with carcinogenesis in GI tract.

One of the strongest evidence that NSAID has the capability of chemoprevention and treatment of colorectal cancer was the obvious effect of sulindac in treatment of FAP[32-33]. Pasricha *et al* reported that the number and size of polyps were reduced in 24 FAP patients after treatment with sulindac and induction of apoptosis was regarded

as the main mechanism. However, there we re no studies about growth inhibition and apoptosis induction of sulindac in gastric cancer. In this study, two gastric cancer cell lines with different status of differentiation were used. The growth inhibition and apoptosis of $HepG_2$ cells were more obvious as compared with MKN45 and MKN28 cells. Since sulindac is a pro-drug and is metabolized to sulindac sulfide and sulfone by the gut flora and in the liver[34], HCC cells might be able to convert sulindac to its metabolic derivative and increase its capability of growth inhibition and apoptosis inducti on. We suggested that the effects of growth inhibition of sulindac on gastric cancer might be increased *in vivo* than *in vitro*. We also concluded that sulindac could induce apoptosis in gastric cancer cells and HCC cells, which may account for its growth inhibitory effects.

Bcl-2 is one of the most important factors in apoptosis process[35]. The elevated expression of COX-2 could increase the level of Bcl-2 in the epithelial cells of rat colon and decrease the apoptotic rate of colonic cells[36]. Prostaglandin E_2, mediated by COX-2 from a rachidonic acid, could inhibit apoptosis of human colon cancer cells *in vitro* and increase expression of Bcl-2 in cancer cells[37]. Liu *et al*[38] also confirmed the relationship between COX-2 and Bcl-2 in prostate cancer. We found that the levels o f COX-2 in MKN45, SMMC7721 and HepG 2 cells as well as Bcl-2 were decreased after treatment with sulindac, whereas both COX-2 and Bcl-2 were unchanged in MKN28 cells. Apoptosis was also more evident in MKN45, SMMC7721 and $HepG_2$ than in MKN28 cells. It was suggested that COX-2 and Bcl-2 were involved in apoptosis of gastric cancer cells and HCC cells induced by sulindac.

Several mechanisms have been proposed affecting the pathways regulating cellular proliferation and apoptosis by NSAIDs. Although parts of the mechanisms were related to COX-2 inhibition, most of them were COX independent[39-44]. Sulindac and its derivatives have different mechanism in inducing apoptosis of cancer cells. Sulfone neither inhibits COX nor has anti-inflammatory properties, but can produce chemopreventive effect similar to that of sulindac[45-46]. The death rate of MKN28 cells was higher than its apoptotic rate in our study, which might be caused by the COX-2 in dependent way.

As COX-2 specific inhibitors, Celecoxib and Rofecoxib have been used clinically with few side effects[47]. The chemopreventive eff ect of COX-2 specific inhibitor has not yet been well studied as compared with COX-2 nonsp ecific inhibitors[48-49]. As one of COX-2 nonspecific inhibitors, sulindac had little effect in renal prostanoid synthesis and provides addtional advantage for its use in clinical trials[50]. The concentration and time course of sulindac in inhibiting growth and inducing apoptosis of gastric cancer cells *in vivo* need further investigations. Other possible mechanisms of action of sulindac need to be further studied.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Association of *H. pylori* infection with gastric carcinoma: a Meta analysis

Fu-Bo Xue[1], Yong-Yong Xu[1], Yi Wan[1], Bo-Rong Pan[2], Jun Ren[2], Dai-Ming Fan[3]

[1]Department of Health Statistics, Department of [2]Oncology and [3]Gastroenterology of XiJing Hospital, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Supported by Funds for University Key Teachers by the Ministry of Education, No. 2000-65
Correspondence to: Fu-Bo Xue, Department of Health Statistics, the Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. alnico@sohu.com
Telephone: +86-29-3374853
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Abstract

AIM: **To follow the principles of evidence based medicine to reach the integrated results of these studies.**

METHODS: **Twenty-one papers of case-control studies were selected, including 11 on gastric cancer, 7 on precancerous lesion of stomach and 3 on lymphoma of stomach. Meta analysis was used to sum up the odds ratios (OR) of these studies.**

RESULTS: ***H. pylori* vsgastric cancer (intestinal and diffuse type): the odds ratio from the fixed effect model is 3.0 016 (95% CI: 2.4197-3.7234, *P*<0.001). *H. pylori vs* precancerous lesion of stomach: a random effect model was used to calculate the summary odds ratio and its value is 2.5635 (95% CI: 1.8477-3.5566, *P*< 0.01). *H. pylori vs* lymphoma of stomach: though the quantity of literature is too small to make Meta analysis, the data of these 3 studies show that lymphoma of stomach is highly associated with *H. pylori* infections.**

CONCLUSION: **Since it had been revealed that *H. pylori* infection pre-exists in gastric carcinoma and precancerous lesions, the results of Meta analysis present a strong evidence to support the conclusion that *H. pylori* infection is a risk factor for gastric carcinoma.**

Subject headings *Helicobacter pylori*; Helicobacter infections; stomach neoplasms/microbiology; evidence-based medicine; meta-analysis

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INTRODUCTION

Since *H. pylori* was founded in 1983, the association of *H. pylori* with *H. pylori* related diseases has become the hot spot of gastroenterological studies. Gastric carcinoma is the most important disease among *H. pylori* related diseases. It is believed that *H. pylori* is one of the important causes of gastric carcinoma. But there is still lack of the final conclusion and the definite mechanisms of their association.

In China, malignant tumor is the second death cause of men and the third death cause of women according to the investigation of death cause in 29 provinces, municipalities and autonomous regions conducted from 1 973 to 1975. The annual average mortality rate of gastric carcinoma is as high as 16 per 100 thousand, which is the leading death cause among malignant tumors, and the rates of *H. pylori* infection are higher than 50 percent in the mainland of China[1]. In t he past two decades, Chinese medical researchers and clinical workers have done much a great amount of studies to reveal the relationship and the mechanisms of the association of *H. pylori* with gastric carcinoma. Most of them agree to the opinion that *H. pylori* is a risk factor for gastric carcinoma, but a certain number of them hold different points of view[2-19].

Eslick *et al*[20] have workedout the summary odds ratio of 2.04 of those same studies made in Western countries. In this paper, we reviewed all the literatures of studies in China on the relationship between *H. pylori* and gastric carcinoma as well as precancerous lesions of stomach published from 1995 in order to reach a summary conclusion using statistical methods.

MATERIALS AND METHODS

Literature

A CBM (Chinese Biomedical Database) search for articles published from 1995 was performed with the MeSH headings "*Helicobacter pylori*," "gastric carcinoma (cancer),""precancerous lesion of stomach" and "lymphoma of stomach." More than 100 papers were retrieved. Since most of them had no appropriate controls or their data did not meet the requirements of Meta analysis, only 21 papers of case-control studies were selected, including 11 about gastric cancer (Table 1), 7 on precancerous lesions of stomach (Table 2) and 3 on lymphoma of stomach (Table 3).

Data

Eleven case-control studies on the relationship between *H. pylori* infection and gastric cancer (intestinal and diffuse type) included totally 820 patients and 11-647 controls. Among them, 7 attained significant results, and 4 did not (Table 1).

Seven case-control studies on the relationship between *H. pylori* infection and precancerous lesions of stomach included totally 1978 patients and 6076 controls. All of them had significant results (Table 2).

Three case-control studies on the relationship between *H. pylori* infection and lymphoma of the stomach included totally 83 patients and 143 controls. All of them had significant results (Table 3).

Methods

In the statistical analysis, Meta analysis method with fixed effect model and random effect model was used to reach the integrated conclusion[42].

Table 1 Eleven case-control studies of *H. pylori vs.* gastric cancer[21-31]

No.	Cases		Controls		OR	95%CI	X^2	*P* value
	Hp (+)	*Hp* (-)	*Hp* (+)	*Hp* (-)				
1	61	13	49	25	2.394	1.110-5.163	4.284	0.038
2	13	3	78	68	3.778	1.033-13.818	3.475	0.062
3	71	21	46	46	3.381	1.791-6.384	13.52	0.000
4	99	23	6	8	5.793	1.841-18.155	8.400	0.004
5	35	16	34	87	5.597	2.746-11.408	22.873	0.000
6	101	35	6236	4628	2.142	1.455-3.151	14.959	0.000
7	16	9	32	43	2.389	0.937-6.092	2.618	0.106
8	21	19	3	12	4.421	1.080-18.093	3.457	0.063
9	55	21	12	10	2.183	0.821-5.805	1.75	0.186
10	90	13	89	50	3.889	1.977-7.653	15.500	0.000
11	60	25	35	50	3.429	1.815-6.475	13.743	0.000
Summary	622	198	6620	5027	3.0016	2.4197-3.7234	99.9483	0.000

*the literatures that did not attain significant results.

Table 2 Seven case-control studies of *H. pylori vs.* precancerous lesion of stomach[32-38]

No.	Cases		Controls		OR	95%CI	X^2	*P* value
	Hp (+)	*Hp* (-)	*Hp* (+)	*Hp* (-)				
1	344	194	1467	1311	1.585	1.309-1.918	22.089	0.000
2	67	29	175	180	2.376	1.466-3.851	11.955	0.000
3	103	38	26	53	5.525	3.036-10.056	31.988	0.000
4	427	252	992	892	1.524	1.273-1.824	20.736	0.000
5	99	41	12	28	5.634	2.614-12.144	20.128	0.000
6	134	35	272	201	2.829	1.870-4.282	24.490	0.000
7	188	27	332	135	2.831	1.805-4.442	20.836	0.000
Summary	1362	616	3276	2800	2.5635	1.8477-3.5566	31.7540	0.000

Table 3 Three case-control studies of *H. pylori vs.* lymphoma of stomach[39-41]

No.	Cases		Controls		OR	95%CI	X^2	*P* value
	Hp (+)	*Hp* (-)	*Hp* (+)	*Hp* (-)				
1	24	5	13	16	5.908	1.762-19.810	7.465	0.006
2	15	0	36	24	10.811	1.347-86.798	5.692	0.017
3	34	5	31	23	5.045	1.709-14.896	8.176	0.004

RESULTS

H. pylori infection vs gastric cancer

All 11 odds ratios were statistically homogenous ($P>0.05$). Summary odds ratio for gastric cancer related to *H. pylori* infection was 3.0016 using fixed effect model (95% CI 2.41 97-3.7234, Table 1). Figure 1 shows the summary odds ratio and odds ratios and their 95%CI of 11 case-control studies on the relationship between *H. pylori* infection and gastric cancer.

H. pylori infection vs precancerous lesion of stomach

Since 7 odds ratios were not statistically homogenous ($P<0.05$), random effect model was used to calculate the summary odds ratio. The summary odds ratio for precancerous lesions of stomach related to *H. pylori* infection was 2.5635 (95% CI 1.8477-3.5566, Table 2). Figure 2 shows the summary odds ratio and odds ratios and their 95%CI of 7 case-control studies on the relationship between *H. pylori* and precancerous lesions of the stomach.

H. pylori vs lymphoma of stomach

Though the quantity of literature is too small to make Meta analysis, the data of these 3 studies show that lymphoma of the stomach was highly associated with *H. pylori* infections. Figure 3 shows the odds ratios and their 95%CI of 3 case-control studies on the relationship between *H. pylori* infection and lymphoma of the stomach.

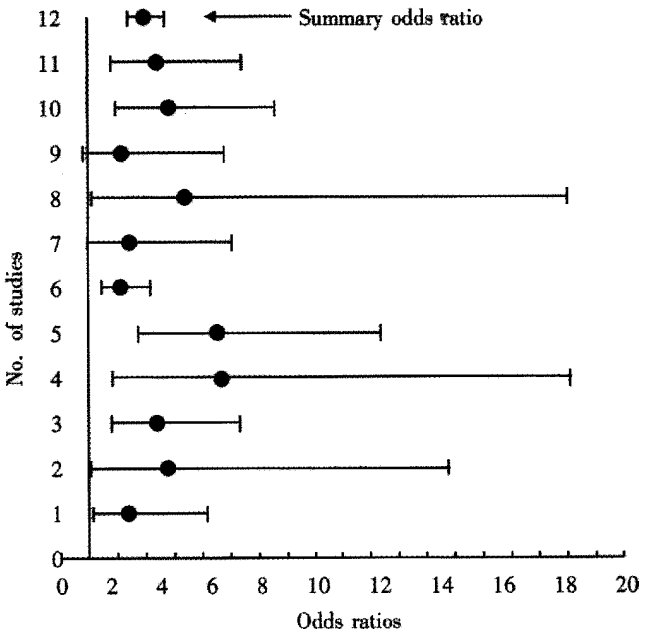

Figure 1 Odds ratios and summary odds ratio with 95%CI of 11 case-control studies.

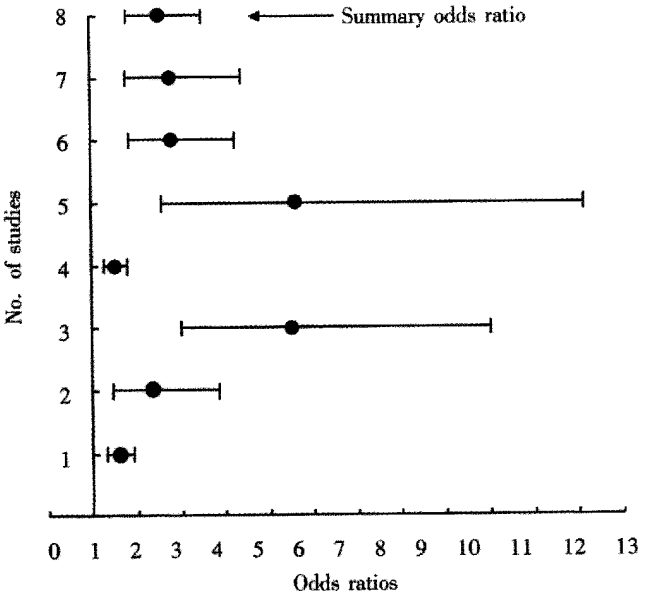

Figure 2 Odds ratios and summary odds ratio with 95%CI of 7 case-control studies.

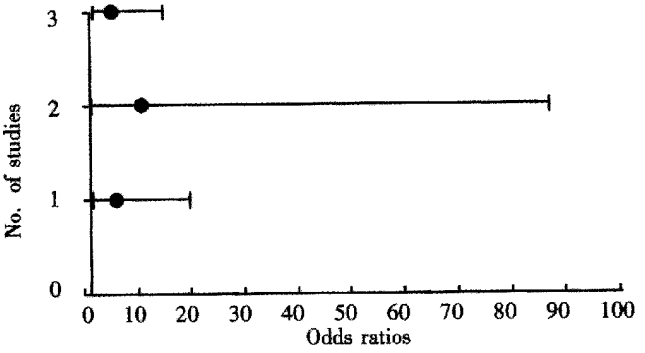

Figure 3 Odds ratios with 95%CI of 3 case-control studies.

DISCUSSION

Since it had been revealed that *H. pylori* infection pre-exists in gastric carcinoma and precancerous lesions, the result of the Meta analysis present a strong evidence to support the conclusion that *H. pylori* infection is a risk factor for gastric carcinoma.

The following statements might explain the association of *H. pylori* infection with gastric carcinoma: ① gastric carcinoma is caused by *H. pylori* infection; ② *H. pylori* infection is caused by gastric carcinoma; ③ there are some certain factors for *H. pylori* infection and gastric carcinoma[9]. As we all know that *H. pylori* infections also exist in the gastric mucosa of nearly normal persons, it is impossible that *H. pylori* infection is caused by gastric carcinoma. Numerous studies support the point that *H. pylori* infection exist before the occurrence of gastric carcinoma[43-48], indicating that *H. pylori* infection is not a secondary infection but a primary infection. It was reported that. Mongolia gerbils have been infected with *H. pylori* through mouth route, which is implanted in their stomach for a long time. Twenty-six weeks after the infection, severe chronic active gastritis, ulcer and intestinal metaplasia occurred in the stomachs of the gerbils. And 37% of the gerbils involved in the study were attacked with gastric adenocarcinoma 62 weeks after the infection[10].

The development and occurrence of gastric carcinoma is a long-lasting process and the effect of multiple factors. It is accepted by numerous scholars that *H. pylori* is an important risk factor for gastric carcinoma. In addition to the studies that support the association of *H. pylori* with gastric carcinoma, some researchers investigated the relationship between *H. pylori* and other kinds of carcinoma such as cancer of the colon, rectum, esophagus etc. and they made a negative conclusion, which proved the effect of *H. pylori* in gastric carcinoma in the other directions. However, *H. pylori* is not the unique etiological factor for gastric carcinoma. The association of the occurrence of gastric carcinoma with *H. pylori* infection should be considered from the angle of the multi-agent compound etiological theory[49-62].

According to the studies that support the opinion of association of *H. pylori* with gastric carcinoma, It can be assumed that t he canceration of gastric mucosa took place under the action of *H. pylori* in the following process: *H. pylori* related gastritis of antrum→ atrophic gastritis→intestinal metaplasia→gastric carcinoma.

There are many problems we are going to face. The infection rate of *H. pylori* is very high in the pop ulation surveys. But why most of those with *H. pylori* infection were not atta cked wit h gastric carcinoma. What are the other factors acting together with *H. pylori* to cause gastric carcinoma? How do they act? We must do more prospective interventional trial to answer these questions. It will be more helpful to do random interventional trial of eradication of *H. pylori* infection among the high-risk groups of gastric carcinoma and precancerous lesions to observe if their incidence rate of gastric carcinoma will decrease. In China, though very few of this kind of prospective studies have been made, an interventional trial of eradication of *H. pylori* is being made in Shandong and Fujian provinces. It will be of great help in revealing the exact mechanism of *H. pylori* in the process of gastric mucosa canceration.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Clinicopathological and molecular genetic analysis of 4 typical Chinese HNPCC families

Qi Cai[1], Meng-Hong Sun[1], Hong-Fen Lu[1], Tai-Ming Zhang[1], Shan-Jing Mo[2], Ye Xu[2], San-Jun Cai[2], Xiong-Zeng Zhu[1], Da-Ren Shi[1]

[1]Department of Pathology, [2]Department of Abdominal Surgery, Cancer Hospital/Cancer Institute, Fudan University, Shanghai 200032, China
Supported by the Shanghai Medical Development Project, No. 993025
Correspondence to: Meng-Hong Sun, Department of Pathology, Cancer Hospital/Cancer Institute, Fudan University, 399 Lingling Road, Shanghai 200032, China. smh9618@public6.sta.net.cn
Telephone: +86-21-64175590 ext.3357, Fax: +86-21-64174774
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Abstract

AIM: **To study the clinicopathological and mole cular genetic characteristics of typical Chinese hereditary nonpolyposis cotorectal cancer (HNPCC) families.**

METHODS: **Four typical Chinese HNPCC families were analyzed using microdissection, microsatellite instability analysis, immunostaining of hMSH2 and hMLH1 proteins and direct DN A sequencing of hMSH2 and hMLH1 genes.**

RESULTS: **All five tumor tissues of 4 prob ands from the 4 typical Chinese HNPCC families showed microsatellite instability at more than two loci (MSI-H or RER+phenotype). Three out of the 4 cases losthMSH2 protein expression and the other case showed no hMLH1 protein expression. Three pathological germline mutations (2 in hMSH2 and 1 in hMLH1), which had not been reported previously, were identified. The same mutations were also found in other affected members of two HNPCC families,respectively.**

CONCLUSION: **Typical Chinese HNPCC families showed relatively frequent germline mutation of mismatch repair genes. High-level microsatellite instability and loss of expression of mismatch repair genes correlated closely with germline mutation of mismatch repair genes. Microsatellite instability analysis and immunostaining of mismatch repair gene might serve as effective screening methods before direct DNA sequencing. It is necessary to establish clinical criteria and molecular diagnostic strategies more suitable for Chinese HNPCC families.**

Subject headings colorectal neoplasm; hereditary nonpolyposis/ genetic; colorectal neoplasms, hereditary nonpolyposis/ pathology; immunohistochemistry; sequence analysis, DNA

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch syndrome, is the most common human hereditary cancer predisposition and accounts for 5%-10% of total colorectal carcinomas (CRCs). Tumors in HNPCC families are characterized by an early age (before the age of 50) of onset, an excess of syn-and meta-chronous colore ctal cancers, nigh occurrence in the proximal colon and an increased risk of neoplasms of other organs including endometrium, ovary, stomach, small intestine , pancreas, biliary tract, bladder and ureter[1-5]. The lack of characteristic diagnostic features has prompted the establishment of the so-called Amsterdam criteria for diagnosis: the histologically verified colorectal cancer must occur in at least three relatives (one of whom is a first-degree relative of the other two); the cancer must occur in at least two successive generations; and at least one case must occur at an onset age of less than 50. In addition, familial adenoma touspolyposis (FAP) must be ruled out[6].

Germline mutations of six genes in volved in DNA mismatch repair (MMR), i.e. hMSH2, hMLH1, PMS1, PMS2, MSH6 (also known as GTBP) and MLH3, have been identified in patients with the disease, and t he former two genes account for the large majority of mutations found in families with HNPCC. hMSH2 is localized to chromosome 2p21-22, contains 16 exons, and is predicted to encode a 935 amino acid protein, whereas hMLH1 is localized to chromosome 3p21 and contains 19 exons encoding a 756 amino acid protein[3,7-12]. These genes are required for the correction of DNA mismatches that occur during replication. Defective DNA mismatch repair genes result in microsatellite instability (or called replication errors, RER+). It has been suggested that the presence of replication errors can be a useful marker for HNPCC[13-25]. More recent studies indicate that immunohistochemistry may be a useful alternative strategy for identifying tumors with mismatch repair deficiency. Additionally, immunohisto chemistry provides information on the specific defective gene involved and may, therefore, be cost-effective by limiting the numbers of genes to be sequenced[26-30]. MSI and immunohistochemical analysis may be useful screening methods before MMR gene mutation analysis[22-23,31-34]. Until now there have only be en some case reports of HNPCC in China and no systemic study of molecular genetic aspects of HNPCC had been presented. We have collected 61 Chinese HNPCC families (reported elsewhere) and conducted clinicopathological and molecular genetic analyses of 4 HNPCC families fulfilling the Amsterdam criteria (referred to as typical H NPCC families).

MATERIAL AND METHODS

Patients

Four typical Chinese HNPCC families were taken into the study after informed consent (Figure 1) was made. One proband was female and the other three were male. The onset age was 38, 29, 58 and 67 years respectively, with a mean age of 48. Metachronous and synchronous tumors and their locations are listed in Table 1. Tumor tissues and peripheral white blood cells were collected for the study.

Table 1 Clinicopathological charcateristics, MSI status, immun ostaining and the affected MMR genes

Family	Onsetage	Number of CRC	Tumor site	MSI status	Immunostaining		Gene
					hMSH2	hMLH1	
H2	29	4	RightColonX2; rectum X2	4/5	—	+	hMSH2
H9	67	2	Hepatic flexure; transverse colon	4/5; 5/5	+	—	hMLH1
H11	58	2	Rectum X2	4/5	—	+	hMSH2?
H27	38	2	Cecum; transversecolon	3/5	—	+	hMSH2

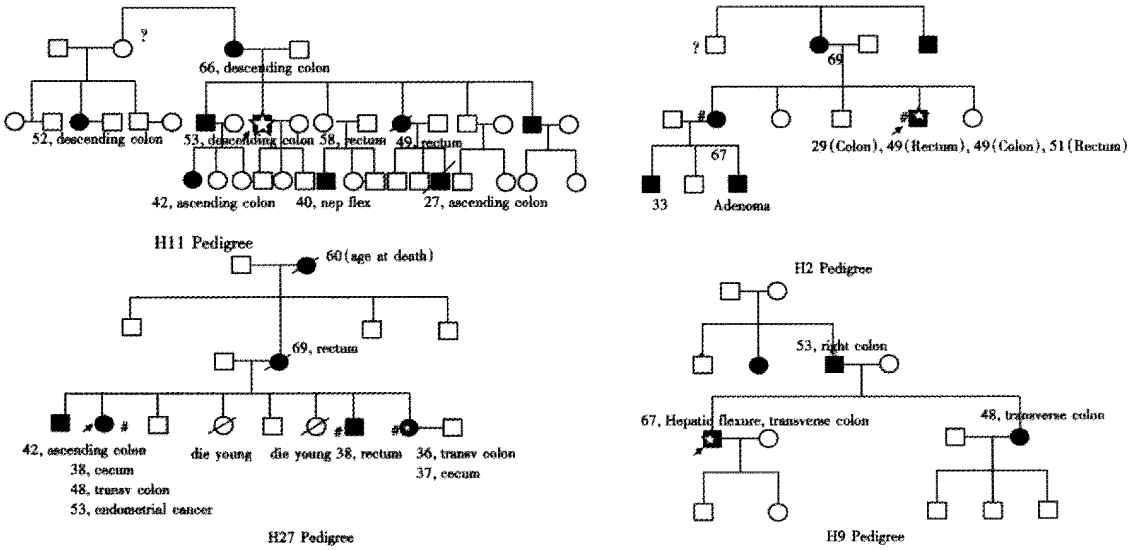

Figure 1 Four typical Chinese HNPCC pedigrees.

Microdissection and minimal amount of DNA extraction

One 5 μm and four 7 μm paraffin-embedded sections were deparaf finized. The 5 μm slide stained with HE served as control. The 7 μm ones were lightly stained with hematoxylin for microdissection. The microdissection was performed under the dissection microscope with a scalpel. Tumor cells should account for at least 80% of the total cells isolated. The microdissected tissues were transferred directly into a centrifugation tube with 150 μL cell lysis buffer (0.5 mol·L^{-1} Tris, 20 mmol·L^{-1} EDTA, 10 mmol·L^{-1} NaCl, 10 g·L^{-1} SDS, 0.5 g·L^{-1} Proteinase K). The subsequent DNA extraction was performed according to the protocol of the DNA extraction kit (Daxia Biotech Ltd, Shanghai). Genomic DNA from per ipheral white blood cells was also extracted with a large volume.

Microsatellite instability analysis

Matched normal and tumor DNA were investigated with a panel of 5 microsatellite markers (mononucleotide repeats BAT26 and BAT25, dinucleotide repeats D5S346, D2S123 and mfd15)[31]. The primer sequences have been published elsewhere[35]. The primer pairs were synthesized by Shenyou Biot ech Ltd. Each forward primer was labeled with a fluorescent dye at 5' end (Fam, Tamara or Joe) to enable the PCR products detectable by an ABI automated DNA sequencer. After successful amplification, the 2 μL PCR product was mixed with 12.5 μL dei one d formamide and 2 μL 350 Rox Sizer. The mixture was denatured, snap cooled and electropheresed on ABI 310 automated DNA sequencer according to the manufacturer' s recommendation. The electrophoresis results were analyzed by GeneScan Software (Applied Biosystems, Incorporated, Foster City, CA). MSI was determined according to Gebert *et al* [36]. Additional peaks (bands) at a microsatellite locus in the tumor compared with the normal tissue from the same patient were interpreted as microsatellite instability (MSI). Cases with MSI in more than 2 of the 5 loci were interpreted as exhibiting high microsatellite instability (MSI-H).

Immunostaining for hMSH2 and hMLH1

Sections of 4 μm were prepared from 100 mL·L^{-1} neutral buffered formalin-fixed and paraffin-embedded tumor tissue. After deparaff inization and rehydration, the sections were pretreated with microwave (4 min×4 at 900 W) in 0.1 mol·L^{-1} citrate buffer and were then incubated overnight at 4℃ with a monoclonal antibody against the hMSH2 prepared with the carboxy-terminal fragment (FE11, Oncogene Research Products, Cambridge, MA) and a monoclonal antibody against the hMLH1 prepared with full-length protein (G168-728, PharMingen, S an Diego, CA) at 1:40 dilutions. The antibodies were detected by the Envison two-step method (Dako, Denmark) using diaminobenzidine as the chromagen. The slides were counterstained with hematoxylin. Diminished expression of hMSH2 or hMLH1 in cancer tissues were demonstrated when there was complete absence of detectable nuclear staining of neoplastic cells. Infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor area served as internal positive controls. Two pathologists assessed all cases without any knowledge of microsatellite instability or germline mutation status.

Sequencing analysis

All 19 exons of hMLH1 gene and all 16 exons of hMSH2 gene (including all intron - exon borders) from proband's genomic DNA were individually amplified in a thermocycler (Perkin-Elmer 9700, Applied Biosystems.). All the primers were kindly provided by Prof. von Knebel in the Division of Molecular Diagnostics and Therapy, Department of Surg ery, University of Heidelberg. Either sense or antisense was anchored with a M13 primer that benefits the subsequent

sequencing. PCR reaction was set in 25 μL volume containing 100 ng genomic DNA. The PCR products were purified using the Q IAquick-spin PCR purification kits (Qiagen Inc., Chatsworth, CA) and were subjected to direct sequencing with M13 forward primers using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The electrophoresis was performed on an ABI 310 automated sequencer. Search of the same mutation in additional family members was performed in the family with a detected mutation.

RESULTS

Clinicopathological characteristics are shown in Table 2.

Microsatellite instability

All 5 tumors of the 4 HN PCC probands showed microsatellite instability at more than two loci (MSI-H, or called RER+ phenotype) (Table 1, Figure 2). One tumor displayed MSI in 5/5 loci, three tumors showed MSI in 4/5 loci and the others had MSI in 3/5 loci.

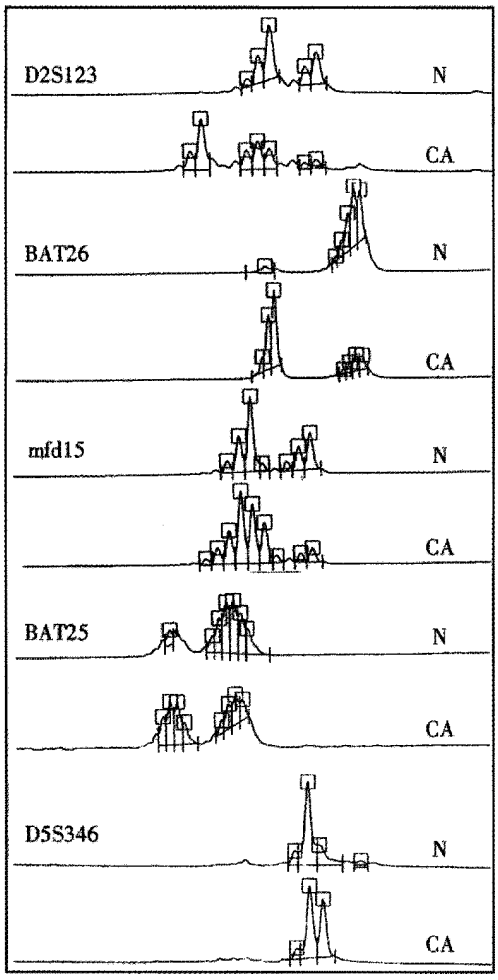

Figure 2 MSI status of H2 proband. Microsatellite analysis of H2 proband with five microsatellite markers, MSI in 4/5 loci

Loss of expression of hMSH2 and hMLH1 protein

Lack of hMLH1 immunostaining was observe d in tumors from H9 proband. Tumors from probands of H2, H11 and H27 were negative for hMSH2 immunostaining (Table 1, Figures 3 and 4).

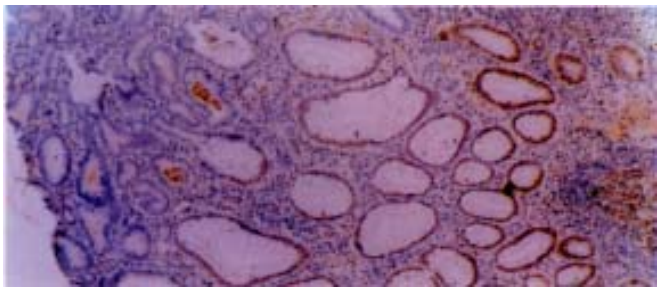

Figure 3 Immunohistochemical staining. No hMSH2 protein expression in adenoma and carcinoma areas of H11 proband tumor section, infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor showed nuclear staining of the hMSH2 protein. ×100

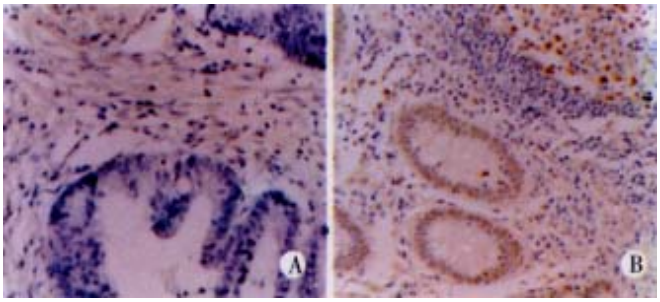

Figure 4 Immunohistochemical staining. A: No hMLH1 protein expression in carcinoma area of H9 proband tumor section. ×400
B: Infiltrating lymphocytes as well as normal colonic crypt epithelium next to the tumor showed nuclear staining of the hMLH1 protein.×200

Germline mutation of hMSH2 and hMLH1 gene

Germline mutations were found in all four probands. Three of four were definitely pathological mutations that had not been reported previously (family H2, H9, H27). The first pathological mutation was a transition of C to T in exon 13 (codon 680) of hMSH2, which leads to a stop codon (CGA-TGA) (family H2)(Figure 5). The second mutation was a 24 bp deletion in exon 11 (codon 305) of hMLH1 (family H9) (Figure 6). The third mutation was one "A" insertion at codon 206 of exon 3 of hMSH2 leading to a stop codon 73 bp downstream (family H27) (Figure 7). All 3 mutations give rise to protein truncation or protein structure alteration. In addition, the affected sister of H2 proband also carried t he same mutation in exon 13 of hMSH2. One sister and one brother of H27 porband also suffered from colorectal cancer at young age (her sister at 36 in transvers al colon and at 47 in cecum; her brother at 38 in rectum), both carrying the sam e germline mutation as their proband does. In H11 proband, a missense mutation in exon 1 of hMSH2 was identified. In order to determine whether this alteration represents a neutral polymorphism or a disease causing mutation, we assessed all possible family members and found no definite relationship between the base change and the disease. So it could not be demonstrated to be pathological and might be a single nucleotide polymorphism (SNP).

Table 2 Pathological germ-line mutations in the hMLH1and hMSH 2 genes

Family	Gene	Exon	Codon	Mutation	Nucleotide Change
H2	hMSH2	13	680	Nonsense	CGA-TGA(stop codon)
H9	hMLH1	11	305	In-frame deletion	24 bp deletion
H27	hMSH2	3	206	Frame shift	1 bp(A) insertion; stop at 73 bp downstream of the mutation

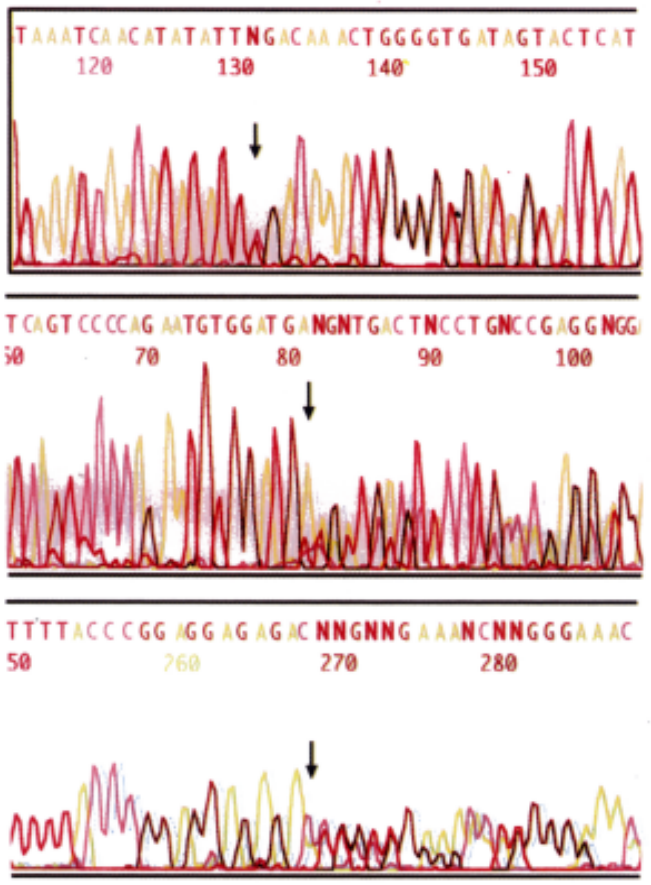

Figure 5 C-T transition (CGA-TGA) at codon 680 in exon 13 of hMSH2 gene in H2 proband, resulting in a stop codon.
Figure 6 A 24 bp deletion at codon 305 in exon 11 of hMLH1 gene in H9 proband.
Figure 7 A 1 bp insertion at codon 206 in exon 3 of hMSH2 gene in H27 proband, resulting in a stop codon 73 bp downstream of the mutation.

DISCUSSION

In China, the first clinical report of HNPCC cases was published by Mo *et al*[37] in 1996. Attention has been paid to this kind of hereditary tumor syndrome thereafter by Chinese sc holars[38-39]. Our study is a postlude of Mo's report. Three male prob ands and one female proband out of the 4 typical Chinese families had a mean onset age of 48 years. Three displayed proximal CRCs and one had a rectum cancer, all showed synch ronous and/or metachronous tumors, with one having metachronous endometrial cancer. These families fulfilled the strict HNPCC criteria. The four probands mentioned above had altogether 10 tumors. The current study is the first report with comprehensive microsatellite analysis, immunohistochemistry and direct mutation a nalysis of mismatch repair genes in Chinese HNPCC study.

Microsatellite is highly polymorphical, thus it has been widely considered as an ideal genetic marker. Microsatellite instability reveals loss of the function of mismatch repair gene s. It can serve as a reliable preliminary screening strategy of HNPCC family as several studies have shown that microsatellite instability occurs in about 80%-90% of HNPCC tumors[13-25]. Microsatellite instability was also found in 15%-20% of sporadic colorectal carcinomas[36,40]. In the current study, we adopted a panel of five sensitive microsatellite markers accepted by the International Collaborative Group for HNPCC and the National Cancer Institute to detect the MSI status[35]. One hundred percent (5/5) of the five tumors displayed high-level microsatellite instability, which suggests that Typical Chinese HNPCC families show high level defection of mismatch repair function in the affected patients.

The majority of HNPCC cases are associated with the mutation of hMSH2 and hMLH1 genes. Recent studies showed that the immunostaining of proteins produced by these two genes could serve as a convenient, rapid and cheap approach in screening HNPCC families[26-30]. In our study, the tumors from H2, H11 and H27 probands lost the expression of hMSH2 protein, while germline mutation of hMSH2 gene was only detect ed in H2 and H27 probands. The tumor of H9 proband showed no hMLH1 protein expression and a germline mutation of hMLH1 gene was identified. Although H11 proband had a tumor displaying no expression of hMSH2 protein, no pathological germline mutation had been detected. In general, microsatellite instability status and immunohistochemical alteration of hMSH2 and hMLH1 proteins correlated closely with each other. Immunohistochemistry is also very useful in screening HNPCC families.

Direct gene sequencing remains the most reliable method for HNPCC diagnosis. Mutations of hMSH2 and hMLH1 accounted for 25%-86% of the total cases[41-46]. Two main reasons were suggested for the discrepancies of mutation detection rate: firstly, different clinical criteria for selecting HNPCC families were adopted in various studies; secondly, the methods used by individual investigators varied. Till now more than three hundred different predisposing mutations have been reported, mainly affecting the MMR genes hMLH1 (about half), hMSH2 (about 40%) and MSH6 (about 10%)[7]. There appeared no hot spot mutations among those found in these mutations. The three pathological mutations (two in hMSH2 and one in hMLH1) found in our collectives are all novel mutations that had not been reported before (http:/ www.nfdht.nl/database/mdbchoice). The rate of mutation is 75% (3/4). The one A-insertion frame shift mutation in H27 proband gave rise to stop codon 73 bp downstream. H9 proband showed 24 bp deletion mutation in exon 11 of hMLH1 gene. The third mutation was a base substitution resulting in a stop codon. All of the detected mutations resulted in the truncation or structure alteration of the proteins. The mutations existed also in genomic DNA from other affected family members. That all mutations in our study appear new demonstrates the wide spectrum of the mutation responsible for HNPCC. The mutation may be different in a variety of races and geographical regions. It is therefore very important to develop HNPCC screening and genetic analysis strategies in China. The remaining family proband (H11) possessed a missense mutation (CCG-CAG, Pro-Glu). Its pathological meaning could not be demonstrated, because the presence and absence of cancer in this family showed no relation with the base change, suggesting that this may be only a polymorphism, i.e., SNP. This result could not explain the phenomenon of the lost of hMSH2 protein in the tumor of H11 proband. Curia et al con sidered that the possible germline mutation of such cases was located outside the coding region and intron-exon borders. Such mutations have the potential to affect the transcription, processing, and/or stability of mRNA encoded by the corresponding allele, resulting in germ-line transcript imbalance that should be detectable in normal tissues or PBLs. Such imbalance could be investigated by primer extension assays[47]. This kind of screening is necessary for the family like this, so as to discover the abnormality not detectable by sequencing.

Since HNPCC has many characteristics different from those of sporadic colorectal cancer, it is necessary to distinguish between them. HNPCC has better prognosis and shows more resistance to the chemotherapeutic drugs (for example, 5-FU, cisplatinum, etc). MMR gene mutation analysis will give both HNPCC proband and his family members better management and surveillance, and it will also support genetic counseling as well as gene therapy in the future. To the proband himself, it is helpful for us to conduct positive and

effective therapy to reduce the occurrence of possible me tachronous multiple colorectal cancer. To the mutation carriers in his family who have not yet suffered from colorectal cancer, close follow-up and early diagnosis are more likely to be performed. Colonoscopy every 1 to 3 years starting at age of 25 is recommended. To the non-mutation carriers, we should free them from unnecessary psychological and economical burden[48-50].

The current report is only a first description of our study at the initial stage. We hope that it could provide a guidance to the surgeons and pathologists in China who are closest to the patients. A wider survey with more kindreds in detail and a deeper analys is of the tumor spectrum of Chinese HNPCC kindreds remain a heavy assignment for us.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Construction and selection of the natural immune Fab antibody phage display library from patients with colorectal cancer

Bao-Ping Wu[1], Bing Xiao[1], Tian-Mo Wan[1], Ya-Li Zhang[1], Zhen-Shu Zhang[1], Dian-Yuan Zhou[1], Zhuo-Sheng Lai[1], Chun-Fang Gao[2]

[1]Institute for Digestive Diseases, Nanfang Hospital, Guangzhou 510515, Guangdong Province, China
[2]Surgical Department of Colon and Rectum, 150 Central Hospital, Luoyang 471031, Henan Province, China
Supported by the Natural Science Foundation of Guangdong Province, China, No.980120 and the Foundation of Excellent Youth Teacher, China, 2001
Correspondence to: Dr. Bao-Ping Wu, Institute for Digestive Medicine, Nanfang Hospital, Guangzhou 510515, Guangdong Province, China. bpwu@263. net
Telephone: +86-20-85141544
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Abstract

AIM: **To construct the natural immune Fab antibody phage display libraries of colorectal cancer and to select antibodies related with colorectal cancer.**

METHODS: **Extract total RNA from tissue of local cancer metastasis lymph nodes of patients with colorectal cancer. RT-PCR was used to amplify the heavy chain Fd and light chain κ and the amplification products were inserted successively into the vector pComb3 to construct the human libraries of Fab antibodies. They were then panned by phage display technology. By means of Dot immunoblotting and ELISA, the libraries were identified and the Fab phage antibodies binding with antigens of colorectal cancer were selected.**

RESULTS: **The amplified fragments of Fd and κ gained by RT-PCR were about 650 bp. Fd and κ PCR products were subsequently inserted into the vector pComb3, resulting in a recombination rate of 40% and the volume of Fab phage display library reached 1.48×10^6. The libraries were enriched about 120-fold by 3 cycles of adsorption-elution-multiplication (panning). Dot immunoblotting showed Fab expressions on the phage libraries and ELISA showed 5 clones of Fab phage anti bodies which had binding activities with antigens of colorectal cancer.**

CONCLUSION: **The natural immune Fab antibody phage display libraries of colorectal cancer were constructed. They could be used to select the relative antibodies of colorectal cancer.**

Subject headings colorectal neoplasms, immunology, bacteriophages, antibody library

Wu BP, Xiao B, Wan TM, Zhang YL, Zhang ZS, Zhou DY, Lai ZS, Gao CF. Construction and selection of the natural immune Fab antibody phage display library from patients with colorectal cancer. *World J Gastroenterol*, 2001;7(6):811-815

INTRODUCTION

The incidence of colorectal cancer is growing in China. It has been clear that the prognosis of colorectal cancer is related to early diagnosis and treatment[1-15]. Since the development and evolution of the colorectal cancer is one kind of complicated pr ocedures involving multi-genes, multi-factors and multi-steps, there have been no highly peculiar molecular pathologic changes and tumor[16-39]. A major focus of cancer immunology is on the isolationof antibodies that react selectively with human tumor cells[40,41], because the antibodies could have important applications for targeting diagnostic and therapeutic agents to tumors and for identifying tumorigenic antigens[42-47]. The established approach has been to generate large panels of monoclonal antibodies (mAb) from mice immunized with human tumor cells and to screen the antibodies for reactivity against the tumor[48-50]. Despite the enormous efforts put on this approach, few antibodies that react preferentially with human tumors, and none that react specifically with one type of tumor (such as colorectal cancer), have been reported. Further attempts to isolate more specific and high affinity antibodies will require improved methods of generating and selecting antibodies against human tumors[51]. One is the introduction of method for synthesizing virtually the entire repertoire of a person's antibody genes of variable regions by PCR technique and for expressing the encoded antibodies on the surface of a phage vector[52]. The resulting phage antibody library can be panned to select and clone rare antibodies on the basis of their binding specificities[53,54]. It can resolve the problems of generating humanization mAb by hybridoma approach. Humans can be immunized or are immune to many antigens (including tumor antigens) but only local lymph nodes, a productive source of antibody-producing cells, is readily available. Here we describe the construction of the natural immune phage display libraries expressing Fab antibodies derived from local cancer metastasis lymph nodes of co lorectal cancer patients and report the initial results of panning the libraries for anti-colorectal cancer antibodies.

MATERIALS AND METHODS

Vector, E.coli and helper phage

The vector (pComb3), about 4894 base pairs, contains ampicillin resistance (Ampr) gene, start sites of plasmid CO1E1 and f1 phage replication. The expression of inserting fragments is control led by the same LacZ promoter on its upstream sequence. *E.coli* XL1-Blue contains tetracycline re sistance (Tetr) gene on its gene type of Tn10. Helper phage (VCSM13) contains kanamycin resistance (Kanar) gene with valency of 10^{15} pfu·L^{-1}. It is amplified in SOC culture medium and preserved in 4℃.

Lymph nodes total RNA preparation

Lymph nodes in mesenterium were resected during surgical operation on patients with colorectal cancer and preserved in liquid nitrogen immediately. The nodes of patients (case number 260280, 260583 and 260476) were defined as tumor metastatic lymph nodes by pathological examination. One hundred mg of each node was used to

extract total RNA by the standard method of guanidinium isothiocyanate.

Amplifying Fd and κ chain genes of antibodies by RT-PCR

Total RNA (20-50 μg) was added to 60pmol primer of *Oligo(dt)* and heated at 65℃ for 10 min. The mixture was then used in a 20 μl reverse transcription reaction containing 200 μmol·L^{-1} each dNTPs and 20 U of reverse transcriptase (Promega), which was incubated at 37℃ for Ih. The RNA-cDNA mixture (5 μL) was then used in 50 μL PCR reaction mixture containing all four dNTPs at 60 μmol·L^{-1}, 5 U of Taq polymerase (Promega), and 50 pmol·L^{-1} of appropriate 5' and 3' primers[55,56]. VK1a and VK3a are 5' primers for amplification of the κ chain with the Sac I site f or cloning into the vector pComb3. CK1a is a 3' primer corresponding to the 3' end of the light chain κ, *Xba* I site. VH1a and VH3a are 5' primers for the heavy chain (Fd), *Xho* I site. CG1z is the 3' primer for the Fd and corresponds to part of the hinge region, *Spe*I site.

$V_κ$5' primers: VK1a, 5'-GACATCGAGCTCACCCAGTCTCCA -3';
$V_κ$3a, 5'-GAAATTGAGCTCACGCAGTCTCCA-3';
$V_κ$3' primers: CK1a,
5'-GCGCCGTCTAGAACTAACACTCTCCCCTGTTGAAGCTCTTTG-TGACGGGCAAG-3';
V_H (Fd) 5' primers: VH1a, 5'-CAGGTGCAGCTCGAGCAGTCTGGG-3';
V_H3a, 5'-GAGGTGCAGCTCGAGGAGTCTGGG-3';
V_H (Fd) 3' primers: CG1z, 5'-GCATGTACTAGTTTTGTCACAAGATTTGGG -3'.

The reaction mixtures were then subjected to 35 rounds of amplification (PE/Cetus thermal cycler) at 94℃ for 1 min, 52℃ for 1.5 min and 72℃ for 2 min followed by a final incubation at 72℃ for 10 min. An aliquot of the reaction mixture (5 μL) was run on a 10 g·L^{-1} agarosegel.

Cloning heavy chain Fd into pComb3

The Fd fragment product of PCR (isolated by agarose gel electrophoresis) was cut with an excess of the restriction enzymes *Xho* I and *Spe* I and typically about 350 ng was ligated with 2 μg of *Xho/Spe* I -linearized pComb3 vector (isolated by agarose gel electro phoresis) in a total volume of 150 μL with 10 U of ligase (Promega) at 16℃ for 15 h. Following ligation, DNA was precipitated at -20℃ for 2 h by the addition of 15 μL of 3 mol·L^{-1} sodium acetate (pH 5.2), and 330 μL of ethanol. DNA was pelleted by microcentrifugation at 4℃ for 15 min. The DNA pellet was resuspended in 20 μL of water and transformed into 150 μL XL1-blue (porated by calcium chloride). After transformation, XL1-blue samples (10, 1, 0.1 μL) were withdrawn for plating to determine the rate of transformation. The insertion of target Fd fragments were detected by PC R from the plasmids extracted from several random XL1-blue monoclones. The plasmids with Fd insertion were named p+Fd.

Cloning light chain κ into p+Fd and antibodies Fab libraries construction

The κ fragment product of PCR and recombined p+Fd (isolated by agarose gel electrophoresis) were cut with an excess of the restriction enzymes *Sac* I and *Xba* I. The ligation, transformation, *E.coli* amplification and determination of the transformation rate were same as described above. The insertion of target κ fragments was detected by digestion with *Sac* I / *Xba* I from the plasmids extracted from several random XL1-blue monoclones. The Fab fragments' insertion was detected by digestion with *Xho* I and *Xba* I. The plasmids with Fd together with κ insertion were named Fd+κ. Helper phage VCSM13 (10^{12} pfu) was added to XL1-blue samples contained Fab gene libraries. Following the superinfection, the primer Fab phage display libraries of two patients were constructed, named K1 and K2, and preserved at 4℃.

Panning of the primer Fab phage display libraries

The colorectal cancer antigens were derived from fresh cancer tissues of patients 1, 2, and 3 by sonicating the tissues (each mass 500 mg). The supernatants were named Ag1, Ag2 and Ag3. Colon cancer cell line of Lovo (10^9 ·L^{-1}) was sonicated also, and its supernatants were named Lovo. The wells of a microtiter plate were coated overnight at 4℃ with the antigen supernatants above respectively (antigens were diluted with 0.1 mol·L^{-1} bicarbonate buffer, pH9.6). Cancer tissue antigens coated the microtiter plate wells in mixture of Ag1 and Ag2. Lovo cells of 200 μL(0.5×10^8·L^{-1}) were attached to the wall of the microtiter plat e wells, and 50 μL mixture of K1 and K2 was added to the wells coated with antigens respectively. The panning procedure is a modification[57] of that originally described by Parmley and Smith. The phage binding with antigens re-infected the XL1-Blue by eluting from the wells. A round of panning was finished after superinfection by VCSM13. Following 3 rounds of panning, the percent yield of phage was determined as (no. of phage eluted / no. of phage applied) ×100.

Dot immunoblotting analysis of Fab displaying[58]

Five μL library of each round of panning was added to nitrocellulose (NC) filter (with diameter of 0.5 cm). After being dried at room temperature, the NC filters were blocked in 50 mL·L^{-1} de-lipid milk and then react with the biotinylated anti-human IgG (1:100 dilution) and alkaline phosphatase (AP)-avidin at room tempe rature successively. Finally the NBT+BCIP was added and color developed. The control unit was the suspension of the phage contained vacant vector pComb3.

ELISA analysis of Fab displaying[59]

The wells of a microtiter plate were coated overnight at 4℃ with the antigen supernatants Ag1, Ag2, Ag3 and Lovo respectively (antigens were diluted in 1:100 with 0.1 mol·L^{-1} bicarbonate buffer, pH9.6). The suspension of K1 and K2 (50 μL each) was added to the wells respectively, and cultivated at 37℃. Following 3 washes with TBS buffer, 25 μL of a 1:100 dilution of biotinylated goat anti-human IgG and HP-conjugated biotin were added succes sively and incubated at 37℃. Finally 50 μL of TMB was added and color development was moni tored at 490 nm. The A490 values of positive clones were higher than that of neg ative clones at least two folds. After 3 rounds of panning, 5 clones of positive XL1-blue were superinfected with VCSM13 and the supernatants of Fab displaying were prepared. FiftyìL of the Fab display supern atants were added to the wells (coated with antigens of Ag1, Ag2, Ag3 and Lovo) respectively, and ELI SA analysis was applied by the method described above.

RESULTS

Amplifying the fragments of Fd and κ chain genes of antibodies by RT-PCR

The total RNAs of the lymph nodes defined as tumor metastasis in mesenterium of patients with colorectal cancer were isolated. The integrity of RNAs was shown by alkaline denaturing agarose gel. Their purity (A_{260}/A_{280}) reached 2.04. Fd and κ immunoglobulin chains were PCR-amplified after reverse transcription from this RNA. For amplifying Fd and κ, two 5'primers and one 3'primer were used respectively which contained some sites of the restriction enzymes corresponding to the vector pCom b3. The PCR amplification of Fd and κ was about 650 bp (Figure 1).

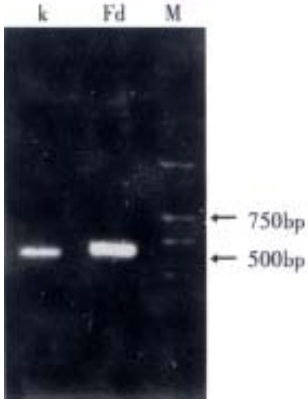

Figure 1 PCR products of Fd and κ.

Construction and identification of recombinant p+Fd

The recombinants of p+Fd were constructed by cloning Fd fragments into pComb3. After transforming XL1-blue, the Fd gene libraries were formed. And the quantity of the transformants reached 5.53×10^6. After extracting the plasmids from eight random XL1-blue monoclones, the insertion of target Fd fragments was detected by PCR with the primers same as that in amplifying Fd described above. Four of eight PCR could amplify the fragments of 650 bp (Figure 2). This meant that the recombination frequency was 50%, so the practical volume of Fd library was 2.77×10^6.

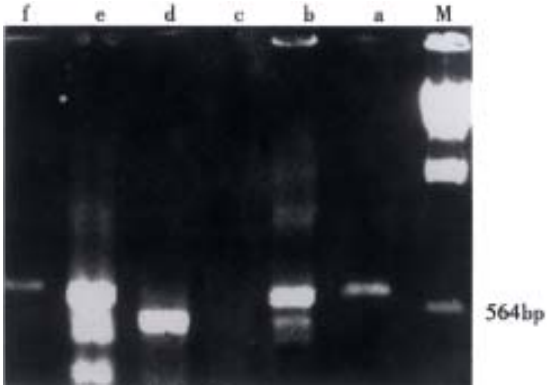

Figure 2 Fd insertion by PCR identification.

Construction and identification of Fab gene recombinant

The recombinants of Fd+κ were constructed by cloning ê fragments into p+Fd. After transforming XL1-blue, the Fab gene libraries were formed and the quantity of the transformants reached 7.4×10^6. Following extracting the plasmids from ten random XL1-blue monoclones, the insertion of target κ fragments was detected by digestion with *Sac* I and *Xba* I, and five of these plasmids discharged the fragments of 650bp (Figure 3). By digesting with *Xho* I and Xba I, four of these plasmids discharged the fragments about 2.4 kb (Figure 4). The recombination frequency of fragments Fd together with κ insertion was 40%, so the practical volume of Fab library was $7.4\times10^6\times50\%\times40\% = 1.48\times10^6$.

ELISA of binding activity of colorectal cancer antigens and Fab displaying on primer phage libraries

The activity of colorectal cancer antigens binding with K1 (or K2) was identified by ways of ELISA with control unit of wash buffer. The primer phage display libraries integrated with the colorectal cancer antigens of not only its immune derivation, but also other immune derivation (Table 1).

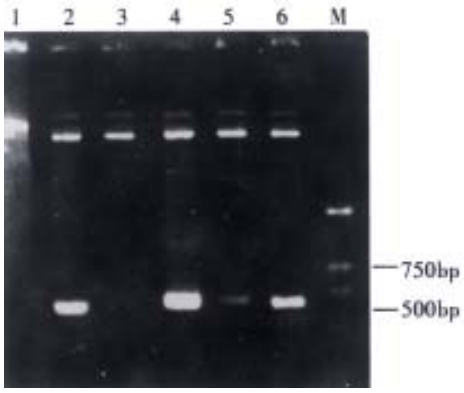

Figure 3 Identification of κ insertion by *Sac* I/*Xba* I digestion.

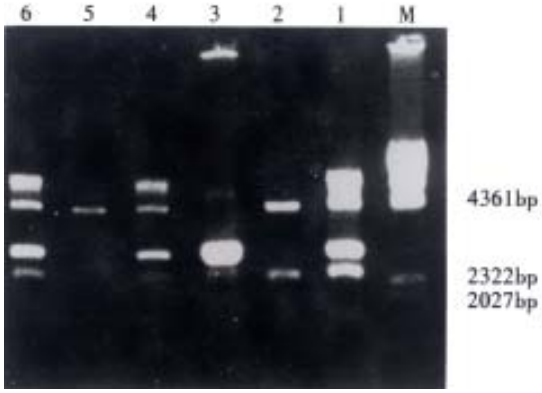

Figure 4 Identification of κ and Fd insertion by *Xho* I/ *Xba* I digestion.

Table 1 Binding of Fab displayed by prime phage display libraries with colorectal cancer antigens (A_{490})

	Ag1	Ag2	Ag3	Lovo	Wash buffer
K1	1.285	1.496	1.332	0.326	0.105
K2	1.324	1.552	1.352	0.322	0.122
BC	0.115	0.105	0.110	0.092	0.087

Panning and identification of the Fab phage display libraries

After 3 rounds of panning, the libraries were enriched about 120-fold. Dot immunoblotting applied by the system of AP-goat anti-human IgG showed that there were Fab expressions on the phage lib raries of each panning round (Figure 5). There was no obvious color development in the control units (C). The supernatants of Fab displaying prepared from 5 clones of positive XL1-Blue after 3 rounds of panning showed the significant binding activity with the antigens related with c olorectal cancer by ELISA analysis (Table 2). The control units were the rat colon antigens and wash buffer.

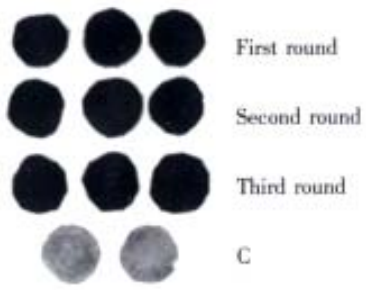

Figure 5 Dot immunoblotting analysis.

Table 2 Binding activity of phage antibodies with antigens (A_{490})

Phage antibodies	Ag1, Ag2 and Ag3	Lovo	Rat colon antigens	Wash buffer
Clone 1	1.236	1.042	0.121	0.096
Clone 2	1.117	0.873	0.110	0.100
Clone 3	1.450	1.329	0.182	0.121
Clone 4	1.106	0.912	0.106	0.087
Clone 5	1.345	1.024	0.090	0.083

DISCUSSION

Fd parts and κ immunoglobulin chains (650 bp) were PCR-amplified after reverse transcription from the RNAs, which were isolated from the lymph nodes de fined as tumor metastasis in mesenterium of patients with colorectal cancer. For mimicking the diversity of the antibody genes, we used 5' primers based on the leader exon and the N terminus of first framework (FR1) sequences according to their relatively constant regions, and 3' primers within the constant regions during amplifying the V genes of immunoglobulin Fab. To maximize complementarity, degeneracy was incorporated into the primers, or different primers were designed for different families of antibody V genes. For cloning of the amplified DNA into expression vectors, rare restriction sites were introduced within the PCR primers. Following mixing the 2-group of Fd PCR products, we cloned them into the vector pComb3. Then the 2-group of κPCR products were inserted into the recombinants of p+Fd. Therefore the gene libraries of humanization antibody were constructed, which contained parts of 2 -subgroup of heavy chains and 2-subgroup of κ chains. Previously reports had shown that the volume of the phage display libraries was 1.0×10^7 at most constructed by this vectors and ways[57]. In review, the volume was 10^6-10^7 according to the reports abroad, and about 10^6 according to the domestic reports[60,61]. We reported the volume being 1.48×10^6.

By antibody technology, the mAbs can be prepared by-passing hybridoma approach. Construction of the natural immune antibody library is the method of by-passing unnatural immunization. The natural immune antibody library is constructed from the natural donors, such as lymphocyte B and plasma cell. In our present study, the natural immune Fab antibody phage display libraries were constructed from the lymphocytes in the lymph nodes of 2 patients with colorectal cancer. There have been several studies on the library of immunity antibody in recent years[60-62], but no reports on construction of the natural immune antibody library of human colorectal cancer. Theoretically the humanization antibody library constructed from the lymphocytes B in lymph nodes defined as tumor metastasis of patients with tumor can aim at screening the antibodies, because the genes of heavy and light chains have been rearranged and ligated *Spe* cifically due to immunizing of tumor antigens. By this way the promiscuous work of preparation of antigens by immunizing rats was relieved and the problems of weak antigens, heterologica l antibodies, low fusion, instability and low antibodies products of hybridoma were resolved. From the natural immune antibody library, a few of phage antibodies immunized by some foreign antigens (such as bovine serum album, lysozyme, bovine thyroid globulin, etc.) were screened, and some self-antigens (such as tumor necrosis factor-α, carcinoembryonic antigen, CD4, thyroid globulin, etc.). Many of these phage antibodies showed high affinity and specificity, and the potential value for application.

It is important to assemble antibody molecules that have correctly folded heavy and light chains and thus retain antigen-recognition capabilities. Previously it had been demonstrated that Fd chains (comprising V_H and C_{H1}, the variable region and constant domain 1 of the immunoglobulin heavy chain) and κ chains targeted to the periplasm of *E.coli* assemble to form functional Fab molecules. If the Fd chain was anchored in the membrane and concomitantly provided with secreted κ chain, functional Fab molecules could form on the membrane surface facing the periplasm. The coexpression behind the PelB leader sequence of Fd fused to cpIII (the major coat protein of M13 phage) and ê chains lead to membrane anchoring of the Fdc hain and compartmentalization of the κ chains in the periplasm. These two chains could a ssemble *in situ* to allow accumulation of functional Fab on the membrane surface, which, by virtu e of the cpIIIsequences, would be incorporated along the entire length of the filamentous ph age particles on subsequent infection with helper phage. These phages displaying Fab could inf ect *E.coli* again. Following rounds of panning by adsorption-wash-amplification, the phages comprising specific antibody molecules could be enriched[63]. Our study showed that the phage Fab libraries were enriched about 120-fold after 3 rounds of panning. It suggested that the colorectal cancer antigens had selected the phages in the libraries displaying Fab. Dot immunoblotting showed that there were Fab expressions on the phage libraries of each panning round. ELISA analysis showed that the phage displaying Fab had the significant binding activity with the antigens related with colorectal cancer. Considering that nowadays there are few and hypo-specific antibodies related with colorectal cancer, the natural immune Fab antibody phage display libraries of colorectal can be used to select the affinity and specificity antibodies related to colorectal cancer. It is important to diagnose and treat colorectal cancer using these antibodies.

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Edited by Xu JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

SF/HGF-c-Met autocrine and paracrine promote metastasis of hepatocellular carcinoma

Qian Xie, Kang-Da Liu, Mei-Yu Hu, Kang Zhou

Experimental Research Center of Zhongshan Hospital, Fudan University, Shanghai, 200032, China
Supported by Natural Science Foundation of China No.39970290
Correspondence to: Dr. Kang-Da Liu, Experimental Research Center, Zhongshan Hospital, Fudan University, 180 Fenlin Road, Shanghai 200032, China. kdliu@shmu.edu.cn
Telephone: +86-21-64041990-2295
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Abstract

AIM: **To explore the role of SF/HGF-Met autocrine and paracrine in metastasis of hepatocellular carcinoma (HCC).**

METHODS: **SF/HGF and c-met transcription and protein expression in HCC were examined by RT-PCR and Western Blot in 4 HCC cell lines, including HepG2, Hep3B, SMMC7721 and MHCC-1, the last cell line had a higher potential of metastasis. sf/hgf cDNA was transfected by the method of Lipofectin into SMMC7721. SF/HGF and c-met antibody were used to stimulate and block SF/HGF-c-met signal transduction. Cell morphology, mobility, and proliferation were respectively compared by microscopic observation, wound healing assay and cell growth curve.**

RESULTS: **HCC malignancy appeared to be relative to its met-SF/HGF expression. In MHCC-1, c-met expression was much stronger than that in other cell lines with lower potential of metastasis and only SF/HGF autocrine existed in MHCC-1. After sf/hgf cDNA transfection or conditioned medium of MHCC-1 stimulation, SMMC7721 changed into elongated morphology, and the a bilities of proliferation ($P<0.05$) and mobility increased. Such bio-activity could be blocked by c-met antibody ($P< 0.05$).**

CONCLUSION: **The system of SF/HGF-c-met autocrine and paracrine played an important role in development and metastas is potential of HCC. Inhibition of SF/HGF-c-met signal transduction system may reduce the growth and metastasis of HCC.**

Subject Headings: hepatocyte growth factor/Scatter factor; c-met; hepatocellular carcinoma; metastasis

Xie Q, Liu KD, Hu MY Zhou K. SF/HGF-c-Met autocrine and paracrine promote metastasis of hepatocellular carcinoma. *World J Gastroenterol*, 200 1;7(6):816-820

INTRODUCTION

The human proto-oncogene c-met encodes a Mr. 190, 000 heterodimeric transmembrane protein with structural features of a tyrosine kinase receptor and is expressed predominantly on epithelial cells. Its ligand HGF is a mesenchymal protein which is identical to Scatter factor (SF), a factor secreted by fibroblasts. It is well known that SF/HGF has a special binding with c-met, mainly attending mitogenic, motogenic and morphogenic effects on normal targeted cells[1-4]. Recently, its signal pathway has been explored a lot, including STAT in tubulogenesis[5], Gab-1 in cell growth[6,7], MAPK in morph ogenesis[8,9], and PI-3K in cell mitogenesis[10,11]. Its relationship with ad hesive factors[12-16] and apoptotic factors[17,18] and the cross-talk between HGF/SF-c-Met and other growth factors[19] and proto-oncogenes[20] have also bee n discussed. However, in cancer research, controversial results have been found in various tumors. Some reports suggested that HGF/SF was potent stimulator for tumor proliferation and motility[21-29], some reports made exactly opposite conclusions[30-33]. Among all the tissues, situation in liver becomes the most complicated, since SF/HGF attends all the stages of liver growth, regeneration, cirrhosis and carcinoma. SF/HGF was first found as a serum derived factor that stimulated proliferation of primary liver cells, acted as one of the initial factors in liver regeneration[34-35] and reversed cirrhosis by suppressing the increased TGF betal, fibrogenesis and hepatocyte apoptosis[36]. It ha s been unexpectedly reported to inhibit the growth of hepatoma cell in many reports[31]. Little has been known about the mechanism.

We studied a HGF/SF autocrine hepatocellular carcinoma (HCC) cell line which has a high potential of metastasis and compared its characteristic with several other HCC cell lines which do not have these features. Gene transfection and other interfering methods were used to further demonstrate the SF/HGF autocrine and paracrine on malignant capability of HCC.

MATERIALS AND METHODS

Cells

SMMC7721, HepG2, and Hep3B human HCC cell lines were purchased from Shanghai Institute of Cell Biology, Chinese Acadamy of Science (Shanghai, China). MHCC-1 was a cell line with high potential of metastasis from a resected lesion of a HCC patient. Cells were cultured in DMEM supplemented with 100 $mL \cdot L^{-1}$ fetal calf serum or AB serum (MHCC-1), penicillin (100 $kU \cdot L^{-1}$), streptomycin (100 $kU \cdot L^{-1}$).

Collection of MHCC-1 conditioned medium (MHCC-1-CM)

Cells were planted in 100 ml culture bottles. When 80% cells were subconfluent, they were washed and replaced with serum free DMEM (0.1 $mL \cdot cm^{-2}$). Three to five days later, conditioned medium was collected, centrifuged under 2000 $r \cdot min^{-1}$ for 20 minutes and stored at -20℃ until use.

Plasmid and DNA transfection

The plasmid pBS7.3 containing human full-length sf/hgf(kindly provided by Dr. George Vande Woode) was cloned into the BamHI-ApaI site of the p cDNA3.0(+) mammalian expression vector. SMMC7721 HCC cell line was transfected overnight with the constructed plasmid, using lipofectamine (Gibco). Cells were selected by G418. Colonies of cells were trypsinized within cloning rings, then they were transferred to 24-well dishes, and grown to confluency. After conditioned medium was changed to serum free DMEM for 3 d, both supertenant and whole lysised cells were screened for SF expression (ELISA, R&D). The highest expressing

clone (SF7721) was selected for further research.

RT-PCR

Total RNA was extracted (QIAGEN) and 1 μg was reversing transcribed in a 25 μL volume using Super rScriptII™ (Gibco), according to the manufacturer's instructions. Five μL reverse transcription product was used for amplification with the following primers: SF: 5'-CAG CGT TGG ATT CTC AGT AT-3', 5'-CCT ATG TTT GTT CGT GT T GGA-3'. c-met: 5'-ACA GTG GCA TGT CAA CAT CGC T-3', 5'-GCT CGG TAC TC T ACA GAT TC-3'. Forty cycles were performed, each consisting of 95℃, 45 s; 60℃, 1 min. There was a time delay for 7 min at 72℃.The reaction products were visualized by 15 g·L^{-1} agarose gel electrophoresis.

Western blotting

Goat anti-human HGF antibody was purchased from R&D. Rabbit anti-human c-met anti body, anti-rabbit IgG-AP and anti goat IgG-AP were purchased from Santa Crus. Cells were washed with PBS, and lysed at 0℃ for 30 min in lysis buffer (TrisCl 50 mmol·L^{-1} pH8.0, NaCl 150 mmol·L^{-1}, NaN_3 0.2 g·L^{-1}, PMSF 100 mg·L^{-1}, Aprotinin 2 g·L^{-1} , TritonX-100 10 g·L^{-1}). In c-met detection, protein content was examined using BCA™ Protein Assay (Pierce), and 20 μg protein per lane was electrophoreses on 80 g·L^{-1} SDS polyacrylamide gels after boiling for 5 min in 2X loading buffer. As to SF/HGF, conditioned medium was mixed with 2X loading buffer and added to 100 g ·L^{-1} SDS polyacrymide gels. Protein was blotted onto nitrocellulose membranes. After electrobloting, the membranes were blocked in PBS-50 mg·L^{-1} non-fat dry milk, washed with PBS-Tween buffer, and incubated with the primary antibody (1:500) diluted in blocking buffer for 2 h. Membranes were then washed, incubated with the appropriate second antibody (1:500) in blocking buffer for 2 h, and re-was hed. Blotted proteins were stained with BICP-DAB (Huamei).

Cell scatter and morphology

5×10^3 cells of SMMC7721 and SF7721 were planted into the wells of 96-wells plates. Three replicated wells. Twenty-four hours later, wells were washed with PBS and fixed with ethanol and stained with Giemsa and examined under an inverted microscope. To explore the role of SF/HGF-c-met paracrine, 5×10^3 cells of SMMC7721 were planted into the wells of 96-wells plates. And 200 μL MHCC-1-CM was added into each well within diluted range 1:2, 1:4, 1:8, 0, respectively. Three replicated wells. Twenty-four hours later, cells were fixed and observed as above.

Cell migration assay

The 'wound assay' of Birch was used to determine the migratory capacity. 5×10^4 SMMC7721 or SF7721 cells were planted into the wells of 24-well plates and cultured 24-48 h until the cultures were subconfluent. A wound track was scored in each well. In paracrine system detection, 5×10^4 SMMC7721 were planted into the wells of 24-well plates and cultured 24-48 h until the cultures were subconfluent. A wound track was scored in each well. Conditioned medium was replaced in the experimental group with 1 mL MHCC-1 - CM and 1 mL control medium, control group with 1 mL surum free DMEM and 1 mL control medium. Replicated wells were terminated at 16, 24 and 48 h after wounding and examined under inverted microscope.

Cell growth curve

5×10^4 SMMC7721 or SF7721 were planted into 24-well plates, respectively. From the next day, cells of triplicate wells were digested by typsin and counted under microscope for 8 d. The average was recorded as cell number of the day. In addition, 2×10^4 SMMC 7721 cells were planted into the wells of 96-wells plates. Twenty-four hlater, 200 μL MH CC-1-CM was added into each well within diluted range 1:2, 1:4, 1:8, 0, respectively. Three replicated wells. The effects of MHCC-1-CM on SMMC7721 were also contrasted using SMMC7721-CM. After 72 h incubation, 20 μL MTT (Methabenzthiazuron, Serva Co, USA) was added into each well to a final concentration at 50 g·L-1. Four hours later, the medium with 100 μL DMSO was replaced, and asorbance (OD) was read under 540 nm (Bio-rad).

Inhibition assay by c-met antibody

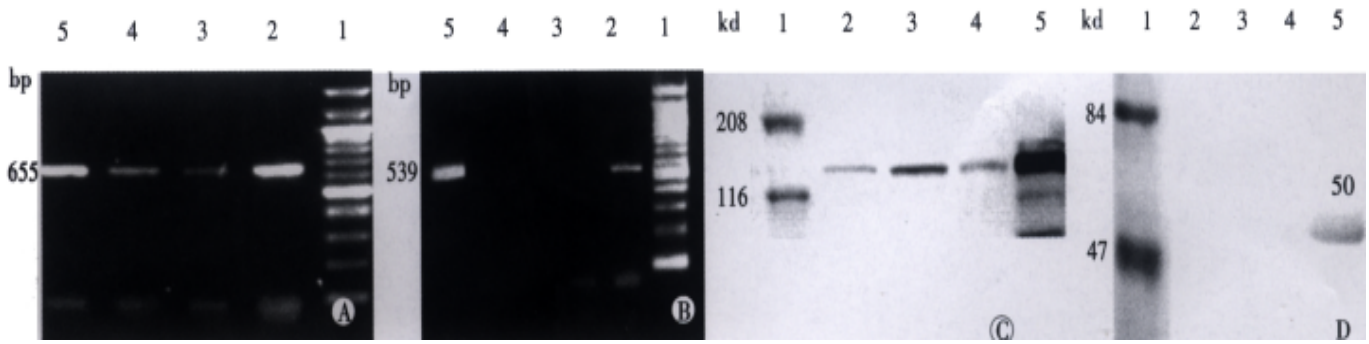
2×10^4 MHCC-1 or S F7721 were planted in 96-well plates, 3 replicated wells. After 24 h incubation, cells were washed by free DMEM, and incubated in 50 μL c-met polyantibody within a concentration range of 20, 10, 5, 2.5, 0 mg·L^{-1}. Rabbit IgG was used as control. Two hours later, 150 ìL control medium was added into each well and 72 h later, cells were examined by MTT. As to SMMC7721, 10^4 cells incubated with 2 μg c-met antibody for 2 h. Afterward, cells were washed by D-Hanks and added into wells of 96-well plates within 100 μL conditioned medium and 100 μL MHCC-1-CM or SM MC7721-CM, respectively. Twenty-four h later, cells were observed under micros cope. Rabbit IgG was used as negative control.

Statistical analysis

In SMMC7721 cell proliferation experiments, Student's *t* test was used to compare the difference of each corresponding dosage groups. Values were expressed as means ± standard. The cell growth curve and c-met blocking assay were tested by two-way anova.

RESULTS

SF and c-met expression in HCC cell lines (Figure 1)



A:c-met transcription B: SF/HGF transcription
1: Mark; 2: MHCC-1; 3: HepG2; 4: Hep3B; 5: SMMC7721; 6: pBS7.3

C: c-met protein expression D: SF/HGF protein expression
1: Mark; 2: SMMC7721; 3: Hep3B; 4: HepG2; 5: MHCC-1

Figure 1 SF/HGF and c-met expression in HCC cell lines.

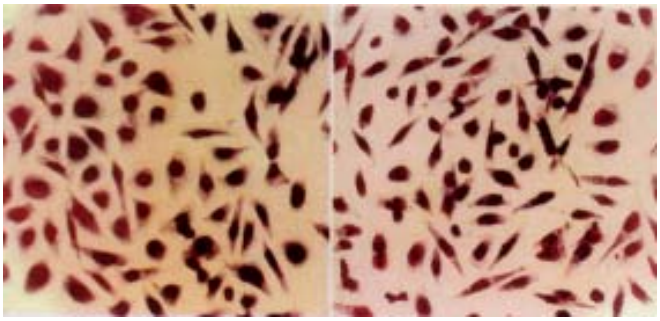
RT-PCR showed that all the cell lines had the transcription and protein expression of c-met, in which MHCC-1 most actively expressed. Only MHCC-1 had the transcription and protein expression of SF/HGF to the medium.

SF/HGF expression in SF7721

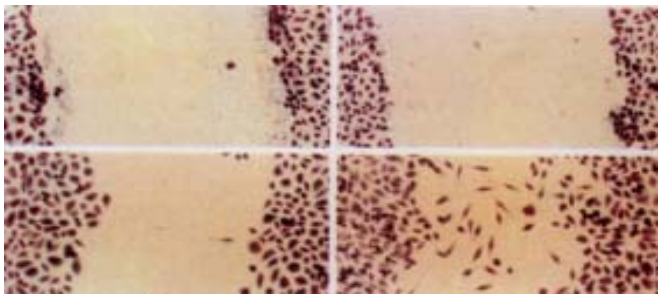
After sf/hgf transfection, in SF7721 cell extracts, the highest expression of SF/HGF reached 692 $\mu g \cdot L^{-1}$, compared with 0.026 $\mu g \cdot L^{-1}$ in control cells. However, no SF/HGF was detected in conditioned medium.

SF/HGF-c-met autocrine stimulate HCC malignancy

After gene transfection, SF7721 cells displayed scattered distribution and elongated morphology (Figure 2A) together with increased ability of proliferation. The cell growth curve showed that after 8 days, cell number of SF7721 reached almost double of that of SMMC7721 (Figure 2C, $P<0.05$). Also, the transfected SF7721 acquired stronger mobility. In "wound healing assay", SF7721 moved faster than SMMC7721 cells into cell free area (Figure 2B). However, the c-met expression in SF7721 and SMMC7721 did not show great difference.



Left:SMMC7721; Right:SF7721
Figure 2A Cell morphology comparison. ×400



Up: Control at "0" time Left down:SMMC7721 at 16 h after wound healing Right down:SF7721 at 16 h after wound helaing
Figure 2 B Cell morphology comparison ×400

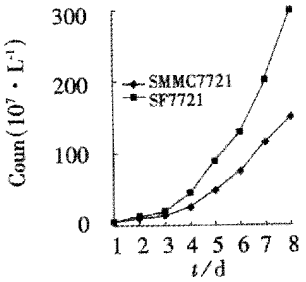


Figure 2 C Cell growth curve before and after gene transfection

Role of SF/HGF-c-met paracrine

The paracrine system of SF/HGF-c-met was examined by stimulating SMMC7721 with MHCC-1-CM. Results were consistent with that in autocrine. The proliferation of SMMC7721 was stimulated by MHCC-1-CM at 1:2 dilution (Table 1, $P<0.01$). In addition, MHCC-1- CM increased the mobility of SMMC7721 cells in wound healing assay and induced the elongated morphology displayed in SF7721 cells. (Data not shown).

Table 1 MHCC-1-CM stimulated proliferation of SMMC7721 (contrasted by SMMC7721-CM, $\bar{x}\pm S_{\bar{x}}$)

Group	Conditioned medium/culture medium (volume ratio) 0	1:16	1:8	1:4	1:2[b]
SMMC7721-CM	0.596±0.090	0.535±0.315	0.568±0.099	0.524±0.053	0.541±0.377
MHCC-1-CM	0.513±0.043	0.581±0.080	0.570±0.061	0.620±0.033	0.853±0.031

[b]$P<0.01$, 0.541±0.377 *vs* 0.853±0.031.

Assay of C-met antibody blocking

Both cell proliferation and motility could be blocked by c-met antibody. After 3-day's incubation with c-met antibody, either MHCC- 1 or SF7721 showed inhibition of growth, and was correlative with antibody concentration (Figure 3, $P<0.05$). As to SMMC7721, the effect of inhibition could be seen under microscope, their shape turned round, and became shrank.

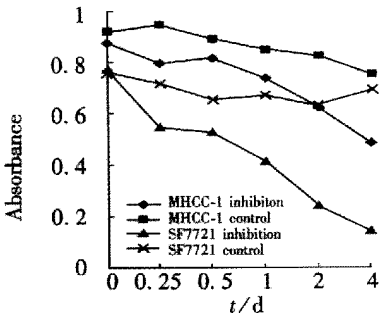


Figure 3 C-met inhibition assay.

DISCUSSION

Metastatic dissemination of solid tumors is a complex patho-physiological process including various factors. When we started to research on a HCC cell line (M HCC-1) with high potential of metastasis, we found that it had a high expression of c-met and with SF/HGF autocrine, which did not exist in other cell lines without or with low potential of metastasis. Thus, it became a promising approach to discuss c-met-HGF/SF signal transduction and tumor metastasis. Our result showed that cell malignancy of HCC is relative to its SF/HGF-c-met expression. The c-met expression of cell line with a higher potential of metastasis is much stronger than those with lower potential of metastasis and appeared with SF/HGF autocrine. Previous studies reported that many cancer cells expressed HGF/SF and c-met *in vivo*, but few of carcinoma cell lines produced HGF/SF *in vitro*, indicating that SF/HGF is a negative regulator in tumor progression. To further eluciate the phenomena, we transfected SF/HGF cDNA into S MMC7 721 cell line, trying to demonstrate that acquired SF/HGF autocrine may increase the malignancy and improve the metastatic potential in less metastatic cells. Our results showed that the proliferation, mobility and cell morphology had greatly changed in

SF7721 cells. Although the c-met expression in SF7721 cell did not increase significantly, it did improved greatly in SF7721 tumors in nude mice assay, later in the *in vivo* research. (Data not shown) When SF/HGF-c-met system was blocked by c-met antibldy, both MHCC-1 and SF7721 were blocked, demonstrating that SF/HGF-c-met was a positive regulator in HCC progression. Thus, we postulated that carcinoma cells may lose the ability to produce HGF/SF during *in vitro* passage, or the expression of HGF/SF need an activation from matrix. The high potential of metastasis of MHCC-1 may, to a great extent, contribute to its preservation of HGF/SF expression and keep the c-met activated all the way. *In vivo*, fibroblasts can produce HGF/SF, which may induce the expression of HGF/SF and c- met in cancer cells, thus establishing an autocrine and paracrine system and promoting cell scatter, proliferation and invasion[37-39]. We also studied the paracrine role of HGF/SF by stimulating SMMC7721 with MHCC-1-CM. Results were cons is tent with that from experiment of autocrine. Cell scatter, proliferation and mobility in SMMC7721 increased after they were stimulated by conditioned medium of M HCC-1. Such biological activities can be blocked by anti c-met polyclonal anti body. However, the influence of SMMC-7721 proliferation by the conditioned medium of MHCC-1 happened only when the medium was in 1:2 dilution, suggesting that the c-met receptor needs a certain amount of SF/HGF to activate. Once activated, the biological activity may depend on the quantity of c-met expression and the ex tent of receptor phosphorylation, thereby initiating downstream regulations. There were two reasons why we choose c-met rather than SF/HGF to be blocked. One was the conflicting reports of SF/HGF. Up to now, various of SF/HGF variants have been found, each having different structure and bioactivity *in vitro* and/or *in vivo*. This could be another reason why different results of SF/HGF are reported[40-47]. Compared with HGF/SF, c-met introduces many biological functions, but all the signal transductions start from a same 'multifunctional docking site'[4,5,48]. The different biological functions come from different signal messages, thus making it a better choice compared with HGF/SF and other members of tyrosine kinase family. Recently, c-met inhibition has become a hot spot in anticancer research[1,2,49-51]. In our research, the tumor cells blocked by c-met antibody shrank in morphology and decreased in cell proliferation. Results suggested that the inhibition of met-SF/HGF could become one of the potential approaches to reduce tumor growth and metastasis. In conclusion, our experiment showed that the system of SF/HGF-c-met autocrine and paracrine play an important role in invasion and metastasis of hepatocellular carcinoma. Inhibition of c-met-H GF/SF system may reduce the proliferation and metastasis of hepatocellular carcino ma by lowering the expression of c-met or its downst ream signal transduction.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Expression of liver cancer associated gene HCCA3

Zheng-Xu Wang[1], Gui-Fang Hu[1], Hong-Yang Wang[2], Meng-Chao Wu[2]

[1]Department of General Surgery, Chinese PLA General Hospital of Lanzhou Military Command, Lanzhou 730050, Gansu Province, China
[2]Eastern Hepatobilliary Surgical Hospital, Second Military Medical University, Shanghai 200438, China
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Correspondence to: Dr. Zheng-Xu Wang, Department of General Surgery, Chinese PLA General Hospital of Lanzhou Military Command, No. 98, Xiaoxihu West Street, Block Qilihe, Lanzhou 730050, Gansu Province, China. zhxwang18@hotmail.com
Telephone: +86-931-8975301, Fax: +86-931-2661767
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Abstract

AIM: **To study and clone a novel liver cancer related gene, and to explore the molecular basis of liver cancer genesis.**

METHODS: **Using mRNA differential display polymerase chain reaction (DDPCR), we investigated the difference of mRNA in human hepatocellular carcinoma (HCC) and paired surrounding liver tissues, and got a gene probe. By screening a human placenta cDNA library and genomic homologous extend, we obtained a full-length cDNA named HCCA3. We analyzed the expression of this novel gene in 42 pairs of HCC and the surrounding liver tissues, and distribution in human normal tissues by means of Northern blot assay.**

RESULTS: **A full-length cDNA of liver cancer associated gene HCCA3 has been submitted to the GeneBank nucleotide sequence databases (Accession No. AF276707). The positive expression rate of this gene was 78.6% (33/42) in HCC tissues, and the clinical pathological data showed that the HCCA3 was closely associated with the invasion of tumor capsule ($P = 0.023$) and adjacant small metastasis satellite nodules lesions ($P = 0.041$). The HCCA3 was widely distributed in the human normal tissues, which was intensively expressed in lungs, brain and colon tissues, while lowly expressed in the liver tissues.**

CONCLUSION: **A novel full-length cDNA was cloned and differentiated, which was highly expressed in liver cancer tissues. The high expression was closely related to the tumor invasiveness and metastasis, that may be the late heredited change in HCC genesis.**

Subject headings Carcinoma, hepatocellular/genetics; DNA, Complementary/genetics; Liver neoplasms/genetics; RNA, messenger/genetics; Gene expression; Polymerase chain reaction

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common fatal malignant tumors in China[1-26]. According to the statistics of our country, primary liver cancer claims 20.40 lives per 100 000 people annually, with 19.98 per 100 000 in cities and 23.59 per 100 000 in rural areas, ranking as the 2nd and the 1st leading cause of cancer death, respectively. Of all the newly enrolled cases in the world each year, 45% are found in the mainland of China. In the southeast areas of high incidence, the situation is even worse with tumors tending to occur in a younger age group. The molecular events for HCC development are very complex, and HCC has proved to be genetically heterogenous neoplasm[27-30]. But to date, the identified genes have not yet fully disclosed the mechanisms of HCC[31-38]. In an attempt to identify HCC susceptible genes, differential display method was employed in this study. In the analysis of altered expression genes between HCC tissues and their nontumor counterparts, we isolated a novel gene named HCCA3 with a full length of cDNA.

MATERIALS AND METHODS

Materials

PCR polymerase is a product of Promega (Madison, USA); isotope α-^{32}P-dATP was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL); Qiaex II gel extraction kit were from Qiagen (Hilden, Germany); pGEM-T vector were from Promega; human placental cDNA library were from Clontech (USA); nitrocellulose were from Amersham Pharmacia Biotech; Prime-a-Gene Labeling System were from Promega; 14-mer anchored oligo (dT) primer (dT_{12}CA) and an arbitrary 10-base olignucleotide A2 (5'-AATCGGGCTG-3') were donated by Professor Pei.

Patients and specimens

Primary HCC and their surrounding liver tissues were obtained from 42 patients who received surgical resection at the Eastern Hepatobilliary Surgical Hospital of the Second Military Medical University, Shanghai, China. These included 41 male and 1 female patients with a median age of 49 years (range 24-72 years, mean age of 49.8 years). Thirty-five (83.3%) patients had serological evidence of hepatitis B virus infection. The serum AFP level was above 25 $\mu g \cdot L^{-1}$ in 23 cases (54.8%). The tumor size was smaller than 5cm (small HCC) in 13 patients and larger than 5 cm in 29. Histologically, 40 patients (95.2%) were complicated with cirrhosis. There were 7 well differentiated (Edmondson's grades I and II) and 35 poorly differentiated (Edmondson's grades III and IV) HCCs. Macroscopic portal vein tumor spread was found in 3 patients, and microscopic surrounding liver vascular cancer thrombi were found in 26. Gross and microscopic intrahepatic adjacent small satellite nodules lesions were found in 28, and tumor capsule invasion of liver cancer in 32. Adult normal tissues were obtained from a healthy young man who died of a traffic accident.

Methods

RNA extraction and differential display Total cytoplasmatic RNA was extracted by the acid guanidinium thiocyanate-phenol - chloroform extraction methods[39, 40]. The differential display method

was perf ormed as described previously[39-41,42]. Amplification consisted of initial den aturation at 94℃ for 4 min, followed by 40 reaction cycles (60 s at 93℃, 2 min at 40℃, and 90 s at 70℃) and a final cycle at 72℃ for 10 min. PCR fragments were then reamplified by the same primer, separated on a 16 g·L^{-1} agarose gel, purified by Qiaex II gel extraction kit, and subcloned into the pGEM -T vector using standard molecular cloning techniques.

Library screening and DNA sequencing Fragment contained in the PCR clone (length in 350 base pairs) from DDPCR served as probe to screen placental cDNA library, using the standard filter hybridization techniques described[43,44]. At the end of the third screening, we got several plagues containing the target DNA sequence and sequenced them by DNA automated sequencing system. To obtain the cDNA in a full length, genomic homologous screening was used through comparing the cDNA sequence obtained by screening the library with the NCBI GeneBank EST database. We used the PCR assay and sequencing to confirm the correct ness of the cDNA sequence.

Northern blot assay For Northern blot analysis[37,40,44], 40 μg of total RNA was denatured, loaded on a 15 g·L^{-1} agarose gel and ran at 5 V·cm^{-1} for about 3 h. The collected gels were then transferred to nitrocellulose. Hybridization of the filters was performed using specific probe of HCCA3 cDNA fragment (length in 1125 bp) obtained from screening the library. The probe was labeled with 50 μCi A-^{32}P-dATP using Prime-a-Gene labeling kit according to the given protocol. After prehybridization at 42℃ for 3 h, the membranes were hybridized in the same solution containing the labeled probe for 6 h at the same temperature, and exposed to X-ray film for 10 d at-70℃. In order to calibrate relative quantities of loaded RNAs, the blot was rehybridized with a cDNA probe of the â-Actin gene.

Statistical analysis

χ^2 test or Fisher's exact test was used to examine the differences and relationship among groups of patients classified by HCCA3 expression. Differences at $P<0.05$ were judged to be statistically significant.

RESULTS

Differential display analysis and library screening

By DDPCR, we found a differentially expressed gene fragment that exclusively present in the liver cancer lane. This fragment (length in 350 bp) was then subcloned into pGEM-T vector and served as specific probe to screen human placental cDNA library. We have o btained the gene fragment of 1125 bp in length, which shortened nearly 600 bp according to the location of Northern hybridization by screening the library. We also obtained the full-length cDNA of 1706 bp, which was in good agreement with the size of the mRNA species observed by Northern blotting through genomic h omologous extend, along with the EST sequence (GeneBank Accession No. AP001077, length in 197663 bp) of NCBI GeneBank EST databases. The sequence of 1706 bp in length was corrected by PCR assay and sequencing. It was named HCCA3 (HCC associated gene 3, also named STW-2) and submitted to EMBL/GenBank/DDBJ nucleotide sequence databases (Accession No. AF27 6707).

Sequence characteristics of the full-length HCCA3

HCCA3 contains a consensus initiation codon[45,46] at position 681 followed by a single open reading frame of 792 bp encoding 264 amino acids. The 3' untranslated region of 230 bp had a consensus polyadenylation signal (AATAAA) beginning 16 bases upstream the poly (A) tail. Alignment at nucleotide and amino acid level showed no significant homologues with known genes. The deduced protein was estimated to be 29 396 dalton and has a pI of 6.93. Amino acid sequence analysis by GCG sequence analysis software package (version 9.1, Genetics Computer Group, Madison, Wisconsin) and PC/GENE (Version 5.03, Geneva University, Switzerland) showed that there were several putative modification sites. These include two N-glycosylation sites at amino acid of 40 and 67, three N-myristoylation sites at amino acid of 183, 185 and 258, five phosphorylation sites for protein kinase: two protein kinase C (PKC) phosphorylation sites at 82 and 250, three cases in kinase II phosphorylation sites at 8, 189 and 209. No transmembrane domain and signal peptide was found. Further·more, there was no nuclear targeting sequence. The schematic presentation of HCCA3 cDNA is shown in Figure 1.

Normal tissue distribution of HCCA3 Mrna

Northern blot analysis showed that HCCA3 mRNA appears to be widely expressed in human normal tissues (Figure 2). The HCCA3 gene was particularly highly expressed in human lungs, brain and colon, moderately expressed in muscle, stomach, spleen and heart tissues, weakly expressed in small intestines, pancreas, and liver tissues. Among the tissues with positive signals, HCCA3 mRNA was observed in a transcript of approximately 1.7 kb which well corresponded to the size of the cloned cDNA.

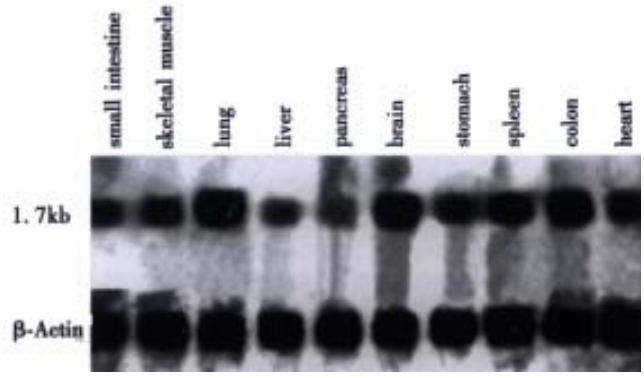

Figure 2 Northern blot analysis of HCCA3 mRNA in human adult normal tissues. Upper panel showed HCCA3 mRNA was highly expressed in human lungs, brain and colon, moderately expressed in muscle, stomach, spleen and heart tissues, weakly expressed in small intestines, pancreas, and liver tissues. Equal a mounts of total RNA loading as indicated by rehybridizing with (-actin cDNA probe (lower panel).

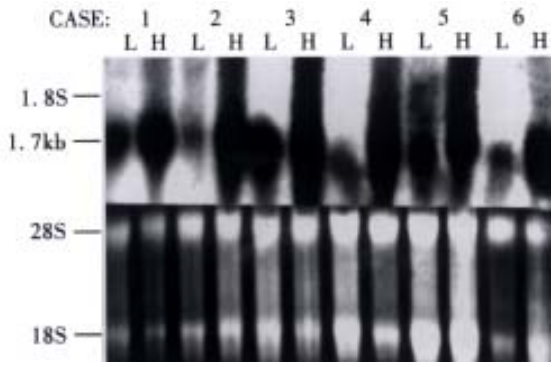

Figure 3 Differential expression of HCCA3 analyzed by Northern b lot. Upper panel shows that HCCA3 mRNA was highly expressed in HCC tissues (H), and lowly in paired surrounding liver tissues (L). A signal transcript of 1.7 kb is shown in six tumors. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading (lower panel).

HCCA3 mRNA expression in HCC and its clinical significance

HCCA3 mRNA expression was noticed in 78.6% (33/42) patients, which was intensively expressed in HCC tissues (Figure 3), while lowly expressed in the surrounding liver tissues. To investigate the potential biological role of HCCA3 in the development of HCC, we further investigated its expression in HCC with extensive samples and compared with the clinically pathological parameters. The mRNA expression level of HCCA3 was associated with the invasion of liver cancer capsule and the adjacant small satellite nodules lesions ($P< 0.05$), but not with tumor size, tumor differentiation, serum AFP, hepatitis B vrius (HBV) infection and microscopic vascular cancer cell thrombi ($P>0.05$). The results are listed in Table 1.

```
                                                                    35
GGCAGGAGGGAAAAGGTTATGGGTCGGCCCGGCGGT
CTCTCCCAGCACCCGCGACTCTCACCTTGCTCAGCTGCTGGTGGA                       80
TGAGCTGCTTCAGCTCGGAGGCTTTCTCCGGAGGCAGCGACATGC                      125
TGGCAGCCGGCGTCTCCCCGCCGCTTCTCCCCGCCTCAGATGCCC                      170
TAACTGCGCGGCCCCAGCCGGGCCAGGGAGCGTTAGGAGCGACTG                      215
GAGCACAAAGCGCCGCAGCCGTTCGCCTAGCGCAGCTCCCGGGGGG                     260
ACGCAACGCCGCGTCAGGCCGGGGGGCTGACCTGGAAATGAGGCCC                     305
CGGCGGCGGCGGCGCCAACTGTTTTCAAACAGTGGCGGACAAACA                      350
GGGCTTGGGGCTGGCCCGCACGCTGCCTGATCGTTTCCGCCCGCC                      395
GCTCCACCTCCCCGCGGGCCCCGCACCCCGAGACCTCAGCGCACC                      440
CCATCGCCTTGCTTGCCAGGGTCTCCGAAAGCGCTGCTGGCCCCT                      485
CTTCGCGGCCACCCGGCGGCCTCTTTCCGCCCTCTGAACCGGCAG                      530
TTAGCTGGCAGCCTCAAGGGCCCGGCGCCCAGGGACTCAAAGGAC                      575
CCTCCCGCGCCCCGCGAGGCTCCGGGGGTCTCGGGCTTCCGCCTTC                     620
TTGCTGCCCTCGTTCTTGCCAGGGCCGCGGTTAGTCCCTGCTGGC                      665
CACCCCACTGCGACC[ATG]TTCGTTCCCTGCGGGGAGTCGGCCCCC                    710

1                                   M   F   V   P   C   G   E   S   A   P
GACCTTGCCGGCTTCACCCTCCTAATGCCAGCAGTATCTGTTGGA                      755
11  D   L   A   G   F   T   L   L   M   P   A   V   S   V   G
AATGTTGGCCAGCTTGCAATGGATCTGATTATTTCTACACTGAAT                      800
26  N   V   G   Q   L   A   M   D   L   I   I   S   T   L   N
AATGTTGGCCAGCTTGCAATGGATCTGATTATTTCTACACTGAAT                      800
41  M   S   K   I   G   Y   F   Y   T   D   C   L   V   P   M
GTTGGAAACAATCCATATGCGACCACAGAAGGAAATTCAACAGAA                      890
56  V   G   N   N   P   Y   A   T   T   E   G   N   S   T   E
CTTAGCATAAATGCTGAAGTGTATTCATTGCCTTCAAGAAAGCTG                      935
71  L   S   I   N   A   E   V   Y   S   L   P   S   R   K   L
GTGGCTCTACAGTTAAGATCCATTTTTATTAAGTATAAATCAAAG                      980
86  V   A   L   Q   L   R   S   I   F   I   K   Y   K   S   K
CCATTCTGTGAAAAACTGCTTTCCTGGGTGAAAAGCAGTGGCTGT                     1025
101 P   F   C   E   K   L   L   S   W   V   K   S   S   G   C
GCCAGAGTCATTGTTCTTTCAAGCAGTCATTCATATCAGCGTAAT                     1070
116 A   R   V   I   V   L   S   S   S   H   S   Y   Q   R   N
GATCTGCAGCTTCGTAGTACTCCCTTCCGGTACCTACTTACACCT                     1115
131 D   L   Q   L   R   S   T   P   F   R   Y   L   L   T   P
TCCATGCAAAAAAAGTGTTCAAAATAAAATAAAGAGCCTTAAGTGG                    1160
146 S   M   Q   K   S   V   Q   N   K   I   K   S   L   N   W
GAAGAAATGGAAAAAAAGCCGGTGCATTCCTGAAATAGATGATTCC                    1205
161 E   E   M   E   K   S   R   C   I   P   E   I   D   D   S
GAGTTTTGTATCCGCATTCCGGGAGGAGGTATCACAAAAACACTC                     1250
176 E   F   C   I   R   I   P   G   G   G   I   T   K   T   L
TATGATGAAAGCTGTTCTAAAGAAATCCAAATGGCAGTTCTGCTG                     1295
191 Y   D   E   S   C   S   K   E   I   Q   M   A   V   L   L
AAATTTGTTTCAGAAGGGGACAACATCCCAGATGCATTAGGTCTT                     1340
206 K   F   V   S   E   G   D   N   I   P   D   A   L   G   L
GTTGAGTATCTTAATGAGTGGCTTCAGATACTCAAACCACTTAGC                     1385
221 V   E   Y   L   N   E   W   L   Q   I   L   K   P   L   S
GATGACCCCACAGTATCTGCCTCACGGTGGAAAATACCAAGTTCT                     1430
236 D   D   P   T   V   S   A   S   R   W   K   I   P   S   S
TGGAGATTACTCTTTGGCAGTGGTCTTCCCCCTGCACTTTTCTGA                     1475
251 W   R   L   L   F   G   S   G   L   P   P   A   L   F   *
TCTAATTTCTGTTTTATACCTTATACCCAAAAACACTTACTACCAA                    1520
CACAGCTGTTAAACATTCTATACAAAAAAAATTGTATGATCTGGTA                    1565
TTAGGAAATTACTTTCACAGTAAATATCAAAGAAAAAAAGATTAAG                    1610
GGTCTCTTTGCCATGCTTTTCATCATATGCACCAAATGTAAATTT                     1655
TGTACAATAAAAATTTTATTTCCTAAGTAAAAAAAAAAAAAAAAAAAAAA                1706
```

Figure 1 Nucleotide sequence and predicted amino acid sequence of HCCA3. HCCA3 nucleotide sequence (1706 bp) and deduced amino acid sequence (264 amino acid). The franking sequence of the predicted initiating methionine which satisfied the Kozak criterion is shown in the box. A consensus polyadenylation signal sequence (AATAAA) be ginning 16 bases upstreams the poly(A) tail shown in black line. The asterisk indicates the stop codon. EMBL / GenBank™ / DDBJ accession number is AF276707.

Table 1 Relationship between HCCA3 mRNA expression and clinic opathological features in HCC

Clinicopathology	*n*	Positive *n* (%)
Tumor size (cm)		
<5	10	6 (60.0)
>5	32	27 (84.4)
Tumor differentiation		
Well	7	4 (57.1)
Poor	35	29 (82.9)
Serum AFP ($\mu g \cdot L^{-1}$)		
⩽25	>19	15 (79.0)
>25	23	18 (78.3)
HBV infection		
Positive	35	28 (80.0)
Negative	7	5 (71.4)
Capsule invasion		
Positive	32	28 (87.5)
Negative	10	5 (50.0)[a]
Cancer thrombi		
Positive	26	21 (76.9)
Negative	16	12 (82.4)
Satellite lesions		
Positive	28	25 (89.3)
Negative	14	8 (57.1)[a]

[a]$P<0.05$, *vs* positive.

DISCUSSION

Differential display polymerase chain reaction (DDPCR) method is a useful tool for detecting and characterizing altered gene expression in eukaryotic cells[39,41,47,48]. Using this technique, we have successfully isolated a gene named HCCA3 with a transcript of 1706 bp. A consensus initia tion coden is at 681 bp and the franking sequence of the predicted initiating me thionine coincides with Kozak[45,46,49] criterion because the nucleot ides at -3, +4 site of start codon are purine, and there is one stop codon at the upstream sequence. The length of HCCA3 cDNA agrees with the size of mRNA by Northern b lot analysis. No significant homologues with known genes at nucleotide and amino acid levels were found[47-50]. No signal peptides searched by GCG and PC/GENE software were found, suggesting that HCCA3 was not a secretive protein[51,52]. This protein also has no transmembrane domain and nuclear targeting sequence indicating that it may not be located on cell member or within nucleus. The putative protein of HCCA3 revealed several phosphorylation sites for protein kinase C and casein kinase II. It is generally accepted that protein phosphorylation-deph osphorylation plays a role in the regulation of essentially all cellular functions, and there is evidence that deregulation of protein phosphorylation is involved in several human cancers[22,53,54]. HCCA2 as a possible oncoprotein may be functionally abnormal, and phosphorylative deregulation may be a mechanism. The function of HCCA3 protein may also largely depend on its phosphorylation status[22,54]. However, this needs further studies.

Studies on the expression of HCCA3 can reveal its potential biological significance[22,36,37,55]. By Northern blot analysis, we noted that HCCA3 mRNA was expressed widely in normal human tissues, indicating that HCCA3 is a normal cellular gene which may be involved in the physical process of the distributed tissues. Although HCCA3 mRNA was lowly expressed in normal liver tissues, it was significantly expressed in HCC tissues, showing that the high expression of HCCA3 might participate in the process of liver on cogenesis[36,37]. Because HCCA3 is a normal cellular gene, increased expression of HCCA3 in HCC implys the genetic abnormality and acts as an oncogene in the dev elopment of HCC. In 42 patients with HCC, HCCA3 mRNA was detected in 33 (78.6%), which was highly expressed in HCC tissues, suggesting that HCCA3 is a very common molecular event involved in the pathogenesis of HCC. These findings indicate that HCCA3 mRNA is overexpressed preferentially in HCC and can serve as a tumor biomarker for HCC[7,18,23,36,37]. The three patients with macroscopic portal vein tumor thrombi all had significantly high expression of HCCA3 mRNA, implying that HCCA3 is a late incidence in HCC carcinogenesis. Compared with pathological features,HCCA3 mRNA expression was associated with the invasion of tumor capsule and the adjacant small satellite nodules lesions ($P<0.0$ 5), indicating that HCCA3 mRNA expression is a factor of HCC invasiveness and metastasis[37,38]. Although the function of HCCA3 is still unknown, our results suggest that the up-regulation of expression of HCCA3 mRNA may play an important role in the development and/or progression of hepatocellular carcinoma[36-38,42,45]. This finding demonstrated that it is possible to identify the pr eviously unknown, differential gene expression from a small amount of clinical samples[39-42,56]. Information about such alteration of HCCA3 in gene expression could be useful in elucidating the genetic events in HCC pathogenesis, developing new diagnostic markers, or determining novel therapeutic targets.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

TECA hybrid artificial liver support system in treatment of acute liver failure

Yi-Long Xue[1], Shi-Feng Zhao[1], Yun-Luo[1], Xin-Jian Li[1], Zhong-Ping Duan[2], Xiao-Ping Chen[1], Wen-Ge Li[1], Xiao-Qiang Huang[1], Yan-Ling Li[1], Xin-Cui[1], Da-Guang Zhong[1], Zuo-Yun Zhang[1], Zhi-Qiang Huang[1]

[1]Chinese PLA General Hospital, Beijing 100853, China
[2]Beijing You-An Hospital, Beijing 100054, China
Supported by the Research Initiation Fund for Returned Students from Overseas, Ministry of Education,No. 94001
Correspondence to: Dr. Yi-Long Xue, Institute of Basic Medical Sciences, Chinese PLA General Hospital, 28 Fu Xing Road, Beijing 100853, China. xueyl@plagh.com.cn
Telephone: +86-10-66937914, Fax: +86-10-68159670
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Abstract

AIM: **To assess the efficacy and safety of TECA type hybrid artificial liver support system (TECA-HALSS) in providing liver function of detoxification, metabolism and physiology by treating the patients with acute liver failure (ALF).**

METHODS: **The porcine liver cells (1-2)×10¹⁰ were separate d from the Chinese small swine and cultured in the bioreactor of TECA-BALSS at 37.0℃ and circulated through the outer space of the hollow fiber tubes in BALSS. The six liver failure patients with various degree of hepatic coma were treated by TECA-HALSS and with conventional medicines. The venous plasma of the patients was separated by a plasma separator and treated by charcoal adsorbent or plasma exchange. The plasma circulated through the inner space of the hollow fiber tubes of BALSS and mixed with the patients' blood cells and flew back to their blood circulation. Some small molecular weight substances were exchanged between the plasma and porcine liver cells. Each treatment lasted 6.0-7.0 h. Physiological and biochemical parameters were measured before, during and after t he treatment.**

RESULTS: **The average of porcine liver cells was (1.0-3.0) ×10¹⁰ obtained from each swine liver using our modified enzymatic digestion method. The survival rate of the cells was 85%-93% by trypan blue stain and AO/PI fluorescent stain. After cultured in TECA-BALSS bioreactor for 6 h, the survival rate of cells still remained 70%-85%. At the end of TECA-HALSS treatment, the levels of plasma NH_3, ALT, TB and DB were significantly decreased. The patients who were in the state of drowsiness or coma before the treatment improved their appetite significantly and regained consciousness, some patients resumed light physical work on a short period after the treatment. One to two days after the treatment, the ratio of PTA increased warkedly. During the treatment, the heart rates, blood pressure, respiration condition and serum electrolytes (K^+, Na^+ and Cl^-) were stable without thrombosis and bleeding in all the six patients.**

CONCLUSION: **TECA-HALSS treatment could be a rapid, safe and efficacious method to provide temporary liver support for patients with ALF.**

Subject headings liver, artificial; liver failure; acute/therapy

Xue YL, Zhao SF, Luo Y, Li XJ, Duan ZP, Chen XP, Li WG, Huang XQ, Li Y L, Cui X, Zhong DG, Zhang ZY, Huang ZQ. TECA hybrid artificial liver support system in treatment of acute liver failure. *World J Gastroenterol*, 2001;7 (6):826-829

INTRODUCTION

Liver diseases are common in China[1-8]. The treatment for acute liver failure (ALF) is still a focus of research[9-15]. Some clinical reports have shown that non-biological-artificial liver support system with charcoal adsorbent or plasma exchange could improve the rehabilitation process in the patients with acute and chronic liver failure[16-25]. Our previous experiments have demonstrated that as a temporary alternative treatment, TECA type bioartificial liver support system (TECA-BALSS) using the swine liver cells was safe and effective in treating the ALF dogs induced by acetaminophen, with the injured liver cells regenerated and repaired, and a long-term survival[26-30]. In order to reduce the damage to the swine liver cells caused by the toxic substances in the ALF patients' blood and improve the efficacy of the treatment, we treated 6 patients with acute and chronic liver failure by our newly developed TECA type hybrid artificial liver support system (TECA-HALSS) using swine liver cells combined with charcoal adsorbent or plasma exchange.

MATERIALS AND METHODS

TECA-BALSS

The swines were purchased from the small swine breeding laboratory of Beijing Agricultural University. The porcine liver cells were separated by the enzyme method from Chinese experimental small swine and the survival rate of the cells was determined by trypan blue stain and AO/PI fluorescent stain. Porcine liver cells (1-2)×10¹⁰ were cultured in the T ECA-BALSS bioreactor at 37.0℃ and circulated through the outer space of the hollow fiber tubes in BALSS[31-32]. One of the femoral veins or subclavian veins of the patient was cut and a tube was inserted to establish the blood circulation pathway. The venous plasma of the patient was separated by a plasma separator and through the inner space of the hollow fiber tubes of BALSS and mixed with the patient's blood cells and flew back to their circulation.

Non-bioartificial liver support system

The plasma was treated by carbon absorption with Gambro Adsorda 300C and mixed with the patient's blood cells and flew back to their venous sys tem for 2-3 h in cases 1, 2 and 3. The patient's plasma was separated and exchanged for 2 to 3 L by PLASAUTO-IQ Plasma Exchanger (Japan) in cases 4, 5 and 6[33,34]. The heparin was administered to all the patients for anticoagulation.

TECA-HALSS

After the treatment with the non-bioartificial liver support system, the

patient's plasma was circulated through the inner space of the hollow fiber tubes in TECA-BALSS for 4-5 h.

Examination indexes

Before, during and after the treatment with BALSS, the porcine liver cells' survival rate in the cell suspension was examined by trypan blue stain and AO/PI fluorescent stain once an hour and the patient's heart rate, blood pressure and respiration condition were measured with multi-functional monitor and the blood biochemical indexes for liver function, kidney function and blood coagulation function were analyzed.

Clinical data

The general condition and the therapeutio methods for the six patients with live r failure are shown in Table 1. Among these patients, cases 1, 3 and 5 were chronic viral hepatitis, their liver function decompensated and developed liver failure; cases 2, 4 and 6 were ALF caused by partialliver excision after surgery, viral hepatitis or drug toxication, respectively. Before the treatment of TECA-HALSS, all the patients suffered from various degree of hepatic coma. They were treated by TECA-HALSS for 6-7 h, and with conventional medicines as well.

Table 1 Clinical data of six patients with liver failure

No	M/F	Age	Diagnosis	General condition	Program of treatment	*t* (treatment)/h
1	M	50	Liver cirrhosis (decomp ensation) HCC, ALF	Hepatic encephalopathy lethargy	Whole blood CA & TECA-BALSS	2.5
2	F	50	Post operation of cancerof biliary duct, ALF, ARF	Hepatic encephalopathy lethargy	Plasma CA & TECA-BALSS	2+4
3	M	32	Hepatitis B Liver failure	Hepatic encephalopathy TECA-BALSS	Plasma CA &	2+4
4	F	43	Acute viral hepatiti sfulminant hepatic failure	Hepatic encephalopathy (stage IV)	PE and TECA-BALSS	2+4
5	M	32	Hepatitis B (decompens ation) Liver failure	Hepatic encephalopathy (stage IV)	PE and TECA-BALSS	2+5
6	F	34	Drug induced hepatic injury, liver failure	Hepatic encephalopathy lethargy	PE and TECA-BALSS	2+5

RESULTS

The swine liver cells obtained and cultured

The obtained average of porcine liver cells was (1.0-3.0)×10^{10} from each swine liver by our modified enzymatic separation method. The survival rate of the cells was 85%-93% by trypan blue stain and AO/PI fluorescent stain. After cultured in TECA-BALSS bioreactor for 6 h, the survival rate of cells still remained 70%-85%.

Changes in basic physiological indexes

During the TECA-HALSS treatment, heart rates, blood pressure and respiration condition in all the six patients remained stable without thrombosis and bleeding. Those who were in the state of drowsiness or coma before the treatment improved their appetite significantly and regained consciousness, some patients resumed light physical work in a short period after the treatment.

Changes in biochemical indexes of blood

At the end of the treatment with HALSS, the patients' liver function related biochemical indexes, such as the levels of NH 3, ALT, TB and DB were significantly decreased. Blood coagulation was improved, the PT was shortened and PTA was raised. There were no significant changes in the levels of the patients' main serum electrolytes (K^+, Na^+ and Cl^-) during the treatment.

Typical cases

Case 2 was a patient with ALF complicated with acute kidney failure after left half liver excision. After twice blood dialysis, the patient's renal function was improved temporarily, but she was in the state of hepatic coma with drowsiness. At the 7th day after operation, she received the treatment of TECA-HALSS with plasma-carbon absorption for 2h and plasma-BALSS treatment for 4h. The patient's blood ammonia level was returned to normal and she regained consciousness (Table 2). Two days after the treatment of HALSS, she had normal liver function and receive d blood dialysis for the renal disfunction. Because of economic reasons, she was discharged from the hospital voluntarily.

Table 2 Changes of pre- and post-treatment by TECA-HALSS in case 2

Parameters	Pre-HALSS	Post CA	4 h post-BALSS	2 d post-HALSS
NH_3(μg/L)	134	93	30	53
ALT (IU/L)	64	27	29	53
AST (IU/L)	69	47	269	97
TB (μmol/L)	495	423	400	405
DB (μmol/L)	240	204	198	350
UN (mmol/L)	41	35.7	36.6	37
Cr (μmol/L)	651	121	407	
K^+(mmol/L)	4.99	4.66	4.47	4.9
Na^+(mmol/L)	132	131	133	131
Cl^-(mmol/L)	99.6	104	104	96
Mentality	Lethargy	Lethargy	Consciousness	Consciousness

Case 4 was a patient with acute severe viral hepatitis complicated with fulminant liver failure and stage IV hepatic coma and PTA 13%. The liver was found shrink and diffused liver damage by ultrasound B examination. After coma for three days, she received T ECA-HALSS with 2.5 L plasma exchanged and 4 h of BALSS treatment. After the treatment, the levels of ALT, TB, DB and ALP were significantly decreased and PTA value increased rapidly (Table 3). The patient experienced superficial coma one day and regained consciousness and could eat food two days after treatment. Five days later, her abilities of calculation and orientation became normal and eight days later, she was discharged from the hospital. Two months' follow up showed that her general condition was good and she could do some light physical work, his liver function parameters were within normal range.

Table 3 Changes pre- and post-treatment by TECA-HALSS in case 4

Pre-HALSS		HALSS			Post-HALSS			
		Post-PE	BALSS 2 h	BALSS 4 h	d 1	d 2	d 4	d 7
NH3 (μg/L)	78	114	101	108				
ALT (IU/L)	1352	408	390	336			225	146
AST (IU/L)	142	45	629	751			45	63
TB (μmol/L)	17.11	7.23	9.99	9.22			16.95	29.18
DB (μmol/L)	12.03	5.08	6.25	5.72			12.74	21.26
TP (g/L)	69.8	54.5	48.1	43.5			48	56.2
ALB (g/L)	34.29	31.8	28.5	25.6			23.3	24.6
ALP (IU/L)	297	144	140	115			176	213
PTA (%)	13.15				21.6	29.6		48.8
Mentality	Deep coma	Deep coma	Deep coma	Deep coma	Superficial coma	Consciousness		

DISCUSSION

It is well known that ALF has a very high morbidity and mortality rate. The conventional medical treatment was hard to achieve satisfied outcomes since liver cells possess the strong ability of regeneration. Therefore, if a full liver support therapy can be provided to keep the patients alive and avoid severe complications to occur, the patients' liver function can recover spontaneously or win the time for liver transplantation. The research about using artificial means to temporarily support the liver function has attracted worldwide attention. Many years of research has been carried out on non-bioartificial liver support systems, which detoxicate nonspecifically or specifically by using absorption, plasma dialysis, blood or plasma exchange and so on. In this way, it can eliminate the possible toxic substances in the blood so as to provide a chance for liver cells to regenerate and repair. But some reports indicated that these methods did not work well in treating liver failure. For example, carbon absorption can only nonspecifically detoxicate part of the toxic substances in the blood and can not greatly increase the survival rate of patients with liver failure. Although replacing a large quantity of patients' plasma (3-4L) within a short time can correct one third of the biochemical indexes related to liver function, this effect can only last 1-3 days and the patients' mental malfunction can not be improved significantly[35-37]. Case 3, received 8 times of plasma exchange. Each time after plasma exchange, the patient was still listless and drowsy, his biochemical indexes of liver function were corrected by only one third to one fourth, and deteriorated again within 1-3 days. At the end of TECA-HALSS treatment, the patient turned from drowsy to conscious, he also asked for food and walked out of the treatment room without help. The patient's blood parameters of liver function remained normal for almost 20 days. Therefore, it is believed that non-bioartificial liver support system could not be enough for substituting the complicated function of liver. In recent years, newly developed bioartificial liver support systems use exnogenous liver cells to provide the functions of biosynthesis, detoxication and biotransformation. Our previously developed TECA-BALSS has been proved to be safe and effective as a temporary replacement of liver function in the treatment of ALF in dogs caused by acetaminophen[26]. The porcine liver cells cultured in TECA-BALSS possess liver cell functions, such as biosynthesis, detoxication and biotransformation. The ALF patient's blood circulated through BALSS and reacted with the porcine liver cells through the semipermeable membrane of the hollow fiber tubes in BALSS[31,38-39]. Our and other studies showed that porcine liver cells in BALSS, functioning as a temporary replacement of liver, could win a period of time for the patients or animals with liver failure to regenerate and repair their liver[29,40]. Our research found that t he toxic substances of the ALF patients' blood can damage the porcine liver cells[41]. In this experiment, we used non-bioartificial liver methods (carbon absorption and plasma exchange) for reducing the toxic substances first and later used BALSS to exert biological function of liver cells, i.e., TECA-HALSS. The results from 6 cases of acute and chronic liver failure showed that TECA-HALSS could significantly improve the liver function by lowering the levels of blood NH 3, ALT, TB and DB and increasing PTA. Our preliminary result indicated that BALSS could significantly improve the patient's consciousness and the effect persisted longer than by plasma exchange.

According to the reports both domestic and overseas, the major use of HALSS is for the patients with ALF caused by various reasons, such as viruses, drugs and ischemia[42-45]. Through temporary liver function substitution, HALSS treatment wins the time for liver cells to regenerate and repair and compensatie liver function. However, for the patients with chronic liver failure, the main propose of HALSS treatment is to provide the bridge to liver transplantation, especially for the patients with hepatic coma staged III-IV[30,46-47]. BALSS treatment can be carried out in two ways: blood perfusion and plasma perfusion. Our results suggested that it preventing thrombosis by the way of plasma infusion is favored in HALSS. This can also reduce the dosage of heparin, which is very important to the patients with liver failure complicated with coagulation malfunction. The times and duration of HALSS treatment depend on the patients' biochemical indexes of liver function, mental consciousness and so on. It was reported that some patients received HALSS treatment as many as over 10 times[48-50]. The results from our experiment and others indicated that HALSS treatment is safe and practical. During the treatment, to monitor the basic physiological indexes of the patients and supplement blood instantly are suggested. Other authors found that after the treatment with HALSS, there were no significant immune reactions and no negative effect on the following liver transplantation. No immune factors directly influence the patients' prognoses. The results suggested that TECA-HALSS could be a rapid, safe and efficacious method to provide temporary liver support for the patients with ALF.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Chiral metabolism of propafenone in rat hepatic microsomes treated with two inducers

Quan Zhou[1,2], Tong-Wei Yao[1], Su Zeng[1]

[1]College of Pharmaceutical Sciences, [2]Second Hospital of Medical School, Zhejiang University, Hangzhou 310031, Zhejiang Province, China

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Correspondence to: Prof. Su Zeng, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310031, China. zengsu@zjuem.zjuen.edu.cn
Telephone: +86-571-87217060, Fax: +86-571-87217086.

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Abstract

AIM: **To study the influence of inducers of drug metabolism enzyme, β-naphthoflavone (BNF) and dexamethasone (DEX), on the stereoselective metabolism of propafenone in the rat hepatic microsomes.**

METHODS: **Phase I metabolism of propafenone was studied using the microsomes induced by BNF and DEX and the non-induced microsome was used as the control. The enzymatic kinetics parameters of propafenone enantiomers were calculated by regress analysis of Eadie-Hofstee Plots. Propafenone enantiomer concentrations were assayed by a chiral HPLC.**

RESULTS: **The metabolite of propafenone, N-desalkylpropafenone, was found after incubation of propafenone with the rat hepatic microsomes induced by BNF and DEX. In these two groups, the stereoselectivity favoring R(-) isomer was observed in metabolism at low substrate concentrations of racemic propafenone, but lost the stereoselectivity at high substrate concentrations. However, in control group, no stereoselectivity was observed. The enzyme kinetic parameters were: ①K_m. Control group: R(-) 83±6, S (+) 94±7; BNF group: R(-) 105±6, S(+)128±14; DEX group: R(-) 86±11, S(+) 118±16; ② υ_{max}. Control group: R(-) 0.75±0.16, S(+) 0.72±0.07; BNF group: R (-)1.04±0.15, S (+)1.0 7±14; DEX group: R(-) 0.93± 0.06, S(+) 1.04±0.09; ③ Cl_{int}. Control group: R(-) 8.9± 1.1, S(+) 7.6±0.7; BNF group: R(-)9.9±0.9, S(+)8.3± 0.7; DEX group: R(-) 10.9±0.8, S(+) 8.9±0.9. The enantiomeric differences in K_m and Cl_{int} were both significant, but not in υ_{max}, in BNF and DEX group. Whereas enanti omeric differences in three parameters were all insignificant in control group. Furthermore, K_m and υ_{max} were both significantly less than those in BNF or DEX group. In the rat liver microsome in duced by DEX, nimodipine (NDP) decreased the stereoselectivity in propafenone metabolism at low substrate concentration. The inhibition of NDP on the metabolism of propafenone was stereo selective with R(-)-isomer being impaired more than S(+)-isomer. The inhibition constant (Ki) of S(+)- and R(-)-propafenone, calculated from Dixon plots, was 15.4 and 8.6 mg·L^{-1}, respectively.**

CONCLUSION: ***CYP*1A subfamily (induced by BNF) and CYP3A4 (induced by DEX) have pronounced contribution to propafenone N-desalkylation which exhibited stereose lectivity depending on substrate concentration. The molecular base for this phenomenon is the stereo selectivity in affinity of substrate to the enzyme activity centers instead of at the catalyzing sites.**

Subject headings propafenone/metabolism; mitochondria; liver; rat; optical rotation

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INTRODUCTION

Propafenone, is a widely used antiarrhythmic agent administered as the racemic mixture of R(-) and S(+) enantiomers. The two enantiomers are equipotent in terms of sodium channel-blocking activity, but the main side effect, ie., β-adrenoreceptor-blocking action resides in the S (+)-isomer[1], and, therefore, information on stereoselective disposition of the racemate is of clinical relevance.

The main metabolic pathways of propafenone in vivo and *in vitro* involve CYP1A2 and CYP3A4 mediated N-desalkylation, CYP2D6 mediated 5-hydro xylation and UDPGT mediated glucuronidation[2-6]. N-desalkylpropafenone has the same electrophysi ological potency as 5-hydroxypropafenone and propafenone, and the plasma concentrations of N-desalkyl propafenone are similar to those of 5-hydroxypropafenone during chronic administration in human, therefore, N-desalkylpropafenone contributes to the antiarrhythmic effects of propafenone, especially in patients with poor metabolizer phenotype of CYP2D6[7-8]. Although stereoselectivities in 5-hydroxylation and glucuronidation *in vitro* have been reported[9-11], whether N-desalkylation exhibits stereoselectivity has not been addressed. Meanwhile, rat liver microsomes pretreated by specific inducers provide sound models to study metabolism *in vitro*[12-16]. Considering that β-nap hthoflavone (BNF) was a typical inducer of CYP1A subfamily and dexamethasone (DEX) was a typical inducer of CYP3A4[17-21], this experiment studied the stereoselective propafenone N-desalkylation in rat hepatic microsomes induced by BNF and DEX.

Material AND METHODS

Chemicals and solutions

Dexamethasone (DEX), β-naphthoflavone (BNF), 7-ethoxyresorufin (ER), triacety loleandomycin (TAO), NADPH, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocynate (GITC), (R,S)-propafenone, R(-) and S(+)-propafenone were supplied by Sigma Chemical Co (St. Louis, MO, US A). N-desalkylpropafenone was a generous gift from Prof. Tang YN (Xinhua Hospital, Shanghai). All other chemicals were obtained from the common commercial sources. Stock buffer (pH7.4): 1 mol·L^{-1} pH7.4 Tris-HCl buffer 25 mL, 1 mol·L^{-1} KCl 75 mL and 1 mol·L^{-1} $MgCl_2$ 5 mL were mixed and diluted with water to 500 mL. NADPH solution: dissolve NADPH in ice-cold 10 g·L^{-1} NaH CO_3

solution to the desired concentration of 25 mmol·L^{-1}. The solution should be freshly prepared just before the incubation.

Preparation of hepatic microsomes

Sprague-Dawley rats (male, 170-210 g) were divided into three groups. One group received three daily intraperitoned injection of 80 mg·kg^{-1} BNF (dissolved in oil); the second group received three daily DEX (132 mg·kg^{-1}·d^{-1}, ig) and the third group was used as the non-treated control. About 24 h after the last treatment and with no food supplied for 16 h before taking the livers, the rats were sacrificed by decapitation. Liver samples were excised and perfused by the ice-cold physiological saline to remove blood and homogenized in ice-cold Tris buffer. Hepatic microsomes were prepared with the ultracentrifugation methods[22,23]. All manipulations were carried out in cold bath. Pellets were re-suspended in sucrose-Tris buffer (pH 7.4)(95:5, mass to volume ratio) and immediately stored at -30℃. Protein and cy tochrome P450 contents were estimated according to the methods of Zeng *et al*[24]and Omura *et al*[25],respectively. Enzymatic activity of CYP1A was measured according to the method of Klotz *et al*[26], and expressed as initial velocity of O-deethylation of 7-ethoxyresorufin (activity of EROD). Enzymatic activity of CYP3A4 was determined according to the method of Wrighton *et al*[27], and expressed as the extent of P450-MI complex (absorbance difference per gram of protein between 456 nm and 510 nm) using triacetyloleandomycin as substrate. Incubation of propafenone with rat hepatic microsomes The incubation mixture contained microsomal protein (1.6 g·L^{-1}), stock buffer (pH 7.4) bubbled with oxygen for 1 min and racemic propafenone as substrate. After 5 min preincubation, reaction was started by adding 10 μL NADPH solution. The final volume was 250 μL. For kinetic experiments, racemic propafenone was used at concentrations of 10, 20, 40, 80, 160, and 320 mg·L^{-1} and the incubation time was 30 min. For the time dependent experiments, the substrate concentration used was 10 mg·L^{-1}. For inhibition experiments, nimodipine was used as inhibitors (at 0, 8, 16, 32 mg·L^{-1}) and incubated simultaneously with racemic propafenone (50, 100 mg·L^{-1}). After the indicated time, the reaction was terminated by adding 750 μL chlorform. The mixture was votexed for 3 min, then centrifuged at 2 000 g for 10 min. The organic layer was transferred to a clean tube and evaporated to dryness under a gentle stream of air. GITC solution (in acetonitrile) and methanol containing 14 g·L^{-1} triethamine were added and the tube was capped and allowed to react for 30 min a t 35℃. After evaporation of organic solvents, the residues were reconstutited with 100 μL methanol, and 20 μL was injected into HPLC system.

HPLC procedure for determining propafenone enantiomer in the rat hepatic incubates

Enantiomers of propafenone were quantitated with an HPLC system with UV detection (λ = 254 nm)[28]. A 5-ìm reverse phase column (Shimpack CLC- ODS 15 cm×4.6 mm) was used with a flow rate of 0.8 mL/min. The mobile phase was a mixture of methanol -water-glacial acetic acid (67:33:0.05).

Statistical analysis

The maximum velocity (υ_{max}) and Michaelis-Menten constant (K_m) values for propafenone enantiomer were determi ned by regress analysis of Edie-Hofstee plots. The $\bar{x}\pm s$ of three determinations of υ_{max} and K_m was calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of υ_{max}/K_m. All statistical difference was tested by unpaired *t* test.

RESULTS

A baseline separation between the diastereomers of S(+) and R(-)-propafenone was achieved, with the retention time being 23 min and 28 min, respectively. The HPLC system also allowed monitoring the formation of N-desalkylpropafenone. The retention time was 8 min and 10 min for diastereomer of N-desalkylpropafenone, respectively. The amount of diastereomers of N-desalkylpropafenone were increasing while those of propafenone were decreasing during 30 min incubation with the rat hepatic microsomes induced by DEX and BNF. Typical chromatograms were showed in Figure 1. Quantitation was performed by external standardization. Calibration curves were linear at a range of 0.5 to 320 mg·L^{-1} for each enantiomer of propapfenone. The LOQ was 0.5 mg·L^{-1} (S/N = 10, *n* = 5) for each enantiomer. The inter-assay and intra-assay variability averaged 8.5% for both enantiomers. The method recovery averaged 77.1% for both enantiomers.

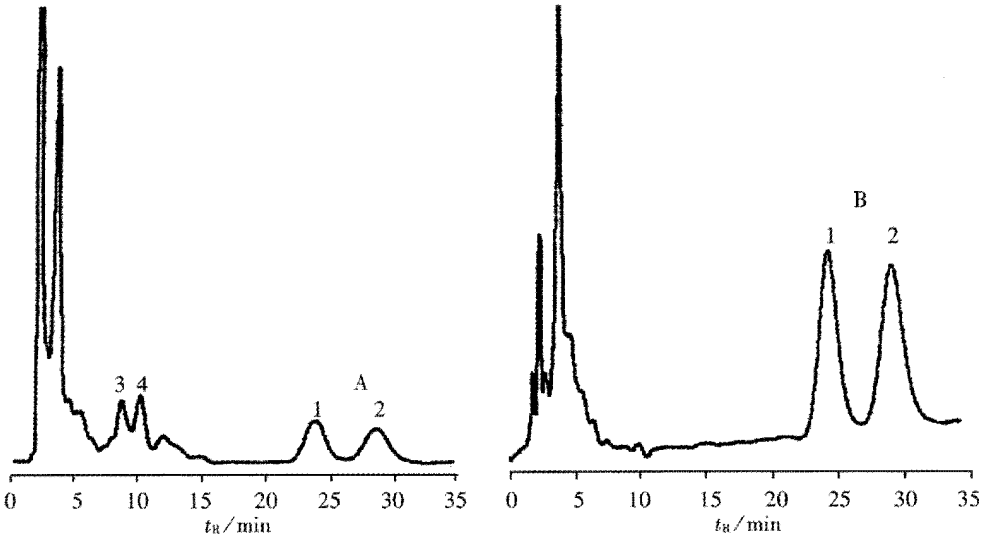

Figure 1 Chiral high performance liquid chromatogram of racemic propafenone in rat liver microsomal incubates after 30 min incubation. A:BNF pre-treated B:without incubation.

Peaks 1,2: Diastereomers of S(+)-propafenone and R(-)-propafenone; Peaks 3,4: Dia stereomers of metabolite (N-desalkylpropafenone)

Induction of rat hepatic metabolizingenzymes

In DEX group, the extent of P450-MI complex (an indicator of activity of CYP3A4) was significantly more than the control or BNF group (P<0.001, Table 1). In BNF group, the initial velocity of deethylation of 7-ethoxyresorufin (an indicator of activity of CY P1A) was significantly more than in the control or DEX group (about 20-fold, P<0.001). Therefore, CYP1A subfamily was successful inducted by BNF and CYP3A4 by DEX, which provided sound enzymatic sources for getting information on CYP1A and CYP3A4 mediated N-desalkylation of propafenone.

Table 1 The amount and activity of P450 in rat liver microsomes ($\bar{x}$±s, n = 3)

Pretreat	P450 in pro /μmol·g^{-1}	Extent of P450-MI complex△A	Activity of EROD /μmol·min^{-1}μg^{-1}
Control	0.95±0.15	0.5±0.2	0.22±0.04
BNF	1.42±0.21	2.2±0.4	3.87±0.20[b]
Dex	1.11±0.17	18.3±3.6[a]	0.18±0.02

[a]P<0.01,*vs* BNF or control, [b]P<0.01,*vs* Dex or control.

Impact of substrate concentration on stereoselective metabolism of propafenone

At 10 mg·L^{-1} concentration of racemic propafenone, stereoselectivity was observed in DEX and BNF group, but not in control group (Table 2). The depletion of R(-)-isomer was faster than that of S(+)-isomer. However, with the substrate concentration increasing, S/R ratios of prop afenone were not altered in control group (P>0.05), but in DEX and BNF group S/R ratios were decreasing from 1.18 to 1.00 (P <0.01), and 1.10 to 1.00 (P<0 .01), respectively.

Table 2 Ratio of S(+)/R(-) propafenone at different concentrations in rat liver microsomal incubates ($\bar{x}$±s, n = 3)

Enantiomer / mg·L^{-1}	Pretreat		
	Control	Dex	BNF
5	1.016±0.016	1.177±0.062[a,b]	1.104±0.019[a,b]
10	1.029±0.012	1.103±0.057	1.069±0.015
20	0.995±0.016	1.088±0.018	1.053±0.002
40	0.974±0.026	1.057±0.030	1.043±0.000
80	0.978±0.024	1.019±0.017	1.027±0.005
160	0.988±0.012	1.003±0.019	1.005±0.005

[a] P<0.01, *vs* control; [b]P<0.01,*vs* 160 mg·L^{-1}.

Concentration-time curves and ratio of S(+)/R(-) propafenone concentration

The ratio of S/R was in unity in control group from the incubation time of 0 to 30 min, whereas in DEX or BNF group, the ratio of S/R increased and was significantly different with the corresponding ratio in control group at 8 and 30 min (P<0.01, 0.05, Table 3). Moreover, the ratio of S/R in DEX group at incubation time of 30 min was significantly higher than that in BNF.

Table 3 Ratio of S(+)/R(-) propafenone concentration in rat liver microsomal incubates ($\bar{x}$±s, n = 3)

Group	t (incubation)/min				
	0	3	8	20	30 (min)
Control	1.000	1.017±0.010	0.997±0.016	1.006±0.012	1.016±0.016
Dex	1.000	1.007±0.003	1.044±0.011[d]	1.076±0.019	1.170±0.050[a,b,c]
BNF	1.000	1.005±0.002	1.031±0.012[d]	1.068±0.023	1.094±0.017[a,c]

[a]P<0.01, *vs* 8 min; [b]P<0.05, *vs* BNF group; [c]P<0.01, [d]P<0.05, *vs* control.

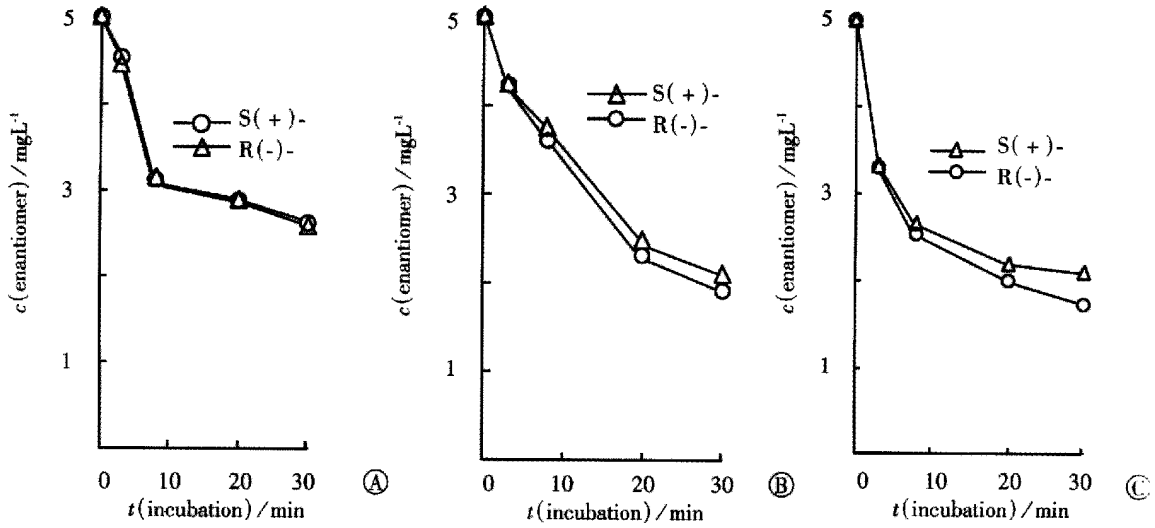

Figure 2 Concentration-time curves for S(+)- and R(-)-propa fenone metabolism in rat hepatic microsomes. A: Control; B: BNF; C: DEX.

Enzymatic kinetic parameters for propafenone metabolism in hepatic microsomes

Depletion of propafenone could be described by Michaelic-Menten kinetics. K_m had no statistical difference between the two enantiomers in control microsomes, whereas the enantiomeric difference in K_m was significant in the microsomes induced with DEX or BNF (S>R, P< 0.05, Table 4). There was signific ant difference for Clint between the two enantiomers (S<R, P<0.05, Table 4) in DEX or BNF group,

but not in control group. The K_m of S(+)-isomer in DEX, or S(+)- or R(-)-isomer in BNF group was significantly higher than the corresponding enantiomer in control group (P<0.05, 0.01, Table 4). The υ_{max} of S(+)-isomer in DEX group, or S(+)- or R(-)-isomer in BNF group, was significantly higher than the corresponding enantiomers in the control group (P<0.05, 0.01, Table 4). Difference for Clint between the two enantiomers in DEX or BNF group and the corresponding enantiomer in control group was insignificant. Moreover, the K_m of R(-)-propafenone in DEX group was significantly lower than that in BNF group (P <0.05, Table 4).

Table 4 Enzymatic parameters in propafenone enantiomer metabolism *in vitro* ($\bar{x}$±s, n = 3)

Pretreat	Enantiomer	K_m /μmol·L^{-1}	υ_{max} /μmol·g^{-1}·min^{-1}	Cl_{int} in prot /Lμmin^{-1}·g^{-1}
Control	S(+)	94±7	0.72±0.07	7.6±0.7
	R(-)	83±6	0.75±0.16	8.9±1.1
Dex	S(+)	118±16[a,b]	1.04±0.09[c]	8.9±0.9[a]
	R(-)	86±11[d]	0.93±0.06	10.9±0.8[b]
BNF	S(+)	128±14[a,c]	1.07±0.20[b]	8.3±0.7[a]
	R(-)	105±6[c]	1.04±0.15[b]	9.9±0.9

[a]P<0.05, *vs* R(-)-propafenone; [b]P<0.05, [c]P<0.01, *vs* corresponding enantiomer in control; [d]P<0.05, *vs* R(-)-isomer in BNF.

Stereoselective inhibition of propafenone metabolism by nimodipine

*K*i for S(+)- and R(-)-propafenone was 15.4 and 8.6 mg·L^{-1}, respectively, which suggested that nimodipine (specific substrate of C YP3A4) inhibited metabolism of propafenone enantiomer stereoselectively (Figure 3). With nimodipine amount increasing, the depletion of propafenone enantiomers and the S/R ratio of the remaining amount o f propafenone enantiomer were decreasing (Table 5).

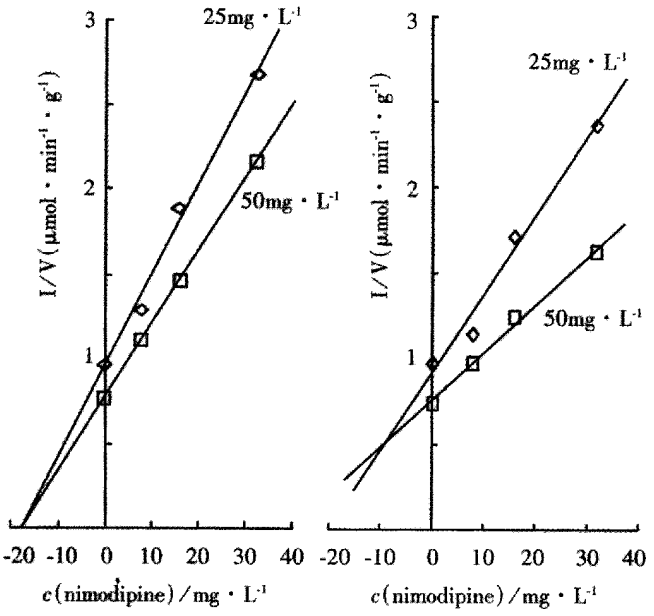

Figure 3 Dixon plot for S(+)-propafenone (Left) and R(-)-pro pa fenone (Right) wit nimodipine as inhibitor at three concentration. Ki for S(+)-and R(-)-PPF was 15.4, 8 mg·L^{-1}, respectively. Each data point represents the mean of duplicate determinations.

Table 5 The stereoselective effects of nimodipine on metabolic depletion of propafenone ($\bar{x}$±s, n = 3)

Group	Nimodipine /mg·L^{-1}	S(+)-propafenone	R(-)-propafenone /mg·L^{-1}	S/R
DEX	0	2.10±0.04	1.75±0.14[a]	1.20
DEX	8	2.32±0.26	2.10±0.21[b]	1.1
0DEX	16	3.81±0.11[c]	3.62±0.13[c]	1.0
6DEX	32	4.30±0.13[c]	4.17±0.26[c]	1.03

[a]P<0.01,*vs* S(+)-propafenone in DEX without nimodipine; [b]P<0.05, [c]P< 0.001, *vs* the corresponding enantiomer in DEX without nimodipine.

DISSCUSSION

Due to the capabilities of highly efficient separation and sensitive determination of enantiomers in microsome incubates, chiral chromatography is extremely valuable to study stereoselectivity of racemate met abolism[29-34]. So far as we are aware, we took the lead in acquiring the information on stereoc hemistry of propafenone metabolism by chiral HPLC method.

Previously, we observed that the glucuronidation of propranolol in rat hepatic microsome has stereoselectivity of S(-)-propranolol, and that the induction of phenobarbital reduced this stereoselectivity[35]. The phase I metabolic stereoselectivity of propranolol was reversed by the induction of BNF and increased by the induction of phenobarbital[36]. Phenobarbital instead of BNF inducted the stereoselective difference of Cl_{int} in glucuroniodation of ofloxacin[37]. However, the induction of DEX or BNF in this study vested propafenone metabolism with stereoselectivity in rat hepatic microsomes. It is thus clear that different inducers may have different impacts on some racemate metabolism.

The enantiomers of a racemic drug may differ in metabolic behavior as a consequence of stereoselective interaction with hepatic microsomes[38-42]. The underlying mechanism of stereoselectivity in metabolism, as many studies have shown, was enantiomeric difference in υ_{max} (an indice of enzymatic catalyzing ability) and/or in K_m (an index of enzyme affinity to the substrate). For example, the stereoselective N-demethylation of chlorpheniramine was due to enantiomeric differences in K_m[43]. Whereas there were little or no difference in K_m of the enantiomers of ofloxacin, the stereoselectivities in glucuronidation were caused by enantiomeric differences in υ_{max}[44]. The υ_{max} of the O-demethylation of (-)-tramadol was 1.6 times that of (+)-isomer, but the Km for both enantiomers was same, thus resulted in its stereos elective O-demethylation[45]. Recently, we have also proved that stereoselectivity of propranolol cytochrome P450 metabolism in the rat hepatic microsomes was due to the stereoselectivity of the catalyzing function in enzyme[35]. In this *in vitro* study, stereoselectivity of propafenone occurred in K_m and Clint in the rat hepatic microsomes induced by DEX or BNF, but not in υ_{max}. Combining with the interesting results of Table 2 that stereoselectivity depends on substrate conce ntration, we suppose that stereoselectivity at low substrate concentration was mainly due to the enantiomeric difference of the enzyme affinity to the substrate, and that insignificant enantiomeric difference in catalyzing abilities resulted in the abolished stereoselectivity at high subs trate concentration. Fujita *et al*[46] also reported that stereoselectivity of propranolol in rat liver microsomes was sometimes altered when the substrate concentration was varied. Augustijns *et al*[38] observed that the enantiomeric ratio (R/S) of desethylchloroquine was dependent on concentrati on, and ranged from 8 at 1 microM to 1 at 300 microM. Mutual enantiomer-enantiomer interacti on studies at low concentration (1-5 microM) revealed that the formation of (R)-desethylchlor oquine was strongly inhibited by (S)-chloroquine. In this *in vitro* metabolism,

enantiomer-enantiomer interaction at enzyme activity centers may also exist at low concentration, resulting in enantiomeric difference of the enzyme affinity to the substrate. This needs to be addressed by additional experiments.

Table 3 indicated that the stereoselectivity in DEX was stronger than in BNF. It maybe explained by the difference in K_m of R(-)-propafenone between DEX and BNF group and that the affinity of R (-)-PPF with CYP3A4 was higher than that with CYP1A, and that of S(+)-PPF with CYP3A4 was similar with CYP1A. Table 1 showed that CYP1A and CYP3A4 were significantly inducted by BNF and DEX, respectively, and this agreed with the well known documents. In BNF or DEX group, the υ_{max} was also significantly higher than that in the control group (about 1.5-fold), which indicated that CY P1A and CYP3A4 contributed to the metabolism of propafenone. This substantiated the methods used by Botsch *et al*[47]. In their study, CYP1A2 and CYP3A4 were identified involved in N-desalkylation using specific antibodies and inhibitors and stably expressed cytochrome P450. K_m in the control group was significantly lower than that in DEX or BNF group, which indicated that other enzyme with high affinity to substrate involved in metabolism of propafenone. CYP2D6 which had very low value of K_m might be one of such enzymes. Due to the lower value of both K_m and υ_{max} in control group, the Clint of propafenone enantiomer was not different from that in DEX or BNF group.

The competitive inhibition model (propafenone/nimodipine) suggested that propafenone and nimodipine were both substrates of the same coenzyme. Because nimodipine was as pecific substrate of CYP3A4[48,49], the results of inhibition experiment also proved that CYP3A4 contributed to propafenone metabolism. Drug interaction of enantiomer with specific inhibitor of P450 is an important tool in the search for detailed information on the stereoselective metabolism of xenobiotics[1]. Because fluoxetine impeded in vivo met abolism of R-methadone more than that of S-methadone, Eap *et al*[50] conclud ed that CYP2D6-mediated methadone metabolism exhibited stereoselectivity. The fact that the AUC ratio for the two enantiomers of reboxetine was minimally affected by ketoconazole treatment indicates similar affinities of the enantiomers for CYP3A4[51]. In the present study, the phenomenon that nimodipine in hibited S(+)-propafenone more than R(-)-isomer also implies that CYP3A4-mediated propafenone metabolism existed stereoselectivity.

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Edited by Xu JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Expression of lipopolysaccharide binding protein and its receptor CD14 in experimental alcoholic liver disease

Guo-Qing Zuo[1], Jian-Ping Gong[2], Chang-An Liu[2], Shen-Wei Li[2], Xin-Chuan Wu[2], Kang Yang[2], Yue Li[2]

[1]Department of Digestive Disease, [2]Department of General Surgery, Second College of Clinical Medicine & the Second Affiliated Hospital, Chongqing Un iversity of Medical Sciences, Chongqing 400010, China
Supported by the National Natural Science Foundation of China (No. 39970719).
Correspondence to: Guo-Qing Zuo, Department of Digestive Disease, Second College of Clinical Medicine & the Second Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400010, China. zgqly@public.cta.cq.cn
Telephone: +86-23-62764578, Fax: +86-23-63822815
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Abstract

AIM: **To evaluate the relationship between the expression of lipopolysaccharides (LPS) binding protein (LBP) and CD14 mRNA and the severity of liver injury in alcohol-fed rats.**

METHODS: **Twenty Wistar rats were divided into two groups: ethanol-fed group (group E) and control group (group C). Group E was fed with ethanol (5-12 g·kg^{-1}·d^{-1}) and group C received d extrose instead of ethanol. Rats of the two groups were sacrificed at 4 weeks and 8 weeks. Levels of endotoxin and alanine transaminase (ALT) in blood were measured, and liver pathology was observed under light and electronic microscopy. Expressions of LBP and CD14 mRNA in liver tissues were determined by RT-PCR analysis.**

RESULTS: **Plasma endotoxin levels were increased more significantly in group E (129±21) ng·L^{-1} and (187±35) ng·L^{-1} at 4 and 8 wk than in control rats (48±9) ng·L^{-1} and (53±11) ng·L^{-1}, respectively (P<0.05). Mean values of plasma ALT levels were (1867±250) nkat·L^{-1} and (2450±367) nkat·L^{-1} in Group E. The values were increased more dramatically in ethanol-fed rats than in Group C after 4 and 8 weeks. In liver section from ethanol-fed rats, there were marked pathological changes (steatosis, cell infiltration and necrosis). In ethanol-fed rats, ethanol administration led to a significant increase in LBP and CD14 mRNA levels compared with the control group (P<0.05).**

CONCLUSION: **Ethanol administration led to a significant increase in endotoxin levels in serum and LBP and CD14 mRNA expressions in liver tissues. The increase of LBP and CD14 mRNA expression might wake the liver more sensitive to endotoxin and liver injury.**

Subject heading lipopolysaccharides/analysis, antigens, cd14/analysis, liver diseases, alcoholic/pathology, liver/pathology, liver/ultrastr ucture, rat, animal

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INTRODUCTION

There is an accumulating evidence suggesting a role for endotoxin or lipopoly-sac charide(LPS) in the cause of alcohol-induced liver disease (ALD)[1-5]. Circulating LPS consists of several compounds including its specific carrier, the LPS binding protein (LBP)[6-8]. The LPS-LBP complex has high affinity to the LPS receptor CD14 located on monocytes/macrophages. CD14 is a 55-kD myeloid membrane glycoprotein, expressed mainly by monocytes and macrophages[7,9-11]. Attachment of LPS-LBP complex to the CD14 initiates a process lead in g to the release of cytokines and liver injury[13-17]. Although indirect evidence cited previously suggested an interaction among LPS, LBP and CD14 during ALD[18-20], a direct link is lacking. To evaluate the role of LBP and CD14 in ALD, the intragastric ethanol porat model for ALD was used to study the relationship between the expression of LBP and CD14 genes and the severity of liver injury in ALD rats.

MATERIALS AND METHODS

Animals and treatments

Twenty adult female Wistar rats weighing 180 and 220 g were fed ad libitum a liquid diet. They were divided into two groups (ten rats/group): et hanol liquid diet group (group E) and control liquid diet group (group C). Group E were fed ethanol, and group C received the same diet but with isocaloric amounts of dextrose instead of ethanol. In the ethanol-fed rats, an initial dose of 5 g·kg^{-1}·d^{-1}. The ethanol concentration within the diet was gradually increased up to 12 g·kg^{-1}·d^{-1} in 8 wk. All diets were kept fresh daily. They were anesthetized with sodium pentobarbital (30 mg kg^{-1} intraperitoneally) and sacrificed at different time points (4 wk and 8 wk). Blood was withdrawn from the tail vein and liver samples were frozen in liquid nitrogen and stored at -70℃ before use.

Blood endotoxin and ALT

For determination of endotoxin, blood was collected into pyrogen-free tubes containing heparin. Plasma was immediately separated at 4℃ by centrifugation at 200 g for 8 minutes and stored in pyrogen-free tubes at -70℃. Plasma en dotoxin levels were measured within a week using the Limulus Amebocyte Lysate assay. Levels of endotoxin in plasma from normal rats were below the limits of detection. Serum alanine transaminase (ALT) was measured by standard enzymatic procedures.

Liver pathology

Liver samples from different liver lobes were fixed with 100 ml·L^{-1} buffered formalin or 25 g·L^{-1} glutaraldehyde immediately. For optical microscopy, the tissue blocks were embedded in paraffin, and stained with hematoxylin and eosin (HE). For electronic microscopy, the tissue blocks were embedded in Epon 618 resin and ultrathin sections

were stained with urany acetate and lead citrate. A H-2000 transmission electron microscope was used.

RNA isolation and complementary DNA synthesis

Total RNA was isolated from rat liver tissue using the TRIZOL Reagent (Life Technologies, USA). The quality of RNA was controlled by the intactness of ribosomal RNA bands. A total of 0.5 mg of each intact total RNA samples was reverse-transcribed to complementary DNA (cDNA) using the reverse transcription polymerase chain reaction (RT-PCR) kit (Roche, USA). cDNA was stored at -70¡æ until polymerase chain reaction (PCR) analysis.

Determination of LBP and CD14 mRNA by RT-PCR

The PCR primers used were LBP: sense (5'-GAGGCCTGAGTCTCTCCATCT-3'), antisense (5'-TCTGAGATGGCAAAGTAGACC -3'); CD14: sense (5'-CTCAACCTAGAGCCGTTTCT-3'), anti-sense (5'-CAGGATTGTCAGACAGGTCT-3'); β-actin: sense (5'-ACCACAGCTGAGAGGGA-A ATCG-3'), antisense (5'-AGAGGTCTTTACGGATGTC-AACG-3'). The sizes of the amplified PCR products were 552 bp for LBP, 267 bp for CD14, and 281 bp for β-actin. The conditions for amplification were as follows: denaturation at 94℃ for 1 min, annealing at 58℃ for 2 min, and extension at 71℃ for 2 min for 28 cycles. The PCR products were electrophoresed in 20 g·L^{-1} agarose gels, and the gels were ethidium bromide stained and videophotographed on an ultraviolet transilluminator.

Statistical analysis

All results were expressed as $X \pm S_x$. Statistical difference between means were determined using two-way ANOVA or Student's *t* test. A *P* value of ≤0.05 was considered significant.

RESULTS

Blood endotoxin and ALT levels

Plasma endotoxin levels in control rats were (48±9) ng·L^{-1} and (53±11) ng·L^{-1} at 4 wk and 8 wk, respectively. Plasma endotoxin levels in ethanol -fed rats were increased significantly by ethanol to values of (129±21) ng·L^{-1} at 4 wk and (187±35) ng·L^{-1} at 8 wk. The Levels of endotoxin were about 2-and 3-fold higher than the values from control rats (Figure 1). Me an values for ALT in the control animals were (517±200) nkat·L^{-1} and (550±150) nkat·L^{-1} at 4 wk and 8 wk, respectively. Plasma ALT levels were increased dramatically to (1867±250) nkat·L^{-1} and (2450±367) nkat ·L^{-1} in ethanol-fed rats after 4 wk and 8 wk, respectively (Figure 2).

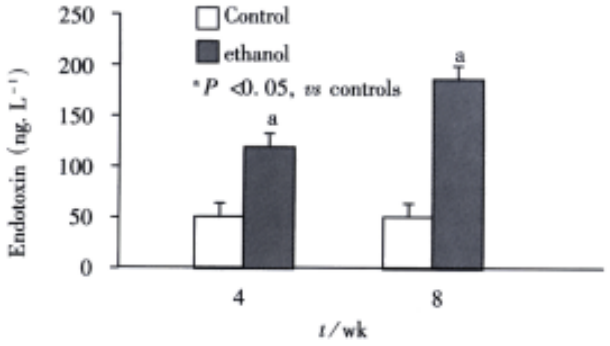

Figure 1 Chenges of endotoxin levels in two groups at different time points.

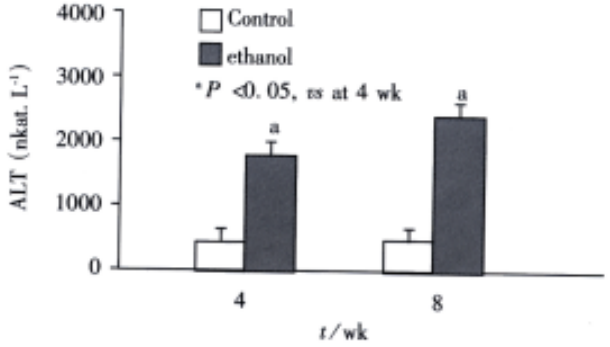

Figure 2 Changes of serum transaminase levels in two groups at different time points.

Pathological changes

Rats in the two groups, increased their body weight. Although the average weight gain was lower in ethanol-fed rats than the control rats, the differences between the two groups were not significant. Histopathological changes of the liver tissues were depicted in rep resentative photomicrographs in Figure 3A and 3B. None of the rats in the control group deve loped pathological changes in the liver at 4 wk or 8 wk. But, in liver section from rats after 4 wk on ethanol liquid diet, steatosis which was both microvesicular and macrovesi cular and few in flammation but accumulation of blood cells in the sinusoidal lining can be seen. In liver section from rats after 8 wk on ethanol diet, there was marked pathological changes (steatosis, cell infiltration and necrosis). Under electron microscopy, focal cytoplasmic degeneration and necrosis could be seen in hepatocytes of ethanol-fed rats(Figure 4A and B).

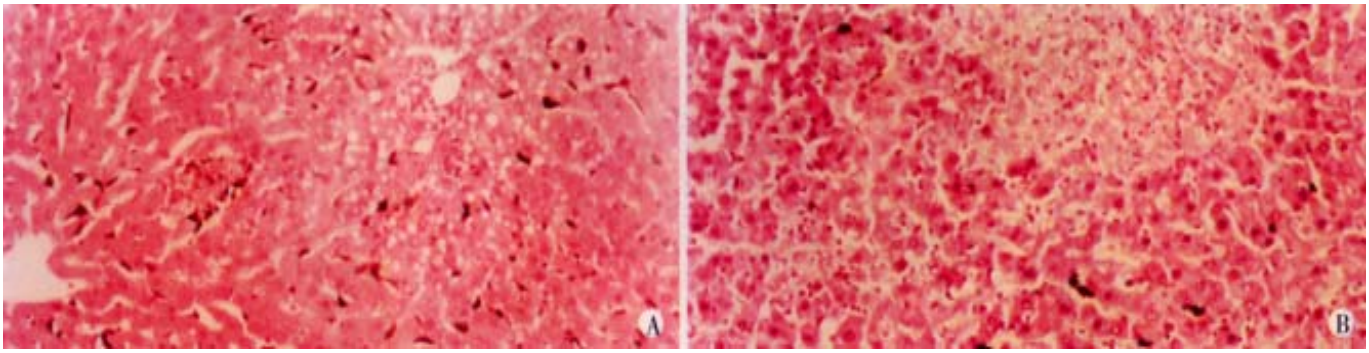

Figure 3 Liver section from rats (HE×200) A: 4 wk after ethanol liquid diet, steatosis and accumulation of blood cells in the sinusoidal lining; B: 8 wk after ethanol diet, steatosis, cell infiltration and necrosis.

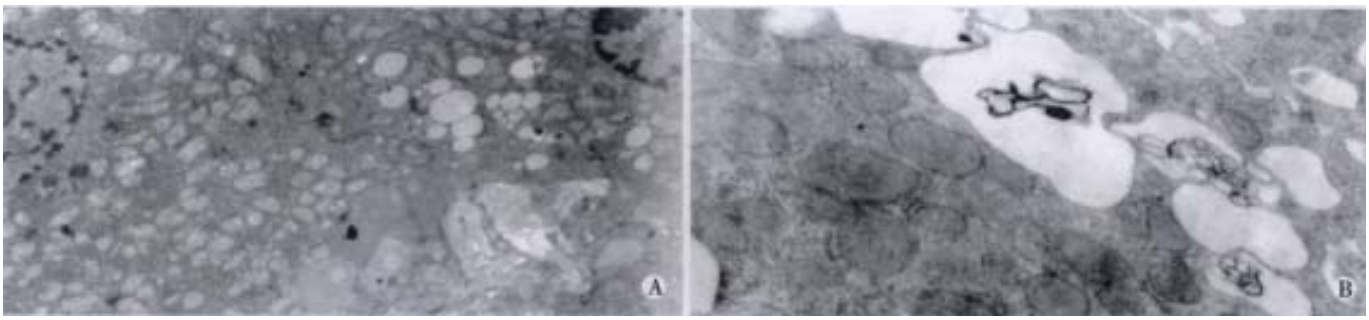

Figure 4 Ethanol-fed rats. A: Steatosis degeneration and necrosis in hepatocytes TEM×5000 B: Focal cytoplasmic degeneration and many myelin figures (TEM×20 000).

Expression of LBP and CD14 mRNA in the liver

The livers of rats in the individual group were examined for LBP and CD14 mRNA expression by RT-PCR (Figure 5). In the control rats, there was no significant difference in the levels of LBP and C D14 at 4 wk and 8 wk. Ethanol administration led to a significant increase in LBP and CD14 mRNA levels compared with the control group ($P<$ 0.05). The levels of LBP and CD14 mRNA in ethanol-fed rats were significantly higher in 8 wk than in 4 wk ($P<0.05$). The highest levels of CD14 mRNA were seen in ethanol-fed rats after 8 wk (Figure 6).

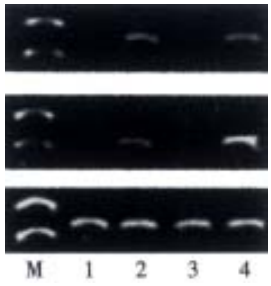

Figure 5 Expression of LBP and CD14 mRNA by RT-PCR analysis. M: Marker; Lane 1, 3: Group C in 4, 8 wk respectively. Lane 2, 4: Group E in 4, 8 wk.

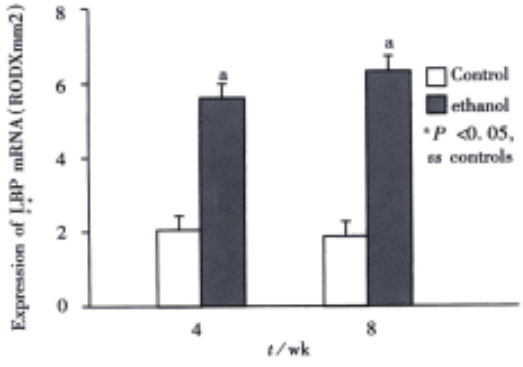

Figure 6 Expression of LBP mRNA.

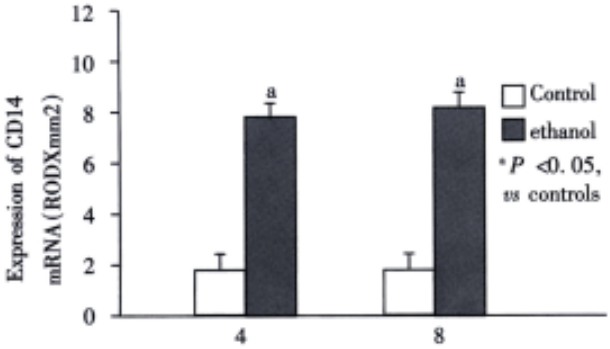

Figure 7 Expression of CD14 mRNA.

DISCUSSION

It is well documented that liver disease can result from the dose- and time-dependent consumption of alcohol[23-25] , female rats exhibit greater susceptibity to early alcohol-induced injury than males[26-28], Glycine prevents alcohol-induced liver injury by decreasing alcohol in the stomach[29]. However, mechanisms re main unclear. There appears to be increasing evidences that ethanol toxicity is associated with increased level of endotoxin in plasma[1,2,31]. Endotoxin or LPS is believed to exert many of its effects on the liver injury via interaction with LBP and CD14[11-18]. LBP and CD14 are clearly implicated in the mo lecular and cellular basis of the interaction between endotoxin and monocytes/ macrophages. LBP in serum can recognize and bind LPS to form LPS-LBP complexes and activate cells through the CD14 receptor on membrane of these cells, initiate a process leading to the release of cytokines (e.g. tumor necrosis factor a and interleukines), prostanoids, and other soluble mediators[24,31-34].The release of these mediators is considered to be an early key step in the pathogenesis of liver disease because they trigger inflammatory events in the liver and alter the parenchymal homeostasis, ultimately initiating liver injury[35-38].

A major goal of this study was to observe the expression of LBP and CD14 mRNA in ethanol-fed rats an d evaluate the in role in ALD. It was found that endotoxin levels in the plasma of rats treated with ethanol were increased significantly when compared with control animals and fatty liver, necrosis, and inflammation were developed in the ethanol treated rat. Control rats showed no liver pathology. In the present study, we found the severity of pathological changes in ethanol-fed rats were accompanied by an increase in intrahepatic LBP and CD14 mRNA levels and serum ALT levels. The increase in LBP and CD14 mRNA levels in the ethanol-fed rats is correlated with the degree of inflammation and necrosis in the livers of these animals. A similar pattern of changes was observed by Yin, *et al*[39]. They found that blood end otoxin and hepatic levels of CD14 messenger RNA and protein were increased by ethanol. Therefore, the sensitivity of rat liver to alcohol-induced injury is directly related to CD14 expression in the liver that lead to increasing the production of TNF-á, free radicals, interleukins and other cytokines[40-44]. The marked increase in CD14 expression suggests a new mechanism by which alcohol increases the LPS-mediated cytokine signaling by the liver macrophages, thus promoting the interaction between alcohol and endoto xins in the development of liver damage[45-50].

It has been well established that the role of LBP is to augment the response of monocytes/macrophages to low levels of endotoxin via interaction with CD14 protein and play an important role in alcoholic liver injury[36,]. The increase in intrahepatic CD14 m RNA expression may represent either an increase in the expression of CD14 within cells that reside in the liver or may represent recruitment of inflammatory cells(e.g., infiltrating mononuclear cells or macrophages) that have high expression of CD14 gene and CD14 protein[39,51-53]. In either case, an increase of C D14 may result in greater sensitivity to

endotoxin and NF-kB activation and production of pro-inflammatory cytokines which mediate liver injury[54-57].

In summary, our results show that ethanol administ ration led to a significant increase in LBP and CD14 mRNA levels in ethanol-fed rats when compared with the control rats. Increase of LBP and CD14 mRNA expression may result in greater sensitivity to endotoxin and liver injury. However, the mechanism of LBP increase and CD14 mRNA express ion is thus as yet unclear and needs further studies.

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Edited by Wang YQ and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Cloning of UGT1A9 cDNA from liver tissues and its expression in CHL cells

Xin Li[1,2], Ying-Nian Yu[1], Ge-Jian Zhu[1], Yu-Li Qian[1]

[1]Department of Pathophysiology, School of Medicine, Zhejiang University, Hangzhou, China
[2]College of Pharmcy, Zhejiang University, Hangzhou, China
Supported by the National Natural Science Foundation of China (C39370805), Zhejiang Provincial Natural Science Foundation (300487) and the Excellent Youth Scientist Fund of Zhejiang Province
Correspondence to: Prof. Ying-Nian Yu, Department of Pathophysiology, School of Medicine, Zhejiang University, Hangzhou, China. zjhsin@yahoo.com
Telephone: +86-571-87217203, Fax: +86-571-87217149
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Abstract

AIM: **To clone the cDNA of UGT1A9 from a Chinese human liver and establish the Chinese hamster lung (CHL) cell line expressing human UGT1A9.**

METHODS: **cDNA of UGT1A9 was transcripted from mRNA by reverse transcriptase-ploymerase chain reaction, and was cloned into the pGEM-T vector which was amplified in the host bacteric *E. Coli* DH5α. The inserted fragment, verified by DNA sequencing, was subcloned into the *Hind* III/*Not* I site of a mammalian expression vector pREP9 to construct the plasmid termed pREP9-UGT1A9. CHL cells were transfected with the resultant recombinants, pREP9-UGT1A9, and selected by G418 (400 mg·L^{-1}) for one mo nth. The surviving clone (CHL-UGT1A9) was harvested as a pool and sub-cultured in medium containing G418 to obtain samples for UGT1A9 assays. The enzyme activity of CHL-UGT1A9 towards propranolol in S9 protein of the cell was determined by HPLC.**

RESULTS: **The sequence of the cDNA segment cloned, which was 1666 bp in length, was identical to that released by Gene Bank (GenBank accession number: AF056188) in coding region. The recombinant constructed, pREP9-UGT1A9, contains the entire coding region, along with 18 bp of the 5′ and 55 bp of the 3′ untranslated region of the UGT1A9 cDNA, respectively. The cell lines established expressed the pro tein of UGT1A9, and the enzyme activity towards propranolol in S9 protein was found to be 101±24 pmol·min^{-1}·mg^{-1} protein (n = 3), but was *Not* detectable in parental CHL cells.**

CONCLUSION: **The cDNA of UGT1A9 was successfully cloned from a Chinese human liver and transfected into CHL cells. The CHL-UGT1A9 cell lines established efficiently expressed the protein of UGT1A9 for the further enzyme study of drug glucuronidation.**

Subject headings UGT1A9; cloning; glucuronidation; cell lines

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INTRODUCTION

Most organisms are exposed to a range of lipophilic compounds and converted them into excretable hydrophilic compounds. This metabolism of foreign compounds (xenobiotics) can be divided into two phases. For phase I metabolism, a reactive group is mostly introduced into the xenobiotic molecule. These reactions are mainly catalyzed by the cytochrome P450 monooxygenase system which consists of cytochrome P450s (CYPs) and cytochrome P450 reductase (CPR). For phase II metabolism, the reactive metabolite is conjugated to small, hydrophilic endogenous molecules such as glucuronic acid. The conjugation of this cofactor to xenobiotics is catalyzed by UDP-glucuronosyltransferases (UGTs). Since xenobiotic metabolizing enzymes have to catalyze the metabolism of structurally very divers e substrates, the various enzyme systems (e.g. CYPs and UGTs) comprise several isozymes that differ in their catalytic properties. The members of a given enzyme system have been grouped into families and subfamilies based on sequence homologies. In UGTs, two enzyme families termed UGT1 and UGT2 have been described.

The UGT1 locus is highly conserved between species[1]. UGT1A is a subfamily of UGT1 gene complex that is located at chromosome 2q37. UGT1A subfamily is encoded by tandem individual promoters and their first exons are linked by differential splicing to four common exons. As one of the isoforms, UGT1A9, is mainly expressed in liver. UGT1A9 can be induced by polycyclic aromatic hydrocarbons (PAHs), and therefore the drug glucuronidation catalyzed by UGT1A9 will be increased in cigarette smokers who inhale PAHs[2].

Human hepatic UDP-glucuronosyltransferases (UGT) is a family of microsomal enzymes that catalyze the glucuronidation of many important drugs, xenobiotics and endogenous compounds. Attempts to characterize the microsomal enzymes by conventional purification technique are often frustrated due to its instability. UGT isoenzyme expressed by cells is a useful tool for characterizing UGT's function. The cDNA cloning of UGTs from various sources (rabbit, rat, monkey, human beings, etc.) and their expression in cell lines were widely used for the gene characterization and function study of UGT isoforms[3-9]. In order to study tie drug metabolisms by UGTs, the cDNA encoding UGT1A9 was cloned from human liver and expressed in Chinese hamster lung (CHL) cell line in this study. The enzyme expressed was extracted and its activity was assayed with a substrate of propranolol which is a nonselective β-adrenergic blocking agent and can be used widely clinically[10].

MATERIALS AND METHODS

Isolation of RNA from human liver tissue

Human liver tissue was obtained from a surgical specimen of Chinese and stored at -80℃ until use. The total RNA was isolated with TRIzol reagent (Gibco Corp, USA)

UGT1A9 cDNA transcription

cDNA was transcr ipted from mRNA by revere transcriptase polymerase chain reaction (RT-PCR). Five μg of the total RNA and 2 μg of random primer (SANGON, Shanghai) in deionized water containing

DEPC (1 g·L^{-1}) were denatured at 65℃ for 15 min, then 4 μL 5 ×reverse transcriptase buffer, 3 μL10 mmol·L^{-1} dNTP, 1 μL M-MuLV reverse transcriptase (200 U) (Fermentas) and essential deionized water containing DEPC (1 g·L^{-1}) were added to have the total volume of 20 μL. The reaction was performed at 25℃ for 10 min, then 42℃ for 1 hour, and 70℃ for 10 min to inactivate the reverse transcriptase. The product was finally held at 4℃. Two μL of the reactant was mixed with 2 μL of 10 mmol·L^{-1} dNTP, 30 pmol of PCR primers and 3.5 U of DNA ploym erase (Perkin-Elmer Corp). The total volume of 100 μL was reached by adding deionized water. Two 26 meroligonucleotides as PCR primers were designed according to the DN A sequence of UGT1A9 (GenBank accession no. AF056188). The sense oligonucleo tides corresponding to base positions 1 to 26 was 5'-CTAAGCTTCAGTTCTCTGATGGCTTG-3 'with a restriction site of *Hind* III, and the anti-sense one, corresponding t o the bases position from 1641 to 1666, was 5'-GTTGGAAATG CCTAGGGAATGGTTC-3'. The poly merase chain reaction (PCR) was performed at 94℃ 2 min, then 94℃ 15 s, 60.1℃ 30 s and 72℃ 2 min for 31 cycles, and 72℃ for 10 min. The product was finally held at 4℃. An agrose gel electrophoresis was carried out with 10 μL of the PCR solution to check the 1666 bp DNA amplified.

Construction of recombinant pGEM-UGT1A9 and sequencing of UGT1A9

The PCR product of about 1.5 kb was isolated and ligated with pGEM-T (Promega) vector by T4 DNA ligase (Fermentas). *E. Coli* DH5α was transformed with the resulted recombinants pGEM-UGT1A9[11] and the positive bacteria colonies were screened by ampicillin resistant and blue-white screening with X-gal and IPTG. The cDNA of UGT1A9 subcloned in pGEM-T was sequenced on both strands by dideoxy chain-termination method marked with BigDye with primers of T7 and SP6 promoters and a specific primer of 5'-CAAGTATCGT GTTGTTCGC-3'. The termination products were resolved and detected using an automated DNA sequencer (Perkin-Elmer-ABI Prism 310, Foster City, CA).

Construction of the pREP9 based expression plasmid for UGT1A9

The *Hind* III-*Not* I fragment of the human UGT1A9 cDNA cleaved from the selected and amplified recombinant pGEM-UGT1A9 by *Hind* III and *Not* I digestion was purified by agarose electrophoresis and cloned directly into a unique *Hind* III-*Not* I site within the multicloning site of the mammalian expression vector pREP9 (Invitrogen, San Diego, CA) with T4 ligase.

Transfection and selection

Chinese hamster lung (CHL) cells were transfected with the resultant recombinants, pREP9-UGT1A9, using a calcium phosphate method[12]. After 24 h incubation at 37℃, the culture was rinsed and re-fed with fresh growth medium. Seventy-two h after transfection, the culture was split and then selected in the culture medium containing the neomycin analogue G418 (Gibco BRL, MD) (400 mg ·L^{-1}). The selective medium was changed every 3-4 d to remove dead cells and allow the growth of resistant colonies. After 1 mon, surviving clonies (termed C HL-UGT1A9) were harvested as a pool and propagated in medium containing G41 8.

Preparation of S9 of CHL-UGT1A9

CHL-UGT1A9 cells grown in the culture medium containing G418 (400 mg·L^{-1}) were rinsed with phosphate balanced solution (PBS), scraped and collected from the bottle with 11.5 g·L^{-1} KCl in aqua solution and t hen sonicated 3 s for 5 times with 5 s of interval break. The resulted homogenate was centrifuged at 9000×*g* for 20 min and the supernatant (S9) was transferred carefully to a clean tube for assay or storage under -70℃. The protein in S9 was determined by the same method that was used in our previous paper[13].

UGT assay

The UGT1A9 activities of S9 fraction were determined by the gl ucuronidation of propranolol. The assay was performed in a total volume of 100 μl containing final concentrations of 0.2 mmol·L^{-1} propranolol, 1 mmol · L^{-1} UDPGA, 1g ·L^{-1} Triton X-100, 50 μg of S9 protein in 50 mmol· L^{-1} Tris-HCl, 10 mmol·L^{-1} $MgCl_2$ buffer, pH 7.8 at 37℃. The mixtures were pre-incubated and the glucuronidation was started by the addition of UDPGA and stopped after 2 h by the addition of 100 μL of methanol. The mixtures were stirred thoroughly and centrifuged at 10 000 r·min^{-1} for 10 min. Un-reacted propranolol in the layer of reactant was d etermined by HPLC and the enzyme activity was calculated according to the amount of propranolol declined after incubation.

HPLC analysis of propranolol metabolized by S9 of CHL-UGT1A9

The concentration of propranolol metabolized by S9 of CHL-UGT1A9 was assayed by the HPLC procedure[13] with modification to the mobile phase. Twenty mL of the sample was applied to a reversed phase column (Shim-pack CLC-ODS15 cm×0.6 cm id, 10 μm particle size). Propranolol was monitored with a UV detector at 290 nm. The mobile phase is made up with ammonium acetate buffer (4.0 g ammonium acetate, 10 mL acetate acid and de-ionized water in 1 L)-methanol-acetonitrile (2:1:1), and to 500 mL mobile phase add 0.7 mL triethylamine as the elution modifier. The flow rate is 1.0 mL·min^{-1}.

RESULTS

Construction of recombinants

The recombinant of pGEM-UGT1A9 (Figure 1) was const ructed with the human UGT1A9 inserted into the cloning site of vector pGEM- T between the promoters of T7 and SP6. Selection and identification of the recombinant was carried out by *Hind* III / *Not* I endonuclease digestion and agarose elector phoresis (Figure 3). The cloned DNA segments in selected recombinants were sequenced completely. According to the results of DNA sequencing, the cDNA in a selected recombinant was identical to the DNA sequence of UGT1A9 reported by Ciotti-M *et al* (GenBank accession no. AF056188) in the reading frame. The restriction sites of *Hind* III and *Not* I in the recombinant were used for the subcloning of insertion fragment into an expression vector.

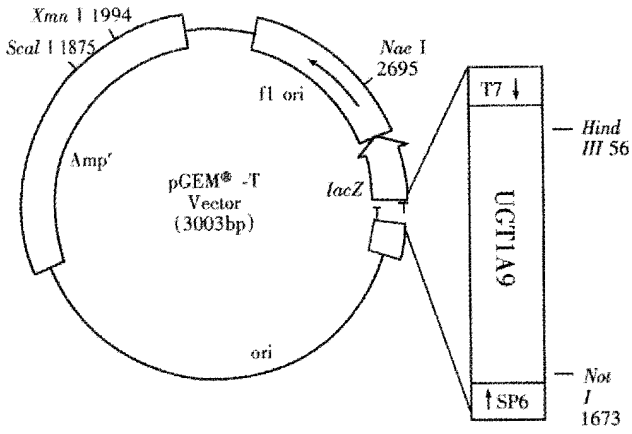

Figure 1 Scheme of pGEM-UGT1A9.

The *Hind* III/*Not* I fragment (1.5 kb) containing the complete *UGT*1A9 cDNA was subcloned into the *Hind* III/*Not* I site of mammalian expression vector pRE P9 (Figure 2). Selection and identification of the recombinants were carried out by *Hind* III/*Not* I endonuclease digestion and agarose electrophoresis (Figure 3). The re sulting plasmid was designated as pREP9-*UGT*1A9 which contained the entire coding region, along with 18 bp of the 5' and 55 bp of the 3' untranslated region of the *UGT*1A9 cDNA, respectively. In addition, the neo gene of the plasmid confers the G418 resistant phe*Not*ype to CHL cells for the selection of transfected cells.

Establishment of recombinant cell lines with UGT1A9 enzyme activity

CHL cells were transfected with pREP9-UGT1A9, and selected with G418 (400 mg·L^{-1}). The surviving clone was propagated and the cell line termed CHL-UGT1A9 was established. The preparation S9 was prepare d from CHL-UGT1A9 cells harvested for UGT1A9 activity assay by HPLC. Figure 4 shows t he typical elution of propranolol in incubation solution. The UGT enzyme activity towards propranolol in S9 protein was found to be 101±24 pmol·min^{-1}·mg^{-1} (n = 3), but was *Not* detectable in parental CHL cells.

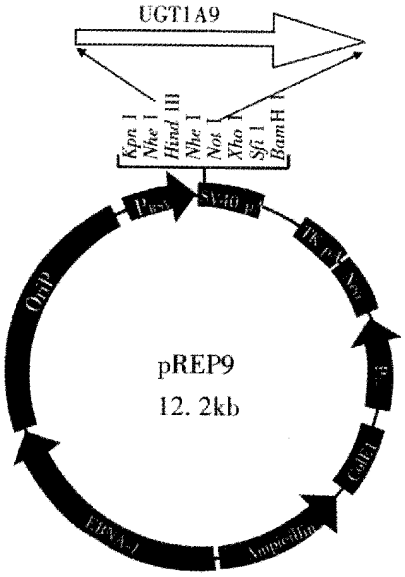

Figure 2 Scheme of pREP9-UGT1A9.

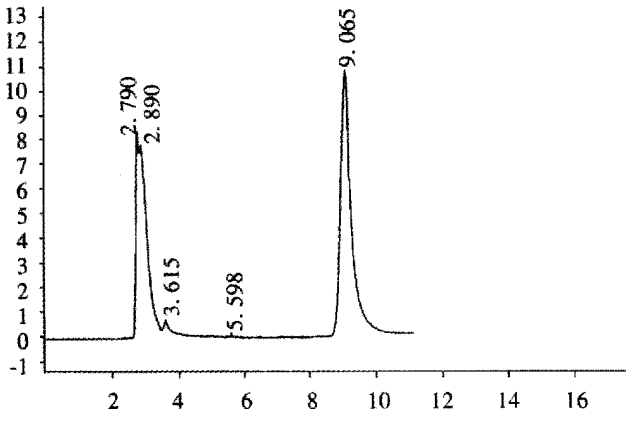

Figure 3 Electrophoresis identification of recombinants constr ucted.

Lanes 1: Marker (λ / EcoR I and *Hind* III); 2: PCR product of *UGT*1A9 from Chinese human liver; 3: recombinant of pGEM-UGT1A9; 4: recombi nant of pGEM-*UGT*1A9 cleaved by *Hind* III and *Not* I; 5: recombinant o f pREP9-*UGT*1A9; 6: recombinant of pREP9-*UGT*1A9 cleaved by *Hind* III and *Not* I; 7:pREP9 expression vector; 8:Marker (λ / *Hind* III).

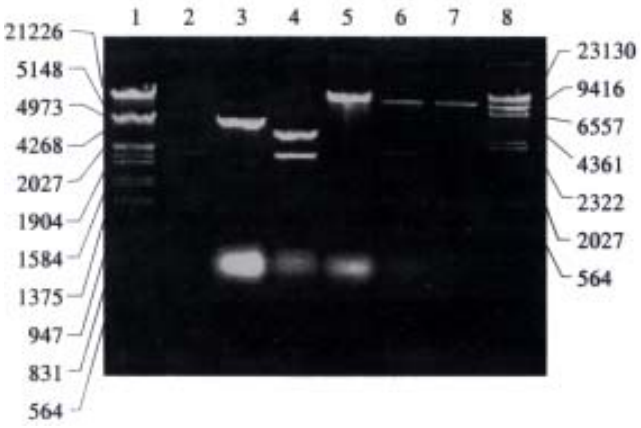

Figure 4 Chromatogram of propranolol after incubation with S9 prepared from CHL-UGT1A9 cell. A Shi m-pack CLC-ODS column (15 cm×0.6 cm i.d.) was used. The mobile phase was constituted with ammonium acetate buffer-methanol-acetonitrile (2:2:1) and 1.4mL·L^{-1} triethy lamine with the flow rate at 1.0 mL·min^{-1}. Propranolol was monitored at 290 nm. propranolol: t_R = 9.065 min.

DISCUSSION

UGTs are involved in the conjugation of UDP-glucuronic acid (UDPGA) to a variety of chemicals, drugs, and endogenous compounds. The elimination of hydrophobic chemicals from cells is aided by their conversion to water-soluble glucuronides. UGTs are closely relatied to the system of cytochrome P450 monooxygenase, and involved in the transportation of carrier and the passage of drugs through cell phospholipid bilayer. In most cases, the lipophilic compounds are converted by phase I metabolism to the substrate f or glucuronidation by obtaining an essential function (such as carbon, nitrogen, sulfur and oxygen), but in many cases xenobiotics and endogenous substances canalso be glucuronidated by UGTs without the phase I metabolism. The xenobiotic metabolizing cytochrome P450 monooxygenase system and the UGTs reside mainly in the endoplasmic reticulum. However, CYPs and the CPR are localized on the cytosolic side of the endoplasmic reticulum, which the UGTs are localized on its luminal side[14]. UGTs are latent enzymes, needing activation (in general by detergents) to express its maximal activity.

Numerous reports revealed that glucuronidation is a major pathway involved in the metabolism of drugs, exogeno us, and numerous endogenous compounds such as bile acids and steroid hormones. E ach UGTs family or subfamily has its own substrates but the substrate spectrum a re partly overlapped. UGT1 has substrates such as thyroid hormone[15], SN-38[16], bilirubin[17,18], opioids, bile acids, fatty acids, retinoids, c iprofibrate , furosemide, dilunisa, catechol estrogens, coumarins, flavonoids, anthraquinone s, EM-652 (an active antiestrogen)[19] and phenolic compounds[20]. UGT2 catalyzes substrates such as estrogens, androgens, morphine, AZT, and retinoicacid, epirubicin[16,21,22,23], etc. UGT1A9 is a member of UGT1A subfamily. The endogenous substrates for UGT1A9 are estrone, 4-hydroxyestrone, ethinylestradiol, retinoic acids, etc, and exogenous substrates include propofol, propranolol, paracetamol, S-naproxen, ketoprofen, ibuprofen, entacapone, some mutagenic arylamines, etc[2,24-26]. UGT1A9 was found to have regioselectivity on the glucuronidation of hydroxyl group of carbohydrate-containing drugs[27].

UGTs are expressed extensively in organs and tissues, and they may play a key role in the regulation of the level and action of steroid hormones in steroid target tissues. Organs that express UGTs include liver, kidney, gastrointestinal tract[28-29], olfactory[30], jejunum,

ileum[31], prostate[32-33], colon[34]. UGT1A9 is mainly expressed in liver, and also expressed in steroid targets[35] and colon[34].

UGTs are inducible enzymes. In most cases this induction is due to increased transcription of the corresponding genes but sometimes it is also due to an improved stability of proteins. The pattern of enzymes affected is dependent on the inducing agent. Usually, phenobarbital induces mainly enzymes within UGT2 family, and methylcholanthrene induces enzymes belonging to the UGT1 family[28]. Other chemicals that induce UGTs include aryl hydrocarbon receptor ligands or oltip raz[36], flavonoid chrysin[37], and t-butylhydroquinone and 2,3,7,8-tetrachloro dibenzo-p-dioxin[38], etc. UGT1A9 can be induced by polycyclic aromatic hydro carbons (PAHs)[39]. On the other hand, UGTs can also be inhibited, for example by uridine diphosphate[40], and N-glycosylation is involved in the functional properties of UDP-glucuronosyltransferase enzymes[41].

To clone and express UGTs in cells can help screen substrates that an isoenz yme is responsible. The prod uction of a UGT enzyme protein using transgenic cell lines is a practical manner to study its function[42-43]. We report here the cloning of UGT1A9 cDNA and establishment of a CHL cell line expressing UGT1A9 from a Chinese human liver. The full-length cDNA, UGT1A9, that encodes for a human UDP-glucuronosyltransf erase protein, was isolated from a Chinese human liver total RNA. To achieve high expression levels of UGT1A9, the UGT1A9 cDNA was cloned into the eukaryotic expressi on vector pREP9, which we had previously used in this laboratory for the express ion of human CYP450 1A1, 2B6, 3A4, etc in CHL cells[44-45]. The salient feature of this vector has an EBV origin of replication and nuclear antigen (EBNA-1) to allow high-copy episomal replication in mammal cell lines. The Rous sarcoma virus long terminal repeat (RSV LTR) early promoter controls the expression of the U GT1A9 cDNA. As *Not*ed under "Results", the isolated clone contains a 1592-nuc leoti de open reading frame flanked by 18 and 55 base pairs of 5' and 3' noncoding sequences, respectively. The DNA sequence in the reading code frame is identical to that reported (GenBank accession no. AF056188). The expression of a protein that catalyzed the glucuronidation of propranolol was proven in the Chinese hamster lung cells transfected with the recombinant plasmid pREP9-*UGT*1A9.

Conjugation with glucuronic acid is an important biotransformation pathway for a large number of clinically used drugs. In human intestinal, UGTs play an important role in the detoxification of xenobiotics compounds and, in some cases, may limit the bioavailability of therapeutic agents[20]. The deficient of a UGTs isoenzyme, may cause disease and clinical incident[46-47], the typical example was serious adverse events associated with chloramphenicol toxicity in neonates. Human UGTs are regulated in cases of healthy condition and exposure of harmful environmenta l carcinogens[48-50]. Moreover, UGT was identified as an antigenic tar get in a subgroup of liver- kidney microsomal auto-antibodies[51]. Hence, it is very necessary to undertake the study of functions and characteristics of UGTs. Over the last decade, some research papers were published about the usage of cloned and expressed human UGTs for the assessment of human drug conjugations and identification potential drug interactions[6-8]. However, the information gap still exists regarding the enzymatic aspects of UGTs to drugs elimination and its potenti al impact on therapy. More researches on the drug metabolism by UGTs are necessary for effective translation of scientific information into clinically applicable knowledge. As has been shown with the CYPs, coupling of basic and clinical science is needed to continually improve our understanding of the UGTs. Many factors are known to influence the activities of UGTs involved in drug metabolism, hence plasma clearances of glucuronidated drugs. Such factors include age (especially neonatal period), cigarette smoking, diet, certain disease states, drug therapy, ethnicity, genetics and hormonal effects. Knowledge of the profile, substrate specificities and regulation of human UGTs remains limited and cons equently it is still generally *Not* possible to predict the effects of specifice nvironmental and genetic factors on the metabolism and pharmacokinetics of individual glucuronidated drugs. Future investigations must define the substrate spec ificities of the various UGTs and investigate mechanisms by which the separate isozymes are regulated. Only then will it become possible to rationalize (and predict) the alterations in pharmacokinetics and response to glucuronidated drugs in specific patient groups.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Copper transportion of WD protein in hepatocytes from Wilson disease patients *in vitro*

Guo-Qing Hou[1], Xiu-Ling Liang[2], Rong Chen[2], Lien Tang[3], Ying Wang[2], Ping-Yi Xu[2], Ying-Ru Zhang[2], Cui-Hua Ou[2]

[1]Department of Neurology, Guangzhou First Municipal People's Hospital, Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China
[2]Department of Neurology, First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou 510080, Guangdong Province, China
[3]Department of Pharmacology, University of Kentucky, Lexington, KY 40506, USA
Supported by Key Clinical Program of Ministry of Ministry of Health (No.37091), "211 Project" of SUMS sponsored by Ministry of Health, and Guangdong Provincial Natural Science Foundation, No.990064
Correspondence to: Dr. Guo-qing Hou, Department of Neurology, Guangzhou First Municipal People's Hospital, Guangzhou Medical College, 1 Panfu Rd, Guangzhou 510180, China. spring-hua@126.com
Telephone: +86-20-81083090 Ext 596, Fax: +86-20-81094250
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Abstract

AIM: **To study the effect of copper transporting P-type ATPase in copper metabolism of hepatocyte and pathogenesis of Wilson disease (WD).**

METHODS: **WD copper trans porting properties in some organelles of the cultured hepatocytes were studied from WD patients and normal controls. These cultured hepatocytes were incubated in the media of copper 15 mg·L^{-1} only, copper 15 mg·L^{-1} with vincris tine (agonist of P-type ATPase) 0.5 mg·L^{-1}, or copper 15 mg·L^{-1} with vanadate (antagonist of P-type ATPase) 18.39 mg·L^{-1} separately. Microsome (endoplasmic reticulum and Golgi apparatus), lysosome, mitochondria, and cytosol were isolated by differential centrifugation. Copper contents in these organelles were measured with atomic absorption spectrophotometer, and the influence in copper transportion of these organelles by vanadate and vincristine were comparatively analyzed between WD patients and controls. WD copper transporting P-type ATPase was detected by SDS-PAGE in conj unction with Western blot in liver samples of WD patients and controls.**

RESULTS: **The specific WD proteins (Mr155 000 lanes) were expressed in human hepatocytes, including the control and WD patients. After incubation with medium containing copper for 2 h or 24 h, the microsome copper concentration in WD patients was obviously lower than that of controls, and the addtion of vanadate or vincristine would change the copper transporting of microsomes obviously. When incubated with vincri stine, levels of copper in microsome were significantly increased, while incubated with vanadate, the copper concentrations in microsome were obviously decreased. The results indicated that there were WD proteins, the copper transportion P-type ATPase in the microsome of hepatocytes. WD patients possessed abnormal copper transporting function of WD protein in the microsome, and the agonist might correct the defect of copper transportion by promoting the activity of copper transportion P-type ATPase.**

CONCLUSION: **Copper transportion P-type ATPase plays an important role in hepatocytic copper metabolism. Dysfunction of hepatocytic WD protein copper transportion might be one of the most important factors for WD.**

Subject headings glucuronosyltranferase/genetics; glucurono syltranferase/biosynthesis; DNA,complementary/genetics; liver/cytology; hasters; lung/cytology; animal

Hou GQ, Liang XL, Chen R, Tang LW, Wang Y, Xu PY, Zhang YR, Ou CH. Copper transportion of WD protein in hepatocytes from Wilson disease patients *in vitro*. *World J Gastroenterol*, 2001;7(6):846-851

INTRODUCTION

Hepatolenticular degeneration (Wilson disease, WD) is an autosomal recessive disorder first described in detail by Wilson in 1912, which is characterized by excessive accumulation of copper in the liver, brain, cornea an d subsequently in kidneys and other organs. The disease has a world prevalence of 5-50 per million and a birth incidence from 17-29 per million[1-6]. In China, WD is one of the most common neurogenetic diseases. According to a survey reported in 1995, WD patients accouted for about 10.14% of the total 957 neurogenetic patients first visiting the Neurogenetic Clinic of the First Hospital affiliated to Sun Yat-Sen University of Medical Sciences, and ranked as the second on the list[7]. The principle of copper metabolic disturbance in WD includes low serum cerulopl a smin levels and low serum copper levels, as well as increased copper excretion in urine. By means of removing the excessive copper, the disease development will be inhibited, and if treatment started before the appearance of neurological manif estations, the latter can be prevented to a large extent[2-3,8]. However, why does the abnormal copper metabolism happen in WD? It has been shown that more than 95% of circulating plasma copper were bound to a blue-copper oxidize cerulopl smin (CP), while the levels of CP was magnificently reduced in the majority of WD patients[1,9]. But no relationship was found between the concentration of cellular copper and the CP gene expression or CP protein with rodent mod el of WD and patients' cultured fibroblasts[10-13]. Therefore, it was suggested that the genetic defect of copper transportation did not alter biosynthetic and secretary of CP. Seemingly, neither the theory of MT (metallothionein) nor lysoso me abnormality could well explain the pathogenesis of WD[14-16]. Recently more concerns were focused on ATP7B, the gene of WD, which was just found in 1993 and has been mapped to chromosome 13q14.3 by three different genetic techniques[17-19]. Many researchers are trying to search for clues to the copper metabolic abnormality from the mutations of this gene, and the latter was predicted to en cod e a putative protein product, the WD copper-transporting P-type ATPase (WD protein), which has 1411 amino acids and a calculated molecular mass of about 159ku[20-28]. But up to now, the cellular localization of WD protein apparently has not yet been documented. There were reports that canalicular membranes, mitochondri a, microsome, or Golgi apparatus had WD

proteins[29-34]. However, all these researches were carried out in animal livers or in abnormal/immortal cell lines, which h ad much more different cell structures and biochemical metabolisms from human beings. We now set up a cultured hepatocyte model for studying WD copper transporting properties in such suborganelles as microsomes, lysosomes, cytosol, and mitochondria of the cultured hepatocytes from WD patients and normal controls under different incubative conditions with copper, ATP or the adjusting agents of WD proteins, and analyzed the cellular localization of WD proteins in hepatocytes.

MATERIALS AND METHODS

Subjects

Five (male 3, female 2) patients, aged 13-31 years, were diagnosed as having Wilson disease patients according to clinical symptoms, signs and copper biochemical laboratory assay by our Neurohereditary Clinic from 1998 to 1999. They had lower levels of serum ceruloplasmin and high levels of urinary copper. They all had liver cirrhosis accompanied by splenomegaly, and intended to receive splenectomy and liver biopsy. Five (male 4, female 1) controls, aged 28-49 years, were patients with hepatolith, cholith, or liver a ngioma, or healthy liver grantors, with normal neurological examinations and nor mal copper chemical tests, and were to receive hepatolobectomy. Immediately after operation, liver samples were rinsed and preserved in 4℃ F12/DMEM culture medium.

Hepatocyte culture and protein blotting

Hepatocytes were separated by 0. 5 $g \cdot L^{-1}$ type IV collagenase digested and cultured according to the methods introd uced by literatures[35-40]. The isolated hepatocytes were seeded and cultured in flasks pre-coated with rat tail collagen at 37℃ 50 $mM \cdot L^{-1}$ CO_2 with F12/DMEM supplemented with 200 $mM \cdot L^{-1}$ fetal bovine serum, 10 $mmol \cdot L^{-1}$ nicotinamide, 5 $mg \cdot L^{-1}$ a mphotericin B, 0.5 $mg \cdot L^{-1}$ glucagon,10 ìg·L^{-1} EGF(epidermal growth factor), 10 $\mu g \cdot L^{-1}$ insulin-transferrin-sodium selenite media supplement, and other growth factors. The cultured cells were observed and photographed and the media were partly changed every day. When they grew to contact with each other, discard the culture media, and cultured the hepatocytes further for 2 or 24 h in all WD patients and controls with culture media containing: copper 15 $mg \cdot L^{-1}$ only; copper 15 $mg \cdot L^{-1}$, ATP 30 $mmol \cdot L^{-1}$ and vanadate 18.39 $mg \cdot L^{-1}$; copper 15 $mg \cdot L^{-1}$, ATP 30 $mmol \cdot L^{-1}$ and vincristihe 0.5 $mg \cdot L^{-1}$; and copper 15 $mg \cdot L^{-1}$ and ATP 30 $mmol \cdot L^{-1}$, respectively. After reincubation, cells were rinsed with D-Hanks solution atroom temperature, harvested by rubber policeman after adding 1 mL of 0.05 $mol \cdot L^{-1}$ Tris-HCl (2.5 $g \cdot L^{-1}$ Nonidet P-40, 0.5 $mmol \cdot L^{-1}$ PMSF, 0.1 $g \cdot L^{-1}$ aprotinin, 1 $mg \cdot L^{-1}$ leupeptin, 1 $\mu mol \cdot L^{-1}$ pepstantin, pH8.6), dissolved for 15 min at 4℃ and disintegrated under ultrasonic (80 W×90 s), and then centrifuged at 16 000× *g* for 20 min. Finally, the protein rich supernatants were transferred to separate vials for testing or preservation at-700℃.

Fifty μg proteins of the supernatants were first separated by SDS-PAGE on 200 volts in 60 $g \cdot L^{-1}$ gels for 45 min, followed by 90 min of electrophoretic transfer to nitrocellulose membranes on 120 volts[31,41] according to the instructions of the Bio-Lab kit (New England Bio-Lab Co.). Transferred membranes were incubated with the primary antibodies and goat anti -rabbit antibodies with HRP (Bio-Lab Co.), respectively. After rinsed with the buffer thoroughly, the membranes were reacted with enhanced chemiluminescence. The primary antibody was a rabbit anti-human WD protein antibody (anti-WD), a gift from Dr. Gitlin and Dr. Lutsenko. The band number, density and molecular weight of specific bands were observed and analyzed by Bio-Rad Gel 2000 Imaging System.

Isolation of organelles

Total homogenates of cytosol, lysosome, microsome and mitochondria were isolated at 4℃ by differential centrifugation (8 000×g, 10 min; 9 000×g, 10 min; 30 000×g, 15 min; 108 000×g, 60 min) using super-high speed centrifuge (Beckman L8-55M, USA). Degree of contamination of cytosol, lysosome and microsome were estimated by measuring the lactate dehydrogenase a ctivities, acid-phosphatase activity and glucose-6-phosphatase activityies, respectively.

Content of copper and protein assay

All samples were assayed for protein concentration by the methods described by Bradford[4], using the bovine serum albumin as a standard. Copper contents were measured with atomic absorption spectrophot ometer, and expressed as copper/protein ratios (Cu/Pr):

$$\text{Cu/Pr } (\mu g \cdot g^{-1}) = \frac{\text{Copper contents } (\mu g \cdot L^{-1})}{\text{Protein contents } (g \cdot L^{-1})}$$

Statistical analysis

Results were given as the mean with the corresponding standard deviation ($\bar{x} \pm s$). Statistical analysis was performed with SPSS/8.0. F test and Student's t test were used to determine the differences between the means of different groups. Statistical significance was considered at the level of $P<0.05$.

RESULTS

Hepatocyte morphology

After the first 24 h culture, viable hepatocytes changed their shape from spherical to flat on the substrate and displayed one or two long cytoplasmic projections onto the substrate, and a ppeared three to six sided in shape. After 4 d, a widespread and a monolayer of hepatocytes could be found. After 7 d, they became smaller and with more granules. After 21 d culture, hepatocytes began to fall from the flasks and died.

Protein blotting

Western blotting analysis of WD protein separated from cultured human hepatocytes two main lanes with molecular mass of M_r 155 000, 90 000 and 80 000 were found in normal human and WD patient hepatocytes, but non could be seen in the blood vessel endotheliocytes of human liver (Figure 1).

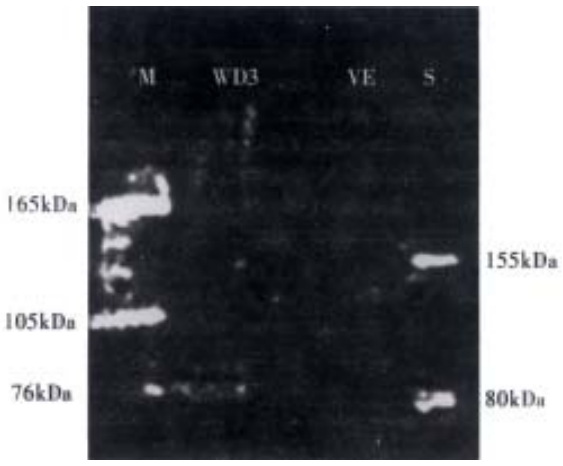

Figure 1 Western blotting analysis of WD protein separated from cultured human hepatocytes. M: Protein molecular mass markers; WD3: The hepatocytes of one WD patient; VE: Blood vessel endotheliocyte; S: Normal human hepatocytes.

Copper transportion of hepatocytes

Normal subjects After 2 h incubation with 15 mg·L^{-1} copper, the copper levels of all organelles increased significantly. When adding 30 mmol ·L^{-1} ATP to the culture media, there were different changes of copper concentrati ons in different organelles. Lysosome and microsome copper contents were much higher with ATP than without ATP, and the cytoplasmic copper level with ATP was lower than that of without ATP, and the differences between each group were not significant by Student's *t* test. The copper level of mitochondria showed no significant change. After 24 h copper incubation, the copper contents of microsome, mitochondria and cytoplasm with ATP became much lower than that without ATP, while the copper contents in lysosome showed no changes (Table 1).

Table 1 Concentrations of copper in organelles of nor mal hepatocytes after 2 h or 24 h culture ($\bar{x}\pm s$, μg·g^{-1})

t (culture) /h	ATP / (30 mmol·L^{-1})	Microsomes	Lysosomes	Cytoplasm	Mitochondria
0		74±13	70±10	526±63	85±11
2	absence	231±31	306±19	1571±115	420±43
2	presence	269±43[a]	342±26[a]	1488±129[a]	395±35
24	absence	346±52	322±40	1589±137	458±68
24	presence	288±39[a]	369±46	1464±110[b]	417±73[a]

[a]*P*<0.05, *vs* ATP absence; [b]*P*<0.01, *vs* ATP absence.

WD patients

Copper contents of cytoplasm after incubation with medium containing 15 mg·L^{-1} copper for 2 h, cytoplasmic copper concentration in WD patients became obviously higher than that of controls under all incubative conditions (*P*<0.05 *vs* control). When co-incubated with 0.5 mg·L^{-1} vincristine, there was no significant change of copper concentration in WD patients, while it decreased in the controls; when adding 18.39 mg·L^{-1} vanadate, there was no significant change of copper concentration in WD patients, while it inicreased in the controls (*P*<0.05). After 24 h culture with copper, cytoplasm copper levels of WD patients were higher than the controls (*P*<0. 01 *vs* control). The adding of 0.5 mg·L^{-1} vincristine decreased its cop per level (*P*<0.01 *vs* no vincrinstine), but there was no difference between the WD group and the controls. When adding 18.39 mg·L^{-1} vanadate, the copper level in WD group increased (*P*<0.05 *vs* no vanadate), while that of the controls did not change, and that of WD group was higher than the controls (*P*<0.01 *vs* control, Figure 2).

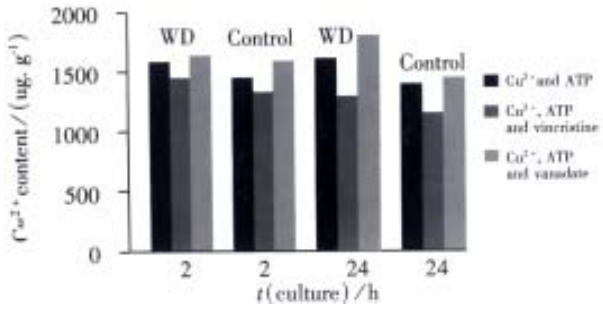

Figure 2 Cu^{2+} levels in hepatic cytosol at different culturing conditions.

Copper contents of microsome Copper concentrations of microsome in WD patients were obviously lower than that of controls after incubation for 2 h under each incubative condition (*P*<0.05 *vs* control). After incubation of copper and 0.5mg·L^{-1} vincristine, copper levels in the microsome were significantly increased (*P*<0.05 *vs* no vincrinstine). And after incubation with copper and 18.39 mg·L^{-1} vanadate, the copper concentrations of microsome in WD patients were obviously decreased (*P*<0.05 *vs* 2 h of no vanadate).

After 24 h incubation with 15 mg·L^{-1} copper and 30 mmol·L^{-1} ATP, copper contents in WD group were lower than the controls (*P* <0.05 *vs* control). When adding 0.5 mg·L^{-1} vincristine, there was no change in the WD group, and when adding 18.39 mg·L^{-1} vanadate, the copper contents increased (*P*<0.05 *vs* 24 h of no vanadate, Figure 3).

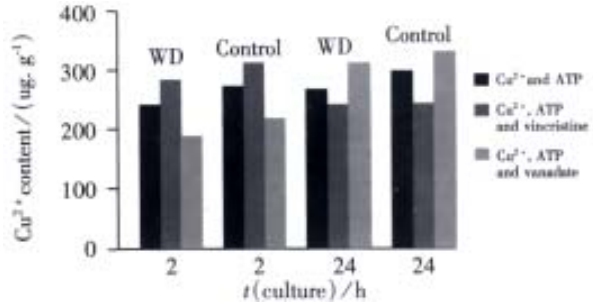

Figure 3 Cu^{2+} levels in hepatic microsome under different culturing conditions

Copper contents of lysosome After incubation with medium containing 15mg·L^{-1} copper and ATP for 2 h, lysosomal copper concentrations in WD patients were lower than that of controls (*P*< 0.05 *vs* control), and when adding vanadate, there was significant decrease of copper concentration in the controls (*P*<0.05 *vs* no vanadate). With incubation up to 24 h, the WD patients' lysosome copper concentrations rose to the same level as the controls. When co-incubated with vincristine or vanadate, there was no change in the copper concentration of WD patients (Figure 4) .

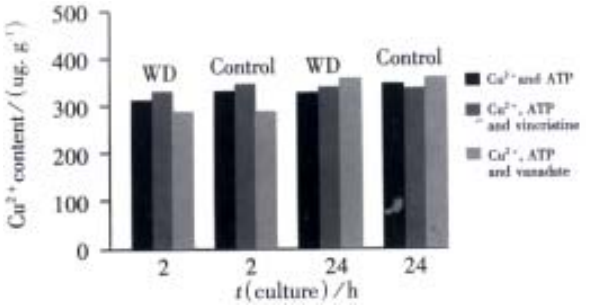

Figure 4 Cu^{2+} levels in hepatic lysosome under different culturing conditions.

Copper contents of mitochondria The copper content in mitochond ria was significantly lower than the controls when cultured 2 h or 24 h with 15 mg ·L^{-1} copper and 30 mmol·L^{-1} ATP. But when adding 0.5 mg·L^{-1} vincristine or 18.39 mg·L^{-1} vanadate, there was no significant change in the copper level of mi tochondria (Figure 5).

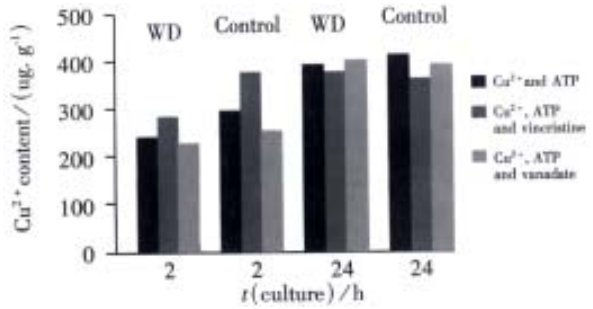

Figure 5 Cu^{2+} levels in hepatic mitochondria under different culturing conditions.

DISCUSSION

Copper is a trace element required by most organisms and is indispensable as a cofactor in a number of proteins, including cytochrome-c-oxidase, superoxide dismutase (SOD), dopamine o-hydroxylase, lysyl oxidase and ceruloplasmin. Both of lack and accumulation of copper may cause diseases[13,20]. One of the most important copper pumps in human body is considered as the copper transporting P-type ATPase, which is considered to play an essential role in cellular copper homeostasis[17-19,42]. In order to study the copper metabolism disorder of WD, Chan *et al* [43] ha d first applied the technique of skin fibroblast culture *in vitro* 20 years a go. Because it is easy to get the skin specimen, skin fibroblasts can be cultured and pass generations successfully, and its culture *in vitro* can be controlled and repeated steadily, this model had been widely used by researchers from all over the world[4,12,44]. In China, Liang and Chen *et al*[4,44] improved the fib roblast culturing model to study the copper metabolism of WD in 1992, and found that incubation with high contents of copper could promote the expression of hereditary abnormalities of copper metabolism in cultured skin fibroblasts of WD patients. They had studied the characteristics of copper uptake and excretion, analyzed the copper transporting manner of suborganelles, probed the actions of zinc on WD, and investigated the possible mechanisms of vanadate and vincristine to regulate the copper metabolism of WD cultured cells. However, as the copper metabolism disorders originated from the liver, and the main lesions were also localized in the liver, the study on the hepatocytes of WD patients can more directly reveal the possible mechanism of abnormal copper metabolism in WD.

Hepatocyte is one of the high-differentiated cells in human body[36-39,45]. Under normal biological conditions, hepatocytes of adult human body remain still and do not divide until stimulated by lesion, inflammation or other pathological factors. Cultured with common medium *in vitro*, hepatocytes can not multiply and divide. The rough endoplasmic recticulums disappear rapidly, cell appearance changed early, and its biochemical functions attenuated simultaneously. The cultured hepatocytes lost their tissue specific functions within 3 d to 5 d[35,36]. So it is important to improve the skills of hepatocyte separation and culture.

We had used thin biopsy liver pieces to culture rat hepatocytes by the method for skin fibroblasts culture[43,45,46]. In the first 3 days culture, there were round cells removing from the liver slices, and the cell number increased gradually. One week later, most of the cells grew to triangles or multipleangles in shape which were hepatocytes, and less cells showed shuttle-shaped, which were fibroblasts or other fibroblastoid. Up to 2-3 weeks, cells spread over the bottom of the flasks, but most of them were fusiform shaped and were fibroblastoid, and the hepatocytes were very rare then. This indicated that hepatocytes needed much higher culture condition than fibroblasts. When we cultured hepatocytes from embryo rats, the hepatocytes could be divided rapidly even if common culture media PMP L 1640 were used. And when various growth factors and mineral metals were added into the media, the embryo hepatocytes could maintain strong capacities of albumin synthesis and secretion up to one month or more. Most researchers agreed that embryo hepatocytes remained immature, so they could divide and be cultured easily[36].

WD patients we studied in this series, all had liver cirrhosis, and their hepatocytes were more difficult to culture. We mimicked the normal natural growth conditions of human liver and supplemented the culture media with fetal bovine serum, nicotinamide, amphotericin B, glucagon, EGF (epidermal growth factor), insulin-transferrin-sodium selenite media supplement. The hepatocytes in WD were then growing as the normal ones except that fewer fibroblasts speckled. We could erase the fibroblasts easily with rubble policeman under microscope or only by prolonging the collagenase digesting period to 60-90 minutes.

We observed that copper uptake by normal hepatocytes, and found that the copper contents in microsomes (endoplasmic reticulum and Go lgi apparatus) and lysosome all increased significantly, while the copper contents in cytoplasm decreased markedly after 2 h of culture with 15 $mg \cdot L^{-1}$ copper and 30 $mmol \cdot L^{-1}$ ATP in the culture media. This indicated that there existed ATP de pendent copper uptake in these organelles of hepatocyte. We could not determine w here the concrete copper absorption took place at that time, because there is a variety of types of ATP-dependent copper transporters in hepatocytes, such as A TP-dependent glutathion coupling copper transporter, canalicular CMOT transporter and copper transporting P-type ATPase. When the culture medium was absent of magnesium, which was a necessary catalyzer to ATPase for the hydrolysis of its terminal phosphate, the copper accumulation in these organelles could not happen. And more importantly, after adding sodium vanadate, the specific antagonist sensitive to copper transporting P-type ATPase, the copper transporting in micro some and lysosome were inhibited markedly (Figures 3, 4). This proved that the A T P-dependent copper transporter was right the copper transporting P-type ATPase and suggested that it is located in both of the above two organelles, and our immunologic al blotting results with specific antibody against WD protein also proved that W D proteins were existing in hepatocytes. These were identical to the results of that of Bingham[48,49], Dijkstra[29], Shah[24] *et al* .

Liver plasma membranes in canalicular and basolateral fractions from Wistar rats were fractionated on discontinuous sucrose gradients by Dijkstra *et al*[29] and Usta *et al*[32], and it was found that there was ATP-stimulated uptake of radiolabeled copper in canalicular membranes, which was consistent with Adachi *et al*' studies[47] about the biochemistry of copper transporting in LEC rat, an animal model of Wilson disease. If this copper transporter functioned abnormally, it would lead to copper acc umulation in the liver as a result of deficient biliary copper excretion. This w ell explained the mechanism of copper excretion disorder of WD, but it could not answer the question of the deficiency of ceruloplasmin (CP), since CP is formed in endoplasmic reticulum where copper was transported to apo-ceruloplasmin. The research of Bingham *et al* (1995)[49] on rat hepatocytes indicated that copper transporting P-type ATPase might exist in endoplasmic reticulum. Furthermore, Shah[24] and other researchers did immunohistochemical studies using antibodies against the cation combining sites or other function domains and also found the specific reaction in endoplasmic reticulum in the transferred cell strains[33,41]. These results all suggested that copper transporting P-type ATPase should be in endoplasmic reticulum.

Several groups had provided evidence which suggested that the copper transporting ATPase transport copper with the oxidation equivalence (I), that is Cu^+ ion. One key evidence is the six conserved metal binding motifs Gly-Met-X-Cys-X-Ser-Cys in the amino terminal of each copper transporting P-type ATPase, where the cysteine residues only bind copper as Cu^+. Other reports an d our study did not support this hypothesis. It was shown that Cu^+ transportion in microsomes was not dependent on ATP, but our data indicated that the copper accumulation in microsomes was ATP-dependent. Moreover, when we detected the concent rations of Cu^+ in each organelles, we did not find any significant changes under different incubation conditions (data not shown), so all the copper contents provided in this paper were the results of Cu^{2+} detected by atomic absorption spectrophotometer. Finally, many papers have shown that the most common gene mutation of WD patients in Western Europe and Northern America is the His1069Glu in the conserved sequence Ser-Glu-His- Pro-Leu of copper transporting P-type ATPase[5,21,32], and we know histidyl residues bind copper as Cu^{2+}[48,49]. All this argued that copper transporting P-type AT Pase might transport copper as Cu^{2+}.

Microsome consists of endoplasmic reticulum and Golgi apparatus. The former mainly functions to synthesize proteins (including ceruloplasmin), and the latter works to process these proteins and make them glycosylated, while the function of lysosome is to digest the endogenous and exogenous fractions of cells, and to join the renewal of cells and tissues of the hepatocytes[48]. Harada *et al*[50] used colchicine to destroy microtubules in lysosome vesicles, and found that the secretion of bile copper was inhibited, therefore indicated that lysosome could work to transport cellular copper. We found that the copper levels in hepat ocyte lysosome of controls could be regulated by the antagonist of copper transporting P-type ATPase (vanadate) after incubation for 2 h, the agonist (vincristine) was not found in stimulating lysosome's copper transportion, and when incubated longer, vanadate did not show the inhibition to copper uptake. It remains unclear whether the copper transportion of lysosome is reached by WD proteins. Yin *et al*[46] regarded that copper transporting P-type ATPase was in both microsome and lysosomes of the cultured fibroblasts of WD patients. Our data showed that lysosome had copper transporting function too, but it did not belong to WD protein, because of its absence of the permanent inhibition by vanadate, the specific inhibitant to WD protein. This disagreement suggested that more evidences are needed to solve the problem.

In cultured hepatocytes of WD patients, the microsome copper contents after being co-cultured with copper and vanadate were significantly lower than that co-cultured with copper only, while being co-cultu re d with copper and vinscrtine, the copper contents in microsome and cytoplasm were significantly higher than that with copper only. This suggested that the agonists and antagonist of P-type ATPase affected the uptake and excretion of copper in microsome by inhibiting or increasing the activation of P-type ATPase. After 24 h of culture with copper and vanadate, the copper contents of microsome and cytoplasm were significantly higher than that with copper only, which indicated that the agonist and antagonist affected the secretion of copper significantly, which further suggested that there was copper transporting P-type ATPase in the microsome of human hepatocytes. Our data showed the significantly different copper levels of microsomes in WD patients' hepatocytes after co-cultured with copper as compared with the controls, which indicated that there were abnormalities of copper transportion in microsomes, that is the disturbance of WD proteins in view of the above results.

WD is one of the rare neurogenetic diseases that can be curable. Because of the frequent side effects (the most often applied medicine), there are about 10 to 30 percent of WD patients who could not tolerate the long-term use of the drug[2,4,51-53]. Much more adverse effects had also been found on the use of DMS, trientene or all other therapeutic drugs. These made it necessary to find new safe and effective alternation to D-Penicillamine to treat WD. When we added agonist (vincristine) to the culture media, the copper levels microsomes in WD patients' hepatocytes in c reased significantly, and showed no difference with the controls. This indicated that the function of the copper transporting ATPase in WD patients' hepatocytes could be promoted by vincristine's activation, and recover to the normal levels. Yin *et al*[46] also found that the agonist of copper transporting AT Pase could modify the impairment of copper excretion from the microsome of WD cultured cells . All the data suggested that it might be a new clinical approach for WD to useagonists of copper transporting ATPase by regulating this enzyme's activity of WD patients. Furthermore, cultured hepatocyte model using high content copper for copper transportion will provide a useful cytological tool for probing the mechanism and therapeutic methods of WD.

In conclusion, this paper used cultured hepatocyte model for WD copper studies, and the data indicated that there is copper transporting P-type ATPase in the microsomes; WD patients had abnormal functions of copper transportion P-type ATPase in the microsomes, and the agonist might correct the defect of copper transporting by increasing P-type ATPase activity.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Distribution of nitric oxide synthase in stomach myenteric plexus of rats

Xi Peng, Jin-Bin Feng, Hong Yan, Yun Zhao, Shi-Liang Wang

Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
Correspondence to: Xi Peng, Institute of Burn Research, Southwestern Hospital, Third Military Medical University, Chongqing 400038, China. pxlr@netease.com
Telephone: +86-23-65317058
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Abstract

AIM: **To study the distribution of nitric oxide synthase (NOS) in rat stomach myenteric plexus.**

METHODS: **The distribution of NOS in gastric wall was studied in quantity and location by the NADPH-diaphorase (NDP) histochemical staining method and whole mount preparation technique.**

RESULTS: **NOS was distributed in whole stomach wall, most of them were located in myenteric plexus, and distributed in submucosal plexus. The shape of NOS positive neurons was basically similar, most of them being round and oval in shape. But their density, size and staining intensity varied greatly in the different parts of stomach. The density was 62±38 cells/mm² (antrum), 43±32 cells/mm² (body), and 32±28 cells/mm² (fundus), respectively. The size and staining intensity of NOS positive neurons in the fundus were basically the same, the neurons being large and dark stained, while they were obviously different in antrum. In the body of the stomach, the NOS positive neurons were in an intermediate state from funds to antrum. There were some beadlike structures which were strung together by NOS positive varicosities in nerve fibers, some were closely adherent to the outer walls of blood vessels.**

CONCLUSION: **Nitric oxide might be involved in the modulation of motility, secretion and blood circulation of the stomach, and the significant difference of NOS positive neurons in different parts of stomach myenteric plexus may be related to the physiologic function of stomach.**

Subject headings nitric oxide synthase/analysis; nitric oxide synthase/physiology; rats; stomach/chemistry; immunohistochemistry; stomach/innervation

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INTRODUCTION

Recent pharmacological and physiological studies indicated that nitric oxide (NO) is a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves in the mammalian gastrointestinal tract[1-15]. It may play a very important role in the neuronal regulation of gut. Previous studies have revealed the distribution, morphological features and projections of nitric oxide synthase (NOS) activity neurons and fibers in the enteric nervous system in intestine[16-25]. However, there have been much fewer studies of NOS activity in stomach wall since it is very difficult to make whole mount preparations of stomach. If NO is a transmitter of NANC inhibitory nerves, it should be present in neurons innervating the muscularis in stomach. What are the regularity of distribution of NOS in rat gastric wall, and the pat tern of innervation of these neurons? To answer these questions, we examined the distribution and morphological feature of NOS positive neurons in the stomach wall using improved whole mount preparation technique.

MATERIALS AND METHODS

Whole mount preparations production

Adult male or female Wistar strain rats (210 g-250 g in body weight) were provided by the Center of Laboratory Animals of the Third Military Medical University. The rats were fasted overnight prior to the experiment, and anaesthetized with sodium pentobarbitone (50 mg·kg^{-1}, ip). Stomach was rinsed with 0.01 mol·L^{-1} phosphate buffered saline (PBS, pH 7.4) and poured into gastric cavity with 40 g.L^{-1} paraformaldehyde (until 3-4 times of normal stomach volume), put in the same fixed liquid for 4 h-6 h (4℃), and soaked in 300 g·L^{-1} sucrose liquid for 24 h (4℃). Thereafter, wash away with PBS, wrap stomach up with bandage, rub gently for 2-3 min, stuff tightly with cotton, then demarcate trace along greater curvature of stomach toward lesser curvature with an extremely thin piece of bamboo, and strip slightly. Finally, trim the longitudinal muscular layer and dip in PBS for staining.

NADPH-diaphorase staining

Whole mount preparations were rinsed with PBS three times, and incubated in a solution of 0.1 mol·L^{-1} PBS (pH 8.0) containing 0.5 g·L^{-1} β-NADPH, 0.2 g·L^{-1} nitroblue tetrazolium (NBT), 2.0 g·L^{-1} L-malic acid, 3g·L^{-1} Triton X-100, hatched at 37℃ for 45 min, and rinsed three times with PBS, then mounted on glass slide, paved nicely (drug ratio less than 1:1.1), dehydrated through graded alcohol, cleared in xylene, and coverslipped with DPX. Incubative solution without adding β-NADPH served as negative control.

Statistical analysis

All results were expressed as the x±s, data were analyzed by Student *t* test. *P* values <0.05 were considered statistically significant.

RESULTS

Our study showed that NOS was widely distributed in rat stomach wall, most of them being localized in myenteric plexus, and distributed in submucosal plexus, gastric mucosal epithelium and gastric gland as well. In the myenteric plexus, NOS positive neurons were clearly identified by their sharply defined dark blue cytoplasmic stain with almost no background, the nuclei appeared as colorless "holes" (Figure 1). The shape of neurons was basically similar, most of them varied from round, oval to fusiform, while their density, size and staining intensity varied greatly in the different parts of stomach.

The density was 62±38 cells/mm^2, 43±32 cells/mm^2 and 32±28 cells/mm^2, respectively in antrum, body and fundus. Two subtypes of NOS positive neurons could be distinguished on the basis of size, staining intensity and progress in number. In fundus, about 75% neurons were large, dark stained and had some long progresses (Figure 2). Neurons of the second subtype were slightly smaller, with some short processes and mainly located in antrum (approximately 65%) (Figure 3). In the body of stomach, the NOS positive neurons were in an intermediate state from fundus to antrum. Moreover, some beadlike structure was strung together by NOS positive varicosities in nerve fibers, and some were colsely adherent to the outer walls of blood vessels (Figure 4).

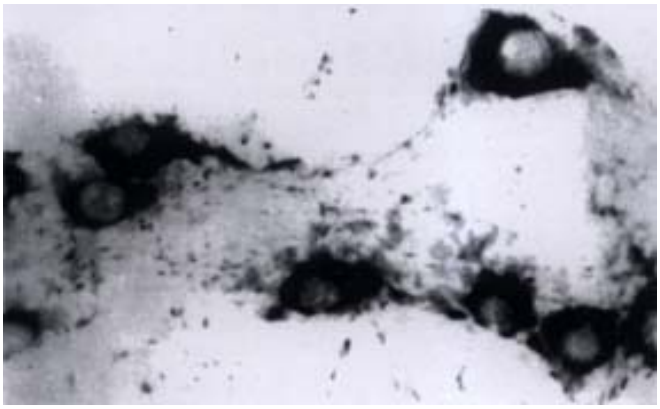

Figure 1 The shape of NOS positive neurons in the myenteric plexus of rats (body). NDP×400

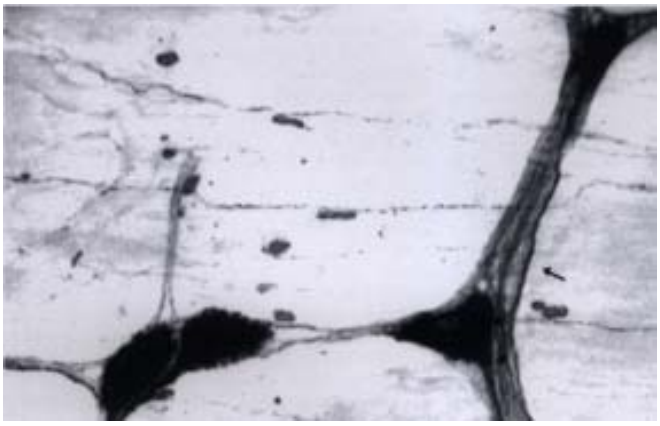

Figure 2 The shape of NOS positive neurons and its long processes (↑) in myenteric plexus of rat fundus. NDP×400

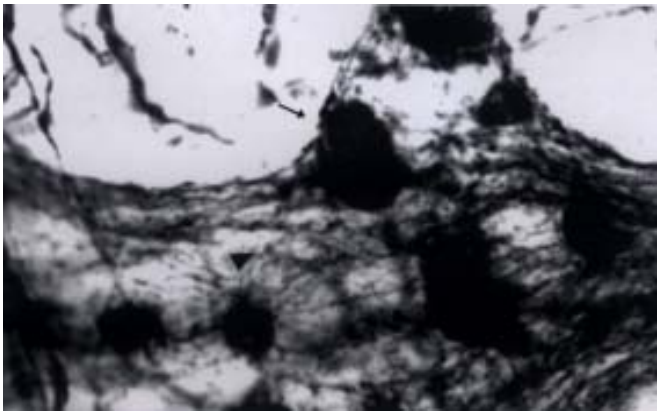

Figure 3 The large NOS positive neurons (↑) and small neurons (▲) in rat antrum. NDP×400

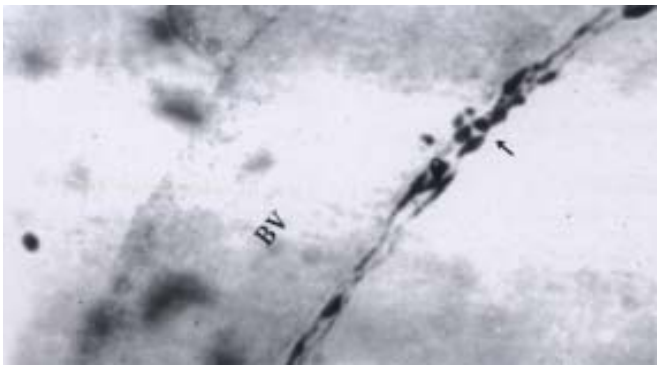

Figure 4 NOS positive varicosities (↑) in nerve fibers closely associated to blood vessels. NDP×1000

DISCUSSION

Since the early 1960s, it has been known that discrete populations of nerve cells can be labeled by an obscure, but simple, histochemical reaction involving the reduction of tetrazolium salts to form dark formazan products. This reaction is catalyzed by an unidentified enzyme contained in these cells and requires NADPH. The enzyme, called NADPH diaphorase, has been identified as being identical to NOS[26-39]. It has been demonstrated that NADPH diaphorase reacticity and NOS immunoreactivity coexist in enteric neurons. The simple histochemical procedure to visualize NADPH diaphorase may be useful to localize NOS in other tissues in view of the currently limited availability of antisera to NOS[40-50]. Therefore, the NADPH-d is now wildly used as a marker for neuronal structures in the CNS and ENS which contain the enzyme NOS.

The result showed that NOS was widely distributed in the gastric wall. Briefly, many NOS positive neurons were present in the myenteric plexus but relatively few in the submucous plexus. The majority of the NOS positive neurons in stomach could be classified as either Dogiel type 1 or type 2 neurons,although they were not as typical as the Dogiel neurons in small intestine, suggesting that NOS is both in the circular muscle motor neurons and in some interneurons. Some NOS positive nerve fibers formed a dense network in the circular muscle and in myenteric ganglia, and few nerve fibers in the mucosa. Based on this evidence, it is clear that the distribution of NOS in the enteric nervous system is compatible with a transmitter role of NO in stomach.

The significant difference of NOS positive neurons in different parts of the stomach myenteric may be related to the physiologic function of stomach. The density of NOS positive neurons was scarce in fundus, it is advantageous to keep tonic contraction at fundus and maintain foundation tension, but the activity of NOS was more powerful than that in antram. It benefits gastric lumen relaxation for food store after diet. Teng *et al*[6] found that involvement of NO in the reflex relaxation of the stomach to accommodate food or fluid, and the NO released from fundus plays a principal role in adjusting, gastric lumen volume and press. We also found that the density of NOS in pyloric part was intensive, it is possibly related to stomach peristalsis since there were not only NOS neurons, but also other types, especially cholinergic neurons[7]. They released transmitters to coordinate each other, adjust stomach smooth muscle to shrink, thus producing peristalsis as a mechanical pump pushing food to duodenum. Meanwhile, the results show that staining intensity of NOS neurons was different in pylorus, hinting that some NOS neurons may be in a reserving condition, once required NOS will be activated and NO sythesized to regulate stomach movement and the tension of pyloric sphincter. But the detailed mechanism is far from clear. We also found that there were many NOS positive varicosities, and bead-

like structure strung together in nerve fibers. Some of them were closely adherent to the outer walls of blood vessels and smooth muscle fibers. This finding has provided morphological evidence of NO involved in the modulation of motility and blood circulation of gastrointestinal tract.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Taxotere resistance in SUIT Taxotere resistance in pancreatic carcinoma cell line SUIT 2 and its sublines

Bin Liu[1], Edgar Staren[2], Takeshi Iwamura[3], Hubert Appert[2], John Howard[2]

[1]Department of General Surgery, the Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China
[2]Department of Surgery, Medical College of Ohio, Toledo, Ohio, USA
[3]Department of Surgery, Miyazaki Medical College, Miyazaki, Japan
Supported in part by Phne-Poulenc Rorer Pharmaceuticals INC.
Correspondence to: Dr. Bin Liu, Department of General Surgery, the Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu, China. liubinxy@pub.xz.jsinfo.net
Telephone: +86-516-5802003
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Abstract

AIM: **To investigate the specific mechanisms of intrinsic and acquired resistance to taxotere (TXT) in pancreatic adenocarcinoma (PAC).**

METHODS: **MTT assay was used to detect the sensitivity of PAC cell line SUIT-2 and its sublines (S-007, S-013, S-020, S-028 and TXT selected SUIT-2 cell line, S2/TXT) to TXT. Mdr1 (P-gp), multidrug resistance associated protein (MRP), lung resistance protein (LRP) and β-tubulin isotype gene expressions were detected by RT-PCR. The functionality of P-gp and MRP was tested using their specific blocker verapamil (Ver) and indomethacin (IMC), respectively. The transporter activity of P-gp was also confirmed by Rhodamine 123 accumulation assay.**

RESULTS: **S-020 and S2/TXT were found to be significantly resistant to TXT(19 and 9.5-fold to their parental cell line SUIT-2, respectively). RT-PCR demonstrated strong expression of Mdr1 in these two cell lines, but weaker expression or no expression in other cells lines. MRP and LRP expressions were found in most of these cell lines. The TXT-resistance in S2-020 and S2/TXT could be reversed almost completely by Ver, but not by IMC. Flow cytometry showed that Ver increased the a ccumulation of Rhodamine-123 in these two cell lines. Compared with S-020 and SUIT-2, the levels of β-tubulin isotype II, III expressions in S-2/TXT were increased remarkably.**

CONCLUSION: **The both intrinsic and acquired TXT-related drug resistance in these PAC cell lines is mainly mediated by P-gp, but had no relationship to MRP and LRP express ions. The increases of β-tubulin isotype II, III might be collateral changes that occur when the SUIT-2 cells are treated with TXT.**

Subject headings pancreatic neoplasms/pathology; tumor cells, cultured/drug effects; paclitaxel/analogs & derivatives; paclitxael/pharm acology; drug resistance, multiple; drug resistance, neoplasm

Liu B, Staren E, Iwamura T, Appert H, Howard J. Taxotere resistance in SUIT Taxo tere resistance in pancreatic carcinoma cell line SUIT 2 and its sublines. *World J Gastroenterol*, 2001;7(6):855-859

INTRODUCTION

Up to now, pancreatic adenocarcinoma (PAC) is still one of the leading causes of cancer death in the world, although meat progress has been made in the treatment of this disease[1,2]. Most patients with PAC are in its advanced stages and surgically unresectable at the time of diagnosis, and for those who are resected, the risk of recurrence is very high[3,4]. Consequently chemotherapy still is an alternative strategy for patients with non-resectable PAC[5-8]. However, the response to most forms of chemotherapy achieved so far is generally quite limited, and is related in part to the resistance to these chemical agents[1,5,9]. To date, some specific mechanisms of drug resistance have been elucidated, among which the best understood are increased expressions of mdr1-encoded p-glycoprotein (P-gp)[10-14], multidrug resistance protein (MRP)[15-17] and lung resistance protein (LRP)[18-20]. These membrane transporter proteins play important roles in multiple drug resistance (MDR) involving increased drug efflux and intracellular drug entrapment and/or redistribution.

Taxotere (TXT) is a member of the family of taxanes and is more potent than paclitaxel with regard to the promotion of the polymerization of tubulin, inhibition of depolymerization, and it inhibits cell replication and has greater antitumor activity in many *in vitro* and *in vivo* tumor model systems[21-24]. The drug has displayed significant antitumor efficacy against breast, lung and ovarian cancer s in clinical trials[25-28], but some clinical studies do not support the use of Taxane in advanced PAC[29,30]. The studies on mechanisms by which cells acquire resistance to Taxane demonstrated that the drug is a substrate for the P170 multidrug resistance pump that is able to confer resistance to a wide variety of naturally derived hydrophobic substances[16,29,31]. Therefore, the cells selected with Taxane were found to exhibit cross-resistance to a variety of other hydrophobic drugs and have elevated levels of P-gp[32]. Other possible mechanisms of resistance to TXT the include the alteration in microtubulin composition and/or dynamics[33,34] increased protein kinase C-α and -γ expression[35] and overexpression of Bc1-2[36], and SP-gp[37]. But the exact mechanism of TXT- resistance in PAC is still not clear[32,38]. The specific mechanisms in different tumors might be different.

The mechanisms of refractoriness to chemotherapy of PAC are not fully understood. Published reports provide confl icting information regarding the expression of this MDR phonotype in human PAC[16,39]. Whether t he intrinsic and acquired TXT-resistance share the same MDR mechanism is unclear, which might be important for designing a chemotherapeutic regiment and investigating appropriate reversal agents. This study was designed to investigate the mechanisms of intrinsic and acquired resistance to T XT in PAC cell line SUIT-2 and its sublines.

MATERIALS AND METHODS

Chemical reagents

Taxotere (Rh0ne-Poulenc Rorer Pharmaceuticals Inc.) was stored as 10 mmol·L^{-1} stock solution in absolute ethanol at -20℃. These solutions were further diluted in the mediu m used in the cell culture immediately before each experiment. Final dilutions of 0.5-3.5 nmol·L^{-1} TXT were used for the experiments to detect the sensitivity of SUIT-2 and its sublines. Verapamil (Ver) was purchased from

American Reagent Laboratory, Inc. and Trypsin, EDTA was purchased from Gibco. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), rhodamine (Rho) 123 (2-[6-Amino-3-imino-3H-xanthen-9-yl] benzoic acid methyl ester), indomethacin (IMC) (1-[P-chl orobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), McCoy's 5A Medium [modified], DMEM (phenol free) medium, Fetal calf serum (FCS), sodium pyruvate, MEM amino acids (50X), MEM amino acids (100X), MEM Vitamins, L-glutamine (200 mmol·L^{-1}) penicillin-streptomycin, serine, a sparagine sodium bicarbonate were purchased from Sigma.

Cell cultures

Human pancreatic cancer cell line SUIT-2 was established and supplied by Iwamura. Its sublines, including S2-007, S2-013, S2-020, S2-028, were cloned by soft agar culture and shown to have different metastatic potential[40]. These cell lines were cultured and the TXT resistant SUIT-2 cell line (S2/TXT) was develop ed as described previously[41].

MTT colorimetric assay

The MTT colorimetric assay was performed as described previously[41]. Briefly, when the cells of SUIT-2 and its sublines reached 85%-90% confluency, they were detached from the flasks with 0.25% trypsin and resuspended in the culture medium. Cells were grown within 9 6-well microtitre plates (Costar) in 2×10^{10} cells·L^{-1} of culture medium each well and acclimated for 6 h, and 100 μL of various concentrations of drugs diluted in culture medium was added. To study the effect of Ver and IMC on TXT cytotoxicity, 1 μmol·L^{-1} Ver or 10 μmol·L^{-1} IMC was added with this drug. Five duplicate wells were used for each determination. The plates were incubated for 72 h when the control cells reached 90% confluency, and 30 μL of MTT in PBS solution was then added to each well and the plates were incubated for another 4 h. The medium and MTT solution were then aspirated and 150 μL of dimethyl sulfoxide (DM SO) (Sigma) was added. The plates were read on Bio-Tek Microplate reader EL 800 (Bio-Tek Instruments, Inc). Fractioo of cell proliferation was defined as the ratio of optical density volume to that of controls. The IC_{50} was defined as the concentration of the drugs required to reduce the absorbance by 50% in treated cells as compared to that of the controls.

Rho-123 accumulation assay

Rho-123 accumulation was determined by flow cytometry. Briefly, SUI T -2, S-020 and S2/TXT cells in logarithmic growth phase were harvested with trypsin and resuspended in phenol red-free DMEM medium at 1×10^{9} cells·L^{-1}. Aliquots of 1 ml cell suspension were preincubated with or without Verapamil for 45 min and then 200 μg·L^{-1} Rho-123 dissolved in DMEM was added and incubated for 40 min. After incubation, cells were washed and resuspended in ice-cold Rho-free DMEM with 5 μmol·L^{-1} Ver. The accumulation of Rho-123 in cells was analyzed with flow cytometry. Ten thousand cells per sample were analyzed. Cells of these cell lines, which had not been exposed to Rho-123, were used to determine the background of autofluorescence.

RT-PCR

The isolation of total RNA was based on the method of Chomczynski *et al*[40]. Equal amounts of RNA were reversed transcribed using SuperScript™ O ne-step™ RT-PCR System (Life Technologies). Twenty-five μL PCR mixed in each tube containing: 0.5 μL RT/Tag Mix, 3 μL of 5 mmol·L^{-1} $MgSO_4$, 5 μL diethy pyrocarbonate (DEPC, Sigma)-treated distilled water, 3 μL mixed primer pairs, 12.5 μL 2X Reaction Mix and 1 μg Template RNA in DEPC water. After an initial denaturation in a programmable thermocycler at 94℃ for 2 min, PCR was carried out for 30 cycles with the thermal profile: denaturing at 94℃ for 30s, annealing at 55℃ for 30 s and extension at 72℃ for 1 min with an extra-10 min extension for the last cycle. After completion of the amplification cycles, 5 μL of each PCR product was electrophoresed at 60 V for 1.5 hrs on a 1.2% agarose gel (GIBCOBRL) in Trizma base and Glacial acetic acid EDTA buffer. Both target and control (β-actin) gene sequen ces were coamplified in the same tube. Gene expression was normalized to β-actin transcript; this was noted for REL for relative expression levelg(REL=densitometric value of studied gene/densitometric value of â-actin). The specific primers for mdr1, MRP and LRP used in this study were as described as before [41]. The beta-tubulin isotype sequences were designed as follows: The sense primer sequence was 5'-CAA CAG CAC GGC CAT CCA GG-3'. The antisense primer sequences were M40 (Cla ss I): 5'-AAG GGG CAG TTG AGT AAG ACG G-3', β9 (Class II): 5'-GTA GAA AG A CCA TGC TTG GG-3', β4 (Class III): 5'- CTT GGG GCC CTG GGC CTC CGA-3', 5β (C lass I Va): 5'-AAG TAG CCA GAG GTA AAG CGA G-3', β2 (Class IVb): 5'-CTT TCC CCA GTG AC T GAA GG-3'. β-actin was used as control. Its sense primer was: 5'- TGA CG G GGT CAC CCA CAC TGT GCC CAT CTA-3'; and antisense primer was: 5'- CTA GAA GCA T TT GCG GTG GAC GAT GGA GGG-3'.

RESULTS

Sensitivity of SUIT-2 and its sublines to TXT

To identify for intrinsic TXT-resistant PAC cell lines, SUIT-2 and its sublines (S2-007, S2-013, S2-0 20 and S2-028)) were investigated as to their sensitivity to TXT as by MTT assay. The in nate sensitivity of SUIT-2 and its sublines to TXT were found. S2-020 cells we re most resistante to TXT compared with its parental cell line SUIT-2 (IC_{50} 0.85 nmol·L^{-1}), and other cell lines. IC_{50} of S2-020 (16.2 nmol ·L^{-1}) was 19-fold to that of SUIT-2. The second most resistant cell line was found to be S2-007 (IC_{50}: 1.82 nmol·L^{-1}). The other cell lines had no significant resistance to TXT. The IC_{50} of S2-013 and S2-028 were 0.75 and 1.2 nmol·L^{-1}, respectively. IC_{50} of the acquired TXT resistant cell line established from SUIT-2 was 8.1 nmol·L^{-1}, 9.5- fold to its parental cell line SUIT-2 (Figure 1).

Reversal effects of Ver and IMC on the resistance to TXT

In order to elucidate the function of drug transporter pump P-gp and MRP expressed in the cell line S2-020 and S2/TXT, their corresponding blockers Ver and IMC were used respectively. Figure 2A shows that Ver at a concentration of 1ìmol·L^{-1} can almost completely reverse the resistance of S2-020 and S 2/T XT to TXT, but the same concentration of Ver had no sensitizing effect to SUIT- 2, which did not express P-gp. IMC, a specific modulator of MRP, had no reversal effect on the TXT-resistance found in these cell lines, although they all expressed MRP (Figure 2B).

Rho 123 accumulation and efflux

Accumulation and efflux of Rho-123, which is related to the transporter activity of P-gp, from SUIT-2, S2/TXT and S-020 cells as tested by flow cytometry. The accumulation of Rho-123 in SUIT-2 cells is much higher than that of S-020 and S2/TXT cells. The addition of 5 μmol·L^{-1} Ver led to significantly increased intracellular Rho 123 levels in the TXT-resistant S2-020 and S2/TXT cells but not in the TXT-sensitive SUIT-2 cells and its other cell sublines.

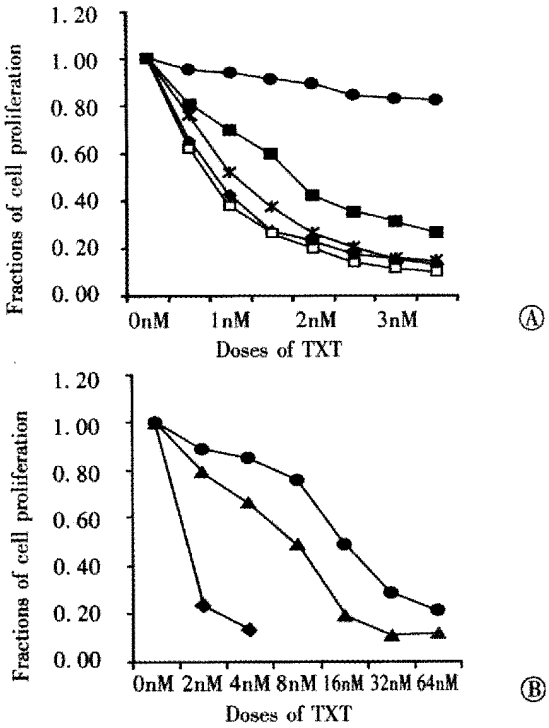

Figure 1 Dose-response curves of SUIT-2 and its subl ines for taxotere. ◆: SUIT-2, ■: S2-007,: S2-013, ●: S2-020, : S2-028, △: S2 /TXT.

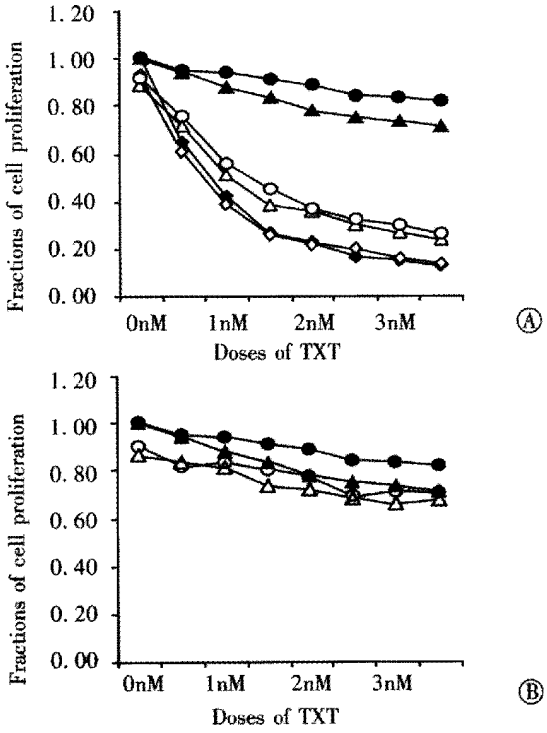

Figure 2 Reversal effects of Verapamil and Indomethacin on TXT -resistance. S2/TXT (▲), and S2-020 cell lines (●) were compared to SUIT- 2 (◆). Ver (Figure 2A) and IMC (Figure 2B) have been shown different sensitizing effects of TXT-resistance in S2/TXT and S2-020 (open symbols).

Expressions of mdr1, MRP, and LRP

The expressions of three major drug transporter pump genes mdr1, MRP, L RP were studied by RT-PCR. Figure 3 shows that there is a strong expression of mdr1 in both TXT-resistant cell line S2-020 and S2/TXT, no expressions in their parental cell line SUIT-2 and other two subline S2-013 and S2-028, which are most sensitive to TXT. MRP and LRP expressed in most of these cell lines, but no relationship was found between their expression and TXT-resistance.

Expressions of β-tubulin isotypes

β-tubulin isotype transcript analysis was performed by using isotype-specific primers and the RNA from SUIT-2 and its two TXT-resist ant cell line S2-020 and S2/TXT. Densitometric analysis of expression levels of each isotype was quantitated relative to the expression of the control gene β actin by calculating the ratio of the target gene to the control gene PCR product. As shown in Figure 4, the βI and β I Vb were the predominant transcript in all the three cell lines. A 2.4-fold increase of β II and a 2.3-fold increase of β III compared to SUIT-2 cell line were seen in S2/TXT cell line.

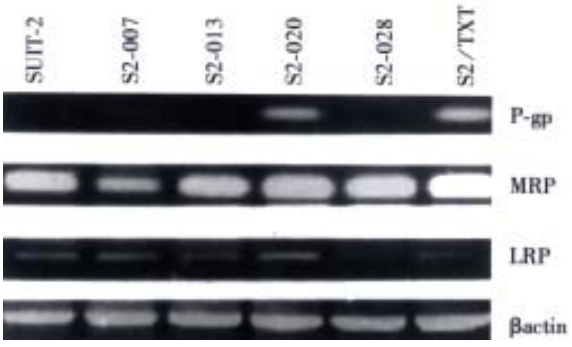

Figure 3 Expressions of mdr1, MRP, LRP in SUIT-2 and its sublines by RT-PCR analysis.

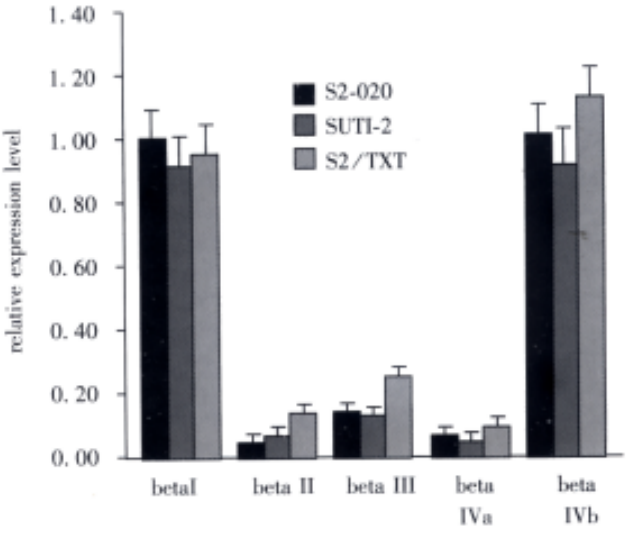

Figure 4 The RT-PCR analysis of the β tubulin isotype transcripts for the SUIT-2 and its two TXT- resistant cell lines. The relative expression levels of transcripts were expressed as a ratio of denitomet ric value of studied gene to that of β actin.

DISCUSSION

An increasing use of Taxanes in the treatment of variety of cancer including advanced PAC, has highlighted the need to elucidate the mechanism responsible for the development of resistance to this kind of drugs. Multidrug resistance may develop through a variety of mechanisms, which may include the alterations of extracellular drug efflux, intracellular extrapment or redistribution, drug detoxification, nuclear target and apoptotic response[42]. With respect to taxane-related resistance, many studies have shown that it is often associated with the expression of mdr1[29,32], but the expression of the mdr1 gene only accounts for part of the resistance mechanisms to taxanes. In

a study by Dumontet *et al*, only 44% of resistant clones were found to express the mdr1 gene, and accumulation studies with labeled PTX did not show altered accumulation in mdr1 negative clones[33]. Such observations indicate that there must be other mechanisms involved in taxanes resistance. Whether or not other drug transporters including MRP and LRP are also involved in this mechanism is not clear. There are reports that cell lines with MRP or LRP phenotype also confer a low level of resistance to taxane[43], though most findings showed that taxane-resistance is independent of MRP expression[44,45].

In this study, both intrinsic and acquired TXT resistant cell lines, which were derived from the same parental cell line, have expression of mdr1 gene, and also these resistances can be reversed almost completely by a P-gp blocker Verapamil. Active drug transporter pump in these TXT-resistant cells was also shown by Rho-123 accumulation assay. Though most of cell lines expressed MRP and LRP as show n by RT-PCR, they had no relationship with the TXT-resistance in these cell lines. To confirm that no MRP mediated TXT resistance exists in these cell lines, we used Indomethacin, a specific inhibitor of MRP[46] and found no sensitizing effect to TXT cytotoxity. LRP positive cell line SUIT-2 had the same sensitivity as LRP negative S2-028 cell lines, showing that there is n o active LRP mediated TXT-resistance. These data indicated that the TXT-related drug resistance in these cell lines is mainly mediated by P-gp. The high incidence of MRP and LRP expressions in these PAC cell lines might be involved in other kinds of intrinsic drugs resistance. Recently, other studies on the mechanisms involve d in taxane-resistance have been focused on the composition and the mutations in â-tubulin isotypes[32,33,47-49]. The possible mechanisms involved in the induction of resistance to taxanes may include altered metabolism and/or subcellular distribution, altered interaction between the tubulin-binding agents and microtubules, and altered response to cell cycle arrest by mitotic blockage.

The taxanes are unique among tubulin-targeted cytotoxins so far as they bind to polymerized tubulin only[24,50]. Direct photoaffinity labeling has demonstrated that taxane binds preferentially to the β subunit of the microtubule[51]. Sequence analysis identified the photoaffinity labeled amino acid residues β270 and β364 as important modulators of paclitaxel's interaction with the tubulin molecule[47]. The direct proofs that altering the isotype composition of a cell affects its sensitivity to any antimitotic drug have been reported, but the results were conflicting[32,33,47,48,52]. Virtually all major β-tubulin isotypes have been reported to be changed in various drug resistant mutants[32-34,4 7-49,52], and this runs counter to the idea that specific isotype of β-tubulin confers altered sensitivity to these drugs. Currently, more direct evidences t hat altered expressions of β-tubulin isotype in taxane-resistance cells contribute to their resistance phenotype have been demonstrated. Blade *et al* [32] by transfecting Chinese hamster ovary cells with cDNA encoding epitopetagged class I,II, and IV b â tubulins found that the production of βI, βII, or βIVb tubulin had no effect on the sensiti vity of the cells to PTX. However, Kavallaris *et al*[53] designed antisense phosphrothioate oligode oxynucleotides targeted against resistant lung cancer cells, and demonstrated that a decrease in class IIIβ tubulin mRNA and protein expression which corresponded to a 39% decrease sensitivity to PTX.

Different mechanism of taxane-related drug resistance may be tumor (or cell line)-dependent or even cell-dependent. Tumor histologic origination and heterogeneity of tumor cells may be responsible for this difference[33]. Since P-gp was often expressed in normal pancreatic tissue[54], P-gp mediated TXT resistance can be found natively in PAC or be readily induced by their subst rates. This study showed the changes of â-tubulin isotype not only in the P-gp-negative taxanes-resistance cells as in other studies[47,48], but also in P-gp-positive cells. However, the S2-020/TXT cells with both P-gp expression and changes of tubulin isotype profile did not show an additive or synergetic effects on TXT-resistance resistant to TXT compared with only P-gp-positive cell line S2-020. On the other hand, this resistance can be fully reversed by P-gp reversal agent Verapamil, suggesting that the change of β-tubulin isotypes might be a collateral change and not necessarily responsible for TXT-resistance in this cell line. Since many critical cellular functions, such as cell movement, mitosis, and maintenance of cell structure are associated with cytoskeletal elements in which the microtubular system plays a major role, it is not clear whether the changes of tubulin in this TXT selected cell line are involved in other biologic behavior of cells, such as cell mobility and inva siveness[55-58], which need to be further investigated.

The different expression of P-gp or tubulin mutation may also be related to the means by which the resistant cells were selected. Multiple step selected cells often present high levels of taxane-resistance mediated by P-gp, while single -step selection yields low level taxane-resistance cells with tubulin mutations[59], since severe tubulin mutations are very likely to affect cell survival and will be lost during the selection. The cells with di fferent mechanisms of taxane-resistance may have different characteristic. TXT-related drug resistan ce mediated by P-gp often presents a cross-resistance to other natural chemical agents, which is different from the taxane-resistance induced by the changes of â-tubulin isotype profile, the latter was often only cross-resistant to other kinds of anti-microtubule agents, such as Vinca alkaloids[33]. However, additive and synergetic effects between Vinorelbine and TXT on human lung cancer[60] seems that their combinations are not contraindicated. Another study has shown that resistance to taxanes can be reduced by increasing the duration of exposure in P-gp expressing cells, but no t in the taxane-resistance cell line which does not express P-gp[61]. Since the resistance was highly drug specific and none of the cell lines was resistant to all drugs, identifying specific mechanism of drug resistance phenotype in certain tumor is helpful in selecting non-cross-resistant regiments and appropriate reversal agents.

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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Fas counterattack in cholangiocarcinoma: a mechanism for immune evasion in human hilar cholangiocarcinomas

Zi-Yu Li, Sheng-Quan Zou

Department of General Surgery of Tongji Hospital, Wuhan, 430030, Hubei Province, China
Correspondence to: Dr. Sheng-Quan Zou, Department of General Surgery of Tongji Hospital, 1095 Jiefang Road, Wuhan 430030, China. Sqzou@tjh. tjmu.edu,cn
Telephone: +86-27-83662851
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Abstract

AIM: **To investigate FasL expression in hilar cholangiocarcinoma tissues and cultured cholangiocarcinoma cells, and to assess its ability to induce apoptosis.**

METHODS: **We studied the expression of FasL by human hilar cholangiocaroinomas tissues by immunohistochemistry, and the QBC939 cholangiocarcinoma cell line by RT-PCR, immunohistochemistry, and Western Blot. TUNEL and flow cytometry were used to detect apoptotic cells.**

RESULTS: **Prevalent expression of FasL was detected in 39 resected hilar cholangiocarcinoma tissues. TUNEL staining disclosed a high level of cell death among lymphocytes infiltrating FasL positive areas of tumor. FasL mRNA and protein expressions in cholangiocarcinoma cells could induce Jurkat cells.**

CONCLUSION: **Hilar cholangiocarcinomas may elude immunological surveillance by inducing, via Fas/FasL system, the apoptosis of activated lymphocytes.**

Subject headings cholangiocarcinoma/immunology; tumor cells, cultured/ immunology; membraneg lycoproteins/ biosynthesis; lymphocytes/immunology; apoptosis

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INTRODUCTION

The evasion, also called 'tumor escape', has been suggested to result from the inability of the immune system to react to the tumor, because of either non-recognition of tumor antigens or non-reactivity secondary to insufficient co-stimulation, anergy, tolerance, or immunosuppression. Recent reports showing the expression of FasL in Sertoli cells of the testis and ocular tissues[1,2], have provided new insights into the concepts of tolerance and immune-privilege. FasL triggers apoptotic cell death of sensitive lymphoid cells which express its cell surface receptor (Apo-1/CD95)[3-5]. FasL has been shown to confer immunological privilege in tissue transplantation experiments[6]. In rodents, successful allograft survival was obtained from FasL expressing tissues[2,7]. Moreover, FasL has been found to be expressed by non-lymphoid tumors as a mediator of immune evasion, which was initially raised by the finding that colon cancer cell lines express functional FasL[8]. Despite being immunogenic, cholangiocarcinoma overcomes antitumor immune responses by the mechanism that has not yet to be fully elucidated. The fact that many diverse tumors have been found to express FasL, suggests that a 'Fas counterattack' against antitumor immune effector cells may contribute to tumor immune escape[9-15]. We studied the expression of FasL on human hilar cholangiocarcinomas and the QBC939 cholangiocarcinoma cell line. We also studied apoptosis of lymphocytes (TILs) in filtrating into tumors. Jurkat cells were cocultured with cholangiocarcinoma cells.

MATERIAL AND METHODS

Human hilar cholangiocarcinoma tissue and cell

Thirty-nine human hilar cholangiocarcinomas of disparate pathological stages were collected from surgical resections performed at Qingdao municipal hospital. The patients were diagnosed as having hilar cholangiocarcinoma by histologic examination, and consisted of 17 males and 22 females. None of the patients have received chemo-, radio- or immuno-therapy before resection. The differentiati on of tumors were moderate (n = 12), poor (n = 18), or high (n = 9). The QBC939 cells (a human hilarcholangiocarcinoma cell line) were a generous gift from Professor Wang (Third Military Medical University, China)[16]. The human T cell line Jurkat was purchased from the American Type Culture Collection (Rockville, MD). QBC939 cells were cultured in DMEM supplemented with 100 mL·L^{-1} FBS. Jurkat cells were maintained in RPMI1640 nutrient medium supplemented with 100 mL·L^{-1} FBS, penicillin (100 KLI·L^{-1}) and streptomycin (100 mg·L^{-1}), and incubated at 37℃ in a 50 mL·L^{-1} CO_2 atmosphere.

Immunohistochemistry for FasL and CD45

Detection of FasL expression and CD45 positive cells was performed using a rabbit polyclonal anti-human FasL specific IgG and a mouse anti-human monoclonal antibody on paraffin sections of human cholangiocarcinoma respectively (Boster Biological Technology Company, Wuhan, China). Five μm thick sections on the slides were deparaffinized, rehydrated and blocked for removing endogenous peroxides activity with 3 mL·L^{-1} H_2O_2 in methanol. Then the sections were washed in PBS and pre-incubated with 50 mL·L^{-1} normal goat serum for 30 min. The slides were incubated with antibodies against FasL and CD45 for 1h at room temperature respectively. After washing, antibody binding was localized using a biotinylated secondary antibody with the ABC detection kit. The slides were counterstained with haematoxylin.

RT-PCR for FasL Mrna

Total RNA was prepared from the QBC 939 cell line with Trizol reagent (Gibco) according to the manufacturer's instructions. RT-

PCR was performed using RT-PCR kit (Promega) according to the manufacturer's protocol. cDNA synthesis was carried out with 2 μg of total RNA. The primers for PCR were 5'-TCCAACTCAAGGTCCATGCC-3' (forward) and 5'-CAGAGAGAGCTCAGATACGTTT-3'(reverse). PCR reactions mith total volume of 50 μL were processed in a MJ. PTC 100 Thermocycler under the following conditions: 94℃ for 2 min; 94℃ for 30 s, 57℃ for 45 s, and 72℃ for 1 min for 35 cycles; and 72℃ at 5 min. The RT-PCR products (342 bp fragments) were analyzed on 20 g·L^{-1} agarose gels. Amplication of human β-actin served as control for sample loading and integrity.

Western blotting

Immunoblotting was performed for detection of FasL. Cells (1×10^6) were scraped, centrifuged briefly, and lysed for 30 min on ice in 50 mmol ·L^{-1} Tris-HCl buffer (pH 8), containing 120 mmol·L^{-1} NaCl and 10 g·L^{-1} lyepal supplemented with the complete-TM mixture of proteinase inhibitors. T h e total protein was collected by centrifugation (14,000 r·min^{-1}, 30 min, 4℃) and assessed for protein concentration. SDS-PAGE (120 g·L^{-1}) was performed, an d the proteins were electroblotted onto nitrocellulose membranes. After 1h incubation in blocking solution (200 mL·L^{-1} IgG-free normal horse serum in PBS), the membrane was exposed to the primary antibody overnight at 4℃. After washing in PBS, the secondary peroxidase-labeled antibody was added at a 1:10 000 dilution for 40 min at room temperature. The proteins were visualized with the enhanced chemiluminescence technique.The primary anti-FasL antibody was the clone 33 (Jingmei Biotech Co. Ltd. China) mAb (1:1 000 dilution). The secondary antibody was peroxidase-labeled anti-mouse IgG antibody.

Cell death detection in situ by TUNEL

Cell death was detected *in situ* in resected tissues by enzymic labelling of DNA strand breaks using a TUNEL assay (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. Only those cells with positive TUNEL staining and of apoptotic morphology were considered apoptotic.

T-cell apoptosis analysis

Cholangiocarcinoma cells were seeded in 6-well tissue culture plates and allowed to grow to 90% confluence. The cells were then washed twice with PBS and fixed with 20 g·L^{-1} paraformaldeyhde at 4℃ for 1 h. After the cells were washed 3 times with PBS, they were layered with 2 mL of Jurkat cell suspension (5×10^8 T cells·L^{-1}) in serum-containing media. After 48 h of coculture, Jurkat cells were collected from the 6-well plates, centrifuged, fixed in 700 mL·L^{-1} ethanol, and stored at -20℃ prior to analysis. Apoptotic cells were detected as a sub-G_1 fraction after propidium iodide staining and analysis using a FACScan.

RESULTS

Immunohistochemical localization of FasL

Paraffin sections from hilar cholangicarcinomas (n = 39) were stained for FasL. Positive staining for FasL was seen in the tissue of all 39 patients with hilar cholangiocarcinomas assessed (Figure 1). Positive staining of neoplastic tissue varied in both intensity and extent from individual tumor cell to cell, region to region in tumor and among tumors. Intensity of staining varied from weakly positive neoplastic areas to in tense regions and was stronger than that observed in local FasL positive TILs staining areas where staining was locally uniform with nests of tumor cells. All tumors examined were predominantly FasL positive (>70% of tumor area). To generate further evidence that FasL is expressed by human hilar cholangiocarcinoma cells, we also identified the protein expression of FasL in QBC939 cholangiocarcinoma cell line (Figure 2).

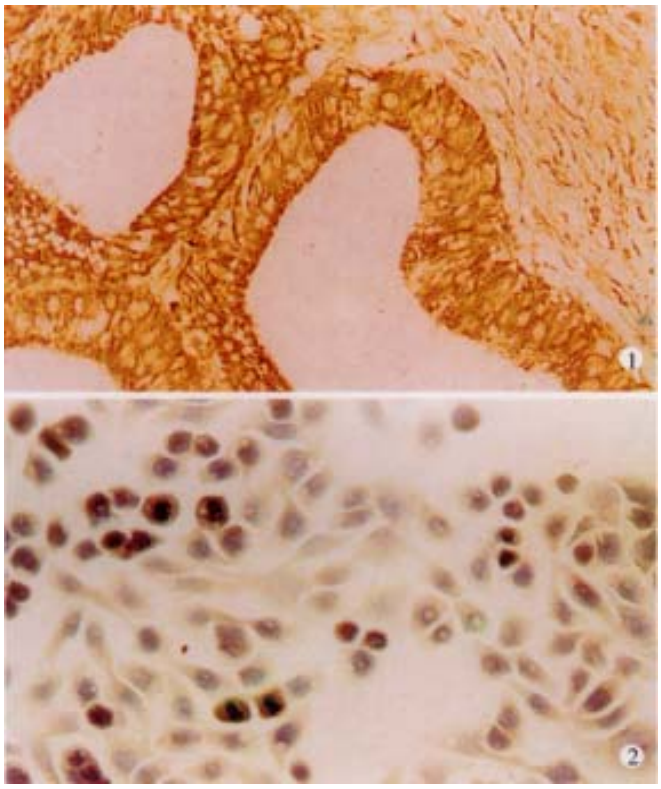

Figure 1 FasL positive in human hilar cholangiocarcinom as (brown). SABC×200
Figure 2 Expression of FasL in cholangiocarcinoma cell line. QBC939×200

Expression of FasL mRNA

To confirm the results obtained from the immunohistochemical studies, we evaluated the expression of FasL mRNA in the QBC939 human cholangiocarcinoma cell line. Total RNA was extracted and tested using RT-PCR. FasL mRNA was identified in QBC939 cells (Figure 3).

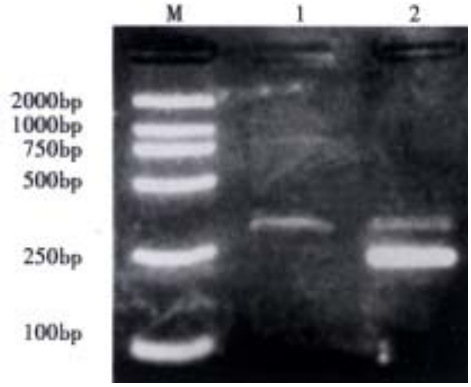

Figure 3 Expression of FasL mRNA in human cholangiocarcinoma cells QBC939. M: DL 2000 Marker; 1: FasL; 2: FasL+β-actin

Apoptosis of TILs

CD45 immunohistochemistry showed immunocyte infiltration in all 39 carcinomas (Figure 4). Most of the CD45 positive cells were of lymphoid morphology. Apoptosis was detected by TUNEL among TILs adjacent to FasL positive areas of the hilar cholangiocarcinomas. These

TUNEL positive TILs exhibited morphological features of apoptosis, including nuclear condensation and fragmentation (Figure 5). This was a consistent finding in all the tumors examined (n = 15).

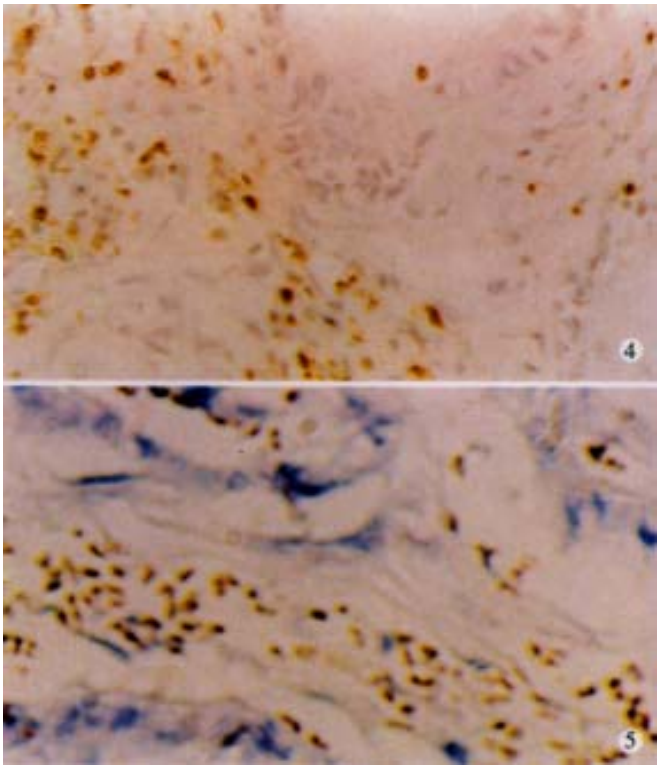

Figure 4 CD45 positive cells(brown) of lymphoid morphology adjacent to carcinoma. ×200

Figure 5 Positive apoptotic TUNEL stainig *in situ* (brown) with apoptotic morphology.

FasL by Western blot

Whole cell lysates from QBC939 cell cultures were electrophoresed in a polyacry lamide gel. We had previously obtained mRNA from the same samples, Mr 37 000 protein was recognized on line 06 clone 33 (Figure 6).

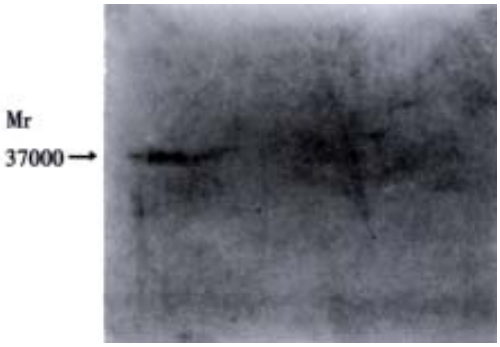

Figure 6 Western blotting of FasL protein with mAb from QBC939 cell cultures clone 33 from QBC939 cell cultures

Is functional FasL expression

To determine if FasL expressed by cholangiocytes in culture was capable of inducing cell death, we cocultured fixed cholangiocacinoma cells with FasL-sensitive (Jurkat) thymocytes. The QBC939 cholangiocarcinoma cell line induced cell death in FasL-sensitive T cells. At 48 h of coincubation, the cellline induced cell death in 50% of FasL-sensitive cells. These data show that the FasL expressed by the cholangiocarcinoma cells is capable of inducing T-cell apoptosis and therefore is functional.

DISCUSSION

It has been shown that hilar cholangiocarcinomas could express FasL, an inducer of immunocyte apoptosis in our study. The expression of FasL potentially enables hilar cholangiocarcinomas to counterattack and kill antitumor immune effector cells that seem Fas sensitive. As an established mediator of immune privilege and immunological tolerance in the eye and testis, functional FasL expressed in hilar cholangiocarcinomas was recognized as a contributor to the im mune evasion of hilar cholangiocarcinoma. Expression of FasL in human hilar cholangiocarcinomas was prevalent; all 39 samples resected from hilar cholangiocarcinomas were found to express FasL protein. FasL staining was variable in both intensity and extent within tumors. The fact that extensive expression (>70% of the tumor area) occurred in all tumors irrespective of tumor stage or degree of differentiation suggest that FasL may be expressed throughout hilar cholangiocarcinoma progression. In contrast with the extensive expression detected in the hilar cholangiocarcinomas, FasL expression was consistently restricted to only those epithelial cells at the luminal surface in control normal biliary epithelial sections (n = 6).It was estimated that a down-regulation and upregulation of FasL expression occurs during the transformation process. Using well-characterized, stable primary culture system of human cholangiocarcinoma cells, we found that cholangiocarcinomas in culture could express mRNA and protein of FasL and induce cell death in T cells. Apoptosis of TILs is an evidence *in vivo* which indicated that FasL expressed by the tumor is functional and can kill Fas sensitive anti tumor immune effector cells.

Several cancers have been reported to express FasL. The tumor derived cell lines in all of the cases could induce apoptosis in Fas sensitive, but not Fas resistant lymphoid target cells *in vitro*[17-30]. Tumors and cell lines themselves usually exhibit resistance to FasL mediated apoptosis because of various defects acquired in Fas signal transduction[31-37,44-48]. Immunogenic tumor cells are probably subjected to a barrage of c ell mediated cytotoxic antitumor immune assaults[38-43,49-52]. The fact that most tumor cells are efficiently killed by LAK cells *in vitro* suggests that cancer cells probably exhibit some degree of susceptibility to cell mediated cytotoxic mechanisms. Expression of a molecule to defuse antitumor immune challenge clearly offers a protective advantage to tumor growth and development. As an established mediator of immunological tolerance and privilege, FasL is such a molecule. Our findings have conclusively show that human hilar cholangiocarcinomas express functional FasL. Hilar cholangiocarcinoma may therefore be added to the growing list of malignancies that appear to be immunologically privileged through FasL expression. The high prevalence of FasL expression in the tumors suggests that this molecule may be critical to tumor immune privilege. In conclusion, the Fas counterattack appears to prevail as a potentially critical mechanism of immune privilege in human hilar cholangiocarcinoma.

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Edited by Wang YQ and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• ORIGINAL RESEARCH •

Relationship between genotype and phenotype of flagellin C in Salmonella

Wan-Sheng Ji[1], Jia-Lu Hu[1], Jun-Wen Qiu[1], Bo-Rong Pan[1], Dao-Rong Peng[2], Bing-Long Shi[1], Shao-Juan Zhou[1], Kai-Chun Wu[1], Dai-Ming Fan[1]

[1]Chinese PLA Institute of Digestive Diseases, [2]Department of Bacteriology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China
Correspondence to: Jia-Lu Hu, Chinese PLA Institute of Digestive Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi Province, China. jiwsh@netease.com
Telephone: +86-029-3375229(lab), Fax: +86-29-7432505(home)
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Abstract

AIM: **To discover the relationship between the genotype and antigen serotype of flagellin C among Salmonella strains.**

METHODS: **Fragment of Salmonella flagellin C in plasmid pLS408 was cloned, sequenced and compared with the corresponding sequence in other strains. Salmonella strains including two typhi strains, one paratyphoid strain, one enteritidis and one typhimurium strain were isolated from outpatients. Genome DNA was purified respectively from these clinical isolates, then the corresponding flagellin C fragment was amplified by polymerase chain reaction, and the amplification products were analyzed by agarose gel electrophoresis.**

RESULTS: **The cloned fragment includes 582 nucleotides encoding the variable region and partial conservative region of Salmonella flagellin C in plasmid pLS408. With comparison to the corresponding sequences reported previously, there is only a little difference from other strains with the same flagellar serotype in both nucleotide and amino acid level. Specific PCR products were amplified in Salmonella strains with flagellar serotype H-1-d including *S. muenchen*, typhi and typhimurium, but not in *S. paratyphoid* C or S. enteritidis strains.**

CONCLUSION: **In this experiment, the specificity of nucleotide sequence could be found in flagellin C central variable regions as it exists in flagellar serotypes in Salmonella. It may be helpful to developing a rapid, sensitive, accurate and PCR-based method to detect Salmonella strains with serotype H-1-d.**

Subject headings: Salmonella; flagellin C; polymerase chain reaction; serotype; genotype

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INTRODUCTION

Bacteria of genus Salmonella are both major and minor pathogens that cause disease outbreaks that arise from single incidents of breakdown in food hygiene. Much effort has been devoted to methods that differentiate these organisms in order that a given outbreak may be traced to its source and breakdown. Serological analysis of the surface antigens, such as flagellar antigen, has been proven to be the most suitable approach and has resulted in the recognition of a large number of serotypes.

In the past decade, the nucleotide sequences of flagellin in several Salmonella strains were published, the information of secondary and tertiary structure of flagella was discovered by chemical methods and X-ray crystallogram, and the antigen variable region was also determined roughly. These results indicate that serotype of flagella is determined by the linear primary structure of flagellin and the strain with different serotypes has different amino acid sequence in the central region of flagellin.

According to the standpoint that phenotype is determined by corresponding genotype, it is reasonable to think that the differentiation in the variable region of flagellin may be reflected by the nucleotide sequence. Detection of nucleotide difference by specific amplification may be helpful for the diagnosis and typing of Salmonella.

MATERIAL AND METHODS

Strains and plasmid

Salmonella 5930, which contains plasmid pLS408, was offered by Professor Stocker (Stanford University School of Medicine). Plasmid pUC18 and Eschrichiacoli. DH-5á was stored in our laboratory. Salmonella strains including two typhi strains, one paratyphoid strain, one enteritidis and one typhimurium strain were isolated from outpatients, and samples were stored in Department of Bacteriology, Xijing Hospital.

Cloning of partial flagellin C gene fragment

Plasmid pLS408, in serted with the complete sequence of flagellin C from *S. muenchen*[1], was prepa red as template by plasmid purification kit (Shanghai Watson Biotech. LTD). Primers, including EcoRV and *KpnI* sites for convenient cloning, were designed referring to the complete sequence of flagellin C in *S. muenchen* and *S. typhi*. Forward primer: 5' GCAGGATATCTTCCTCGAGACCACAGTTGCGGCTC 3'; reverse primer: 5' TGCGCC AGAACGGAGGTACC 3'. PCR product was digested by EcoRV and *KpnI*, ligated to pUC18 digested by *HinCII* and *KpnI* and sequenced in Sangon.

Amplification of flagellar serotype specific fragment in Salmonella strains

Genome DNA was prepared by genome purification kit according to the Watson's Handbook for DNA Isolation an d Purification. Amplification was performed by two-step polymerase chain reaction, i.e. preheating at 94℃ for 5 min, then denaturing at 94℃ for 45 s and an nealing at 68℃ for 1 min, 30 cycles with a final extension at 72℃ for 10 min. Amplification products were analyzed by 1.5 $g \cdot L^{-1}$ agarose gel electrophoresis.

RESULTS

The map of plasmid pLS408 and the result of agarose gel electrophoresis of fliC PCR product were demonstrated (Figure 1).

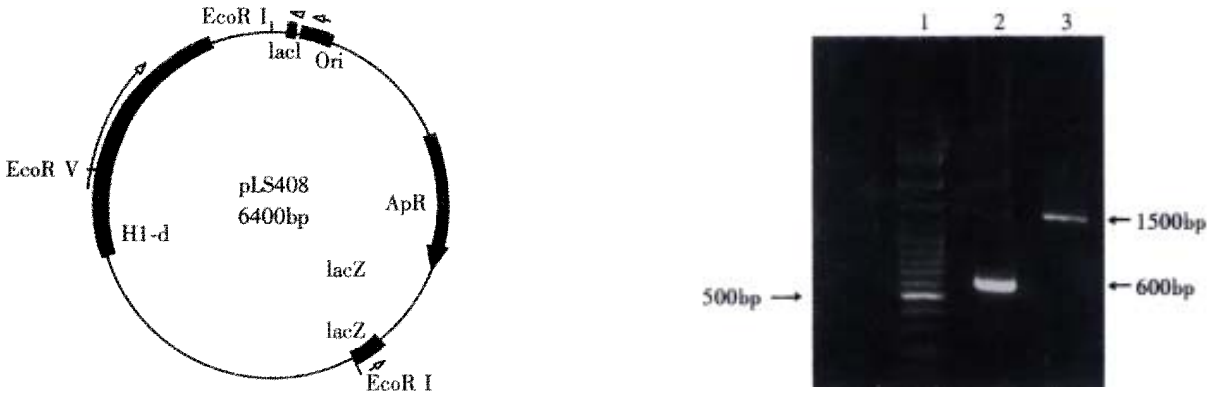

Figure 1 Map of pLS408 (left) and gel electrophoresis of PCR p roduct (right).
Lane 1: 100 bp DNA ladder, lane 2: PCR product of partial flagellin C gene, lane 3: PCR product of flagellin C gene.

The amplification product includes 582 nucleotides encoding variable region and partial conservative region of flagellin C. In comparison with the corresponding sequences reported before, there is only a little difference from other strains with the same flagellar serotype in both nucleotide and amino acid level (Figures 2 and 3). Compared with the known *S. muenchen* sequence, there are three single-nucleotide insertions, which cause frame shift and ten amino acid changes (166-195). T here are three single-nucleotide mutations, which cause two missenses (23:C to T, 136:G to A) and one silent mutation (216: G to T), and a double-nucleotide mutation (281, 282:TA to AT) and a three-nucleotide short fragment mutation (217, 218, 219: CTC to GCT), which causes one missense mutation, respectively. Though t here are some differences between the sequence here and that derived from the other *S. muenchen* strain, it is conservative highly with the corresponding sequence in Salmonella strains with the same flagellar serotype.

1	ACCACAGTTGCGGCTCAACTTGTTGCTGCAGGGGTTACTGGTGCCGATAAGGACAATACT
	ACCACAGTTGCGGCTCAACTTGCTGCTGCAGGGGTTACTGGTGCCGATAAGGACAATACT
61	AGCCTTGTAAAACTATCGTTTGAGGATAAAAACGGTAAGGTTATTGATGGTGGCTATGCA
	AGCCTTGTAAAACTATCGTTTGAGGATAAAAACGGTAAGGTTATTGATGGTGGCTATGCA
121	GTGAAAATGGGCGACAATTTCTATGCCGCTACATATGATGAGAAAACAGGTACAATTACT
	GTGAAAATGGGCGACGATTTCTATGCCGCTACATATGATGAGAAA_CAGGTACAATTACT
181	GCTAAAACAACCACTTATACAGATGGTGCTGGCGTTGCTCAAACTGGAGCTGTGAAATTT
	GCTAAA_CAACCAC_TATACAGATGGTGCTGGCGTGCTCCAAACTGGAGCTGTGAAATTT
241	GGTGGCGCAAATGGTAAATCTGAAGTTGTTACTGCTACCGATGGTAAAACTTACTTAGCA
	GGTGGCGCAAATGGTAAATCTGAAGTTGTTACTGCTACCGTAGGTAAAACTTACTTAGCA
301	AGCGACCTTGACAAACATAACTTCAGAACAGGCGGTGAGCTTAAAGAGGTTAATACAGAT
	AGCGACCTTGACAAACATAACTTCAGAACAGGCGGTGAGCTTAAAGAGGTTAATACAGAT
361	AAGACTGAAAACCCACTGCAGAAAATTGATGCTGCCTTGGCACAGGTTGATACACTTCGT
	AAGACTGAAAACCCACTGCAGAAAATTGATGCTGCCTTGGCACAGGTTGATACACTTCGT
421	TCTGACCTGGGTGCGGTACAGAACCGTTTCAACTCCGCTATCACCAACCTGGGCAATACC
	TCTGACCTGGGTGCGGTACAGAACCGTTTCAACTCCGCTATCACCAACCTGGGCAATACC
481	GTAAATAACCTGTCTTCTGCCCGTAGCCGTATCGAAGATTCCGACTACGCGACCGAAGTC
	GTAAATAACCTGTCTTCTGCCCGTAGCCGTATCGAAGATTCCGACTACGCGACCGAAGTC
541	TCCAACATGTCTCGCGCGCAGATTCTGCAGCAGGCCGGTACC*
	TCCAACATGTCTCGCGCGCAGATTCTGCAGCAGGCCGGTACC#

Figure 2 Comparison of nucleotide sequence between PCR product and the corresponding sequence reported previously (all differences are shadowed or dashed).
*Sequence of partial flagellin C PCR product, #Sequence from Genbank X0 3395

1	TTVAAQLVAAGVTGADKDNTSLVKLSFEDKNGKVIDGGYAVKMGDNFYAATYDEKTGTITAKTTT
	TTVAAQLAAAGVTGADKDNTSLVKLSFEDKNGKVIDGGYAVKMGDDFYAATYDEKQVQLLLNN
	TTVAAQLAAAGVTGADKDNTSLVKLSFEDKNGKVIDGGYAVKMGDDFYAATYDEKTGAITAKTTT
66	YTDGAGVAQTGAVKFGGANGKSEVVTATDGKTYLASDLDKHNFRTGGELKEVNTDKTENPLQKID
	YTDGAGVLQTGAVKFGGANGKSEVVTATVGKTYLASDLDKHNFRTGGELKEVNTDKTENPLQKID
	YTDGTGVAQTGAVKFGGANGKSEVVTATDGKTYLASDLDKHNFRTGGELKEVNTDKTENPLQKID
131	ALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLSSARSRIEDSDYATEVSNMSRAQILQQAGTS*
	AALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLSSARSRIEDSDYATEVSNMSRAQILQQAGTS#
	AALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLSSARSRIEDSDYATEVSNMSRAQILQQAGTS&

Figure 3 Comparison of amino acid sequences among different strains with (all differences are shadowed).
*Amino acid sequence of PCR product. #The corresponding sequences of *S. muenchen* (Genbank X03395). &The corresponding sequence of *S. typhi* (Genbank L21912)

By polymerase chain reaction, amplification products appeared only in those salmonella strains with specific flagella antigen type (H-1-d) as *S. muenchen*, typhi or typhimurium, but not in *S. paratyphoid* C or *S. enteritidis* (Figure 4).

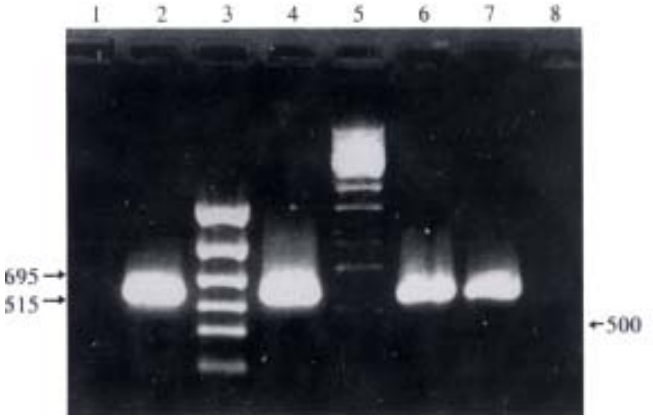

Figure 4 Amplification of flagellin C gene fragment in different Salmonella strains.
Lane 1: S. enteritis; 2,4: *S. typhi*; 3: PCR DNA marker; 5: 100 bp DNA lad der; 6: *S. typhi*murium; 7: *S. muenchen* (plasmid pLS408); 8: S. typhoid.

DISCUSSION

Flagella is a necessary organelle for bacterial motility and its biological behavior is well controlled[2-23]. The flagellar filament is composed of a single protein, flagellin. Serological analysis has proved that diverse types exist among different Salmonella strains[24-26]. The a minoacid sequence of flagellin has been assigned to four domains of flagellin subunit structurally identified in the filament structure, based on biochemical, immunological and structural information[27,32]. The terminal regions form the c ore of t he filament and the central regions form the outer part. Despite the high conservatism in terminal regions, the great divergence in central regions was discovered in both amino acid and nucleotide sequence among the different Salmonella strains. This point was also strongly supported by our experiments.

By comparison with other sequences in Genbank, we found the conservatism of flagellin C central regions among the different strains with the same flagellar antigen type. It is the foundation for the design of primers. In this experiment, primers were designed by software (primer 3 on the Internet) to amplify the flagellin C gene with specific antigen type H-1-d. The sequence of flagellin C variable region in *S. muenchen* was sequenced and compared with other sequence. The cloned fragment was highly aligned with other strains. In fact, the specific amplification occurred in all isolates with H-1-d serotype like *S. muenchen*, typhi or typhimurium, but not in other serotypes like *S. enteritidis* or typhoid C strain and the negative amplification was found in other bacteria like *Helicobacter pylori* is olates or *Eschrichia Coli.* strains (data not shown).

As a developed tool, PCR-bas ed technique has been widely used in both basic and clinical research[33-58]. In bacterial taxonomy, PCR-based genotype typing is complementary to serological typing[59-61]. Furthermore, as a rapid and convenient method, polymerase chain reaction is very helpful to the detection of many microbes like *H. pylori*, Salmonella, crypt ococcal neoformans and many other microbes [62-66]. In this experiment, the specificity of nucleotide sequence could be found in central variable regions of flagellin C as it exists in flagellar serotypes in Salmonella.

In summary, the specificity of nucleotide sequence was found in central variable regions of flagellin C as it exists in flagellar serotypes in Salmonella in this experiment. It may be helpful in the develop ment of a rapid, sensitive and accurate method to detect Salmonella strains with serotype H-1-d.

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Edited by Xu XQ and Wang JH

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF PEPORT •

Preliminary research on myosin light chain kinase in rabbit liver

Bin Ren[1], Hua-Qing Zhu[2], Zhao-Feng Luo[1], Qing Zhou[2], Yuan Wang[2], Yu-Zhen Wang[1]

[1]Department of Biochemistry and Molecular Biology, University of Science and Technology of China, Hefei 230027, Anhui Province, China
[2]Laboratory of Molecular Biology, Anhui Medical University, Hefei 230032, Anhui Province, China
Supported by the National Natural Science Foundation of China (39870324, YW), Key Innovation Project of Chinese Academy of Science, P.R. China (KSCX 2-2-01, XB Yao), Grant for Excellent Young Teachers of Ministry of Education of China (99044312, YW) & Natural Science Foundation of Anhui Province (99044312, YW)
Corresponding to: Prof. Yu-Zhen Wang, School of Life Science, University of Science and Technology of China, Hefei 230027, Anhui Province, China. yzwang@ustc.edu.cn
Telephone: +86-551-5118963, Fax: +86-551-3601441
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Abstract

AIM: **To study preliminarily the properties of myosin light chain kin ase (MLCK) in rabbit liver.**

METHODS: **The expression of MLCK was detected by reverse transcription-polymerase chain reaction (RT-PCR); the MLCK was obtained from rabbit liver, and its activity was analyzed by γ-^{32}P incorporation technique to detect the phosphorylation of myosin light chain.**

RESULTS: **MLCK was expressed in rabbit liver, and the activity of the enzyme was similar to rabbit smooth muscle MLCK, and calmodulin- dependent. When the concentration was 0.65 mg·L^{-1}, the activity was at the highest level.**

CONCLUSION: **MLCK expressed in rabbit liver may catalyze the phosphorylation of myosin light chain, which may play important roles in the regulation of hepatic cell functions.**

Subject headings myosin light chain kinase; liver, rabbit; enzyme activity; reverse transcription-polymerase chain reaction

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INTRODUCTION

Protein kinases perform important regulatory roles in response to both intra cellular and extracellular signals. Specific protein kinases are thought to control various cellular functions including glycogen metabolism, muscle contraction , and growth. Myosin light chain kinase (MLCK) is an enzyme of the kinase family, which phosphorylates the light chain of smooth muscle, skeletal muscle and no n-muscle myosin in the presence of Ca^{2+} and CaM, id it requires the C a^{2+} and the Ca^{2+} binding protein CaM for the activity. Phosphorylation of the light chain in skeletal muscle is involved in modulating the tension produced during contract ion, whereas in smooth muscle it appears to be required for initiation of contraction. Phosphorylation of myosin regulatory light chain (RLC) by smooth muscle myosin light chain kinase is a key event initiating smooth muscle contraction. However, the roles of myosin phosphorylation in non-muscle function is not well understood. However, present researches show that a variety of non-muscle processes are associated with MLCK, including endothelial cell retraction, fibroblast con traction, mast cell secretion, receptor capping in lymphocytes, and platelet aggregation, contraction, etc. That MLCK controls endothelial Ca^{2+} entry in endothelial cells not through myosin light chain phosphorylation suggests its role in vasodilation through its action in endothelial cells[1]. The activation of volume-regulated anion channels in macrovascular endothelium is modulated by myosin light chain phosphorylation through the action of MLCK or myosin light chain ph osphatase[2].

Ma *et al*[3] incubated the filter-grown Caco-2 intestinal monola yers in Ca^{2+} free solution (CFS) and found an increase in tight junction p ermeability of the monolayer. The CFS-induced modulation of tight junction barrier was related with the activation of MLCK activity and centripetal retraction of peri-junctional actin and myosin filaments. Ueno *et al* reported that a Ca^{2+}-CaM- dependent protein kinase purified from rabbit liver phosphorylated the regulator y light chain of hepatocyte myosin. The kinase catalyzed the incorporation of ph osphate into the 22 ku light chains of hepatocyte myosin, which resulted in a 7 - fold activation of the Mg (2+)-ATPase activity by F-actin. MLCK is possibly involved in many Ca^{2+}-dependent activities of monocytes or macrophages[4]. It is found that there are at least two different stress fiber systems in human foreskin fibroblasts including central stress fiber system and peripheral stress fiber system, and the latter system depends on MLCK[5]. MLCK plays an important role in the development of neuron. Preventing calcium influx through blocking of MLCK activity selectively decreased dendritic branching[6]. A novel MLCK cDNA was isolated from a HeLa cell cDNA library. The deduced amino acid sequence was identical to that of a zipper-interacting protein kinase, which mediates apoptosis. With the fragment of the bovine stomach MLCK gene including kinase and calmodulin regulatory domains as a probe. Murata-Hori *et al*. screened a HeLa cell cDNA library. They found that one serine/threonine kinase, HeLa zipper-interacting protein kinase, from non-muscle cells phosphorylated regulatory chain of myosin II[7]. Is there any novel MLCK in hepatocytes? How is MLCK involved in cellular functions of hepatocytes? In order to investigate the roles of MLCK in the maintenance of liver functions and its association with some liver diseases in the future study, we preliminarily studied the expression of the enzyme in the rabbit liver by reverse transcription polymerase chain re action (RT-PCR) and observed some of its properties preliminarily through assaying its catalytic activity of the phosphorylation of myosin light chain by γ-^{32}P incorporation method. Our preliminary research provides basis for our further investigation of functions of MLCK in the liver and its relation with the pathology of some hepatic diseases.

MATERIALS AND METHODS

Reagents and instruments

CaM, rabbit smooth muscle MLCK positive control and myosin

regulatory light chain were the gifts from Dr. Zhi at University of Texas Southwestern Medical Center, USA. EGTA and MOPS were purchased from Sigma Chemical Company, and DTT, phenylmethy sulfonylflouride (PMSF) and proteinase inhibitors from Sino-America n Biotechnological Corp, Shanghai. HEPES was produced by MERCK; EDTA was obtained from Life Technology, GibicoBRL. [γ-^{32}P]ATP (radio activity > 185 PBq ·mol-1·L^{-1}) was purchased from Yahui Biomedical Engineering Corp of Beijing. Other reagents were made in China and they were of analytical purity. Backman LS1701, Liquid Scintillation System was made in USA and DYY-III type-2 electrophoresis and transfer system were made in June 1 Instrument Factory of Beijing and idEA Ideal Sci Co. UV-754 Spectrophometer was made by The Third Factory of Analytical Inst ruments of Shanghai.

Tissue samples and hepatocyte isolation

Fresh liver tissues were obtained from New Zealand white rabbits and hepatocytes were isolated in reference as described elsewhere[8]. For liver tissues they were rinsed with cold Hanks solution (NaCl 137; KCl 5.0; $CaCl_2$ 1.3; $MgSO_4 \cdot 7H_2O$ 0.8; Na_2HPO_4 0.6; KH_2PO_4 0.4; $NaHCO_3$ 3.0; glucose 5.6 mmol·L^{-1}; pH7. 4). Blood vessels and other tissues were removed carefully. After washing with Hanks solution, the liver was cut into about 1 mm^3 slices and homogenized or stored at -80℃ for further processing.

RT-PCR of MLCK fragments[9,10]

For RT-PCR, hepatocytes were directly lysed with TRIZOL Reagents (GibcoBRL), and total cellular RNA was isolated according to the manufacturer's instructions. First-strand cDNA was generated in the presence of 0.5 μg·L^{-1} Oligo (dT) 12-18 from 5 μg total RN A with reverse transcriptase (Superscript™ Preamplification System, GibcoBRL). The paired primers to detect endogenous MLCK fragments were designed according to the r eported MLCK sequence, and they are paired primer one: 5'> AAGAATTCGATGTCAGCTGAAC <3' and 5' CTTCTCCAGAAGCTTATAGGA <3', and paired primer two: 5'> CCACTGGTG AAGCTTAAAATC<3' and 5'> TGGAATTCCATGGGGGACGTGAA <3'. The PCR was performed in reference as described elsewhere[9]. The PCR products were examined by 20 g·L^{-1} agarose gel and ethidium bromide staining. The photos were taken for the analysis of the PCR products.

Preparation of MLCK from rabbit liver

Using metho d described by Ramji *et al* and Tang *et al*[11], the liver tissues were obtained. Briefly, the liver slices removed blood vessels and other tissues were put into glass homogenizer with addition of suit amount of tissue buffer for homogenizat ion on the ice block, and the homogenized tissues were thawed for three times (5 min once) after homogenizing. 16 000 r·min^{-1} centrifugation for 30 min at 4℃, and MLCK was prepared by the method described by Wang *et al* and Bartelt *et al*. The purified MLCK was used for activity assay

Assay of calmodulin- and calcium-dependent MLCK activity

Ca^{2+}/CaM-dependent activity of MLCK was measured by rates of [γ-^{32}P]ATP incorporation into myosin light chain as substrate referring to Wang *et al* and Blumenthal *et al*. Briefly, the relation of MLCK activity with time, regulatory light chain and CaM concentrations as well as with MLCK concentrations were analyzed. Maximal activity was determined in the reaction buffer containing 50 mmol·L^{-1} MOPS in mmol·L^{-1} at pH 7.0: magnesium acetate 10, dithiothereitol 1, $CaCl_2$ 0.3; 1 mmol·L^{-1} [γ-^{32}P] ATP (200-300 cpm·pmol^{-1}) ; 1.2 μmol·L^{-1} CaM, 25 μmol·L^{-1} regulatory light chain of myosin, and diluted MLCK at room temperature. MLCK was freshly diluted in 10 mmol·L^{-1} MOPS (pH 7.0), 1mmol·L^{-1} dithiothreitol, and 1 g·L^{-1} bovine serum albumin and a dded to the reaction mixture. Final MLCK concentrations used in kinetic measurements showed linear phosphorylation rates with respect to time and enzyme concentration in a certain range.

Protein contents determination

Lowry's method was used for the determination of contents of various proteins, in which bovine serum albumin was used as the standard.

Statistical analysis

Data of MLCK activity were collected and processed through analysis of difference of MLCK activity by Student's *t* test with SPSS 8.0 Windows software. $P<0.05$ or $P<0.01$ was considered as statistically significant in the difference of MLCK activity.

RESULTS

Confirmation of MLCK expression in rabbit hepatocytes by RT-PCR

Two pairs of specific primers were used to detect the expression of MLCK in freshly is olated hepatocytes. The total cellular RNA was isolated and first-strand cDNA was generated. The results showed that the RT-PCR products were 400 bp and 450 bp respectively, confirming with the expected molecular size (Figure 1), which suggests that there is expression of MLCK in rabbit liver.

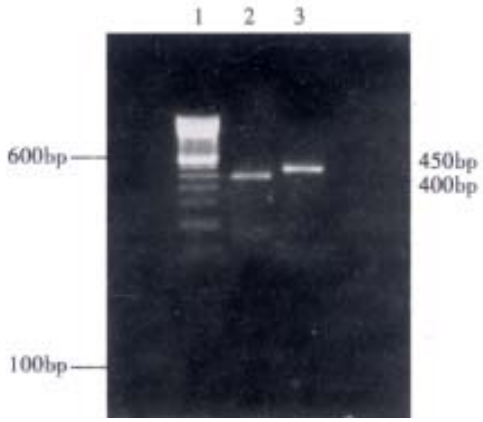

Figure 1 Reverse transcription polymerase chain reaction of cellular total RNA from hepatocytes in New Zealand rabbit. 1.100 bp DNA ladder; 2-3, PCR products: amplified MLCK DNA fragments.

Features of rabbit liver MLCK activity analysis

Comparison of activity of rabbit liver MLCK with that of smooth muscle MLCK To observe the activity level of our isolated MLCK in rabbit liver, we assayed the activity of the MLCK and smooth muscle MLCK. I t was found that both kinds of MLCK had similar catalytic activity, there is no statistically significant difference between two kinds of enzymes ($P<0.01$ £½ by Student's t test (Figure 2 A).

MLCK from rabbit liver was CaM-dependent To confirm the MLCK isolated from the rabbit liver is dependent on CaM, we assayed the effect of phosphorylation of MLC K on regulatory light chain of myosin. It was found that the light chain was obv iously phosphorated when CaM was added into the reaction buffer at the suitable concentration of Ca^{2+}. The MLCK activity increased markedly with CaM added. Ther e is significantly statistical difference when compared with the activity of the MLCK without adding CaM ($P<0.01$) through Student's t test analysis with SPSS 8.0 Windows software. See Figure 2 (B).

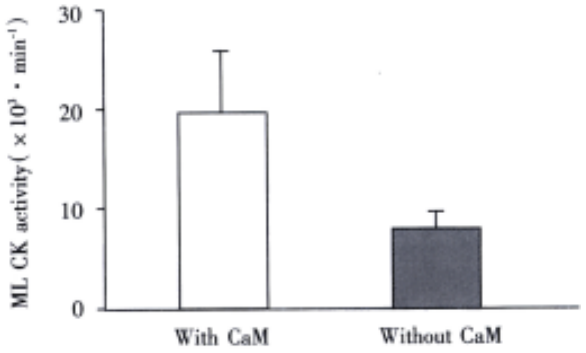

Figure 2 (A) Comparison of enzymatic activity between smMLCK and rabbit liver MLCK (P>0.05)

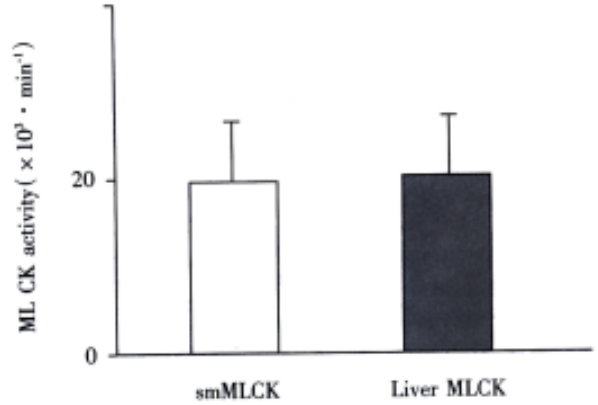

Figure 2 (B) Effect of CaM on MLCK in rabbit liver activity (P<0.01)

Action characteristics of MLCK of rabbit liver In the reaction system, enzymatic catalytic action of the MLCK increased gradually at first three min and the extent of phosphorylation of the regulatory light chain of myosin was at the highest level at about three min on the suitable concentrations of substrate, Ca^{2+} and CaM. In addition, the activity of MLCK of rabbit liver changed with the changes of the substrate concentrati on or the concentration of light chain of myosin. When the concentration was 0.65 mg·L^{-1}, the activity of MLCK was at the highest level, the most amount of light chain was phosphorated at this time. Finally, MLCK concentration influenced t he activity of the enzyme itself. On the suitable substrate, CaM and calcium ion concentrations, the activity of MLCK from the rabbit liver was basically at the higher level when MLCK ranged from 15 mg·L^{-1} to 25 mg·L^{-1}.

DISCUSSION

MLCK is the key regulator of cell motility and smooth muscle contraction in higher vertebrates. MLCK expression shows a complex pattern. In undifferentiated myoblasts, 220-kulong or non-muscle form of MLCK is expressed during differ entiation of skeletal muscle. During myoblast differentiation, expression of the 220-ku MLCK declines and expression of this long-form is replaced by 130 ku smooth muscle MLCK and a skeletal muscle-specific MLCK. In fact, 130 ku smooth muscle MLCK is not a smooth muscle-specific protein, it is ubiquitous in all adult tissues[12]. A prerequisite for vertebrate smooth muscle contraction, potentiation of skeletal and cardiac muscle contraction and various non-muscle motile events in response to intracellular Ca^{2+} signaling is the phosphorylation of myosin II RLC at Ser-19 by MLCK. The phosphorylation of RLC increases the Mg^{2+}-ATP activity of myosin, which catalyses cyclic conversion of ATP chemical energy into mechanical work through the reversible actomyosin interactions. Ca^{2+}-calmodulin -dependent phosphorylation of RLC of myosin by the catalytic COOH-terminal half of MLC K activates myosin II in smooth and non-muscle cells. Three-dimensional reconst ructions showed MLCK density on the extreme periphery of subdomain-1 of each actin monomer forming a bridge to the periphery of subdomain-4 of the azimuthally adjacent actin. There is interaction of MLCK-147 close to the COOH terminus of the first actin and near residues 228-232 of the second. The unique location ensure that MLCK binds to actin without interfering with the binding of any other key actin-binding proteins, including myosin, tropomyosin, caldesmon, and calponin[13]. In addition, the unique sequence of MLCK-210 is involved in its interacti on with the microfilaments and contributes to its tighter association with the actin cytoskeleton[14]. Non-muscle cells may use different mechanisms for targe ting the long-form MLCK to actomyosin structures during interphase and mitosis. MLCK and myosin II phosphatase act cooperatively to regulate the level of Ser 19 -phosphorylated myosin II during mitosis and initiate cytokinesis through the a ctivation of myosin II motor activity[15].

Ca^{2+}/CaM forms a ternary complex with MLCK, facilitating in activation of the kinase and phosphorylation of RLC. Ph osphorylation induces a conformational change, which allows myosin crossbridges to along actin filaments. Some non-muscle processes are also regulated by RLC phosphorylation of the myosin, while smooth muscle MLCK plays important roles in contractile-motile processes of a variety of cells[16]. MLCK has an actin-binding activity in addition to its kinase activity, which assembles actin filaments in to bundles morphologically and biochemically. There are two actin-binding sites on MLCK, including calcium- and calmodulin-sensitive site and insensitive site . The cross-linking between this two sites assembles actin into bundles[17]. Recently, it is found that a novel approximately 60 ku MLCK immunogen contributes to the aberrant contractility associated with preterm labour[18], while Sohn *et al* reported that calmodulin and MLCK play a role in Ach-induced lower esophageal sphincter contraction, whereas the classical MLCK may not be the major kinase responsible for contraction and phosphorylation of myosin light chain in esophagus. Esophagus contraction is protein kinase C dependent contraction[19]. Various new functions of MLCK have been found recently, activation of myosin II by MLCK produces force for many cellular processes including mitosis, migration, and other cellular shape changes. Inhibition or potentiation of myosin II activation via over-expression of a dominant negative or wild type MLCK can delay or accelerate tumor necrosis factor-á induced apoptotic cell death in cells [20]. MLCK specific ally mediates agonist-induced sarcomere organization during early hypertrophic response[21]. During vascular injury, the expression of MLCK decreased[22]. In rabbit portal vein myocytes, MLCK mediates noradrenaline-evoked non-selective cation current[23]. In the liver, agents that elevate intracellular free Ca^{2+} concen tration increase tight junctional permeability and stimulate bile canalicular contraction. Myosin phosphorylation is probably responsible for the tight junction al permeabilization caused by elevation of intracellular Ca^{2+} in hepatocytes. Moreover, the integrity of the phosphorylation system of myosin is essential for normal bile flow. In addition, hepatic sinusoidal Ito cells (fat storing cells) play a regulatory role on hepatic sinusoidal blood flow through their contraction, while the integrity of myosin light chain kinase is essential for Ito cell con traction and normal sinusoidal blood flow. However, the roles of myosin phosphor ylation by MLCK in non-muscle tissues is not well characterized but correlates with important activities such as cell division, receptor capping, etc. Recently, the study showed the existence of a 208 ku protein, named embryonic MLCK because its expression can be detected in early embryonic

tissues, stem cells, and in proliferating cultured cells. In the liver, down-regulation of this 208 ku embryonic MLCK is not so dramatic, and the less dramatic decline in the expression of embryonic MLCK may possibly reflect the high regenerative capacity of liver tissue. It is now found that MLCK is associated with non-muscle cells closely[1,2,4-7]. MLCK activation is also a critical step in the cytoskeletal changes causing pseudopod formation during polymorphonuclear leukocyte phagocytosis[24]. MLC K immunoreactivity was found to be colocalized with the insulin granules which suggests that it increases insulin granules in the ready-releasable pool by acting on different steps in the secretory cascade[25]. In 3T3 fibroblasts, MLCK is responsible for phosphorylation of MLC at the cell periphery, showing its unique spatial regulation of myosin RLC[26]. From the gene of vertebrate smooth muscle and non-muscle MLCK there are at least four proteins expressed. Two high molecular weight MLCK splice variants, EC MLCK-1 and EC MLCK-2 (210-214 ku) in human en dothelium are identical except for a deleted single exon in MLCK-2 encoding a 69 - amino acid stretch that contains potentially important consensus sites for phosphorylation by p60 (Src) kinase, while p60 (Src)-mediated tyrosine phosphorylation represents an important mechanism for splice variant-specific regulation of n on-muscle and cascular cell function[27]. MLCK is also associated with the gap fomation and endothelial hyperpermeability of coronary venular endothelial cell monolayers[28]. Our preliminary study revealed that MLCK in the rabbit liver could phosphorate myosin light chain obviously, and was calmodulin-dependent, which may play an important role in maintaining the normal functions of the tissue. Bu t in the liver, what form of MLCK expressed more, long form MLCK (embryonic MLCK) or short form MLCK with the molecular weight about 130-150 ku? What are their exact roles in the liver? What roles it will play in liver regeneration, in liver injury or in hepatic carcinoma? And what are the action mechanisms? These remain to be elucidated in our further study.

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Edited by Xu XQ and Wang JH

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Influence of BOL on hyaluronic acid, laminin and hyperplasia in hepatofibrotic rats

Li Yao[1], Zhen-Min Yao[2], Tao Yu[2]

[1]Pharmacology Department of Zhejiang College of Traditional Chinese Medicine (TCM), Hangzhou 310053, Zhejiang Province, China
[2]Basic Medicine Department of Zhejiang College of TCM, Hangzhou 310053, Zhejiang Province, China
Supported by the Natural Science Foundation of Zhejiang Province, No.398402
Correspondence to: Li Yao, Pharmacology Department of Zhejiang College of TCM, Qingnian Road, Binjiang township, Hangzhou 310053, Zhejiang Province, China. ylyj@mail.hz.zj.cn
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Abstract

AIM: **To study the anti-hepatofibrosis mechanism of Bie Jia Jian oral liquid (BOL).**

METHODS: **The model was induced by subcutaneous injection of CCl_4. BOL was administered and the change of serum hyaluronic acid (HA) and lamin in (LN) was observed and the degeneration of liver cells and the degree of fibre hyperplasia analyzed. Changes of ultra micro-structure in liver cells were observed in some samples.**

RESULTS: **HA was reduced in both the groups with low and high dosage of BOL, which showed a remarkable difference as compared with that of the model group (low dosage group: 376.15 μg/L±35.48 μg/L *vs* 806.07 μg/L±98.49 μg/L, *P*<0.05; high dosage group: 340.14 μg/L±30.18 μg/L *vs* 806.07 μg/L±98.49 μg/L, *P*<0.05). The LN content of low and high dosage group of BOL was lower than that of model group (low dosage group: 71.99 μg/L±8.15 μg/L *vs* 133.94 μg/L±14.45 μg/L, *P*<0.01; high dosage group: 71.68 μg/L±11.62 μg/L *vs* 133.94 μg/L±14.45 μg/L, *P*<0.01) and colchicine group (low dosage group: 71.99 μg/L±8.15 μg/L *vs* 118.28 μg/L±16.13 μg/L, *P*<0.05; high dosage group: 71.68 μg/L±11.62 μg/L *vs* 118.28 μg/L±16.13 μg/L, *P*<0.05). Examined by Ridit, BOL could reduce the degeneration and necrosis of liver cells (χ^2 = 11. 99 *P*<0.05), the degree of fibre hyperplasia (χ^2 = 13.24 *P*<0.05) and the pathological change of ultra micro-structure as well.**

CONCLUSION: **The BOL has certain therapeutic effect on the exp eriment hepatofibrosis. Its mechanisms might include: protecting the function of liver cells, inhibiting excessive synthesis and secretion of extracelluar matrix from hepatic stellate cells, relieving the capillarization of hepatic sinusoid, improving liver micro-circulation, and regulating immune function.**

Subject headings BIEJIA JIAN PILL/administration dosage ; BIEJIA JIAN PILL/pharmacology; peroral liquids; liver cirrhosis, experimental/TCD therapy; laminin/analysis; hyaluronic acid/analysis

Yao L, Yao ZM, Yu T. Influence of BOL on hyaluronic acid,laminin and hyperplasia in hepatofibrotic rats. *World J Gastroenterol*, 2001;7(6): 872 –875

INTRODUCTION

Since 1994, our research group has studied on anti-hepatofibrotic effect of the Biejiajian pill and Biejiajian oral liquid (BOL) in rats. It verified that both of them had fair effect on anti-hepatofibrosis[1-3]. Supported by the Natural Scientce Foundation of Zhejiang Province in 1999, we studied the anti-hepatofibrotic mechanism of BOL in rats again. Now we report the results below.

MATERIALS AND METHODS

Materials

Eighty SD rats, weighing (180±20) g, offered by Animal Centre of the Academy of Medical Sciences of Zhejiang Province. Fodder: maize powder (produced by Hangzhou Sijiqing Feed Factory); Lard (commercially supplied); Cholesterol (produced by Chemical Branch of Guangzhou Medicinal Company, batch number: 980503). Ethanol (A.R) (produced by Changyuan Chemical Plant of Changshu City, batch number: 980630). Medicines: All of the 23 ingredients of BOL are purchased from the Out patient Department of Zhejiang College of TCM, such as Biejia, Danpi, etc. appraised by the Department of Pharmacy. Colchicine (produced by Kunming Pharmacy Company, Limited. batch number: 980536). Reagents: CCl_4(A.R) (produced by Fenshui Synthesis Chemical Plant of Yixing City, batch number: 1995122). Liquid paraffin (C.P) (produced by Hangzhou Chemical Reagents Factory , batch number: 971020). HA and LN radioimmunoassay kits (afforded from Shanghai Navy Institute of Medical Science); main instruments: SN-688γ -counter (Shanghai Atomic Energy Institute); UV-754 ultraviolet-visible spectrophotometer (Shanghai 3rd Analytic Instrument Factory); MR4100 enzyme mar ker (American Dynatach Company); H-500 transmission electricmicroscope (Japanese Hitachi, Co).

METHODS

Animal model

All groups but the normal group received sc CCl_4 (A.R) 5 mL·kg^{-1} in the first day of experiment, afterwards sc 400 mL·L^{-1} CCl_4-liquid paraffin mixture 3 mL·kg^{-1} every 3 days; the normal group sc equal amount of 9 g·L^{-1} NaCl (NS), lasted 6 weeks. Except the normal group, every group was fed with mixed fodder (maize powder with 5 g·L^{-1} cholesterol and 200 g·L^{-1} lard) and drank 200 g·L^{-1} ethanol only. The normal group was fed with general fodder and water. The time required to complete the induction of model is 6 weeks.

Grouping

Thirty-seven established rat models were grouped into II-V groups randomly i.e. group II model control (n = 10), group III colchicine (n = 9), group IV low dosage of BOL (n = 9) and group V high dosage of BOL (n = 9). Another 11 normal rats served as normal control in group I.

Therapy

The BOL afforded by Zhejiang College of TCM, according to the ingredient of Jingui Biejiajian pill, which is recorded in an ancient medical book of TCM (Jinkui Yaolue). It contains crude Chinese

medicine 0.92 g per millilitre (that is 20 times the dosage used in adults). I, II group ig NS 10 mL/kg, III group ig 0.001% colchicine in distilled water of the dosage of 0.1 mg·kg^{-1}, IV group ig BOL 4.6 g·kg^{-1} and V group ig BOL 9.2 g·kg^{-1}. All groups had been treated once a day for 5 weeks and took water and fodder ad lib. For maintaining pathological attack, 400 mL·L^{-1} CCl_4-liquid paraffin mixture 3 mL·kg^{-1} once a week until the end of treatment was injected subcutaneously to the models of II-V groups. After the treatment, venous blood was collected from retrobulbar vein, serum was separated and preserved under -70℃ for test. HA and LN were examined by radioimmunoassay. The left liver lobe was dessected and fixated by 100 mL·L^{-1} formalin and embedded in paraffin, serially sectionalize d and then stained with HE observed under optical microscope. In case of observing the collagenous fiber hyperplasia Masson staining was employed. The standards for interpreting the degree of hyperplasia under microscope are in accordance to the criteria of the 5th National Academic Conference of Infection and parasitosis[4], and the degree of degeneration refers to the standard of reference[5].

Statistical analysis

Data described as $\bar{x}\pm s$ and analysed by F test, and *q* test was used to compare the data between 2 groups of multiple groups. The semiquantitative data was analysed by Ridit test. $P<0.05$ regarded as significant.

RESULTS

During the period of treatment, in gruop II2 rats died, 1 rat each died in group I, IV and V, and in group III no rat died.

Influence of BOL on serum HA and LN

Compared with model group, the serum HA of each treated group was lowered significantly ($P<0.05$). There was no significant difference between each treated group ($P>0.05$). This is in accordance with the results of other article[6]. Results of the serum LN: the difference between the colchicine group and the model group was not significant ($P>0.05$). Compared with the normal group, the LN of each treated group was raised signific antly ($P<0.01$). The LN in both low-and high-dose BOL groups were higher significantly than that of normal group ($P<0.01$) and colchicine group ($P<0.05$, Table 1). The difference between high-and low-dose BOL groups was not significant.

Table 1 Serum HA and LN in BOL treated SD rats ($\bar{x}\pm s$, μg·L^{-1})

Groups	*n*	HA	LN
Normal	10	197.77±26.02	42.78±10.05
Model	8	806.07±98.49^{b}	133.94±14.45^{b}
Colchicine	9	402.53±50.78^{a}	118.28±16.13^{b}
Low-dose BOL	8	376.15±35.48^{a}	71.99±8.15^{bdc}
High-dose BOL	8	340.14±30.18^{a}	71.68±11.62^{bdc}

$^{b}P<0.01$ *vs* normal group; $^{a}P<0.05$ *vs* model group, $^{d}P<0.01$ *vs* model group; $^{c}P<0.05$ *vs* colchicine.

Influence of BOL on liver hyperplasia and degeneration

Being analysed by Ridit ($\chi^2 = 13.24$, $P<0.05$, Table 2), we found that the treatment was relevant with the extent of liver hyperplasia. According to the average Ridit, the high-dose BOL group did the best in improving liver hyperplasia.

According to the results of statistical analysis ($\chi^2 = 11.99$, $P<0.05$, Table 3), we considered that the extent of liver cell degeneration was related to the treatment. Referring to the average Ridit, the effectiveness of improving the degeneration in high dosed BOL group was best, and model group had the worst result.

Table 2 Extent of liver hyperplasia in each treated group

Groups	*n*	Degree of liver fibrosis					Average of Ridit
		-	+	++	+++	++++	
Model	8	0	0	1	4	3	0.7247
Colchicine	9	0	1	2	4	2	0.5894
Low-dose BOL	8	0	2	1	4	1	0.5176
High-dose BOL	8	0	5	2	1	0	0.2364

$\chi^2 = 13.24$, $\chi^2_{(4)} = 9.49$.

Table 3 Liver cell degeneration in each treated group

Groups	*n*	Extent of hyperplasia					Average of Ridit
		-	+	++	+++	++++	
Model	8	0	0	2	3	3	0.7408
Colchicine	9	0	1	4	3	1	0.5584
Low-dose BOL	8	0	1	2	4	1	0.5599
High-dose BOL	8	0	4	3	1	0	0.2576

$\chi^2 = 11.99$, $\chi^2_{(4)} = 9.49$.

Electron microscopy

The nucleuses in liver cell of normal SD rats were circular or elliptic and located in the center of cell, the mito chondria were circular or elliptic, the ridges were clear, the Ito cell could be found in Disse's spaces, its cytoplasm contained plenty of fat droplets. Howeic, the nucleoluses disappeared, rough endoplasmic reticulums expanded obviously, the mitochondria were tumid, the ridges were broken or lacked, there were a great deal of fat droplets in the cytoplasm. Many collagenous fibers accumulated in Disse's spaces. The number of stellate cells and rough endoplasmic reticulum increased and surrounded by a large number of collagenous fibers. Compared with the model group, the fat droplets in liver cell of the high-dose BOL group decreased, the structure of nucleus was clear and the mitochondria were tumid lightly. Being observed under the electronic microscope the results of low dosed BOL group were similar to those of colchicine group. Their damage as lighter and more serious than that in model group and high-dose BOL group respectively (Figures 1-5).

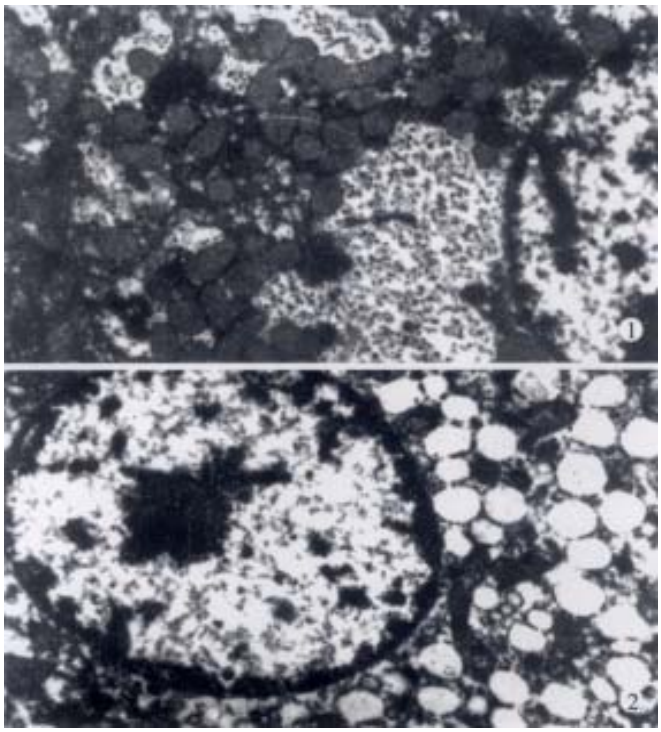

Figure 1 Normal group Showing clear liver cell structure with plenty of mitochondria and clear ridges, no fat droplets in cytoplasm. EM×6 000

Figure 2 Model group Showing few mitochondria without nucleolus, cell structure is not clear, a lot of fat droplets in cytoplasm. EM×4 000

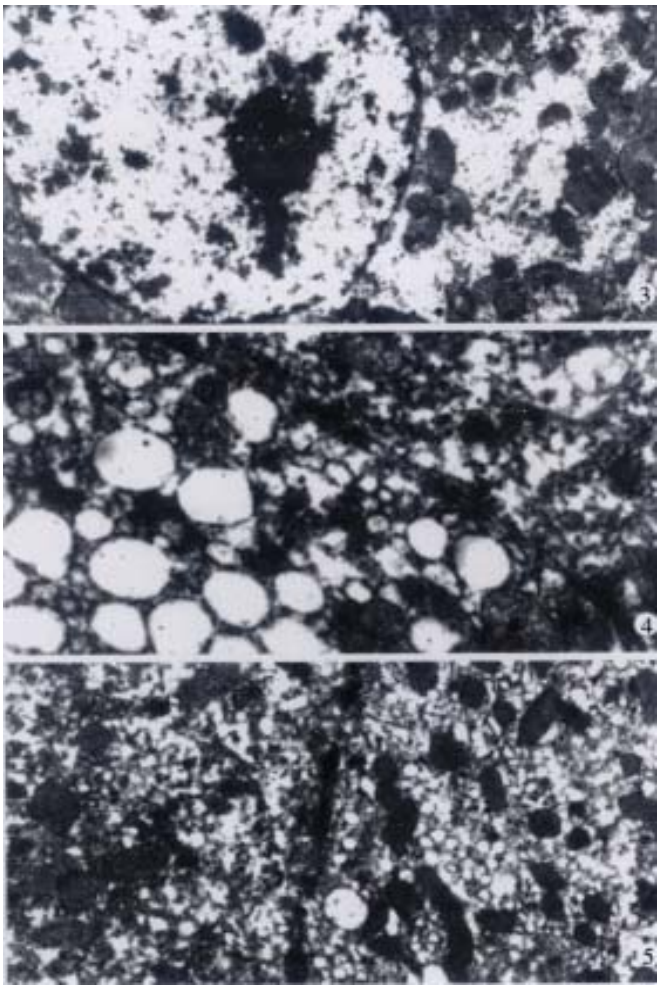

Figure 3 High dosed BOL group Showing clear nucleus structure, lightly tumid mitochondria and few fat droplets in cytoplasm. EM×4 000
Figure 4 Model group Showing many deposited fibers. EM×8 000
Figure 5 High dosed BOL group Showing few deposited fibers. EM×5000

DISCUSSION

Recently, studies on the prevention and treatment of liver fibrosis are very popular in China. Besides western medicine[7-11], single Chinese drug and its effective ingredients[12-19], anti-hepatofibrotic effect of Chinese complex formula[20-27] are also the main project in researches. These studies illustrate the mechanism and clinical effectiviness[28-32] in anti-hepatofibrosis from different point of view. Now, we will especially discuss the anti-hepatofibrotic mechanism of BOL.

The knowledge of liver fibrosis in TCM

Though the term, liver fibrosis, is not mentioned in the ancient medical documents of TCM, the corresponding clinical symptoms of this disease in TCM terms such as abdominal mass, costal pain, flatulence, jaundice, blood stasis, etc. are commonly noted. The clinical symptom of liver fibrosis is intricate. According to the theory of TCM, its basic pathogenesis is that: the condition is manifested as body resistance weakened while pathogenic factors prevailed, damp-heat and blood stasis coexisted, liver depressed and deficiency of *qi* and blood in spleen and kidney. Liver depression and *qi* stagnation can result in blood stasis, blood fails to nourish liver, and the key pathogenetic mechanism is blood stasis[33]. At the beginning, the pathogenetic mechanism is qi stagnation and blood stasis. If being treated unproperly, the disease will evolve into blood stasis mainly, then into a condition which manifests as blood stasis, body resistance weakened and excess in superficiality. The therapy is promoting the circulation and relieving the stasis, strengthening the body resistance to eliminate pathogenic factors, resolving and softening hard mass.

BOL is an improved preparation from Biejiajian Pill which is recorded in an ancient medical book (Jinkui Yaolue). Its ingredients include twenty-three herbs, such as biejia, taoren, danpi, shaoyao, dahuang, caihu, guizhi, banxia, houpu, renshen, e-jiao, huangqin etc. The combination of ingredients has the effect on promoting circulation and relieving stasis, strengthening body resistance and eliminating pathogenic factors simultaneously, resolving and softening the hard mass, which is conformable with the principle of treating liver fibrosis. The therapy of promoting blood circulation and cleminishing stasis has been adopted in clinic[34].

Inquiring into the anti-hepatofibrotic mechanism of BOL

At present, the mechanism of liver fibrosis is inferred that the pathogenic factors injure the liver cell and activate Kupffer's cells to secrete certain kinds of cytokines, which act jointly with other cytokines secreted by the platelets, hepatic sinusoid endotheliocytes, hepatocytes and some chemical intermediates to activate stellate cells, converting them into myofibroblasts, secreting a great deal of extracellular matrix(ECM). The increase of secretion and decrease o f degradation result in accumulation of ECM in liver, forming liver fibrosis gradually[35-39]. According to this experimental result, we suggest the main effect of BOL on treating liver fibrosis in four aspects as follows. ① Preventing the hepatocyte from degeneration and necrosis. The degeneration and necrosis of liver cell are the factors inducing excessive deposition of ECM, which takes an important role in the progression of liver fibrosis[40,41]. So, preventing the degeneration and necrosis of liver cell after being hurt can eliminate the promoting factor of liver fibrosis, preventing Kupffer's cells and stellate cells from being activated, inhibiting the progression of liver fibrosis. This experimental result shows that BOL can protect the liver cells from being harmed, reduce their d egeneration and necrosis, clean or partly clean the inflammatory reaction (also a promoting factor of liver fibrosis), all these demonstrate the good effect on treating liver fibrosis. ② Inhibiting the liver stellate cells to synthesize and secrete ECM. It is well known that liver stellate cells are the interstitial cells served as the main source of ECM. As the liver cells are injured, they will be activated, and increased, and then convert into the transitive type and myofib roblast which synthesize and secrete the ECM excessivly[40,42-44]. The collagens are the main elements of ECM. They are formed in rough endoplasmic reticulums of fibroblasts and are secreted as collagenous fibrils[45]. Both the number of stellate cells and rough endoplasmic reticulums of high dosed BOL group are less than those of model group, which indicates that inhibiting the activation and hyperplasia of the liver stellate cells maybe one of the effects of BOL to prevent and treat liver fibrosis. ③ Relieving the capillarization of hepatic sinusoid, improving the microcirculation of liver. The fenestrate on the hepatic sinusoid endotheliocyte is the site for exchanging materials between the liver cells and plasma. When liver fibrosis developed, ECM synthesized profoundly, deposited in Disse's spaces, forming the membranous spiral lamina under the hepatic sinusoid endothelium, fenestrate of hepatic sinusoid decreased or vanished and then forming capillarization, resulting in impairment of microcirculation, subsequently, affecting the exchange of the oxygen and substance of the liver cell seriously, thus bringing out or aggravating the damage of liver cell[45-48]. LN is the main glycoprotein of the membranous spiral lamina and distributes together with the IV type collagen in the liver. So its serum content is considered as a renewal index of membranous spiral lamina[49-51]. This experimental result indicated that: BOL can decrease the LN content in liver fibrosis, thus relieving the capillarization of hepatic sinusoid, improve the microcirculation of liver, reducing the damage of liver cell and accelerating the repairment of liver. This may be an

important effect of BOL on promo ting the circulation and transforming stasis. ④ Regulating the immune function, reducing the immuno-damage of liver cell. Some researchers[52] complain that the liver cirrhosis is the result of the immune function disturbance. According to our previous report[2], we suggest that BOL can adjust humoral immunity, reduce the immuno-damage of liver cell, improve the substance metabolism and protect the liver tissue from being injured.

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Edited by You DY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Clinical application of serial operations with preserving spleen

Hong-Chi Jiang[1], Bei Sun[1], Hai-Quan Qiao[1], Jun Xu[1], Da-Xun Piao[1], Hang Yin[2]

[1]Department of General Surgery, First Clinical Hospital, Harbin Medical University, Harbin 150001,China
[2]Department of General Surgery, Heilongjiang Provincial Hospital, Harbin 150001,China
Supported by Awarded the Second class Prize of National Science and Technology Advance in 2000 (J-233-2-08).
Correspondence to: Bei Sun, Department of General Surgery, First Clinical Hospital, Harbin Medical University, Harbin 150001, China.
sunbei70@ 163.net
Telephone: +86-451-3600283, Fax: +86-451-3670428
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Abstract

AIM: **To evaluate the clinical application of serial operations with preservation of spleen.**

METHODS: **Serial operations with preserving spleen were performed on 211 cases in our hospital from 1980 to 2000. The patient's age ranged from 13 to 56 years, averaging 38 years. Diseases included splenic injury in 171 cases, portal hypertension in 9 cases, splenic cyst in 10 cases, and the lesion of pancreatic body and tail in 21 cases.**

RESULTS: **All the cases were cured, and 129 patients were followe dup from 3 months to 3 years with the leukocyte phagocytosis test, detection of immunoglubin, CT, ^{99m}Tc scanning and ultrasonography. The results were satisfactory.**

CONCLUSION: **The operations with preserving spleen were safe, feasible, and worth of clinical application.**

Subject headings spleen; spleen-preservation operation; splenic injury; splectomy; methods; human; clinical application; portal hypertension

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INTRODUCTION

Serial operations with preservation of spleen, which are mainly performed to treat splenic injury, in addition to portal hypertension, splenic cyst and lesion of body and tail of pancreas, were performed in our hospital on 211 cases from the early 80s to January 2000. The clinical application study on preserving spleen is reported below.

CLINICAL MATERIALS

Among the 211 patients, including 165 males and 46 females, with average age of 38 years (range 13-56 years), 171 had splenic injuries complicated with hepatic lacerations in 9 and 18 hemorrhagic shock in 17 and mesentery lacerations in 21, portal hypertensions in 9, 10 splenic cysts in 10 and lesions of body and tail of pancreas in 21 which included 8 pancreatic cyst adenom as and 3 pancreatic pseudocysts and 3 insulinomas and 7 pancreatic injuries. All the injuries were abdominal closed injuries. All the patients were cured, and 129 patients were followed up from 3 months to 3 years with the leukocyte phagocytosis test, detection of immunoglubin, splenic CT scan, ultrasonography and Technetium ^{99m}sp lenic scan. The results of following up were satisfactory (Table 1).

Table 1 Operative procedures of preserving spleen

Diagnosis	Operative procedures	*N*	Rate (%)
Splenic injury	Arresting bleeding by adhesion	9	4.3
	Suture and repair	19	9.0
	Ligation of splenic artery	7	3.3
	Partial splenectomy	27	12.8
	Ligation of splenic artery plus Suture and repair	23	10.9
	Suture and repair or adhesives plus partial splenectomy	28	13.3
	Autosplenosis after splenectomy	58	27.5
Splenic cyst	Partial splenectomy	5	2.4
	Autosplenosis after splenectomy	2	1.0
	Paracentesis under BUS	3	1.4
Portal hypertension	Subtotal splenectomy and splenorenal venous shunt	9	4.3
Lesions of body and tail of the pancreas	Resection of the body and tail of the pancreas with preservation of spleen	21	10.0
Total		221	100

METHODS, RESULTS and DISCUSSION

Spleen is not an essential organ to life, though it has many important functions[1,2]. Tempestuous bleeding and h emorrhagic shock are the most commont manifestation of splenic laceration, which are often complicated with other organ injuries and need to be rescued rapidly and decisively following the fundamental treatment principle (or golden standard) of splenic injury-saving life first, preserving spleen second. It is the modern viewpoint that we should do our best to preserve the spleen (tissue) if it permits. In this study, 58% of the cases with splenic injury underwent total splenectomy, which was nearly 61% reported by Livingston, and 63% of the m underwent autosplenosis after splenectomy when the patients' vital signs were permitted. We realized that in treating splenic injury, the fundamental principle is "saving life first, preserving spleen second"; the younger the patient is, the more precedent the spleen-preserved operation is chosen; choose the best operative procedure according to the degree and type of splenic injury; and it is more safe and practical to combine with several operative procedures in preserving spleen.

It is controversy whether the spleen of the patient with portal hypertension should be preserved[3-8]. It was reported that 31% of patients with portal hypertension had immunologic function[9-12]. It has more practical significance for patients with portal hypertension to undergo the operation that can not only protect the immunologic function of spleen but also eliminate the hypersplenism, which is the source of Warren's operation[13-16]. The effect of Warren's operation is not satisfactory in China because post-hepatitis cirrhosis is more

frequently encountered and splenomegalia and hypersplenism are more severe[17-20]. It is difficult to improve the hypersplenism if the spleen has been preserved[21-26]. According to this point of view, we designed the subtotal splenectomy and splenoren al venous shunt by artificial vessel bypass to treat portal hypertension. In all the 9 cases, 1-3 years' postoperative follow-up gave no evidence of recurrent bleeding. It also showed that the hypersplenism relieved and the residual spleen had immunologic function. The significance of this operative procedure is: This operative procedure of preserving spleen is also fit for portal hypertensio n provided we follow the principle of partialsplenectomy, This method does not only eliminate hypersplenism but also preserve the spleen and its immunologic function; and this gives a new way to treat portal hypertension.

Spleen is located near the body and tail of pancreas. When we dealt with the lesion of the body and tail of pancreas previously, we always resected the spleen, though the spleen was healthy, which is called "innocent splenectomy". In fact, the blood supply and function of spleen can b e preserved even if splenic artery is ligated or resected. In this study, 21 cases of distal pancr eatectomy with preservation of spleen were performed. This operative procedure can be classified into two types, one is conservation of splenic vessels (14 cases), the other is conservation of short gastric vessels and left gastropepipolic vessels (7 cases). The former operative procedure is the first choice, because the original blood supply and anatomy of spleen are intact. The latter is the second choice, because the splenic blood supply is influenced and the splenic clearance ability to bacteria is decreased.

Splenic cyst is a benign lesion of spleen, so we should try our best to preserve the spleen and its function[27-30]. If the congenital or acquired splenic cyst is small or symptomless, usually no treatment is needed. If the splenic cyst is big or located at the hilus of spleen or complicated with secondary infection or might autorupture or causes symptoms, it should be treated with cystectomy which mainly includes partial splenectomy (5 cases in our group) and autosplenosis in omental bursa after splenectomy (2 cases). The former operative procedure can preserve the splenic function better. If the patient does not accept the operation or cannot tolerate the operation, we can use the method of splenopuncture drainage under BUS guide (3 cases), its therapeutic effect is definite and can be performed repeatedly. But splenic cyst is liable to relapse (2 cases) and to be infected secondarily, which influences the late results. This method is the second choice and it is especially prohibited when applied for parasitic splenic cyst[31-36].

We should master the types of spleen-preserving operation and choose suitable operative procedures according to splenic injury degree correctly[37-39].The degree of splenic injury is the pathological foundation of spleen -preserving operative procedure[40]. Classification and standard for splenic injury[41-44] varied among different countries. We should set up a new classification of splenic injury degree based on our national conditions and the development of splenic surgery soas to facilitate the academic exchange and the statistics and analysis of document. By synthesizing 121 cases of splenic injury treated in our hospital, we put forward the IV degree clinical classification of splenic injury and corresponding spleen-preserving operative procedures (Table 2). Degree I:rupture of splenic capsule or solitary and multiple splenic laceration , length<5.0 cm, depth<1.0 cm; Degree II: solitary or multiple splenic lacerat ion with intact splenic hilus, length >5.0 cm, depth>1.0 cm; Degree III: irregular laceration, with destroyed splenichilus or a part of spleen broken; Degree IV: spleen is widely broken, capsule is widely peeled, the trunk of splenic vessels are destroyed or broken. The characters of this classification are: simple, convenient and practical; including all the injury types from capsule to parenchyma, from branch to trunk of splenic vessels; the splenic injury degree is classified by quantified index and can be judged promptly; it meets with the common mechanism of splenic injury in our country to date; it stems from practice and can help choose treatment principle and spleen-preserving operative procedures clinically. In clinical practice, the degree of splenic injury can not be as classical as the standard of splenic injury classification. No operative procedure can fit all the patients with splenic injury. We should flexibly choose and use several operative procedures jointly based on the indication and basic skill of the operative procedure. And we should not use some spleen-preserving operative procedure mechanically. Among all the 211 cases of spleen-preserving operations performed in our hospital, 171 cases (81.0%) were splenic injuries, including 9 cases by arresting bleeding by adhesion, 19 cases by suture and repair, 7 cases by ligating splenic artery, 23 by ligating splenic artery and suture and rep air, 28 by suture and repair and arresting bleeding by adhesion plus partial splenectomy, 27 by partial splenectomy, and 58 cases by autosplenosis after total splenectomy.

Table 2 The degree of splenic injury and the choice of therape utic method

I	Nonsurgical treatment
	Arresting bleeding by adhesion
	Suture and repair
II	Partial splenectomy
	Binding the rupture of spleen
III	Ligation of splenic artery
IV	Splenectomy plus autosplenosis

Grasping the main technical points of spleen-preserving operation is the most important factor in the serial operations with preserving spleen.

Suture and repair of the rupture of spleen

The parenchyma of spleen is fragile like bean curd so that suturing and knotting often tear it open, which is liable to cause bleeding or impediment of blood supply[38,39]. We met two cases. One bled to bleed more and more seriously during suturing, so we had to perform total splenectomy because of the turbulent life sign. The other case had to undergo upper-half splenectomy because of the impediment of blood supply res ulting from wide and deep suturing. The width and depth of the suture should be proper, the strength knotted should be well distributed, drawing dexterously and knotting slowly. In order to prevent the second knot from cutting the spleen tissue, or the first knot sliding and loosing while making the second knot, the first knot must be pressed with forceps. To prevent the thread cutting the spleen tissue, a pad of galform or with a piece of omentum beneath the rupture should be used for the knotting. If the suture and repair of the spleen failed or new rupture and bleeding occurred, we should change the operative procedure decisively instead of sticking to suturing and repairing.

Partial splenectomy

Partial splenectomy means less than half of splenectomy, half splenectomy and subtotal splenectomy. We should handle the corresponding blood vessels near the rupture of spleen with each bundle, and observe the boundary of blood supply of spleen, which is the relative avascular plane[40,41]. The "U" shaped interlock suture was performed withdrawing 0.5cm from the boundary to blood supply side. The spleen was excised with forcipressi on. If blood still oozes from the splenic cross-section, wet gauze was used to hemostasis by compression, or "8" shaped suture was made. We covered the cross-section with the capsule pealed off from the cut spleen, and immobilized the capsule on the cross-section with round needle and 1# thread. The advantages of this method are: transplanting splenic capsule can prevent bleeding or liquefaction and necrosis of the cross-

section; the transplanted capsule made the cross-section peritoneal metasplasia, which can decrease the chance of abdominal adhesion; omentum was not used to cover the cross-section rountinely, which reduced the interruption of the abdominal cavity, pre serving the function of the omentum. The splenic capsule is a tissue containing serous coat of its off, so transplanting splenic capsule has a high success rate; the collagen of the transplanted capsule is exposed, which facilitate activating coagulation system to hemostasis effectively and eliminate the dead space. This method has been used in handling the cross-section of partial spleen transplantation. The cross-section is still in good condition after rejection occurred several times. In this group, 41 cases underwent partial splenectomy, including 27 cases with splenic injury, 5 cases with splenic cyst and 9 cases with portal hypertension.

Autosplenosis

After the spleen is excised, it is washed with cold normal saline and put into the 4℃ Hartmann solution[45]. The abdominal cavity was washed and the splenic capsule pealed off and the slice of spleen tissue prepared. The spleen slices of 2.0 cm×2.0 cm×0.4 cm made from 1/3 of the spleen were put into the space between the anterior and posterior lobe of great omentum rich in blood supply. We found that peeling off the capsule facilitates the establishment of blood supply between the graft and the transplantion space, making the hormone from the spleen come into blood cir culation easily. We suggest that autosplenosis should be performed in the omental bursa by transplanting the little splenic cubes peeled off the capsule. In our study, 60 cases of autosplenosis after splenectomy were performed, including 58 cases with splenic injury and 2 cases with splenic cyst .

Resection of the body and tail of the pancreas with preservation of spleen

Dissociating the posterior pancreas space: Dissect the gastrocolic ligment out of the gastro epiploic vessels, then come into the lesser omental bursa, and expose the body and tail of pancre as and the pedicle of spleen[46-49], and protect the short gastric vessels extremely[50,51]. In the avascular area between the upper and lower edge of the body and tail of the pancreas, incise the posterior peritoneum along the pancreas longitudinally, an d dissociate the soft tissue behind the pancreas bluntly to join together with right index finger[52-55]. And then the body and tail of the pancreas and the splenic vessels are dissociated completely with the tissue around them[56-58]; Exsecting the pancreas: Exsect the pa ncreas at the proximal end of the pancreas where you want to cut, and ligate the pancreatic duct, then suture the proximal end of the pancreas with silk thread interruptedly; Dissociating splenic vessels: Clamp the distal end of the pancreas with Kocher's forceps, dissociate the splenic vessels from the pancreas tissues towards the tails of pancreas and protect the splenic vessels, which is called antidromic dissociated method (10 cases)[59-62], on the cont rary, dissociate the splenic vessels from the tail to the proximal end of pancreas, which is called anterograde dissociated method (4 cases). The former method is more commonly used in clinic, because it is difficult to recognize the tail of the pancreas from the fat tissue around t he pedicle of spleen, and the vessels of the tail of the pancreas remified to many thin branches. The resection of the body and tail of the pancreas comes to an end when the splenic vessels are dissociated from the pancreas tissues completely. When the adhesion between the splenic vessels and the pancreas tissue is wide and inseparable and it is too difficult to preserve the spleen vessels, the trunk of splenic vessels far away from the splenichilus and near the tail of the pancreas (1.0 cm from the branch of splenic vessels at least) should be ligated. At this moment, the preserved short gastric vessels and left gastroepiploic vessels supply the blood of spleen (7 cases). To the resectied body and tail of the pancreas with preservati on of spleen and its vessels, the splenic vessels should be immobilized properly because the ve ssels losing the support of the pancreas are easy to twist and bend. For those with preserved short gastvic vessels and left blood vessel of gastric omentum for the blood supply of pancreas, the breaking end of splenic vessels should be ligated exactly. Finally, check the colour and activity of spleen before close the abdomen[63,64].

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Percutaneous transsplenic embolization of esophageal and gastrio-fundal varices in 18 patients

Gao-Quan Gong, Xiao-Lin Wang, Jian-Hua Wang, Zhi-Ping Yan, Jie-Min Cheng, Sheng Qian, Yi Chen

Department of Radiology, Zhongshan Hospital, Fudan University, Shanghai 200032, China
Supported by tackling key problems in science and technology from the State Science and Technology Ministry, TJ99-LA01, No. 96-907-03-01
Correspondence to: Gao-Quan Gong, Department of Radiology, Zhongshan Hospital, Fudan University, Shanghai 200032, China. ggqjj@online.shanghai.cn
Tel: +86-21-64041990 Ext: 2533
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Abstract

AIM: **Clinical application and potential complication of percutaneous transsplenic varices embolization (PTSVE) of esophageal or gastrio-fundal varices in patients with hepatocellular carcinoma (HCC) complicated with portal vein cancerous thrombosis (PVCT).**

METHODS: **18 patients with HCC complicated with PVCT and esophageal or gastrio-fundal varices who underwent PTSVE were collected. The rate of success, complication, mortality of the procedure and postoperative complication were recorded and analyzed.**

RESULTS: **PTSVE were successfully performed in 16 of 18 cases, and the rate of success was 89%. After therapy erythrocyte counts decreased in all of the natunts. 5 of patients needed blood transfusion, 2 patients requiredsurgical intervention because of and 11 patients with ascites were alleviated by diuresis. Among these 18 patients, the procedure-related mortality was 11% (2/18), one died of acute hepatic failure on the forth day after procedure, another died of acute renal failure on the fifth day. The patients were follow up for 1-12 mon exceptone. 13 of them died of their tumors but none of them experienced variceal bleeding.**

CONCLUSION: **PTSVE is a relatively safe and effective method to treat esophageal or gastrio-fundal varices in HCC patients with PVCT when percutaneous transhepatic varices embolization (PTHVE) of varices is impossible.**

Subject headings Esophageal or gastric varices/ complications; Gastrointestinal hemorrhage/ etiology; Gastrointestinal hemorrhage/therapy; Embolization, therapeutic; Radiology, interventional/methods

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INTRODUCTION

Hepatocellular carcinoma (HCC) is common in china[1-8] and many complica tions occur[9-13] including ascites, portal hypertension and splenomegaly. Reviewed literatures, we found there are many articles about treatment of HCC[2,4,11,14-32] or varices[33-38], but few relering the treatment of HCC complicated varices[39-41]. The percutaneous transhepatic variceal embolization (PT HVE) of gastroesophageal varices is a highly promising procedure for controlling acute variceal hemorrhage and decreasing the mortality in patients with portal hypertension. However there are limitations of PTHVE application, because the natench of the main trunk of portal vein is reauired, which was limited its use. Thus, it is very important to find another route to catheterize into portal vein in patients whose portal vein were invaded by HCC. In order to control variceal hemorrhage in these patients it is very important to find another approach for catheterizing into their portal vein. From 1999-08 to 2001-01, 18 HCC patients with portal vein cancerous thrombosis (PVCT) underwent percu taneous transsplenic embolization (PTSVE) of esophageal or gastrio-fundal varices in our institution as reported here.

MATERIALS AND METHODS

Patients

18 patients (17 male, 1 female, mean age 48 yr, aged from 29-72 yr) of HCC with PVCT enrolled in to this study. The diagnosis of HCC with esophageal or gastrio-fundal varices was made by clinical history, alpha-fetoprotein (AFP), ultrasound, CT or MRI. 4 of the patients experienced variceal hemorrhage in different degree and 1 patient had seven episodes of serious variceal bleeding before therapy.

Varices classification

According to Burcharth *et al*, varices were classified into 4 grades: 0 means no varices were visible; 1 grade: only tiny va rices were visible; 2 grade: varices were distinctly visible, and 3 grade: severe varices were seen.

Clinical data

According to the Child-Pugh scores, 10 patients were Child A and 8 patient s were Child B. Patients' average platelet counts were 83. 2×10^9, ranged from (11 to 195)$\times1\ 0^9\cdot L^{-1}$, and 3 patients had accepted 4 units blood transfusion due to decreased erythrocyte before thera py. The slight or moderate ascites were observed in 10 patients.

Pre-operative preparation

The patients with ascites were given albumin and diuretics. The patients have not underwent PTSVE until the ascites were alleviated. CT angiography (CTA) or MR angi ography (MRA) of portal venous system was necessary to show patients' splenic vein and its tributary[42], main trunk of portal vein and esophageal or gastrio-fundal varices in order to obtain the inlormations of the site, direction, and depth of puncture before therapy (Figure 1).

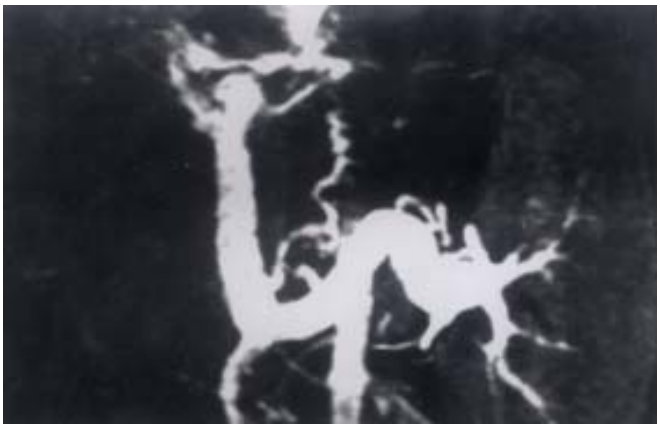

Figure 1 CTA of portal vein that show tributary of splenicve in clearly.

Procedure

For clearly visualization of portal venous system and varices, indirect or direct portography was performed on Natie nt them.

Indirect portography

The indirect portography was performed after 5F RH catheter was catheterized into patient's splenic artery via left or right femoral artery approach. 40 mL cont rast medium (Ultravist 300, Schering AG or Omnipaque 300, Nyegarrd) were injected into the splenic artery at a speed of 7 mL·s^{-1}. The film were exposed 1 picture·s^{-1} for 10 s until the main stem of patients' splenic vein and portal vein were clearly displayed. The tip of catheter was to be kept in the site for localization of the puncture.

Direct portography

The puncture site was determined on the basis of CTA or MRA images of portal vein s. Usually the seventh to ninth intercostal space on the left midaxillary line was selected as the puncture site. Local anaesthesia was followed by percutaneous transsplenic puncture using a 21 G Chiba needle. The core of needle was removed and the needle was pulled back slowly with intermittent aspiration. When blood can be freely aspirated, 5 ml diluted contrast medium (1:3) was injected as test. If patient's splenic vein was displayed clearly, a 0.018" (0.457 mm) guidewire was introduce d and manipulated into the main stem of portal vein through the needle. Then the needle was withdr awn and replaced by a COPE puncture cannula system. A 5F catheter's sheath was pushed into patient's splenic vein through the exchanged 0.038" (0.968 mm) guidewire. After a 5F pigtail catheter was put in patient's superior mesenteric vein (SMV), the direct portography was performed with 40 ml contrast medium injected into patient's SMV at a ratio of 7 mL·s^{-1}.

Variceal embolization

A 5F Cobra or Simmons I catheter was catheterized into patient's varices. Abs olute alcohol, gelfoam and steel coils were used as embolizational material. When the variceal vessels were occluded or the blood flow in varices was very slow, the varices were considered completely embolized. Then, the catheter was removed and the sheath was pulled back to the edge of the parenchyma of spleen. The duct of puncture was embolized by gelfoam and steel coil till the main stem of splenic vein was not displayed when the contrast medium was injected.

Post-operative management

Routine post-operative management included hematischesis, hepatic function protection and anti-infection. Hepatic function and hematocyte were examined d 2 and d 5 after procedure. Abdominal symptom and body temperature were recorded too. B ultrasound, CT or MRI was followed in 9 patients 1 wk after operation. It was considered the proof of infection that high body temperature (> 38.5℃) and high leucocytic counts (>10×10^{9}·L^{-1}), persisted longer than 7 d.

Follow-up

Survival time and post-operative bleeding rate were recorded in 15 patients in whom the follow up was done.

RESULTS

Rate of success

The branches of splenic vein have been successfully punctured in 16 of 18 patients and esophageal or gastrio-fundal varices were embolized in the patients except 1 one patient with slight varices. The success rate of PTSVE was 89% (16/18). The other 2 patients whose PTSVE was unsuccessful didn't undertake the CTA or MRA examination before treatment.

Portography

Hepatofugal blood flow in portal vein was observed in 16 patients. The main stem of portal vein obstructed and collateral circulation occurred in 14 patient s. Grade 1 varices in 1 patient, Grade 2 in 4 patients, and grade 3 in 11 patients were observed (Figure 2-4). Among 3 patients whose varices fallen in grade 2, 2 patients had spontaneous splenorenal shunt and another one had spontaneous portacaval shunt.

Complication and mortality

All patients' erythrocyte counts were decreased after the procedure and 5 of them needed blood transfusion. 2 patients experienced hemoperitoneum and 1 patient had mild reactive pleural effusion. All of these counlications were alleviated by conservative treatment. 11 patients had mild or moderate ascites because of severe hypoproteinemia which was controlled by supplying albumin and diuresis (Table 1). Hepatic functions didn't deteriorate in the patientexcept one who had thrombosis in the main stem of his portal vein and died on the forth day after therapy. Another patient died of acute renal failure on the fifth day after therapy. The procedure-related mortality was 11%(2/18). No severe infection was observed. 9 patients undertook B ultrasound, CT or MRI 1 wk after procedure and no hamatoma in spleen surrounding area was displayed.

Figure 2 indirect portography via transsplenic artery show splenicvein and its tributary.

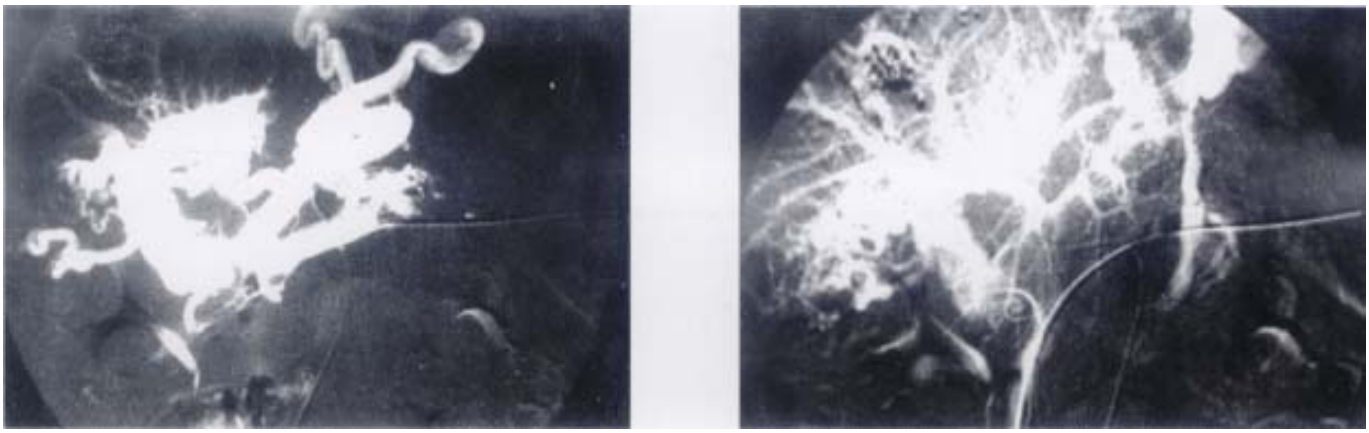

Figure 3 direct portography show main portal vein obstruction and the varices.

Figure 4 the same patient as figure 3, the variceal blood flow was turned slowly after embolization.

Table 1 Postoperative data of 18 HCC patients received PTSVE

Code/Survival	Reconstitu-tion of the portal vein	Operative Success	Blood infusion (Unit)	Ascites	Post-operative bleeding	Other complications
1/lost tofollow up	no	yes	none	negative	unknown	none
2/dead	yes	yes	none	negative	no	pleural effusion
3/dead	yes	yes	yes/4	moderate	no	none
4/ suicide	yes	yes	none	negative	no	none
5/dead	yes	yes	none	slight	no	none
6/dead	yes	yes	none	moderate	occurred	hemoperitoneum
7/dead	no	no	none	moderate	no	none
8/alive	yes	yes*	none	moderate	no	none
9/lost to follow up	no	no	none	negative	unknown	none
10/dead	no	yes	yes/2	moderate	no	none
11/alive	yes	yes	none	negative	no	none
12/dead	no	yes	yes/4	slight	no	none
13/dead	no	yes	yes/2	slight	occurred	none
14/alive	no	yes	none	negative	no	hemoperitoneum
15/dead	yes	yes	yes/2	moderate	no	Acute hepatic failure
16/dead	yes	yes	none	slight	no	none
17/dead	yes	yes	no	sliight	no	none
18/dead	no	yes	no	negative	no	acute renal failure

*Patient with slight varices which needn't to be embolized.

Follow up

15 of 16 patients whose varices were successfully embolized had been followed up 1 to 12 mo. During the follow-up 13 of these 15 patients died. The cause of death was hepatic failure in 7 patients (1 in 4 d after procedure, 1 in 1 mo, 2 in 3 mo, 2 in 6 months, 1 in 8 mo). One died of acute renal failure in the fifth day after therapy, 2 died of metastasis, 2 died of ulcer bleeding which were conformed by gastroscope, 1 committed suicide. In the 2 failed cases, one was dead, the other was beyond follow up.

7 of the Nationls was died of hepatis failure which was the main ocuse of the do nth the causes of the ather 6 Natient were different. They night died of acute read failuisinth fifth day after the rany (Icase), of metastasis (2 case), of wher bleading (2 case) and committted suiside (1 case).

DISCUSSION

Interventional treatments include two different way, one is shunt (for example: transjugular intra-he patic portosystemic shunt, TIPSS), and the other is varices embolization[33-36]. There are some defects of TIPSS: proximal shunt can decrease hepatic blood flow in portal vein, which makes hepatic function deteriorated and restenosis rate high. Traditional embol ization route includes transhepatic and transumbilical vein and celiotomy incision to puncture branches of SMV. The latter two have been abandoned because of difficult operation and traumatic problem.

Percutaneous transhepatic variceal embolization (PTHVE) may be useful to control acute variceal bleeding and decreasing mortality. Since PTHVE is less traumatic and it s success rate is high, some authors apply PTHVE as a prophylactic management for the patients wit h severe varices in whom the rate of bleeding is high. However, the premise to perform PTHVE is that main portal vein must be patent and there is no tumor on the route of puncture. HCC has a high in cidence in our country. Advanced HCC usually complicated with portal vein cancerous thrombosis (PVCT) which deteriorated the previous portal hypertension. In such patient there was a high incidence of esophageal or gastrio-fundal variceal bleeding. Since main portal vein is invaded and obstructed by HCC and the tumor always impedes the puncture route, the utility of PTHVE is limited. Basing on PTHVE and percutanous transsplenic portography[43], we designed PTVSE to avoid puncturing obstructed main stem of portal vein and to treat embolus in portal vein in addition.

In 16 of 18 patients, the splenic vein was punctured, and superselective catheterization into varices was done successfully. In order to determine the puncture site, most of the patients undertook pre-operative CTA or MRA of portal vein and indirect portography via splenic artery. The rate of success of PTSVE in this group was 89%

(16/18), similar to PTHVE and Liang's report in which 16 of 17 transsplenic portography were done susccsshully under B ultrasound guidance[43]. We find that only Rasinska has reported one case treated by PTVSE in 1987 through our review.

It is suggested that transsplenic puncture might lead to hamorrhage because of d ecrease of erythrocyte counts after procedure in all patients. But in most of cases, it didn't need blood transfusion except 5 patients who accepted 2 to 4 units of blood transfusion. Hemoperitoneum occurred in two cases and slight reactive pleural effusion occurred in one. Both of the complications could be alleviated by conservative treatment. Portal vein thrombosis developed in 1 case within 4 d and led to acute hepatic failure. 1 case experienced acute renal failure within 5 d. Both of them died. Procedure- related mortality was 11% (2/18). Examination in 9 patients by imaging modalities 1 wk after procedure, no hamatoma was found around spleen. In addition, 11patients developed slight or moderate ascites after procedures that were alleviated by heteropathy. No severe infection occurred in all 18 patients. It is suggested that PTSVE be a safe technique relatily[43]. However PTSVE is not the first choice of treatment when PTHVE can be performed because its mortality was higher and may lead to hamorrhage compared with.

No esophageal or gastrio-fundal variceal bleeding occurred in the 15 patients during follow-up. Only two cases had bleeding due to gastric ulcer verified by gastroscope. It is also suggested that PTSVE be relatively effective in short term and similar to PTHVE. Since the life expectancy of patients of HCC complicated with PVCT is short, the long-term follow up seems to be unnecessary.

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Edited by Wang YQ

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORT •

Rapid donor liver procurement with only aortic perfusion

Qi-Yuan Lin[1], KK Chui[2], AR-Nitin Rao[3]

[1]Department of General Surgery, First University Hospital, West China Medical Central of Sichuan University Chengdu 610041 China
[2]Department of surgery, The Price of Wales Hospital, The Chinese University of Hong Kong
[3]Department of surgery, The Price of Wales Hospital. The Chinese University of Hong Kong
Correspondence to: KK Chui, Department of surgery, The Price of Wales Hospital, The Chinese University of Hong Kong.
qiyuan@mail.sc.cnifno.net
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Abstract

ATM: **To describe a rapid technique for procurement of donor liver with aortic perfusion only (APO).**

METHODS: **Only the aorta is cannulated and perfused with chilled preservation solution.**

RESULTS: **The quality of donor liver can ensure the grafted liver functions.**

CONCLUSION: **The method of APO can simplify the operative procedure, compared with the dual cannulation. It also can minimize the danger of injuring vascular structures and involve less dissection.**

Subject beading liver; transplantation; aorta perfusion; donor

Lin QY, Chui KK, Rao AN. Rapid donor liver procurement with only aortic perfusion. *World J Gastroenterol*, 2001;7(6):884-886

INTRODUCTION

A rapid technique of harvesting the donor liver helps to ensure the quality of the grafted liver. The aim of this report is to describe a simple and rapid method for procurement of the donor liver using aortic perfusion only.

Operative procedures

Organ preparation

At induction of anaesthesia, cefuroxime and methyprednisolone are given intravenously to the donor. A midline incision is made with electrocautery from the suprasternal notch to pubis. The sternum is spilt with a sternal saw, and hemostasis is obtained. Self-retaining retractors for the sternum and the abdominal wall provided excellent exposure. At this point the liver is evaluated, with particular attention paid to its consistency, color, and size. The liver is mobilized first by dividing the falciform ligament down to the suprahepatic vena cava. The left lateral section of the liver is mobilized by dividing the left triangular ligament and lesser omentum. At this stage it is important to check for a accessory left hepatic artery (HA) coming off the left gastric artery (LGA). The right colon and the caecum are mobilized together with the mesentery of the intestine to expose the aorta, superior menteric artery (SMA), inferior vena cava (IVC), left renal vein and inferior mesenteric vein (IMV). The IMV is ligated and divided. Both ureters are identified and slung with vascular loops. The first step is the dissection of the IVC from above its bifurcation to the left renal vein level, with a No. 1 silk encircled above the bifucation. A small segment of the aorta just above the bifurcation is cleared, and the No. 1 silk ties are looped at two sites above the bifurcation. The aorta is dissected to view the SM A. The inferior mesenteric artery (IMA) may be ligated and divided with 3-0 vicryl sutures to prevent loss of cold perfusion. The fundus of the gallbldder is incised and the bile is washed out with saline using a 50-mL bladder syringe. The duodenum is Kocherised then a search is made for an anomalous right hepatic artery arising from the SMA, which occasionally can be the only artery to the liver[1-3]. This is found by palpating for a pulse behind the portalvein. If the arterial anatomy is normal, the gastroduodenal artery is then ligated and divided, thereby bringing into view the portal vein. The oesophagus is taped with a red rubber for retraction t o facilitate the dissection of the supracoeliac aorta, which is mobilized by incising the right d iaphragmaticcrus between the oesophagus and the inferior vena cava. The SMA is dissected to its origin from the aorta. It is essential to examine again for an abnormal left hepatic artery arising from left gastric artery[2] before dissecting the tissues around the oesophagus. A tape is then passed around infradiaphragmatic aorta. 3 bowls with ice slush are prepared on the bench table, two containing one sponge and the other containing four sponges. If cardiac transplantation is also occurring, the heart teams come in at this stage and prepare their organ for retrieval.

Cannulation

The donor is systemically heparinised at a dose of 300 U/kgbody weight, lasting about 3 minutes. A large cannula (Wire Reinforced Venous Return Catheter with TF 28 diameter and 35 cm in length) previously flushed with preservation solution is p laced in the distal aorta and fixed; care should be taken not to insert the tip of the cannul a beyond the origins of the renal arteries. The lower end of the IVC is tied off with the silk tie in place, and a large aortic clamp is simultaneously used above supraceliac artery to cross clamp. The aorta line clamp is then opened to flush the liver with 4 liters Ross solution. The suprahe patic inferior vena cava is opened at its entrance to the heart and blood is vented into the pericardium and pleural space. Cold packs are put over and under the liver and also over the kidneys and slush are poured into the abdominal cavity to facilitate rapid cooling of the viscera. Four litres of Ross solution and two litres of UW solution (each liter of UW solution containing 15mg of dexa methasone and 40 units of insulin) are used for the perfusion.

Organ retrieval

When the effluent clears and the liver is cold, in the bloodless field, the infr ahepatic IVC is opened and transected above the renal veins. The diaphragm is divided on the left side down to the aorta avoiding the oesophagus. The pericardium and the posterior wall of the suprahepatic IVC are divided. The hepatic hilar dissection commences with dissection of the common bile duct (CBD) inferiorly. It is divided low behind the head of the pancreas, the stump being marked with a 5-0-prolene stitch. The SMV is followed behind the head of

pancreas tow ards the portal vein. The branches of SMV are dissected and ligated. The lower part of SMV is divided, and the origin of spleen vein is ligated and dissected. The PV and SMV are harvested tog ether. The aorta is opened from the bifurcation to the origin of SMA. The LGA and the splenic artery are ligated and divided close to the stomach and the splenichilum, respectively. The possible existence of an abnormal right hepatic artery from the SMA still needs to be ruled out by careful examination. The duodenum is then dissected off the pancreas. The origin of the coeliac artery is identified within the opened aorta and cannulated with an infant feeding tube probe to ensure the supply to the liver. The presence of any accessory hepatic arteries from either the SMA or the aorta must be excluded again[3]. If no abnormal arterial anatomy is found, a patch of aorta and SMA combining the coeliac artery and the common hepatic artery with a part of pancre as are completely retained until the next step that is to remove en bloc. The right diaphragm is incised from anterior to posterior down towards the upper pole of the right kidney, a generous patch of diaphragm left attached to the liver. The right side is protected by gentle down ward traction, with careful division of the hepatorenal ligament. When the diaphragmatic attachment of the liver freed, the liver vasculature is delineated; the remaining diaphragamatic and peritoneal attachments are divided. The liver is removed and put into an ice bowel over a cold abdominal pack containing cold normal saline solution. The portal vein is cannulated and perfused with some UW solution, and the liver is put into a bag and packed for transport in this UW solution. Both iliac arteries and veins are routinely removed from the donor for possible use in artery or portal vein reconstruction in the recipient. These vessels can be preserved in specimen bottles containing UW solution and transported in the same ice chest as the liver.

Table work and liver storage

Trimming of the liver is carried out under in a sterile operating theatre, the liver being kept in the same basin containing cold UW solution. The aorta is first divided and infant-feeding tube 5F is used to delineate arterial anatomy from the aortic patch towards the liver. The tissue around the vessels is excised, and all of the major arterial branches are ligated except for one or two, which may be left to check after the anastomosis. The divided common bile duct is flushed with cold saline using the larger infant feeding tube until the effluent clear. The biliary tract is not usually dissected to prevent devascularisation of the bile duct. Further preparation of the common bile duct is left until biliary reconstruction is being performed in the recipient. The inferior vena cava is prepared by excising the patch of diaphragm around the suprahepatic vein lumen. Excising the adrenal tissue and carefully ligating the right adrenal veins similarly cleans the infrahepatic ven a cava. Sometimes it is helpful for the operator to insert their finger into the lumen of inferior vena cava to provide guidance to the dissection. All of the redundant tissues attaching the liver should be cleaned up, and the opening of the vessels should be sutured with 5-0 prolene sutures. Liver biopsy is routinely done. The Donor liver is also weighed and recorded. The first bag containing the donor liver is tied and put into another bag containing cold saline and the 2nd bag is tied again and put into the 3rd bag and tied. The bags are placed in a box containing ice with the lid tightly seale d and the bag placed into a large box containing ice.

DISCUSSION

Since the earliest description of a standardized technique for multiple organ procured by Starzl *et al*[4], modifications have been suggested to simplify the operative methods and minimize the risk of damage to the graft[5-7]. Ville de Goyet[8] *et al* have previously reported that APO has no detrimental affect on the graft liver function in either adult or pediatric transplant patients. Effective liver perfusion occurs in the APO via the aorta and hepatic artery, but also via the portal vein after the fluid has traversed the intestinal circulatory bed (Figure 1). The donor liver is in fact perfused with the cold solution from both of HA and PV, thereby rende ring the liver with APO safe for grafting as shown in our previous report[9].

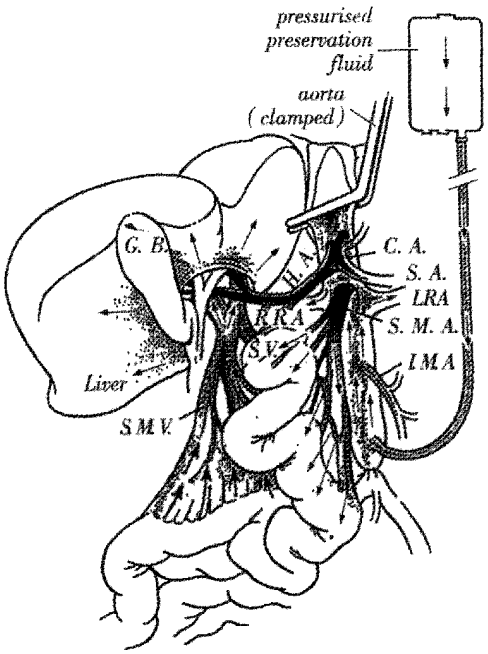

Figure 1 Effective liver perfusion occurs in the APO via the aorta and hepatic artery

The advantage of the technique of aortic perfusion only is the avoidance of the need to undertake the additional dissection and vessel cannulation required for a technique involving portal vein perfusion. The method has simplified the operative procedure, as it involves flushing through an aortic cannula only, instead of having the two cannulae and perfusion sets needed for the portal vein perfusion technique. In the critically unstable donors these methods have been shown to be beneficial.

The APO method can minimize the risk of damage to the graft. The conventional portal vein perfusion technique requires dissection especially around the SMA prior to its ligation. Such dissection may result in a higher incidence of hepatic arterial injury[1-3,10]. The risk of surgical error is especially significant when the right hepatic artery arises from the SMA. This vascular anomaly occurs in about 9%-15% of the population[1,6]. With regard to the other abdominal organs retrieved during multiorgan procurement, the vascular anatomy can be defined on a side table allowing the best setting for the most appropriate division of common vascular supplies, which reduce the danger of injured vessels as well. Another advantage of the APO method is to decrease the risk of manipulative arterial spasm with resulting ischaemic damage to the organs.

The method also allows the other organs of donor to be utilized. The procedure of perfusing the liver allows the other abdominal viscera, including the pancreas, intestine and kidneys, to be flushed with a perfusion solution at the same time. The procurement of abdominal multiorganen bloc combined liver; pancreas, kidneys and intestine can be performed in a single donor.

The presence of a normal blood supply is very important for a liver following transplantation. Any procedure resulting in damage to the arterial sup ply should be avoided at all costs. Special attention should be paid to the possible presence of accessory hepatic arteries from the SMA, LGA and aorta. During prolonged back-table

procedures involving arterial reconstructions, hypothermia should be also strictly maintained.

CA	-	celiac artery	SA	-	splenic artery
HA	-	common hepatic artery	SMA	-	superior mesenteric artery
SMV	-	superior mesenteric vein	IMA	-	inferior me senteric artery
LRA	-	left renal artery	RRA	-	right renal artery
SV	-	splenic vein	GB	-	gall bladder

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