

On the cell biology of pit cells, the liver-specific NK cells

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INTRODUCTION

Natural killer (NK) cells are functionally defined by their ability to kill certain tumor cells and virus-infected cells without prior sensitization^[1]. NK cells comprise about 10% to 15% of lymphocytes in the peripheral blood and most of these cells in human and rat have the morphology of large granular lymphocytes (LGL)^[2]. However, recent studies have demonstrated that small agranular lymphocytes, lacking CD3 expression, have cytolytic activity comparable to NK cells^[3]. These variations may be related to the stage of NK cell differentiation or heterogeneity^[4]. Moreover, some cytotoxic T lymphocytes (CTL) also display LGL characteristics^[4]. Besides NK cells in peripheral blood, NK cells are also found in tissue compartments, such as the spleen, lung, intestine, lymph nodes, bone marrow and liver^[4]. NK cells in the liver, also called pit cells^[5], constitute a unique, resident NK population in the liver sinusoids. Their immunophenotypical, morphological and functional characteristics are different from blood NK cells^[6]. Presently, several

names, such as pit cells, hepatic NK cells and LGL are used to describe pit cells, referring to different aspects of their morphology or function^[6]. We prefer to use the first-given name of pit cells for these cells in the liver, because it is not related to the ever varying levels of function and morphology or a person (like Kupffer cells)^[7]. In addition, liver-associated lymphocytes (LAL), in some instances, are used to describe the total population of lymphoid cells in the liver^[8,9]. However, LAL contain about 30% T lymphocytes, 3% B lymphocytes besides 43% pit cells in a human liver washout^[9]. Rat liver washouts contain 43% T lymphocytes, 16% of B lymphocytes, 3.3% of monocytes and 26% pit cells^[10].

The present review will discuss the biological relevance of pit cells with emphasis on rat liver.

IDENTIFICATION, STRUCTURE AND TISSUE DISTRIBUTION OF PIT CELLS

Pit cells were firstly described in 1976 by Wisse *et al*^[5]. The name pit cell was introduced because of the characteristic cytoplasmic granules, which in Dutch language are called pit, resembling the pits in a grape^[5]. The hypothesis that pit cells might possess NK activity was suggested by Kaneda *et al*^[11], because of their morphologic resemblance to LGL. The development of a method for the isolation and purification of pit cells from rat liver and the evidence of pit cells possessing spontaneous cytotoxicity against NK sensitive YAC-1 lymphoma cells confirmed these cells to be hepatic NK cells^[12,13].

Pit cells exist in the liver sinusoids and often adhere to endothelial cells (Ec), although they incidentally contact Kupffer cells (Kc) (Figure 1). They face the blood directly. Pseudopodia of pit cells can penetrate the fenestrae of the Ec, and enter the space of Disse and can directly contact the microvilli of hepatocytes^[5,14]. Their appearance in the space of Disse is not a common feature^[15]. By morphological investigation, the frequency of pit cells in liver tissue is about an average of 1 pit cell per 10 Kupffer cells. The number of pit cells, in untreated rats, is therefore estimated to be $(1.4-2) \times 10^6$ cells per gram liver weight^[12]. By immunohistochemistry, using mAb 3.2.3 against NKR-P1A (a specific marker of NK cells), the

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number of pit cells in frozen sections of rat liver is about 13.7 per mm²^[16]. After intravenous injection of biological response modifiers (BRM), the number of pit cells increases 4-fold to 6-fold in rat liver treated with zymosan^[17] and 43-fold with interleukin-2 (IL-2)^[18]. The surplus of pit cells is considered to originate from local proliferation and from the bone marrow^[17,18]. Pit cells were found to be more numerous in the periportal than in the pericentral region of the liver lobule^[11,16].

Pit cells have essentially the same morphology as NK cells from blood and other organs, *i.e.* LGL (Figure 1). LGL morphology is characterized by a relatively large size, the presence of granules in the cytoplasm, a pronounced asymmetry of the cell and an indented or kidney shaped nucleus of high density^[10]. Pit cells in the rat are about 7µm in diameter and vary in shape, while possessing well developed pseudopodia. They show a pronounced polarity with an eccentric nucleus and most organelles lying at one side of the nucleus.

The most conspicuous organelles are the electron-dense granules. These granules have several characteristics. They are azurophilic, therefore, Giemsa staining of a cell smear or cytopsin preparation reveals the presence of the granules in pit cells with light microscopic examination. As measured by electron microscopy, the granules differ in size between different pit cell subpopulations (LD and HD pit cells, see next page), but within one cell type the granules are very homogeneous with respect to size, shape and electronic density^[19]. The granules are membrane bound and range in size between 0.2µm in LD pit cells and 0.5µm in LAK cells. These granules contain a number of lysosomal enzymes, such as acid phosphatase^[12,20]. Although perforin and granzymes, which have been isolated from NK cell granules^[21,22], are not yet been identified in pit cell granules, it is believed by analogy that these molecules are present in the granules of pit cells.

Rod-cored vesicles are small inclusions, ranging in diameter from 0.17 µm to 0.2 µm, and are exclusively found in LGL^[11]. They contain a straight rod structure which is 30 nm-50 nm in length, that bridges the entire diameter of the vesicle^[11,20]. Rod-cored vesicles derive from and distribute preferentially around the Golgi apparatus. Possibly rod-cored vesicles may also contain cytotoxic factors functioning in natural cytotoxicity^[15].

Pit cells also exist in human and mouse liver, but the identification of pit cells in human and mouse liver is more difficult than in rat because they contain a lower number and smaller size of the typical dense granules and very few rod-cored vesicles^[9,23,24]. On the other hand, 5% to 25% of human pit cells contain 'parallel tubular arrays' (PTA), that were also reported in human blood NK

cells and considered as a characteristic of these cells^[6,23].

SURFACE PHENOTYPE OF PIT CELLS

Extensive phenotypic analysis has shown that no unique NK cell marker has been identified yet, but expression of a set of differentiation antigens in the absence of antigen-specific receptors of T and B lymphocytes serves to identify NK cells. NKR-P1 was first identified in the rat^[25] and has now been shown to be expressed also by mouse and human NK cells^[26,27]. NKR-P1 is a type II membrane glycoprotein of the C-type lectin superfamily^[28]. The NKR-P1 genes are located on mouse chromosome 6^[29], human chromosome 12 p 12-p13^[27], and rat chromosome 4 in a region designated as the 'NK gene complex' (NKC)^[28]. NKR-P1 antigen is present on 94% of rat LGL and serves as a triggering structure on these cells^[25]. NKR-P1 is considered to be a useful marker for NK cell identification^[25]. However, a subset of T lymphocytes and polymorphonuclear leukocytes also express NKR-P1^[25,27]. CD56 and CD16 are expressed, either alone or in combination, on the majority of human NK cells and are most extensively used as human NK cell 'markers' for clinical and basic research purposes^[2]. Other surface antigens expressed on NK cells are: CD2, CD8, CD11a-c, CD18, CD45, CD54, CD56, CD58 and CD69^[2,4,30,31].

Most surface antigens found on rat pit cells are similar to that found on spleen or peripheral blood NK cells (Table 1)^[13,16,32]. All LGLs from a rat liver washout, in preparations for light and electron microscopy, were found to express NKR-P1^[16] (Figure 2) as recognized by using the monoclonal antibody (mAb) 3.2.3^[25]. CD11a is present on 90% of rat pit cells, which is different from rat peripheral blood NK cells (54%)^[32]. Approximately 90% of rat pit cells express CD18, 35% express CD54 and 80% express CD2^[32]. Asialo-GM1, which is expressed by all rat blood NK cells^[19], is present on 36% of LD pit cells and 70% of HD pit cells^[13]. CD8, a marker of NK cells and cytotoxic T lymphocytes^[2], is present on all rat pit cells^[13]. However, the composition of CD8 in NK cells and T lymphocytes is different. Most CD8⁺ NK cells express CD8 α /CD8 α homodimers rather than the CD8 α /CD8 β heterodimers prevalent on cytotoxic T cells^[3,3]. In addition, rat pit cells do not express T cell receptor and CD5 antigen (a pan T cell marker)^[13,15].

LAL from human liver contain about 35% CD56⁺ cells, in which three subsets are found: ① CD3⁺/CD16⁻, ② CD3⁻/CD16⁻, ③ CD3⁻/CD16⁺^[8]. Moreover, all the CD56⁺ LAL express CD11a and CD18, and partly express CD2, CD11b, CD11c, CD54 and CD58^[8,34,35].

Table 1 Characteristics of LD, HD pit cells and peripheral blood NK cells

Item	LD pit cell	HD pit cell	Blood NK cell
Morphology*			
Size(μm^2)	27.7	24.5	24.8
Rod-cored vesicles per cell	1.0	0.8	0.5
Microvilli per cell	5.2	5.9	7.1
Size of granules (μm^2)	Smaller(0.09)	Intermediate(0.1)	Larger(0.14)
Number of granules per cell	Higher(50)	Intermediate(20)	Lower(10)
Surface antigens [†]			
CD2	80	80	80
CD8	100	100	40
CD11a	90	90	54
CD18	90	90	90
CD54	35	35	35
Asialo-GM1	36	70	100
NKR-P1	95	95	94
Functional features			
NK activity	High	Intermediate	Low
P815 cell killing	Yes	No	No

*Data from reference 19.

[†]Approximate % of cells that express antigen; data summarized from references 2, 13, 16, 19, 25, and 32.

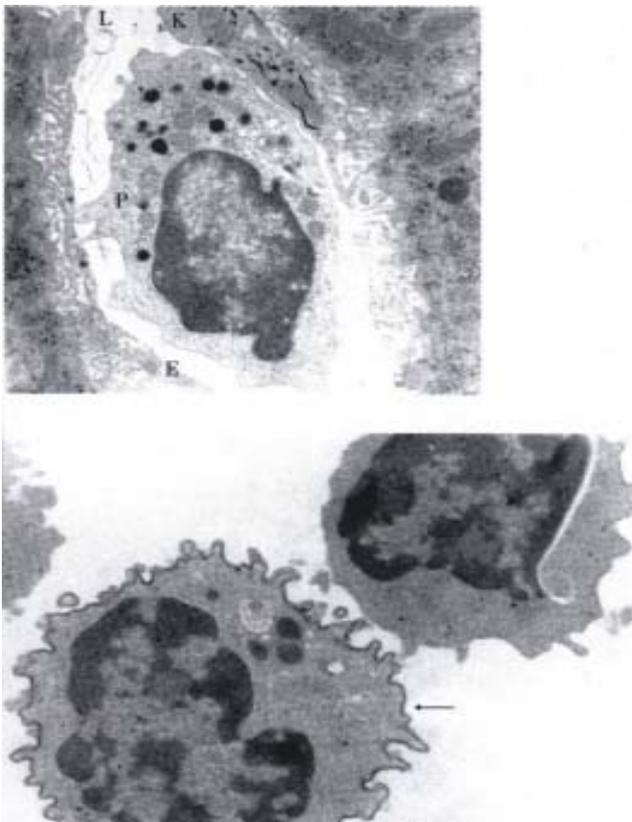


Figure 1 Transmission electron micrograph of a pit cell in a rat hepatic sinusoidal lumen (L). The pit cell shows polarity with an eccentric nucleus. The cytoplasm is abundant and contains characteristic electron-dense granules and other organelles lying mainly on one side of the nucleus. The cell contacts an endothelial cell (E) and a portion of a Kupffer cell (K) with a positive peroxidase reaction product in the rough endoplasmic reticulum. Bar = 1 μm . (from *Hepatology*, 1988;8: 46-52, with permission)

Figure 2 Immunotransmission electron micrograph showing a 3.2.3 positive LGL (pit cell) (arrowhead) and a 3.2.3 negative agranular cell. The 3.2.3⁺ pit cell shows characteristic electron-dense granules in the cytoplasm and immunoperoxidase reaction product on the surface. Bar = 1 μm . (from *Hepatology*, 1995;21: 1690-1694, with permission)

ISOLATION AND PURIFICATION OF PIT CELLS

Our isolation method for rat pit cells is based on a washout technique^[12] followed by purification, based on the magnetic negative-selection of cells using mAbs against surface antigens and receptors found on T and B cells^[6,36]. Since pit cells are apparently not heavily anchored in the liver sinusoids, the cells could be washed out by this non-enzymatic, high-pressure (50cm water) perfusion of the liver via the portal vein with phosphate-buffered saline supplemented with 0.1% EDTA^[12]. The washout was collected from the vena cava. The erythrocytes, granulocytes and cell debris in the washout were removed by Ficoll-Paque gradient centrifugation. The mononuclear cells recovered from the interface of Ficoll-Paque gradient contained T cells, pit cells, B cells, monocytes, and a few Ec and Kc. Adherent monocytes and B cells in this population could be selectively removed in a nylon wool column^[12]. Pit cells were further purified by magnetic cell sorting^[36]. With this system, a highly purified population of pit cells was obtained by negative selection, *i.e.* by elimination of remaining monocytes, T and B cells using specific antibodies and immunomagnetic beads. By this method, we obtained pit cells with a purity of more than 90% and a viability of more than 95% (Figure 3). Moreover, this non-enzymatic method does not destroy cell surface molecules.

Alternatively, pit cells could be isolated by enzymatic methods^[5]. However, this method is time consuming, labor-intensive and only provides pit cells with 30% purity and 90% viability^[6,12].

HETEROGENEITY AND ORIGIN OF PIT CELLS

A considerable set of data indicates that rat pit cells constitute a heterogeneous population. Based on the cell density, pit cells can be separated into a low density (LD) and high density (HD) fraction by 45% iso-osmotic Percoll gradient centrifugation^[19]. These two cell populations have been shown to differ immunophenotypically, morphologically and functionally from each other and from blood LGL (Table 1)^[19,37]. LD pit cells (Figure 4A) contain more rod-cored vesicles and more, but smaller granules than blood NK cells (Figure 4B)^[19,37]. The number of rod-cored vesicles and granule composition (number and size) of HD pit cells are intermediate between LD pit cells and blood NK cells^[19,37]. Immunophenotypically, almost all blood NK cells are asialo-GM1 positive, and 70% of HD pit cells are strongly positive, whereas only 36% of LD pit cells are weakly positive^[19]. Furthermore, functional differences have been observed among these three populations. The LD pit cells are five to eight times more cytotoxic against YAC-1 cells and

colon carcinoma cells than blood NK cells^[37]. The HD pit cells have intermediate cytotoxic activity between LD pit cells and blood NK cells^[37]. In addition, LD pit cells are able to lyse LAK-sensitive P815 mastocytoma targets, which are resistant to normal blood NK cells and hepatic HD pit cells^[37].

Pit cells are considered to originate from blood NK cells^[38,39]. Several evidences support the concept that blood NK cells immigrate into the hepatic sinusoids to become HD pit cells, which further differentiate into LD pit cells. Importantly, the characteristics and functions of HD pit cells are intermediate between blood NK cells and LD pit cells^[19]. Kinetic experiments with sublethal total body irradiation (700cGy) showed that blood NK cells and HD pit cells were depleted in about one week after irradiation, whereas LD pit cells totally disappeared at two weeks after irradiation^[39]. Shielding of the liver gave similar results and splenectomy did not affect pit cell number^[39]. By using intravenous anti-asialo-GM1 antiserum injection, blood NK and HD pit cells totally disappeared within one week of treatment, whereas LD pit cells disappeared from the liver one week later^[39]. The direct evidence for LD pit cells originating from asialo-GM1 positive precursors (blood NK and HD pit cells) was given by the adoptive transfer of fluorescent-labeled HD pit cells into syngeneic rats^[39]. After three days, 5% of labeled cells were recovered in the LD fraction and these cells displayed typical LD pit cell morphology^[39]. These observations also indicate that the life span of pit cells in the liver is about two weeks^[6,39].

The mechanism behind the migration of blood NK cells to the liver sinusoids is not fully understood. Several adhesion molecules were found to be involved in the process^[32]. Rat blood NK and pit cells express LFA-1 (CD11a/CD18) and CD2 (LFA-2) adhesion molecules^[32]. Their ligands, CD54(ICAM-1) and CD58 (LFA-3) were found to be present on liver Ec^[40]. After intravenous injection of antibodies against CD2, CD11a and CD18 into rats, the number of pit cells in the liver decreased significantly, indicating that the interactions of LFA-1/CD54 and CD2/CD58 are involved in the recruitment of pit cells in the liver^[32].

Once marginated in the liver sinusoids, blood NK precursors further differentiate into HD pit cells, then into LD pit cells. The microenvironment of the liver sinusoid is believed to be responsible for this differentiation process^[41]. Vanderkerken *et al* found that Kc were selectively eliminated 3 days after intravenous injection of liposomes containing the cytotoxic drug dichloromethylene

diphosphonate^[41]. The number of HD pit cells declined 3 days after the injection. Conversely, the LD pit cell population showed no change in number after 3 days, but a decline of about 80% was seen 7 days after the injection^[41]. These data indicate that pit cells constitute a Kupffer cell-dependent population and that Kc play an essential role in the differentiation of pit cells in the liver. However, it remains unclear what factor(s) secreted by Kc is responsible for this differentiation. On the other hand, other conditions present in the liver microenvironment, *i.e.* Ec and their secreted factors, may work synergically with Kc to contribute to pit cell differentiation, since cocultivation of HD pit cells with Kc failed to induce the full differentiation of HD into LD pit cells^[41].

FUNCTIONS OF PIT CELLS

NK cells were initially defined as lymphoid cells capable of mediating spontaneous killing of target cells, including tumor and virus-infected cells^[1]. Such NK cytotoxic activity is mediated without prior sensitization and any obvious stimulation or activation^[1]. In addition to this natural spontaneous pathway of tumor killing, NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC) by a mechanism involving CD16, an IgG Fc receptor^[42]. Most human and mouse NK cells express CD16^[2]. Rat NK cells contain genes with a high level of homology to human and murine Fc receptors^[43] and are able to display ADCC^[44]. Unfortunately, no antibodies against rat CD16 are available yet.

Although the cytotoxic function of NK cells is spontaneous, it can be significantly augmented by several cytokines^[2]. One of these, IL-2, has been shown to play a central role in the regulation of NK cells, including augmenting NK cell cytotoxicity, expanding NK cell antitumor spectrum and inducing NK cell proliferation^[4].

NK cytotoxic (*i.e.* cytolytic) activity is usually determined by measuring the release of radiolabeled chromium from target cells after been exposed to effector cells^[2]. A new assay using flow cytometry to assess NK cell activity, in which various dyes are used to differentiate viable from dead target cells, has recently been described^[45]. Initial studies have shown that this method is quick, reliable, and correlates well with the standard ⁵¹Cr release assay^[45].

Besides the cytotoxic function, NK cells can produce various cytokines^[46], regulate the growth of hemopoietic tissues and bone marrow transplants^[47], and participate in the resistance to microbial pathogens^[2].

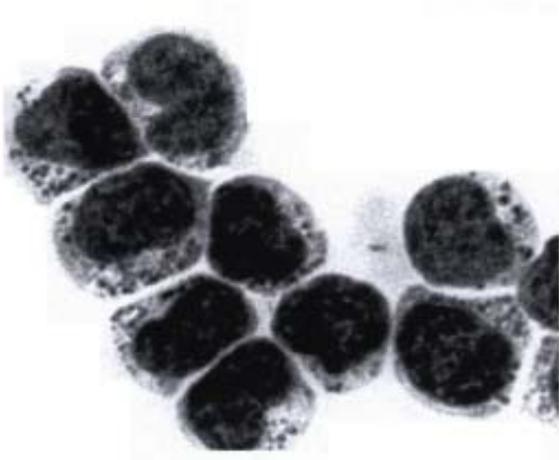


Figure 3 Light micrograph of an isolated and purified pit cell population in a May-Grünwald-Giemsa-stained cytospin. The cells contain cytoplasmic granules, which can be used to recognize and count the number of pit cells in freshly isolated liver-associated lymphocyte population. Bar = 5 μm .

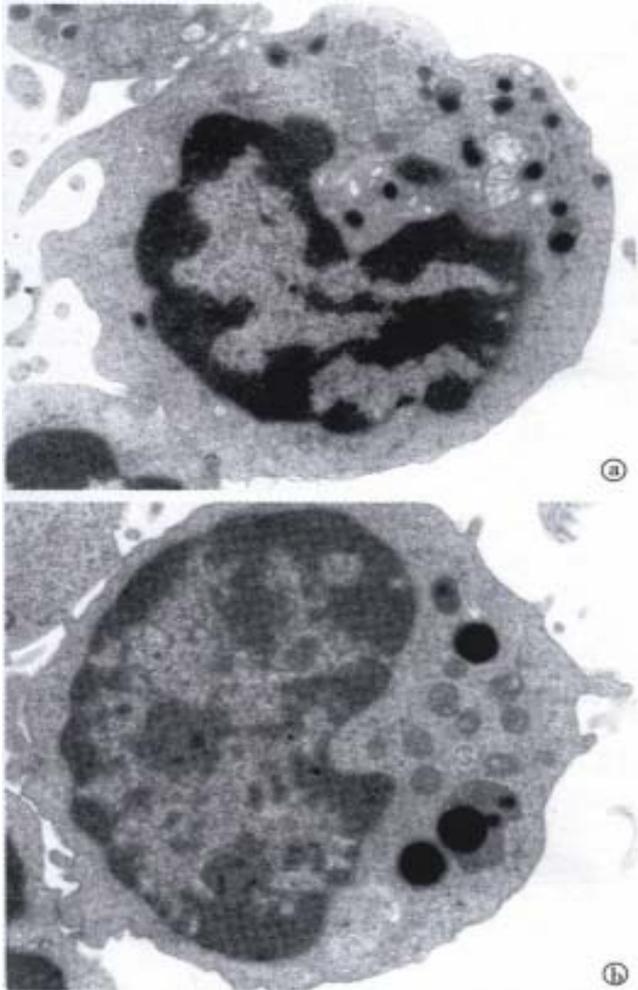


Figure 4 Transmission electron micrographs of a typical LD pit cell and a blood NK cell (B).

(A) The main morphological characteristic of LD pit cells, compared to HD cells and blood NK cells, is the presence of numerous small cytoplasmic granules.

(B) Note the few, but large granules in blood NK cell. Bar = 1 μm . (from *Hepatology*, 1990;12:70-75, with permission)

Most investigations on pit cell functions focus on cytotoxic activity. Rat pit cells have high spontaneous cytotoxic activity against various tumor cell lines, such as YAC-1, P815, CC531s, DHD-K12, L929, 3LL, and 3LL-R^[10]. Compared with blood NK cells, pit cells are four to eight times more cytotoxic against YAC-1 and CC531s cells, and are able to kill the NK-resistant but lymphokine activated killer (LAK)-sensitive P815 cells (Figure 5)^[19,48]. This evidence seems to support the idea that pit cells become activated once they become liver residents. However, it is not understood yet what kind(s) of factor(s) is (are) responsible for the activation of pit cells in the liver, although it has been shown that pit cells are dependent on the presence of a healthy Kc population^[41]. Furthermore, NK activity in the liver could be augmented by BRM, like *Propionibacterium acnes* or maleic anhydride divinyl ether^[24]. Interestingly, an increase in function seems to coincide with a large increase in the number of LGL^[17,24]. IL-2 treatment results in a dramatic accumulation of pit cells in the hepatic sinusoids *in vivo*^[18], induces HD cell proliferation and augments liver HD pit cell cytotoxic activity *in vitro*^[49]. In contrast, IL-2 treatment does not induce liver LD pit cell proliferation^[49].

MOLECULAR MECHANISMS IN NK CELL-MEDIATED CYTOTOXICITY

It is believed that the cytotoxic function of NK cells is mediated by multiple pathways, and each pathway, in principle, encompasses a cascade of events, including recognition of target cells, binding of effector cells to target cells (conjugation), activation of effector cells, delivery of the lethal signal to target cells, and effector cell detachment and recycling^[2,4,50]. Although the precise mechanisms of individual steps in this process have not been fully elucidated, significant progress has been made recently in identifying a number of molecules participating in NK cell-mediated cytotoxicity.

CONJUGATION

The prerequisite of NK cell killing is the binding of one or more effector cells to a target cell, that is, conjugation^[6]. Several adhesion molecules on the NK cell, such as CD2, CD28 and LFA-1, and on the target cell, such as CD58, B7 and CD54, participate in this process, and some of them may also possess co-stimulatory or even triggering capacity in the cytotoxic cascade^[28,50-52].

CD2 is an adhesion molecule of the immunoglobulin (Ig) superfamily expressed on T cells and NK cells^[53]. Approximately 80% of rat pit

cells express CD2^[32]. Although CD2 is a well-known activation structure on T cells^[53], mAbs against CD2, depending on experimental conditions, either induce^[54,55] or inhibit NK activity^[31,56]. Anti-CD2 mAb had no effect on the binding of pit cells to rat colon carcinoma cells (CC531s), or on the cytotoxicity against CC531s^[57]. However, the anti-CD2 mAb enhanced the cytolytic function in rat pit cells against FcγR⁺ P815 target cells^[57]. The ligand of CD2 is another adhesion molecule, CD58 (LFA-3) that is widely expressed on various cell types^[53]. Transfection of CD58 into murine cell lines increased the lysis of these targets by some human CD2⁺ NK cell clones^[58]. However, expression of CD58 alone is insufficient to confer cells sensitive to NK cell-mediated lysis, indicating that CD2 may serve as a costimulatory receptor that augments, but not initiates, the primary activation of NK cells^[58].

The interaction between β2 integrins (CD11a-c/CD18) and ICAMs (in tercellular adhesion molecules) has been found to be important in the binding of NK cells to their targets^[31,51,56]. β2 integrins are heterodimers containing a common β-chain (CD18) and one of three different β-chains (CD11a,CD11b,CD11c). β2 Integrins are expressed only in leukocytes, including NK^[56] and pit cells^[32]. Besides the effect on the binding to target cells, LFA-1 (CD11a/CD18) also participates in signal transduction in NK cells required for NK cell activation^[59]. Cross-linking of LFA-1 on NK cells with its antibody is known to induce a calcium influx, phosphoinositide turnover, tumor necrosis factor-α (TNF-α) production^[59], and to inhibit the target cell killing by NK cells^[60]. LFA-1 was also found to be involved in pit cell-mediated cytotoxicity. The antibody against LFA-1 inhibits not only the binding of pit cells to target cells, but also the killing of target cells by pit cells^[57]. Taken together, this information suggests that LFA-1 on effector cells may have a dual function of binding to target cells and of triggering cytolysis.

Studies have shown that conjugation between NK cells and target cells is essential but not sufficient for NK activity^[50]. After conjugation, further recognition events mediated by triggering and inhibitory receptors on NK cells are required to trigger NK cell cytotoxic activity^[2,51].

NK CELL RECEPTORS INVOLVED IN MHC CLASS I RECOGNITION

NK cell-mediated cytotoxicity was originally thought to be spontaneous and major histocompatibility complex (MHC) class I-unrestricted. However, increasing evidence indicates that NK cells preferentially kill cells

lacking MHC class I. Expression of MHC class I on a number of target cells is correlated with target cell resistance to natural killing^[61-65]. Masking of MHC class I by a n mAb, enhances pit cell-mediated cytotoxicity against CC531s cells, indicating that MHC class I on CC531s cells protects these cells from being killed by rat pit cells^[66].

An explanation for these observations is, that the cytotoxic activity of NK cells is regulated by positive and negative signals from triggering and inhibitory membrane receptors. The final outcome, *i.e.* triggering of cytotoxic activity or inhibition of cytotoxicity, appears to depend on the balance between the positive and negative signals^[51,67]. An increasing number of triggering and inhibitory receptors have been described in recent years^[28,68,69]. In hibitory receptors on NK cells recognize MHC class I, and these generally inhibit the lysis of MHC class I⁺ cells^[28,67-71]. Three receptor families, Ly49, CD94/NKG2 and Killer-cell inhibitory receptors (KIRs), are reported to be involved in the recognition of MHC class I molecules on target cells^[28]. The Ly49 family is the product of at least nine highly related genes (Ly 49A-Ly49I) present on mouse chromosome 6 in the NKC^[72]. Ly-49 homologies have been identified on rat chromosome 4 in the 'NKC'^[73], but have not been found in human. The Ly-49 molecules are type II membrane glycoproteins and belong to the C-type lectin superfamily^[72]. Ly-49 receptors recognize a trimeric MHC class I complex composed of the H-2D or H-2K heavy chain, β2-microglobulin, and a bound peptide. However, the composition of the bound peptide does not appear to influence the interaction to a large extent^[68]. Most, but not all, Ly-49 receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains^[28,67]. Ly-49 receptors containing the ITIM sequence inhibit NK cell effector function^[28,67], whereas Ly-49 lacking ITIM, such as Ly-49D and Ly-49H, can activate NK cell-mediated cytotoxicity when the receptor is ligated by anti-Ly49D mAb^[74].

KIRs, the inhibitory receptors recognizing MHC class I in human NK cells, are monomeric type I glycoproteins that contain Ig domains^[67]. They are encoded by genes located on human chromosome 19q13.4^[28]. Two subfamilies of KIRs can be identified by the number of Ig-like domains in the extracellular regions of the molecules^[28]. The KIR3D subfamily contains three Ig-like domains, whereas the KIR2D contains two Ig-like domains^[28]. A remarkable feature of both KIR2D and KIR3D is the heterogeneity in the length of the cytoplasmic domains. KIRs with long cytoplasmic

domains, *i.e.* KIR2DL (p58) and KIR3DL (p70), contain two ITIM sequences that are responsible for the inhibitory function of these molecules^[67,75]. KIRs containing short cytoplasmic domains, *i.e.* KIR2DS (p50) and KIR3DS, lack ITIM and potentially activate NK activity^[76,77]. Both KIR2D and KIR3D molecules bind to HLA class I trimers, composed of a class I heavy chain, β 2-microglobulin, and a bound peptide^[28].

In addition to KIRs, human NK cells also express another type of receptor capable of recognizing MHC class I, namely CD94/NKG2^[78-80]. This receptor is a heterodimer and is composed of CD94 glycoprotein that is disulfide-bonded to either a NKG2A or a NKG2C subunit^[58]. CD94 and NKG2 genes are present on human chromosome 12p12.3 - p13.1 in the 'NKC'^[81]. Both CD94 and NKG2 molecules belong to the C-type lectin superfamily^[81]. CD94 lacks a cytoplasmic domain, thus lacking intrinsic signal transduction capacity^[81]. However, CD94 is required for the transport and membrane expression of the NKG2A or NKG2C glycoproteins^[78,80]. Since NKG2A possesses an ITIM sequence in the cytoplasmic domain and NKG2C lacks an ITIM, the CD94/NKG2A complex acts as inhibitory receptor, whereas CD94/NKG2C complex acts as a noninhibitory receptor for MHC class I on NK cells^[28,68].

Triggering NK cell receptors

Several membrane molecules are described to serve as triggering receptors on NK cells, including CD16, NKR-P1, NK-TR1, 2B4, P38 and Lag3^[28,51,69]. Only CD16 and NKR-P1 can be regarded as 'established' triggering receptors, while the role of the others is still undefined or controversial^[51]. However, CD16 is responsible and necessary for ADCC and is not involved in natural killing activity^[28].

NKR-P1, a marker of NK cells^[25], is expressed by rat^[25], mouse^[82] and human NK cells^[27], including pit cells^[16]. There are three homologous NKR-P1 genes, NKR-P1A, NKR-P1B and NKR-P1C, in mice and rats^[26,82,83], while only one human NKR-P1 gene has been found^[27]. MAbs against mouse and rat NKR-P1 were found to trigger NK cell-mediated lysis of FcR⁺ target cells, termed re-directed ADCC^[25]. This action also involves a rise in intracellular Ca²⁺ levels^[84] and cytokine production^[85]. Furthermore, mAbs to NKR-P1 stimulate phosphoinositide turnover^[84], arachidonic acid generation^[86] and granule exocytosis^[25]. NKR-P1 on pit cells is involved in pit cell-mediated cytotoxicity against FcR⁺ P815 target, but not in

FcR⁺ CC531s target killing^[66]. However, the function of NKR-P1 on human NK cells appears more complex. Treatment of human NK cells with anti-NKR-P1 mAb gives controversial results, such as activation, inhibition or no effect, depending on the NK cell population studied^[27,87]. The conditions determining the outcome of the engagement of NKR-P1 in human NK cells are not known. When human NKR-P1 is compared with the corresponding rat and mouse proteins, it was found that all rodent NKR-P1 has the C \times CP motif that interacts with phosphorylated P56^{lck}^[88], whereas human NKR-P1 lacks this motif^[28].

THE TWO MAJOR PATHWAYS OF NK CELL-MEDIATED CYTOTOXICITY

CTL and NK cells, including rat pit cells, kill target cells by one of two distinct mechanisms or both: necrosis and apoptosis^[19,48,50,89]. Necrosis or cytolysis is characterized by swelling of the cell and organelles, and results in disruption and leakage of the cell membrane and in lysis^[6]. Cell membrane damage is a key event in cytolysis and release of the cytoplasmic contents possibly leads to an inflammatory response *in vivo*^[6]. The ⁵¹Cr-release assay is thought to reflect this type of damage^[6].

Apoptosis or programmed cell death is morphologically recognizable by membrane blebbing, chromatin condensation, nuclear fragmentation, shrinking, condensation of cells and their organelles, and fragmentation of the cells into apoptotic bodies (Figure 6). The cellular remains are phagocytosed by neighboring cells or macrophages. When phagocytosing cells are absent, apoptotic bodies progress to secondary necrosis^[6,90].

Recent studies have demonstrated that NK cell-mediated apoptosis can mainly be implemented by two pathways, *i.e.* the perforin/granzyme (granule exocytosis) pathway and the Fas/FasL (Fas ligand) pathway^[91,92]. NK cell-mediated lysis is believed to be mainly based on granule exocytosis^[91], whereas Fas-mediated necrosis has been recently reported when caspases are blocked^[93].

The Fas pathway of apoptosis is mediated by the interaction of CD95 ligand (CD95L, FasL) with the apoptosis-inducer CD95 (Fas/APO-1) molecule expressed on target cells^[91,94,95]. CD95 is a member of the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family^[95,96]. CD95 is widely expressed on lymphoid and nonlymphoid tissues, and some tumor cells^[89,95]. The expression of CD95 can be up-regulated by interferon γ (IFN- γ) in various cell lines^[96,97]. The cytoplasmic tail of CD95 contains a motif called 'death domain', that is essential for transmitting the apoptotic signal^[98].

CD95L is a type II transmembrane protein of the TNF family^[95]. CD95L is expressed by activated T cells, NK cells and pit cells^[89,95,99,100]. The binding of CD95L to its receptor CD95 induces apoptosis of CD95-bearing cells^[94]. It is demonstrated that CD95/CD95L plays an important role in the killing of virus-infected cells and tumor cells by CTL and NK cells^[98]. Although CD95 is expressed on CC531s cells and CD95L is expressed on rat pit cells, pit cell-mediated CC531s apoptosis was found to be exclusively implemented by the perforin/granzyme exocytosis pathway^[89].

The perforin/granzyme pathway is a Ca^{2+} -dependent pathway and is mediated by the pore-forming protein perforin and granzymes, especially granzyme B, both of which are stored in NK cell granules^[92]. After the contact between effector and target cells, perforin and granzymes are released in a directed manner into the intercellular space between these cells. Perforin alone induces lysis without inducing apoptosis, *i.e.* fragmentation of target cell DNA. Granzymes play a critical role in the rapid induction of DNA fragmentation by CTL, NK cells and pit cells (Figure 7)^[89,101]. Entrance of granzymes into target cells is postulated to occur through pores produced in the target cell membrane by perforin. Recent studies have shown that granzyme B is endocytosed by target cells independently of perforin, possibly through saturable high affinity cell surface binding sites. In the absence of perforin, granzyme B shows a cytoplasmic localization. When perforin is added, granzyme B relocates to a nuclear position, rapidly inducing apoptosis^[102,103]. These data indicate that the cooperation of the two molecules is necessary to induce apoptosis including DNA fragmentation.

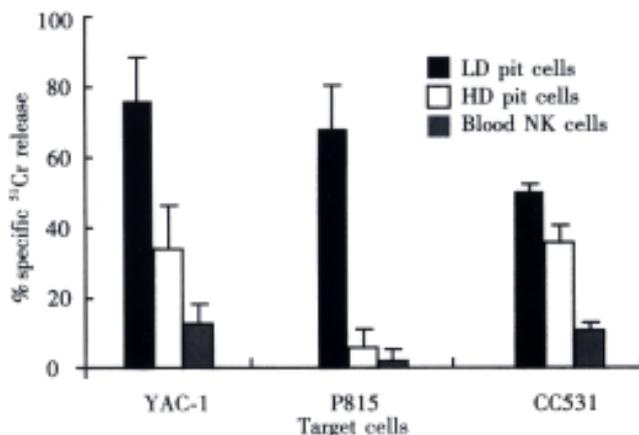


Figure 5 Comparison of cytotoxicity between rat blood NK, HD and LD pit cells. The ratio of freshly isolated effector cells to target cells was 20:1. The cytotoxicity was measured in a 4 hour ⁵¹Cr-release assay for YAC-1 and P815 cells and a 16 hour ⁵¹Cr-release assay for CC531s cells. The data show that LD pit cells are more cytotoxic against YAC-1, P815 and CC531s than HD cells and blood NK cells. Values were means±SD of three to five independent experiments. (Hepatology, 1990;12:70-75, with permission)

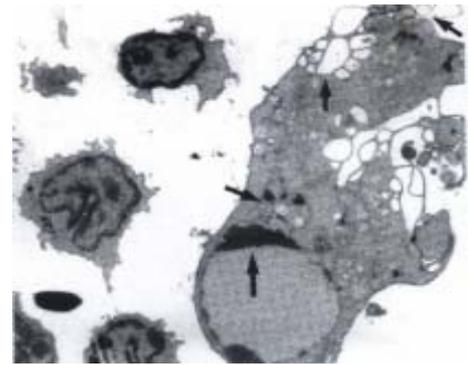


Figure 6 Transmission electron micrograph of an apoptotic CC531s cell (T) coincubated with pit cells (E) for 3 hours. The apoptotic CC531s cell (T) shows vacuolization (large arrowhead), blebbing of the cell surface (small arrowhead), chromatin condensation (thin arrow), and fragmentation of the nucleus (thick arrow). Bar: 2µm. (Hepatology, 1999;29:51-56, with permission)

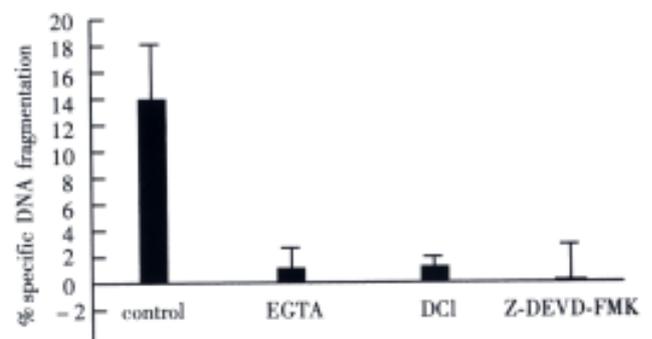


Figure 7 The involvement of the perforin/granzyme pathway in pit cell-induced CC531s apoptosis. The ratio of freshly isolated pit cells to CC531s cells was 10:1. Apoptosis was measured in a 3 hour DNA-fragmentation assay. EGTA is a Ca^{2+} chelator that blocks granule exocytosis and the action of perforin. DCI is a granzyme inhibitor. Z-DEVD-FMK is an inhibitor of caspase 3. These treatments completely inhibit pit cell-induced CC531s apoptosis. Values were mean±SD of three independent experiments. (Hepatology, 1999;29:51-56, with permission)

SUMMARY

There is growing evidence that pit cells are highly active, liver-specific NK cells. Pit cells are located in the liver sinusoids and can be easily isolated and purified by liver sinusoidal lavage and a magnetic separation method. Furthermore, pit cells can be separated into a LD and HD fraction by 45% isotonic Percoll gradient centrifugation. These two populations are shown to differ morphologically, phenotypically and functionally from each other and from blood NK cells. LD pit cells contain more rod-cored vesicles and more, but smaller granules than blood NK cells although both of them share LGL morphology. Phenotypically, LD cells have a higher expression of LFA-1 and a lower expression of asialo-GM1 molecules compared to blood or spleen NK cells. Functionally, pit cells are more cytotoxic against several tumor cell lines as

compared to blood NK cells, and are able to kill-NK-resistant but LAK-sensitive P815 cells. These data indicate that pit cells are a kind of naturally activated NK cells and their cytotoxic function is comparable to IL-2 *in vitro* activated NK cells. The characteristics of HD cells are intermediate between LD pit cells and blood NK cells. Pit cells most probably originate from blood NK cells, although they show mitosis in the liver after certain stimuli. The recruitment of pit cells in the liver is mediated by adhesion molecules. A major challenge is to achieve a better understanding of the mechanisms of pit cell cytotoxicity and the cooperation between pit cells and other cells in the liver, *i.e.* Kc, Ec and LAL. Moreover, since pit cells are located in a strategic position in the hepatic sinusoids, they represent a first line of cellular defense against metastasizing colon cancer cells. The role of pit cells in a number of liver pathologies deserves more attention.

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Dynamic and ultrastructural study of sphincter of Oddi in early-stage cholelithiasis in rabbits with hypercholesterolemia

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Subject headings hypercholesterolemia; cholelithiasis; sphincter of Oddi/ultrastructure

Abstract

AIM To study the relationship between pre-formation of gallstone and the kinetics and ultra-structure of sphincter of Oddi.

METHODS Adult female rabbits were used and divided into 3 groups, and fed with either normal or high cholesterol diet for four or eight weeks. Each group contained eight rabbits. The manometry of sphincter of Oddi, biliary cineradiography, gallbladder volume measurement and ultrastructure observation under electron microscope were performed.

RESULTS In groups I and II, the basal pressure in low-pressure ampulla or high pressure zone of sphincter of Oddi was elevated, the amplitude of phasic contraction was decreased and the volume of gallbladder were increased, with a significant difference ($P < 0.01$, from those of control). Gallstones were found in group II rabbits (7/8). Under cineradiography, low-pressure ampulla showed a spasmodic status without apparent peristaltic contraction. Under electron microscope, inside the muscular cells of sphincter of Oddi, loosening of microfilament and swelling of plasmosomes which congregated at the top were observed. The amount showed no obvious change under nitric oxide synthase (NOS) stain.

CONCLUSION Twisting of the microfilament and disarrangement of kink macula densa inside the muscular cells suggested that the sphincter of Oddi was under spasmodic status. The impaired diastolic function caused and aggravated the stasis of cystic bile. The swelling plasmosome could be one of the important factors in elevating the tonic pressure of sphincter of Oddi.

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INTRODUCTION

Gallstone formation results from many complex factors working together. Among them the bile stasis caused by impaired gallbladder emptying is thought to be the fundamental kinetic factor. Because the sphincter of Oddi (SO) is the only gate through which bile discharged into the duodenum, the bile filling and excretion of the gallbladder are closely related with the motility state of SO. Hypercholesterolemia is a crucial factor of lithogenesis. But up to date, there has been no report on the effect of hypercholesterolemia on SO and the causal relationship between abnormality of SO and gallstone formation. The aim of this study is to investigate the relationship between SO motility and its ultra-morphology, and gallstone formation under the influence of hypercholesterolemia.

MATERIAL AND METHODS

Animal experiment

Twenty-four adult female rabbits weighing between 2.0 kg and 2.5 kg were provided by the Laboratory Animal Center of the Fourth Military Medical University. They were divided randomly into three groups ($n = 8$ each). Control group, fed with standard diet; Group I, standard diet plus cholesterol 1 g/d, 6 times/week, sacrificed after 4 weeks; and Group II standard diet plus cholesterol with 1 g/d, 6 times/week, sacrificed after 8 weeks.

The cholesterol (98% purification, 0.1% phosphatide) was purchased from Changsha Biochemical Pharmaceutical Factory, China. Before the study, the serum cholesterol was tested and only animals with a concentration lower than 3 mmol/L were included in the study. After feeding, a concentration higher than 10 mmol/L was evaluated as hypercholesterolemia. During laparotomy, if crude cholesterol crystal was observed in naked eye in the bile and the surface of gallbladder mucosa, it was interpreted as gallstone formed^[1].

Manometry and equipment

Manometry catheter was modified from Cook's 5F (outer-diameter 1.6 mm) angiographic catheter. The tip hole was blocked, and a side-hole (0.4 mm diameter) was made from 10 mm of the tip. During

manometry, the catheter lumen was infused with 9 g/L saline at 0.25 mL/min using a miniature complaint hydraulic infusion pump^[2]. The baseline resistance for duodenum pressure was set to zero during infusion. Recordings were obtained using a 4-channel RM-6200C polygraphic recorder (Chengdu Instrument Inc., Chengdu, China) with a PT-14M transducer (Gaolian Transducer Science Inc., Shanghai, China). Following an overnight fast (except water), the animals were anesthetized with ketamine (30 mg/kg) (Shaanxi Medical Research Institute) and maintained with 15 mg/kg per 30 min. After anesthesia the animals were tied supine on a board and underwent laparotomy. The extra-hepatic bile duct system was readily visible. The duodenum was cut open, and the catheter was cannulated into common bile duct through the duodenal papilla of biliary duct. The catheter was withdrawn at 1 mm-2 mm increments, and measurement was started after 5 min pause until the tip of the catheter dropped out into the duodenum.

After manometry, 300 g/L meglucamine diatrizoate was injected into biliary tract of controls and group I animals. The cineradiographic recording of biliary tract was obtained at a frame rate of 25/s when the tract was full of contrast.

Nitric oxide synthetase histochemistry and imaging analysis

SO specimens from three rabbits of each group were subjected to histochemistry and image examinations after manometry. Each specimen was fixed in 40 g/L paraformaldehyde PBS solution at 4 °C for 16-18 hours, then put into 100 g/L sucrose solution and preserved at low temperature according to the method of Thune A^[2]. After NADPH stain, the total number of NADPH, mean string length and spacing were measured with MPIAS 500 Multimedia Color Pathologic Image Pattern Analysis System (Qing Ping Imaging Corp.).

Statistical analyses

All results were expressed as $\bar{x} \pm s$. Differences were tested for significance by Student's *t* test, and $P < 0.05$ was considered as significant difference.

RESULTS

Serum cholesterol

Before the cholesterol-feeding, the total serum cholesterol had no difference between control and groups I and II (< 3 mmol/L), while the concentration was elevated significantly (> 10 mmol/L) after four or eight weeks of cholesterol feeding, reaching the standard of hypercholesterolemia. There was significant difference between control and experimental groups ($P < 0.01$), but no difference was observed between

groups I and II ($P > 0.05$), (Table 1).

Table 1 Serum cholesterol concentration (mmol/L)

Group	Pre-feeding	Post-feeding
Control	1.43±0.61	1.36±0.42
Group I	1.57±0.56 ^a	30.61±6.32 ^a
Group II	1.40±0.68 ^b	29.03±2.59 ^b

Post ^a $P < 0.01$, $t = 13.06$, ^b $P < 0.01$, $t = 32.17$ vs control.

Gallbladder volume and bile characteristics

Gallbladder volume of control, group I and II was 2.0 mL ± 1.0 mL, 3.4 mL ± 1.0 mL, 3.9 mL ± 0.9 mL respectively, and volume of gallbladder of group I and II increased more significantly than control group ($P < 0.01$, $t = 2.8$ vs control. ^{II} $P < 0.01$, $t = 3.96$ vs control), and there was no difference between group I and II ($P > 0.05$, $t = 1.04$ vs group I).

Cineradiography

The calibra of low-pressure ampulla segment in control animals was uneven, and enlarged significantly at the site where contrast media concentrated, while in group I it was smaller than that of the controls and the changes of caliber were not prominent during excretion of contrast (Figures 1, 2).

Manometry

In the control, manometry showed a constant high-pressure zone (HPZ) at the terminal of SO segment. It featured a high basal pressure and inconspicuous amplitude fluctuation. A low-pressure zone existed beyond the HPZ (proximal to the SO), with a lower basal pressure but relatively more prominent rhythmic peristalsis. These results coordinated with Toouli's report^[3].

The results obtained in groups I and II are compared and listed in Table 2. The basal pressure in proximal segment of groups I and II was higher than that of controls ($P < 0.01$), but there was no difference between the two groups II ($P > 0.05$). The amplitude of peristalsis in these segments were lower than the controls ($P < 0.01$), with no difference between groups I and II.

The basal pressure in HPZ of groups I and II was higher than the controls, but the amplitudes were lower than the control. There was no difference in basal pressures between groups I and II ($P > 0.05$), (Figures 3-5).

NADPH stain imaging analysis

As shown in Table 3, there was no difference in the total number, total area, area density, area percentage, mean string length and mean spacing among the groups ($P > 0.05$).



Figure 1 The cineradiographic recording of same biliary tract of one control. The contrast clustering the proximal segment (low-pressure ampulla) which enlarged significantly during excretion of contrast indicated that part was diastoled. (↑ → ↑: External segment of SO)

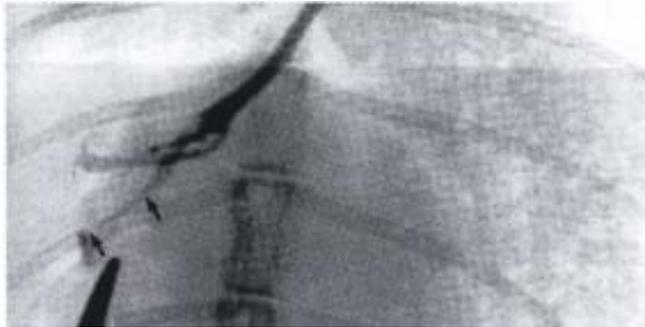


Figure 2 Biliary tract cineradiographic image of group I rabbit. The caliber of proximal segment decreased, retained a fixed spasm state. (↑ → ↑: External segment of SO)

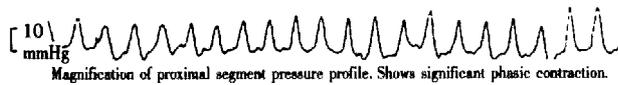
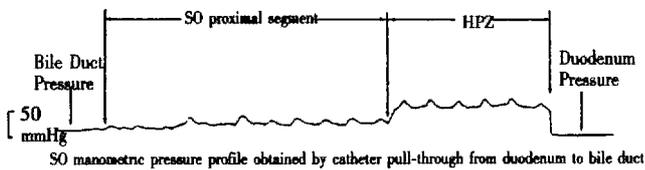


Figure 3 Pressure curve of SO segment of control rabbit.

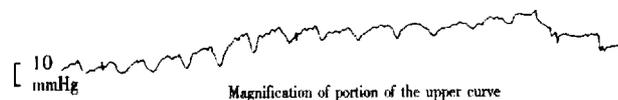
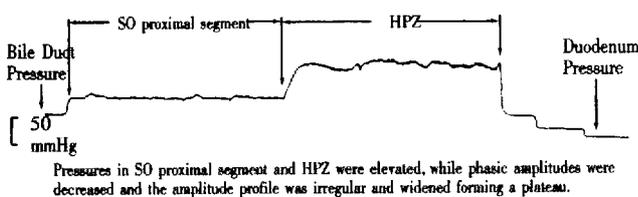


Figure 4 Pressure curve of SO segment of group I rabbit.

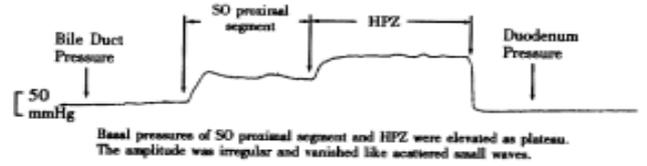


Figure 5 Pressure curve of SO segment of group II rabbit.

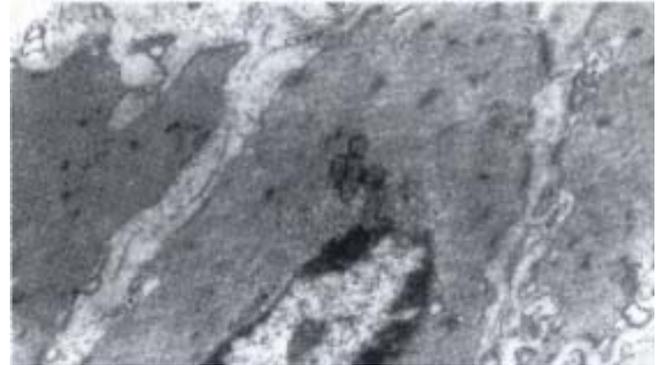


Figure 6 Electron-microscopic scanning of a control 's SO segment smooth muscle. The myofilaments were regularly arranged, kink macula densa clear and dense, plasmosome was normal.



Figure 7 Electron-microscopic scanning of a group I rabbit's SO segment smooth muscle. Image showed the swelling of plasmosome and disappear of intercrystal space, decrease of kink macula densa and twisting of myofilaments.



Figure 8 Electron-microscopic scanning of a group II rabbit's SO segment smooth muscle. It shows the swelling of plasmosome, irregular arranged or fragmented intercrystal space, some vacuolized, congregated at one end of nuclear, and disarrangement of kink macula densa.

Table 2 Manometry of rabbits SO (mmHg)

	Proximal segment		HPZ	
	Basal pressure	Amplitude	Basal pressure	Amplitude
Controls	11.7±2.8	18.1±5.9	69.3±9.8	8.7±3.6
Group I	20.9±6.1	6.4±4.5 ^a	138.4±45.5	4.2±2.2 ^a
Group II	25.6±9.1	6.7±4.8 ^b	144.5±40.5	3.4±2.2 ^b

Proximal: Basal pressure ^a $P < 0.01$, $t = 4.20$, ^b $P < 0.01$, $t = 5.11$ vs controls; Amplitude ^a $P < 0.01$, $t = 4.47$, ^b $P < 0.01$, $t = 4.24$ vs control.

HPZ: Basal pressure ^a $P < 0.01$, $t = 4.20$, ^b $P < 0.01$, $t = 5.11$ vs control; Amplitude ^a $P < 0.01$, $t = 3.15$, ^b $P < 0.01$, $t = 3.17$ vs control.

Table 3 Rabbit's smooth muscle of SO NADPH stain imaging analysis (μm)

	Total (number)	Total (area)	Area (density)	Area (percentage)	Mean (string)	Mean (spatial range)
Controls	107.6±64.4	330.5±133.4	0.0079±0.0048	2.4±1.0	1.6±0.4	77.2±67.7
Group I	117.6±43.4	319.5±126.5	0.0087±0.0032	2.4±0.9	1.5±0.3	69.7±26.2
Group II	109.6±45.4	360.4±190.4	0.0081±0.0033	2.7±1.4	1.6±0.4	79.9±61.7

Reference area is 13550.6μm.

Pathologic examination

Two SO specimens of each group were examined histologically. Each specimen was fixed in formalin and then HE stained. Under light microscope, the smooth muscle arranged regularly. There was no increase of inflammatory cell and collagenous or elastic fibers.

Three SO smooth muscle specimens of each group were collected randomly. They were cut into 1mm small specimens, soaked in 30g/L glutaraldehyde promptly and pre-fixed for 2-4 hours under 4 °C, then post-fixed, dehydrated, embedded, ultra-thin sliced, lead/uranium double stained and observed under JEM-200EX transmission electronic microscope. It was found that SO smooth muscle cells' microfilament was loose and irregularly arranged, kink macula densa reduction, swelling and intercrystal space disappeared or vacuolized and congregated at the top of the nuclear of plasmosome in groups I and II, but no such changes in the controls (Figures 6-8).

DISCUSSION

The biliary tract system can be treated as a half-opened and low-flow system according to its anatomic features, and SO is the only excreting gate to the duo denum. According to Dodd WJ *et al*^[4], at fasting state only 50%-75% of hepatic bile enters the gallbladder while the remainder flows directly into the duodenum. Obviously, SO plays an important role in the participating in hepatic bile flow and remaining the pressure gradient of biliary system, consequently the regulating gallbladder filling and emptying.

The pathologic agents that related to the gallstone formation were still the hot spot of research. Most researchers hold that hypotonus of gallbladder and bile stasis are the main factors of chololithiasis while the dyskinesia may be the result of gallbladder pathologic lesion itself^[5]. However, Lange K *et al*^[6] found that the tension of gallbladder was not decreased, but increased during the early stage of chololithiasis formation. Pitt A *et al*^[7] and Li YF *et al*^[8] had shown that sphincterectomy may prevent the formation of gallbladder stone and improve the contractility of gallbladder partially, but has no effect on the cholesterol crystal genesis. All these suggested that the dysfunction of the SO may result in the abnormal motility of gallbladder.

Toouli J *et al*^[3] and Wei JG *et al*^[9] reported their findings on SO dynamics anatomy, which indicated that choledochus sphincter's physiological anatomy consisted of a high pressure distal segment and a low-pressure proximal ampulla segment. The low-pressure ampulla segment's rhythmic contractile and diastole activity are the primary kinetic sources during fasting. Our results showed that there was no difference in basal pressure of low-pressure segment and total pressure (basal+phasic amplitude) of HPZ between experimental groups and the control. It suggested that hypercholesterolemia did not impair the construction foundation of the contractile activity of SO. But the contractile amplitude of functional ampulla in the experimental rabbits was decreased to those of the controls. The amplitude of low-pressure ampulla/ total (basal + amplitude) was 60.7% in the control rabbits, but was only 23.4% and 25.5% in groups I and II. Obviously that

elevated basal pressure of low-pressure ampulla of SO significantly decreased the phasic amplitude in the experimental animals. The cineradiography showed that the low-pressure ampulla retained a fixed spasm state, thus, the diastolic and peristaltic functions were impaired. According to the hydrodynamic laws, $Q_{out} = -dv/dt$, i.e. excretion volume of liquid is equal to the shrunken volume of the elastic vessel. This suggested that the effective excretion of each pumping (peristaltic wave) per unit time of low-pressure ampulla was impaired in groups I and II.

Additionally, all experimental animals characterized by a more distinctly elevated basal pressure in HPZ than SO proximal segment, and the manometric curve appeared almost like a plateau, on this basis, the phasic amplitude significantly decreased. The HPZ is not merely a passive "valve", but also an intrinsic conducting tube of bile, so that the increased basal pressure may enhance the excreting resistance of the bile significantly. This study demonstrated that the volume of gallbladder increased in experiment animals, suggesting that the tonus alteration in ampulla and HPZ of experimental rabbits may play an important role in the bladder bile stasis, subsequently that may be the pathologic motility function of cholithiasis formation.

There were many reports about nitric oxide (NO) regulation of SO smooth muscle. Some authors confirmed that NO may be the terminal neuron transmitter of SO smooth muscle^[2]. Several studies showed that there were abundant NO synthetase (NOS) in SO, which had tissue-specific characteristics. The results may demonstrate that relaxation of SO is related to the quality and quantity of NOS directly. But our data showed no difference in quantities of NOS between the experimental and control animals, indicating that the decreased diastolic activity of the sphincter may not be the result of reduced quantities of NOS in sphincter tissues.

Gaasch WH *et al*^[10] reported that diastolic deficiency is the early change of heart dysfunction.

This may be related to malfunctioning energy metabolism of the heart muscle cell. This study showed that the cell of SO underwent changes of plasmosome swelling which may affect the energy metabolism of sphincter cell, while the swelling of myofilament may indicate the intracellular edema of sphincter. Zhang JS *et al*^[11] reported that during sphincter cell culture *in vitro* in high cholesterol solution, the cell underwent similar alteration. The sensitivity of cell membrane to digestive synthetase was increased. According to these data, the changes of sphincter cells of experimental animals may relate to the alteration of cell membrane biological characteristics due to hypercholesterolemia. Since the sphincter functional ampulla (low pressure segment) acts like kinetic heart pump during bile excretion, we conclude that diastole of functional ampulla may be an active movement and energy consumption process, the swelling of plasmosome may result in decrease of energy supply during diastole, leading to diastole deficiency eventually.

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The relation between HLA-DQA1 genes and genetic susceptibility to duodenal ulcer in Wuhan Hans

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Subject headings duodenal ulcer; HLA-DQA1 gene; polymerase chain reaction; restricted fragment length polymorphism; genetic susceptibility

Abstract

AIM To study the genetic susceptibility of HLA-DQA1 alleles to duodenal ulcer in Wuhan Hans. **METHODS** Seventy patients with duodenal ulcer and fifty healthy controls were examined for HLA-DQA1 genotypes. HLA-DQA1 typing was carried out by digesting the locus specific polymerase chain reaction amplified products with alleles specific restriction enzymes (PCR-RFLP), i.e. *Apal-I*, *Bsaj-I*, *Hph-I*, *Fok-I*, *Mbo-II* and *Mnl-I*.

RESULTS The allele frequencies of DQA1*0301 and DQA1*0102 in patients with duodenal ulcer were significantly higher and lower respectively than those in healthy controls (0.40 vs 0.20, $P = 0.003$, $P_{\text{correct}} = 0.024$) and (0.05 vs 0.14, $P = 0.012$, but $P_{\text{correct}} > 0.05$), respectively.

CONCLUSION DQA1*0301 is a susceptible gene for duodenal ulcer in Wuhan Hans, and there are immunogenetic differences in HLA-DQA1 locus between duodenal ulcer patients and healthy controls.

INTRODUCTION

Duodenal ulcer is a set of extensive heterogeneous hereditary diseases with varied pathogeny and pathogenesis^[1], there were some immunological changes in some patients with duodenal ulcer^[2-4]. Immunopathogenesis may play a role in the pathogenesis of duodenal ulcer. Human leukocyte antigen (HLA-DQA1 gene) contributes to the pathogenesis of some diseases. Investigating the correlation between HLA-DQA1 gene and duodenal ulcer may afford important clues to reveal duodenal ulcer pathogenesis. HLA-DQA1 genotypes are highly polymorphism and can not be determined by ordinary serological methods. We applied polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) nucleotide typing technique to study the genetic susceptibility of HLA-DQA1 alleles to duodenal ulcer in Wuhan Hans.

MATERIALS AND METHODS

Subjects

We examined 70 patients with duodenal ulcer (53 male, 17 female; mean age 38 years) and 50 healthy controls (38 male, 12 female; mean age 36 years) for HLA-DQA1 genotypes. All these subjects were from the Digestive Department, Second Hospital of Hubei Medical University. Duodenal ulcer was diagnosed endoscopically. The ulcerogenic drugs, such as aspirin, indomethacin or corticosteroids were ruled out as the etiological factors in all of the duodenal ulcer patients. Patients with combined duodenal and gastric ulcers were excluded from this study. All patients and controls were Chinese Hans living in Wuhan.

Primer synthesis and reagents

HLA-DQA1 locus specific PCR primers were designed by Ota M^[5]. Primer GH26: 5'-GTGCTGCAGGTGTAAACTTGTACCAG-3', primer GH27: 5'-CACGGATCCGGTAGCAGCGGTAGAGTTG-3', they were synthesized by Shanghai Branch, Canadian Sangon Company. Alleles specific restriction enzymes (*Apal-I*, *Bsaj-I*, *Hph-I*, *Fok-I*, *Mbo-II* and *Mnl-I*) were all purchased from New England Biolabs Company. Taq DNA polymerase and DNA dNTP were purchased from Canadian Sangon Company. Proteinase K and pBR322/*Hae*-III Mark were provided by Huamei Bioengineer Company.

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Methods

DNA extraction Leukocytes from anticoagulated whole blood were isolated with hypo-osmotic haemolytic method. DNA was extracted by phenol-chloroform extraction method from leukocytes.

PCR amplification A total amount of 100 μ L reaction solution contained 1 μ g DNA sample, 200 μ mol/L dNTP and 50 pmol each of primers 1 and 2. The amplification of HLA-DQA1 exon 2 was carried out in one reaction system and PCR procedure was as follows: pre-denaturation at 94 $^{\circ}$ C for 5 min, addition of 2.5 μ Taq DNA polymerase, denaturation at 94 $^{\circ}$ C for 1 min, annealing at 62 $^{\circ}$ C for 2 min and extension at 72 $^{\circ}$ C for 2 min repeated 30 cycles and finally extension at 72 $^{\circ}$ C for 5 min to terminate the reaction. Because some alleles of HLA-DQA1 locus had a 3 bp deletion in the PCR-amplification the size of the amplified region of the DQA1 exon 2 was 239 bp or 242 bp as indicated (Figure 1).

Digestion with restriction endonucleases After amplification, aliquots (7 μ L) of the reaction mixture were digested with restriction endonucleases (Apa-I, Hph-I, Fok-I and Mbo-II: 5 units) at 37 $^{\circ}$ C for 1 h after addition of appropriate incubation buffer. When digested by Bsa-I, the reaction mixture was incubated at 60 $^{\circ}$ C for 1 h.

Acrylamide gel electrophoresis Samples of the restriction enzyme cleaved product amplified DNA were usually subjected to electrophoresis in 12 % polyacrylamide gel in a horizontal minigel apparatus. They were expected to allow discrimination of seven alleles among the eight DQA1 alleles because the DQA1*0101 and DQA1*0102 alleles gave the same restriction map. However, when digested with Mnl-I enzyme, DQA1*0101 and DQA1*0102 can be discriminated with 15% polyacrylamide gel. Cleavage or no cleavage of amplified fragments was detected by staining with ethidium bromide. Eight alleles of HLA-DQA1 locus were determined by this method^[5]. The restriction map of the DQA1 alleles concerning these enzymes is shown in Figure 2.

Statistical analysis

Chi-square test or Yate's correction Allele detect frequencies (f) were made by the direct count. According to Hardy-Weinberg theorem, gene frequencies (AF) were calculated by $AF = 1 - f$. Comparisons of allele frequencies were made by the exact probabilities in 2×2 table P test. Relative risk frequencies (RR) were made by Wolf formula. The corrected P value was P times the number of alleles in comparison^[6].

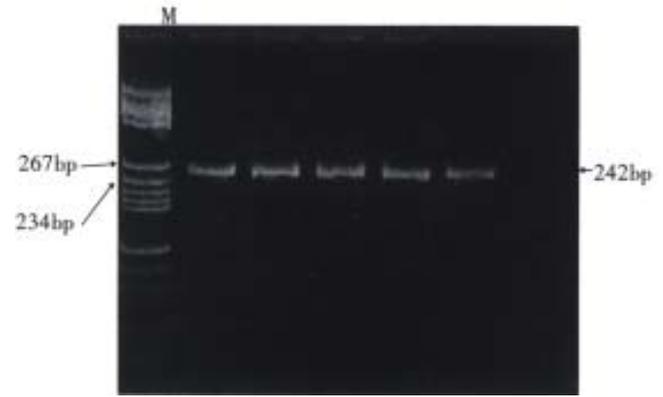


Figure 1 The size of the amplified region of the HLA-DQA1 exon 2 is 242 bp as indicated (12% PAGE). M: pBR322/Hae-III Mark

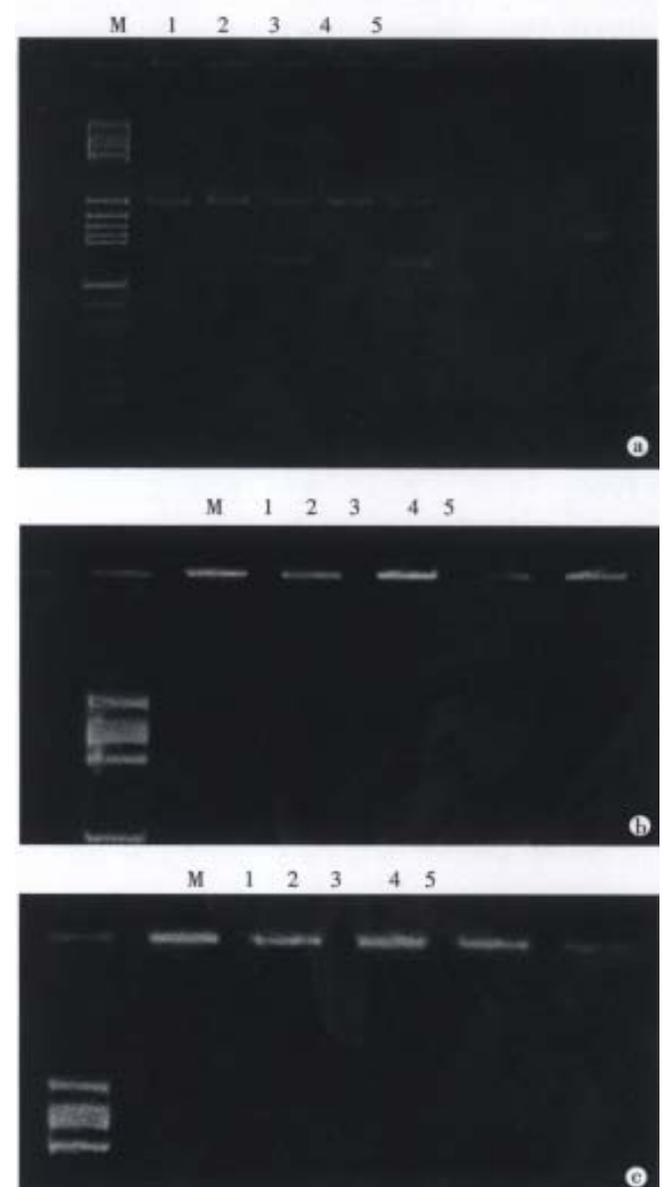


Figure 2 Cleavage patterns of polymorphic restriction fragments in the PCR-amplified DQA1 genes obtained with restriction enzymes (12% PAGE). M: pBR322 / Hae-III Mark; 1: Apa-I; 2: Hph-I; 3: Bsa-I; 4: Fok-I; 5: Mbo-II; a: 0301 / 0501; b: 0103 / 0501; c: 0102 / 0601

RESULTS

The distribution of HLA-DQA1 alleles is shown in Table 1. DQA1*0301 is the allele commonly seen in healthy controls. The detect frequency and the allele frequency of DQA1*0301 was 0.36 and 0.20 in healthy controls, and 0.64 and 0.40 in patients with duodenal ulcer, respectively. The increased allele frequency of DQA1*0301 in patients with duodenal ulcer (0.40) was statistically significant ($RR = 3.20$, $P = 0.003$, $P_{correct} = 0.024$) compared with healthy controls (0.20). In contrast, the detect frequency and the allele frequency of DQA1*0102 was 0.26 and 0.14 in healthy controls, and 0.09 and 0.05 in patients with duodenal ulcer, respectively. The decreased frequency of DQA1*0102 in patients with duodenal ulcer (0.05) controls (0.14) was statistically significant compared with healthy ($RR = 0.27$, $P = 0.012$, but $P_{correct} > 0.05$). There were no significant differences in other allele frequencies between duodenal ulcer patients and healthy controls.

Table 1 Distribution of DQA1 allele frequency in duodenal ulcer patients and healthy controls from Wuhan Hans

HLA-DQA1 alleles	Controls (n = 50)			DU (n = 70)		
	PN	PF	AF	PN	PF	AF
0101	10	0.20	0.11	9	0.13	0.07
0102	13	0.26	0.14	6	0.09 ^a	0.05
0103	8	0.16	0.08	12	0.17	0.09
0201	7	0.14	0.07	10	0.14	0.07
0301	18	0.36	0.20	45	0.64 ^b	0.40
0401	2	0.04	0.02	1	0.01	0.01
0501	12	0.24	0.13	22	0.31	0.17
0601	7	0.14	0.07	12	0.17	0.09

PN: positive number; PF: phenotype frequency; AF: allele frequency. Compared with controls: ^a $P = 0.012$, $RR = 0.27$, $P_{correct} > 0.05$; ^b $P = 0.003$, $RR = 3.2$, $P_{correct} < 0.05$.

DISCUSSION

Various factors are accepted as the causes of duodenal ulcer disease. Not only inherited factor, but also immuno-dysfunction is associated with duodenal ulcer. Since the discovery of *Helicobacter pylori* (*H. pylori*)-its infection has been widely accepted as the predominant cause of duodenal ulcer disease. The pathogenic mechanisms that *H. pylori* causes human disease remain poorly understood. The human leukocyte antigen (HLA) DQA1 gene contributes to the host response against *H. pylori*. Many immune responses are controlled by genes of the major histocompatibility complex which encode HLA. Individuals with different HLA types may differ in susceptibility or resistance to particular infectious pathogens, and associations between HLA polymorphism and susceptibility or resistance to infectious or autoimmune diseases have been

identified^[7,8]. The polymorphic HLA-DQA1 genes encode polypeptides which fold together to form a receptor that specifies T-lymphocyte recognition of self and foreign peptides^[9]. Therefore we examined the HLA DQA1 locus in patients with duodenal ulcer in an attempt to investigate immunogenetic differences in the host.

Researches in the correlation between HLA and peptic ulcer disease were started sixty years ago. The finding showed that HLA-B5 antigen was associated with peptic ulcer^[10-13]. However, traditional serological method was used in some investigations, but it was obsolete and inaccurate. The individual genetic difference of HLA at the level of DNA was produced by encoded gene^[14]. To understand the disease essence, correlation between peptic ulcer and HLA should be further studied with nucleotide typing technique.

DQA1 gene is the most polymorphism in HLA Class II gene and is strongly associated with some diseases. We analysed nucleotide sequences of PCR-amplified regions in the DQA1 genes for allele specific restriction sites to study DQA1 genes involved in the genetic susceptibility to duodenal ulcer in Wuhan Hans. The result of the present study indicated a significant difference in the frequencies of HLA-DQA1 alleles between duodenal ulcer patients and healthy controls. The allele frequency of DQA1*0301 was significantly higher ($RR = 3.20$, $P = 0.003$, $P_{correct} = 0.024$) in duodenal ulcer patients (0.40) than in healthy controls (0.20). In contrast, the allele frequency of DQA1*0102 was significantly lower ($RR = 0.27$, $P = 0.012$) in duodenal ulcer patients (0.05) than in healthy controls (0.14), but $P_{correct} > 0.05$. These results suggest that the HLA-DQA1*0301 may contribute to the susceptibility to duodenal ulcer.

DQA1 chain is not on isolated entity and is tightly linked with DR and DQB locus genes in the all genes region of the major histocompatibility complex. The result of the present study may provide a clue that HLAII genes are more strongly associated with duodenal ulcer than HLAI genes. The clue could not affirm which locus gene of HLAII Class genes is more important. Further genetic analysis of DRB1 and DQB1 genes, using various DNA markers to clarify the host genetic factors of susceptibility or resistance to duodenal ulcer, is required.

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The relationship of *Imp2* and DR3 genes with susceptibility to type I diabetes mellitus in south China Han population

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Subject headings diabetes mellitus; *Imp2* genes; DR3 genes; polymerase chain reaction; restriction fragment length polymorphism; genetic susceptibility

Abstract

AIM To study the relationship of *Imp2* and DR3 genes with type I diabetes mellitus.

METHODS *Imp2* genotypes and DR3 were identified in 68 patients with type I diabetes mellitus (I-DM) and 71 healthy controls. Then, I-DM patients and controls were respectively allocated into DR3-positive and DR3-negative groups. The frequencies of *Imp2* and DR3 gene in random subjects, and *Imp2* genotypes in DR3-matched subjects were compared between I-DM patients and controls. At the same time, I-DM patients were divided into 3 groups based on the onset age of diabetics: group A ≤ 14 years, group B 15-30 years and group C ≥ 31 years.

RESULTS The frequency of DR3 in I-DM patients was significantly higher than that in controls (47% vs 21%, $P < 0.005$), and it was significantly higher in group A than that in group B+C (70% vs 36%, $\chi^2 = 7.07$, $P < 0.01$). There was a significant difference among groups with different onset age of diabetics ($\chi^2 = 8.19$, $rp = 0.33$, $P < 0.05$). In random subjects, the frequency of *Imp2* R/R in I-DM patients was lower (43% vs 61%, $P < 0.05$) and *Imp2*-R/H higher (53% vs 28%, $P < 0.05$) than that in controls, and there was no significant difference among groups with different onset age of diabetics. In DR3-positive subjects, the frequency of *Imp2*-R/R in I-DM patients was lower (47% vs 87%, $P < 0.05$) and *Imp2*-R/H

higher (47% vs 13%, $P < 0.05$) than that in controls. In DR3-negative subjects, the frequency of *Imp2*-R/H in I-DM patients was higher than that in controls (58% vs 32%, $P < 0.01$), but the frequency of *Imp2*-R/R and *Imp2*-H/H was not significantly different between these two groups.

CONCLUSION DR3 gene may be one of the susceptible genes of I-DM, and significantly related to the onset age of diabetics, and the persons with DR3 may have a younger onset age of diabetes. The *Imp2*-R/R may be the protective genotype of I-DM, and *Imp2*-R/H the susceptible genotype. These were not affected by DR3 gene. *Imp2* genotypes were not related with the onset age of diabetics.

INTRODUCTION

Type I diabetes mellitus (I-DM) is an autoimmune disease due to insufficient insulin secretion resulting from immunologically mediated destruction of pancreatic beta cells. Previous studies suggested that some genes (including DR3 gene) within Major Histocompatibility Complex (MHC) class II region determined the susceptibility to I-DM in other populations^[1-4]. We investigated the relationship of DQA1 and DQB1 with I-DM^[5,6], in south China Han population. However, the relationship between DR3 and I-DM has not yet been studied. *Imp2* is another gene locus within MHC class II region, its polymorphism site is at R/H-60. When the amino acid at position 60 is arginine (R) or histidine (H), the allele will be *Imp2*-R or *Imp2*-H. *Imp2* has 3 genotypes, i.e., *Imp2*-R/R, *Imp2*-R/H and *Imp2*-H/H. The relationship between *Imp2* and I-DM is still controversial. This study aims at investigating the relationship of *Imp2* and DR3 gene with I-DM in south China Han population.

MATERIALS AND METHODS

Subjects

Sixty-eight I-DM patients and 71 healthy persons (as controls) were included in this study. All the subjects were Han population without relatives from southern China. The controls were the healthy

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persons having no family history of autoimmune or hereditary diseases. The diagnosis of I-DM was based on 1985 WHO criteria. Both IDM patients and controls were subdivided into DR3 positive and DR3-negative groups. The I-DM patients were divided based on the onset age of diabetics into 3 groups: group A ≤ 14 years, group B 15-30 years and group C ≥ 31 years.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes treated with protease-K, saturated phenol/chloroform extraction and collected by cold ethanol precipitation.

Identification of DR3 gene

DR3 gene was identified by the nested-PCR^[7]. First, the exon2 of DRB1 was amplified from genomic DNA, and the PCR primers were exon2.1, 5'-CCGGATC CTTCGTGTCCCCACAGCAC-3' and exon 2.2, 5'-TCGCCGCTGCACTGTGAAG-3'. Then, DR3 gene was amplified from exon2, the PCR primers were DR3.1, 5'-TACTTCCATAACCA GGAGGAGA-3', DR3.2, 5'-TGCAGTAGTTGTCCACCCG-3'. The primer amplifying DR3 was used to amplify all the alleles (except for DR10) within exon2 of DRB1 to justify its specificity.

Genotyping of *Imp2*

Imp2 was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)^[2].

PCR amplification The PCR primers were *Imp2*-1, 5'-GCCAGCA AGAGCGGAAACAAG-3' and *Imp2*-2, 5'-GTGAACCGAGTGTTTGAGAAGC-3'. The PCR product was a DNA fragment of 252bp containing the *Imp2* polymorphism site R/H-60. PCR was performed in 50 μ L of reactive volume containing 100 ng of genomic DNA, 0.8 μ mol/L primer, 0.2 μ mol/L dNTP, 5 μ L 10 \times PCR buffer and 1.5 u Taq DNA polymerase. The samples were subjected to 35 thermal cycles of 50 s at 94 $^{\circ}$ C for denaturing, 60 s at 52 $^{\circ}$ C for annealing, and 60 s at 72 $^{\circ}$ C for extension, 7 min at 94 $^{\circ}$ C for denaturing before the first cycle, and 5 min at 72 $^{\circ}$ C for extension after the last cycle.

Hha-I digestion of PCR production The reactive volume 20 μ L contained 10 μ L of PCR product, and 10 u of Hha-I (Gibco). The samples were incubated in warm water bath at 37 $^{\circ}$ C overnight. The allele *Imp2*-R contained the *Hha*-I site, however, the *Imp2*-H did not. Therefore, the polymorphism of *Imp2* can be revealed by *Hha*-I. When *Hha*-I digestion was performed, the PCR

products with known *Imp2* genotype were used as controls.

Statistical analysis

The χ^2 test in the 2 \times 2 table was used to compare the frequencies of *Imp2* genotypes and DR3 gene between I-DM patients and controls, and if the results were significant, the odds ratio (Ψ) would be calculated. The frequencies of *Imp2* genotypes and DR3 gene were compared among groups with different onset age of diabetics by the χ^2 test in the R \times C table, and if the results were significant, the Pearson's *rp* would be calculated.

RESULTS

Genotyping of *Imp2* and identification of DR3 gene Figure 1 shows the DR3 gene by nested-PCR. Figure 2 shows the various genotypes of *Imp2* and the product of PCR. The identification of DR3 was made twice in 90 samples, getting 99% (89/90) precision. Genotyping of *Imp2* was performed twice in 70 samples with 100% coincidence. The primer amplifying DR3 and Hha-I digestion had excellent specificity.

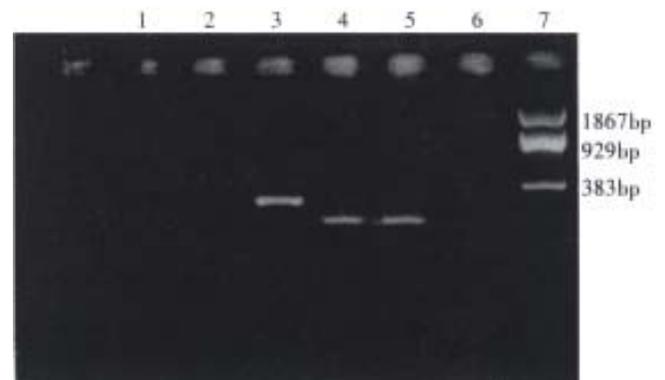


Figure 1 PCR products of DR3 and exon2 of DRB1. 1. negative control of PCR; 2. exon2; 3. homozygous cell line of DR3; 4. DR3-positive sample; 5. DR3-negative sample; 6. pBR322DNA/-BstNI Marker.

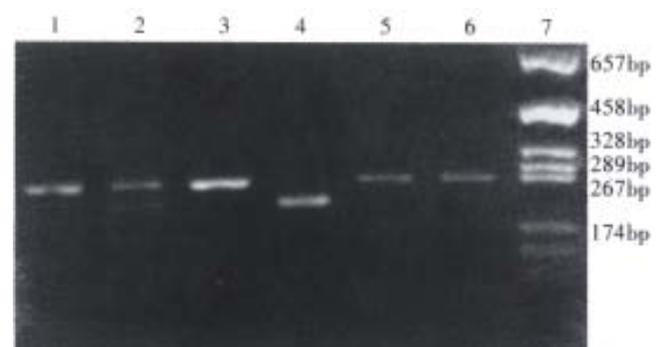


Figure 2 Genotyping of LMP2 gene by PCR-RFLP. 1, 3, 5. products of PCR; 2. LMP2-R/H; 4. LMP2-R/R; 6. LMP2-H/H; 7. Pge m7Zf (+) Hae-III DNA marker.

Distribution of DR3 and *Imp2* in random subjects

The frequency of *Imp2*-R/R in 1-DM patients was significantly lower, but the frequency of *Imp2*-R/H and DR3 was significantly higher than that in controls. And *Imp2*-H/H showed no significant difference between these two groups (Table 1).

Table 1 Comparison of frequencies of *Imp2* genotypes and DR3 gene between I-DM patients and controls in random subjects

	n	DR3	<i>Imp2</i>		
			R/R	R/H	H/H
1-DM	68	32(0.47) ^a	29(0.43) ^b	36(0.53) ^c	3(0.04)
Controls	71	15(0.21)	43(0.61)	20(0.28)	8(0.11)

Compared with controls: ^a $\chi^2 = 10.44$, $\psi = 3.32$, $P < 0.005$; ^b $\chi^2 = 4.47$, $\psi = 0.48$, $P < 0.05$; ^c $\chi^2 = 8.86$, $\psi = 2.87$, $P < 0.005$.

Distribution of *Imp2* in DR3-matched subjects

In DR3-positive subjects The frequency of *Imp2*-R/R and *Imp2*-R/H in I-DM patients was respectively lower and higher respectively than that in controls with significant difference, but *Imp2*-H/H had no significant difference between these 2 groups (Table 2).

In DR3-negative subjects The frequency of *Imp2*-R/H in I-DM patients was significantly higher than that in controls, while *Imp2*-R/R and *Imp2*-H/H had no significant differences between these two groups (Table 2).

Table 2 Frequencies of *Imp2* genotypes compared between I-DM patients and controls in DR3-matched subjects

	Positive DR3		Negative DR3	
	1-DM (n = 32)	Controls (n = 15)	1-DM (n = 36)	Controls (n = 56)
R/R	15(0.47) ^a	13(0.87)	14(0.39)	30(0.54)
R/H	15(0.47) ^b	2(0.13)	21(0.58) ^c	18(0.32)
H/H	2(0.06)	0(0.00)	1(0.03)	8(0.14)

Compared with controls: ^a $\chi^2 = 7.50$, $\psi = 0.14$, $P < 0.01$; ^b $\chi^2 = 4.98$, $\psi = 5.74$, $P < 0.05$; ^c $\chi^2 = 6.15$, $\psi = 2.96$, $P < 0.01$.

Distribution of *Imp2* and DR3 in groups with different diabetic onset age

There was a significant difference in DR3 frequency among different groups of diabetic onset age. The frequency of DR3 in group B was not significantly different from that in group C, thus, groups B and C were merged into group B+C, and its frequency of DR3 was 36% (16/45). When group A was compared with group B + C, the χ^2 was 7.07, and $P < 0.01$, indicating that, the younger the age of diabetic onset, the higher the DR3 frequency (Table 3).

There were no significant differences in *Imp2* frequencies among different groups of diabetic onset age (Table 3).

Table 3 Frequencies of *Imp2* genotypes and DR3 gene compared among different age groups of diabetic onset

	n	DR3		<i>Imp2</i>		
		Positive	Negative	R/R	R/H	H/H
Group A	23	16(70%)	7(30%)	11(48%)	10(43%)	2(9%)
Group B	26	11(42%)	15(58%)	8(31%)	17(65%)	1(4%)
Group C	19	5(26%)	14(74%)	10(53%)	9(47%)	0

Difference of DR3 frequency among different age groups of diabetic onset was $\chi^2 = 8.19$, $rp = 0.33$, $P < 0.05$, *Imp2* was $\chi^2 = 4.53$, $P > 0.05$.

DISCUSSION

The relation between DR3 and I-DM Previous studies showed that DR3 is one of the susceptible genes of I-DM in some populations^[1]. We studied the Han population in south China, and found that the frequency of DR3 in I-DM patients was significantly higher than that in controls. It suggests that DR3 may be one of the susceptible genes of I-DM, and the persons with DR3 have a higher risk of suffering from I-DM.

Relation between *Imp2* and I-DM The *Imp2* encoded product is LMP protease, which is responsible for processing antigen, and may play an important role in antigen presentation^[8]. Therefore, *Imp2* may be an attractive candidate as a gene related with susceptibility to I-DM. Studies on the relation between *Imp2* and I-DM were still controversial. A recent study by Deng *et al*^[9] suggested that *Imp2*-HR may be the protective genotype, and *Imp2*-R/H the susceptible genotype of I-DM, and they may have no linkage disequilibrium to HLA-DR/DQ. Undlien *et al*^[10] divided the subjects into many subgroups according to HLA-DRB1-DQA1-DQB1, and found that *Imp2* genotypes had no association with I-DM, but, the sample size was too small after divided into subgroups. The studies by Van Endert *et al*^[11], Kawaguchi *et al*^[12] and Chauffert *et al*^[13] yielded the similar results.

Our data indicated that, in random subjects, the frequencies of *Imp2*-R/R and *Imp2*-R/H in I-DM patients were significantly lower, and higher than those in controls respectively (Table 1). Therefore, *Imp2*-R/R may be the protective genotype, and *Imp2*-R/H the susceptible genotype of I-DM. In order to make sure that the effect of *Imp2* on I-DM will be affected by DR3, we investigated the frequency of *Imp2* genotypes in DR3-matched subjects. We divided the subjects into DR3 positive and DR3 negative groups and compared the frequencies of *Imp2* genotypes between I-DM and controls respectively in these groups, and obtained nearly the same results as

those in random subjects (Table 2). Therefore, our data suggested that the relationship between *lmp2* and I-DM may not be affected by DR3 gene. However, we have not study the relationship between *lmp2* and other protective and susceptible genes of I -DM within MHC class II region, so we can not confirm whether *lmp2* genotype s have independent effects on I-DM or not. Nevertheless, our data show that *lmp2* genotypes can predict the risk of I-DM occurrence. The persons with *lmp2*-R/R have a decreased risk, and those with *lmp2*-R/H a increase d risk of suffering from I-DM.

Relation among *lmp2* genotype, DR3 gene and the onset age of diabetics Recently, many studies have shown that I-DM is a heterogeneitic disease. The study by Caillat-Zucman *et al*^[8] showed that DRB1, DQA1 and DQB1 were not only associated with the predisposition to I-DM, but also with the onset age of diabetics, and the younger the onset age of diabetics, the higher the frequencies of these genes. Some recent studies by my colleagues showed the same results in DQA1 and DQB1^[9,10]. This study also indicated that DR3 was associated with the age of onset in I-DM and the frequency of DR3 in patients with I-DM developed in childhood (≤ 14 years) was significantly higher than that in adulthood (≥ 15 years), suggesting that the persons with DR3 may have an earlier diabetic onset.

However, our data did not show any differences in frequencies of *lmp2* genotypes among various age groups of diabetic onset, suggesting that the *lmp2* genotypes may not have any association with the age of diabetic onset, and the distribution of *lmp2* genotypes may not be the same as that of DR3, D QA1 and DQB1 in I-DM patients.

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Experimental study of "Tong Xia" purgative method in ameliorating lung injury in acute necrotizing pancreatitis

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Subject headings pancreatitis/ therapy; lung disease; tumor necrosis factor; Tong Xia method

Abstract

AIM To investigate the role of tumor necrosis factor (TNF) in lung injury during acute necrotizing pancreatitis (ANP), and the therapeutic effect of "Tong Xia" purgative method in minimizing the severity of lung injury. **METHODS** Fourteen canines were randomly divided into 3 groups: the "Tong Xia" treatment group ($n = 5$) using Dachengqitang; saline control group ($n = 5$), and the sham operation group ($n = 4$). TNF activity in serum and in bronchoalveolar lavage fluid (BALF), the serum endotoxin levels were measured, and the severity of lung injury evaluated.

RESULTS Elevation of TNF activity was more prominent in BALF than in serum. TNF activity in serum at 6 and 12 hours and in BALF was significantly decreased in the "Tong Xia" treatment group than in the saline control one ($q = 21.11$, $q = 12.07$, $q = 9.03$, respectively, $P < 0.01$) and the lung injury was significantly alleviated at 12 hours as compared with that in the saline group, manifested as amelioration of the lung wet/dry weight ratio, decrease in protein concentration and neutrophils count in BALF, and improvement of pulmonary inflammatory changes. A positive correlation was demonstrated between serum TNF activity

and endotoxin level.

CONCLUSION Hypersecretion of TNF is shown to be one of the major causes of lung injury during ANP; "Tong Xia" purgative method could alleviate the degree of lung injury mediated by TNF.

INTRODUCTION

In the process of the multiple organ dysfunction syndrome (MODS) caused by severe acute pancreatitis, acute lung injury (ALI) occurs most frequently. A number of cytokines are involved in ALI caused by severe acute pancreatitis due to excessive stimulation of monocyte macrophages provoked by endotoxin. Dachengqitang can inhibit the absorption of enterogenous endotoxin and relieve endotoxemia so as to prevent and ameliorate the MODS including the lungs. The purpose of this study is to investigate the effects of TNF on ALI in acute necrotizing pancreatitis (ANP) and to determine whether the herb mixture could relieve the ALI, and investigate its mechanism of action.

MATERIALS AND METHODS

Grouping of experimental animals

Fourteen healthy adult canines, male and female, weighing 7.7 kg-9.9 kg (8.8 ± 1.1), were used. The ANP model canines were divided randomly into 3 groups, *i.e.* saline control group ($n = 5$), Dachengqitang treatment group ($n = 5$), and sham operation group ($n = 4$).

Preparation of animal models

ANP models were made by the retrograde injection of 5% sodium taurocholate into the pancreatic duct. The canines were only accessible to drinking water, 24 hours later were anesthetized with 3% pentobarbital sodium (1 mL/kg) intraperitoneally

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in normal saline ($10 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) dripping continuously. A median incision was made in the upper abdomen to expose the duodenum, then a transverse incision (2 cm) was made at the level of the orifice of the main pancreatic duct. A catheter was inserted and 5% sodium taurocholate (1 mL/kg) was injected into the pancreatic duct at a pressure of 16 kPa, then pulled out, the duodenal dwelling catheter was placed, and Chinese herbal medicine or normal saline could be given via the duodenum. In sham operation group, the catheter was pulled out and no medicine or saline was given.

Preparation of Chinese herbal medicine

Dachengqitang consisted of -Radix at Rhizoma Rhei 30 g, Natrii Sulfas 20 g, Cortex Magnoliae Officinalis 30 g, and Fructus Aurantii Immaturus 15 g. The latter two were decocted for 25 minutes with 500 mL water, then added with Radix at Rhizoma Rhei, decocted for another 3 minutes, and then filtered to yield 150 mL medicinal fluid. Finally, the Natrii Sulfas was added. In treatment group, 50 mL of the medicinal fluid and saline were given through the duodenal fistula tube at 0.5, 4 and 8 hours, respectively.

Observation indices

Serum amylase It was measured before the model was made and at 2, 6 and 12 hours after.

Serum endotoxin It was measured with the of azo coloration before the model preparation and at 2, 6 and 12 hours after, 2 mL blood was drawn from the femoral vein, and the serum was isolated for measurement. The standard curve correlation coefficient was $r = 0.999$.

Serum TNF activity It was measured with the method of the mouse fibroblast L_{929} biological activity quantitative analysis, the same procedure was carried out before and after.

Broncho-alveolar lavage fluid (BALF) The canines were exsanguinated to death 12 hours after the model preparation. After thoracotomy, the whole lungs together with the trachea were taken out, the left bronchus was ligated and the left lung was removed and weighed; the right lung together with the trachea was used for lavage purpose. The surface blood was washed away with normal saline. A total of 100 mL of normal saline was repeatedly lavaged for 5 times, and 80 mL of the lavage fluid was collected. Ten mL was centrifuged, and 2 mL of supernatant was obtained for protein content measurement; another 2 mL was used for the TNF

activity measurement. The sediment was stained for the counting of all kinds of nucleated cells.

Pathological examination The lung and the pancreatic tissues were re sectioned and preserved in 10% formalin fluid.

The lung wet/dry weight ratio The wet left lung was weighed and placed in the oven for 24 hours at 60°C , and weighed when it was dry. The wet/dry weight ratio was calculated.

Statistical analysis

All data were placed in the computer for the statistical analysis with the STATA statistics software.

RESULTS

Autopsy findings

Abdominal cavity In both saline control group and treatment group, as much as 300 mL-500 mL of bloody ascitic fluid were present. There was no statistical significance between them. In sham operation group, only a small amount of clear ascitic fluid was noted. In saline control group and treatment group, the pancreas appeared violet black and enlarged, subserous hemorrhage and a little saponification were observed.

The lungs In saline control group, there were various degrees of pulmonary edema and extensive patchy or petechial hemorrhage. In treatment group, the pulmonary edema was less severe without any patchy hemorrhage. In sham operation group, the lung tissue was normal.

Blood amylase (Figure 1).

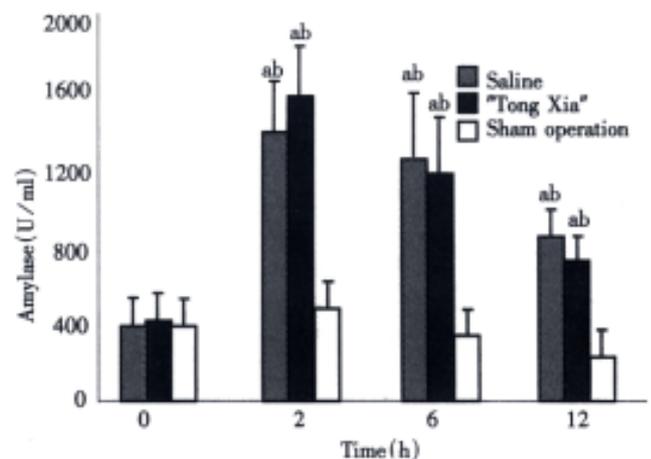


Figure 1 Serum amylase.

^a $P < 0.01$ vs preoperation; ^b $P < 0.01$ vs sham operation.

Serum endotoxin(Figure 2).

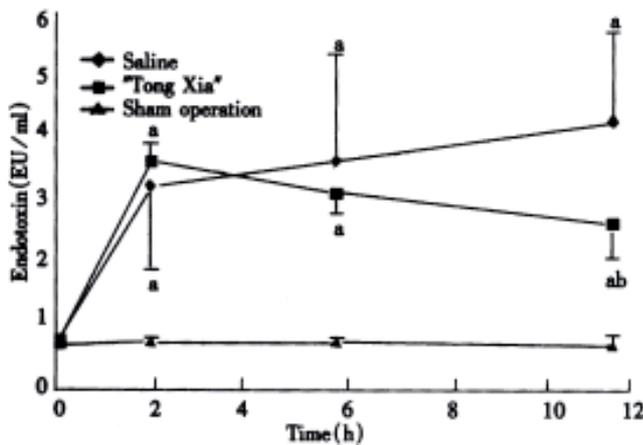


Figure 2 Serum endotoxin.
^a*P*<0.01 vs sham operation; ^b*P*<0.05 vs saline.

Serum TNF activity(Figure 3).

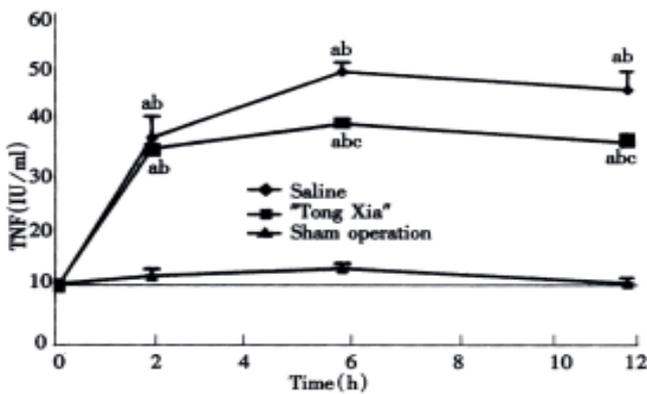


Figure 3 Serum TNF activity.
^a*P*<0.01 vs preoperation; ^b*P*<0.01 vs sham operation; ^c*P*<0.01 vs saline.

Cell changes in BALF(Table 1).

Table 1 Cell changes in BALF ($\bar{x}\pm s, \times 10^6/L$)

Group	Total cell count	Differential count		
		Polymorphonuclear	Mononuclear-macrophage	Other
Saline	36.4±8.2 ^{ac}	3.1±1.2 ^{ac}	11.2±3.5 ^a	22.1±7.6
"Tong Xia"	20.7±10.3 ^b	0.8±0.2 ^b	5.3±2.3	14.6±6.3
Sham operation	7.2±4.1	0.2±0.07	4.2±2.3	2.80±1.8

^a*P*<0.01, ^b*P*<0.05 vs sham operation ; ^c*P*<0.05 vs "Tong Xia".

Protein content and TNF activity in BALF (Table 2).

Table 2 Protein content and TNF activity in BALF ($\bar{x}\pm s$)

Group	TNF activity (IU/mL)	Protein content
Saline	58.75±4.65 ^{ab}	2.53±0.83 ^{ac}
"Tong Xia"	49.00±1.83 ^a	1.42±0.31
Sham operation	17.00±0.82	0.93±0.21

^a*P*<0.01 vs sham operation; ^b*P*<0.01, ^c*P*<0.05 vs "Tong Xia".

Lung wet/dry weight ratio (Table 3).

Table 3 Lung wet/dry weight ratio ($\bar{x}\pm s$)

Group	Lung wet/dry ratio
Saline	4.23±0.74 ^{ab}
"Tong Xia"	3.24±0.34
Sham operation	2.80±0.17

^a*P*<0.01, vs sham operation; ^b*P*<0.05, ^c*P*<0.05, vs "Tong Xia".

The correlation analysis between serum TNF and endotoxin

The linear correlation analysis showed that there was a positive correlation between serum TNF activity and endotoxin level in saline control group (*r* = 0.9706, *P*<0.05).

Microscopic changes

The pancreatic and pulmonary lesions in the three groups (Table 4) The pancreatic lesion was graded according to Meng's criteria^[2] whereas the pulmonary lesion was graded on basis of Lei's criteria^[3].

Table 4 The pancreatic and pulmonary lesions

Group	Number	Pancreatic lesion				Pulmonary lesion			
		Negative	Mild	Moderate	Severe	0	I	II	III
Saline	5			2	3		1	3	1
"Tong Xia"	5			3	2		4	1	
Sham operation	4	4				4			

DISCUSSION

MODS is frequently seen in severe acute pancreatitis. It is probably due to the cytokines produced by excessive activation of the monocyte-macrophage system, and the influx and infiltration of polymorphonuclear leukocyte cytokines as TNF, IL, inflammatory mediators, phospholipase A₂, oxygen free radicals, cathepsin may all contribute^[4]. Among them TNF plays an important role in the processes. Many authors reported that TNF activity was increased in the serum and BALF from the patients with severe acute pancreatitis and adult respiratory distress syndrome^[5]. Majority of the authors believed that the increase in TNF activity was due to excessive stimulation of mononuclear-macrophage by endotoxin. In this study, serum TNF activity was remarkably increased, which indicated and was involved in the pathophysiological processes of ANP. TNF activity was also increased more markedly in the BALF than that in the serum, which suggested that TNF also came from pulmonary alveolar macrophages. There was a positive correlation between serum TNF activity and endotoxin level, which indicated that

the increase in TNF activity was caused by the stimulation of endotoxin. In BALF, polymorphonuclears and macrophages were both increased more remarkably than those in sham operation group, indicating the involvement of polymorphonuclears in the lung injury. In treatment group, at 6 and 12 hours after the model preparation, TNF activities in both serum and BALF were lower than those in saline control group, and the lung injury was also much milder, indicating that Dachengqitang could ameliorate the lung injury mediated by TNF.

“Tong Xia” therapeutic method, is an important part of traditional Chinese medicine in treating acute pancreatitis, and has been studied extensively by Chinese scholars. As regards to the therapeutic mechanism, the herb mixture by purgation could decrease the absorption of the gut endotoxin, ameliorate the endotoxemia and translocation of gut bacteria, and protect the gut barrier function. In this study, at 6 hours after

ANP, the serum endotoxin level was a little lower in the treatment group than that in saline control one, and the serum TNF activity was lowered very much, suggesting that Dachengqitang inhibited TNF activity via inhibiting the absorption of endotoxin.

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Prevalence of primary *Helicobacter pylori* resistance to metronidazole and clarithromycin in Singapore

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Subject headings *Helicobacter pylori*; triple therapy; metronidazole; clarithromycin

INTRODUCTION

Eradication of *Helicobacter pylori*, a bacterium residing in stomach and causing peptic ulcer disease, can be achieved by using combination therapies consisting of one or two antibiotics with a proton pump inhibitor (PPI). The major antibiotics widely used in the regimens to eradicate *H. pylori* are metronidazole and clarithromycin^[1-3]. However, resistance to these antibiotics by *H. pylori* affects the effectiveness of treatment. Treatment failure is often associated with resistance to metronidazole and clarithromycin^[4-7]. In the United States, the frequency of resistance to metronidazole is about 25% with range from about 20% to more than 50%^[8]. In the Netherlands and Germany, the incidence of metronidazole resistance is 17%^[9] and 32%^[10], respectively. In contrast, the prevalence of metronidazole resistance in developing countries was reported to be as high as 70%-90%^[4].

Compared to metronidazole resistance, clarithromycin resistance is low with a range of 7%-14% in the United States^[8], 1% in the Netherlands^[9] and 3% in Germany^[10]. Data on clarithromycin resistance in developing countries are rare.

The rise in antibiotic resistance emphasizes the need for surveillance of *H. pylori* sensitivity to antibiotics as in other infectious diseases. These data will allow clinicians to choose suitable therapy

for their patients. The present study provides recent data on the prevalence of primary metronidazole and clarithromycin resistance of *H. pylori* in Singapore.

MATERIALS AND METHODS

H. pylori strains isolated from 282 consecutive of *H. pylori* positive patients (108 females and 174 males) undergoing routine endoscopy with informed consent for dyspepsia at the Singapore National University Hospital were included in this study. None of the 282 patients had been previously treated for *H. pylori* or had known exposure to antibiotics, bismuth compound or proton pump inhibitor in the past four weeks. Methods for isolation and culture of *H. pylori* were described previously^[11,12]. Briefly, 2 gastric biopsies were obtained from the gastric antrum within 2cm of the pylorus from each patient. The biopsies were transported in 0.85% sterile saline to the laboratory for processing within 24h. The two biopsies were smeared onto a chocolate blood agar plate (blood agar base No.2 supplemented with 5% horse blood) without antibiotics followed by smearing onto a chocolate blood agar plate supplemented with antibiotics (vancomycin 3mg/L, colistin methane sulphate 7.5mg/L, nystatin 12500U/L and trimethoprim 5mg/L). The plates were incubated at 37°C in a humidified incubator (Forma Scientific) with 5% CO₂. Identification of *H. pylori* isolates was based on the results of Gram staining, cell morphology and positive reaction for catalase, oxidase and urease activity. These isolates were further confirmed by API ZYM Kit (BioMérieux)^[13], a semiquantitative micromethod for the rapid detection of the presence of 19 preformed enzymes.

The disk diffusion test was used for the testing of bacterial sensitivity to antimicrobial agents. An inoculum of 0.2 mL of *H. pylori* suspension equivalent to McFarland 3 turbidity standard was spread onto the chocolate blood agar plate. The plates were dried completely for 5 min - 10 min before a metronidazole disk (5µg, Oxoid) or clarithromycin disk (15 µg, Oxoid) was placed on the surface of each dried agar plate. These plates were incubated at 37°C in 5% CO₂ atmosphere for

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2-4 days. The inhibitory zone around each antibiotic disk was recorded accordingly. Inhibitory zones of less than 15mm for metronidazole^[14,15] and 30mm for clarithromycin^[16] were considered resistance.

Fisher's exact test was used for statistical analysis. *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

In a total of 282 *H. pylori* isolates, resistance to metronidazole was found in 130 isolates (46%; 95% confidence interval, 40.3% - 51.9%) and clarithromycin in 18 isolates (6%; 95% confidence interval, 3.2%-8.8%), respectively. Eight of 18 isolates (3%; 95% confidence interval, 1.92%-3.68%) resistant to clarithromycin were also resistant to metronidazole.

Of the 20 isolates obtained in 1995, 4 (20%; 95% confidence interval, 2.5% - 37.5%) isolates were resistant to metronidazole. Of the 36 isolates obtained in 1996, 19(53%; 95% confidence interval, 36.7%-69.3%) isolates were resistant to metronidazole. In 1997 and 1998, 40/83 (48%; 95% confidence interval, 37.8%-58.7%) and 67/143 (47%; 95% confidence interval, 39.9%-54.1%) isolates were found to be resistant to metronidazole, respectively (Table 1).

Of 282 isolates, 50/108(47%; 95% confidence interval, 37.5%-56.4%) and 80/174 (46%; 95% confidence interval, 38.6%-53.4%) isolates from females and males, respectively, were found to be resistant to metronidazole. No statistical difference was found between two genders ($P>0.05$).

Table 1 Prevalence of metronidazole resistance to *H. pylori*

Year	No.	Resistant isolate	Resistance %
1995	20	4	20
1996	36	19	53
1997	83	40	48
1998	143	67	47
Total	282	130	46

DISCUSSION

This study showed that during the 4 years period of investigation the metronidazole resistant rate increased from 20% in 1995 to 47% in 1998 with an average of 46% in 282 *H. pylori* isolates from Singapore. Our previous investigation in 1994 revealed 13% metronidazole resistant rate in 43 isolates^[17]. It is, therefore, believed that resistance to metronidazole in Singapore rose to reach a platform of about 50%. On the other hand, clarithromycin resistance was 6% in the total of 282 isolates from Singapore in this study.

Since metronidazole attains high concentration in the stomach and is not influenced by pH, it is among the most antibiotics to be used to eradicate *H. pylori*. However, the effectiveness of treatment was compromised by emergence of metronidazole resistance^[4-7]. Thus, it is of great importance to monitor the resistance. The prevalence of metronidazole resistance varies widely from country to country. A study of multicentre in Europe showed that metronidazole resistance is 28% with large variation from 7% in Spain to 49% in Greece^[8]. Recent studies from Germany^[10] and the Netherlands^[9] reported the prevalences of metronidazole resistance are 32% and 17%, respectively. The finding of 46% of metronidazole resistance in Singapore in this study is relatively higher than those of developed countries, but the 6% of clarithromycin resistance in Singapore is similar to those in developed countries, such as 10% in France^[19], 5% in Ireland^[16], 1% the Netherlands^[9], 3% in German^[10] and 7%-14% in the United States^[8]. This may be due to the fact that the history of metronidazole application in treating infectious diseases other than *H. pylori* in Singapore is much longer than that of clarithromycin. However, if the use of clarithromycin increases, the clarithromycin resistance could pose a serious problem in eradication of *H. pylori* in the future.

In 8 of 18 clarithromycin resistant isolates, metronidazole resistance was also found. The combination of resistance to 2 antibiotics has been reported elsewhere^[9,10,18]. The data remind gastroenterologists to be cautious in the use of triple therapy comprising both clarithromycin and metronidazole when they treat patients with peptic ulcer disease. Such therapy if administered to patients infected with *H. pylori* which is resistant to both clarithromycin and metronidazole may result in treatment failure. On the other hand, the treatment may overcome the problem if the isolate is resistant only to one of these two antibiotics.

It is interesting to note that there is no discrimination of metronidazole resistance in terms of gender distribution. This is in contrast to the reports from Europe which state that women are more likely to harbour resistance metronidazole than men^[8-10]. This difference possibly reflects equal exposure of metronidazole to males and females in Singapore.

In conclusion, in Singapore the prevalence of resistance to metronidazole is high (46%) as compared to developed countries. However, the prevalence of clarithromycin resistance is comparatively low (6%). The surveillance of *H. pylori* susceptibility to antibiotics is critical in guiding the clinicians on the effectiveness of

treatment regimens. If antibiotic susceptibility testing of *H. pylori* is not available, it is suggested that the clarithromycin-contained triple regimen be preferred to metronidazole-contained triple regimen in local population.

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Schistosomiasis: progress and problems

Wu GY and Halim MH

Subject **headings** schistosomiasis;
development; immune response; diagnosis;
treatment

INTRODUCTION

Schistosomiasis, also commonly known as bilharziasis, is one of the most significant parasitic diseases of humans. A report of World Health Organization in 1996 estimated that over 200 million people were infested worldwide, mainly in rural agricultural and periurban areas. Of these, 20 million suffer severe consequences from the disease and 120 million are symptomatic. Symptoms range from fever, headache and lethargy, to severe sequelae including ascites, hepatosplenomegaly and even death^[1]. More than 600 million people in the tropics are at risk for developing schistosomiasis. Schistosomiasis is the major public health problem in rural Egypt, with almost six million Egyptians are infested^[2].

Schistosomiasis is caused by digenetic trematodes belong to phylum platyhelminthes, super family schistosmatoida, genus schistosoma. It is usually attributed to three species, subdivided into intestinal *Schistosoma mansoni* and *Schistosoma japonicum* or urinary *Schistosoma hematobium* types, according to the site preferred by the adult worms. In Egypt, the two species of bilharziasis are *Schistosoma mansoni* and *hematobium* whose intermediate hosts are fresh snails, *Biomphalaria alexandra*, and *Bulinus truncatus* respectively^[3].

In humans, these blood flukes reside in the mesenteric and vesical venules. They have a life span of many years and daily produce large numbers of eggs, which must traverse the gut and bladder tissues on their way to the lumens of the excretory organs. Many of the eggs remain in the host tissues, inducing immunologically mediated granulomatous inflammation and fibrosis. Heavy worm burdens may produce hepatosplenic disease in schistosomiasis

mansoni and *japonica* and urinary tract disease in schistosomiasis hematobia. Because both the schistosomes and the eggs utilize host metabolites, and because the host responses to the parasite are affected by its nutritional status, malnutrition may strongly affect both the parasite and the complex host-parasite relationship^[4].

HISTORICAL REVIEW

Egyptians have had a long history of symptoms caused by schistosomiasis, notably hematuria, which appeared classically in young boys and was once deemed to be a sign of puberty. It was in Egypt in 1851 that Theodore Bilharz discovered, in autopsy material that the causative agent of hematuria was *schistosoma*^[2].

In 1903, Manson^[5] observed lateral spined eggs in the feces of a patient who had no hematuria. He suggested that more than one species of the worm was involved in the vesical and intestinal forms of the disease on grounds of dissimilar geographical distribution of both types of infestation.

In 1907, Sambon^[6] verified Manson's suggestion and named the worm that produced lateral spined eggs and caused intestinal infestation as *Schistosoma mansoni*.

In 1915, Leiper^[7] discovered the two genera of snails in Egypt (*Bulinus* and *Biomphalaria*) that transmitted the two species *S. hematobium* and *S. mansoni*, respectively.

In 1937, Scott^[8] reported on the prevalence of schistosomiasis in 100 Egyptian Villages. At that time, *S. hematobium* infestations were common, while *S. mansoni* infestations were rare in the Nile delta. Since 1977 this pattern of schistosomiasis in Egypt changed as the prevalence of *S. mansoni* infestation increased and of *S. hematobium* decreased. This change has important public health implications, because the hepatosplenic schistosomiasis caused by *S. mansoni* is more difficult to trace and is associated with more morbidity and mortality than the urinary schistosomiasis caused by *S. hematobium*^[9].

LIFE CYCLE OF-SCHISTOSOMA-PARASITE

The three species of schistosomes that commonly affect human (*S. hematobium*, *S. mansoni* and *S. japonicum*) have similar life cycles and develop by a succession of stages: the egg, miracidium, first stage sporocyst, second stage sporocyst, cercariae, schistosomule and adult.

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All the species of schistosomes are contracted in the same way: by direct contact with infested surface water containing free-living forms of the parasite known as cercariae, which can penetrate the skin. Schistosome cercariae consist of a tail, used for motility in the water, and a head region, which is used for attachment to host skin, and glands containing proteolytic enzymes to facilitate penetration of the skin.

During penetration of the skin, the tail is shed and several other major changes accompany transformation into a new form called the schistosomulum. After penetrating the wall of a nearby vein, schistosomula are carried in the host blood flow, eventually reaching the liver where they grow and reach sexual maturity. The mature male and female worms pair, and then, depending on species, migrate to the vessels of the bowel or bladder where egg production occurs. Many eggs pass through the intestinal or bladder wall and are excreted in the feces or urine. The schistosome life-cycle is completed when the eggs hatch, releasing free-swimming miracidia, which in turn, reinfect freshwater snails.

Rather than being excreted, however, some of the eggs may lodge in the tissues of the host. It is the presence of these retained eggs, rather than the worms themselves, that causes the pathology of schistosomiasis. In intestinal schistosomiasis, eggs lodged in the mucosa or submucosa of the gut cause granulomatous reactions, which may extend into the gut lumen as pseudopapillomas, resulting in colonic obstruction and blood loss. Eggs lodged in the liver result in portal fibrosis, leading to portal hypertension, splenomegaly, ascites, esophageal and gastric varices. Exsanguination from bleeding esophageal varices is the major cause of death^[10].

DEVELOPMENT OF SCHISTOSOMA

In vitro cultivation methods can provide useful insights into the biology, nutrition and immunology of schistosomes. Among the key issues in parasite cultivation is the degree to which cultured organisms resemble their counter parts reared in normal hosts^[11].

Trials to cultivate *S. mansoni* from cercariae have led to production of nonviable eggs by worm pairs grown entirely *in vitro*^[11].

In 1974, Tiba *et al*^[12] showed that artificially prepared schistosomules can develop to maturity when injected into mice shortly after preparation. Basch *et al*^[13] demonstrated that 2 hour and 13 day old schistosomules grown *in vitro* from *S. mansoni* cercariae can complete normal development successfully after implantation into mouse mesenteric veins.

Clemens *et al*^[14] also studied the rate

development of *S. mansoni* schistosomules *in vitro*, as determined by developmental milestones and thymidine incorporation into DNA.

An alternative approach, the study of egg production by pairs of mature worms maintained *in vitro*, has not been productive. In general, egg laying has been observed for only a few days after adult worms were transferred from the host to cultures and ceased after the 10th day^[15].

In 1986, Wu *et al*^[16] demonstrated that the portal serum from various mammalian sources have components that stimulated *S. mansoni* oviposition *in vitro*.

In 1993, Hobbs *et al*^[17] established protocols for the initiation and maintenance of cultures from juvenile worms of *S. mansoni*. These cultures exhibited properties characteristic of the organism from which they originated and could be maintained for as long as 6 months *in vitro*.

The work of Taylor *et al*^[18] and Taylor^[19] showed that in single-sex infestations, schistosomes migrated to the portal- mesenteric venous system, indicating that each sex is capable of locating the preferred site independent of the other sex. Blood draining to the portal vein is derived from the gastrointestinal tract. Therefore, it is different from the peripheral blood in many respects^[20]. The site preference of *S. mansoni* could be dependent on a constituent of portal blood that is not present in the periphery. This might take the form of a substance that the parasite recognizes or requires to develop.

It has been shown that egg production can be stimulated by portal serum components added to culture media, but not by serum from peripheral blood^[21]. This occurred regardless of whether the host is a natural or not. More recently, immature schistosomes in culture have been shown to have enhanced cellular proliferation when portal serum was added to the medium. This effect could not be reproduced by serum obtained from the vena cava. Furthermore, when the serum was fractionated, the size of the stimulatory substances was estimated to be larger than would be expected for simple nutrients absorbed from the gut^[22].

In experimental animals, granuloma formation has been shown to be induced and elicited by soluble egg antigens (SEA) secreted by the miracidia within eggs^[23]. Over the years, several laboratories have isolated antigenic fractions from crude egg homogenates. A number of partially purified glycoproteins have been shown to possess serological, dermal, lymphocyte-stimulating, hepatotoxic, and granuloma inductive properties^[24]. However, the relative importance of the various fractions as granulomatogenic agents remains unexplored. More recently, the differential

responsiveness of acute-versus chronic-infestation murine lymphocytes to a panel of SEA-derived fractions has been demonstrated. A 38-kDa fraction was found to be egg stage specific, to elicit strong lymphokine production *in vitro*, and to induce granuloma formation *in vivo* during the acute stage of murine schistosomiasis^[25].

THE IMMUNE RESPONSE IN SCHISTOSOMIASIS

The immunology of schistosomiasis is largely dependent on the biological characteristics of the parasite itself. After skin penetration, schistosome undergo a complex migratory life cycle in the vertebrate host before they settle, in the case of *S. mansoni*, in the blood vessels of the portal and mesenteric system. In this intravascular situation, the adult worms release a large amount of excretory or secretory material, which elicits a strong antibody response. Antigens may be found in the serum and various body fluids in the form of free antigens, and more generally as immune complexes^[1]. This continuous release of soluble antigens has important implications in the regulation of the immune response, both in terms of antigenic competition and as direct factors of immunosuppression or tolerance. The major role of antibodies in protective immunity is to induce cytotoxic destruction of schistosome targets, and antibody-cell mediated cytotoxicity appears to be the main mechanism for destruction of parasites both in rat and human schistosomiasis^[26].

The persistence of the trematodes in an immunologically hostile environment has been attributed to their ability to acquire or synthesize, during their maturation, surface antigenic determinants (host antigens) to which the animal is unresponsive^[27]. The worm tegument, which undergoes a continuous and rapid turnover, acquires numerous host molecules ranging from various serum proteins or glycolipid to major histocompatibility antigens. This phenomenon has been considered as an essential escape mechanism^[28-30].

It has been assumed from epidemiological studies in endemic areas that age-dependent immunity may develop against infestation, or against reinfestation after treatment, with *S. mansoni*^[31] or *S. hematobium* infestation^[32]. Using a mathematical model, it has also been shown that predicted patterns of variation in age-related changes in the intensity and prevalence of *S. hematobium* infestation are consistent with the epidemiological effects of acquired immunity^[33]. At present, however, there is no effective vaccine against schistosomiasis or any other human parasitic disease. In order to develop such vaccines, it is obviously important to elucidate mechanisms involved in protective immunity at the cellular and

molecular levels because of the complex life cycle and stages of parasites which occur in the human body.

CLINICAL MANIFESTATIONS

Clinical manifestations reflect developmental stages of the parasites and host responses to toxic or antigenic substances derived from the parasite and eggs.

During the early stage of infestation, the patient may present with signs caused by cercarial penetration of the skin (cercarial dermatitis), followed by bronchopulmonary manifestations attributed to the passage of schistosomes through the lungs. Approximately five weeks after infestation, more dramatic symptoms, often known as Katayama Disease consist of malaise, weight loss, gastrointestinal symptoms, eosinophilia and fever. They are caused by the initial deposition of eggs by female worms^[34].

In the case of *S. japonicum* and *S. mansoni*, female worms lay eggs in the mesenteric branches of the portal vein along the intestinal wall and although a relatively large part of the eggs are carried into the liver and other organs by the blood flow, the remainder of them may stay in the small venules until the embryo they contain develop in to miracidia within 10 days. Antigenic substances excreted from miracidia diffuse out through submicroscopic pores in the egg shell, and elicit an acute inflammation in the surrounding tissues, resulting in the rupture of the vascular wall and escape of the eggs from the venules through the intestinal submucosa and mucosa into the intestinal lumen. The inflammation causes recurrent daily fever, abdominal pain and enlarged tender liver and spleen, and discharge of eggs into the intestinal canal is accompanied by dysentery or diarrhea^[35]. Blood chemistry may reveal a transient elevation of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase and alkaline phosphatase 5-6 weeks after infestation. Eosinophilia may be observed in most of the patients with or without increase of leukocyte counts. Serum level of IgE may increase as observed in other helminth infestations^[36].

Chronic schistosomiasis is characterized by a series of chronic inflammatory lesions produced in and around blood vessels by the eggs or their product^[37]. The chronic manifestations in *S. japonicum* and *S. mansoni* infestations is characterized by hepatosplenomegaly, although development of polyps or mucosal proliferation of the intestine may also be observed in most cases. Egg granulomas are replaced by fibrotic tissues, which are prominent in the periportal areas and lead to the development of pipestem fibrosis^[38].

Hepatosplenic schistosomiasis refers to the

major complication of chronic infestation with *S. mansoni* and *S. japonicum*. Hepatosplenic schistosomiasis is usually, but not invariably, associated with enlargement of the liver and spleen, and reversible hepatosplenomegaly may occur in early infestations not complicated by the development of portal hypertension^[39].

The liver is invariably involved in intestinal schistosomiasis, but the extent of such involvement depends on many factors including intensity of infestation and duration of infestation which are mainly responsible for the changes produced. The liver gradually decreases in size, but increases in hardness as fibrosis is gradually extended into the parenchyma, resulting eventually in liver cirrhosis in severe cases. The enlarged spleen may reach the level of the umbilicus or even at times expand to fill most of the abdomen^[35].

End stage hepatosplenic schistosomiasis may be complicated by features of hepatocellular failure, ascites often being the most obvious clinical sign. While this may all result from severe schistosomiasis, the possibility of other coexistent liver disease must be considered. In Nairobi, two of 25 patients considered to have schistosomal portal hypertension also had histological evidence of cirrhosis^[39].

The portal hypertension of schistosomiasis is presinusoidal and presumably related to the portal zone reaction^[40]. In advanced schistosomiasis, hepatic arterial hypertension contributes to increased sinusoidal pressure^[41]. Retrograde flow develops in the portal vein. Hepatic blood flow is not significantly reduced.

At the stage when hemorrhage occurs from varices, the granulomatous reaction may have subsided and the picture is predominantly that of fibrosis^[42]. Portal hypertension is considered present when the portal vein pressure is raised to 5 mmHg above inferior vena caval pressure, when the intrasplenic pressure is above 15 mmHg, or when the portal vein pressure measured directly at surgery is above 30 mmHg^[43]. While portal hypertension is a prerequisite for the development of a collateral circulation, in cirrhosis the risk of bleeding cannot be directly correlated with the exact portal vein pressure, although hemorrhage is unlikely in cases where the portal vein pressure is less than 10 mm Hg above inferior vena caval pressure^[44].

Cirrhosis is defined anatomically as a diffuse process with fibrosis and nodule formation^[45]. It has followed hepatocellular necrosis. Although the causes are many, the end result is the same.

Fibrosis is not synonymous with cirrhosis. Fibrosis may be in zone 3 in heart failure, or in zone 1 in bile duct obstruction and congenital hepatic

fibrosis or interlobular in granulomatous liver disease, but without a true cirrhosis^[42].

Urinary schistosomiasis is caused by *S. hematobium* and affects the genito-urinary system. The stage of oviposition is manifested by genito-urinary trouble such as cystitis, dysuria with terminal hematuria, dull suprapubic pain, spermatorrhea and hemospermia. Spontaneous recovery is rare and the condition may be complicated by the bladder ulcer, calculi, polyps, fistulae, hydronephrosis or hydronephrosis or carcinomatous changes of the bladder^[46].

The association of bladder cancer with *S. hematobium* has been discussed in the context of the involvement of urinary tract infestations by species of nitrate-reducing bacteria. The urine of patients infected with *S. hematobium* contained higher levels of nitrosamines, in association with nitrate-reducing bacteria, than the urine of either Egyptian or German controls, and this may result in the endogenous formation of carcinogenic N-nitrosocompounds in the urine^[47].

The involvement of gynecological organs may be observed in *S. hematobium* infestation. As a disease entity, female genital schistosomiasis has been neglected, despite the fact that vaginal schistosomiasis was reported from Egypt as early as in 1899. It has generally been considered that the presence of *S. hematobium* eggs is not as common in female genital organs as in male genital organs, although in the female lesions are found in the vulva, vagina, cervix and less commonly the ovaries, fallopian tubes or uterus^[48]. However, *S. hematobium* may migrate through the network of female pelvic vasculature during puberty and especially during pregnancy make ectopic localization of the parasites possible^[49]. Because sexually transmitted disease increase the probability of HIV transmission, presumably through lesions in the genital mucosa, female genital schistosomiasis may be an important risk factor for transmission of HIV^[50].

PATHOGENESIS AND PATHOLOGY

The pathological changes in schistosome infestations are caused mainly by the deposition of the eggs into various tissues and organs where granulomas or pseudo tubercles are formed around them. In primary infestations, the granuloma is composed of aggregations of mononuclear phagocytes, neutrophils, lymphocytes, plasma cells and fibroblasts. Giant cells are also frequently observed in the granulomas. Granulomas may vary in size and cellular components with the immune status of the host in experimental infestations in immunized animals, a dominant cellular infiltration of eosinophils and lymphocytes is observed around the

eggs and the egg granuloma is smaller^[35].

Granuloma formation around schistosome eggs has been considered to be the result of delayed-type hypersensitivity reactions mediated through a T cell mediated immune response to soluble egg antigens^[51]. However, recent studies have demonstrated that there exist at least 2 subsets of T helper cells with a CD4 phenotype, termed Th1 and Th2 cells, which can be distinguished from each other by their cytokine production^[52]. The cytokines derived from Th1 cells, such as IL (IL)-2, interferon or tumor necrosis factor (TNF), may be responsible for activation of macrophages and cell-mediated immunity, whereas IL-4 or IL-5, the cytokine produced by Th2 cells, stimulates IgE production or eosinophilia, respectively^[53].

DIAGNOSTIC TESTS

Decisions on individual and community treatment, estimations on prognosis and assessment of morbidity, evaluation of chemotherapy and control measures all depend on the results from diagnostic tests. Selection and application of methods should, therefore, correspond to the type of information sought by the clinician or the epidemiologist^[54].

Specific diagnosis of schistosomiasis can be made by detection of the characteristic eggs in the stools or urine under microscopic examination.

In *S. mansoni*, where eggs are excreted in feces, simple concentration and sedimentation of fecal specimens are reliable. Many concentration techniques have been described^[55]. These involve removal of fat, fecal debris and mucus and require more sophisticated laboratory facilities. They find their optimum use in the detection of "light" infestations where few eggs are excreted or, in some cases, eggs are excreted intermittently.

Currently, the cellophane thick fecal smear, the Kato technique^[56], or one of its numerous modifications^[57,58] have become standard diagnostic tools in epidemiological studies. They are simple microscopic methods which examine about 50 mg of stool and are quantitative thus permitting comparison of data.

Infestation with all human schistosome species are efficiently diagnosed through microscopic examination of minute biopsies of the rectal mucous. Snips are taken from suspicious lesions or if absent, from the plica transversalis recti. Even in infestation with *S. hematobium*, eggs are frequently detectable in rectal snips^[59,60]. Since rectoscopy is an invasive technique, its application is limited to the hospital or the experienced gastroenterologist^[61] has, therefore, advocated rectal swabs with for patients in areas that lack these resources.

It is rarely necessary to resort to liver biopsy

for diagnosing infestation with intestinal schistosomes, but where this has been done, the examination of hepatic tissue in crush preparations is more efficient than sectioning of the material. The probability of aspirating tissue that contains egg granulomas is rather low in conventional and even ultrasound-guided fine needle liver puncture^[62].

Moreover, diagnosis of peripheral fibrosis is made with similar efficiency by means of ultrasonography and, therefore, does not require biopsies with histological sectioning^[63]. Except when carcinoma of the bladder is considered as differential diagnosis, cystoscopy and bladder biopsy seem wholly unjustified. In contrast, filtration of several 24 h urine samples is commonly available in hospital and frequently leads to the detection of ova in urine^[64]. Furthermore, Burki *et al*^[65] have demonstrated that ultrasonography compares favorably with cystoscopy and pyelography to detect specific pathology.

Indirect methods for the diagnosis depend on clinical symptoms and signs, and biochemical or immunological analyses. Especially for urinary schistosomiasis, hematuria is a suggestive sign and microhematuria or proteinuria may correlate well with the intensity of infestation in endemic areas^[66]. In intestinal schistosomiasis, the repeated presence of blood in stool is indicative of high intensity of infestation^[67].

Immunodiagnosis may be useful for demonstration of active or chronic schistosomiasis. A unique immunological method for the diagnosis of schistosomiasis is the circumoval precipitin (COP) test in which precipitate is formed around the eggs containing live miracidia after incubation in the serum of infected individuals^[68].

The enzyme linked immunosorbent assays (ELISA) is also widely used in diagnosis. Furthermore, ELISAs for the detection of circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine have been developed and applied as an epidemiologic tool in a recent, intense focus of *S. mansoni* in Senegal^[69]. CAA and CCA in serum and CCA in urine were found in 94%, 83% and 95%, respectively, of the population of which 91% were positive on stool examination. Circulating antigens were also detectable in sera and urine of most egg-negative individuals. The sensitivities of the urine CCA and serum CAA ELISA were substantially higher than that of a single egg count and increased with egg output. The CAA and CCA levels correlated well with egg counts and with each other. The age related evolution of antigen levels followed a similar pattern to egg counts, providing supplementary evidence for a genuine reduction of worm burden in adults in spite of the supposed

absence of acquired immunity in this recently exposed community^[70].

TREATMENT OF SCHISTOSOMIASIS

There have been great advances in chemotherapy of schistosomiasis during the past 2 decades. Compared to antimonials, which were the only available chemotherapeutic agents for schistosomiasis from the 1920s to the 1960s, new drugs are more consistently effective, less toxic and applicable to oral rather than parenteral administration, thereby making field trials of mass chemotherapy feasible^[35]. The major antischistosomal drugs that have been or still are in use against infestation with schistosomes are metrifonate, oxamniquine and praziquantel and all three are included in the World Health Organization list of essential drugs^[70].

The classification of antischistosomal drugs can now be simplified into two categories^[71]:

1-The one drug effective against all species of schistosomes infecting man (praziquantel).

2-The other drugs effective against one species of schistosomes *i.e.* the monospecific drugs: oxamniquine, effective only against *S. mansoni* and metrifonate, used in *S. hematobium* infestations.

Praziquantel is the newest and most effective drug for treating schistosomiasis occurring in man^[72]. It is effective orally in a single dose (40 mg·kg⁻¹) yielding 70% to 95% cure rates against all species of schistosomes infecting man. With few significant side effects and no adverse reactions on liver, renal, hematopoietic or other body functions, praziquantel is undoubtedly the most advanced in antihelminthic chemotherapy of recent decades.

The exact mode of action of praziquantel is unknown. Most evidence implicates the susceptibility of muscle and tegumental systems as important sites of action. Praziquantel's effect on worms is very dramatic. Worms exposed to 1μM praziquantel *in vitro* show almost an instantaneous and sustained contraction with a half-maximal effect time of 12 sec. This contraction results in paralysis of the parasite leading to the hepatic shift observed *in vivo* which is 95% complete within 5min after a single oral dose for infested mice^[73].

The effect of praziquantel on worm muscle tension seems to result from the ability of the drug to increase the permeability of the worm muscle cells to calcium ions. Praziquantel also, causes severe destruction of the worm's tegument^[74].

Recently, however, the possible existence of an *S. mansoni* isolate tolerance to praziquantel has been reported from Senegal where the parasitologic cure rate 12 weeks after treatment was as low as 18%^[75]. The tolerance of the Senegalese isolate to praziquantel may be defined as tolerance, indicating

an innate insusceptibility of a parasite to a drug to which it has never been previously exposed^[76]. In contrast, a genetically transmitted loss of susceptibility in a parasite population that was previously susceptible to a given schistosomicidal drug has been termed resistance^[70]. Indeed, in recent work carried out in Egypt, where praziquantel has been extensively used, it has been demonstrated that a small percentage (1%-2.4%) of villagers may harbor parasites which cannot be killed even after repeated administration of high doses of praziquantel^[77]. When isolates obtained from these uncured individuals were examined in the mouse model, the ED⁵⁰ values of the isolates were found to be 3 times higher than that of one reference control isolate^[76]. The reduced susceptibility of *S. mansoni* to praziquantel in infected human populations has important implications for current schistosomiasis control programs.

Oxamniquine is widely used in the treatment of infestation due to *S. mansoni*. It is a well known, highly useful drug for the treatment of all forms of *S. mansoni* infestation including many complicated syndromes^[78].

Recent studies showed that oxamniquine irreversibly inhibits nucleic acids and protein synthesis in adult worms. Male were more susceptible than females to the drug and showed a higher degree of inhibition of protein synthesis^[79].

Metrifonate, an organophosphorus cholinesterase inhibitor, is used for the treatment of urinary schistosomiasis. Metrifonate, like other organophosphorus compounds, inactivates the enzyme that destroys acetylcholine. Because this action allows the chemical neurotransmitter to persist, cholinergic symptoms might be expected during treatment. These include fatigue, muscular weakness, abdominal colic, nausea, diarrhea and vomiting. All of these symptoms are a reflection of stimulation of cholinergic synapses in the autonomic nervous system, ganglionic sites in both parasympathetic and sympathetic divisions, the neuromuscular junction and several sites in the cardiovascular system^[71].

CONCLUSION

In conclusion, control of schistosomiasis is not an easy task. Even after successful treatment, reinfestation easily takes place in most of endemic areas, unless transmission is cut off somewhere between the intermediate hosts and the final hosts in the life cycle of the parasites.

Much work has concerned the immunopathology of the disease, particularly granuloma formation. Although the granulomas contribute significantly to the pathology, they do

seem to protect the host liver from the toxic secretion of the egg. Reduction in granuloma size, without affecting its protective function, would seem to be a desirable aim. A number of approaches are currently under investigation, including the use of live cercariae and schistosomules, and the use of more or less purified antigens.

A wide range of approaches are being taken towards the development of an effective vaccine for schistosomiasis. These range from basic research into schistosome biology through to human epidemiological and immunogenetic studies, construction of a variety of different types of vaccine including native or recombinant proteins, peptide constructs and nucleic acid vaccines, as well as vaccination trials utilizing these in experimental animals ranging from mice to water buffalo. Taken together, the breadth of research into schistosomiasis vaccine development is substantial. Hopefully, these efforts will result in a successful outcome.

Schistosomiasis can be treated with relative ease today since a number of good drugs, several of which are taken orally, have become available. The response to some of the drugs may differ markedly according to geographic location. The emergence of poorly susceptible (tolerant) strains is an area of concern, and that deserves further research to develop new agents for control of the disease.

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Expression of the CagA gene of *H. pylori* and application of its product

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Subject headings *Helicobacter* infection; *Helicobacter pylori*; CagA; gene expression; polymerase chain reaction; immunogold

INTRODUCTION

Helicobacter pylori (*Hp*) plays an important role in the upper digestive tract diseases. It can be divided into two main groups (toxic and non-toxic *Hp*) according to the production of vacuolating cytotoxin (*VacA*). The toxic bacteria also produce cytotoxin associated protein A (*CagA*) which might have something to do with the transcription, folding, transportation or the function of *VacA*. Studies showed that *CagA* positive *Hp* (*CagA*⁺*Hp*) accounted for more than 50% of all kinds of *Hp*, and peptic ulcer and gastric cancer were closely related to their infection^[1-7]. Therefore, detection of the infection of *CagA*⁺*Hp* is of great significance. This study was carried out to express *CagA* gene in *E.coli* and develop an immuno-assay for the rapid detection of *CagA*⁺*Hp*.

MATERIAL AND METHODS

Material

pMC3, plasmid that contains most parts of the 5'-end of *CagA* was kindly given by Dr. Tummuru (Vanderbilt University, USA); pBV220 (preserved in our institute); superdex HR75 (Pharmacia, USA); Wizard plus milipres DNA purification kits (Pr omega, USA); ELISA detecting kits for anti-*CagA* antibody (Jing Ying Biotech Company, Shanghai); thermolyne PCR Amplitron (USA); Hermle Z323K refrigerated high speed centrifuge (Germany); DU-640-nucleic acid protein analyser (Bechman, USA); 373 Auto-DNA sequencer, (Bechman Company, USA); bio-Rad mini-protein II electrophoresis (Joss Bio-Lab Company); sera from patients and healthy people (Xijing Hospital, Xi'an).

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Methods

CagA gene cloning The oligonucleotide primers were designed and synthesized to amplify a 2544bp fragment at the 5'-end of *CagA* according to its sequence^[8]. The primers were *CagAp1* (5'-TCGCCC-GGGATGACTAACGAACTATTGACC-3') and *CagAp2* (5'-CAGGTCGACTTAGACTAGGG-TTCCGTTACAC-3'). At the 5'-end of each primer, there was a restriction endo-nuclease site (*SmaI* or *SalI*), which was favorable for the cloning of PCR products. Fifty microlitre polymerase chain reaction mixture contained 0.4 μmol/L primers, 0.2 mmol/L dNTPs, 2 units Taq-DNA polymerase, 1 ng pMC3, 100 mmol/L Tris-HCl, 50 mmol/L KCl, and 15 mmol/L MgCl₂. The *CagA* was amplified by denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extending at 72 °C for 3 min, and the procedures were repeated 30 times. The PCR product was analyzed by 15g/L agarose gel containing 0.5mg/L ethidium bromide (EB). The bands were examined under ultraviolet light and recovered by low melt point agarose gel. By preparing the pUC19 with alkaline dissolving method, we cloned the *CagA* gene fragment into it for sequencing.

CagA gene expression First, we digested pUC19/*CagA* with *SmaI* and *SalI* separately, and cloned the 2544 bp fragment into pBV220, but this gene fragment failed to express in DH5α. We then digested the pUC19/*CagA* with *EcoRI* and *BamHI*, and recovered the 5'-end fragment of *CagA* (FCagA, 854 bp), which was then subcloned into vector pBV220 to form pBV220/FCagA. After transforming the competent DH5α with the pBV220/FCagA, the clones that could grow in Luria-Bertani (LB) plate containing ampicillinum (100 mg/L) were selected and cultured in LB solution at 30 °C overnight. We diluted (1:100) the cultured mixture of a positive clone in LB and let it grow at 30 °C till the late stage of logarithmic. Then we immediately raised the temperature to 42 °C and kept for 4 h. The heat induced fragment of *CagA* (FCagA) was analyzed by 12% sodiumdodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Purification of FCagA Differential test showed that most of the FCagA existed in inclusion body, of which only 2% was in the supernatant after the

lysate was centrifuged at 12000×g for 10 min. The soluble protein was precipitated by 50% saturated ammonium sulphate and resuspended in phosphate buffered saline (PBS) containing 1.3 mmol/L NaH₂PO₄, 11 mmol/L Na₂HPO₄ and 140 mmol/L NaCl. Three millilitre mixture was applied to Superdex HR75 column (100 mL volume bed), and eluted with PBS in a HPLC protein purification system. The active peak was collected after being tested by dot immunogold filtration assay (DIGFA), with staphylococci protein A as the second antibody and colloidgold as the marker^[9]. The solution was then lyophilized and dialysed against PBS to eliminate extra salt.

Rapid detection anti-CagA antibody by DIGFA The colloidgold and immunogold were prepared according to the published articles^[10,11]. The purified FCagA was immobilized on the nitrocellulose membrane. To block non-specific conjugate sites, the membrane was immersed in 5% bovine serum albumin in (BSA) for 2 h, then washed with PBS three times and laid to dry automatically. Later, we assembled the DIGFA detecting kits. After a drop of serum was added to the membrane and passed through it, the immunogold was added. If the serum contained anti-CagA antibody, there would be a red dot on the membrane, otherwise, there would be no dot.

RESULTS

Cloning and sequencing of CagA

With pMC3 used as template, CagAp1 and CagAp2 as the primers, the 5'-end of CagA was amplified by polymerase chain reaction. The PCR products were analyzed under ultraviolet light after 1.5% agarose gel electrophoresis (Figure 1). We then cloned the 2544 bp CagA fragment into pUC19 for sequencing. The results showed that the PCR product was indeed a portion sequence of CagA, from which the 854 bp upstream fragment of CagA was achieved when digested with Bam-HI (Figure 2).

Expression of CagA and purification of its products

Subcloning FCagA into vector pBV220 and transforming DH5 α with pBV220/FCagA permitted the expression of FCagA with a MW of 38 kDa when the culture temperature reached 42 °C (Figure 3). The new protein mainly existed in inclusion body with only 2% soluble. When the supernatant of the bacteriumlysate was precipitated with ammonium sulphate and the FCagA was purified by Superdex HR75 column, only one protein band of 38 kDa could be seen when the collected fraction correspondent to the active peak was analyzed by 12% SDS PAGE (Figure 4).

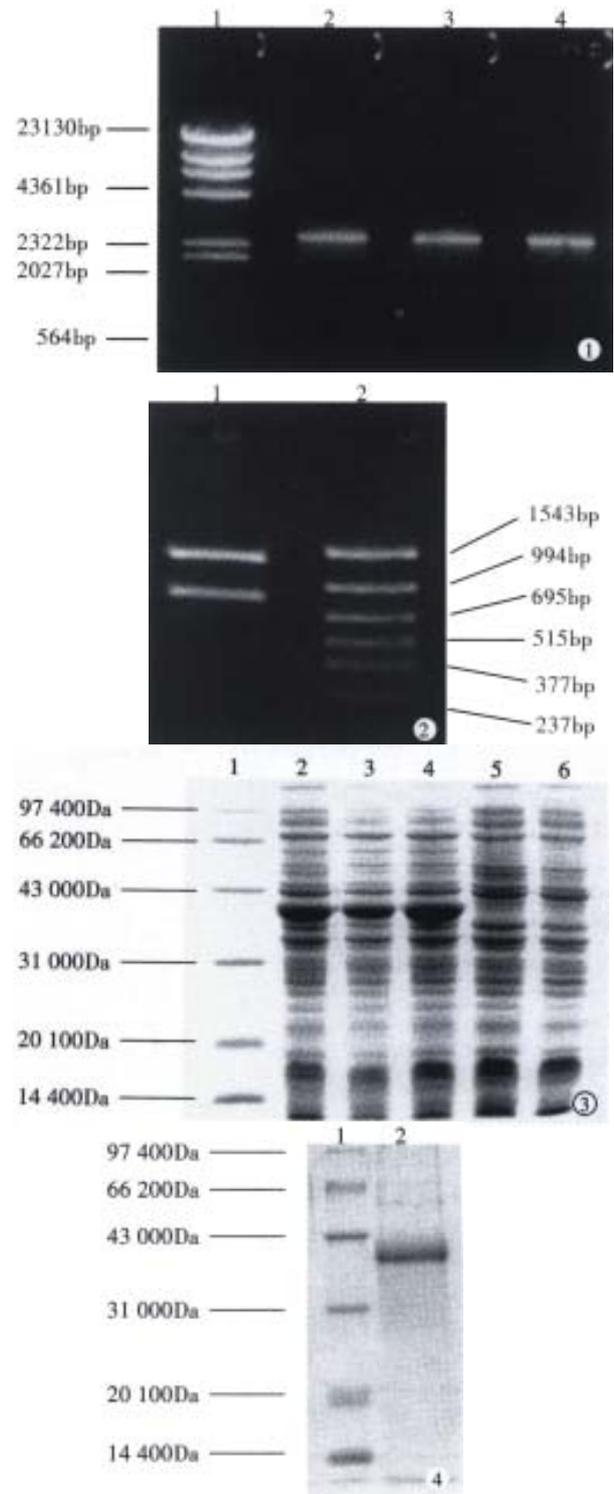


Figure 1 Analysis of the PCR product of CagA by agarose gel electrophoresis.

1: λ DNA/Hind-III markers; 2: PCR product of CagA.

Figure 2 Analysis of CagA fragment by agarose gel electrophoresis.

1: CagA fragments digested by Bam-HI; 2: PCR markers.

Figure 3 Analysis of FCagA expression by 12% SDS-PAGE.- 1: Protein markers; 2, 3, 4: Heat induced pBV220/FCagA-DH5 α ; 5: pBV220/FCagA-DH5 α ; 6: Heat induced pBV220/DH5 α .

Figure 4 Determination of the purified FCagA by 12% SDS-PAGE.

1: Protein markers; 2: FCagA.

The antigenicity of FcagA

To identify the antigenicity of FCagA, DIGFA was performed with FCagA acting as antigen. The results showed that FCagA had the same antigenicity as CagA when 20 samples of anti-antibody positive serum were tested.

Specifications of the DIGFA kits

① Tests of specificity and sensitivity. Using the DIGFA kits, we detected the anti-CagA IgG in 262 cases. Comparing with ELISA, the specificity of DIGFA was 98.5%, the sensitivity was 96.8%, and the coincident rate was 97.7% (Table 1). ② Duplicate tests. From the 262 serum samples, we randomly selected 100 sera for the detection of anti-CagA IgG. The experiment was repeated 5 times and the same result was got: 42 serum positive, 58 serum negative. ③ Blocking tests. When 20 anti-CagA IgG positive sera were randomly chosen, a mixture of 10 μ L serum and excess amount of FCagA (10 μ g) would make the result negative, and a mixture of serum and BSA did not change the positive result, indicating that FCagA can specifically bind anti-CagA IgG. ④ Stability. The stability tests showed that the specifications of the kits were stable for at least 6 months at 4 °C.

Table 1 Results of anti-CagA IgG in sera detected by DIGFA and ELISA

		ELISA		Total
		Positive	Negative	
DIGFA	Positive	123	2	125
	Negative	4	133	137
Total		127	135	262

DISCUSSION

Hp CagA is a bacterium surface protein with a MW of 120 kDa-128 kDa. A dominant characteristic of this protein is its high antigenicity^[1]. The infection of CagA positive *Hp* results in local and systemic humoral immuno-reaction, and there is high concentration of anti-CagA in the patient's blood and gastric juice^[3,6]. This is the base of immuno-detection of anti-CagA antibody. Most recombinants of CagA previously expressed were at its carbonic end and the antigenicity of its amino end has not been reported. Since the 5'-end of CagA had no usable restriction endonuclease sites, we used PCR to amplify the 5'-end fragment of CagA, and cloned it into pBV220^[12]. Subsequently the DH5 α was transformed by pBV220/FCagA, and a 38 kDa recombinant was induced by heat when temperature reached 42 °C. The new protein accounted for 33% of the total bacterium proteins and could combine with anti-CagA IgG, indicating that the recombinant had the antigenicity of CagA. Purification of the soluble part of the recombinant

protein made it possible to develop immuno-detecting method for CagA+Hp.

At present, the detecting methods of *Hp* infection can be divided into two categories: injury and non-injury. Since the injury methods are based on the gastrointestinal endoscope, the non-injury methods are preferred, among which the rapid urease test has low specificity, and the ELISA takes too much time. The same problems exist in the detection of CagA+Hp. Since the infection rate of CagA+Hp is high and its harmful effects on human being are great, it is necessary to develop rapid, simple, and non-injury approaches to detect the infection of CagA+Hp.

DIGFA is an immuno-method developed in recent years. In this method, the red immunogold is used as the marker and nitrocellulose as the substrate, which has the function of absorption, filtration, concentration, and capillary, and can let the antigen-antibody reaction take place rapidly. In this study, we established an anti-CagA assay and developed the test kits which have great applicable value and high sensitivity and specificity. This may contribute to the epidemic study of the infection of CagA+Hp in a large scale.

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The expression and clinical significance of CD44v in human gastric cancers

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Subject headings CD44v gene; stomach neoplasms; neoplasm metastasis; gene expression

INTRODUCTION

CD44 was originally implicated as a "homing" receptor directing the migration of recirculating lymphocytes^[1]. CD44 expression has been confirmed not only in lymphocytes but also in a wide variety of epithelial tissues. It is considered as an important cell adhesion molecule for cell to cell interactions^[2]. Molecular cloning and analysis of the genetic structure have revealed that the CD44 gene has at least 19 exons, of which 12 can be alternatively spliced to make up a wide variety of CD44 splice variants which have been found in various types of human malignancies and have been considered as a marker in tumor progression and metastasis^[3]. We analysed the expression of aberrant CD44 transcripts with RT-PCR method in gastric carcinomas, and discussed the clinical significance of metastasis-related CD44v gene.

MATERIALS AND METHODS

Materials

Fresh tissue specimens including normal gastric mucosa, gastric tumor and mucosa adjacent to tumor were tissues taken from 20 patients with gastric cancers who underwent surgical treatment in the Department of General Surgery in our hospital from November 1997 to February 1998. The diagnosis was confirmed before operation, by endoscopic and pathological examinations. The normal mucosae were taken from mucosae 10 cm away from carcinomas, and the adjacent tissues 2 cm away from the carcinomas. The specimens were derived in 30 minutes after surgical resection, and were frozen in liquid nitrogen prior to store below -70 °C.

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There were 15 males and 5 females, aged 30-79 years, with an average of 58. Pathological examination showed two types of tumors according to *Laureu's* classification division of Finland: intestinal type (well-differentiated) and diffuse type (poorly-differentiated). All the gastric mucosae, gastric cancers and their adjacent tissues expressed CD44s: 16 of 20 gastric cancer cases including 14 cases of diffuse type and 2 cases of well-differentiated type had positive expression of CD44v; 5 of 10 adjacent tissues had positive expression of CD44v. In contrast, there was no positive expression of CD44v in all 10 normal gastric mucosae.

RT-PCR method

Primers sp1 and sp2 were taken from the cDNA sequence described by Matsumura^[4]. sp1: 5'GACACATATTGCTTCAATGCTTCAGC3', sp2: GATGCCAAGATGATCAGCCATTCTGGAAT3', pointing to part of exons of standard CD44s, the amplified product was CD44s; primer p1 and p2 were taken from No.1 and No.5 exons in human cDNA sequence described by Hofmann^[5], p1: 5'GACAGACACCTCAGTTTTTCTGGA3', p2: 5'TTCCTTCGTGTGTGGGTAATGAGA3', pointing to the abnormal splice variants of CD44 gene containing v7 exon, the amplified product was CD44v. The two couples of primers were both synthesized by SAGON Co, Canada, kits and TaqDNA polymerase were purchased from PE Co. USA.

Total RNA extraction was prepared according to AGPC single-step method^[6]. RT-PCR: model RNA 3 µL, down primers (sp1, p1) 2 µL (25 pmol) and 2 µL AMV-RTase were put into 20 µL reaction system to synthesize cDNA. Putting 10 µL cDNA into a reaction tube, the primers sp2, p2 and 2 µL TaqDNA polymerase were added, then the amplification happened in 50 µL reaction system. The conditions of PCR reaction was 94 °C for 50", 52 °C for 50" and 72 °C for 1'33" undergoing 35 cycles. For negative controls, template cDNA was not added to the reaction mix. The amplified product was electrophoresed in a 2% agarose gel, observed and photographed with ultraviolet radiography.

Statistical analysis

Data were analyzed for Fisher exact examination by

running statistical software SPSS for Win95. *P* value was set at 0.05 for statistical significance.

RESULTS

Amplified with sp1 and sp2 as primers, the CD44s specific 482 bp product was obtained from all the gastric cancers, the adjacent tissues and normal gastric mucosas (Figure 1).

With p1, p2 as primers, 10 normal gastric mucosas showed no RT-PCR fragment; 5 of 10 adjacent tissues showed specifically the 740 bp CD44v product of RT-PCR fragment, these 5 cases with positive CD44v expression were diffuse-type gastric cancers with distant lymph node metastasis; 16 of 20 gastric cancers expressed specific 740 bp band, of which, 14 of 15 were diffuse cancers, only 2 of 5 cases were well-differentiated cancers; 15 of 16 cases with lymph node metastasis had CD44v expression, only 1 case had CD44v expression in all 4 cases without lymph node metastasis. All the cases with CD44v expression in adjacent tissues were diffuse-type gastric cancer with distant lymph node metastasis (Figure 2).

The relationship between the biological behaviors of gastric cancer and CD44v mRNA expression is presented in Table 1.

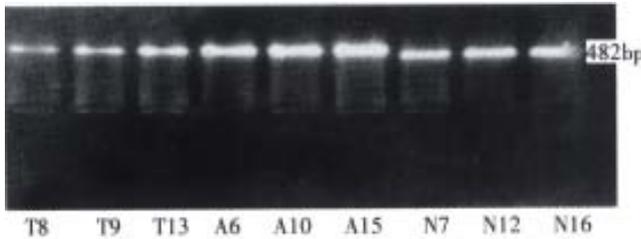


Figure 1 Electrophoretic pattern of RT-PCR product for CD44s in gastric tissues.

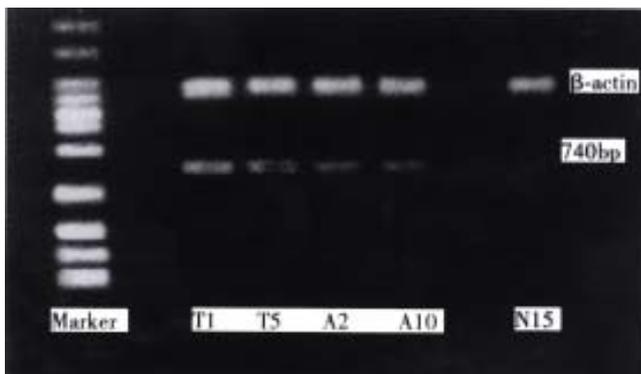


Figure 2 Electrophoretic pattern of RT-PCR product for CD44v in gastric tissues.

Table 1 Relationship between CD44v expression and histological classification of gastric cancer, lymph node metastasis, clinical pathology and tumor size

	<i>n</i>	CD44v mRNA		<i>P</i> value
		+	-	
Histological types				
Diffuse type	15	14	1	
Well-differentiated type	5	2	3	<i>P</i> <0.05
Lymph node metastasis				
Positive	16	15	1	
Negative	4	1	3	<i>P</i> <0.05
Tumor size				
>5 cm	13	12	1	
<5 cm	7	4	3	<i>P</i> >0.05
Clinicopathology				
Ulcerative type	13	12	1	
Infiltrative type	7	4	3	<i>P</i> >0.05
Stage				
I-II	7	4	3	
III-IV	13	12	1	<i>P</i> >0.05

DISCUSSION

As an adhesive molecule, CD44 exists on the surface of cells. The NH₂-terminal function area of CD44 can join the hyaluronate in the basement membrane to extracellular matrix, thus to regulate the movement and function of cells. By this mechanism, neoplastic cells can adhere itself to the extracellular matrix and basement membrane of the host cell, resulting in invasion and metastasis of malignancy. On the other hand, the degraded products of hyaluronic acid can motivate the growth of local vessels providing the basis for invasion and metastasis. This varied CD44v is a promising marker in diagnosing gastric cancer clinically^[4,7].

Yokozaki^[8]'s research suggests that the rate of abnormal expression of CD44 gene whether in well or poorly-differentiated gastric cancer may approach to 100%. In gastric cancer cells, CD44 gene generates abnormal splice variant CD44v during transcription, whereas in normal gastric mucosa cells, CD44 gene only expresses CD44s protein. In metastatic cells of gastric cancer the abnormal expression of CD44v is strengthened more than those in primary cancer cells. Therefore, CD44 gene's abnormal expression may not only serve as a good marker for identifying gastric cancer in normal mucosa, but also predict cancer metastasis.

We have got similar result to Yokozaki's. The expression rate of CD44v in gastric tissue is up to 80% with difference between two types of gastric cancer, but not high in the adjacent tissue and normal mucosa. It shows that the variant expression of CD44v is related to the phenotype of gastric malignancy, and may serve as a useful indicator in tumor metastasis, and have potential significance in diagnosing gastric cancer.

The formation and metastasis of gastric cancer are related to the abnormal expression of onco-gene (c-erb2, c-myc, ras) and suppressor onco-gene (p53). CD44's abnormal expression may be an earlier event than that of ras, p53 and other genes, and may be related to the activation of ras gene. The mechanism in promoting the formation of cancer cell and metastasis may probably be related to the function of CD44. The cancer cell with CD44v expression may get lymphocyte's guise so as to escape the recognition and execution from human immune system and easily invades lymph node, resulting in metastasis^[9].

Our results show that 16 of 20 gastric cancer specimens and 5 of 10 adjacent tissue specimens had positive CD44v expression, and none of the normal mucosa had CD44v expression (Chi-square $\chi^2 = 17.160$, $P < 0.05$). There was no statistical significance as comparing the former two with the normal mucosa. The CD44v expression rates in two types of gastric cancer were different significantly ($P < 0.05$), the rates were also markedly different ($P < 0.05$) in cases with or without lymph node metastasis. Expression of CD44v mRNA was related to the genesis and prognosis of gastric cancer, and is one of the biological markers indicating metastasis and poor prognosis. However the expression of CD44v had nothing to do with clinical stage or size of the cancer. Considering the limited data available, further investigations are needed.

No specific strand was discovered in the 20 cases of normal gastric mucosal tissues, Northern blot-hybridization method has also been used for CD44 variant gene expression product in different tumors, *i.e.* gastric cancer, lung adenocarcinoma, colon cancer and esophagus cancer tissues.

However, the method is less sensitive than RT-PCR method, it is also time-consuming and needs large quantities of RNA and tissues. RT-PCR technique can be used to detect CD44 gene's variant expression product rapidly with a tiny amount of tissues in batches, so it is highly valuable in clinical practice. False negative result is possible if only RT-PCR is used for detecting CD44, thus further researches are needed to improve the detecting sensitivity, when RT-PCR with Southern blot-hybridization technique or semiquantitative density technique are used in combination.

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Study of primary leiomyosarcoma induced by MNNG in BALB/C nude mice

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Subject headings leiomyosarcoma; stomach neoplasms; MNNG; BALB/C nude mice

INTRODUCTION

It has been well known that MNNG is one of the strong and multipotential carcinogens that have been frequently reported inducing malignant peptic tumors. We have successfully induced rat and dog gastric adenocarcinomas, squamous cell carcinomas of rat forestomach and gastric leiomyosarcoma of rats since 1979. In order to replicate the model of human gastric carcinoma in rats, large doses of MNNG was administered to BALB/C nude mice, to which human embryonic mucosa had been transplanted. During the experiment, the primary leiomyosarcoma (PLS) of the nude mouse was unexpectedly obtained.

MATERIALS AND METHODS

Experimental animals and human embryonic gastric mucosa Twenty-two ZZ BALB/C nude mice (offered by the Center of Experimental Animals of Chinese Academy of Medical Sciences), 6-8 weeks of age and 16 g-20 g in weight, half and half female and male, were raised in the "SPF" environment (constant temperature and humidity, and sterilized water, food and padding).

Human embryonic gastric mucosa was obtained from 3-8 month old 31 fetuses (12 males, 19 females) of induced labor and the mucosa was cut into lumps of 0.5 cm³ and sewed in a bag-like shape for transplantation.

Reagent MNNG (product of Sigma Chemical Co.) was dissolved in DW to form 10 µg/L solution which was then filtered with 0.25 µ filter-membrane. Vimetin, desmin, actin and cytokeratin were products of Maixin Co.

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Mucosa transplantation The nude mouse was anesthetized and its peritoneal cavity was opened on a superclean working table. Human embryonic gastric mucosa was transplanted into nude mice's peritoneum by sewing it with surgical threads. The cut healed in 5-7 days. When human embryonic gastric mucosa was wrapped by peritoneum into a tumor, each mouse was injected with 0.1 mL of MNNG at an interval of 15 days. The injection was repeated four times in all. In the meantime, immunosuppressant was administered. Those experimental nude mice were killed at different intervals 5 weeks later.

Pathological observation and general staining

Autopsy was made on the killed animals, and the transplants and adjacent organs were observed. The transplants and adjacent organs were fixed in 10% neutral-buffered formalin, dehydrated according to the routine and embedded in paraffin wax and sectioned. Finally, the sections were stained with H. E. and observed microscopically.

Special staining "Tumor-like" tissues were sectioned and then stained with AB/PAS, Argyrophil, VG, Masson's and PTAH.

Immunohistochemical method SP method has been applied in this research.

Electron microscopy The tumor tissues were cut into small pieces (0.5 mm in diameter) and first fixed with 2.5% glutaraldehyde and then postfixed with 1% osmic acid and dehydrated in a graded series of acetone and embedded in epoxy resin 812. The ultra thin sections were stained with lead acetate followed by lead nitric acid. The grids were observed under a EM109 electron microscopy and photographed.

Test of DNA repetitive sequence DNA was extracted from the tumor and NIH/3T3 cells were then hybridized by A/u probe.

RESULTS

PLS induction and gross inspection Except that four nude mice which died in the early stage of the experiment, the rest of 18 nude mice were kept under observation for 9 months. Three (16.7%) cases of PLS were induced successfully. The

duration for forming a tumor was 3-9 months, averaging 6 months. Exploratory laparotomy was performed and the tumors localized in the right abdominal wall were partly connected with tissues of the right ilium. The surface of the tumor was nodal-like, smooth and dark-red. The size of the three tumors was 2.5cm × 2.0 cm × 2.0 cm, 3.0 cm × 2.5 cm × 2.0 cm and 3.0 cm × 2.5 cm × 2.5 cm, respectively. The tumor looked like sarcoma.

Light microscope There was a large number of tumor cells characterized by their interlaced cell

arrangement (Figure 1), their irregular cell shape such as fusiform or oval forms and their various sizes in the neoplastic tissues. The rich cytoplasm was stained red, part of that was in the form of granules or vacuoles. The nucleuses were seen to exist in polymorphic shapes such as oval or stab form with unusually thick nuclear membrane.

Hyperchromasia, nucleolus, giant nucleus, multinucleus tumor giant cells, karyokinesis (0-3/HPF) and pathological karyokinesis could be seen (Figure 2). Necrosis could be seen also. Besides, surrounding tissues were invaded by tumor cells.

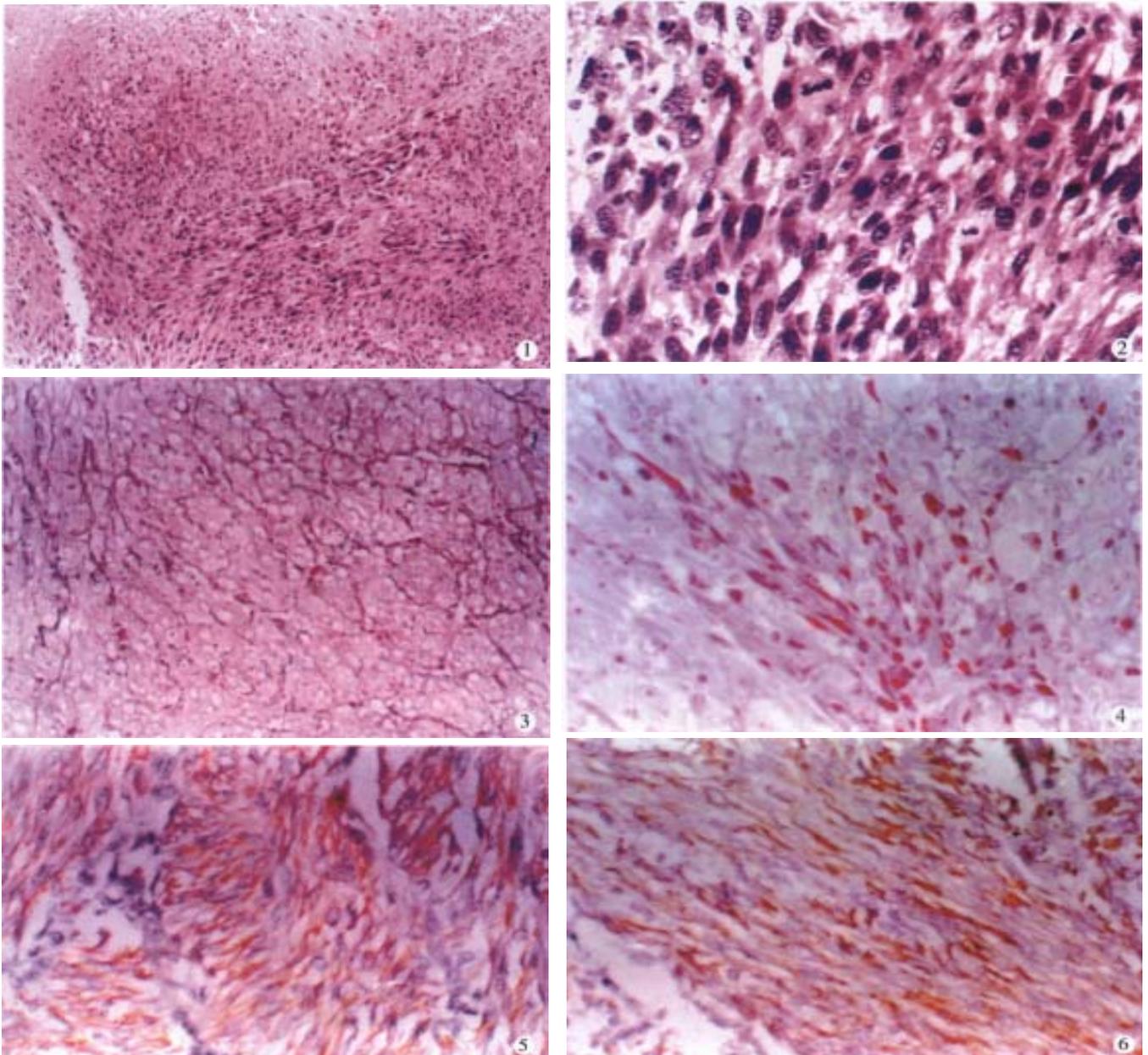


Figure 1 Interlaced arrangement of leiomyosarcoma cells stained with HE. ×20

Figure 2 Karyokinesis of leiomyosarcoma cells stained with HE. ×40

Figure 3 Reticular fibers of leiomyosarcoma cells stained with silver. ×40

Figure 4 Leiomyosarcoma cells stained red by Masson's method. ×40

Figure 5 Micrograph of positively stained desmin of cytoplasm of leiomyosarcoma cells by immunohistochemical method. ×20

Figure 6 Micrograph of positively stained actin of cytoplasm of leiomyosarcoma cells by immunohistochemical method. ×20

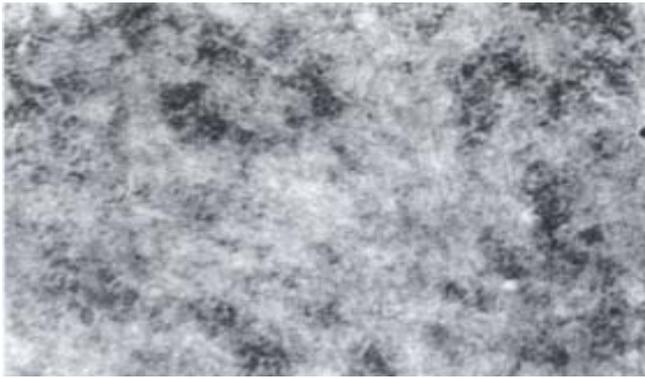


Figure 7 Electron micrograph of dense bodies and myofilaments of leiomyosarcoma. E.M.×20000

Special staining Tumor cells stained with AB/PAS were negative. Argyrophil staining showed that reticular fiber surrounded the tumor cells (Figure 3). The tumor cells were stained in yellow by VG and were red by Masson's staining (Figure 4). Cross striation was seen in the tumor cells by PTAH staining.

Immunohistochemistry Cytokeratin of the tumor cells was negative. Vimetin, desmin(Figure 5) and actin-(Figure 6)of the tumor cells were positive.

Electronic microscope The tumor cells had thick membrane, dense patches, dense bodies and myofilaments, with oval nucleuses. Concave nuclear membrane was very thick and the heterochromosome under the membrane was very large. The entosthoblast was quite clear (Figure 7).

Tests of human DNA repetitive sequence A/u test showed that the DNA of both tumor cells and NIH/3T3 cells had no human A/u repetitive sequence, suggesting that the gene of the tumor cells belonged to nude mice and not human being.

DISCUSSION

MNNG is a very powerful carcinogen, which can not only induce tumor of mouse, cancer of dog's gland stomach, squamous cell cancer of non-gland stomach of mouse, but also PLS of mouse, nevertheless, it has not been reported that MNNG can induce PLS of nude mice.

Our research aims at inducing PLS of nude mice to duplicate human stomach tumor model after

human embryonic gastric mucosa was transplanted into BALB/C nude mice which were then injected MNNG. The pathoanatomy of PLS of nude mice looked like that of PLS of mouse cervix or PLS of human soft tissue^[1-3]. It was unexpected that the tumor originated from soft tissue of abdominal wall. The special staining of AB/PAS, VG, Argyrophil, Masson's and PTAH and the results of immunohistochemistry indicate that tumor tissues originate from the smooth muscle cells^[2,3], but A/u test shows that the tumor cells do not originate from human being. We, therefore, conclude that it is PLS of nude mice.

The mechanism of MNNG inducing PLS in nude mice is still unclear, but it can be inferred that a large amount of MNNG that has affinity to smooth muscle cells continues to act on smooth muscle cells and damages the DNA of these cells, and then activates oncogene or inactivates suppressive gene and finally results in formation of sarcoma. In addition, the lack of T cells and the use of immunosuppressant amplify the effect of carcinogen. It was recently reported that MNNG inducing PLS was a very complicated process of multistage and multigene variation. A researcher found that MNNG damaged DNA and the damaged DNA was not repaired on time and then ras oncogene produced point mutation to activate ras oncogene^[4]. Wang B *et al*^[5] reported that MNNG induced gastric epithelium cells strain of GES₁ with point mutation at codon 12 of C-Ha-ras oncogene, rearrangement of C-met oncogen and increase of C-erbB-2 oncogene.

In brief, further study of MNNG inducing PLS in nude mice is significant so that we can learn more about the mechanism of the molecular pathology of oncogene and suppressive gene.

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The value of postoperative hepatic regional chemotherapy in prevention of recurrence after radical resection of primary liver cancer

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Subject headings liver neoplasms/drug therapy; neoplasm recurrence/prevention and control; regional chemotherapy

INTRODUCTION

In China, primary liver cancer (PLC) ranks second in cancer mortality since the 1990s. In the field of PLC treatment, surgical resection remains the best, which includes large PLC resection, small PLC resection, re-resection of subclinical recurrence, as well as cytoreduction and sequential resection for unresectable PLC. However, recurrence and metastasis have become the major obstacles for further prolonging survival after resection. In authors' institute, as reported in 1984, the 1-, 3-, and 5-year recurrence rates after curative resection were 17.1%, 32.5% and 61.5%, respectively^[1]. Similar results were reported in 1993, being 15.0%, 45.5% and 55.3%^[2]. Even after curative resection of small hepatocellular carcinoma (HCC), the recurrence rate remained high being 6.5%, 25.7% and 43.5% (Tang *et al*, 1989). Therefore, recurrence and metastasis might be the next important targets to be studied. New strategies for the prevention of recurrence after curative resection become a key point to further improving the results of operation for PLC patients. In the literature, the opinion was preoperative transcatheter arterial chemoembolization (TACE) for resectable PLC could result in intrahepatic spreading and lung metastasis and could not improve the survival. Herein, the efficacy of postoperative hepatic regional chemotherapy in prevention of recurrence after radical resection for PLC was evaluated.

MATERIALS AND METHODS

The criteria for the curative resection were listed as follows: a. No distance metastasis was found in patients preoperatively. b. The tumor should be completely removed with the surgical margin from the tumor edge to the cut surface for over 0.5cm. c. The α -fetoprotein (AFP) level should return to normal within 2 months after resection for AFP positive patients and no residual tumors were detected by B-model ultrasonography and/or lipiodol-computed tomography (L-CT) at 2 months after resection for AFP-negative patients.

A total implantable port system was used for chemoembolization. The top of the system was inserted into hepatic artery proper via gastroduodenal artery without hepatic artery ligated during operation. Another system should be put into the main trunk of portal vein for patients with tumor embolus in portal vein or hepatic vein system. A four-day course of chemoembolization with 5 fluorouracil 750 mg-1000 mg, mitomycin C 8 mg-12 mg, cisplatin 80 mg and Ultralipiodol 5 mL was performed at 4-6 weeks postoperatively. For patients with tumor embolus in the portal vein or hepatic vein system, the drugs were equally injected via the two catheters, while the lipiodol was given via the hepatic artery only. The regional hepatic chemotherapy will be repeated at regular interval of 2-4 months thereafter.

After discharge from the hospital, all the patients were closely followed up: every 2 - 3 months during the first 2 postoperative years and 6-months intervals thereafter. Follow-up comprised a clinical examination, conventional liver function tests, serum AFP level assay, ultrasonography and chest X-ray. Lipiodol-CT were done at 2-3 weeks after each time of chemoembolization. When recurrence and/or metastasis were suspected, more detailed examinations including CT, MRI and bone scan were taken. The diagnosis of recurrence or metastasis were decided according to the positive imaging examinations.

A total of 105 patients having a histologically verified PLC met all the above-mentioned criteria from January 1995 to December 1997 at Shanghai Liver Cancer Institute. The median age of the entire series was 52 years (range, 30-73 years) and the median diameter of the tumor was 6.8 cm (range,

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1cm - 18.8 cm). The male to female ratio was 5.5:1. Among these patients, ninety two patients (87.6%) combined with posthepatic cirrhosis and seventy patients had positive AFP level. As to the number of tumor, it was found 76 patients with single nodule, 19 with two nodules and 10 with three to six nodules. Five patients had visible tumor embolus in the second branch of portal vein and one patient had tumor embolus in the right hepatic vein. The average time of chemoembolization given to patients was 3.4 times (range, 1-7 times).

Statistical analysis: the difference between different groups were tested by χ^2 test.

RESULTS

All patients were followed up for 4 - 40 ($\bar{x} \pm s = 22.7 \pm 9.7$) months. Two patients without recurrence died of severe hepatitis at 12 or 13 months, respectively. In the remaining 103 patients, recurrence in the remnant liver was found in 11 patients, metastasis to the lung in 1 patient and implantation in the abdominal cavity also in one. The recurrence rates of 1-, 2-, 3- years were 1%, 15% and 18%, respectively. The interval between recurrence and operation varied from 10 to 20 ($\bar{x} \pm s = 15.2 \pm 2.9$) months. Another two patients had a second primary tumor in the breast and lung. For the treatments of the 13 recurrent patients, a second resection was done in 9 patients, hepatic regional chemoembolization plus percutaneous intratumor ethanol injection was done in the other four patients, who could not tolerate the second operation. Neither recurrence nor metastasis was found in six patients with tumor embolus in portal vein or hepatic vein.

The recurrence rates of different groups are shown in Table 1.

Table 1 The recurrence rates of different groups of PLC

Items	No.	Recurrence rate (%)	
AFP	Positive	70	12.9
	Negative	35	11.4
Tumor	>5cm	45	13.3
	<5cm	60	11.7
Nodules	Single	74	12.2
	Multiple	31	12.9

DISCUSSION

The following causes might be involved for the recurrence after curative resection for PLC: a: There existed undetectable intrahepatic spreading or long distance metastasis. b: A metachronous and unrecognized synchronous multifocal primary tumor may be responsible for recurrence. c: The procedure of operation can make tumor cell

detached and spread in the liver. So, curative resection doesn't mean all the tumor cells being totally removed. The depressed immune system's function of PLC patient can not guarantee that all the residual tumor will be killed even under the condition of combining with oral or one shot of hepatic chemotherapy via the hepatic artery^[3,4]. Systemic chemotherapy is abandoned in prevention of recurrence for it can not only damage the immune function of the patient but also can not guarantee the effective drug concentration at the target organs. Contrast to the systemic chemotherapy, hepatic regional chemotherapy given via hepatic artery and/or portal vein can result in a high drug concentration in the target organ without severe damage to the immunologic function.

No significance difference was found between AFP-positive patients and AFP-negative patients and between large PLC and small one and between patients with single or multiple tumor nodules (Table 1). Take into consideration that the short time recurrence rates of large PLC, multiple PLC and PLC with tumor embolus in the portal vein or hepatic vein were not higher than those of small PLC, PLC with single nodule and PLC without tumor embolus, our results indicated that postoperative hepatic regional chemotherapy can significantly decrease the short time recurrence rate after curative resection for these patients.

The peak recurrence time for PLC was within two years after curative operation. The resection for PLC and depress the patients' immune function and stimulate secretion of some regeneration related factors^[5]. These changes can make residual tumor cells and precancerous cell fast dividing and more sensitive to chemotherapy. So, it is a good chance for patients to receive postoperative hepatic regional therapy once the liver function and immune function return to normal level. It should be emphasized that if the liver function and immune function have not returned to normal, too early chemotherapy could result in further damage to patients. In general, the first time chemoembolization should be taken at 4-6 weeks after curative resection and repeated at regular interval of 2-4 months thereafter until the patients survive over the peak recurrence time. The interval of chemoembolization can be prolonged under the closing of follow up and without positive signs which indicating recurrence.

Our results indicate that auxiliary hepatic regional chemotherapy after curative resection can significant decrease the recurrence rate during the peak recurrent time. Considering that chemotherapy can neither kill tumor cell completely

nor can permanently prevent new tumor foci occurring in the remnant liver. It is no doubt that hepatic regional chemotherapy can't effectively prevent recurrence of PLC permanently. Repeated chemotherapy via hepatic artery can occlude the hepatic artery and damage the liver function. Most patients' port system can maintain in good condition for only about one year, this may contribute to the higher recurrence rates in the later two years as compared to that of the first year. Other multimodality treatment, such as immunotherapy, should be used in combination to further reduce the recurrence rate after curative resection.

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Overexpression of P-glycoprotein in hepatocellular carcinoma and its clinical implication

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Subject headings liver neoplasms; carcinoma, hepatocellular; P-glycoprotein; multidrug resistance-1 gene; immunohistochemistry

INTRODUCTION

Most advanced hepatocellular carcinoma (HCC) is insensitive to most anticancer drugs which might be related to the high frequency of expression of the multidrug resistance-1 (MDR1) gene^[1] and its product, P-glycoprotein (P-gp)^[2]. P-gp expression may also be concerned with tumor progression and differentiation^[3]. In the present study, we investigated P-gp expression and assessed the relationship between expression level of P-gp and the clinico-pathological parameters of HCC by immunohistochemistry in combination with computer-imaging analysis.

MATERIALS AND METHODS

Patients and specimens

The data were from 47 HCC resected specimens, from June 1996 to July 1997, including 47 tumor tissues and 33 adjacent tissues, 39 men and 8 women, age 25-74 years (mean 49 years). No one received preoperative chemotherapy. Of the 47 tumors, 72% were >5 cm, and 28% were ≤5 cm, 40% and 26% were accompanied by involvement of portal veins with presence of satellite nodules and intrahepatic metastasis, respectively. The HCC consisted of well and poorly differentiated types and 11 normal liver tissues were obtained at surgery from patients without chronic liver disease as control.

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Immunohistochemical stainings for P-gp

Serial sections of 4μm in thickness were prepared and immunohistochemical examination was performed by the streptavidin-biotin-complex (SABC) method using monoclonal antibody JSB1 (Boehringer Mannheim). The negative control was processed by substituting PBS for the primary antibody. The positive control was a breast cancer sample shown to express P-gp by SABC method.

Quantitative analysis

Immunoprecipitates were analyzed using an Axiotron microscope (Zeiss, Germany) and a 3CCD camera (JVC, Japan), and then processed by an image analysis system (IBAS, Kontron, Germany). According to Shen's method^[4], only the gray level and the area of defined area A and the phase α of positive products in A need to be tested. Positive unit (PU) was tested and calculated with the formula in A: $PU = 100 \times |G_{\alpha} - G_A / (1 - A_{A\alpha}) \times G_{max}$. G_{α} and G_A denote respectively the mean gray level of the studied structure α and the test area A. $A_{A\alpha}$ means the area density of phase α in A. G_{max} is the maximum gray level of the instrument. PU that reflected intensity and area of the phase α of positive products in A represented P-gp expression level. Five times were performed at random in a section to obtain the mean PU.

Statistical analysis

PU of P-gp was expressed as mean±SD. The data were analyzed statistically by means of Student's *t* test. Statistical significance was defined as a two-sided *P* value of *P*<0.05.

RESULTS

Positive expression of P-gp in HCC, adjacent to tumor and normal liver tissues

P-gp positive cells were not uniformly distributed, the intensity of immunoreactivity was also variable, staining was mainly on the cellular membrane. In normal liver, P-gp was present on the bile canalicular surface of hepatocytes and the luminal surface of bile duct epithelial cells. In P-gp-positive HCC, the staining was observed on the contact surface between tumor cells resembling the canalicular staining pattern (Figure 1). P-gp expression was found in 70% (33/47) of HCC tissues and 66% (22/33) of adjacent tissues. The staining was mild in three normal liver tissues

specimens. A significant increase in P-gp expression level was observed in HCC tissues ($P < 0.01$) and those adjacent to the tumor ($P < 0.01$) but without significant difference ($P > 0.05$).

P-gp expression level in relation to clinico-pathological parameters in HCC

The expression level of P-gp in HCC with local invasion was significantly higher than that in HCC without ($P < 0.05$). Moreover, it was significantly higher in HCC of the well differentiated type than that of poorly differentiated ($P < 0.01$). Recurrence of HCC also had a significantly higher P-gp expression than that without ($P < 0.01$). On the other hand, there were no relationships between P-gp expression level and the tumor size ($P > 0.05$).

DISCUSSION

Hepatocytes exclusively express P-gp on the bile canalicular surface, it is a $M_r 170\ 000$ membrane protein responsible for pumping lipophilic anticancer drugs such as doxorubicine and vincristine out of tumor cells^[5], resulting in chemotherapeutic effect poor. Recent reports showed increased expression of P-gp in HCC was significantly associated with non-responders^[6]. Our results indicated more than two thirds of the samples were positive for P-gp in HCC and adjacent tissues, this suggested that P-gp overexpression be probably responsible for the so-called innate drug resistance of HCC or non responder.

The fact that P-gp expression level was significantly higher in well differentiated than poorly differentiated HCC ($P < 0.01$), suggested the well differentiated HCC have a stronger potential of P-gp expression and a tendency to produce resistance to anticancer drugs more easily. It is worth trial to treat with anticancer drugs in

combination with a reverse agents of MDR1 gene or P-gp modulator as verapamil, preferably supported by data on P-gp expression.

The intrahepatic spreading, metastasis and postoperative recurrence were closely related to the biologic behavior of tumor cells, etc, about 43.5% of patients with small HCC recurred within 5 years after surgical resection^[7]. Recently, P-gp overexpression had been demonstrated in cancer with recurrence^[8]. So far there have been no reports on P-gp expression in HCC recurrence. In this study, hepatic recurrence had a significantly higher P-gp expression level than that without ($P < 0.01$). These results revealed that recurrence of hepatoma probably more easily to acquire multidrug resistance, thereby, the response to chemotherapy was likely unsatisfactory.

Our study further showed P-gp expression level of invasive HCC was significantly higher than that of non-invasive HCC ($P < 0.05$), suggesting that a high P-gp expression level was well associated with the invasiveness of HCC, and not related to the size of the tumor whether large or small.

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Localization of HCV RNA and capsid protein in human hepatocellular carcinoma

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Subject headings hepatitis C virus, liver neoplasms; carcinoma, hepatocellular; in situ hybridization; immunohistochemistry

INTRODUCTION

The relation of HCV to hepatocytic carcinoma (HCC) has been emphasized recently in low HBV infection countries and regions. The distribution patterns of HCV in liver tissues are not well understood although studies on HCV infection in blood and hepatocytes have been conducted by PCR. In this study, 42 liver cancers and surrounding liver tissues were detected for HCV RNA and HCAg using photosensitive biotin-labeled HCV cDNA probe and Immuno-gold-silver stain (IGSS) method.

MATERIALS AND METHODS

Human HCC and paratumor tissues

Fourty-two samples including cancers and their surrounding tissues were collected and processed into formalin-fixed, paraffin-embedded blocks.

Probe preparation

NS5 cDNA probe PCR product from NS₅ region was inserted into charomid 9-42. This resulted in recombinant HCV plasmid which could be spliced into specific cDNA fragment with DNA length 534 bp^[1].

HCV cDNA probe HCVC cDNA probe was the PCR product of C region.

The preparation of photosensitive biotin-labelled probe was as described in literature^[2].

In situ hybridization

Five μm sections were dewaxed, rehydrated in buffered solutions, then digested in proteinase K,

and fixed in polyformaldehyde. After dehydration, prehybridization was performed and then the sections were denatured (steaming bath at 100 °C, 10 min, then rapidly cooling down in ice bath), and hybridized again (42 °C, overnight). After rinse, the sections were conjugated with gold-labeled strepto-biotin, stained with AgNO₃ buffered solution, then restained in hemoxylin and eosin or eosin. Positive stain should be present as intracytoplasmic or intranuclear black granules.

Immunohistochemical detection of HCAg PAP method was used. The specific human anti-HCV antibody was from Laboratory of Immunology at Tongji Medical University.

Negative control Sections were treated with RNase (0.1 g/L), 1 hour, at 37 °C.

RESULTS

HCV RNA in liver tissues

HCV RNA positivity was detected in 8 cancers and their surrounding tissues including 2 positivity in cancers, 1 in both cancer and their surrounding tissue, 5 in surrounding liver tissues. Positive cells appeared diffuse, clustered, or discrete in cancer and its surrounding tissues. Three types of positive granules were noted: ① cytoplasmic, diffuse granules in cytoplasm with prominent perinuclear staining (Figure 1); ② nuclear, evenly diffuse granules in nucleus; ③ nuclear-cytoplasmic, positive granules present in both cytoplasm and nucleus. Detection by NS₅ cDNA probe revealed that 7 out of 42 were positive; detection by C cDNA probe showed one nuclear positive in 2 cases.

HCAg expression in liver tissues

Ten out of 42 stained positive for HCAg and positive cells were distributed in cancers and its surrounding tissues. HCAg positive cells appeared diffuse, clustered or discrete. Two distributing patterns of intracellular positive granules: ① cytoplasmic type: diffuse positive granules in cytoplasm; ② inclusion type: positive granules present as inclusions in cytoplasm (Figure 2).

DISCUSSION

HCV is a single-stranded RNA virus which reproduces itself on the template of negative strand and its copies are rarely present in tissues. It has been established that replication of HCV RNA occurs mainly in liver tissues. How it infects liver cells and at which point it starts duplication remains unknown.

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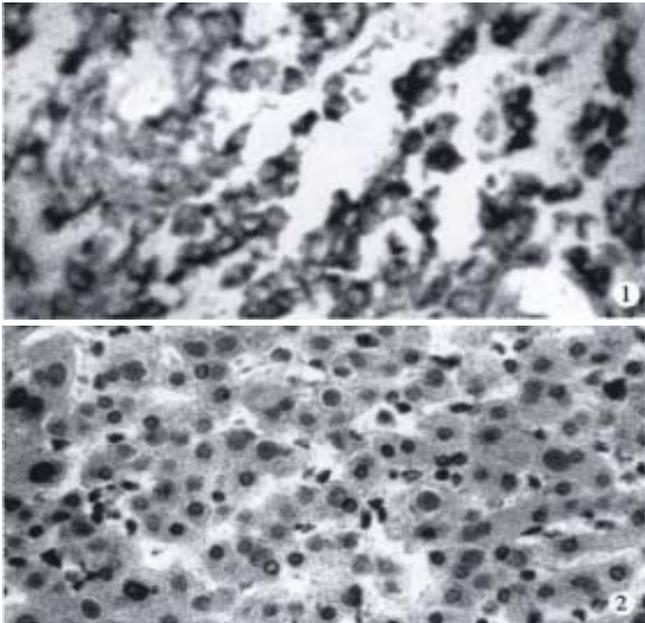


Figure 1 HCV-ISH (cytoplasmic type), biotin marker, ABC methods, HCCII, $\times 132$

Figure 2 HCAg (inclusion pattern), cancerous surrounding liver, PAP method, $\times 200$

Since 1992, there have been some reports on detection of HCV RNA in hepatic tissues using ISH, and HCV RNA was present in cytoplasm or nucleus^[3-8]. However, it is still unclear whether HCV RNA is present in hepatocytic carcinoma. Using ISH, we found that HCV RNA did infect cancers and their surrounding tissues, which was in good agreement with other authors' reports. HCV infected cells appeared diffuse, clustered or discrete in cancers and their surrounding tissues.

There is a viewpoint that the reverse transcriptive form of HCV RNA does not exist at all during its replication. Therefore, it is disputable on the significance of its presence in nucleus. Kayhan^[8] reported that HCV RNA was present in nucleus and nucleoli, and suggested that HCV duplication might use its host's genes or intranuclear apparatus, or HCV granules accumulated in infected cells like HBV. Our results were in accordance with the above findings. The studies on yellow fever virus also supported the above viewpoints: NS5 protein could be detected in nucleus and nucleoli, indicating that viral replication could occur in nucleus^[9].

In 42 liver cancers, 10 were positive for HCAg, while only 8 expressed HCV RNA by ISH, indicating that positivity of viral genes was lower than that of viral antigen. It is suggested that this phenomenon could be accounted by the variations of viral genes, and the fixing time by formalin. HCV is a variable virus, it may differ individually and

may vary at different stages of infection. Therefore, the variation of gene sequence may result in less sensitivity of ISH detection. In addition, longer fixation may cause loss of viral genes or false negativity. The above defects could be overcome by using probes of different fragments of the same gene. Two cases with HCAg(+), NS5 cDNA(-) were hybridized again and revealed positive signals of NS5 cDNA in one case. This agreed well with Haruna's^[7] findings. Although ISH is not as sensitive as PCR, it is a promising technique in the study of relation of HCV RNA and infected cells^[10-12].

The significance and mechanism of continual variation of HCV gene *in vitro* remain to be resolved in recent years. It was reported that HCVs continual variation *in vivo* may be related to virus escape from host immune deletion and virus configurative change brought about by immune injury introduced by the host^[13-15].

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Expression of IGF-II, p53, p21 and HBxAg in precancerous events of hepatocarcinogenesis induced by AFB1 and/or HBV in tree shrews

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Subject heading liver neoplasms; carcinoma, hepatocellular; hepatitis B virus; IGF-II; p53 gene; p21 gene; HBxAg; aflatoxin B1

INTRODUCTION

In order to study the relationship between oncogene expression and HCC generation, we observed the precancerous hepatic GGT foci, IGF-II, p53 and p21 expression during hepatocarcinogenesis of tree shrew induced by hepatitis B virus (HBV) and/or aflatoxin B1 (AFB1).

MATERIALS AND METHODS

Materials

One hundred and twenty tree shrews (Tupaia Belangeri Chinese) were purchased from Yunnan Province of China. Their body weight was 100g-159g.

HBV infected serum was got from the patients with hepatitis B, positive for HBsAg and HBeAg. Monoclonal antibodies (MoAb) to HBsAg, p21 (MAB-0143), p53 D0.7 (MAB-0142) and S-P Kit were purchased from Maxim Technic Co. Anti-HBxAg MoAb was presented by Professor Wu GH in Liver Disease Institute, General Hospital of PLA Beijing Military Area Command. IGF-II (Lot 12850) RbAb was from Ups tate Co. HBV DNA-Bio probe and *in situ* hybridization kit were provided by Liver Disease Institute of Beijing Medical University.

Methods

HBV infection marks were negative in serum of the animals before inoculation. Liver biopsy (LB) was taken from each animal which was negative for

HBV-infected marks in serum before inoculation. It was used as self-control. Each of the 80 tree shrews was inoculated with HBV-infected serum, injected through the femoral vein. Blood was taken from the animals were from wk2 to wk8 after inoculation. The animals positive for HBsAg, HBeAg and anti-HBcAg were divided randomly into group A and group B. The remaining 40 animals that did not receive inoculation of HBV were divided into group C and group D. Group A was HBV positive and fed with AFB1; group B was HBV infected; group C was fed with AFB1 and group D was as control. AFB1 mixed in the milk was freely lapped by animals, 6 d/w at a dose of 150 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. LB was taken from all animals anaesthetized by ketamini. The size of the tissue s was about 0.8 cm \times 0.4 cm \times 0.4 cm, and they were fixed immediately. LB was taken repeatedly in every other 15wk after fed with AFB1.

HBsAg, HBxAg, IGF-II, p53 and p21 were detected by immunohistochemistry. Microwave citrate buffer strengthened HBxAg and p53. After testing suitable concentration of antibodies, slides were stained according to the S-P kitins truction. Positive and negative controls were set in each test to differentiate false reaction. GGT was stained by the method of Rutenberg. *In situ* hybridization of HBV- DNA was made according to the kit information.

The data was statistically evaluated by using *t* and χ^2 tests.

RESULTS

HCC was not found after 75 wk of observation. The average amount of AFB1 taken by each animal in groups A and C was 9.9 mg and 10.4 mg respectively ($P>0.05$).

The number of GGT positive foci was larger in group A than in groups B and C ($P<0.05$). GGT positive foci in groups A, B and C were more and bigger than in group D ($P<0.01$) (Table 1).

Table 1 GGT positive foci at wk75

Group	HBV	AFB1	Animal No	GGT positive foci ($\bar{x}\pm s$)		
				No/cm ²	mm ² /cm ²	mm ² /focus
A	+	+	15	143.3 \pm 96.4	7.12 \pm 7.50	0.04 \pm 0.03
B	+	-	14	71.1 \pm 72.0	1.90 \pm 2.09	0.03 \pm 0.01
C	-	+	16	72.9 \pm 70.4	2.22 \pm 2.42	0.23 \pm 0.01
D	-	-	10	19.4 \pm 19.9	0.35 \pm 0.36	0.01 \pm 0.01

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The HBsAg positive rates were 86.7% and 95.7%, and HBxAg rates were 92.9% and 82.6% respectively in groups A and B (Table 2).

Table 2 HBsAg, HBxAg and HBV DNA in group A and B

Group	HbsAg		HBxAg		HBV DNA	
	Animal <i>n</i>	Positive <i>n</i> (%)	Animal <i>n</i>	Positive <i>n</i> (%)	Animal <i>n</i>	Positive <i>n</i> (%)
A	15	13(86.7)	14	13(92.9)	10	7(70.0)
B	23	22(95.7)	23	19(82.6)	16	12(75.0)
Total	38	35(92.1)	37	32(86.5)	26	19(73.1)

At wk 15, IGF-II positive liver cells in brown color were dispersely distributed in plasma. At wk 75, however, the positive cells were found to be near the margin of the proliferation foci of liver cells. The positive rate was quite different between wk 45 and wk 75 ($P < 0.05$, Table 3). Nuclear p53 was not found at wk 75. p21 positive rate was about 5% in groups A, B and C, with small brown grains in the plasma of liver cells.

Table 3 IGF-II at wk 45 and wk 75

Group	wk 45		wk 75	
	Animal <i>n</i>	Positive <i>n</i> (%)	Animal <i>n</i>	Positive <i>n</i> (%)
A	15	13(86.7)	15	5(33.3)
B	23	17(73.9)	20	13(65.0)
C	18	18(100.0)	17	11(64.7)
D	11	6(54.5)	11	4(36.4)

DISCUSSION

Hepatocarcinogenesis was found only in the precancerous phase of the tree shrews. The reason for the unequal numbers among the tests was that the LB tissue was too small to detect the marks.

Both HBsAg and HBxAg were found in the liver of tree shrew by immunohistochemistry. HBV-DNA was detected by *in situ* hybridization. These results showed that the tree shrew model for HBV infection was reliable.

GGT positive foci did not form in the early stage of the experiment. Small and irregular GGT

positive foci were found at wk 75. There were more GGT foci in group A (treated by HBV and AFB1) than in groups B and C (treated by HBV or AFB 1), indicating the synergistic effect of HBV and AFB1 in hepatocarcinogenesis, which was also reported by other researchers. Group B (infected with HBV) had more GGT positive foci than group D (control), indicating that the effect of HBxAg trans-activation could induce precancerous lesions in the liver of tree shrew.

IGF-II was over expressed in human liver cancer and its surrounding tissues. Usually the expression of cancer-surrounding tissues is higher and is almost the same as that in fetal liver. In normal liver tissue, however, IGF-II was negative^[1,2]. IGF-II has high expression rate at wk 45, but significantly low at wk 75. In the HCC tissue of tree shrew, IGF-II was 100% positive^[3]. This revealed that IGF-II expression did not persist in a high level during hepatocarcinogenesis of tree shrew. IGF-II in group D probably caused by LB might repeatedly activate the expression.

Over expression of oncogenes and anti-oncogenes was found in AFB1 and HBV induced liver cancer. Many studies showed p21 in the early stage of carcinogenesis, and it was related with liver cell transformation. High rate of p21 was not observed. The expression of p21 in the later stage of the experiment is to be studied. p53 was not found in the precancerous tissues, which is in agreement with that reported in the literature^[4].

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Antitumoral activity of low density lipoprotein-aclacinomycin complex in mice bearing H₂₂ tumor

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Subject headings hepatoma cells; lipoprotein, low density; a clacinomycin; LDL receptor; mice

INTRODUCTION

Cancer cells, which proliferate rapidly need large amounts of cholesterol for new membrane synthesis, and high LDL receptor (LDLR) activity. LDL has been proposed as a useful discriminatory vehicle for the delivery of cytotoxic drugs to tumor cells^[1,2].

LDL presents many advantages as drug carriers that may circumvent many problems encountered with synthetic carriers^[3,4]: ① LDL may deliver highly lipophilic compounds with promising cytotoxic effect. On the other hand, the sequestered drug is protected from enzymatic action. ② Furthermore, LDL is not cleared by the monocytic macrophagic system and may prolong the serum half-life of antineoplastic drugs. ③ Tumor cells internalise and degrade LDL by the LDL receptor pathway. This potentially high efficient process may lead to different pharmacological effects.

It is possible to incorporate aclacinomycin (ACM) into LDL to form low-density lipoprotein-aclacinomycin (LDL-ACM) complex^[5,6]. In our recent study, the therapeutic activity of LDL-ACM complex and that of ACM were compared in experimental mice tumor with the potential effect of LDL-ACM complex.

MATERIALS AND METHODS

Materials

Male or female KM mice, 6-8 weeks old, were

purchased from Experimental Animal Center of Shandong Medical University. Murine Hepatoma 22 (H₂₂) cells were bought from Pharmaceutical Institute, Shandong Academy of Medical Sciences, and ACM from Shenzhen Wanle Pharmaceutical Co., Ltd.

Lipoproteins

Human LDL (density, 1.019 kg/L - 1.063 kg/L) was isolated by ultra centrifugation of serum from healthy blood donors^[7]. The isolated LDL was dialyzed against LDL buffer (Na₂HPO₄ 50 mmol/L, NaCl 100 mmol/L, Na₂EDTA 0.1 g/L, pH7.4) for 48 h, filtered through a membrane filter (Millipore, 0.22 μm pore size) and stored at 4 °C. Lipoprotein purity was assessed by agarose gel electrophoresis. All concentrations of LDL given refer to protein. The protein concentration was determined according to the method of Lowry *et al.*

Incorporation of ACM into LDL^[5]

ACM was incorporated into LDL by incubating 36 mg LDL with 40 mg ACM in LDL buffer for 3 h at 40 °C. The mixture was then subjected to extensive dialysis for 48 h against LDL buffer to remove free drug and thereafter filtered and stored at 4 °C. The LDL-ACM complex obtained had a drug: LDL protein weight ratio 0.35. The concentrations of LDL-ACM complex given in the text refer to ACM.

Assay for ACM

ACM was assayed fluorometrically in a HITACHI spectrophotofluorometer model F-3000 using 430 nm and 580 nm as excitation and emission wavelengths, respectively^[5]. ACM was extracted with chloroform: methanol (4:1 V/V). When LDL-ACM complex was assayed, the standards also contained LDL.

Therapeutic activity of LDL-ACM complex

On day 0, H₂₂ cells (2 × 10⁶) were injected subcutaneously in the right upper armpit of KM mice. Free ACM (3.5 mg/kg) or LDL-ACM complex (3.5 mg/kg) was given intravenously in the tail vein q.d. on days 1-7. After 9 days, the animals were killed. Tumor and body weights were

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recorded.

On day 0, H₂₂ cells (2×10⁵) were injected intraperitoneally in KM mice. Free ACM (3.5 mg/kg) or LDL-ACM complex (3.5 mg/kg) was given intraperitoneally q.d. on days 1-7. Then survival times and body weights of mice were recorded.

In both types of experiments, eight mice were used for each schedule of administration and controls.

Statistical analysis

Results are expressed as $\bar{x} \pm s$. Difference between mean values was compared by the Student's *t* test.

RESULTS

Antitumoral activity of LDL-ACM complex in mice bearing subcutaneous H₂₂ tumor (Table 1)

When given intravenously, the complex had a marked therapeutic effect on mice with subcutaneous H₂₂ tumor ($P < 0.05$), whereas ACM had not. The average weight for mice in the group of LDL-ACM complex or ACM lost less during the treatment period.

Table 1 Antitumoral activity of LDL-ACM complex and free ACM against murine subcutaneous H₂₂ tumor

Drugs	Mean weight change (g)	Tumor weight ($\bar{x} \pm s$, g)	Inhibition rate (%)
Control	+5.4	1.74±0.60	
Free ACM	+3.2	1.30±0.57	25.3
LDL-ACM	+3.5	0.86±0.44	50.2

Antitumoral activity of LDL-ACM complex in mice bearing intraperitoneal H₂₂ tumor (Table 2)

The survival times of mice bearing intraperitoneal H₂₂ tumor were all significantly prolonged in the groups of ACM ($P < 0.05$) and LDL-ACM complex ($P < 0.01$) when compared with that in the group of control. Administration of LDL-incorporated ACM resulted in a significant increase in antitumoral activity at the same dose as compared with the free ACM ($P < 0.05$). Abnormality in body weight has been noted in any lot of mice.

Table 2 Antitumoral activity of LDL-ACM complex and free ACM against murine intraperitoneal H₂₂ tumor

Drugs	Survival time ($\bar{x} \pm s$, d)	Life-prolonging rate (%)
Control	17.1±3.44	
Free ACM	24.6±7.50	43.9
LDL-ACM	33.4±7.67	95.3

DISCUSSION

An LDL-ACM complex containing 212 drug molecules per LDL particle could be obtained by incubating LDL with a large excess of ACM at 40 °C. High-performance liquid chromatography studies of the mixture before and after the incubation showed that ACM was stable under these conditions^[5].

We investigated antitumoral activity of LDL-ACM complex in mice bearing H₂₂ tumor. Results showed that entrapment of ACM into LDL particles increased the antitumoral activity as compared with the free ACM in H₂₂ tumor model. The improved antitumoral activity could be the fact that the catabolism of the complex was related to the LDLR pathway. By LDLR pathway, tumor tissue took up more ACM by the LDL-ACM complex than normal tissue. Such targeting could lead to increased exposure of ACM to the tumor and consequently, increase the antitumoral efficacy.

LDL-drug complex might result in lesions in the organs like the liver and adrenals known to be the normal tissues with the highest LDL uptake. This problem might be circumvented since animal studies indicate that it is possible to down-regulate the LDL-uptake in these organs by pretreatment with bile acids and steroids without affecting the uptake by the tumor^[8]. Acetyl LDL (Ac-LDL) and Methyl LDL (Me-LDL) are rapidly cleared from the plasma. The endothelial cells of the liver have scavenger receptors and can remove these modified lipoproteins from the circulation. The endothelial cells of the spleen, bone marrow, adrenal, and ovary also participate in this rapid clearance. Internalization of modified LDL leads to the accumulation of large amounts of cholesteryl esters in the cells, which decreases the LDLR activity of these cells. Modified LDL does not affect the tumor LDLR activity. So giving Ac-LDL or Me-LDL in advance may protect the normal tissue having rich LDL receptors from LDL-drug complex during the treatment of tumors^[9,10].

This study has been performed with human LDL. Even if human LDL is recognised by the animal's receptor^[11], the competition from endogenous LDL is very different from the one in the human situation since there are pronounced species differences in the plasma lipoprotein pattern and metabolism. Therefore, the results must be interpreted with caution. It would be suitable to use an animal model close to the human one for the lipoprotein metabolism such as hamster or guinea pigs.

We used a technique which allowed a high entrapment level of lipophilic ACM into LDL. The

cytotoxic LDL-ACM complex so formed exhibited an increasing antitumoral activity in a murine tumor model. Further human clinical trials will depend on a better knowledge of the expression and the regulation of the LDL receptors by the tumor cells. Studies in this optic are now in progress in our department.

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Effects of somatostatin on splanchnic hemodynamics in cirrhotic patients with portal hypertension

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Subject headings hypertension, portal; liver cirrhosis; somatostatin; hemodynamics

INTRODUCTION

Esophageal variceal bleeding (EVB) is one of the most common complications of cirrhosis with portal hypertension. In recent years, great progress has been made in medicinal treatment. Somatostatin has been widely used in clinics, for it can effectively lower the portal venous pressure (PVP) with little side effect. The aim of this study is to assess the effect of somatostatin on portal venous pressure and splanchnic hemodynamics in patients with liver cirrhosis and portal hypertension.

MATERIALS AND METHODS

Subjects

The study subjects were 20 cirrhotic patients with portal hypertension, including 12 men and 8 women. Their mean age was 46.6 ± 13.4 years. All patients had a history of hepatitis B with positive HbsAg and were pathologically diagnosed as having liver cirrhosis.

Methods

All patients were asked to fast and lie supine for 12 hours before somatostatin infusion. The inner diameter and blood velocity of the portal, left hepatic, middle hepatic and right hepatic veins were measured. Using ACUSON 128 × P/10 color Doppler ultrasonography. A continuous infusion of somatostatin was then administered via the peripheral vein at a rate of 250 μ g/h. The measurement was repeated after an hour. The blood flow was calculated according to the formula $Q = 60 \pi r^2 V$. The πr^2 in the formula is the sectional area (cm^2) of the vein, V represents the mean value of

maximum blood velocity (cm/sec) and Q is blood flow (mL/min). Seven days after the operation, portal venous pressure, blood pressure (BP) and heart rate (HR) were measured in 15 of the 20 patients who had undergone right gastroepiploic venous catheterization. The measurement was taken before infusion of somatostatin and after 1 and 1.5 hours. Student's *t* test was used to compare the data collected before and after the treatment.

RESULTS

Portal venous pressure was measured via the catheter in the right gastroepiploic vein of the 15 portal hypertensive patients at 0, 1.0, and 1.5 hours after somatostatin administration. The portal venous pressure decreased from 20.8 ± 2.0 mmHg (0h) to $18.2 \text{ mmHg} \pm 2.0 \text{ mmHg}$ (1h) and $18.0 \text{ mmHg} \pm 2.0 \text{ mmHg}$ (1.5h), respectively, the differences being statistically significant ($P < 0.01$). However, the difference between 1h and 1.5h was insignificant ($P > 0.05$). The infusion of somatostatin did not affect the systolic arterial pressure (SAP), diastolic pressure (DAP) and HR (Table 1).

Table 1 Effect of somatostatin on PVP, BP and HR

	<i>n</i>	0 h	1 h	1.5 h
PVP (mmHg)	15	20.8 ± 2.0	18.2 ± 2.0^b	18.0 ± 2.0^b
SAP (mmHg)	15	130.0 ± 14.0	134.1 ± 12.0	132.0 ± 16.0
DAP (mmHg)	15	82.0 ± 9.2	84.0 ± 14.0	83.1 ± 14.0
HR (beat/sec)	15	81.2 ± 7.3	79.1 ± 6.2	80.7 ± 6.9

^b $P < 0.01$ as compared to 0 h.

The hepatopetal flow was measured by color Doppler ultrasonography in all the 20 patients. The sectional area of portal vein decreased by 7.28% after the infusion of somatostatin but the difference being insignificant ($P > 0.05$). The average value of portal vein maximum blood velocity decreased by 18.96% from 19.72 ± 7.75 cm/sec to 15.98 ± 7.26 cm/sec, and the average total portal vein blood flow was decreased by 19.72% from $1643.21 \text{ mL/min} \pm 757.25 \text{ mL/min}$ to $1319.49 \text{ mL/min} \pm 622.39 \text{ mL/min}$ after the infusion, the difference being very significant ($P < 0.01$).

In 19 of the 20 patients, the total sectional area and the total blood flow of the three hepatic veins were calculated after somatostatin infusion. The

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former increased from $0.72 \text{ cm}^2 \pm 0.21 \text{ cm}^2$ to $0.76 \text{ cm}^2 \pm 0.24 \text{ cm}^2$, and the latter increased from $1786.22 \text{ mL/min} \pm 923.37 \text{ mL/min}$ to $1836.17 \text{ mL/min} \pm 844.24 \text{ mL/min}$. The changes were not significant ($P > 0.05$).

DISCUSSION

It has been shown that humoral substances play important roles in the pathogenesis of portal hypertension. Due to liver function damage and the shunt of collateral circulation, changes occurred in the blood levels of these vasoactive humoral substances and some of them can regulate the portal venous pressure by interfering with blood vessel resistance or blood flow of the portal vein^[1]. The medicinal therapy is somewhat based on this hypothesis.

Somatostatin, one of the peptide hormones originating from neural-ectoderm, is able to inhibit the release of some hormones *in vivo* and lower the portal venous pressure by changing splanchnic blood flow. Treatment of variceal hemorrhage with somatostatin in portal hypertension has been successful according to many recent reports^[2,3]. Seven days after the 8 mm H-graft portacaval shunts (HGPCS), we measured the portal venous pressure via the catheter in the right gastroepiploic vein before and after the infusion of somatostatin and found that somatostatin could lower the portal venous pressure by 2.6 mmHg-2.7 mmHg. There was no significant difference in the portal venous pressure 1 or 1.5 hours after the administration. The results indicated that continuous infusion of somatostatin could decrease the portal venous pressure. At the same time, there was no significant changes in BP or HR. We concluded that somatostatin had fewer side effects than other drugs used to lower the portal venous pressure and had little influence on systemic hemodynamics. It appears that somatostatin can be used clinically to treat bleeding from esophageal varices of portal hypertension.

In order to find out the mechanism of how somatostatin lowers the portal venous pressure, we measured the sectional area and maximum blood velocity, and flow through the portal, left hepatic, middle hepatic and right hepatic veins using color Doppler ultrasonography before and after somatostatin administration. The color Doppler flowmetry in the study of portal hypertensive hemodynamics proved to be an accurate, simple, non-invasive and easily repeated method^[4]. Michel^[5] reported that there existed consistent results by both the pulsed Doppler and electromagnetic flowmetry methods in measuring the portal vein blood flow ($r = 0.918$). Under these

fixed conditions, the data has shed light on the changes in the portal venous blood flow in a same patient observed by a same examiner^[4-6]. Our study indicates that the inner diameter changed slightly and that the sectional area of the portal vein decreased by 7.28% with no significant difference after the infusion, suggesting that somatostatin itself can not directly constrict the portal vein. Our study also indicates that the maximum blood velocity and the maximum portal vein blood flow decreased significantly by 18.96% and 19.72% respectively after the use of somatostatin. This suggests that somatostatin can lower portal venous pressure by reducing blood velocity and blood flow, which was commonly found in other reports^[7]. However, how somatostatin reduces portal vein blood flow remains unclear. Glucagon is reported to be able to increase blood flow and portal venous pressure^[8] while inhibiting the effect of somatostatin in reducing portal vein blood flow^[9]. This suggests that somatostatin inhibits the release of glucagon. Somatostatin was also reported to be able to inhibit the renin-angiotensin-aldosterone system and lessen Na^+ retention^[10,11]. We also discovered that the summation of the three hepatic venous sectional areas and blood flow through these areas increased slightly without any significant differences. In conclusion, the role of somatostatin in splanchnic hemodynamics in cirrhotic patients with portal hypertension should be further investigated.

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Radiotherapy of double primary esophageal carcinoma

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Subject headings esophageal neoplasms/
radiotherapy; neoplasms, double primary/
radiotherapy

INTRODUCTION

Double primary esophageal carcinoma is defined as having two foci of squamous cell cancer simultaneously or consecutively developing in different sites of esophagus. This rare disease appears mostly in the literature as case reports^[1-4], reports about its treatment are even more infrequent. Here we present our experiences of radiation therapy in 37 patients with this disease and focus the discussion on the optimum method of treatment and complications.

MATERIALS AND METHODS

Materials

Criteria for diagnosing double primary esophageal carcinoma: ① typical findings seen in barium esophagograms, ② two separate lesions spaced ≥ 4 cm apart from each other, with intervening normal mucosa, ③ with endoscopic, pathologic or cytological proof of malignancy, ④ hypopharyngeal or gastric cardia lesions are excluded.

There were 25 males and 12 females, aged 41-80 years, with a peak range of 51-70 years (67.6%). Thirteen of 37 (35.1%) had supraclavicular lymph node metastasis before or during the treatment, 35.1% of patients had lesions in the upper-middle and 64.9% were in the upper-lower segments. The intervening normal mucosa varied from 4 to 13 cm. Length of both lesions was ≥ 5.0 cm in 12 patients, ≥ 5.0 cm in one and < 5.0 cm in the other in 21 cases, both lesions < 5.0 cm in four. The double primaries: developed simultaneously or within 6 months in 48.6%, those

developed beyond 6 months to 3 years in 35.1% and over three years in 16.2%. The longest interval of developing a second primary was 12 years and 8 months. All these were squamous cell carcinoma, among them, 33 were proved by pathology or cytology and 4 by pathology singly.

Treatment

Thirty-three patients received radiation therapy (separate field irradiation) for both lesions and 4 patients refused to have irradiation of the second lesion because of psychological reasons. Among them, two-thirds were treated by rolling technique, and one-third by antero-posterior irradiation, at 30-40 Gy/wk. The rest of the dose was delivered by two posterior oblique technique. In 59.5%, each individual lesion received a curative dose of 60GY-70GY/30F-35F/6 wk-7 wk and 40.5% received a palliative dose (≤ 40 Gy/20 F/4 wk) or with one lesion left untreated. Statistical calculation was done by Chi-square test.

RESULTS

The 1, 3 and 5-year survival rates were 27%, 5% and 0%, different from those with single lesion (38%, 13% and 8.4%) in 3798 patients treated in our hospital but with no statistical significance ($P > 0.05$, $\chi^2 = 1.854$, $\chi^2 = 2.1$). The failure due to metastasis was very high, giving no 5-year survivor.

The 1- and 3-year survival rates of patients who received curative doses were 27.2% and 9.1% and 26.7% and 0%; for those who received palliative doses. Only those with two lesions irradiated were able to survive more than one year, even with absence of supraclavicular lymph node metastasis, the curative group had no 5-year survival.

In the present series, 62.1% failed by local recurrence, 10.8% by pulmonary radiation injury and 29.8% by distant metastasis.

DISCUSSION

The 1-, 3- and 5-year survival rates of double primary esophageal cancer treated with radiation therapy were 27%, 5% and 0% as against 38%, 13% and 8.4% with only one lesion, without statistical significance ($P > 0.05$). The 1- and 3-year survival rates of curative dose were 27.3% and 9% vs 26.7% and 0% of those given palliative dose. The 1- and 3-year survival rates of those having no supraclavicular lymph node metastasis

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were 38.5% and 15.4%.

In debilitated or those who already had supraclavicular node metastasis, palliative dose would give symptomatic relief and prolongation of life. But in cases in which one of the two lesions was left untreated, none lived for over one year.

To improve its prognosis, early diagnosis is essential. During the follow-up examination of post-irradiated esophageal cancer patients, care should be directed not to miss a second primary focus. About 83.3% of the second primary would appear

within 3 years after the detection of the first primary focus.

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bFGF and TGF β expression in rat kidneys after ischemic/ reperfusional gut injury and its relationship with tissue repair

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Subject headings ischemia-reperfusion injury, intestinal; basic fibroblast growth factor; transforming growth factor β ; gene expression

INTRODUCTION

Intestinal ischemia/ reperfusion (I/R) occur commonly in critically ill patients. It is well recognized that gut I/R may cause tissue damage and dysfunction of intestine, and induce remote organ injury including kidney, lung, and liver^[1]. It may also lead to complications after severe burn or injury. Previous studies have focused on cellular elements, cytokines and inflammatory mediators. Relatively little attention has been paid endogenous protective mechanisms, i.e. the growth factors.

Both bFGF and TGF β are important growth factors involved in tissue repair, these involve dermal and epidermal wound healing via promoting the initiation and regeneration of capillary vessels, effective chemotactants and increasing deposition of extracellular matrix^[2]. Recent studies demonstrated that given exogenous growth factors could accelerate internal organ repair after gut I/R injury^[3,4], however, little is known about its molecular mechanism.

The present study was carried out to evaluate endogenous bFGF and TGF β expression of renal origin in a gut I/R rat model, and to explore the role of bFGF and TGF β 's release in active repair.

MATERIALS AND METHODS

Animal model Male, pathogen-free Wistar rats were purchased from the Animal Center, Academy of Military Medical Sciences, weighing 200 g to 250 g. Animals were allowed water only for 24 hours before use, and anesthetized with pentobarbital sodium (30 mg/kg). Following midline laparotomy, intestinal ischemia was

achieved by placing a microvascular clip across the proximal superior mesenteric artery (SMA) for 45 min, animals were then allowed reperfusion for different periods after removal of the clip and randomly divided into ischemia group (IR0; $n = 12$), reperfusion for 6 hours (IR1; $n = 12$) and 24 hours (IR2; $n = 12$) groups. Time-matched, sham-operated animals underwent laparotomy and dissection of the proximal SMA without occlusion served as controls (control; $n = 12$). Experimental animals were sacrificed and kidney tissues were fixed in 4% paraformaldehyde in PBS and 10% formalin respectively for analysis.

In situ hybridization

Tissues were dehydrated in an ascending series of ethanol and were embedded in paraplast. Five micro-thick sections were cut and prehybridized sequentially in PBS, 0.2N HCl for 20 min, Proteinase K, 1 mg/L (Sigma) for 20 min in 42 °C, 2% glycine 15 min, post-fixation was performed with 4% paraformaldehyde in PBS for 20 min. Slides were dehydrated and prehybridized for 2 hours without probe and hybridized for 20 hours with probe at 42 °C bFGF and TGF β cDNA probes were obtained by polymerase chain reaction (PCR) and confirmed by sequencing analysis. The probes were labeled with DIG Labeling and Detection Kit (Boehringer Mannheim Co., Germany) with random primers. After hybridization, slides were washed with following solutions, 2 \times SSC, 0.1 \times SSC, maleic acid 0.1 mol/L, NaCl 0.15 mol/L, and 10% blocking solution. Sections were stained with NBT/BCIP. The positive staining was examined and photographed by a microscope equipped with camera.

Immunohistochemistry

Immunostaining was performed using polyclonal anti bFGF or TGF β antibody by an indirect Streptavidin / Peroxidase (SP) technique. Antibodies and immunohistochemical SP kit were products of Santa Cruz Co. and Zymed Co.. Experiments were performed following the manufacturer. Briefly, sections were re-incubated with polyclonal anti-rat bFGF or TGF β antibody for 12 hours at 4 °C. Slides were washed with phosphate buffered saline (PBS) solution. Biotinylated

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secondary antibody IgG was added for 30 min, followed by horse radish peroxidase labeled streptomycin-avidin complex for 30 min. Slides were stained with diaminoben zidine and counterstained with Harris hematoxylin/eosin and examined under a light microscope equipped with a camera.

Statistical analysis

The percentage of positive staining cells of immunohistochemistry and *in situ* hybridization were expressed as mean±SD. Statistical analyses were performed using paired Student's *t* test. $P<0.05$ was considered significant .

RESULTS

Pathological alterations of kidney tissue

Multiple cross-sections of hematoxylin and eosin stained sections of kidneys were examined for pathological changes. Compared with control group, IR0 had slight alteration, while IR1 showed significant injury. In IR1 sections, clearly tubulointerstitium fibrosis and inflammatory cells infiltrating could be seen, accompanied with vessel wall thickening, tubule narrowing and glomeruli basement membrane destruction, etc. After reperfusion for 24 hours, IR2 recovered gradually.

Immunohistochemical localization of bFGF and TGFβ

Both *in situ* hybridization and immunohistochemistry found expression of bFGF and TGFβ in kidney tissues. bFGF was localized in cortex and glomeruli, particularly in mesangial cells, glomeruli epithelial cells and podocytes. TGFβ, however, was found predominantly in mesenchymal cells and glomeruli epithelial cells. Both bFGF and TGFβ positive staining of ISH were localized in the cytoplasm. While positive staining of immunohistochemistry was in cytoplasm or/and membrane.

Detection of bFGF and TGFβ expression

Immunohistochemistry and *in situ* hybridization showed both bFGF and TGFβ were expressed in controlled rat kidneys, and their expression level was enhanced slightly after 45min ischemia. While significant elevation of bFGF and TGFβ expression was observed after reperfusion, peaking at 6 hours (IR1) and declined at 24 hours (IR2). Both bFGF and TGFβ expression changes have significant difference after reperfusion compared with control ($P<0.05$), and positive correlation was found between bFGF and TGFβ expression ($r = 0.98$, $r = 0.97$). Results of their expression of different groups are shown in Table 1.

Table 1 bFGF and TGFβ expression levels after I/R injury in rat kidneys ($\bar{x}\pm s$)

Group	bFGF positive staining rate (%)			TGFβ positive staining rate (%)		
	<i>n</i>	mRNA	Protein	<i>n</i>	mRNA	Protein
Control	12	0.19±0.03	0.12±0.05	12	0.09±0.01	0.11±0.04
IR0	12	0.24±0.07	0.21±0.06	12	0.18±0.07	0.15±0.08
IR1	12	0.49±0.06 ^b	0.71±0.08 ^b	12	0.61±0.05 ^b	0.35±0.07 ^b
IR2	12	0.35±0.05 ^a	0.50±0.05 ^b	12	0.28±0.08 ^b	0.32±0.05 ^b

^a $P<0.05$, ^b $P<0.01$ (compared with controls).

DISCUSSION

Gut I/R has been implicated in the pathogenesis of intestine and other remote organs, and may potentiate the development of systemic inflammatory response syndrome (SIRS) and even multiple organ dysfunction syndromes (MODS). Much has been learned about the mechanisms that contribute to this injury process, which shows that this process is associated with activation of systemic inflammatory mediators including complement, cytokines, neutrophils, oxygen free radicals, gut derived bacteria and endotoxin. Great attention has been paid to the potential therapeutic effect of antibodies to these elements. Relatively little is known about the endogenous protective mechanism responding to the gut I/R injury. bFGF and TGFβ are important growth factors involved in wound healing and tissue repair and may be important mediators for renal repair after intestinal I/R injury.

bFGF is a potent mitogen, angiogenic agent, and chemoattractant. It is expressed in many kinds of cells, including macrophages, smooth muscle cells, vascular endothelial cells and fibroblasts, bFGF accelerate wound healing through promoting the initiation and regeneration of capillary vessels, accelerating the growth of epithelial cells and fibroblasts, and formation of granulation tissue. It does not have a signal sequence and thus is released independently of endoplasmic reticulum-Golgi pathway. bFGF is stored in inactive form in cell cytoplasm and is activated by cell injury. TGFβ is a growth factor having various functions, its biological activity depends on its concentration and cell types. TGFβ is a potent chemoattractant for inflammatory cells and fibroblasts. It can promote connective tissue formation and accelerate collagen synthesis, induce the release of many other growth factors, such as bFGF, vascular endothelial growth factor (VEGF) in wound healing and tissue repair^[5,6].

In our present study, bFGF and TGFβ mRNA and protein expressed after I/R injury in rat kidneys, and had close spatial and temporal association with the development of renal injury. Their expression levels were induced after I/R injury, peaking at 6 hours and decreased after 24

hours, correlated with the degree of tissue injury and compatible with other experiments of internal organ injury models. Iwata A *et al*^[7] found in a model of transient focal ischemia, bFGF mRNA was markedly induced in the peri-infarcted white matter after reperfusion, persisted for 2 days and disappeared by 5 days. In rat livers after gut I/R injury^[8], endogenous bFGF and TGF β mRNA expression increased after reperfusion, and persisted for 24 hours. These results showed endogenous bFGF and TGF β mRNA in internal organs were induced after injury, peaking at the point of severe damage. At this time point, rat kidneys showed markedly tubulointerstitium fibrosis and glomeruli basement membrane destruction. Whereas developmental assembly of endothelial, mesangial and epithelial cells into glomerular requires a coordinated, temporally defined series of steps occurred in an ordered sequence, growth factors and their receptors are important mediators of many of these events. Our results showed that bFGF and TGF β might participate in this process. Experimental studies had also found that bFGF is a most potent angiogenic factor and TGF β played an important role in angiogenesis. The significantly elevated expression of bFGF and TGF β after renal damage suggests bFGF may be involved in renal blood vessel development. Furthermore, in inflammation reaction after injury accumulation of inflammatory cells, such as monocytes, neutrophils and macrophages etc results in release of large amount of TGF β , and high local concentration of TGF β induces the high expression of bFGF mediating the tissue repair. Our study shows that

I/R injury up-regulate endogenous bFGF and TGF β expression, and suggests this may serve as a compensatory protective response to remote organ injury.

Recent studies have demonstrated the feasibility of using exogenous growth factors *in vivo*, of which VEGF and bFGF can improve internal organ injury. Though the precise underlying mechanisms remain to be determined, the use of growth factors may represent a new therapeutic strategy for patients with gut I/R injury.

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A chronic ulcerative colitis model in rats

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Subject headings colitis, ulcerative; disease model, animal; rats

INTRODUCTION

In recent years, there have been many reports about animal model to investigate drugs for inflammatory bowel diseases (IBD). The experimental animal model often used is acetic acid-induced damage of colonic mucosa. In the present study, this animal model was investigated by administering various concentrations of TNBS.

MATERIALS AND METHODS

Materials

Animals Wistar male and female rats weighing 258g±25 g were used in this study. They were provided by the Department of Experimental Animals of Beijing Medical University. They were housed in rack-mounted cages with a maximum of 6 rats, and were fasted for 12h with access to water ad libitum before experiment.

Reagent Fifty mmol/L (pH=6.0) phosphate buffer, 0.5 % hexadecyltrimethylammonium bromide (HTAB, Beijing Xizhong Chemical Plant) in 50mmol/L (pH=6.0) phosphate buffer, 50mmol/L (pH=6.0) phosphate buffer containing 16.7% (g/L) o-dianisidine Dihydrochloride (Sigma chemical Co.) and 0.0005% hydrogen peroxide, TNBS (Sigma chemical Co. 5% w/v solution) solution of 30% ethanol, 20% (w/v) ethyl carbamate in 0.9% saline.

Instrument T25 Ultra-tukrax (German, JANKE & KUNKEL IKA-Labortechnik). 4710 series

Ultrasonic homogenizer (Cole-Parmer Instrument Co. America), GL20A Refrigerated Centrifuge (Hunan Instrument and Meter Plant China), UV-260 Spectrophotometer (Shimuduzu Co. Japan), PHSJ-4 pH meter (Shanghai Leici Instrument Plant China), Libror EB-2080M Electronic Animal Balance (Shimuduzu Co. Japan).

Methods

Effect of various TNBS doses on myeloperoxidase (MPO) activity, colon damage and weight A total of 30 rats were randomized into five groups, 6 rats each group (in a cage), consisting of a 30% ethanol control group as well as four dose TNBS groups. The animals were anesthetized with 20% ethyl carbamate (ip, 6mL/kg), and 0.5mL of either 30% ethanol (controls) or various concentrations of TNBS was slowly administered into the lumen of the colon via the anus using a rubber catheter (12cm long, external diameter 2mm). The rats were killed after 3wk, and the distal colon (8cm) was removed, opened longitudinally and washed to remove lumina contents, colon wet weight was weighed, and colonic injuries were evaluated. The excised colon was pinned out on a wax block washed with 0.9% saline and assigned a code number. The colon was immediately examined under a stereomicroscope and any visible damage was scored on a 0-5 scale (Table 1). Small sections of colon were taken from two distinct areas from each colon and placed in 10% formalin for histological examination. The colon was fixed, cut longitudinally into 5mm sections, stained with hematoxylin and eosin. The second segment (200mg-400mg) was immediately frozen for subsequent estimation of MPO activity^[1].

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Table 1 Criteria for scoring the gross morphologic damage

Score	Gross morphology
0	No damage
1	Localized hyperemia with no ulcers.
2	Liner ulcers with no significant inflammation.
3	Liner ulcers with inflammation at one site.
4	More sites of ulcers and inflammation, the size of ulcers <1cm.
5	Multiple inflammations and ulcers, the size of ulcers ≥1cm.

The relationship of MPO and time-course Based on the results of dose-response studies, the dose of

TNBS used in subsequent experiments was 100 mg/kg per rat. A total of 40 rats (5 rats per cage) administered a single intracolonic dose of TNBS ethanol solution (0.5 mL/rat). In control experiments, 5 rats received 0.5 mL 30% ethanol. At various times (24 h and 1 wk-8 wk) after intracolonic administration of TNBS or one of the control solutions, 5 rats from each treatment group were randomly selected and killed, the colon tissue MPO activity was determined as the indices of inflammation.

Statistical analysis The data were expressed as $\bar{x} \pm s$, and analyzed using the Student's *t* test to compare the difference.

RESULTS

Assessment of MPO activity, colonic weight and damage score

The severity of colonic damage induced by TNBS increased with the dose (Figure 1). Rats that received the lowest dose of TNBS (25 mg/kg) had damage scores, colon weights and tissue levels of MPO activity were not significantly different from the control animals treated only with 30% ethanol vehicle ($P > 0.05$). When doses of TNBS (50 mg/kg - 150 mg/kg) were used, the damage scores, colon weights and tissue levels of MPO activity increased in a dose-related manner and there was a significant difference compared with control (30% ethanol).

Histological examination

Three weeks after TNBS/ethanol administration, the bowel wall was basically normal in the 25 mg/kg group, and "string of beads" was found in 1 rat. Medium hemorrhage, edema and ulcers, cryptoabscess in the mucosa were observed in animals that received 50 mg/kg of TNBS, in TNBS group (100 mg/kg), the bowel lumen became narrow with thickened wall (2 mm-3 mm), on the bowel lumen mucosal surface area there was adherent membrane with brown black, liner ulcers (1 mm-6 mm), proliferous lymphocyte tissue, inflammatory granulomas and submucosal neutrophils infiltration. Macrophages, lymphocytes, fibroblasts, and cryptoabscess were also observed. The TNBS (150 mg/kg) group had noticeable ulcers and inflammatory granulomas in their colon, neutrophil infiltration was obviously observed in mucosa and sub mucosa extensive necrosis of the colonic mucosa and exfoliation of the epithelia were found in other rats with intact

muscularis. In cases of severe ulcers, the colon had often adhered to surrounding intestinal tissues and abdominal wall (Figure 2).

Time-course study

A single instillation of TNBS at the dose of 100 mg/kg into the rat colon produced chronic ulcers and inflammation which had persisted for up to 7wk. MPO activity reached a maximum value at 3wk after TNBS, and was followed by a gradual reduction in activity. At 3wk the MPO value was at near baseline level (Figure 3).

DISCUSSION

TNBS is a hapten, when it is bound with a substance of high molecular tissue proteins, it will turn into an antigen. It has been shown that it can elicit immunologic responses, induce generation of colitis^[2,3]. The histological features of the animals received TNBS (50 mg/kg-150 mg/kg) were chronic inflammation, relatively long duration of inflammation and changes in various inflammatory mediators such as prostaglandin E₂, thromboxane B₂, leukotriene B₄, 6-keto-prostaglandin F_{1 α} , leukotriene C₄, platelet-activating factor and interleukin. This model is characterized by the simple process and reproducible colonic damage, inexpensive and short duration of the experiment, long-lasting damage with inflammatory cell infiltration and ulcers. Thus, the model is rather suitable for the assessment of the effects of potential agents. In the present study, the dose of TNBS producing a moderate colonic inflammation and ulcers was about 100 mg/kg, the severity of colonic inflammation induced by TNBS increased with the dose administered. So a TNBS dose of 100 mg/kg was chosen for an appropriate experimental dose, the results were similar to the reports in the literature^[4,5].

There was extensive colonic mucosal and submucosal damage characterized by infiltration of inflammatory cells and ulcers after different doses of TNBS (50 mg/kg - 150 mg/kg) were administered into the colons of rats. After the animals received TNBS (100 mg/kg), in acute phase, extensive infiltration of inflammatory cells constituted the main part; in chronic phase, the inflammatory granulomas and ulcers induced by TNBS made up the main part. MPO is an enzyme found in the neutrophils, and can be used as a quantitative index of inflammation in colonic tissue^[1]. MPO activity may be regarded as an index of inflammation damage^[6].

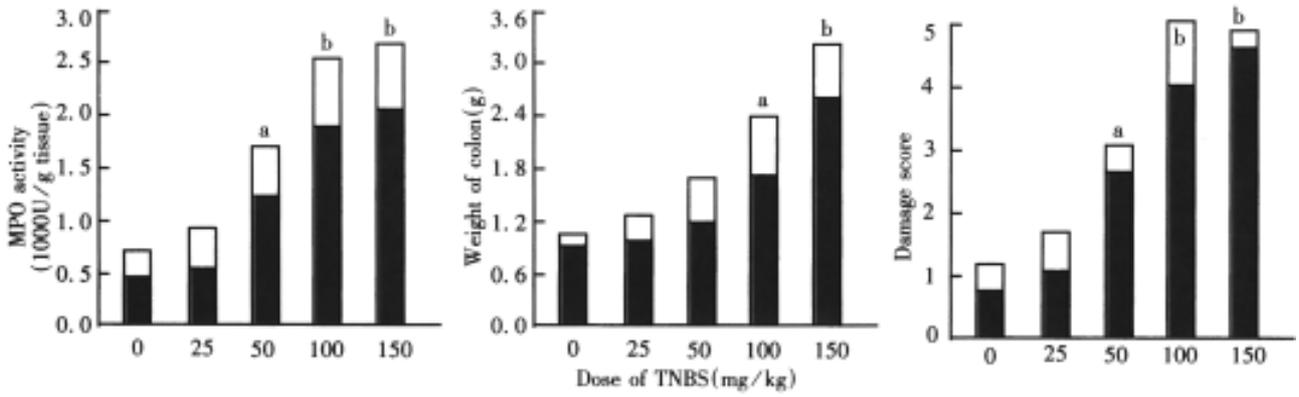


Figure 1 Rats were killed 3wk after intracolonic administration of 25 mg/kg-150 mg/kg of TNBS. Colonic damage, colon weight and tissue MPO activity were assayed. Data of TNBS-treated rats were compared with control (30% ethanol) group by Student's *t* test, ^a*P*<0.05, ^b*P*<0.01.

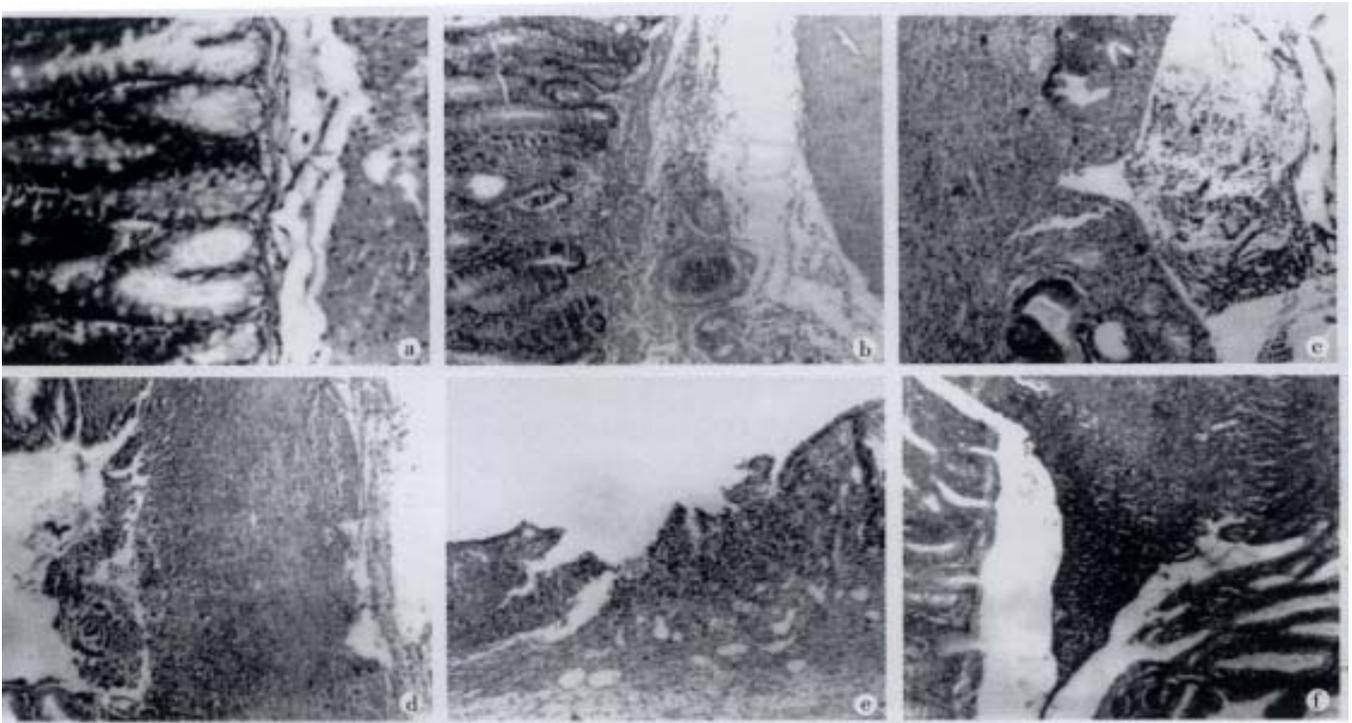


Figure 2 Histological findings in ulcerative colitis induced by different doses of TNBS ethanol. a: TNBS 25 mg/kg, the mucosa is normal. b: TNBS 50 g/kg, small mucosal ulcers and crypt abscess formation. c-d: TNBS 100 mg/kg, mucosal ulcers, inflammatory exudate, proliferous granulomas and cells infiltration. e-f: TNBS 150 g/kg, gross ulcers and proliferous granulomas, necrosis of epithelium at mucosal surface and inflammatory cell infiltration (He, ×100)

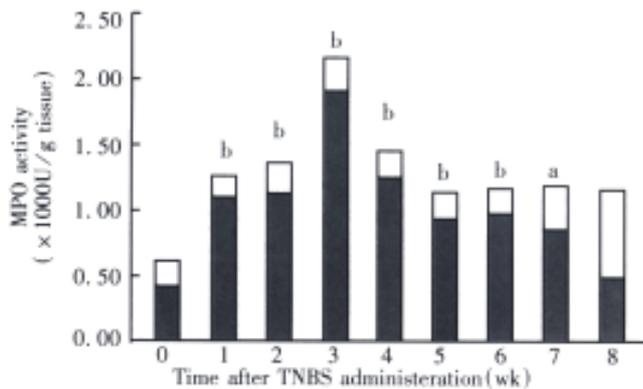


Figure 3 The effects of intracolonic administration of 100mg/kg of TNBS on MPO activity 1 wk-8 wk after administration. Each bar represents the $\bar{x} \pm s$ of 5 animals. All data for TNBS-treated rats were compared with 0 wk (control group). ^a*P*<0.05, ^b*P*<0.01.

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Tracing method study of bacterial translocation *in vivo*

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Subject headings PUC19 plasmid trace; bacterial translocation; restriction map analysis; fluorescence labeling

INTRODUCTION

Endogenous infection plays an important role in nosocomial infection^[1-3]. By studying progress of bacteria translocation from intestinal tract, the concept of gut origin infection has been accepted gradually^[4-6]. Because of no ideal tracing method, there were some controversies. In order to solve the problem, the PUC19 plasmid vector tracing method with restriction map analysis and fluorescence labeling method were used to study gut-origin bacterial translocation. According to the characteristic of PUC19 plasmid, a special animal model was designed and two methods were compared.

MATERIALS AND METHODS

Material

PUC19 plasmid vectors (Promega): amplification of plasmid was in LB culture medium containing 100mg/L ampicillin. Fluorescence-labeling bacteria: 1/10000 acridine orange was added in culture fluid of *E. coli* CMCC 44102. The cells were grown in broth overnight at 37°C. Restriction DNA endonucleases: *Hind*-III, *Eco*R-I (Promega Co.). Plasmid was isolated as described by Kado *et al*^[3]. A total of 110 male Wistar rats, weighing 226 g±64 g were used. The animals were divided into PUC19 plasmid group and fluorescence-labeling one. The animals in fluorescence labeling group were sacrificed and examined at 4, 12, 24 and 48 h postburn with 10 rats at each time point, the normal control group contained 10 rats, with a total of 50

rats. The rats in PUC19 group were treated the same as those described above, another 10 rats were added at the time point of the 12th day postburn.

Methods

Fluorescence labeling group: 10¹²/L fluorescence-labeling *E. coli* were introduced into the stomach of rats by gastric tube. After 8 hours the animals had 30% TBSA (total burned surface area) full thickness burns. Three mL/100 g body weight saline was injected into rat's abdominal cavity for anti-shock. The rats were sacrificed at 6, 12, 24 and 48 h after burn. Mesentery lymph nodes (MLN), liver and subeschar tissue were collected by aseptic technique. The homogenates were divided into two parts, the first part cultured for enumerating microorganisms and second part examined under fluorescence microscope.

PUC19 plasmid tracer group: before burn, animals drank 300 mg/L ampicillin fluid for 3 days to "clean up" the intestinal tract. The PUC19 plasmid vector was introduced into the stomach by way of gastric tubing. Then the animals were again given ampicillin fluid (100 mg/L ampicillin) and examined to confirm whether PUC19 plasmid vector had colonized in the rat's intestinal tract. The animals were inflicted with 30% TBSA third degree burn and sacrificed at 6, 12, 24, 48 h and 12 d postburn. The methods were the same as those described above. Homogenates of mesenteric lymph nodes, liver, and subeschar tissues were incubated in LB broth containing 100 mg/L ampicillin (18 hours) at 37 °C with shaking at 50 min⁻¹-100⁻¹rpm.

Plasmid isolation of the bacteria was made according to Kado's method. The product was digested 1h at 37 °C by restriction enzymes *Eco*RI and *Hind* III. Ten g/L agarose gel with well sufficient to accommodate 40 µL samples was prepared and the gel was stained with ethidium bromide. Photographs of gel were taken and positioned over an ultra-violet (UV) ray source.

RESULTS

Fluorescence-labeling bacteria

Labeling bacteria were found to pass through damaged intestinal mucosal barrier and escaped from cleansing effect of liver and lymph nodes and finally reached the subeschar area.

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Detection rates of fluorescence-labeling bacteria from mesenteric lymph nodes, liver and subeschar tissues were enumerated (Table 1). The quantity and rate of bacteria by fluorescence-labeling at different time points after burn are shown in Table 2.

Table 1 Detection rate of fluorescence-labeling bacteria

Organ and tissue	No. of samples	Positive rate (%)	No. of FB/ ^a 10×100
Mesenteric lymph nodes	40	38(95.0)	3.8
Liver	40	23(57.5)	2.1
Subeschar	40	13(32.5)	1.1

Table 2 Quantity and rates at different periods after burn

Group	t/h	Mesenteric lymph node		Liver	
		Rate	Quantity(cfu/g)	Rate	Quantity(cfu/g)
Burn	6	100	3.0×10 ⁴	70	2.1×10 ⁴
	12	100	8.5×10 ⁴	70	3.1×10 ³
	24	90	7.4×10 ⁴	50	2.6×10 ³
	48	70	8.2×10 ³	40	1.5×10 ³
Normal	6	40	8.0×10 ³	0	0

PUC19 plasmid tracing

Bacteria that resist to ampicillin (AMP) could be separated from mesenteric lymph nodes, liver and subeschar tissues after burn. Isolation of plasmid and restriction enzymes analysis indicated that bacteria resistant to ampicillin that separated from intestinal and subeschar tissue had the same DNA restriction map (Figure 1).

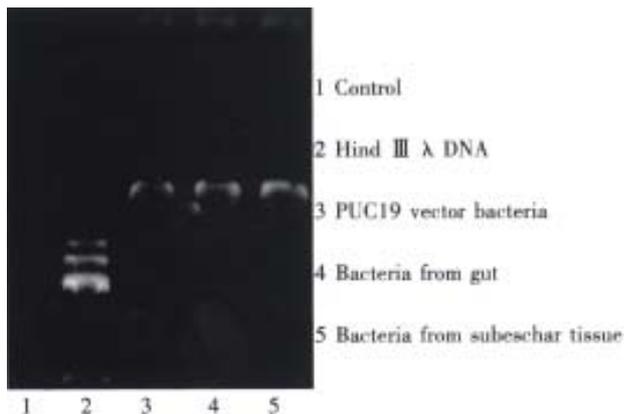


Figure 1 Plasmid fragment generated by restriction endonuclease. 1. Control; 2. HindIII λ DNA; 3. PUC19 vector bacteria; 4. bacteria from gut; 5. bacteria from subeschar tissue.

The detected quantity and positive rate of PUC19 tracing bacteria after burn are shown in Table 3. The positive rate of PUC19 bacteria in early and late stages after burn is shown in Table 4.

Table 3 Quantity and positive rate of PUC19 bacteria after burn

Group	t/h	Mesenteric lymph node		Liver	
		Quantity(cfu/g)	Rate(%)	Quantity(cfu/g)	Rate(%)
Burn	6	5.0×10 ⁴	89.	2.3×10 ³	60.
	12	4.0×10 ⁴	80.	2.4×10 ³	60.
	24	3.1×10 ⁴	80.	2.6×10 ³	50.
	48	8.0×10 ³	62.	8.0×10 ³	37.
	12(d)	5.1×10 ³	25.	0	0
	6	1.0×10 ⁵	20.	0	0

Table 4 Comparison of positive rates of PUC19 in early and late stages postburn

Organ	Early stage within 48 h postburn		Late stage 12 d postburn	
	No. of samples	Positive (%)	No. of samples	Positive (%)
Mesenteric lymph nodes	37	29(78.4)	8	1(12.5)
Liver	37	19(51.4)	8	0 (0.0)
Subeschar	37	4(10.8)	8	5(62.5)

DISCUSSION

In recent years, more and more studies in gut-origin infection have been reported. Gut-origin infection as an important way of infection in burn had been established. But there were still some disputes about its verification by tracing methods^[7-15]. In the past, these included the fluorescence and isotopes labeling methods. PUC19 plasmid was constructed in the 1980s, and was used extensively in molecular cloning and discrimination of gene recombination^[16]. The characteristics of the PUC19 plasmid were: ① plasmid vector carried ampicillin resistance gene; ② the plasmid possessed polyclonal restriction endonuclease sites, panning positive bacteria became easier to be discriminated; and ③ it lacked nic/bom site, hence gene transduction from conjugate of bacteria was impossible. These features indicated that PUC19 plasmid could be an ideal tracer for studying endogenous bacterial translocation.

Comparing the results of the two tracing methods used in our experiment, we could find the positive rate of fluorescence-labeling organisms was higher than that by PUC19 plasmid tracing method in bacteria reaching mesenteric lymph nodes and liver after serious burn. Because many factors can cause nonspecific reaction in fluorescence-labeling method, some false positive results may be present, but we think that fluorescence-labeling bacteria tracing method is still useful for gut bacterial translocation study, although not for quantitation study and long period study.

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Helicobacter pylori infection

Vandenplas Y

Subject headings *Helicobacter pylori*, gastritis; Helicobacter infection

IS THERE ANYTHING NEW?

Helicobacter pylori has been for many years a forgotten bacterium, since the first report on this spiral organism dated from the 19th century^[1]. As early as in 1906, an association between a spiral organism and gastric carcinoma was suggested^[2]. Doenges reported in 1938 that on autopsy not less than 40% of human stomachs were found to be invaded by spiral organisms^[3]. In 1940, the therapeutic effect of bismuth in patients with peptic ulcer in the presence of spiral bacteria in the stomach was reported^[4]. Then, interest in this bacterium decreased. In 1982, two Australian researchers, Marshall and Warren, rediscovered the microbe^[5], and called it at first *Campylobacter pylori*, later *Helicobacter pylori*. Today, the complete genome (1590 genes) of *H. pylori* has been unmasked^[6] and published on the internet (<http://www.tigr.org>), probably paving the way for sequencing the genome of other organisms, including that of humans within a few years^[7].

WHY IS *Helicobacter pylori* SO IMPORTANT?

There is unequivocal evidence that *H. pylori* can be considered as a healthcare issue because of the mortality associated with the infection, due to the risk of ulcer bleeding and gastric cancer. Infection with *H. pylori* results in the development of gastritis in all infected humans, including children and adolescents^[8]. Peptic ulcer disease is a major cause of morbidity and distal gastric adenocarcinoma, which is the second biggest cancer killer worldwide^[9]. However, the majority of infected individuals remain free of symptoms throughout their lifetime; only a small number present with peptic ulcer disease (lifetime risk 15%), and an even smaller proportion will develop

gastric neoplasms including (mucosa-associated lymphoid tissue) lymphoma and adenocarcinoma (lifetime risk 0.1%)^[10]. Overall, *H. pylori* can be discovered in 92% of children with duodenal ulcers and in 25% of children with gastric ulcers^[11]. *H. pylori* infection is contracted primarily in childhood, and infection from childhood appears to enhance the risk for carcinogenesis^[12].

ABOUT THE BACTERIUM: VIRULENCE

The microaerophilic, gram-negative, urease-producing *H. pylori* fulfills each of Koch's postulates^[8]. In the normal living form, it is a spiral-shaped bacterium, but the coccoid form can also cause lesions. The bacterium colonizes the stomach of man and induces severe mucosal inflammation and a local and systemic immune response. It is capable of changing its membrane potential at external pH from 3.0 to 7.0 in order to maintain a neutral internal pH^[13]. Not all *H. pylori* strains are created equally^[14]; and not all are associated with clinical symptoms.

Some virulence factors such as urease and flagella are present in all strains and are necessary for pathogenesis and colonization. Flagella, and thus motility, are needed for persistent gastric colonization^[15]. The gene FlbA is needed for flagellar expression^[16]. Enzymes produced by *H. pylori* have mostly metabolic, antioxidant and toxic properties^[17]; most of these are produced by all isolates tested. Urease is required to establish infection, and is located intra and extracellularly^[15]. It is a nickel-containing metallo-enzyme, consisting of two structural subunits, UreA and UreB^[18]. Urease is primarily a cytoplasmic enzyme^[19], and hydrolyzes urea to bicarbonate and ammonia, resulting in a net increase in the ambient pH. Ammonia is a nutrient for the bacteria, and causes lesions to the gastric epithelium by many different mechanisms^[20]. Surface urease helps protect against acid exposure, but it is as yet unclear why it is found on bacteria deep underneath gastric mucus where the pH is thought to be neutral. Because there is no obvious urease export machinery, it has been suggested that some bacteria undergo autolysis, which released proteins, including active urease are absorbed onto the surface of remaining intact bacteria^[19,21]. Urease might function as an adhesin, although this suggestion has also been contradicted^[22]. Urease stimulates the

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release of a variety inflammatory cytokines including interleukin- beta, interleukin-6, tumor necrosis factor-alfa, and chemokines such as interleukin-8^[23]. Although the exact mechanism by which urease functions in the pathogenesis of gastric disease remains unclear, it is likely that urease is an important virulence factor.

H. pylori can produce different kinds of phospholipases weakening the hydrophobicity of the gastric mucous and mucosa. Phospholipase can also generate ulcerogenic substances^[24]. Many other enzymes, such as mucinase, neuraminidase, fucosidase, alcohol dehydrogenase, etc. have been reported^[20].

The vacuolating cytotoxin A (VacA) gene is present in all strains, but is only expressed in 50% of *H. pylori* isolates^[25]. The vacuolating activity of VacA is increased by exposure to acidic pH values^[26]. The vacuoles are formed by merging of late endosomes and the mechanism causing this has been determined^[27]. The VacA gene exhibits different allelic combinations. Strains with the gene s1/m1 have the highest levels of cytotoxic activity; colonize the stomach more densely and are correlated with peptic ulcer, atrophic gastritis and gastric cancer; s2/m2 strains have no toxic activity^[20,28].

Other virulence factors, such as “cytotoxic-associated gene A” (cagA) encoded proteins are only found in a proportion of the strains. This might explain why not all strains are associated with clinical symptoms, although both cagA and the s1 vacA allele are unreliable as single markers in determining the risk of developing peptic ulcer disease^[29]. The cagA protein is a cryptic 128 kDa immunodominant antigen produced by *H. pylori*. CagA is a marker for a larger cluster of genes (40 different genes^[20]) carried on a pathogenicity island that exhibits variability between strains^[30]. CagA+ strains produce increased amounts of interleukin 8^[30]. Gastric atrophy, duodenal ulceration and gastric carcinoma are more common in patients infected with CagA+ than with CagA- strains^[31]. CagA negative strains are very rare in some Far East countries such as China and Korea but frequent in some other areas such as Hong Kong, and are reported to be not a marker of specific disease in these regions^[32]. However, allelic variations in the cagA protein are found in different parts of the world. In Western countries, cagA positive strains are associated with gastric atrophy and peptic ulcer disease^[33]. But, there appears also to be no association between cagA and clinical symptoms or ulcers in children^[32,34]. Other putative virulence determinants are being discovered, such as the neutrophil-activating protein (napA) gene, a gene

“induced by contact with epithelium” (iceA1), etc^[20]. However, according to the recent data, it is suggested that there is no correlation between the degree of inflammation and the presence of the cag-pathogenicity island, cytotoxin production, vacA alleles associated with cytotoxin expression in children^[35].

Auto-immunity and host mimicry by expression of blood group antigens may be a relevant phenomenon. Adhesion of *H. pylori* is nonspecific although preferential to epithelial cells and is enhanced at low pH, inducing epithelial cell reorganization and causing deep invagination of the apical membrane, explaining resistance to topical antibiotic treatment^[36]. One host receptor for adhesion appears to be a blood group O antigen, possibly explaining why ulcers are more common in people with this blood group^[37]. The *H. pylori* lipopolysaccharide (LPS) or endotoxin is unusually biologically inert compared with that from other bacteria. However, the mechanisms by which *H. pylori* LPS stimulates cells appears similar to that of other types of bacterial LPS^[38]. *H. pylori* LPS often contains Lewis x and Lewis y blood group antigens that are identical to those occurring in the gastric mucosa^[39]. *H. pylori* presents bacterial epitopes to the host which are similar to the structure on host gastric epithelium; therefore, the host reacts with an auto-antibody response recognizing gastric mucosa inducing atrophic gastritis^[40]. Patients with a large parietal cell mass and high acid secretion will have a predominantly antral gastritis, predisposing to duodenal ulcer^[41]. People with a small parietal mass and low acid output (or people on proton pump inhibitors) will be more prone to develop atrophic gastritis and gastric malignancy^[41]. The variability in occurrence of gastric cancer in different parts of the world may only be partly explained by the prevalence of *H. pylori*. Apoptosis of gastric epithelial cells is increased in *H. pylori* infection, stimulating crypt cell proliferation, increasing the risk for mutagenesis^[42]. Atrophic gastritis enhances the development of intestinal metaplasia, and is related to the intestinal type of gastric carcinoma but not to diffuse gastric carcinoma^[43]. Intestinal metaplasia is related to atrophic gastritis, which is in its turn related to *H. pylori* infection.

Symptoms

H. pylori infection in children is mostly asymptomatic and not associated with specific gastrointestinal symptoms^[44]. *H. pylori* gastritis, in the absence of duodenal ulcer, does not appear to be associated with specific symptoms^[11,45-49]. After eradication of *H. pylori* infection, symptoms are

improved only in those children with duodenal disease^[46]. Children with *H. pylori* gastritis cannot be distinguished from noninfected children on the basis of initial symptoms^[45,49]. Many studies failed to demonstrate a difference in *H. pylori* infection rate in children with or without recurrent abdominal pain^[11,49-51], although others did find this association^[52]. Recurrent abdominal pain would occur during the acute phase of *H. pylori* infection^[52]. It is unclear whether children with recurrent abdominal pain with *H. pylori* represent a different entity to those without *H. pylori*. *H. pylori* positive children might more often have pain related to meals than *H. pylori* negative children. Ulcer-like symptoms may be more closely associated with the infection than other symptom complexes^[53].

In adults, a significantly lower *H. pylori* prevalence was reported in patients with gastroesophageal reflux disease^[54]. The role of *H. pylori* in duodenogastric reflux is unclear. A decreased mean acid output in subjects with *H. pylori* gastritis might explain the inverse relation between reflux and *H. pylori*. Heartburn and epigastric pain might be more frequent in *H. pylori* infected patients. Pooled data from 18 studies suggest that the prevalence of *H. pylori* was greater in patients with dyspepsia than in controls^[53]. It is unclear whether *H. pylori* changes gastric emptying rate or not, although most data have suggested that gastric emptying is normal^[55-57]. In adults, *H. pylori* is also beyond any doubt associated with an increased incidence of gastrointestinal cancer. However, the high prevalence of early *H. pylori* infection and chronic gastritis in children contrasts with the rarity of gastric cancer in black African^[58]. Nevertheless, acquisition in infancy is in general considered to be a significant risk factor to develop gastric carcinoma^[48].

Similar to other chronic inflammatory conditions, infection with *H. pylori* has been linked to reduced growth^[59-65], although socioeconomic factors confuse the issue. Tumor necrosis factor- α is inversely correlated with growth, and is increased in *H. pylori*^[66]. However, studies have also failed to find differences in hemoglobin, leukocytes, thrombocytes, weight and height^[50,67]. Differences in growth seem to be limited to the developing countries^[62,63]. It has been speculated that *H. pylori* acquired in infancy could be "the key that opens the door" to enteric infection leading to recurrent diarrhea, malnutrition and growth failure^[62]. There is, however, no difference in diarrhea prevalence in relation to *H. pylori* status^[68]. After control for socioeconomic status,

there is no difference in the height of adults with and without *H. pylori*. *H. pylori* seropositivity is related to a late menarche^[69,70]. Socioeconomic status and malnutrition does not explain late menarche, since elevated body mass index is also independently associated with *H. pylori* in the same population^[70]. Incidentally, anemia, hemoptysis and vertigo have been reported^[71,72].

The association of *H. pylori* with extra-digestive diseases, such as functional vascular diseases and skin and endocrine autoimmune diseases, has been described^[73-76]. An interesting relationship between seropositivity to *H. pylori*, serum glucose and non-insulin dependent diabetes mellitus is worthy of further attention. Recent studies suggest that the association between *H. pylori* and coronary heart disease is rather weak^[65,73]. Primary Raynaud's phenomenon, observed in young women, which is defined by an intermittent vasospasm of the arterioles of the distal limbs that occurs mostly following exposure to cold or emotional stimuli, may be related to *H. pylori* in some cases^[74]. *H. pylori* may in addition cause headache^[74]. Vasoactive substances, such as cytokines (interleukins, interferon, gamma, TNF- α), prostaglandins, leukotrienes, oxyradicals, C-reactive protein and fibrinogen are released in chronic infection^[74]. Henoch-Schönlein purpura and Sjögren's syndrome have been correlated with the bacterium. Many patients with autoimmune thyroid diseases are reinfected with type I cytotoxic cagA-positive strains^[75]. Rosacea and recurrent urticaria may also be associated with *H. pylori* infection^[76]. Alopecia areata is related to atrophic gastritis and pernicious anemia, and thus with *H. pylori*^[74]. Until now, *H. pylori* has not yet been reported to cause hepatitis in human. Although a mice *Helicobacter* species has been reported to cause hepatitis in germfree mice, and *H. pylori* has been identified in the gallbladders of human^[77,78]. An Italian group showed a positive correlation between food allergy and *H. pylori*^[79].

H. pylori in infants and children

Independent risk factors for *H. pylori* infection in infants and children include living in lower socioeconomic and overcrowded circumstances and sharing a bed with a parent. Human lactoferrin can support *H. pylori* growth *in vitro* and *H. pylori* binding lactoferrin has now been identified. Infants born to seropositive mothers passively acquire maternal *H. pylori* IgG^[80,81]. Transplacentally transferred maternal anti-*H. pylori* IgG lasts until about the third month of life in most infants and disappears from nearly all by 6 months^[80]. IgA in mother's milk can protect the infant from *H. pylori*

infection^[82]. However, whether breast feeding is related to a low or high prevalence of *H. pylori* infection in infants remains unclear^[80,83,84] (protective effect of mother's milk versus intimate contact between infant and *H. pylori* positive mother). At the age of 14 months, 7.5% of infants in a population with a seroprevalence in 62% of young adults, had acquired *H. pylori*, an event demonstrated by a rise in IgM, quickly disappearing and preceding IgG^[80,85,86]. In Belgium, less than 1% of infants are seropositive at the age of 1 year^[87] (seropositivity in young adults is about 30%)^[87,88]. In Finland, 4% of children under the age of 7 years have a positive serology^[89].

Approximately 30% of 53 children (16/53) with dyspepsia were infected with *H. pylori* in the antrum, and about half of them a cytotoxic strain was present (anti-Cag A antibodies in 64% and anti-vacA antibodies in 43%)^[90]. In only 6/53 children, the *H. pylori* was also detected in the gastric body^[90]. Clinical evaluation showed a significant difference in favor of subjects positive for *H. pylori* only for epigastric burning and/or pain^[90].

Clinical symptoms associated with *H. pylori* infection have been reported in patients with human immunodeficiency virus (HIV)-1^[91,92]. Although *H. pylori* has been said to be rare in HIV-1 infected individuals (e.g., because of the repetitive and multiple administrations of antibiotics and immunoglobulins)^[92], recent data suggest that the prevalence of *H. pylori* infection in HIV-1 infected children is comparable to the prevalence in the non-infected control population^[91].

Diagnostic and screening tools

A large number of invasive and non-invasive methods have been used to diagnose *H. pylori* infection in humans^[93]. Culture of the organism is a standard method for the diagnosis of bacterial infection. *H. pylori* can be cultured from gastric biopsies. Culture of *H. pylori* requires a microaerobic atmosphere of 5% oxygen with 5%-10% CO₂. When *H. pylori* is cultivated on biopsy, sensitivity to antibiotics should be tested^[94]. Whether coccoidal forms also grow in blood agar or not is controversial^[95,96].

Histologic examination of Giemsa or Warthin-Starry stained gastric biopsy specimens is widely used for the diagnosis. The Sydney criteria for the classification of gastritis have been revised^[97]. Gastric biopsy urease tests make use of a change in color of phenol red which is present in the medium because of a pH increase related to the digestion of urea by the urease. Four rapid urease tests are available commercially: CLO-test (Delta West Ltd, Bentley, Australia), Hpfast (GI Supply,

Philadelphia, USA) and PyloriTek (Serin Research Corporation, Elkhart, USA), Jatrox (Röhm Pharma GMBH, Weiterstadt, Germany), although many hospitals prepare their own urease tests. These commercial tests have a high specificity and sensitivity and provide comparable results^[98]. PyloriTek has a shorter reading time than CLO-test^[99]. Antral and corpus biopsies provide comparable results, and in combination they increase the sensitivity by 4.3%^[100]. Although these biopsy urease tests have a high degree of sensitivity in adults, false negative results are common in children, possibly because of a smaller bacterial load^[101].

Molecular methods for biopsy material or other biological samples and current PCR methods for molecular fingerprinting of *H. pylori* have been developed^[102,103]. PCR techniques can quantitate the bacterial load in gastric samples^[103,104]. Several molecular methods have been applied to typing *H. pylori* isolates and demonstrating their genomic diversity. Unfortunately, all these tests necessitate endoscopy^[103].

Magnetic beads coated with anti-*H. pylori* rabbit antibodies permit detection of less than 10-million organisms per gram of feces^[105]. PCR-detection of *H. pylori* in feces is hindered by the presence of inhibitors of Taq polymerase, complex polysaccharides which can be eliminated by filtration on Qiagen and dilution. But, immunomagnetic separation-PCR is recently reported to be simple, rapid and reliable^[106]. Nevertheless, the technique is not available as routine.

The ability to detect antibodies in saliva rather than in serum would improve antibody tests by avoiding the need for blood collection^[107]. Sensitivity (84% - 93%) and specificity (70% - 82%) are too low, but comparable to rapid whole blood diagnostic tests^[107-109]. The discovery of the potential importance of the cagA-pathogenicity island has stimulated interest in the specific detection of the CagA protein.

Serologic testing for IgG antibodies against *H. pylori* requires validation of the assay in children, since antibody levels differ in children and adults, probably because of the duration of infection and the differences in bacterial load^[85,106,110,111]. In addition, commercially available serologic tests demonstrate lower accuracy compared with testing in a research setting^[112], with sometimes up to 33% false positive and 25% false negative results^[111,113,114]. Serology is more and more frequently reported to be unsatisfactory for screening for *H. pylori* infection in children^[106,111]. Testing should not rely on office

tests^[94]. After eradication, there is a slow decline in antibody titer. Many patients remain seropositive 1 year after eradication^[115]. At acquisition of the infection, there is a temporary rise in IgM^[80,85,86,111,116]. IgA is also reported to be a useful serologic screening tool^[117]. Immunoblot has become the reference method used to confirm doubtful results^[118]. Specific serologies for cytotoxic strains may be helpful in selecting patients for treatment^[90].

Carbon-13 and C-14 breath tests are based on the fact that urease from *H. pylori* will hydrolyze the ingested labeled urea into ammonia and labeled bicarbonate, which is exhaled as labeled carbon dioxide^[18]. Whether a test meal should be given, or whether the labeled urea should simply be given after a period of fasting, or whether addition of citric acid would be beneficial is not clear^[119-121]. A standardized and simplified C-13 breath test was recently described in children^[122]. The high sensitivity and specificity of the 13 C-breath test in the detection of *H. pylori* infection in children has been unequivocally demonstrated^[90,123,124]. The best cut off value is obtained after 30 minutes^[120]. False positive results can occur because of the presence of other urease containing gastric bacteria or because of extra-gastric bacterial urea metabolism (seldom). False negative results are mainly due to fast gastric emptying or previously administered urease-inhibiting drugs, such as antibiotics or bismuth-containing salts. There is a close correlation between the urea breath test and the intragastric bacterial load^[100,125], which is on its turn related to the severity of the gastritis. Unfortunately, carbon-13 breath tests are still expensive in many parts of the world. A less expensive method for the analysis of 13C-labeled carbon dioxide is nondispersive infrared spectrometry, with a comparable sensitivity and specificity^[126]. But, infrared spectrometry necessitates larger volumes of expired air, making the technique less suitable for (small) children. Alternatively, measurement of 14C-labeled carbon dioxide with a scintillation counter is relatively inexpensive^[127]. Although the dose required for one test is not greater than the natural background radiation, the use of 14C is considered unethical in pregnant women, adolescents and children because of its extremely long half-life, since 14C may be incorporated into the bicarbonate pool^[128]. The urea breath test is in general accepted to be the most reliable noninvasive diagnostic method^[90,129]. The urea breath test detects only current infection and can be used to screen for *H. pylori* infection and as the sole method for assessing eradication, and to evaluate treatment efficacy^[129].

Epidemiology

The prevalence of *H. pylori* infection in many populations and/or subgroups is currently well documented. The overall prevalence of *H. pylori* in children is 10% in developed countries but can be as high as 30%-40% in children from lower socioeconomic classes^[48]. In developing countries, the prevalence of *H. pylori* in children ranges from 80% to 100%^[48]. Like many other childhood bacterial infections, *H. pylori* is most frequently acquired in the preschool age group, with the associated effects of family size, clustering in families, low socioeconomic status and education and variable risks associated with gender^[89,130,131]. Recent sociocultural changes may result in changes in infection rates in children^[132], which is an important argument for the cohort effect. In general, it is thought that spontaneous eradication of *H. pylori* infection is extremely rare^[133,134]. However, recently some authors have suggested that from 1.5% up to 10% or even 20% of spontaneous eradication occurs in a period of 6 months during childhood^[111,135,136]. Although, others still report a zero incidence of seroreversion^[89]. These discrepancies may, however, be related to the methodology (serology versus urea breath test). Recent epidemiologic data suggest that serology underestimates *H. pylori* infection in children^[106,111], and antibodies may persist although *H. pylori* disappeared.

In the developed world, acquisition by adults and children is approximately 1% to 3% per decade^[131,136,137], which will result in a dramatic decrease in *H. pylori* infection in that part of the world in the coming decades. In The Netherlands, about 40% of the 60-69-year-old population is seropositive^[137]; since the prevalence in adolescents is below 10%^[138], it can be speculated that the seropositivity of this cohort will probably not be higher than 25% when reaching the age of 70 years. In Gambia, the prevalence of a positive breath test at the age of 3 months is about 19%, increasing to 84% at 30 months^[111].

Re-infection probably does not occur frequently and is, in many cases, considered recrudescence after treatment failure^[139]. Re-infection rates vary strongly with the effectiveness of the treatment protocol^[139,140]. In Chile, re-infection occurred in 4.2% after 1 year, with a treatment protocol that was 82% effective^[141,142]. Annual user-relapse rate in children with duodenal ulcer in whom *H. pylori* was eradicated was reported to be 9%^[143]. The percentage of re-infection does not appear to be much higher in developing countries than in developed regions. As a consequence, there is little reason for treating an entire family to prevent re-

infection, although spread from one adult to another has been suggested^[144]. Others do suggest family treatment^[183]. It seems more likely that re-infection comes from an external source. Of course, a more detailed specification of the *H. pylori* strain will contribute to the answer whether re-infection rather than relapse occurs. Repetitive extragenetic palindromic-PCR can group isolates into clusters that appear to have a different clinical expression^[102]. Oligonucleotide probes containing short repetitive sequence motifs can differentiate between different isolates of *H. pylori*^[146].

The major mode(s) of transmission of *H. pylori* are still unknown, oral-oral, gastro-oral and fecal-oral have been proposed^[147,148]. Infected parents, especially mothers, may play a key role in transmission of *H. pylori* within families^[81,149]. Houseflies could serve as vectors for *H. pylori*^[150]. Pets have been suggested as well as contradicted to be vectors^[151]. There is considerable evidence of transmission of oral bacteria between spouses and between family members^[144]. Vomiting and gastroesophageal reflux might also be a mode of oral-oral contamination^[152]. Mode of spread remains an active area of study, with water as a source of contamination still of potential interest. The coccoid form can cause cellular changes similar to the spiral form^[153], and may serve as the infectious form in environmental sources such as water^[154]. Studies on external water sources in Peru revealed PCR products of *H. pylori* in the municipal water, increasing 12 -times the risk for infection^[155]. The examples of studies in Peru and Chile suggest a role for water as a vehicle, but it does not seem to be the main route of acquisition since many studies in Korea^[132], Taiwan, or Turkey do not support this hypothesis.

Host response to *H. pylori*

Another factor contributing to the heterogeneity of *H. pylori* associated symptoms is the variability in host response to the infection. Duodenal bicarbonate secretion is decreased in ulcer patients, and returns to normal after eradication of *H. pylori*^[156,157]. Acute *H. pylori* infection has been associated with hypochlorhydria, possibly by stimulating the production of a histamine-3 receptor agonist, which inhibits gastric acid output. In contradiction to this finding is the observation that the same histamine-3 receptor agonist can stimulate parietal cells to produce acid via the histamine-2 receptor^[158]. Identification of Lewis carbohydrate structures on *H. pylori* lipopolysaccharide may provide an explanation for the development of autoantibodies, reacting with gastric mucosa ("molecular mimicry")^[159].

Treatment

Currently, there are no guidelines on the need to treat children^[48]. The regimens that have been studied to date have used bismuth preparations, H₂-receptor antagonists, ranitidine bismuth citrate, proton pump inhibitors and various antibiotics. The goal of any treatment should achieve an eradication rate of over 80% on a rigorous intention-to-treat basis^[94]. Most European *H. pylori* study groups now recommend (in adults) a triple regimen: a twice daily dose of proton pump inhibitor (PPI) in combination with two antibiotics [from the following 3 groups: clarithromycin; amoxicillin; nitroimidazoles (metronidazole or tinidazole)] for 1 week^[94,160,161]. There are no specific recommendations for children yet^[162]. It has been hypothesized that combination therapy is more effective because of the synergistic mechanisms between different drugs. The requirement is for a simple, well-tolerated regimen, with which it is easy to comply with, and is cost-effective. In a recent Irish study in children, the therapeutic approach consisted of colloidal bismuth subcitrate (480 mg/1.73 m² body surface for 4 weeks) in combination with amoxicillin (750 mg/day for 2 weeks) or metronidazole (20 mg/kg/day for 2 weeks)^[120]. In most European countries, eradication treatment in children consists usually of a PPI in combination with amoxicillin and either clarithromycin or nitroimidazole, based on the sensitivity of the prevailing strains. Although antibiograms are needed, there seems to be major discrepancy between *in vitro* testing and *in vivo* efficacy. Resistance to amoxicillin has recently been reported, but seems rare^[163]. Resistance to macrolides is rising with increasing use of the drugs, and for both macrolides and nitroimidazoles there is a huge regional variation in resistance patterns. Especially the determination of resistance to metronidazole may be relevant in regions with a high percentage of resistance^[164].

Bismuth triple therapy continues to achieve high eradication rates worldwide (78%-89%). Side effects leading to diminished patient compliance and the marked decline of eradication efficacy in cases of metronidazole resistance are considered to be the major drawbacks of this therapy. PPI dual therapy is better tolerated with fewer side effects than is bismuth triple therapy. The mean eradication rates vary from 55% to 75%, and the extremes lie between 24% and 93%. PPI triple therapies have been shown to be very effective against *H. pylori* with an eradication rate 80% - 90%. Eradication rate in children with 2 weeks of treatment with clarithromycin, amoxicillin and proton pump inhibitors (omeprazole or lansoprazole) is reported to be 75% and 92%, respectively^[165,166].

Omeprazole, clarithromycin and metronidazole or tinidazole for 7 days are reported to cause eradication in 87% and 89%^[167,168]. Dual therapy for 2 weeks with omeprazole and amoxicillin causes eradication in 70% of infected children, whereas a addition of clarithromycin for 2 weeks increases the eradication rate up to 92%^[169]. Amoxicillin, bismuth and metronidazole were reported to eradicate *H. pylori* in 96% of infected children^[143]. Quadruple therapy leads to a mean eradication rate of 96%. Thus, based on efficacy PPI triple or bismuth triple therapy are recommended as first-line treatment^[170]. The cost of PPI versus bismuth should be considered. However, compliance strongly influence the eradication rate, and may explain why, in contradiction to experience in adults, in children two drugs for 2 weeks are sometimes found to be equally effective than triple therapy for 1 week^[171].

Eradication therapy is not recommended for all *H. pylori* infected adults and children^[162]. However, the complex relationship between *H. pylori* and gastrointestinal cancer might stimulate physicians to prescribe eradication treatment, even in the absence of scientific evidence, especially in countries with a strong impact of legislation on health care, as in the case in the US in children, not one randomized prospective placebo-controlled study had been conducted. Whether children with symptomatic *H. pylori* gastritis alone will benefit from treatment is debated^[90,121]. Well-designed clinical trials showing a therapeutic gain of *H. pylori* treatment over placebo are still missing, with the exception in duodenal ulcer patients. The cost-benefit ratio of avoiding endoscopy in dyspeptic patients is only worthwhile considering if the cost of endoscopy is greater than \$500 USD^[172], as is the case in the USA; while the cost of upper gastrointestinal tract endoscopy in Belgium and Finland is only about \$100 and \$170 USD, respectively^[173].

Nevertheless, recommendations differ in Europe and the USA. The European consensus states that scientific evidence for the improvement of functional dyspepsia is equivocal, but the overall evaluation taking into account the expected benefit on the gastritis status makes it worthwhile to consider eradication therapy in such patients. In Europe, it is accepted, although not unanimously, that young patients, aged below 45 years, without alarm symptoms (anemia, weight loss, dysphagia, palpable mass, malabsorption, etc.) and who test positive for *H. pylori* for the first time with validated serology or breath test, can be treated with eradication therapy without further investigations (thus without endoscopy)^[94,160]. However, in the USA, the present consensus states

that there is no scientific evidence to recommend treatment for *H. pylori* in the absence of an established peptic ulcer disease^[156]. As a consequence, according to the North American consensus, non-invasive testing cannot replace endoscopy in the initial diagnosis of *H. pylori* related gastrointestinal diseases (not in children either)^[174]. Eradication of *H. pylori* in patients who do not benefit from it may unnecessarily increase the risk of resistance of *H. pylori* to antibiotics.

H. pylori and non-steroid anti-inflammatory drugs (NSAIDs) are both ulcerogens; however, NSAIDs are not frequently prescribed in children, and moreover, there seems to be no cooperative effect between them. Consequently, eradication of *H. pylori* prior to NSAID administration is not recommended in the USA. In Europe, eradication of *H. pylori* before NSAIDs is considered "advisable".

Elimination of *H. pylori* increases the risk of developing gastroesophageal reflux and reflux esophagitis^[157,175]. *H. pylori* eradication results in a marked decrease in the pH-increasing effect of omeprazole and ranitidine^[176]. Nevertheless, long-term acid suppressive therapy with proton pump inhibitors (and to a lesser extent with H₂-antagonists) for reflux disease in *H. pylori* positive patients enhances the development of atrophic gastritis if *H. pylori* has not been eradicated beforehand^[177,178].

Eradication of *H. pylori* can be demonstrated by normalization of histology and negative culture of gastric biopsies, or with the use of urea breath tests^[94]. With respect to serology, a 50% fall in antibody titers is indicative of successful elimination. However, this usually requires up to 6 months to occur^[179]. When follow-up tests for eradication of *H. pylori* are necessary, they should not be made earlier than 4 weeks after cessation of treatment^[94]. The bacterial load could influence the success rate of eradicating treatment^[180].

Knowledge of the *H. pylori* genome provides major new insights into many aspects. Conversion of pyruvate to acetyl-CoA uses an unusual enzyme, only previously found in free-living bacteria from extreme environments^[181] and the genome sequence shows that acetyl-CoA is likely to be a crucial intermediary in several biosynthetic pathways. Therefore, blocking the enzyme should allow effective and selective drug activity against *H. pylori*. The same is true for many other enzymes.

Vaccination

Study of the *H. pylori* outer membrane is important for both understanding the pathogenicity but also

for development of vaccines since the outer membrane is involved in adherence to the host epithelium and stimulation of the host immune response. Vaccines should be able to confer preventive and curative immunity on humans. Oral immunization with a recombinant urease given in the absence of a mucosal adjuvant has been assessed unsuccessfully in *H. pylori* infected volunteers^[182]. However, recently, the recombinant *H. pylori* urease was given with an *E. coli* heat-labile toxin, provoking diarrhea in the majority of the volunteers, a side-effect which disappeared when the dose was reduced, but also showing an increase in urease specific IgA producing cells and a decrease in the density of gastric colonization by *H. pylori*^[183].

IgA antibodies are expected to play a prominent role in protection, since *H. pylori* is a non-invasive pathogen at the luminal surface of the gastric mucosa. This hypothesis has been supported by the observation that milk IgA protects infants against *H. pylori* infection^[82]. IgA and immunoglobulin G1 (I gG1) depend on T-helper type 2 (Th2) cells. According to different recent experiments, immunization is associated with an elevation of IgG1 levels, indicative of a Th2 cellular immune response, which might be a significant mechanism^[184-186]. The field of vaccination is still very controversial, and is being extensively studied.

CONCLUSION

Helicobacter pylori infection is worldwide one of the most frequent infectious diseases. There is a huge discrepancy in prevalence and incidence between the industrialized countries and the rest of the world (Africa, Asia, South-Americ a). Infection occurs mainly in children. Well-designed studies to identify those infected children who are at risk of developing complications or have symptoms due to the infection are still lacking^[171]. Because of the cohort-effect which is related to the socio-economic status and/or hygienic circumstances, the annual infection rate in the Western world is dramatically decreasing. If this observation is confirmed, it can be speculated that a decrease in incidence of peptic ulcer disease and gastric cancer may occur in the more industrialized countries during the next decades. However, duodenal ulcer and gastric cancer are only related to some more virulent strains. Many children remain asymptomatic, and a clear relation between *H. pylori* and symptoms has only been demonstrated for ulcer-related symptoms. In addition, peptic ulcers are rare in childhood. Treatment of *H. pylori* is indicated in duodenal ulcer diseases. The relation between chronic abdominal pain, functional dyspepsia, and *H. pylori* is unclear.

Screening tests, including serology or the urea

breath test, are of interest for epidemiological studies. The urea breath test evaluates the actual colonization; serum antibodies might persist after eradication, which is only rarely spontaneous. According to the European consensus, eradication therapy can be considered in a child with functional dyspepsia and positive screening test. According to the North American consensus, treatment is only recommended in the presence of ulcer, necessitating endoscopy.

H. pylori strains are not created equal since important virulence factors are not detectable in all strains. The continuous decline of *H. pylori* prevalence as a result of changes in living conditions and active treatment is not unanimously considered to be beneficial^[14]. Unfortunately, screening tests rely on virulence factors which are detectable in all strains. Vaccines are not expected to be available in the near future.

Nevertheless, improvement of the socio-economic status and hygienic circumstances in all countries will result in a dramatic decrease of *H. pylori*.

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Current concept of pathogenesis of severe acute pancreatitis

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Subject headings pancreatitis/etiology; pancreatitis/diagnosis; pancreatitis/pathophysiology

The pathogenesis of severe acute pancreatitis is very complicated. It is a multifactorial as well as multifaceted disease. First of all, the etiologic agents initiate the pancreatic acinar injury by release of pancreatic enzymes and overstimulation of macrophages and neutrophils, then the cytokines and inflammatory mediators are liberated. There is also interaction between neutrophils and endothelial cells producing free radicals, the cytokines cause increasing vascular permeability, activating complement component, resulting in microcirculatory impairment and imbalance of thrombo-fibrinolytic system. Many of these events occur not only in the pancreas itself, but also in the other vital organs and tissues, leading to severe acute pancreatitis and complications. The sequential events are as follows.

PANCREATIC ACINAR INJURY

Pancreatic duct obstruction and bile reflux

Gallstone incarcerated in the distal common duct or biliary-pancreatic duct common pathway initiates bilereflux, activates pancreatic trypsin, produces acinar injury which is the traditional viewpoint, but is unexplainable in case when biliary and pancreatic ducts open into the duodenum individually and in those so called idiopathic acute pancreatitis. It had been found that 91% of such cases had microlithiasis or biliary sludge as the cause of transient incarceration. In animal experiments, ligation of pancreatic duct can also induce acute pancreatitis without having bile reflux, showing that pancreatic duct obstruction is also an important pathogenetic factor^[1]. Such microlithiasis could not be disclosed by the conventional cholecystography, but could be shown from the bile taken from the recovered patients. After centrifuged and observed under

microscope, clustered cholesterol crystals, bilirubin and calcium bicarbonate microgranules were found in 67% of cases^[2]. Many recurrent pancreatitis patients when followed up by ultrasound for 12 months, there were cholesterol crystals and microlithiasis, the biliary sludge and microlithiasis could also be seen in the gallbladder specimens, indicating there had been pancreatic obstruction^[3]. Pancreatic obstruction caused impedance of pancreatic fluid outflow resulted in elevation of pancreatic pressure and produced pancreatic acinar injury.

High fat, high protein diet

High fat and high aminoacid content in the duodenum can stimulate cholecystokinin (CCK) release, which promotes the acini to secrete enzymes. The zymogen granules located at the fusion of the apical cell membrane dislocate and scatter throughout the cell cytoplasm after stimulation. At this stage, the cells show vacuolation because the zymogen granules have been exocytosed. Alcohol can also sensitize the acini to CCK, activate the intracellular and intraluminal pancreatic enzymes, and initiate the catabolism of intracellular protein under the condition of low pH and ionic changes. Two mechanisms have been proposed to account for the intracellular activation of trypsinogen and the zymogen cascade: trypsinogen autoactivation and trypsinogen activation by the lysosomal enzyme cathepsin B. These are the earliest changes in acute pancreatitis^[4], then activated trypsin again activates other pancreatic enzymes, including pancreatic lipase, amylase, chymotrypsin, phospholipase A₂(PLA₂), elastase, carboxylase, nucleotidase, etc. which induce autodigestion of the pancreas. In animal experiments, administering CCK or CCK analogue i.e., cerulein into ligated pancreatic duct, can induce edematous pancreatitis; and injecting taurocholic acid into ligated pancreatic duct can cause necrotizing pancreatitis.

Alcohol

Alcohol has deleterious effects on both pancreatic acini and Oddi's sphincter^[5]. ① It stimulates gastrin secretion and via the cholinergic pathway to stimulate pancreatic secretion, simultaneously, it can cause spasm of Oddi's sphincter, the two in combination can lead to pancreatitis. ② Alcohol

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may change the composition of proteins secreted by the pancreas, resulting in the formation of protein plugs within small pancreatic ductules, this insoluble protein is formerly called stone protein. ③ It increases the amount of lysosomal enzymes and increases trypsinogen/pancreatic trypsinogen inhibitor ratio. ④ Ethanol can change systemic and pancreatic lipid metabolism with accumulation of lipid droplets within the acinar cells, alter membrane fluidity and integrity. ⑤ Ethanol even sensitizes the acinar cells to CCK-stimulated intracellular zymogen proteolysis. In presence of combined-stimulation of CCK and alcohol, it is easier to induce acute pancreatitis.

Ischemia, decreased perfusion

Transient ischemia enhances enzymatic degradation of acinar cells which become more vulnerable. Hypotension during bypass surgery, pancreatic atherosclerosis with hyperfunction of sympathetic nervous system in the elderly, in combination with the above factors can result in acute pancreatitis with necrosis. Animal experiments on ligation of pancreatic duct and injecting cerulein or taurocholic acid, with additional clamping of upstream of pancreatic artery, that is the celiac artery and superior mesenteric artery for 40 min, can cause pancreatic necrosis, edema and inflammation.

Based on the above facts, severe acute pancreatitis can be induced by a combination of multifactors, which include pancreatic obstruction with or without bile reflux, high fat, high protein diet (hyperstimulation of CCK), alcohol, and ischemia, this is what we encounter in our clinical practice, these factors interact with one another and result in this disease.

CHANGES AFTER ACINAR INJURY

Activation and release of pancreatic enzymes

Activated trypsin again activates other pancreatic enzymes, among which, chymotrypsin promotes hydrolysis of tryptophan, tyrosine and phenylalanine peptide chain; elastase hydrolyzes elastic fibers of extracellular matrix, promotes spreading of protein degradation; carboxypeptidase A and B and RANase, each acts on the components of pancreatic tissue; pancreatic lipase hydrolyzes the lipids, triglyceride, produces free fatty acid which is toxic to the capillaries, leads to lipoperoxidation; and PLA² hydrolyzes phospholipids including cephalin, sphingophospholipids and lysocephalin, destroys structural phospholipid and microvasculature, activates platelet activating factor and increases vascular permeability and ischemia^[6]. The kallikren-kinin system produces bradykinin which dilates the blood vessel, increases vascular permeability and produces

hypotension, these are the earliest events occurring in acute pancreatitis. It had been found that injection of ascitic fluid containing high concentration of bradykinin to healthy animal produces hypotension^[7]. But by giving bradykinin antagonist, HOE 140, the pancreatic edema caused by bradykinin can be reduced^[8]. The above pancreatic enzymes not only induce autodigestion of pancreas, inflammation of peripancreatic fat, but also circulate in the blood together with the activated cytokines to the remote organs including the lung and brain causing damages. Besides, the release of pancreatic enzymes also activate complement system and thrombo-fibrinolytic system, producing thrombosis in the microvessels. In the ascitic fluid of severe acute pancreatitis patients, activated protease, phospholipase A₂, bradykinin, complement component, histamine and some inflammatory mediators as platelet activating factor (PAF) and prostaglandin are found.

Release of cytokines

During pancreatic necrosis and inflammation, IL-1 β , IL-6, TNF- α -mRNA expression can be detected in the pancreas. The macrophages release IL-1, IL-6, TNF- α and IL-8, among which, IL-6 produces acute phase proteins, IL-8 chemotacts neutrophils to the inflamed areas, including the pancreas and the lung. The macrophages are the sources of proinflammatory cytokines (IL-1, IL-6, TNF- α) and anti-inflammatory cytokines (IL-4, IL-10, IL-1ra), IL-1ra is IL-1 receptor antagonist, the IL-6 produced in severe acute pancreatitis is greater in amount than that produced in mild acute pancreatitis, and also persists longer^[9]. These proinflammatory cytokines induce intercellular adhesive molecules (ICAM 1) and vascular adhesive molecules (VCAM) expression, promote the spread of inflammation, also augment the elastase of neutrophils to produce free radicals damaging the endothelial cells, causing endothelial swelling, and circulatory stasis^[10]. In severe acute pancreatitis, increase of proinflammatory cytokines and decrease of anti-inflammatory cytokines are crucial factors in its progression, experimental study revealed that using TNF- α monoclonal antibody or recombinant IL-10, either of them could reduce the severity of the disease and increase the survival rates^[11-14].

PAF is the structural component of membrane lipid, it can be synthesized by endothelial cells, macrophages and platelets, and activated by PLA₂, producing chemotaxis, aggregation, releasing superoxides, initiating the interaction of neutrophils and endothelial cells and favoring the entrance of neutrophils into the tissue space. It also causes increased vascular permeability of the capillary network of the lung, kidney, heart and the GI

tract^[15]. Moreover, it enhances the tissue damage by the endotoxin liposaccharide, thus playing a crucial role in the pathogenesis of severe acute pancreatitis, it not only has autocrine and paracrine function, with high content it also has endocrine function, producing multiorgan dysfunction syndrome (MODS)^[11]. In cerulein and taurocholic acid experimental models, its concentration is increased in the blood, ascitic fluid, pancreas, and lung tissue, producing pancreatic ischemia and increasing the inflammatory cellular infiltration. Recently it is claimed by using PAF antagonist, Lexipafant could decrease vascular permeability, diminish IL-6 and IL-8, and decrease the severity of ARDS, and turn the blood amylase and lipase to normal. In neutropenic patients or using neutrophilic cell antibody, the inflammation in the lungs could be attenuated^[16]. 5-fluorouracil formerly used in the treatment of severe acute pancreatitis acted mainly via its upregulating effect of cytokine IL-4 and IL-10^[17].

Increased vascular permeability and Pancreatic microcirculatory impairment

During early stage of acute pancreatitis, the arterioles in the pancreatic lobules constrict, with stasis of neutrophils in the postcapillary venules^[18], in addition, there is increased vascular permeability, these further decrease the pancreatic perfusion. Ischemia causes Ca^{2+} ion influx, aggravating rupture of lysosomal membrane and release of lysosomal enzymes. On the other hand, there is increase of TXB_2 and TXB_2/PGI_2 imbalance which constricts further the vessels and endothelin liberated by the damaged endothelium also decreases pancreatic blood flow. The release of $TNF-\alpha$ also promotes vascular permeability, enhances platelet aggregation, stimulates excessive production of nitric oxide. The vessels in the region dilate, hence, the overall effect is the decrease of pancreatic vascular perfusion and blood stasis in the venular net work, leading to local intravascular coagulation. One can see fibrin deposition in the pancreatic capillaries under microscope, the interstitial pressure become elevated, the red blood cells migrate outward to extravascular space, contributing to the hemorrhage, ischemia, necrosis, inflammation and edema^[18]. But the degree of edema is milder in the severe form. Not only is such in the pancreas substance, similar changes also occur in the pulmonary alveoli, the type II alveolar cells can be destroyed resulting in loss of pulmonary surfactant. Low molecular weight dextran and Dan Shen (*Salvia Miltiorrhiza*) liquid can improve the microcirculation and prevent pancreatic necrosis and lung changes^[19].

Infection-the second attack

In severe acute pancreatitis patients, the cellular immunologic function is compromised, and the CD_4^+ and CD_8^+ lymphocytes are markedly diminished, but their ratio remains unchanged, which is quite different from that seen in mild acute pancreatitis^[20]. Furthermore, because of increased vascular permeability due to PAF, there is loss of gastrointestinal epithelial barrier function^[21], the gut bacteria translocate from the colon to mesenteric lymph node, peritoneal cavity and blood circulation, IL-6 and IL-8 are further released by endotoxin, $TNF-\alpha$ and IL-1 induction, IL-8 chemotacts and attracts more neutrophils accumulating at the pancreas, lung and other vital organs and tissues. Because of the insufficient endogenous superoxide dismutase, the deletion of glutathione, and presence of various oxygen free radicals destroy the cellular membrane, plasmalemma and the organelles^[22]. Procoagulating factors are released leading to cascade of thrombofibrinolytic reaction, and produce inflammatory mediators from eicosanoid products. Moreover, the neutrophilic elastase is more destructive, causing multiorgan failure. Experimental studies showed that plasma neutrophilic elastase level paralleled the multiple organ failure in severe acute pancreatitis which were positively correlated. This is the so-called second attack theory^[23].

Serious complications of severe acute pancreatitis are mainly adult respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), infected necrosis and pancreatic encephalopathy, their pathogenesis are described as follows (Table 1).

Interstitial pulmonary edema and ARDS are caused by many factors: ① phospholipase A_2 arrives at the lung by way of circulation, and destroys the type II alveolar cells, which are then unable to produce surfactant; ② the macrophages become vasuolated simultaneously, being unable to phagocytose and digest the protease and clear away the fibrin; ③ neutrophils being chemotacted and accumulated in the lungs, release the destructive elastase and oxygen free radicals; ④ PAF activated by PLA_2 can damage the endothelium and increase the vascular permeability, causing ischemia, interstitial pulmonary edema and ARDS. If properly treated, this complication is now rarely seen.

DIC is often due to massive exudation in the peritoneal cavity and retroperitoneal space, hypoalbuminemia and hypovolemic shock, if colloid has not been instituted instantly, hypercoagulation might appear and followed by increased hematocrit, microcirculatory stasis and imbalance of thrombofibrinolysis. By given the author's treatment regime^[24], this complication has not been seen in recent years.

Table 1 Complications of severe acute pancreatitis and their mechanisms

Complications	Mechanisms
1. Local complication	
Intra and retro-peritoneal fluid collections	Bradykinin, TNF- α , PAF increase vascular permeability with fluid exudation Hypoalbuminemia
Pseudocyst	Unabsorbed fluid collection for long duration
Pancreatic fistula, transient	Pancreatic duct rupture, communicating with pseudocyst
2. Systemic complications	
Hypotension, hypovolemic shock	Increased vascular permeability, profuse exudation into peritoneal cavity Hypovolemia Hypoalbuminemia
Intestinal ileus, dehydration, hypokalemia	Peritonitis; loss of peristaltic function; large quantity of digestive fluid sequestered in intestinal lumen; infusion of large volume of crystalloid solution
Hypocalcemia	Formation of calcium soap plaques with fats on peritoneum and mesentery
Renal insufficiency	Hypotension, low blood volume, decrease of renal blood flow (PAF further decreases renal blood flow)
Gastric hemorrhage	Acute gastric mucosal bleeding
Jaundice	Pancreatic head edema in mild jaundice, choledocholithiasis in severe jaundice cases
Interstitial lung edema, ARDS	PLA ₂ destroys structural phospholipid; PAF and TNF- α increase vascular permeability; Neutrophils release elastase and free radicals damage type I & II lung epithelial cells with disability of producing surfactants, alveolar atrophy and interstitial edema
Disseminated intravascular coagulation (DIC)	Shock, hypercoagulable state; microcirculatory stasis; imbalance of thrombo-fibrinolytic system; deletion of antithrombin III
Pancreatic encephalopathy	PLA ₂ damages structural phospholipid of brain cell membrane; PAF increases intracerebral vascular permeability with brain edema and demyelination of grey and white matter
Transient blindness	Retinal ischemia; white cell emboli with exudation, increased vascular permeability
Infection, bacteremia, sepsis	Gut barrier dysfunction with translocation of gut bacteria and endotoxemia, bacteremia
Infected necrosis	Cellular immunity decreases, sepsis
Pancreatic abscess	Same as above, hemodynamic changes caused by inflammatory cytokines and inflammatory mediators
Heart failure	Underlying ischemic heart disease; overloading of circulation by massive infusion or too rapid intravenous dripping

Pancreatic encephalopathy is primarily due to the demyelination of the cerebral grey and white matter caused by PLA₂, which can induce increased vascular permeability, the intravascular osmotic pressure decreases and the brain becomes more vulnerable to transudation, resulting in brain edema. With proper treatment, the recovery in patients aged below 40 is uneventful, those older than 60 especially those with previous history of cerebral infarction may have some sequela.

Infected necrosis and pancreatic abscess. The former usually occurs two weeks after the onset of the disease, the latter occurs four to five weeks after the onset. The invading bacteria usually derive from one's own GI tract, including the bacilli and cocci. The first five bacteria are *B. Coli*, *Klebsiella*, *Enterobacillus*, *Streptococcus faecalis* and other *Streptococci*, there can also be *Staphylococcus*, *Pseudomonas aeruginosa* or *Bacteroid fragilis*^[25]. *E. coli* usually derives from

the colon, biliary tract, urinary tract or respiratory tract through hematogenous route, *Staphylococcus epidermis bacteremia* comes from venous catheterization or urinary catheterization. Pancreatic abscess rarely contains necrotic tissue, but frequently is composed of pure pus. If preventive measures are taken beforehand, sepsis and septicemia can be prevented and are now rarely seen.

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Potential roles of tumor suppressor genes and microsatellite instability in hepatocellular carcinogenesis in southern African blacks

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Subject headings liver neoplasms; carcinoma, hepatocellular; tumor suppressor gene; microsatellite instability

MAJOR POINTS OF THE COMMENTED ARTICLE

Cumulative loss of heterozygosity (LOH) of chromosomal regions and tumor suppressor genes has been reported in hepatocellular carcinomas (HCCs) from China, Japan, and Korea. In this issue of the World Journal of Gastroenterology, Martins *et al*^[1] report an analysis of LOH and microsatellite instability in HCCs from a group of 20 southern African blacks. Six known tumor suppressor genes-p53, RB1, BRCA1, BRCA2, WT1, and E-cadherin-were analyzed for LOH. In addition, the p53 gene was analyzed for the codon 249 mutation that is commonly found in subjects exposed to high levels of dietary aflatoxin B1. The authors found LOH at the RB1 locus in 17% (3 of 18 informative subjects), at the BRCA2 locus in 10% (2 of 20 informative subjects) and at the WT1 locus in 8% (1 of 13 informative subjects). Two of the subjects had LOH at both the RB1 and BRCA2 genes. Thus, overall, LOH was found in 20%(4/20) of the HCCs. No LOH was found at the p53, BRCA1, or E-cadherin loci. In contrast to reports from other populations, mutations of the p53 and RB1 genes in combination were not seen in this population. Nine of 10 microsatellite loci examined showed changes in microsatellite repeat number in different HCCs, and changes at two or more loci were found in 15% (3/20) of the subjects. The p53 codon 249 mutation was found in 25% (5/20) of the subjects. Four of the 5 subjects with p53 codon 249 mutations had active or previous hepatitis B infection; the hepatitis B status of the fifth subject with a p53 codon 249 mutation was

unknown. These results provide initial information about the potential role of specific tumor suppressor genes and low level microsatellite instability mechanisms in the pathogenesis of HCCs in southern African blacks.

COMMENTARY

Different views of a single disease

HCC is a major cause of cancer death worldwide, particularly in Asia and Africa. The major risk factors for development of HCC are chronic hepatitis B virus and chronic hepatitis C virus infection, high dietary exposure to fungal aflatoxin B1, and other disorders causing cirrhosis such as hemochromatosis, alpha 1 antitrypsin deficiency, primary biliary cirrhosis, non-alcoholic steatohepatitis, and alcoholic cirrhosis. Over the past few decades, a number of approaches have been explored in an attempt to elucidate the details of hepatocarcinogenesis. These approaches have increased in their sophistication with advances in cell and molecular biology and genetics and have borrowed from and contributed to our general understanding of tumor biology. Techniques and experimental systems that have improved our understanding of the hepatocarcinogenic process include: ① the use of chemical tumor initiators and promoters in animal models; ② studies of growth factors and their signaling pathways such as insulin like growth factor 2 (IGF-2) and its intracellular mediator, insulin receptor substrate 1 (IRS-1); ③ transgenic mouse models overexpressing cytokines, growth factors, or oncogenes such as tumor necrosis factor alpha (TNFalpha), transforming growth factor beta (TGFbeta), or c-myc, expressed in isolation or in combination; ④ studies of immune-mediated mechanisms of hepatocellular injury; ⑤ analysis of the molecular genetic changes that occur in HCCs, including hepatitis B virus integration; and ⑥ studies of the protein products of the hepatitis B and C viruses and their interaction with host cell processes. More recently, there has been mounting evidence that common fragile sites, which are unstable regions in the genome, may also be involved in hepatocarcinogenesis.

These studies have contributed to our growing appreciation of the multiplicity of mechanisms and pathways that may contribute to the carcinogenic process in toxin affected, chronically inflamed or otherwise injured liver tissue. Unfortunately, we

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are still far from a unified, comprehensive understanding of hepatocarcinogenesis. This is in part because HCC, although quite uniform in its final phenotype, is initiated in multiple genetic and environmental contexts and almost certainly emerges as a consequence of multiple possible pathways. However, the lack of a comprehensive view of the pathogenesis of HCC also prevents us from developing effective, targeted, preventive or therapeutic interventions that are elegant and also simple enough to be applicable to the vast majority of patients with this disease. In this commentary we will review current concepts of the mechanisms of human hepatocellular carcinogenesis and address the issue of geographic variation in carcinogenic mechanisms.

Tumor suppressor genes

Current concepts in molecular oncogenesis suggest that the chromosomal breaks and rearrangements or gene mutations found in cancer lead to activation of oncogenes or cause disruption of tumor suppressor genes. Tumor suppressor genes are normal cellular genes that, when homozygously deleted, can contribute to tumor development. Tumor suppressor genes generally operate in a recessive manner, requiring loss of both copies for tumorigenesis, in contrast to oncogenes, which can exert their effects in a dominant fashion. Loss of heterozygosity (LOH) is loss of one allele in a tumor cell from a chromosomal region for which the -individuals'-normal cells are heterozygous. LOH is detected using polymorphic DNA markers that can distinguish between the two alleles^[2]. If one allele of a tumor suppressor gene is inactivated by mutation, then deletion of the second allele, detected by LOH, is associated with loss of expression of the tumor suppressor gene. In human HCCs, LOH has now been reported in multiple chromosomal regions, including 1p, 1q, 2q, 4q, 5q, 6q, 8p, 8q, 9q, 10q, 11p, 12p, 13q, 14q, 16q, and 17p^[3,4]. In a number of these regions, there are known or putative tumor suppressor genes, such as at 17p13.1 (p53), 6q26-27 (mannose-6-phosphate/insulin-like growth factor 2 receptor), 8p21.3-22 (DLC-1), and 13q12-q32 (RB and BRCA2). For a few of these chromosomal loci, clinico-pathologic associations have been demonstrated, such as the association of LOH on chromosome 1p with early stage HCC^[5] and that of LOH on chromosome 16q with progression of HCC^[6]. In addition, patients with LOH at multiple regions have more advanced stage disease, less well-differentiated tumors, higher serum alpha-fetoprotein levels and a worse prognosis^[7]. Chronic hepatitis B viral infection and integration of the hepatitis B genome have also been associated with high rates of genomic instability^[8,9]. The use of the technique of comparative genomic hybridization (CGH) allows the identification of gains and losses of DNA

sequences across the entire tumor genome. Analysis of HCC tumors and cell lines by CGH has revealed genomic DNA copy number gains at 1p34.3-35, 1p33-34.1, 1q21-23, 1q31-32, 6p11-12, 7p21, 7q11.2, 8q24.1-24.2, 11q11-13, 12q11-13, 12q23, 17q11.2-21, 17q23-24, and 20p11.1-q13.2. Recurrent losses were found at 3p12-14, 3q25, 4p12-14, 4q13-34, 5q21, 6q25-26, 8p11.2-23, 9p12-24, 11q23-24, 13q12-33, 14q12-13, 15q25-26, 16q, 17p, 18q11.2-22.2, and 21q21-22^[10,11]. Significantly, a majority of the over-represented regions harbor known proto-oncogenes, and half of the under-represented regions coincide with sites of known or putative tumor suppressors^[11]. Notably, the recognition that there is gain of region 17q11-21, which harbors the ERBB-2 proto-oncogene, has led to the development of early clinical trials of anti-p185HER2/neu monoclonal antibody in appropriately selected HCC patients.

Investigation of p53 gene mutations in HCC has been particularly revealing, as HCCs from patients with high dietary exposure to fungal aflatoxin B1 have a high frequency of point mutations at the third position of codon 249 of the p53 gene, resulting in a G:C to T:A transversion^[12]. This mutational target appears to be specific for liver tumors of hepatocellular origin. A G to T transversion at the second position of codon 249 is commonly found in patients with chronic hepatitis B and C infection. The occurrence of this mutation correlates with oxyradical exposure. Both of these mutations lead to decreased binding of p53 to its nuclear DNA targets^[13]. p53 mutations have been demonstrated in nonneoplastic liver cells in subjects from communities with high dietary AFB1 exposure, suggesting that the mutations are early events in neoplastic transformation. However, p53 mutations can also occur late in tumor progression. The hepatitis B virus HBx gene product has also been shown to interact with p53 and strongly inhibit p53 sequence-specific binding, leading to inhibition of p53-mediated apoptosis. Abnormalities in the retinoblastoma gene have been noted in association with p53 mutations in advanced HCCs, particularly in poorly differentiated tumors, and a possible additive effect of p53 and Rb mutations during the progression of hepatocarcinogenesis has been suggested^[14].

Oncogenes

Proto-oncogenes are cellular genes involved in the control of cell growth. Mutation, overexpression, or amplification of these genes leads to oncogenic activity that contributes to neoplastic transformation. The oncogenes c-fos and c-myc are both overexpressed in HCC^[15]. c-myc overexpression may be a consequence of amplification of the c-myc locus in HCC^[10,11]. Activation of the c-myc and c-fos also occurs as a consequence of the transactivating function of the

hepatitis B virus x protein (HBx) and the carboxyterminal truncated middle hepatitis B surface protein (MHBs-t)^[16]. Oncogene activation may occur through reactive oxygen species mediated pathways or through protein kinase C or mitogen activated protein kinase pathways. Reactive oxygen species or lipid peroxidation mediated processes may also be important in the pathogenesis of chronic liver injury induced by alcohol, genetic hemochromatosis, and alpha 1 antitrypsin deficiency^[17,18].

Growth factors and growth factor signaling pathways

Insulin and insulin-like growth factors (IGF) 1 and 2 promote hepatocyte growth. IGF-2 is frequently over expressed in HCCs. In addition, insulin receptor substrate 1, a cellular mediator of insulin-like growth factor signaling, is also frequently overexpressed in HCCs^[19]. Signaling events downstream of IRS 1 lead to cell proliferation through upregulation of cellular growth genes and inhibition of apoptosis. Transforming growth factor alpha (TGF alpha) and the structurally related epidermal growth factor (EGF) are another potent class of hepatocellular growth factors. TGF alpha is overexpressed in the liver of patients with chronic hepatitis. As TGF alpha levels are often increased in HCCs, it is likely that it contributes to cellular proliferation in cancer, and may be a factor in tumor initiation or progression in patients with chronic hepatitis^[20]. Transforming growth factor beta 1 (TGF beta 1) inhibits cell proliferation and promotes cellular differentiation, fibrogenesis, and apoptosis. Increased TGF beta 1 levels may create an environment in which selection of hepatocyte clones resistant to TGF beta 1-induced apoptosis occurs.

Microsatellite instability

Microsatellite instability (MSI) is defined as a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue. This form of genomic instability is associated with defective DNA mismatch repair in tumors, which is important in the pathogenesis of the hereditary non-polyposis colon cancer syndrome (HNPCC) and associated malignancies^[21]. To date, six mismatch repair genes have been identified in humans; hMLH1, hMSH2, hPMS1, hPMS2, hMSH3 and hMSH6. Colorectal cancers may be characterized as having high-frequency MSI (MSI-H) if greater than 30%-40% of more than 5 microsatellite loci analyzed show instability, low-frequency MSI (MSI-L) if less than 30%-40% of more than 5 loci analyzed show instability, or as being microsatellite stable (MSS) if 0% of loci show instability^[21]. Of sporadic colorectal cancers, 70% are MSS, 15% are MSI-L, and 15% are MSI-H. MSS or MSI-L colorectal cancers do not have an associated

defective mismatch repair phenotype or the clinicopathologic features of HNPCC tumors; instead they behave in the same manner as sporadic colon cancers. Clear criteria have not been defined for MSI in noncolonic tumors. To date, a number of studies, including the study by Martins *et al*^[1], have demonstrated a relatively low frequency of low-level MSI in HCC^[22,23]. As is the case with other tumors, it is unclear whether this finding is of significance in the etiology or pathogenesis of HCCs or is instead simply a consequence of the generalized genomic instability found in cancer. It has been suggested that cumulative low-level MSI at multiple loci leads to tumor progression. Further investigation is needed to resolve this potentially important question.

Chromosomal fragile sites

Chromosomal fragile sites are specific genetic loci which are susceptible to forming gaps, breaks, and rearrangements in metaphase chromosomes of cells cultured under conditions that inhibit DNA replication, such as treatment with the DNA α polymerase inhibitor, aphidicolin. Fragile sites are grouped into "common" or "rare" classes based on their frequency of occurrence and the culture conditions required for their expression. Thus far, 89 common and 28 rare chromosomal fragile sites have been identified. Common fragile sites are present in all individuals. The most frequently observed common chromosomal fragile sites occur at 3p14.2 (FRA3B), 16q23 (FRA16D), 6q26 (FRA6E), 7q32 (FRA7H), and Xp22 (FRAXB)^[24].

Common fragile sites span a distance of 250-500 kilobases and display characteristics of unstable, highly recombinogenic DNA *in vitro*. In particular, they are preferred sites for sister chromatid exchanges, chromosomal deletions and rearrangements, integration of viral sequences and transfected plasmid DNA, and initiation of bridge-breakage-fusion cycles, which lead to gene amplification. Due to these characteristics and the frequent coincidence of fragile sites with chromosomal breakpoints in malignant cells, it has been hypothesized that fragile sites are involved in carcinogenesis^[25]. The most convincing evidence of the potential significance of the fragile sites in carcinogenesis is the location of FRA3B, the most highly inducible common fragile site, at chromosome 3q14.2. This chromosomal region is frequently deleted in lung cancer, renal cell carcinoma, and pancreatic cancer, and is also the location of the fragile histidine triad (FHIT) gene^[26]. The FHIT gene has been proposed to be a tumor suppressor and has recently been identified as a preferential target in HCC^[27]. The cloning of additional fragile sites, particularly ones such as FRA16D (16q23) and FRA6E (6q26) that are located in regions at which there is known LOH in HCC, should provide additional information about

the potential role of fragile sites in hepatocarcinogenesis.

Telomere length and telomerase activity

Telomeres are specialized protein-DNA structures at the ends of chromosomes that contain long stretches of TTAGGG hexameric repeats. Telomeres are thought to prevent degradation of chromosome ends and end-to-end fusion with other chromosomes. Aging of somatic cells is associated with reduction in telomere length. In contrast, germ line and neoplastic cells express telomerase, an enzyme that restores telomere length. There is progressive shortening of telomeres with progression from chronic hepatitis to cirrhosis and eventually to HCC^[28]. This is thought to occur as a consequence of the multiple cycles of cell injury, death, and regeneration that occur in injured liver, leading to premature hepatocellular senescence. It is presumed that telomere shortening beyond a critical length leads to genomic instability of hepatocytes and the evolution of clones of hepatocytes with increased telomerase expression and an immortalized phenotype^[29].

Geographic variations in hepatocarcinogenic mechanisms

Because of the small number of subjects in many studies and the heterogeneity of etiologies of HCC, it has been difficult to conclusively identify geographic variations in hepatocarcinogenic mechanisms. The best evidence so far is for the role of aflatoxin B1 in generation of the G-to-T transversion at codon 249 of the p53 gene. The frequency of this mutation in HCCs increases proportionally to the level of dietary exposure to aflatoxins. LOH at different loci, including the p53 locus, does not show as clear a pattern. Fifty-eight % of 64 heterozygous Chinese patients had a tumor-specific p53 allele LOH^[30]. Reported rates of p53 LOH from Japan are 69% (55 of 80 informative cases) and 95% (34 of 36) in cases with a p53 mutation^[31]. Previously high reported rates of p53 LOH in HCCs from southern African blacks contrast with the results reported in this issue by Martins *et al*^[1,32]. It is unclear whether this represents an artifact due to the small numbers in both studies or is reflective of a real phenomenon. Higher rates of LOH of the retinoblastoma gene than were found in this study have also been previously found in HCCs from Korea, Japan, and Australia. In addition, coincident mutation of the p53 and RB1 genes has been observed in 25% of advanced HCCs from Japan and 12.9% of advanced HCCs from Australia. No coincident mutations of the p53 and RB1 genes were identified in this population of southern African blacks. For the BRCA1 gene, LOH has been reported only once, in 11.5% (3 of 16) HCCs in a Korean population. LOH of the region containing the E-cadherin gene has been reported in 64%-91% of HCCs from China

and Japan^[6,33]. No LOH in this region was found in the study by Martins *et al*^[1]. Further study of larger numbers of advanced HCCs from southern Africa will be needed to confirm the possibility of a significant geographic variation. Homozygous deletions of the tumor suppressor gene p16/CDKN2A, which is located on chromosome 9p, have been shown to be frequent in HCCs from South Korean patients (61%) but uncommon in HCCs from Australian and Japanese patients. CGH analysis of a small number of HCC cell lines derived from different geographic regions showed loss of the 9p12-14 region in all 6 of 6 HCC cell lines established in South Korea^[11]. This may reflect an association between hepatitis B virus infection, the major risk factor for HCC in Korea, and deletions of 9p.

In general, studies of different groups of patients from the same country have shown fairly high rates of variability of LOH. It appears that some patient populations have (or experimental methods result in) lower levels of LOH overall than other patient populations (or methods). In spite of this variability, there is remarkable consistency in the chromosomal locations of LOH across tumors and populations, suggesting that these are targets of the carcinogenic process of represent areas that are preferentially affected by genomic instability in HCC.

SUMMARY

Studies such as the one reported here by Martins *et al*^[1], in which limited numbers of HCCs are examined at a limited number of loci provide tantalizing clues to the potential genetic mechanisms of hepatocellular carcinogenesis, and point out areas where further work is needed. A few groups have recently reported studies with comprehensive coverage of the genome with several markers on each chromosome. However, even the most detailed of these genomic screens has used approximately twenty markers per chromosome. For chromosomes that have 100 megabases of DNA sequence, this translates into a marker every 5 megabases. Since the average gene stretches over from less than ten to a few tens of kilobases, it is clearly impossible with current technology to achieve a comprehensive mapping of all gene deletions or rearrangements occurring in HCC. However, the advent of techniques such as comparative genomic hybridization and array based genomic DNA and RNA expression technologies has greatly expanded our ability to determine the genomic and expression differences between normal, precancerous, and cancerous tissues. Studies comparing genomic and gene expression changes in enough patients from different geographic regions on whom detailed clinical information is available will be invaluable in improving our understanding of the role of particular genomic targets and changes in gene expression in the development of HCC. A number

of secular trends will also affect our ability to elucidate the path ways underlying development of HCC. First, there is a trend towards using ablative therapies and liver transplantation for treatment of HCC and away from the use of surgical resection. This trend, if it continues, may reduce the availability of tissue samples for use in research. It is therefore important that priority be given to the establishment of tissue banks and databases of clinicopathologic information on patients with HCC. Second, it is clear that few research groups have the clinical capacity and resources to independently perform the basic, translational, and clinical research initiatives that are needed to address the pressing questions of prevention, diagnosis, and treatment of HCC. There is therefore a clear need for the formation of international collaborative groups to cooperate in determining the molecular pathogenesis of HCC, to help us better understand the geographic differences in the pathogenesis of HCC, and also to collaborate in developing effective, technologically appropriate preventive, diagnostic and therapeutic alternatives. Major steps in this direction have included the development of joint projects between scientists in China, Africa and the United States and Europe and further collaborations should be actively encouraged.

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Endoscopic treatment of non-variceal gastrointestinal bleeding: hemoclips and other hemostatic techniques

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Subject headings gastrointestinal bleeding/therapy; endoscopic /therapy; hemoclip/therapy; hemostatic technique

Although the number of hospitalizations for non-variceal gastrointestinal bleeding has decreased in recent years, acute upper gastrointestinal hemorrhage continues to be a common reason for hospital admission, and peptic ulcers account for at least fifty percent of all cases. Despite the fact that bleeding from ulcer ceases spontaneously in approximately 80% of patients, it is still a diagnosis associated with substantial medical costs and significant morbidity and mortality, the latter ranging between 8 and 14%^[1], especially in the elderly.

The most common risk factors associated with upper gastrointestinal bleeding are *Helicobacter pylori* infection and the use of nonsteroidal anti-inflammatory drugs and alcohol. The eradication of *H. pylori* decreases the rate of rebleeding in peptic ulcer disease with absolute reductions that range from 2.5% to 33% according to different studies^[2]. The eradication of *H. pylori*, the increased awareness of potential dangers of nonsteroidal anti-inflammatory drugs and the widespread use of H2 receptor blockers and proton pump inhibitors in recent years account for the reduction in the number of patients hospitalized for upper gastrointestinal bleeding from ulcers^[3].

Nonetheless, no proven effective medical therapy exists for treatment of active peptic ulcer bleeding. In randomized trials, patients have demonstrated better outcomes from endoscopic therapies than from medical therapies. Emergency endoscopy should be performed as soon as safely possible after resuscitation to detect the bleeding lesion, to define stigmata of recent hemorrhage and

to perform endoscopic therapy when required. The value of endoscopic hemostatic therapy in patients with active arterial bleeding and non bleeding visible vessel has been firmly established with 75% decrease in rebleeding and operation rates and a 40% reduction in mortality^[4]. It has been shown that shock on presentation, bright red blood in the nasogastric aspirate or in the stomach that fails to clear with lavage, the number and severity of concomitant illnesses, and the age of the patient as well as the presence of rebleeding after successful endoscopic therapy all can predict rebleeding and mortality.

Lau *et al* showed that the stigmata of hemorrhage in bleeding peptic ulcers are prognostic, and that they allow the risk of rebleeding to be quantified^[5]. As a basis for the diagnosis of endoscopic appearance of lesions, the Forrest's classification is commonly used:

FIA: active bleeding (spurting)

FIB: active bleeding (oozing)

FIIA: non-bleeding visible vessel

FIIB: adherent clot

FIIC: presence of many brown or red, flat pigmentations or only one large dark area on the ulcer surface (black base)

FIII: white flat base without any signs of recent bleeding.

Unfortunately, the disagreement on the classification of ulcer features may happen in more than 25% of cases, as shown by Laine *et al*^[6]. Mondardini *et al* showed that good agreement was obtained on bleeding ulcers, both spurting and oozing^[7]. In Lau *et al* study, good agreement among international experts was obtained only on active spurters, but agreement was poor for visible vessels^[8].

Endoscopy is advocated for the treatment of active bleeding (spurting and oozing) and nonbleeding visible vessels and, in some cases, non bleeding adherent clots. Endoscopic treatment controls bleeding in up to 90% and reduces significantly the rates of further bleeding, the need for blood transfusions, hospital costs and emergency surgery. A second endoscopic treatment can be attempted in patients with further hemorrhage after the initial endoscopic therapy, and permanent hemostasis can be achieved in half of these cases.

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A variety of endoscopic treatment methods for bleeding peptic ulcers have been tested in trials. These methods include thermal application (laser, heater probe and bicap), local injection (hypertonic saline, epinephrine or various sclerosing agents such as absolute alcohol and polidocanol) and mechanical hemostasis. Each method has both merits and problems related to the hemostatic mechanism and technical procedure itself, and which one of these methods is superior remains controversial, since comparative studies have in general failed to show any superiority of one technique over another.

Although laser photocoagulation was one of the earliest forms of effective endoscopic hemostasis, currently it is being used mainly for treatment of vascular lesions or tumors, given its limitations of high cost, lack of portability and technical difficulty^[9].

Injection therapy is a low cost effective and safe procedure that is easy to implement in a variety of clinical settings. No one hemostatic agent has been shown to be clearly superior and the main difference between the available agents is probably the safety profile. Complications are less common after epinephrine injection than a sclerosant injection, the latter being associated with transmural tissue necrosis and perforation.

Heater probe and bicap achieve hemostasis through direct coaptive application of the thermal probe to the bleeding site. Thermocoagulation probes can seal arteries as large as 2mm in diameter and contain irrigation devices, which are helpful during hemostasis. Limitations include the requirement of a therapeutic endoscope, probe sticking and precipitation of treatment induced bleeding from a nonbleeding visible vessel. Repeat therapy increases the risk of perforation^[9].

Argon plasma coagulation (APC) results in evenly applied uniform surface coagulation and the noncontact mode is an advantage since there is no adhesion to the tissue. The probe can be used tangentially on lesions and the depth of tissue injury is quite superficial, minimizing the risk of perforation. Cipolletta *et al* demonstrated faster hemostasis with APC when compared to heater probe^[10], but at this time there is not enough literature to justify its routine use.

Recent studies have reported that the use of a combination of epinephrine injections and thermocoagulation for initial endoscopic control of bleeding ulcers yields significantly better results than the use of either treatment alone^[11-14].

The use of metallic endoscopic clips for hemostasis was developed in the mid 1970s. Until recently, however, due to cumbersome application of the technique, there has been limited use in the hemoclip. Improvements in design of the device

have led to increased ease of use. The bleeding vessel is ligated, achieving immediate hemostatic effect, which is definitive if the vessel is properly ligated. When compared to thermal methods and injection of sclerosing agents, both of which may cause excessive tissue injury leading to necrosis and perforation, injury to the surrounding tissue is minimized with hemoclips. Hachisu reported permanent hemostasis of upper gastrointestinal bleeding in 84.3% of 51 patient treated with hemoclips^[15]. A prospective study from Binmoeller *et al* confirmed the efficacy and safety of hemoclips (clip application device HX- 3L Olympus Corp.) to a wide range of bleeding sources. Initial hemostasis was achieved in all 88 patients, and rebleeding rate was 5%^[16]. Lee *et al* applied the hemoclip in 139 patients with a variety of bleeding sources. Recurrent bleeding was seen in 24% of patients presenting with active spurting, 4% of those with oozing and 6% of those with nonbleeding visible vessels. Permanent hemostasis was obtained in over 85% of patients with no major complications^[17]. Ohta *et al* achieved hemostasis in all 10 critically ill patients with severe gastrointestinal bleeding from spurting and oozing gastric ulcers^[18].

In this present uncontrolled prospective study, Lai *et al* used a new rotatable clip device (HX-5LR-1, Olympus) for the application of hemoclip (MD 850, Olympus) to 40 patients with active bleeding (spurting or oozing) from peptic ulcers. The overall hemostatic rate was 93%, hemostasis failed in two patients due mainly to the location of the bleeding source, which made clipping difficult to perform. Rebleeding after hemoclip treatment occurred in three cases secondary to dislodging of clips associated with difficult to approach location when applying the clips in two patients and use of anticoagulants in the other. Hemoclip treatment reduced the rates of rebleeding to 15% in the spurting group and 4% in the oozing group. The study did not include non bleeding visible vessels and did not compare the efficacy of this new improved hemocliping device to other forms of endoscopic therapy.

Villanueva *et al* compared injection with epinephrine alone versus injection combined with hemoclip in a randomized study with 78 patients with peptic ulcer bleeding. Rebleeding occurred in only 5% of patients with hemoclip and injection compared with 19% in the group with injection therapy alone^[19].

Takahashi *et al* have reported that hemocliping is as effective as the pure ethanol injection or heater probe methods in controlling the bleeding from primary gastric lesions^[20].

Nagayama *et al* recently compared the efficacy of endoscopic clipping with topical ethanol injection

in a retrospective study. Endoscopic clipping was performed using the clipping devices Olympus HX-3L or HX-5LR and clips Olympus MD-750. Endoscopic clipping improved therapeutic outcome as determined by rebleeding rates, need for blood transfusion and duration of hospital stay^[21].

These studies demonstrate the efficacy and safety of hemoclips. The advantage of clips is that they do not result in chemical or electrical damage to the surrounding tissue, do not appear to impair healing of ulcers, the hemostatic rate is good and similar to other methods and recurrent bleeding and complication rates are low. There are virtually no limitations to the number of clips to be applied, and therefore, the technique can be used repeatedly.

Because hemoclippping is a local form of mechanical hemostasis, it cannot be performed without identification of the bleeding point. Loading of the clip onto the application device is cumbersome and time consuming. It is usually technically more difficult to clip a vessel when the angle of approach is tangential (upper two thirds of the posterior wall or the lesser curvature of the gastric body and the posterior wall of the duodenal bulb). This technical difficulty persisted even in the rotatable clip device used by Lai *et al.*

Besides hemostasis of peptic ulcers, clips can also be applied to Dieulafoy's lesions^[22], colonoscopic diverticular bleeding^[23], Mallory-Weiss tears, postpolypectomy bleeding, post sphincterotomy bleed^[16] and gastric cancers. Radiopaque clipping serves as a good landmark for radiologists to identify the bleeding point during angiography when clipping fails in hemostasis. The clips can also serve as markers for proper esophageal stent placement.

In summary, endoscopic hemoclip treatment provides an effective and safe modality for hemostasis in gastrointestinal bleeding. Controlled prospective studies comparing hemoclip with other endoscopic methods are still required before this modality becomes widely used.

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Post-gastrectomy patients need to be followed up for 20-30 years

Tovey FI and Hobsley M

Subject headings gastrectomy; nutrition; iron; vitamin B₁₂; vitamin D

Abstract

AIM To investigate the incidence and management of nutritional deficiencies following a gastrectomy.

METHODS A gastrectomy population of 227 patients in London was followed up for 30 years after operation to detect and treat nutritional deficiencies.

RESULTS By the end of the first decade iron deficiency was the commonest problem. Vitamin B₁₂ deficiency became more important in the second decade. During the third decade both reached equal prevalence, being found in some 90% of the female and 70% of the male residual population. Vitamin D deficiency was a lesser problem, reaching its climax in the second decade. Overall, all women fared worse than men.

CONCLUSION The importance of long-term follow-up of gastrectomy patients for iron, Vitamin B₁₂ and Vitamin D deficiencies is emphasised.

INTRODUCTION

In 1981 and 1984, through the courtesy of The Chinese Academy of Medical Sciences, the first author visited centres in the north and south of China to gather information about the prevalence of duodenal ulcer and its relationship to the staple diets. It was noted that the standard operation for duodenal ulcer in many centres was either a Billroth II (gastrojejunal) or a Billroth I (gastroduodenal anastomosis) gastrectomy (Figure 1). This raises the possibility that at the present time there might be a gastrectomy population in China of 25-30 years standing, who may have developed nutritional disorders as a result of their operation. Our experience with the study of patients 25-30 years after gastrectomy and on a Western diet may serve as a guide to the frequency of these problems.

MATERIALS AND METHODS

Patients

We report the outcome of a longitudinal study in the UK. The study was performed at University College Hospital in London on patients who underwent a gastrectomy between 1955 and 1960^[1]. In 1969 contact was made with 227 patients, and although the number diminished from movement elsewhere or deaths, the remainder were followed up regularly until 1990. The population included 186 patients who had undergone a Billroth II gastrectomy (male 141, female 45) and 41 who had undergone a Billroth I gastrectomy (male 24, female 17). After an interval of 10-15 years following the operation they were screened annually, or more often when indicated, and the following investigations were made to detect possible nutritional disorders^[1].

Method

Clinical investigation (on first attendance) The patients were weighed. Compared with the patient's ideal pre-operative weight, a loss of up to 4.5kg was regarded as moderate loss and a greater loss as severe.

A record was made of any post-prandial symptoms including reduced capacity for food, early dumping and late dumping. A moderately reduced capacity was regarded as being able to take one-half of what the patient would normally expect to eat at a meal and severe as one third or less. A record was made of those with a reduced capacity who showed

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discomfort or vomiting if the amount was exceeded. Early dumping consisted of weakness, fainting, sweating and palpitation 10-20 minutes after food. Those with late dumping had similar symptoms occurring about 30-60 minutes after the end of the meal.

Persistent diarrhoea was described as moderate if there were up to 3 loose stools a day and as severe if more. All of the 9 patients with diarrhoea had 24-hour faecal fat estimations and also as many of the other patients who were willing (total 158). A faecal fat output 6g/day-12g/day was regarded as a moderate steatorrhoea and above 12g/day as severe.

Nutritional deficiencies

Iron deficiency Full blood count included blood picture, serum iron and total iron binding capacity (TIBC). Iron deficiency was defined as an iron saturation (serum iron/total iron binding capacity) below 16%.

Vitamin B₁₂ deficiency Vitamin B₁₂ deficiency was diagnosed when two separated bioassays repeated one month apart showed a value of less than 110pmol/L.

Vitamin D deficiency Serum calcium, phosphate and alkaline phosphatase. The first sign was a rising serum alkaline phosphatase estimation. If this was found, liver function tests were done to exclude a hepatic cause. Other causes such as Paget's disease, a recent fracture or bony secondaries were excluded. A 24 h urinary calcium output below 2mmol/24 h supported the diagnosis^[2]. A therapeutic trial of calcium and vitamin D was then given as a diagnostic measure and a sustained fall in serum alkaline phosphatase levels gave confirmation of the diagnosis. Calcium and vitamin D BPC tablets (calcium lactate 300 mg, calcium phosphate 150mg, calciferol 12.5 µg), were given in a dose of 2 tablets, 4 times a day, and the dose was reduced to 2 tablets, 3 times a day when the serum alkaline phosphatase levels fell to normal.

Osteoporosis Until 1989 the right second metacarpal had been X-rayed and measurements taken from the X-ray of the second right metacarpal were used to calculate the Exton-Smith Index:

$$\frac{T^2 - M^2}{TL}$$

where T is the thickness of the bone, M is the medullary thickness at the mid-point and L is the overall length^[3].

After 1989 dual energy X-ray absorptiometry (DEXA) became available and was used to screen the remaining population. Only males were chosen because by then, all the female patients were

postmenopausal, introducing another potential factor for osteoporotic changes.

Statistics Statistical analysis was done using the Student *t* test or Fisher's exact test as appropriate.

RESULTS

Clinical findings

At the first follow-up consultation in this study 66 (29%) of the 227 patients had a moderate and 15 (7%) a severe loss of weight. 107 (47%) patients complained of a reduced capacity for food, which was severe in 41 (18%)

Early dumping was diagnosed in 39 (17%) and late dumping in 7 (3%). Persistent diarrhoea occurred in 9 of the 186 Billroth II patients (being severe in 1) but in none of the 41 with a Billroth I gastrectomy. The difference was not significant (Fisher's exact test $P = 0.2089$). Five of these 9 patients had moderate and 2 severe steatorrhoea.

Moderate steatorrhoea was found after both operations [Billroth I, 9 (24%) of 37; Billroth II, 14 (12%) of 121; not significant, $P = 0.2292$].

However, severe steatorrhoea only occurred after the Billroth II procedure [32 (26%) of 121, this was significantly different from the zero incidence in the Billroth I group, $P < 0.0001$].

Nutritional findings (including management)

Iron deficiency. The first sign was a rising TIBC, which often preceded a fall in serum iron by several months. Actual iron-deficient anaemia developed about 6 months later.

Ferrous gluconate was found to be well tolerated and the patients were given 300mg thrice daily until the iron deficiency was corrected and then a maintenance dose of 300mg daily.

The prevalence of iron deficiency is shown in Table 1. In the men the prevalence was significantly higher in those showing weight loss ($P < 0.02$) or reduced capacity for food ($P < 0.05$), but these differences were not seen in the women.

Vitamin B₁₂ deficiency In most patients a fall in serum B₁₂ concentration preceded any macrocytosis, neutrophil shift or anaemia. Patients were treated by intramuscular injections of 1000 µg hydroxocobalamin in alternate months. The prevalence in the remaining population is shown in Table 1. It can be seen that iron deficiency occurred much earlier than B₁₂ deficiency, appearing in many patients during the first 10 years after operation. Vitamin B₁₂ deficiency developed mostly 10-20 years after operation and its prevalence slowly increased to equal that of iron deficiency by the end of 25-30 years, when approximately 70% of men and 90% of women had developed either iron or B₁₂ deficiency, the deficiencies being combined in 51% and 70%, respectively.

Vitamin D deficiency Vitamin D deficiency occurred in 7.5% of Billroth II and 7.3% of Billroth I gastrectomies and was predominantly a problem of female patients. (F:M=19%:4%). It became apparent in many patients during the first 10 years after operation (Table 1). Of those investigated, 50% had severe and 28% moderate steatorrhea as compared with 20% and 14% respectively for the whole series.

Osteoporosis Osteoporotic changes in excess of normal ageing were seen in 24%, 20% and 22% of men and in 35%, 51% and 86% of women in 1969, 1974 and 1982, respectively. None of these had evidence of vitamin D deficiency. These measurements, however, were not sensitive enough to monitor any treatment over a short term^[4].

Dual energy X-ray absorptiometry was used in 16 active male patients, with no evidence of vitamin D deficiency, who were still attending the clinic. Six (37.5%) were found to have reduction of bone mineral density of the lumbar spine and upper left femur of more than 2 standard deviations. Initially they were treated with a calcium supplement (microcrystalline hydroxyapatite) 16g/day-32g/day and calciferol 0.25 mg daily but with no response. Following this they were given intermittent cyclical etidronate 400 mg nightly for 2 weeks, followed by calcium carbonate equivalent to 500mg calcium daily for 10 weeks. This 12-week cycle was repeated over 2 years. Only 2 patients responded with a return to within the normal range of values. So far no totally satisfactory treatment has been reported for postgastrectomy osteoporosis^[5].

Billroth I versus Billroth II gastrectomies There was no significant difference in overall, moderate or severe weight loss between the two operations in women (BI6/12:BI26/45, $P = 0.1567$). In men, although there was no significant overall difference in weight loss (BI10/24:BI37/141, $P = 0.1438$), significantly more patients showed a moderate weight loss after a Billroth II gastrectomy (BI1/24:BI31/141, $P = 0.0491$), by contrast more showed severe weight loss after a Billroth I procedure (BI9/24:BI6/141, $P < 0.0001$).

There was no significant difference with regards to capacity for food, early or late dumping. The difference in persistent diarrhoea was not statistically different, but in severe steatorrhea the difference between the two operations was significant (BI0/37:BI32/121, $P < 0.0001$).

No difference between the two operations was found in the incidence of nutritional deficiencies.

Sex differences Women on the whole fared less well than men. They had significantly more overall weight loss (F26/45:M37/141, $P = 0.0002$) after a Billroth II operation. There was no significant

difference in severe loss, but the difference in moderate loss was significant (F21/45:M31/141, $P = 0.0021$). Overall, they showed a significant difference in reduced capacity for food (F43/62:M64/165, $P < 0.0001$) and much more women showed a severely reduced capacity (F20/62:M21/165, $P = 0.0016$). Early dumping was more common in women than in men after the Billroth II operation (F15/45:M5/141, $P < 0.0001$). More women complained of discomfort and vomiting, if the irreduced intake was exceeded, after a Billroth II (F15/45:M10/141, $P < 0.0001$). They also showed more aversions to vitamin D containing food such as butter, cream, milk and eggs. Women fared worse with regards to the incidence of iron and vitamin B₁₂ deficiencies and more markedly in the occurrence of vitamin D deficiency (Table 1).

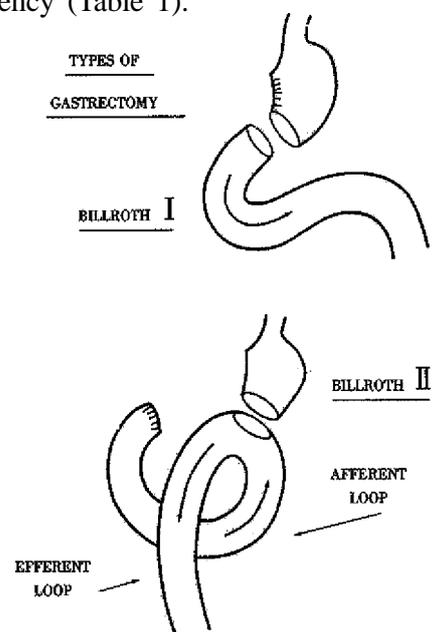


Figure 1 Types of gastrectomy.

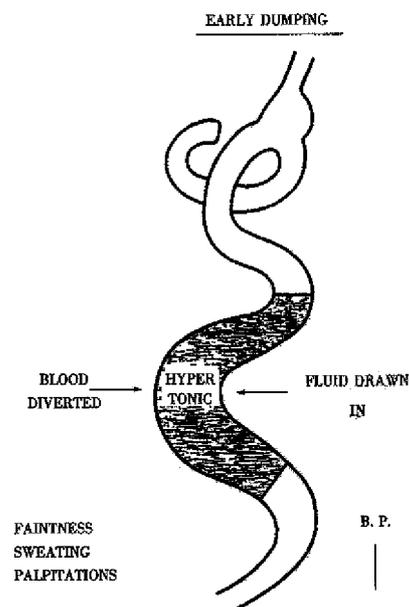


Figure 2 Early dumping.

Table 1 Prevalence of iron, B₁₂ or vitamin D deficiency in male and female patients

Parameter and sex	1969	1978	1984	1988
Gastrectomy population				
Male	165	99	59	40
Female	62	42	20	12
Iron deficiency %				
Male	31.5 ^a	61.6	62.7	67.5
Female	61.3	59.5	80.0	91.7
B ₁₂ deficiency %				
Male	3.0	19.2	59.3 ^b	70.0
Female	0	28.6	90.0	83.3
Vitamin D deficiency %				
Male	1.8 ^c	4.0 ^d	1.7 ^e	0 ^f
Female	11.3	26.2	25.0	33.3

^a*P* < 0.0001, ^b*P* = 0.0131, ^c*P* = 0.0005, ^d*P* = 0.0003,

^e*P* = 0.0039, ^f*P* = 0.0018, males vs females.

DISCUSSION

Several factors^[6-11] contribute to nutritional disorders after a gastrectomy. With the loss of the pyloric sphincter there is uncontrolled gastric emptying and the capacity for food becomes dependent on the ability of the small intestine to accommodate the meal. The rapid emptying stimulates peristalsis and there is rapid passage of food through the small intestine. Small molecules such as those of sugars and starches which are rapidly broken down in the small intestine, produce a severe osmotic effect which leads to the drawing into the gut of extracellular fluid amounting to 2-3 litres, resulting a fall in plasma volume and rise in haematocrit. This ingress of liquid distends the gut and there may be early satiety and reduced capacity for food. When the fall in plasma volume exceeds 7%, certain patients will develop early dumping with hypotension according to their vascular tolerance (Figure 2). In other patients the rapid absorption of glucose from the intestine leads to an oversecretion of insulin followed by hypoglycaemia and the symptoms of late dumping.

The increased water content of the material entering the large intestine may give rise to diarrhoea unless the colon is able to absorb the fluid. The presence of undigested sugars and starch may also act as irritants. The rapid passage of food through the small intestine results in a reduced mixing with the pancreatic and intestinal enzymes. This leads to impaired digestion and absorption of proteins and fats as shown by the presence of steatorrhoea in some patients. Short circuiting of the duodenum in the Billroth II operation (Figure 1) may cause pancreatic juices to lag behind the food as manifested by the presence of severe steatorrhoea in this group.

Absorption of vitamin D is dependent on fat solubility and the combination of steatorrhoea and reduced vitamin D intake may lead to vitamin D deficiency. As mentioned many patients, especially women, develop a selective aversion to certain food, particularly sources of vitamin D: this would explain the increased incidence of vitamin D deficiency in this group.

Iron metabolism may also be impaired. The intake of iron-containing foods may be reduced. Much of the intake is in the form of ferric iron or of iron

combined with protein. Acid is needed to convert ferric iron to ferrous, acid and pepsin are needed to convert organic to inorganic iron. Both acid and pepsin are reduced by a gastrectomy. In addition, most of the iron is absorbed in the duodenum and upper jejunum.

Vitamin B₁₂ deficiency may also develop. One factor is loss of the intrinsic factor that had been secreted by gastric mucosa removed by the gastrectomy. Rapid passage through the small intestine leads to less absorption.

Calcium absorption also occurs principally in the duodenum and upper jejunum and is impaired by intestinal hurry and loss of duodenal continuity. In addition, in the presence of steatorrhoea, calcium absorption is further impaired by the formation of insoluble calcium soaps.

As a result of all these factors, postgastrectomy patients may develop iron deficiency anaemia, vitamin B₁₂ deficiency anaemia, vitamin D deficiency and osteomalacia, or osteoporosis in excess of normal ageing.

Conclusion This study in particular demonstrates the increasing prevalence of iron and vitamin B₁₂ deficiency in a population after gastrectomy, reaching approximately 75% in 20-30 years. This stresses the increasing importance with passing years of regularly monitoring iron saturation and B₁₂ levels. In addition, the increased serum alkaline phosphatase levels may indicate vitamin D deficiency and need to be investigated. Now that gastrectomy is rarely performed for peptic ulcer it is important to remember that there is still a large number of patients who underwent gastrectomy 20-30 years ago and are at risk of developing nutritional deficiencies.

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Elevated basal intestinal mucosal cytokine levels in asymptomatic first-degree relatives of patients with Crohn's disease

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Subject headings crohns disease; cytokine/analysis; intestinal mucosa

Abstract

AIM To determine levels of cytokines in colonic mucosa of asymptomatic first degree relatives of Crohn's disease patients.

METHODS Cytokines (Interleukin (IL) 1-Beta, IL-2, IL-6 and IL-8) were measured using ELISA in biopsy samples of normal looking colonic mucosa of first degree relatives of Crohn's disease patients ($n = 9$) and from normal controls ($n = 10$) with no family history of Crohn's disease.

RESULTS Asymptomatic first degree relatives of patients with Crohn's disease had significantly higher levels of basal intestinal mucosal cytokines (IL-2, IL-6 and IL-8) than normal controls. Whether these increased cytokine levels serve as phenotypic markers for a genetic predisposition to developing Crohn's disease later on, or whether they indicate early (pre-clinical) damage has yet to be further defined.

CONCLUSION Asymptomatic first degree relatives of Crohn's disease patients have higher levels of cytokines in their normal-looking intestinal mucosa compared to normal controls. This supports the hypothesis that increased cytokines may be a cause or an early event in the inflammatory cascade of Crohn's disease and are not merely a result of the inflammatory process.

INTRODUCTION

Numerous studies have proven that cytokines play an integral role in the pathogenesis of inflammatory bowel disease (IBD). These protein mediators have been shown to regulate the immune response, induce tissue injury, and mediate complications such as fibrosis and obstruction in patients with IBD^[1]. In our institution, we have measured cytokines IL-1B (Interleukin-1 Beta), IL-2, IL-6, and IL-8 from mucosal biopsies obtained during colonoscopy in patients with IBD and other colitides to see if there is any predictive pattern of cytokine elevation^[2-5]. Since there is evidence for a genetic predisposition in IBD^[6-10], we were also interested in determining if asymptomatic first-degree relatives of Crohn's disease (CD) patients exhibit elevated intestinal mucosal cytokine levels as well. If they do, is there any pattern to the cytokine elevations?

To our knowledge, there are no studies examining specific cytokine levels in first-degree relatives. Some studies suggest there is increased intestinal permeability in healthy relatives of CD patients^[11-14], pointing to a possible genetic defect in these patients. This study may add that even in asymptomatic relatives, there is evidence of subclinical expression of the disease at a cellular level. Elevated cytokine levels would also support the hypothesis that cytokines are a cause or an early event in the inflammatory cascade of CD and not a result of the inflammatory process, since these patients have no pathological signs or symptoms of disease.

MATERIALS AND METHODS

After obtaining prior approval of the Institutional Review Board in our hospital, we studied ten people without a family history of IBD who acted as controls, and nine first-degree relatives of patients with Crohn's disease, who did not exhibit signs or symptoms referable to CD.

The control patients included four males and six females ranging in age from 40 - 82 years (mean-64.3). Each patient underwent a routine colonoscopy. (Olympus, Lake Success, NY) after appropriate consent. Table 1 shows further data regarding the group. These patients underwent colonoscopy for causes other than IBD such as screening, Guaiac positive stools, abdominal pain (Table 1).

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Eleven first-degree relatives were initially studied but only nine were used in the final analysis. Two relatives were excluded as one had a prior history of radiation proctitis and the other had a prior sigmoid biopsy consistent with nonspecific inflammation. These relatives included two males and seven females ranging in age from 21-79 years (mean-50.1). Two relatives were from the same family (i.e. brother and sister of a CD patient). Otherwise, the relatives were unrelated to each other. After obtaining informed consent each underwent a flexible sigmoidoscopy (Olympus) upto splenic flexure without a prior bowel prep. (Refer to Table 1). There was one patient in this group with abdominal pain and weight loss who was diagnosed with depression and anxiety but no pathologic condition was found. Also, as shown in the table, endoscopic findings were of minimal significance in both groups.

Demographic data of the eight CD patients (the index cases of the first-degree relatives) were analyzed and compared (Table 2).

Table 1 Study population: demographic data

Clinical data	Control group	First degree relative
Number (n)	10	9
Mean age (Range)	64.3 (40-82)	50.1 (21-79)
Males	4	2
Females	6	7
Indications		
Anemia	1	None
Abdominal pain	1	Pain/Weight loss (1)
Guaiac (+)	3	None
Surveillance	5	None
Study	0	3
IBS	0	3
Endoscopic findings		
Normal	5	6
Hemorrhoids	3	3
Polyps	2	None
Family His of colitis	None	All
History of IBD	None	None

Table 2 Demographics of Crohn's disease patients (n = 8)*

Clinical data	Number
Mean age	44 (26.93)
Males	2
Females	6
Extent of the disease	
Ileum and colon	5
Ileum alone	2
Colon alone	1
Duration of the disease	
<10yrs	1
10-30yrs	6
>30yrs	1
Present medications	
6MP, steroids, ASA	4
ASA, steroids	2
ASA	1
None	1
History of Surgery	
Yes	6
No	2
Number of first degree relatives	
One relative	7
Two relatives	1

ASA = Aminosalicic acid; 6MP-6 Mercaptopurine

*One of the patients had two first-degree relatives.

Tissue collection and analysis

Three mucosal biopsies were obtained from the sigmoid or descending colon in each patient in both groups. The biopsy samples, weighing between 15 mg and 30 mg each, were immediately wrapped in aluminum foil, placed in a container of liquid nitrogen and stored at -70°C entigrade until they were processed. Tissue was crushed and homogenized in diluent from IL-kits (Quantikine, R & D systems) for 30 seconds, then centrifuged for 15 minutes at 3000 r/min. The supernatant fluid was used for assaying interleukins. Histologic evaluation was only performed if endoscopic abnormalities were noted as it would have, otherwise, delayed the procedure.

A solid phase ELISA using Quantikine kits (Research and Diagnostic Systems, Minneapolis, MN, USA) was used to measure IL-1B, IL-2, IL-6, and IL-8. Results were expressed as mean \pm SEM. The Quantikine method had a sensitivity of 0.03 pg-0.08 pg and a standard curve linearity of 0.03 pg to 3.0 pg. A Student's independent test was used to compare data from the two groups and P value <0.05 was considered as statistically significant.

RESULTS

The concentrations of IL-2, IL-6, and IL-8 were significantly ($P < 0.05$) higher in the colonic mucosa of first-degree relatives (3.40 ng/g \pm 0.56 ng/g, 1.19 ng/g \pm 0.21 ng/g, 11.98 ng/g \pm 2.62 ng/g, respectively, Figure 1) when compared to controls (1.86 ng/g \pm 0.30 ng/g, 0.69 ng/g \pm 0.01 ng/g, 5.41 ng/g \pm 1.87 ng/g, respectively). No significant difference was found in the concentration of IL 1B between relatives (0.85 ng/g \pm 0.17 ng/g) and controls (1.03 ng/g \pm 0.15 ng/g).

Individually, seven of the nine relatives had significantly higher levels of IL -2 and IL-6 and six relatives had significantly higher IL-8 levels than the mean value of controls. Interestingly, mean IL-1B and IL-6 levels of the two relatives from the same family (0.23 ng/g \pm 0.09 ng/g and 0.21 ng/g \pm 0.09 ng/g, respectively) were significantly lower ($P < 0.05$) than that of the other relatives (1.03 ng/g \pm 0.16 ng/g and 1.47 ng/g \pm 0.14 ng/g, respectively).

We also compared various subsets of the CD index cases to see if there were any differences in the corresponding first-degree relative cytokine levels (Table 2). In analyzing age, severity, duration or extent of disease, medications, and history of surgery, there were no significant differences between the subsets of the relative group in any category.

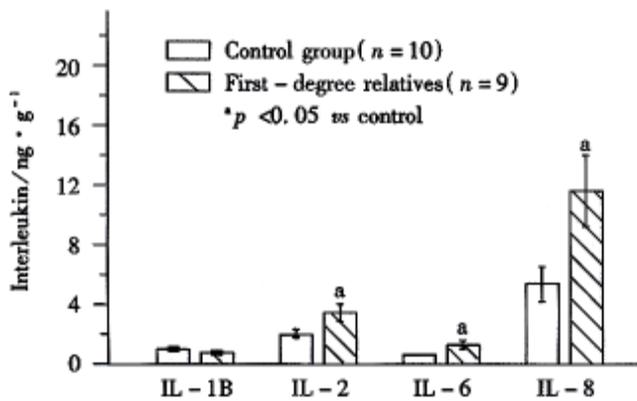


Figure 1 Interleukin levels of first-degree relatives and controls.

DISCUSSION

There is evidence that inflammatory bowel disease is determined by genetic predisposition. Supporting data include familial aggregation^[6,15] and an increased concordance rate in monozygotic twins compared with dizygotic twins^[16]. CD patients have a more frequent positive family history than ulcerative colitis (UC) patients^[17]. Relatives of CD patients have a higher risk of IBD than those of UC patients^[18,19]. There is also a higher concordance rate for CD than for UC among either monozygotic or dizygotic twins^[16]. This suggests that hereditary predisposition may play a more important role in CD than UC.

Genetic studies have used subclinical markers in unaffected family members of IBD to either indicate the genetic abnormality predisposing to a disease or identify those in whom a subclinical phase of the disease process is occurring. These include serum antibodies (i.e. ANCA^[20,21], antibodies to viruses, bacteria, mycoplasma, food antigens^[22], and colonic epithelial cells^[23]), colonic mucin abnormalities^[24], obligate anaerobic fecal flora^[25], mucosal production of IgG subclasses^[26], and C₃ dysfunction^[27]. Abnormal elevations of these markers have been seen in healthy relatives of either CD or UC patients or both.

Most studies on relatives of CD patients have analyzed intestinal permeability in patients with CD and their first-degree relatives by measuring urinary excretion of poorly absorbed, water-soluble compounds such as polyethylene glycol (PEG-400) and large sugars, including lactulose, mannitol, and rhamnose^[28], 51 Cr-labelled ethylene diaminetetraacetic acid (EDTA)^[29] and luminal prostaglandin^[30] and hyaluronin^[31] release. Although initial studies have shown increased permeability in patients with CD^[29,32-34] and their first-degree relatives^[35,36], several additional studies^[37-39] have been inconsistent, showing no increased permeability in either group.

Hollander^[40] and May *et al.*^[41] in 1993,

reviewed earlier studies and found that most studies showed a significant increase in permeability in 10% of first-degree asymptomatic relatives as it is possible that only a subgroup of relatives of CD patients are genetically susceptible. A recent study^[42] has confirmed this by showing that the subset of asymptomatic relatives with increased intestinal permeability had increased CD45 (common leukocyte antigen) isoform in peripheral blood cells similar to CD patients. These findings suggest that the permeability defect in CD patients may serve as a subclinical and genetic marker for CD and is not secondary to intestinal inflammation^[34] or may indicate early intestinal damage (preclinical expression).

Our study is the first to our knowledge to determine cytokine levels, known to be elevated in the colonic mucosa of CD patients and in asymptomatic relatives of CD patients. The fact that IL-2, IL-6, and IL-8 were significantly elevated suggests a possibility of early intestinal damage in the relatives group similar to the studies of increased permeability in these patients. Although the number of cases were small ($n = 9$), six of the nine patients had significant elevations of all three cytokines (IL-2, IL-6, and IL-8) versus controls (the others had significant elevations of at least one or two of these cytokines). Whether a subset of these relatives are actually more predisposed to the disease than controls, as suggested in the permeability studies, has yet to be known. It is also of interest that we could not find any significant differences in cytokine levels between subsets of the relatives with regard to the various parameters of the index CD patients (listed in Table 2). The fact that the two relatives from the same family had significantly lower levels than the rest of the group is of questionable value. Future studies including more patients and the evaluation of other cytokines would be of interest and would help in determining the significance of these alterations in this group.

This study shows that asymptomatic relatives of CD patients have increased levels of cytokines in normal-looking colonic mucosa. Studies with intestinal permeability have been inconsistent. It may be possible that increased cytokines are an early event in the pathogenesis of the disease. Whether these increased cytokines lead to increased permeability and then to CD is something that needs further investigation.

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Endoscopic hemoclip treatment for bleeding peptic ulcer

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See invited commentary on page 42

Subject headings endoscopic hemoclippping; gastrointestinal hemorrhage/therapy; peptic ulcer; hemostasis

Abstract

AIM To evaluate the efficacy of endoscopic hemoclip in the treatment of bleeding peptic ulcer.

METHODS Totally, 40 patients with F1a and F1b hemorrhagic activity of peptic ulcers were enrolled in this uncontrolled prospective study for endoscopic hemoclip treatment. We used a newly developed rotatable clip-device for the application of hemoclip (MD850) to stop bleeding. Endoscopy was repeated if there was any sign or suspicion of rebleeding, and re-clipping was performed if necessary and feasible.

RESULTS Initial hemostatic rate by clipping was 95%, and rebleeding rate was only 8%. Ultimate hemostatic rates were 87%, 96%, and 93% in the F1a and F1b subgroups, and total cases, respectively. In patients with shock on admission, hemoclippping achieved ultimate hemostasis of 71% and 83% in F1a and F1b subgroups, respectively. Hemostasis reached 100% in patients without shock regardless of hemorrhagic activity being F1a or F1b. The average number of clips used per case was 3.0 (range 2-5). Spurting bleeders required more clips on average than did oozing bleeders (3.4 versus 2.8). We observed no obvious complications, no tissue injury, or impairment of ulcer healing related to hemoclippping.

CONCLUSION Endoscopic hemoclip placement is an effective and safe method. With the improvement of the clip and application device, the procedure has become easier and much more efficient. Endoscopic hemoclippping deserves further study in the treatment of bleeding peptic ulcers.

INTRODUCTION

Upper gastrointestinal bleeding is a frequently encountered clinical problem for both the endoscopist and surgeon^[1]. Acute hemorrhage from duodenal and gastric ulcers stops spontaneously in approximately 70% to 80% of cases^[2]. The remaining patients represent a high risk group requiring prompt identification and treatment to improve the high morbidity and death rate. The consensus is that patients who have peptic ulcer with spurting or oozing hemorrhage need active treatment^[3,4]. The death rate of patients with persistent or recurrent bleeding is 12% to 18%^[5-8].

There have been many endoscopic techniques, including thermal application (laser, heater probe, and bicap) and local injection (hypertonic saline, epinephrine, and ethanol), advocated effectively for the control of gastrointestinal bleeding^[9]. But, their results vary depending on the operators or the patients^[10,11]. Although the success rate of initial hemostasis is high, rebleeding has been reported to occur in 10% to 30% of patients^[12-14]. Thermal methods and injection of hemostatic agents can cause tissue injury leading to necrosis and possible perforation^[15-17]. Mechanical hemostasis by the application of a metal hemoclip to a bleeding vessel is an appealing alternative to the currently available techniques. It was first introduced in 1975 by Hayashi *et al*^[18]. However, the initial experience was discouraging because of its complexity and low retention rate. In 1988, Hachisu introduced a modified hemoclip for upper gastrointestinal hemorrhage with a permanent hemostatic rate of 84.3%^[19]. A newly improved rotatable clip-device with better grasping capability has been developed recently, which can make the procedure easier and save much time^[20]. We therefore used this newly improved metallic clip to prospectively evaluate its role in the hemostatic effect on bleeding peptic ulcers.

MATERIALS AND METHODS

From January 1997 to December 1998, totally 40 hospitalized patients were enrolled. All patients were proved to have active hemorrhage from peptic ulcer by endoscopic examination at Cathay General Hospital. All cases had F1a (spurting) or F1b (oozing) hemorrhagic activity by Forrest classification^[21]. Patients who had multiple bleeding sites or gastric cancer were excluded. Since active hemostatic treatment has been recommended

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for acute bleeding from the upper gastrointestinal tract^{3,4}, the present study did not include a control group which was treated conservatively.

Endoscopies were carried out using an Olympus GIF-XQ 200 endoscope (Olympus Corp., Tokyo, Japan). We used a hemoclip (MD 850, Olympus) device, which has a new rotatable clip-device (HX-5LR-1, Olympus) for hemostasis. The hemoclip was applied directly to the bleeding vessel. Vessels traversing the surface were clipped at both ends of the bleeding point. Initial hemostasis was defined as no bleeding from the ulcer for at least 5 minutes.

Patients were treated with intravenous H₂-blockers and oral antacid suspensions after endoscopic examination. A nasogastric tube was inserted to observe the bleeding condition. Blood transfusion was given if the hemoglobin level dropped to less than 90 mg/L, or if vital signs deteriorated.

Shock was defined as systolic blood pressure less than 13.3 kPa (100 mmHg) and a pulse rate greater than 100 beats/min, accompanied by pallor, cold sweating, and oliguria. Rebleeding or recurrent bleeding was defined as blood in the stomach 24 h after treatment, presence of unstable vital signs, and continued tarry or bloody stools, or hematemesis after treatment.

Endoscopic examination was performed again whenever there was evidence or suspicion of rebleeding. Hemoclips were used again if necessary and feasible. Surgical intervention was considered if re-clipping still could not control the bleeding. Ultimate hemostasis was defined as lack of rebleeding for 7 days after treatment. Follow-up endoscopy was performed 1 week after initial hemoclip treatment if patients agreed.

We used Student's *t* test or Wilcoxon 2-sample test for the analysis of continuous variables. Chi-square test and Fisher's exact test were applied for the analysis of nominal variables. A *P* value of less than 0.05 was considered significant.

RESULTS

Totally, 40 patients were included in this study over a 2-year period. There were 29 males and 11 females with a mean age of 62.3±3.3 years (range 26-85 years). Locations of peptic ulcers and their hemorrhagic activity by Forrest classification are listed in Table 1.

Two of our 40 cases failed to terminate hemorrhage in response to hemoclips. One failed case was due to torrential active bleeding from the posterior wall of the mid-gastric body, which prevented treatment feasibility. This patient subsequently received surgical intervention. Another case with F1a activity had hemorrhage from the lesser curvature of the high gastric body, which made clipping difficult to perform, and we were unable to clip this area efficiently. The patient

could not undergo surgery due to end-stage renal disease and sepsis, and he expired in spite of treatment. Rebleeding after hemoclip treatment occurred in three cases. The first patient had chronic liver disease, diabetes mellitus, and renal insufficiency. He had a gastric ulcer (F1b) on the posterior wall of the high body, where the application of clipping was difficult. Massive hemorrhage occurred 2 days after the first endoscopic clipping. Emergent operation was performed and clips were found to have dislodged during the operation. The second patient underwent anti-coagulant treatment for rheumatic heart disease. He had a gastric ulcer (F1a) on the lesser curvature of the antrum. Follow-up endoscopy revealed active oozing from the edge of the clips. Re-clipping successfully stopped the hemorrhage. The third case had a duodenal ulcer (F1a) on the posterior wall. Follow-up endoscopy revealed that the clip had dislodged. The rebleeding rates of F1a and F1b subgroups were 15% and 4%, respectively (Table 2). This difference is not statistically significant.

The final results of our study are shown in Table 3. The overall hemostatic rate was 93%. In patients with F1a ulcers, the rate was 87%, and in F1b ulcers was 96%; the difference between them is not significant. Two cases received emergent operation, and their post-operative courses were smooth and uneventful. Mortality occurred in only one patient who had serious underlying disease (end-stage renal disease and sepsis) and was unable to receive surgical intervention.

Table 4 shows the relationship between shock and the rebleeding rate. After hemoclippping, the rebleeding rate in the F1a shock subgroup was 20%, a rate not different from that 13% in the F1a non-shock subgroup. In the F1b group, the rebleeding rates did not differ between the shock and non shock groups (17% versus 0%, *P* = 0.07). In those patients without shock, the hemostatic rate was 100% in both F1a and F1b subgroups. In patients with shock, the hemostatic rates after hemoclippping were 71% in F1a cases and 83% in F1b cases (Table 5).

The number of clips used per patient in each subgroup is shown in Table 6. The average number of clips used in all cases was 3.0 (range 2-5). The average number was higher in the F1a subgroup (3.4, including gastric and duodenal ulcers) than in the F1b subgroup (2.8, including gastric, duodenal, and marginal ulcers, *p* = 0.04 by Wilcoxon 2-sample test). The number of clips (including F1a and F1b) did not differ between gastric and duodenal ulcers. We also tried to analyze the difference of clip number used in various locations of gastric and duodenal ulcers, but were unable to reach any definite conclusion because of limited case numbers.

Table 1 Number of patients by ulcer type and Forrest classification

Type	F1a	F1b
Gastric ulcer	10	14
Duodenal ulcer	5	9
Marginal ulcer	0	2
Total	15	25

Table 2 Number of rebleeding cases in each subgroup

Type	F1a	F1b
Gastric ulcer	1/8 ^a	1/14
Duodenal ulcer	1/5	0/9
Marginal ulcer	0/0	0/2
Total	2/13 ^b	1/25

Data are expressed as rebleeding/subgroup case number. ^aTwo cases without initial hemostasis are excluded. ^b $P = 0.27$.

Table 3 Outcome of endoscopic hemoclip treatment

Treatment	F1a (n = 15)	F1b (n = 25)	Total (n = 40)
Ultimate hemostasis ^a	13(87%)	24(96%)	37(93%)
Emergent surgery	1	1	2(5%)
Mortality	1	0	1(3%)

Data are presented as case number (percentage).

^aTwo patients with re-clipping are included. ^b $P = 0.28$.

Table 4 The relationship between shock and rebleeding rate

Subgroup	F1a	F1b
Shock ^a	1/5(20%) ^b	1/6(17%) ^c
Non-shock	1/8(13%)	0/9 (0%)

Data are expressed as rebleeding/subgroup case number. ^aTwo cases without initial hemostasis are excluded. ^b $P = 0.77$; ^c $P = 0.07$ compared with non-shock subgroups.

Table 5 The relationship between shock and ultimate hemostatic rate

Subgroup	F1a	F1b
Shock	5/7(71%) ^a	5/6(83%) ^b
Non-shock	8/8(100%)	19/19(100%)

Data are expressed as ultimate hemostasis/subgroup case number. ^a $P = 0.11$, ^b $P = 0.07$ compared with non-shock subgroups.

Table 6 Number of clips used per case in different subgroups

Type	F1a	F1b
Gastric ulcer	3.5 (n = 8)	2.9 (n = 14)
Duodenal ulcer	3.2 (n = 5)	2.7 (n = 9)
Marginal ulcer	—	2.5 (n = 2)
Average ^a	3.4 (n = 13)	2.8 (n = 25)

^a $P = 0.04$.

DISCUSSION

The rotatable clip-device we used was developed in 1995. This device has a dial at the center of the handle, which can rotate the clip and open at the tip of the device. The working length of HX-5LR-1 is 165 cm for an upper GI endoscope. Use of a special wire resistant to rotational distortion and a special coating facilitate rotation of the clips and markedly reduce the force required for clipping, as compared to the conventional device (HX-3/4 clip-device). As a result, precise clipping with application of a smaller force has become possible^[20]. HX-5LR-1 has advantages over the older HX-3/4 clip-device in its rotatability, which

can make clipping more accurate, and its durability including the ability to withstand sterilization by autoclaving^[20].

Mechanical clipping of a bleeding ulcer is appealing because the bleeding can be stopped immediately^[22]. This technique was once abandoned due to its complexity. In 1993, Binmoeller *et al* in Germany evaluated an improved metallic clip for endoscopic treatment of non-variceal hemorrhage from various sources in the upper gastrointestinal tract and concluded its highly effective hemostatic effect^[9]. Our results confirm the efficacy and safety of hemoclips for the treatment of peptic ulcer with active hemorrhage.

It is well recognized that about 50% of high risk patients had continuous hemorrhage or rebleeding during hospitalization^[23]. In our cases, hemoclip treatment reduced the rates of rebleeding to 15% in the F1a subgroup and 4% in the F1b subgroup. The ultimate hemostasis rate was 93%. The tangential application of clips is sometimes difficult, which is the same as for other therapeutic endoscopic modalities (e.g. laser or injection therapy). Application of clips should ideally be performed while approaching the bleeding spot *en face*^[22]. In two of our patients who failed to terminate the hemorrhage by using hemoclip treatment, the locations of the ulcers were such that it was difficult to use an *en face* approach.

Dislodging of clips is another cause of rebleeding. Two of our patients had clips which dislodged, one on the posterior wall of the high gastric body and another on the posterior wall of the duodenal bulb. The locations of the ulcers were difficult to approach when applying the clips. Underlying diseases with bleeding tendency may influence the effect of endoscopic hemostatic procedure^[24]. In one patient with oozing from the site of clipping, the hemorrhage might be related in part to the use of anti-coagulant medications.

In those patients with hemorrhage from the upper gastrointestinal tract, the presence of shock on admission and visible vessel significantly predict rebleeding^[1,25,26]. Hsu *et al* reported that an adherent clot associated with hypovolemic shock had a 50% rebleeding rate. On the contrary, the presence of a clot without hypovolemic shock had a rebleeding rate of only 17%. Non-bleeding visible vessel associated with shock had a 40% rebleeding rate; without shock it decreased to 25%^[7]. The presentation of active hemorrhage, shock on admission, and low hemoglobin concentration predict a poor outcome^[23]. In our series, neither the rebleeding rate nor the ultimate hemostatic rate was influenced by the presence of shock. In our study, the F1a with shock subgroup had the highest rebleeding rate of only 20%, which suggests that endoscopic hemoclippping be a useful and effective

hemostatic method.

Clipping allows clamping of bleeding vessels and achieves mechanical sealing without affecting the ulcer's depth or size. Clips dislodge spontaneously and pass through the gastrointestinal tract safely within 3 weeks^[22]. The damage to surrounding tissue was reported to be limited^[27,28]. In the present study, follow-up endoscopy showed no obvious clip-related tissue injury or impairment of ulcer healing. Our findings confirm that no obvious complications resulted from clip placement.

Both heater probe and injection therapies are widely used. Their hemostatic rates are about 75% to 95%^[1,2,5,29,30]. Our study shows that the hemostatic effect of hemoclips is 93% in peptic ulcers, which is comparable to the above reports. Our data are consistent with those of Takahashi's in which the outcomes of bleeding ulcers did not differ between injection and hemoclipping^[31].

The number of clips required for hemostasis depends on the bleeding activity, endoscopic accessibility of the bleeding site, and the characteristics of the vessel^[9]. Spurting lesions generally require more clips to achieve hemostasis than do oozing lesions. In our series, the average number of clips was higher in the F1a subgroup than in the F1b subgroup. Our results are comparable to the number of Binmoeller *et al*, who used an average of 3.2 clips per case for spurting bleeders and 2.7 clips for oozing bleeders^[9].

In conclusion, endoscopic hemoclip treatment for bleeding peptic ulcer is an effective and safe modality. It has a high initial hemostatic rate (95%) and a low rebleeding rate (8%). Ultimate hemostasis reached 93% in our study with no obvious complications. With the development of newly developed clips and clip application devices, the endoscopic hemoclip treatment has become easier and much more efficient. Endoscopic hemoclip treatment deserves further comparative studies with other hemostatic methods.

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Cloning and expression of MXR7 gene in human HCC tissue

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Subject headings human HCC tissues; MXR7 gene; gene expression; cDNA; mRNA

Abstract

AIM To clone and identify the whole cDNA of MXR7 gene and to find out its expression in human HCC, and normal tissues.

METHODS The DNA primers were designed and synthesized according to the whole cDNA sequence of MXR7 gene. The cDNA of human HCC was taken as the template while the cDNA of MXR7 gene was synthesized by polymerase chain reaction (PCR). Recombinant DNA conforming to reading frame was constructed by connecting purified PCR product of the cDNA of MXR7 gene with expression vector pGEX-5X-1 of fusion protein. The plasmid MXR7/pGEX-5X-1 was identified by sequencing. Using ³²P labeled MXR7 cDNA as probe, MXR7 mRNA expression was detected by Northern blot analysis in 12 different human normal tissues, 7 preoperatively untreated non-liver tumor tissues, 30 preoperatively untreated HCC, the paracancerous liver tissues and 12 normal liver tissues samples.

RESULTS Restriction enzyme and sequence analysis confirmed that the insertion sequence in vector pGEX-5X-1 was the same as the cDNA sequence of MXR7 gene. Northern blot analysis showed no expression of MXR7 mRNA in 12 kinds of normal human tissues including liver, 7 tumor tissues in other sites and 12 normal liver tissues, the frequencies of MXR7 mRNA

expression in HCC and paracancerous liver tissues were 76.6% and 13.3%, respectively. The frequency of MXR7 mRNA expression in HCC without elevation of serum AFP and in HCC <5 cm was 90% (9/10) and 83.3% (5/6), respectively.

CONCLUSION MXR7 mRNA is highly expressed in human HCC, which is specific and occurs at an early stage of HCC, suggesting MXR7 mRNA can be a tumor biomarker for HCC. The detection of MXR7 mRNA expression in the biopsied liver tissue is helpful in discovering early subclinical liver cancer in those with negative serum AFP.

INTRODUCTION

The cloned mitoxantrone-resistant 7 (MXR7) gene contains the whole open reading frame (ORF) screened by differential hybridization from λ complementary DNA expression library, from the mitoxantrone-resistant human gastric carcinoma cell line EPG85-257RNOV and is a full-length cDNA of 2263bp encoding a putative protein of 580 amino acids^[1]. With MXR7 mRNA expression at high level in hepatocellular carcinoma (HCC), its temporospatial expression in human resembles α -fetoprotein (AFP) gene expression^[2]. To probe into and clarify the relation between MXR7 gene and the tumorigenesis and progression and clinical diagnosis of HCC, we analyzed 30 human HCC and the corresponding paracancerous liver tissues, 12 normal liver tissues, 12 different normal tissues and 7 non-liver tumor tissues.

MATERIALS AND METHODS

Specimens

Tissue specimens used in the present study were sampled from 30 preoperatively untreated patients with pathologically confirmed HCC and the non-tumorous liver tissue (2 cm away from the carcinoma), including 28 males and 2 females, aged 21-70 years with a mean of 50.1 years. The controls were normal liver tissues from 12 patients with hepatic hemangioma, 12 different normal tissues from 2 accidental deaths, and 7 preoperatively untreated non-liver tumor tissues. The surgical specimens were immediately cut into small pieces under aseptic condition, snap frozen and stored in liquid nitrogen until use.

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Plasmid

Expressing vector pGEX-5X-1 (4.9 kb) of fusion protein was provided by Max-Planck Institute.

Cell

E. coli DH5 α was preserved in our laboratory.

PCR primers design and synthesis

The sequences of the sense and antisense primers of *MXR7* were 5' GCGAATTCTCCTGCGAAGCAGGATG3' with the *EcoRI* (GAATTC) restriction site and 5' CGCTCGAGTCAGTGCACCAGGAAGAA3' with the restriction site *XhoI* (CTCGAG), respectively. The amplifying DNA fragments of above primers were 1774 bp and all primers were synthesized by Shanghai Biochemistry Institute, Chinese Academy of Sciences.

Plasmid construction

The 1774bp fragments of *MXR7* gene were amplified from the cDNA of human HCC by PCR and cloned into the expressing vector pGEX-5X-1. The positive clones selected from the transfected DH5 α were performed as described in the reference^[3]. The constructed plasmids (designated as *MXR7*-pGEX-5X-1) were identified by the restriction enzyme analysis and verified by sequencing.

Preparation and labeling of the probe

The purified PCR product was labeled with ³²P-dCTP as the probe by random primer method as described in the reference^[4] with Prime-a-Gene Labeling System (Promega) and purified with QIAquick Nucleotide Removal Kit Protocol (Qiagen).

Northern blot analysis

RNA extraction Total RNA was extracted using TRIZOL reagent (Gibco, BRL). The RNA from about 1.0 g tissue mass was dissolved in 0.5 mL water pretreated with diethylpyrocarbonate (DEPC) and then stored at -85 °C for use.

Preparation of the hybridization membrane Each 50 μ g denatured total RNA was transferred to nitrocellulose filters (BA85, Schleicher Schuell) by electrophoresis performed on 1% agarose-formaldehyde gels. The filters were dried in a vacuum drying oven at 80 °C for 2 h and then sealed in a plastic bag for use.

Northern blot analysis Northern blotting was performed as described in the reference^[5]. The amount and quality of the loaded RNA samples were evaluated carefully by ethidium bromide-stained 28S and 18S rRNAs, RNA samples with evidence of degradation and blots that fail to hybridize normally expressed genes were discarded.

Statistical treatment

Percentage of the specimens was compared by χ^2 test.

Follow-up

Through phone calls or by mails and re-examination at the outpatient department, we had followed 22 of 30 patients for more than 2 years or until death for further analysis.

RESULTS

Results of cloning and identification

The expected size of the amplified *MXR7* fragment was about 1800 bp. The expected size of the recombinant plasmid *MXR7*/pGEX-5X-1 after enzyme-cut by *EcoR*-I and *Xho*-I was about 4900 bp and 1800 bp, respectively. Sequence analysis of *MXR7*/pGEX-5X-1 confirmed that the insertion sequence of vector pGEX-5X-1 was the same as the translational region sequence of *MXR7* cDNA.

Result of Northern blot analysis

The results of Northern blot analysis were classified into positive and negative.

Expression of *MXR7* mRNA in HCC, normal tissue and tumor of other anatomical sites

Northern blot analysis showed: ① No expression of *MXR7* mRNA on 12 normal liver tissues of patients with hepatic hemangioma, *MXR7* mRNA was detected at low level in only 4 (13.3%) of 30 corresponding paracancerous tissue (all were cirrhosis), all of which had intrahepatic portal vein tumor thrombus and multiple daughter modules. By comparison, the frequency of expression of 2.3kb *MXR7* mRNA at high level was 76.7% (23 of 30 cases) in HCC samples, significantly higher than that with elevated serum AFP $\geq 400 \mu\text{g/L}$ (43.3%, 13 of 30 cases, $P < 0.01$) in this group (Figure 1, Tables 1 and 2). ② *MXR7* mRNA was undetectable in 12 different normal tissues, including liver, lung, kidney, heart, brain, small intestine, colon, testis, spleen, gastric, cyst and pancreas (Figure 2). ③ Among tumors of other anatomical sites including 3 gastric adenocarcinomas, 1 sigmoid adenocarcinoma, 1 malignant mesothelioma, 1 uterine myo-adenoma and 1 familial colonic adenomatosis, *MXR7* mRNA was undetectable too (Figure 3).

Clinicopathological profiles of 30 studied cases

with HCC These included 28 men and 2 women, aged 21-70 years with a mean of 50.1 years. Serum HBsAg was positive in 24 cases (80%). The serum AFP level was above 400 $\mu\text{g/L}$ in 13 (43.3%) and below 30 $\mu\text{g/L}$ in 10 (33.3%). The tumor size was $< 5 \text{ cm}$ (small HCC) in 6 and large in 24 (large

HCC). Histologically, all 30 tumors had invasion (invasive HCC), with portal vein thrombus in 24 (80%) and distant satellite nodule in 17 (56.7%). The differentiation of all tumors were Edmondson III-IV grade.

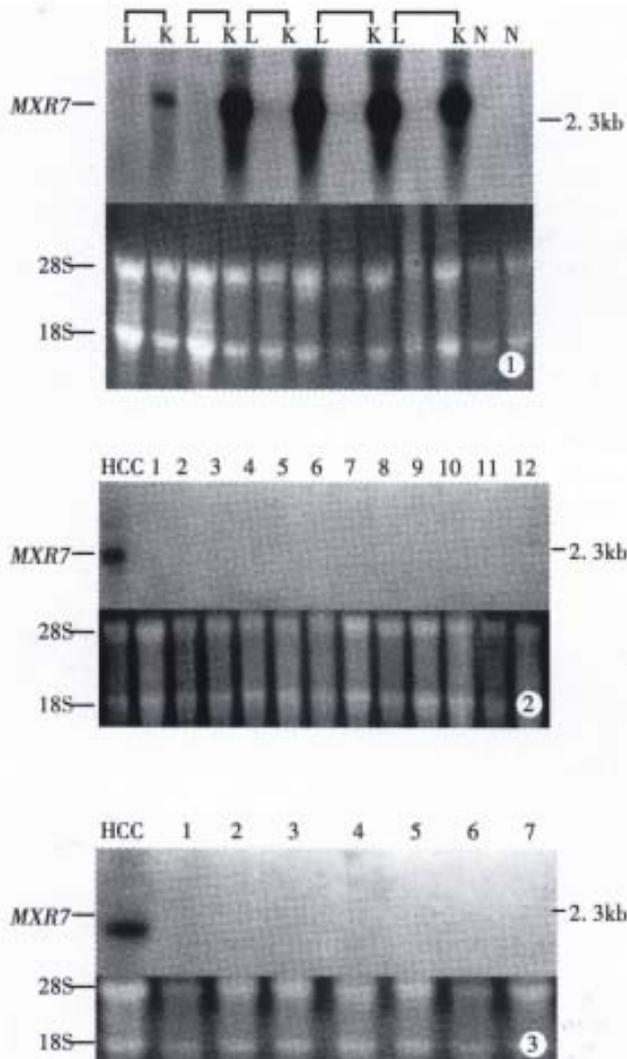


Figure 1 Northern blot analysis of *MXR7* in human HCC, paracancerous hepatic and normal liver tissues. L: paratumor tissue; K: hepatoma tissue; N: normal liver tissue. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading.

Figure 2 Northern blot analysis of *MXR7* in 12 different human normal tissues. Lane 1: liver; Lane 2: lung; Lane 3: kidney; Lane 4: heart; Lane 5: brain; Lane 6: small intestine; Lane 7: colon; Lane 8: testis; Lane 9: spleen; Lane 10: stomach; Lane 11: cyst; Lane 12: pancreas; HCC as a positive control. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading.

Figure 3 Northern blot analysis of *MXR7* in 7 non-liver tumor tissues. Lane 1: gastric adenocarcinoma; Lane 2: sigmoid adenocarcinoma; Lane 3: gastric adenocarcinoma; Lane 4: left colon-ileal malignant mesothelioma; Lane 5: gastric adenocarcinoma; Lane 6: uterus adenomyoma; Lane 7: colic familial polyadenomatosis; HCC as a positive control. 28S and 18S rRNAs were used for evaluating the quality and quantity of RNA loading.

Table 1 Expression of *MXR7* mRNA in human HCC, and the paracancerous liver and normal liver tissues

Surgical specimen	n	<i>MXR7</i> mRNA overexpression (%)
HCC	30	23(76.7)
Paracancerous tissue	30	4(13.3) ^a
Normal liver	12	0(0.0) ^b

^a $P < 0.005$ ($\chi^2 = 24.31$); ^b $P < 0.005$ ($\chi^2 = 19.32$), vs groups HCC.

Table 2 The correlation of *MXR7* mRNA expression and clinicopathological features in 30 patients with HCC

Clinical feature	n	<i>MXR7</i> mRNA overexpression (%)
Serum AFP ($\mu\text{g/L}$)		
≥ 400	13	10(76.9)
30-400	7	4(57.1)
≤ 30	10	9(90.0)
Tumor size (cm)		
≥ 5	24	18(75.0)
< 5	6	5(83.3)
Portal vein tumor thrombus		
Yes	24	17(70.8)
No	6	6(100.0)
Daughter tumor		
Yes	17	12(70.6)
No	13	11(84.6)
Serum HBsAg		
Yes	24	19(79.2)
No	6	4(66.7)
Age (years)		
≥ 50	16	12(75.0)
< 50	14	11(78.6)

***MXR7* mRNA expression in relation to clinicopathological features** As shown in Table 2, *MXR7* mRNA expression did not correlate with serum AFP elevation, tumor size, portal vein tumor thrombus, daughter nodules, HBsAg seropositivity and age. The frequency of *MXR7* mRNA expression in HCC was 70% (14 of 20 cases) with elevated serum AFP $> 30 \mu\text{g/L}$, but 90% (9 of 10 cases) with serum AFP $\leq 30 \mu\text{g/L}$. In HCC $< 5\text{cm}$, the frequency (83.3%, 5 of 6 cases) of *MXR7* mRNA expression was higher than that with elevated serum AFP (33.3%, 2/6 cases).

Association between *MXR7* mRNA expression in surgical specimens of HCC and prognosis of the patients

In our study, 12 out of 22 patients survived for 2 years postoperatively, 10 of whom had no signs of recurrence nor metastasis, in the other two, there had been one recurrent tumor in the right and left lobes, respectively. The average survival period of the 10 deceased of 22 patients was 8.0 months (2-25 months). The main causes of death were tumor recurrence, portal vein tumor thrombosis and ascites. The survival rates for 1 year and 2 years were 59.1% and 54.5%, respectively. Our data did not show that *MXR7* mRNA expression was correlated with the prognosis of patients.

DISCUSSION

Although an elevated serum AFP level is regarded as a tumor marker for HCC, the frequency of elevated serum AFP in HCC is about 60% up to date, which is much lower in small HCC. The study showed no detectable expression of *MXR7* mRNA in 12 different normal tissues including liver, 7 non-liver tumor tissues and 12 normal liver tissues and the frequencies of *MXR7* mRNA expression in HCC and the corresponding paracancerous cirrhotic tissues were 76.7% and 13.3%, respectively. These findings indicate that *MXR7* mRNA overexpression in HCC is common and specific, suggesting that *MXR7* gene served as a sensitive marker for HCC.

Our observations confirmed only 33.3% (2 of 6 cases) of the patients with small HCC (<5 cm) had an elevated serum AFP level (>30 µg/L), which was lower than that of *MXR7* mRNA overexpression (83.3%, 5 of 6 cases), and the frequency of *MXR7* mRNA overexpression in HCC was 70% (14 of 20 cases) with serum AFP elevation but 90% (9 of 10 cases) without serum AFP elevation, suggesting that *MXR7* gene may be a sensitive early tumor marker for HCC and the detection of *MXR7* mRNA expression in liver biopsied tissues was able to discover small HCCs in the subclinical stage with negative serum AFP.

Four cases of *MXR7* mRNA expression in paracancerous cirrhotic tissues were 1 with two 4cm tumor masses in left-external and right-posterior hepatic lobe and 3 cases with >10 cm tumor masses accompanied by multiple daughter tumors, from which we can presume that retained carcinoma cells in the paracancerous hepatic tissue of the surgical specimens with large tumor mass and tumor invasion may be the cause of *MXR7* mRNA expression in the paracancerous liver tissue, this suggests that detection of *MXR7* mRNA expression in the paracancerous liver tissue can serve as one of indicators whether the tumor is completely resected or not and also as referential value in deciding further treatment.

The cDNA sequence of *MXR7* is 100% homologous to the cDNA of Glypican 3 (GPC3)^[1], which is a developmentally regulated gene localized to chromosome X_q^{26[6]}, GPC3 sequences are very well conserved through evolution, being highly

homologous among mice, rats and human being^[7]. GPC3 is believed to be involved in morphogenesis and growth control during development regulated by cell morphology and cell density at the transcription level^[8].

The familial aggregation and the heredity of susceptibility of the patients with HCC is well documented and the frequency of HCC among males is as about 10 folds that of females. The epidemiology study showed that the effect of heredity on maternal side is much higher than that of paternal one. Clinically, HCC responds poorly to the chemotherapy, which might be correlated with the common overexpression of *MXR7* mRNA in human HCC.

MXR7 mRNA expression is closely related to oncogenesis and progression, the heredity of susceptibility and the poor therapeutic effect of chemotherapy of HCC, but the molecular mechanism remains unclear. The construction of recombinant plasmid *MXR7/pGEX-5X-1* expressing fusion portion is useful for studying the correlation of *MXR7* and HCC with the structure and function of the gene product.

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Recurrence or metastasis of HCC: predictors, early detection and experimental antiangiogenic therapy

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Subject headings carcinoma, hepatocellular; neoplasm metastasis; angiogenesis; liver neoplasms

Abstract

AIM To investigate the predictors for recurrence or metastasis of HCC, and to evaluate the effect of antiangiogenic therapy on the growth of transplantable human HCC in nude mice.

METHODS RT-PCR was used to measure the expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) in 56 pairs of nontumorous liver and tumor samples. Sixty blood samples from human HCC were examined by nested RT-PCR to find out AFP mRNA. Recombinant human endostatin and polyclonal antibody against VEGF were administered to treat human HCC transplanted in nude mice.

RESULTS Thirty of 56 HCC samples showed stronger expression of MMP-9 in tumorous tissues than in nontumorous tissues. Fifteen of the 26 patients with relative expression level of MMP-9 more than 0.34 developed tumor recurrence or metastasis, whereas only 7 of 30 patients with relative expression level less than 0.34 developed tumor recurrence ($P < 0.05$). There was no significant difference in the relative expression level of VEGF between patients with postoperative recurrence or metastasis and those without recurrence. AFP mRNA was detectable in 53.3% of patients with HCC. The sensitivity and specificity of AFP mRNA as a marker to detect hematogenous dissemination of HCC cells was 81.8% and 84.4%, respectively. Recombinant human

endostatin and polyclonal antibody against VEGF inhibited the growth of transplantable HCC in nude mice by 52.2% and 45.7%, respectively.

CONCLUSION MMP-9 expression in HCC correlates with the postoperative recurrence or metastasis of HCC. Patients with high level of MMP-9 expression in HCC are susceptible to metastasis. AFP mRNA could serve as an indicator of hematogenous spreading of HCC cells in circulation and a predictor of recurrence or metastasis of HCC. Antiangiogenesis may be an adjuvant therapy for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer in China and in other Asian countries or in south part of Africa. Although some advances have been achieved in the diagnosis and treatment of HCC, the long-term outcome for patients with HCC is still very poor^[1]. The prognosis for HCC depends mainly on the clinico-pathological characteristic regarding invasion and metastasis. The major obstacle to the improvement of the prognosis for HCC is the high incidence of recurrence or metastasis after routine surgical treatment or transcatheter arterial chemoembolization (TACE). Therefore, the following prospective study was designed to investigate molecules responsible for postoperative recurrence of HCC by focusing on matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF). Furthermore, we also studied the detection of hematogenous spreading of HCC cells at a relatively early stage, and the experimental anti-angiogenic therapy for HCC in an animal model.

MATERIALS AND METHODS

Materials

Tumorous and nontumorous liver samples were obtained from 56 HCC patients who underwent hepatectomy. Peripheral venous blood samples were collected from 60 patients with HCC and 30 subjects as control (10 patients with liver cirrhosis and 20 healthy donors). Recombinant human endostatin and polyclonal antibody against VEGF were used to treat HCC transplanted in nude mice.

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Methods

RNA preparation The total tissue RNA was extracted with TRIzol (Life Technologies, Inc. Gaithersburg, USA), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -70°C .

Reverse transcription Moloney murine leukemia virus reverse transcriptase (M-MLV RT) was used to synthesize a complementary DNA strand in the presence of random primer from $6\mu\text{g}$ single stranded RNA.

PCR amplification Primer sequences. Sense primer for VEGF: 5'-TTGCTGCTCTACCTCCAC-3'. Antisense primer for VEGF: 5'-AATGCTTTCTC-CGCTCTG-3'. Sense primer for MMP-9: 5'-CG-GAGCAGGAGACGGGTAT-3'. Antisense primer for MMP-9: 5'-TGAAGGGGAAGACG-CACGCACAGC-3'. Sense primer for internal control of β_2 -MG: 5'ACCC CCACTGAAAAAGATGA-3'. Antisense primer for β_2 -MG: 5'-ATCTTCAAACCTCCATGATG-3'. All primers were synthesized by Shanghai Sangon Biotec hnique Company. PCR reaction: the $25\mu\text{L}$ of PCR mixture contained $2\mu\text{L}$ of the synthesized cDNA solution, $2.5\mu\text{L}$ of $10\times$ polymerase reaction buffer, 1.5mM MgCl_2 , $200\mu\text{M}$ -each of dCTP, dATP, dGTP, dTTP, 10pmol of each primer (sense and antisense); and 1 unit of Taq DNA polymerase. The PCR mixture for VEGF was subjected to 40 cycles PCR amplification using protocol TOUCHDOWN in PTC 100 programmable thermal cycler (MJ Research, USA). Cycle conditions for amplifying MMP 9 included a 94°C denaturation (30 s, first cycle 1 min), a 60°C annealing (30 s), and a 72°C extension (60 s). After the final cycle, tubes were placed at the extension temperature for 5 min.

Assay of PCR production A volume of $7\mu\text{L}$ PCR products was added in 1.5% agarose gel containing $0.5\mu\text{g/mL}$ EB, after electrophoresis, the gel was placed under ultraviolet ray to analyze the results. The density and area of each band were measured using Image Master VDS software (Pharmacia, Sweden). The relative mRNA level of VEGF of MMP-9 gene in tumor or nontumorous tissues was calculated using the house-keeping gene β_2 -MG as an internal control.

Nested RT-PCR amplifying human AFP A 5-mL heparinized blood sample from each patient was taken for AFP mRNA determination. Cell pellets were obtained from heparinized blood samples. The total RNA was extracted with TRIzol and resuspended in RNAase-free water. Reverse transcription was performed using random primers

and PCR using specific AFP primers. β_2 -microglobulin mRNA was co-amplified during the RT-PCR test as an internal control. The $25\mu\text{L}$ of first PCR mixture containing external sense and antisense primer for AFP were subjected to 40 cycles PCR amplification using protocol TOUCHDOWN in PTC-100 programmable thermal cycler (MJ Research, USA). A volume of $8\mu\text{L}$ PCR products was added in 2% agarose gel for electrophoresis. If no specific band of 176 base pairs was observable, $2\mu\text{L}$ PCR product was reamplified with internal primers. The final product was electrophoresed on 2% agarose gel for the specific band of 101 base pairs.

Experimental antiangiogenic therapy of HCC

Preparation of recombinant human endostatin. The human endostatin cDNA which encoded 184 amino-acids was cloned from human fetal liver. The recombinant human endostatin was expressed in a prokaryotic system. The recombinant endostatin underwent denaturation in 8mol/L urea and was refolded in Sephadex G-100 column.

The polyclonal antibody against human VEGF was prepared in our lab. Briefly, the recombinant human VEGF165 was expressed in a prokaryotic system, which was administered as antigen to stimulate the production of antibody against VEGF in rabbits. The antiserum was purified using affinity chromatography column of Sepharose CL-4B-VEGF.

RESULTS

Postoperative recurrence or metastasis of HCC patients

In the total of 56 HCC patients, 22 (39.3%) had relapsed within 20 months after operation. Fifty percent of the recurrence occurred within 6 months after operation. Tumor recurrence had no significant correlation with tumor size or degree of pathological differentiation ($P>0.05$, Table 1).

Table 1 Relationship between clinicopathological characteristics and recurrence or metastasis of HCC

Parameter	n	Recurrence of metastasis	
		Yes	No
Tumor size (cm)			
≤ 5 cm	29	10	19
> 5 cm	27	12	15
Liver cirrhosis			
Absent	8	3	5
Present	48	19	29
Tumor differentiation			
Well	12	4	8
Moderate	23	8	15
Poor	9	5	4
Not determined	12	5	7
Clinical staging			
Stage I	2	1	1
Stage II	33	10	23
Stage III	18	8	10
Stage IV	3	3	0

MMP-9 expression in HCC correlated with tumor recurrence

In 35 of the 56 HCC samples, transcripts of MMP-9 were detected, 27 of 35 these samples showed significant elevation of MMP-9 expression (> 2 fold) compared with nontumorous liver tissues. Among the 30 patients with a relative expression level of MMP-9 in HCC less than 0.34, only 7 cases developed tumor recurrence, whereas 15 of the 26 patients with relative expression level of MMP-9 more than 0.34 had developed tumor recurrence or metastasis. Moreover, among the 22 HCC patients who suffered from postoperative recurrence or metastasis, 16(88.8 %) tumors (T) had significantly elevated level of MMP-9 expression compared with nontumorous liver tissues (N) ($T/N > 2$), while only 11(32.3%) of 34 HCC patients who had not yet relapsed had significantly increased level of MMP-9 expression in HCC. Patients with high level of MMP-9 expression in HCC were susceptible to tumor recurrence or metastasis.

VEGF expression in HCC not correlated with tumor recurrence

Transcripts for VEGF were detected in 48 of 56 HCC samples and in 36 of 53 nontumorous liver tissues. The level of VEGF expression was elevated significantly in 52% HCC samples compared to nontumorous liver tissues (Figure 1). The level of VEGF expression in HCC was not correlated with tumor size or the degree of pathological differentiation. Among the 25 patients with significantly elevated level of VEGF expression in HCC, 12 cases (48%) had tumor recurrence, whereas 10 of 31 patients with similar or decreased level of VEGF expression had tumor recurrence.

AFP mRNA in peripheral venous blood from clinical samples

The frequency of positive cases in 60 patients with HCC was 53.3% (32/60) (Figure 2). The frequency of AFP mRNA positivity in patients with liver cirrhosis and in healthy donors was 10% and 5%, respectively.

Relationship between AFP mRNA expression and intra or extra-hepatic metastasis

In 32 patients with detectable AFP mRNA in peripheral blood, 27 patients (84.4%) were accompanied with metastasis. Of the 11 patients with detectable AFP mRNA in peripheral blood but without metastasis at collected samples, 6 cases developed metastasis or tumor recurrence later. Six or 28 patients without detectable AFP mRNA developed metastasis. Serum AFP level was not correlated with cancer metastasis or recurrence.

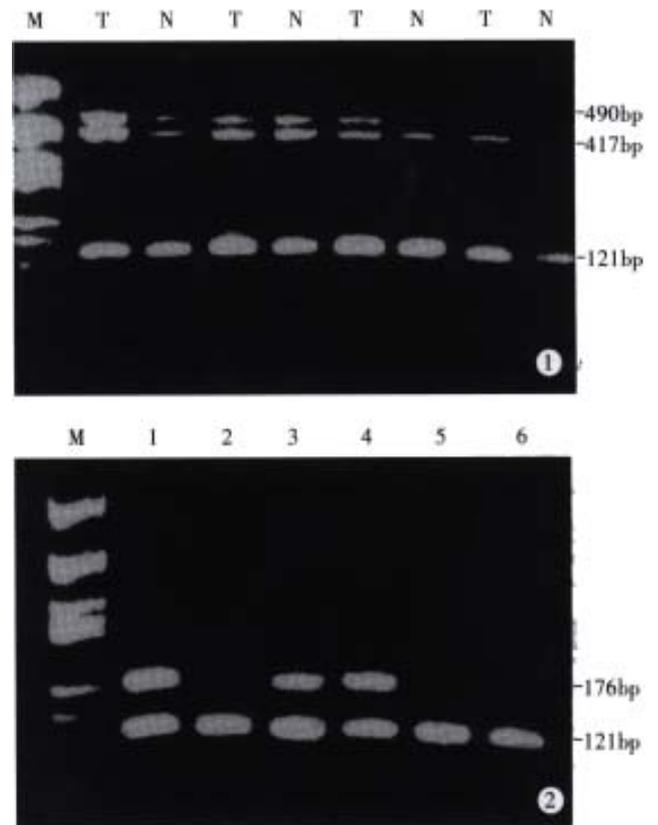


Figure 1 Electrophoresis analysis of RT-PCR product sampled from cDNA obtained from HCC and nontumorous liver tissues. M, DNA marker (PGEM-7ZF(+)/Hae-III); T, tumor; N, nontumorous liver tissues. The positive bands of 417bp, 490 bp, and 121 bp represent VEGF165, VEGF189, and β_2 -MG, respectively.

Figure 2 Electrophoresis analysis of RT-PCR products from cDNA obtained from peripheral venous blood of patients with HCC. M, DNA marker (PGEM-7ZF(+)/Hae-III). Lane 1, lane 3, and lane 4 showed positive bands for AFP (176bp). All lanes showed positive bands for β_2 -MG (121bp).

Relationship between AFP mRNA expression and therapy

Of 60 patients with HCC, 14 cases underwent surgical treatment, 35 received transcatheter arterial chemoembolization (TACE). Fifteen of 33 samples collected before operation or TACE showed detectable AFP mRNA in peripheral venous blood. Twenty-one of 35 samples collected after therapy had detectable AFP mRNA ($P > 0.05$).

Recombinant human endostatin and polyclonal antibody against VEGF inhibited the growth of HCC

We used *E. coli*-derived human endostatin to study the effect of endostatin therapy on primary liver cancers. BEL-7402 hepatocellular carcinoma was implanted into nude mice. The refolded protein of recombinant endostatin was administered to the

mice via peritoneal injection once daily. The growth of primary tumors was inhibited by 52.2% at a dose of 7.5 mg/kg as compared with control mice treated with saline alone. The effect of polyclonal antibody against VEGF on the growth of primary liver cancers was also studied. The polyclonal antibody against VEGF was administered to the mice via peritoneal cavity injection at a dose of 10 mg/kg once daily. The growth of primary tumors was inhibited by 45.7%.

DISCUSSION

The long-term outcome of patients with HCC is still very poor. The major obstacle to the improvement of prognosis for HCC patients is the high incidence of postoperative recurrence or metastasis. The five year recurrence rate in liver cancers is as high as 40%-70%^[2]. Therefore, it is very important to investigate the molecular changes that correlate with recurrence or metastasis of HCC, which is useful to screen HCC patients with high risk of recurrence. Furthermore, the early detection of hematogenous spreading of HCC cells or recurrent lesions and the effective management of recurrent lesions are also important steps to improve the therapeutic effects. We performed a series of study on the recurrence or metastasis of HCC by focusing on some key steps mentioned above.

Metastasis is the spread of cancer from a primary tumor to distant sites of the body and is a defining feature of cancer. Escape of cells from the primary tumor, intravasation and extravasation are some necessary steps in the process of cancer metastasis^[3]. There are a series of collagen containing structural barriers that cancer cells must pass in all steps mentioned above. MMPs are a family of secreted or transmembrane proteins that are capable of digesting extra cellular matrix and basement membrane. MMP-9 is believed to be capable of degrading type IV collagen, which is a major constituent of basement membrane^[4]. Highly invasive tumor cells then, would be expected to secrete large amounts of proteolytic enzyme. The results presented here indicated that MMP-9 mRNA was expressed more frequently in HCC tissues than in corresponding nontumorous ones, and that the degree of MMP-9 mRNA expression in tumors was elevated in 30 of 38 samples with transcripts for MMP-9 compared with corresponding nontumorous tissues. With regard to the correlation of MMP-9 mRNA and tumor recurrence or metastasis, the tumors with high level of MMP-9 expression were more susceptible to relapse or metastasis than those with low level of MMP-9 expression. These results revealed that MMP-9 is an important molecule which participates in the invasion of HCC. MMP-9 expression in HCC is of prognostic significance.

MMP-9 can also serve as a potential target for prevention and treatment of tumor recurrence or metastasis.

Angiogenesis, the recruitment of new blood vessels, is required for the primary and metastatic tumors to grow beyond minimal size^[5]. Vascular endothelial growth factor (VEGF) is an important factor which can promote the proliferation of endothelial cells and the development of new blood vessels^[6]. In the present study, we investigated the expression of VEGF mRNA in HCC, we also examined the correlation between VEGF mRNA expression and the recurrence or metastasis of HCC. We found that VEGF mRNA was expressed in most of HCC specimens and nontumorous liver tissues (87.3% vs 67.9%). Fifty-two percent HCC specimens exhibited stronger expression of VEGF mRNA in tumorous tissues than in nontumorous ones. No apparent correlation was observed between VEGF expression and tumor size or the grading of tumor differentiation. There was also no significant correlation between VEGF mRNA expression level and tumor metastasis. In addition, the growth of HCC could be inhibited by antibody against VEGF. All these data indicated that VEGF may be involved in the growth of HCC, however, the level of VEGF mRNA expression cannot reflect the potential metastasis of HCC, namely, even if VEGF expression is not strong in HCC, tumor recurrence or metastasis may also occur.

Escape of cells from primary tumor into blood circulation is an indispensable step in the process of blood borne metastasis. The detection of tumor cells in peripheral blood by means of RT-PCR is a very attractive hypothesis^[7]. Theoretically, the test could be useful in assessment of prognosis and in predicting the increased probability of metastases. We choose the RT-PCR mRNA AFP as an indicator of liver cells to determine the clinical relevance of the test. Thirty-two of sixty HCC patients involved in this study has positive AFP mRNA test result. One tenth patients with liver cirrhosis and 1/20 healthy donors had positive AFP mRNA in peripheral blood. These data suggested that AFP mRNA in peripheral blood is a sensitive marker of presence of HCC cells in circulation, although false positive results may appear occasionally. The frequency of positive cases in patients with metastases was significantly higher than that in patients without occult metastases at collected samples (93.9% vs 29.7%), which indicated that the presence of AFP mRNA in peripheral blood correlated with metastasis of HCC. Six of 11 patients who were AFP mRNA-positive and metastasis-free at collected samples had clinically evident recurrence or metastasis later. Among 26 negative AFP mRNA, metastasis-free patients, 22

patients remained recurrence-free during the period observed. These data suggest that the detection of AFP mRNA in peripheral blood by means of RT-PCR was useful in predicting the increased probability of metastases, and in identifying a subpopulation of patients with HCC who are at high risk of recurrence. With no significant difference in AFP mRNA expression before and after surgical treatment or TACE, local treatment is insufficient to prevent tumor recurrence. For patients with tumor cells in circulation, more vigilant follow-up or more aggressive management, such as immunotherapy or systemic chemotherapy, should be made.

The effect of conventional chemotherapy on preventing cancer metastasis is not satisfactory mainly due to the multi-drug resistance^[8]. Therefore, it is highly necessary to search for new modalities in treatment of cancer metastasis. It is well known that the development of a tumor requires oxygen and nutrients, which are supplied through neovascularization. Therefore, antiangiogenesis and the suppression of the development of neovascularization may offer a novel strategy in overcoming the development and the metastasis of solid tumors^[9]. VEGF can promote the development of neovascularization, the antibody against VEGF may inhibit angiogenesis and consequently suppress the growth of tumor. We found that polyclonal antibody can inhibit the growth of human HCC transplanted in nude mice by 45.7%, but not inhibit the growth of tumor thoroughly possibly due to the fact that VEGF was not the only growth factor in the development of neovascularization. Endostatin is an endothelial-specific negative regulator of angiogenesis, previous report suggested that it inhibit the growth of some types of tumor in animal model^[10]. We found that the recombinant human endostatin inhibited the growth of human HCC by 52.2%. In addition, both endostatin and antibody against VEGF could

inhibit the lung metastasis of murine breast cancer dramatically in an animal model by 68.9% and 71.4%, respectively. It was also reported that antibody against VEGF could inhibit the experimental liver metastasis of human colon cancer in a mouse model^[11]. These data suggest that endostatin and antibody against VEGF are useful in prevention or treatment of tumor recurrence or metastasis of HCC. It warrants further research to investigate whether antiangiogenic therapy combined with routine chemotherapy or radiotherapy can improve the effect of treatment dramatically.

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Expression of gap junction genes connexin 32, connexin 43 and their proteins in hepatocellular carcinoma and normal liver tissues

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Subject headings connexin; gap junction; liver neoplasm; immunohistochemistry; *in situ* hybridization; carcinoma, hepatocellular; gene expression

Abstract

AIM To investigate the significance and mechanism of cx-32 mRNA, cx-43 mRNA and their proteins in hepatocarcinogenesis.

METHODS Sixty-one cases of HCC and 14 cases of normal liver tissues were detected by immunohistochemical and *in situ* hybridization (ISH) methods.

RESULTS In HCC grades I, II, III and normal liver tissues, the positive rates of Cx32 protein were 55.6%, 42.1%, 18.2% and 92.9%, respectively. The detection rates of Cx43 protein were 44%, 26.3%, 12.1% and 78.6%, respectively. There was significant difference in Cx32 and Cx43 protein between HCC and normal liver tissues ($P < 0.01$). ISH the positive rates of cx 32 mRNA shown by ISH in HCC grades I, II, III and normal liver tissues were 88.9%, 84.2%, 87.9% and 92.9%, respectively. Those of cx43 mRNA were 77.8%, 78.6%, 78.8% and 85.7%, respectively. There was no statistical difference in the positive rates of cx32 mRNA and cx43 mRNA between HCC and normal liver tissue ($P > 0.05$).

CONCLUSION The aberrant location of Cx32 and Cx43 proteins could be responsible for progression of hepatocarcinogenesis, and the defect of cx genes in post-translational processing might be the possible mechanism.

INTRODUCTION

Gap junctions are clusters of intercytoplasmic channels connecting neighboring cells which are composed of proteins called connexins (cx). Gap junction intercellular communication (GJIC) mediated by gap junction channels has been believed to be an important mechanism for the maintenance of tissues homeostasis and metabolic cooperation^[1]. Carcinogenesis is one of the pathological processes in which disorders of GJIC may play an important role^[2]. This study was designed to investigate the significance and mechanism of cx32 and cx43 genes and their proteins in hepatocellular carcinoma (HCC).

MATERIALS AND METHODS

Clinical data

The specimens of surgically removed 61 cases of HCC and 14 cases of normal liver tissues were collected at Xijing Hospital during the period of 1996-1998. The samples had not been treated with chemotherapy or radiotherapy before tumor excision. The pathological diagnosis was verified on the respective paraffin embedded material by histologic examination (HE). The specimens were derived from 9 cases of grade I HCC, 19 of grade II and 33 of grade III, and these were 4 μ m thick sections.

SP immunocytochemistry

SP immunostaining was performed as described by SP immunocytochemistry kit and DAB kit with mouse anti-cx32 mAb, mouse anti-cx43 mAb of Zymed Lab. Inc., USA. Diagnosis was made by brown or yellow coloration with varied intensities. Negative (-): stained cells < 5%, positive (+): stained cells 5%-50%, strongly positive (++) : stained cells > 50%.

Probe labeling

pGEM3-cx32 and pSG5-cx43 plasmids were kindly given by Prof. Gui Yuan Li in Hunan Medical University. After amplification, isolation and purification were done, pGEM3-cx32 plasmid was digested by Ecor-I (Gibco BRL, USA) and pSG5-cx43 by BamH-I (Gibco BRL, USA). Electroporate the digested plasmids on 7 g/L

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agarose gel with λ DNA/Hind-III+-EcoR-I marker. Extract and purify the cx32 cDNA and cx43 cDNA from the gel as the protocol of Advantage™ PCR-Pure Kit from Clontech Lab. Inc., USA. Label the cx32 cDNA and cx43 cDNA using Dig DNA Labeling and detection kit of Boehringer Mannheim, Germany.

mRNA in situ hybridization

Slides were incubated in 0.2 mL/L DEPC at RT for 10 min, in 0.2 mL /L HCl for 10 min and in 5 mg/L PK at 37 °C for 10 min and in 0.1 mol/L glycine to stop the digestion reaction, and then fixed in 40 g/L PFA for 10 min in sequence. After being washed in PBS, the sections were dehydrated in ethanol and air dried. Prehybridized at 42 °C for 30 min, the labeled cDNA probes were denatured in hybridization buffer at 100 °C for 10 min, then -20 °C for 3 min, added on tissues and coverslipped at 42 °C overnight. Sections were washed with 2×SSC, 1×SSC, 0.5×SSC and buffer I, incubated in NSS at 37 °C for 30 min, and then Dig-Ap (1:500) for 2 h, and finally detected with NBT/ BCIP of Dig DNA labeling and detection kit. Diagnosis was made by blue colorat ion with varied intensities and compared with control sections. All results were analyzed by χ^2 test.

RESULTS

SP Immunochemical results

Cx32 and Cx43 proteins appeared as numerous individual spots intracytoplasmically (Figures 1,2), and in some as parts of the plasma membrane. There was fairly intense immunoreactivity in nearly all of the 14 normal liver samples (Cx32 13/14, 92.9%, Cx43 11/14, 78.6%).

In contrast, a clear difference was noted between that in normal tissue and in the HCC, the Cx32 and Cx43 positive spots decreased in the latter especially in grade III HCC, only 18.2% (Cx32) and 12.1% (Cx43) specimens exhibited very weak stainings. The expression of Cx32 and Cx43 proteins in normal liver tissue and I, II and III HCC grades were significantly different ($P<0.01$) (Table 1).

mRNA in situ hybridization results

After restriction digestion, 1.5 kb cx32 cDNA probe was obtained from pGEM3 -cx32 plasmid (Figure 3), and 1.11 kb cx43 cDNA probe from pSG5- cx43 plasmid (Figure 4).

Bright blue specific hybridization appearanc of cx32 mRNA and cx43 mRNA were observed intracytoplasmically in normal liver or HCC tissues (Figures 5-8). The detection rates of cx32 mRNA and cx43 mRNA in normal liver an dI, II and III HCC grades tissues were not significantly different ($P>0.05$).

Table 1 Positive rates of Cx32 and Cx43 proteins in HCC and normal liver

Group	n	Cx32			Cx43		
		+	++	%	+	++	%
HCCI	9	2	3	55.6 ^a	2	2	44.4 ^a
HCCII	19	6	2	42.1 ^a	3	2	26.3 ^a
HCCIII	33	5	1	18.2 ^a	4	0	12.1
Normal liver	14	4	9	92.9	4	7	78.6

^a $P<0.01$ vs normal liver.

DISCUSSION

Since the first cloning of *cx* gene in 1986, there have been rapid progress in identifying and characterizing a multigene family (including at least 13 members) which codes the gap junction proteins^[3]. The cloning sequence of cDNA shows that there is a highly homogenic and strict gene structure among these family members.

The expressions of *cx* genes in different tissues are specific. GJIC plays an important role in the rapid progress of cell society. Gap junctions contain channels that connect contacting cells, and have an apparent selectivity based principally on molecular size, allowing the movement of molecules smaller than M_r1000 , such as cAMP, but preventing the movement of proteins or nucleic acids.

Gap junction mediated GJIC has been postulated to be an important mechanism to maintain tissue homeostasis and to control growth and differentiation. The decreased level of GJIC has been found in many kinds of tumor cells, and this can be important in neoplastic progression by allowing tumor cells to escape local control mechanisms^[4].

Cx32 is widely expressed in many tissues, especially in normal liver, Cx43 is also involved in the gap junctional pathway in liver. This study revealed that Cx32 protein and Cx43 protein expressed at a high level in normal liver and even in pericancerous liver tissues, but at a decreased level in HCC tissues. The lack of Cx32 and Cx43 proteins reduced the number of GJIC between HCC cells and surrounding non-tumorous cells, finally resulting in progression of hepatocarcinogenesis.

In some studies, Cx43 protein was not detectable in normal liver, but could be found in HCC cells. These results suggested that certain tumors or transformed cells have normal levels of gap junction or GJIC and lack of functional gap junctions is not a general feature of malignancy^[5], the exact mechanisms remain to be elucidated.

Because there was no decrease in levels of *cx32* mRNA and *cx43* mRNA in HCC and normal liver tissues, the aberrant localization of Cx32 and Cx43 might be responsible for the reduced GJIC in HCC^[6]. It appears that the control mechanisms of *cx32* and *cx43* genes transcription are not affected during human liver tumorigenesis.

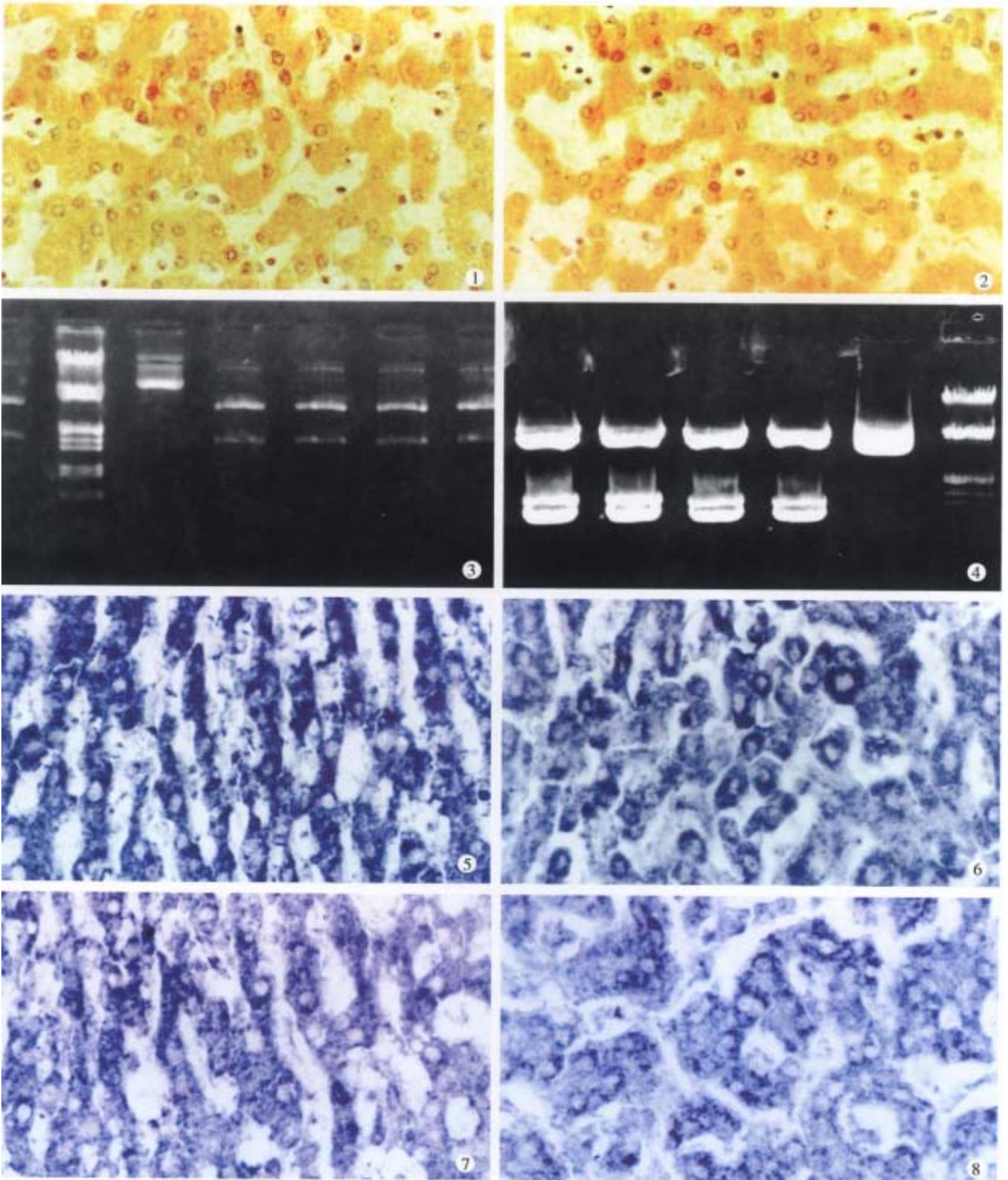


Figure 1 Cx32 protein is positive in normal liver. SP×400

Figure 2 Cx43 protein is positive in normal liver. SP×400

Figure 3 Digestion of pGEM3-cx32 by EcoR-I. A. λ DNA/Hind-III+ EcoR-I marker; B. pGEM3-cx32 plasmid; C. restriction digestion of 1.5 kb cx32 cDNA.

Figure 4 Digestion of pSG5-cx43 by BamH-I. A. λ DNA/Hind-III+ EcoR-I marker; B. pSG5-cx-43 plasmid; C. restriction digestion of 1.11 kb cx43 cDNA.

Figure 5 cx32 mRNA is positive in normal liver. ISH×400

Figure 6 cx32 mRNA is positive in HCC. ISH×400

Figure 7 cx43 mRNA is positive in normal liver. ISH×400

Figure 8 cx43 mRNA is positive in HCC. ISH×400

Not any mutation in the coding sequence of cx genes from any of the human tumors has been found. It is likely that the aberrant location of Cx32 and Cx43 in HCC cells is due to disruption of the mechanisms for construction of these proteins into gap junction plaques rather than to structural abnormality of the Cx32 and Cx43 themselves. The possibility is that there is defect in post-translational processing of Cx32 and Cx43 proteins, which may be essential for their transport to membrane. Post-translational phosphorylation^[7] may be the important factors controlling the GJIC mediated by Cx32 and Cx43 in HCC, it is also responsible for the assembly or function of these proteins.

In conclusion, the aberrant localization of Cx32 and Cx43 proteins in HCC is noteworthy, which has direct biological significance in the process of hepatocarcinogenesis. Recent results demonstrate that the cx gene family is a class of non-mutant tumor-suppressive gene^[8], it should be regarded as an effectual marker of early diagnosis or treatment for liver cancer.

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TT viral infection through blood transfusion: retrospective investigation on patients in a prospective study of post-transfusion hepatitis

Yang SS, Wu CH, Chen TH, Huang YY and Huang CS

Subject headings blood transfusion; TT viral infection; hepatitis C; antibody, viral

Abstract

AIM To investigate the role of blood transfusion in TT viral infection (TTV).

METHODS We retrospectively studied serum samples from 192 transfusion recipients who underwent cardiovascular surgery and blood transfusion between July 1991 and June 1992. All patients had a follow-up every other week for at least 6 months after transfusion. Eighty recipients received blood before screening donors for hepatitis C antibody (anti-HCV), and 112 recipients received screened blood. Recipients with alanine aminotransferase level >2.5 times the upper normal limit were tested for serological markers for viral hepatitis A, B, C, G, Epstein Barr virus and cytomegalovirus. TTV infection was defined by the positivity for serum TTV DNA using the polymerase chain reaction method.

RESULTS Eleven and three patients, who received anti-HCV unscreened and screened blood, respectively, had serum ALT levels >90IU/L. Five patients (HCV and TTV:1; HCV, HGV, and TTV:1; TTV:2; and CMV and TTV:1) were positive for TTV DNA, and four of them had sero-conversion of TTV DNA.

CONCLUSION TTV can be transmitted via blood transfusion. Two recipients infected by TTV alone may be associated with the hepatitis. However, whether TTV was the causal agent remains unsettled, and further studies are necessary to define the role of TTV infection in chronic hepatitis.

INTRODUCTION

After hepatitis B screening in blood donors, the addition of antibody against hepatitis C virus (anti-HCV) has further reduced the occurrence of post-transfusion hepatitis dramatically^[1,2]. However, there still exists some post-transfusion hepatitis, that may be caused by cytomegalovirus (CMV) and other viruses. Among them hepatitis G virus (HGV) infections once had been considered^[1,3,4]. In 1997, a novel virus named TT virus (TTV) was reported by Japan^[5], and the virus is known to be an unenveloped, single-stranded DNA virus with a sequence of 3739 bases. The virus can transmit through blood transfusion^[5]. In Japan, 12% of blood donors and 46% of chronic non-A-G hepatitis patients have detectable TTV DNA in their serum^[6]. Taiwan is an area prevalent for viral hepatitis^[7,8], and the role of TTV has not documented. We therefore studied the role of TTV infection in those patients who received blood transfusions, using serum samples in a previous prospective study of post-transfusion hepatitis.

MATERIALS AND METHODS

We retrospectively studied stored serum samples, which were collected in a prior prospective study for post-transfusion HCV infection^[1] for TTV infection. These serum samples were collected from 192 blood recipients who underwent cardiovascular surgery at the Cathay General Hospital, Taipei, Taiwan from July 1991 to June 1992, before universal screening of blood donors for anti-HCV that was implemented in July 1992. All serum samples were stored at -70 °C. Among them, 19 recipients were healthy hepatitis B surface antigen (HBsAg) carriers with serum alanine aminotransferase levels (ALT) <45IU/L. No recipient had had a blood transfusion within 12 months before recruitment. Recipients already positive for anti-HCV before transfusion, and those having alcoholic, drug-related, autoimmune, or ischemic hepatitis were excluded.

Eighty and 112 recipients received anti-HCV unscreened and screened blood, respectively. For anti-HCV screening in the study, a second-generation enzyme immunoassay (EIA-III, UBI HCV EIA, United Biomedical, Inc., New York,

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NY) was used. All recipients were tested for serum ALT levels and anti-HCV to exclude the presence of possible viral hepatitis C before transfusion. After transfusion, all patients had a follow-up every other week until 6 months after transfusion, and blood samples were also collected.

Recipients with two successive ALT > 2.5 times the normal upper limit were tested for serologic markers for viral hepatitis A, B, C, E, G, TTV, Epstein-Barr virus (EBV), and CMV as well as antinuclear antibodies. Acute viral hepatitis A, B, and E were defined if the recipients were positive for immunoglobulin M antibodies to hepatitis A virus (IgM anti-HAV; HAVAB-M EIA, Abbott Lab., Abbott Park, IL), hepatitis B core antigen [IgM anti-HBc; Corzyme-M (rDNA) Abbott Lab.], and hepatitis E virus (IgM anti HEV; HEV IgM ELISA, Genelabs Diagnostics PTE Ltd, Singapore Science Park, Singapore). Sero-conversion of anti-HCV using the EIA method and/or HCV ribonucleic acid (HCV RNA) using reverse transcription-nested polymerase chain reaction (RT-PCR) assay with primers derived from the 5'-untranslated region of HCV genome was used to define acute viral hepatitis C^[9]. A titer of $\geq 1:64$ for IgM antibodies to Epstein-Barr virus (IgM anti-EBV, IP Azyme, EB/VCA IgM, Savyon Diagnostic Ltd., Beer Sheva, Israel) and CMV (IgM anti-CMV, IP Azyme CMV IgM, Savyon Diagnostic Ltd.) using the immunoperoxidase assay were defined as having acute EBV and CMV hepatitis. Sera were tested for antinuclear antibodies using the immuno-fluorescent method (Fluoro HEPANA, Medical & Biological Lab., Nagoya, Japan).

Patients with positive HBsAg (Auszyme, Abbott Lab.) were also tested for hepatitis Be antigen [HBeAg; HBe (rDNA) EIA, Abbott Lab.] and antibody to hepatitis delta virus (anti-HDV; Wellcozyme, Wellcome Diagnostics, England) using the EIA method.

The occurrence of sero-conversion of GBV-C/HGV RNA was defined as acute HGV infection. The GBV-C/HGV RNA was identified with RT-PCR using nested primers from the 5'-untranslated region of the viral genome as previously described^[10].

The diagnosis of acute TTV infection was based on the occurrence of sero-conversion of TTV DNA determined using PCR method with semi-nested primers as previously described^[6,11]. Briefly, DNA was extracted from 100 μ L of serum using QIAMP Blood kit (QIAGEN Ltd., Crawley, UK) and re-suspended in 50 μ L of elution buffer. For the first round of PCR, 25 μ L of reaction mixture containing 2 μ L of the cDNA sample, 1 \times PCR buffer (10 mM tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂,

0.01% gelatin, and 0.1% Triton X-100), 10 mM of each dNT P, 100ng of each outer primer T-1 (sense: ACA GAC AGA GGA GAA GGC AAC ATG -3') and T-2 (anti-sense : 5'-CTA CCT CCT GGC ATT TTA CC-3'), and 1 unit of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. On a microliter of the PCR products was re-amplified for another 30 cycles with 100 ng of inner primers, T-3 (sense: 5'-GGC AAC ATG TTA TGG ATA GAC TGG-3') and T-4 (anti-sense: CTG GCA TTT TAC CAT TTC CAA AGT T-3'). The amplified products were separated by 3% agarose gel electrophoresis and stained with ethidium bromide.

Statistical analysis was performed using Student's *t* test, Yates' corrected Chi-square, and one-tailed Fisher's exact test where appropriate.

RESULTS

In the 80 and 112 recipients who received anti-HCV unscreened and screened blood, respectively, the gender (male/female = 43/37 vs 66/46, $P = 0.48$), age [mean \pm SD, (range)] = 44 \pm 20 years (4-76 years) vs 45 \pm 22 years (4-75 years, $P = 0.52$), number of HBsAg carriers (9 vs 10, $P = 0.60$), and volume of blood transfused [mean \pm SD, (range)] = 18.0 \pm 14.9 units (2-67 units) vs 18.8 \pm 12.7 units (1-70 units), $P = 0.58$ were not significantly different between the two groups. Eleven (13.8%) and three (2.7%, $P = 0.004$) subjects who received unscreened and screened blood had serum ALT levels >90 IU/L, respectively (Table 1). Among them, four (36.4%) and one (33.3%, $P = 0.72$) patients were positive for TTV DNA, respectively.

Table 1 Clinical and laboratory data of 14 Patients with Post-transfusion hepatitis

Patient	Age (yr)/gender	Peak ALT (IU/L)	Hepatitis
Unscreened Blood			
SYS	58/F	1043	HCV
CLT	66/M	527	HCV
LCSG	74/F	264	HCV
PTT	76/M	109	HCV, TTV
SGM*	43/M	93	HCV
LWG	67/M	257	HCV, HGV
STS	60/M	455	HCV, HGV, TTV
CSPC**	48/F	218	HBV, CMV
CST	30/M	236	CMV, HGV
LYY	13/F	103	TTV
CSG	64/M	159	159
Screened Blood			
CPL	65/F	645	CMV, TTV
HWL	65/F	541	CMV
CHL***	63/M	101	HBV

*Patients were treated with interferon alfa 2b; **HBsAg (+), HBeAg (-), anti-HBe (+), anti-HDV (-); ***HBsAg (+), HBeAg (+), anti-HBe (-), anti-HDV (-);

#Positive for TTV DNA before transfusion.

One patient (CSG) who received unscreened blood was positive for TTV DNA before transfusion (Figure 1). His maximum serum ALT level was 159 IU/L, and maximum serum total bilirubin level was 18.8 $\mu\text{mol/L}$. He was negative for any markers of active hepatitis A-G.

The remaining four subjects had a sero-conversion of TTV DNA. Only one (LYY) of them was negative for markers of hepatitis A-G, and her abnormal serum ALT level and TTV DNA were detected in the 3rd and 6th weeks after transfusion, respectively (Figure 2). Her maximum serum ALT level was 103 IU/L, and maximum serum total bilirubin level was 8.6 $\mu\text{mol/L}$. Two other patients (CSG, LYY) had abnormal serum ALT levels and positivity for TTV DNA until 6 months after transfusion.

The remaining three patients all had a co-infection with other types of hepatitis. All three patients had a transient appearance of TTV DNA lasting only 2 weeks. The first patient (PTT) had HCV and TTV co-infections. His HCV RNA, TTV DNA, abnormal serum ALT activity, and anti-HCV were detected at the 12th, 12th, 18th, and 18th weeks, respectively. His maximum serum ALT level was 109 IU/L, and maximum serum total bilirubin level was 13.7 $\mu\text{mol/L}$. He continued to have abnormal liver tests and positivity for HCV RNA until 27 weeks after transfusion when he finished the follow-up.

The second patient (STS) had HCV, HGV, and TTV co-infection (Figure 3). His HCV RNA, abnormal serum ALT level, anti-HCV, HGV RNA, and TTV DNA were detected at the 2nd, 2nd, 8th, 8th, and 12th weeks after transfusion, respectively. His maximum serum ALT level was 455 IU/L, and maximum serum total bilirubin level was 1.5 mg/dL. His HGV RNA lasted for 24 weeks, and his HCV RNA and abnormal serum ALT levels remained until 37 months after transfusion when he expired from congestive heart failure.

The third patient (CPL) had CMV and TTV co-infection (Figure 4). Her abnormal serum ALT level and IgM and immunoglobulin G (IgG) anti-CMV and TTV DNA were detected at the 2nd, 3rd, and 6th weeks after transfusion, respectively. Her maximum serum ALT level was 645 IU/L, and maximum serum total bilirubin level was 6.8 mg/dL. Her IgM anti-CMV lasted 4 months, and IgG anti-CMV lasted 38 months. Her serum ALT levels returned to normal at the 13th month after transfusion.

None of our patients with post-transfusion hepatitis developed fulminant hepatic failure. Of the five patients with TTV infection, only one patient (CPL), who was co-infected with CMV hepatitis, developed jaundice clinically.

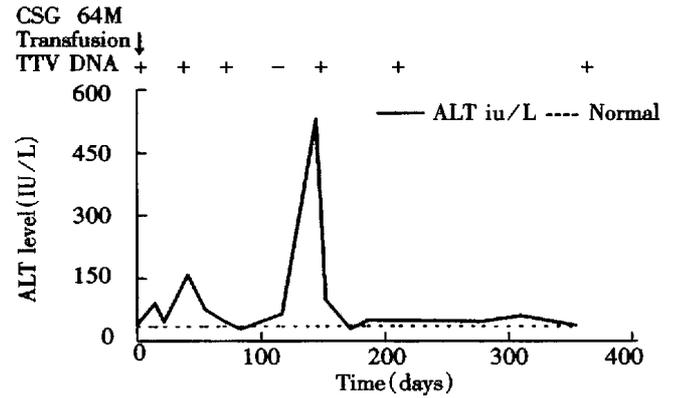


Figure 1 A 64-year-old man who received unscreened blood was positive for TTV DNA before and after transfusion.

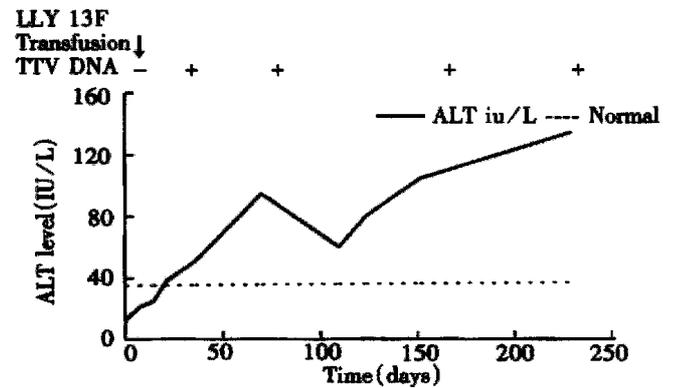


Figure 2 A 13-year-old girl who received unscreened blood had sero-conversion of TTV DNA after transfusion.

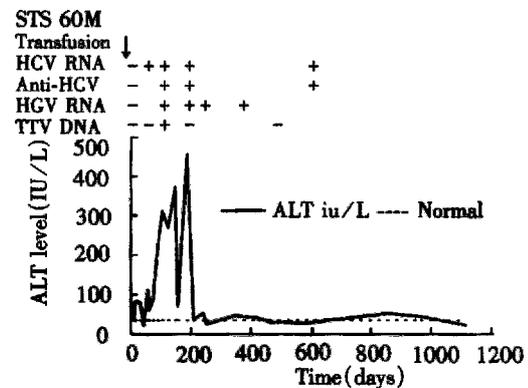


Figure 3 A 60-year-old man who received unscreened blood had sero-conversion of TTV DNA after transfusion. He was co-infected with viral hepatitis C and G.

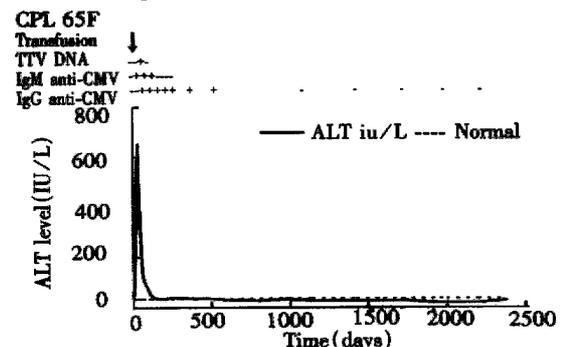


Figure 4 A 65-year-old woman who received anti-HCV screened blood had sero-conversion of TTV DNA after transfusion. She was co-infected with cytomegalovirus infection.

DISCUSSION

In the present study, four patients experienced sero-conversion of TTV DNA after receiving blood unscreened or screened for anti-HCV. Our findings are consistent with recent studies that TTV infection can be transmitted through blood transfusion^[6,12-14]. Although the screening of blood donors with anti-HCV had dramatically reduced the post-transfusion hepatitis from 13.8% to 2.7%, one recipient who received anti-HCV-screened blood developed post-transfusion TTV infection. Our data also showed that the rate of TTV infection is not different between patients who received unscreened and screened blood. Therefore, anti-HCV screening in donor blood does not seem to affect the rate of TTV infection.

Three of our patients had co-infection of TTV infection and other types of hepatitis, and the appearance of serum TTV DNA was only transient. Although all three patients had abnormal serum ALT levels lasting more than 6 months, their chronic hepatitis was not necessarily related with TTV infection. In Japan, the prevalence of TTV infection was 12% in blood donors and was 46% in chronic hepatitis and/or cirrhosis sufferers^[6]. The prevalence of TTV infection in Taiwan was 10% in healthy adults and was 15%-36% in those with chronic hepatitis B or C^[11], findings similar to those in Japan^[6,14]. These findings indicate that TTV infection is common in East Asia. But, the rate of TTV-related post transfusion hepatitis in the present study was only 2.1%. The relative low prevalence of TTV-related post-transfusion suggests that most TTV infections are mild beyond detection clinically. This is further supported by only one patient with TTV infection developing jaundice clinically. Our findings are consistent with recent reports that most post-transfusion TTV infections are likely to be mild clinically^[11,12,15,16].

In the present study, all three patients with HGV infection were co-infected with other types of hepatitis. This is consistent with the findings that risk factors of HGV carriage to other body-fluid transmitted viruses. One percent of healthy adults in Taiwan have detectable HGV RNA^[3,4,17,18].

Our data also show that two patients, who were previously diagnosed as non-A-G hepatitis, were positive for TTV DNA. The one patient with detectable serum TTV DNA before and after transfusion most likely had TTV infection before transfusion. Another patient with sero-conversion of TTV DNA after transfusion is likely to have had an acute TTV infection. Both of these patients had chronic hepatitis with abnormal serum ALT levels for more than 6 months, and their chronic hepatitis may have been related to TTV infection. One patient (LYY) had a recent followed-up 7 years

after the blood transfusion. She had a serum ALT level, non-detectable serum TTV DNA, and positivity for anti-HCV (authors' unpublished observation). She has been a nurse for one year, and she becomes positive for anti-HCV 6 months ago. The blood transfusion 7 years ago was not likely the cause of HCV infection. Although we were determined to exclude other kinds of hepatitis, it is possible that these two patients may have developed hepatitis other than TTV infection. Further studies are necessary to understand the role of TTV infection in chronic hepatitis.

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Stereoselective propranolol metabolism in two drug induced rat hepatic microsomes

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Subject headings propranolol; enantiomers; rat hepatic micro some; phenobarbital; β -naphthoflavone

Abstract

AIM To study the influence of inducers BNF and PB on the stereoselective metabolism of propranolol in rat hepatic microsomes.

METHODS Phase I metabolism of propranolol was studied by using the microsomes induced by BNF and PB and the non-induced microsome as the control. The enzymatic kinetic parameters of propranolol enantiomers were calculated by regression analysis of Lineweaver-Burk plots. Propranolol concentrations were assayed by HPLC.

RESULTS A RP-HPLC method was developed to determine propranolol concentration in rat hepatic microsomes. The linearity equations for R(+)propranolol and S(-)propranolol were $A = 705.7C + 311.2C$ ($R = 0.9987$) and $A = 697.2C + 311.4C$ ($R = 0.9970$) respectively. Recoveries of each enantiomer were 98.9%, 99.5%, 101.0% at 60 $\mu\text{mol/L}$, 120 $\mu\text{mol/L}$, 240 $\mu\text{mol/L}$ respectively. At the concentration level of 120 $\mu\text{mol/L}$, propranolol enantiomers were metabolized at different rates in different microsomes. The concentration ratio R(+)/S(-) of control and PB induced microsomes increased with time, whereas that of microsome induced by BNF decreased. The assayed enzyme parameters were: 1. Km. Control group: R(+) 30 ± 8 , S(-) 18 ± 5 ; BNF group: R(+) 34 ± 3 , S(-) 39 ± 7 ; PB group: R(+) 38 ± 17 , S(-) 36 ± 10 . 2. Vmax. Control group: R(+) 1.5 ± 0.2 , S(-) 2.9 ± 0.3 ; BNF group: R(+) 3.8 ± 0.3 , S(-) 3.3 ± 0.5 ; PB group: R(+) 0.07 ± 0.03 , S(-) 1.94 ± 0.07 . 3. Clint. Control group: R(+) 60 ± 3 , S(-) 170 ± 30 ; BNF group: R(+) 111.0 ± 1 , S(-) $84 \pm$

5; PB group: R(+) 2.0 ± 2 , S(-) 56.0 ± 1 . The enzyme parameters compared with unpaired *t* tests showed that no stereoselectivity was observed in enzymatic affinity of three microsomes to enantiomers and their catalytic abilities were quite different and had stereoselectivities. Compared with the control, microsome induced by BNF enhanced enzyme activity to propranolol R(+)enantiomer, and microsome induced by PB showed less enzyme activity to propranolol S(-)-enantiomer which remains the same stereoselectivities as that of the control.

CONCLUSION Enzyme activity centers of the microsome were changed in composition and regioselectivity after the induction of BNF and PB, and the stereoselectivities of propranolol cytochrome P450 metabolism in rat hepatic microsomes were likely due to the stereoselectivities of the catalyzing function in enzyme. CYP-1A subfamily induced by BNF exhibited pronounced contribution to propranolol metabolism with stereoselectivity to R(+)-enantiomer. CYP-2B subfamily induced by PB exhibited moderate contribution to propranolol metabolism, but still had the stereoselectivity of S(-)-enantiomer.

INTRODUCTION

Propranolol is a nonselective β -adrenergic blocking agent and widely used in clinic as a racemic mixture of R(+) and S(-) enantiomers. It is extensively metabolized and only a small amount of the drug is excreted unchanged^[1,2]. As a beta blocking agent, the optical isomers of propranolol exert different beta receptor blocking and membrane stabilizing effects^[3], therefore its stereoselective metabolism is of clinical importance. Propranolol is metabolized into a number of products *in vivo*. These products arise from naphthalene-ring hydroxylation^[1], N-dealkylation of the isopropanolamine side-chain and side-chain o-glucuronidation^[4,5]. When the influence by the hepatic blood flow^[6] and oxygen delivery^[7] *in vivo* is not considered, the metabolism by monooxygenation is mainly responsible for propranolol elimination in hepatic microsomes and O-glucuronidation was shown to be a minor pathway *in vivo*^[2] and *in vitro*^[5].

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The oxidative metabolism of propranolol is catalyzed by cytochrome p-450. Experiments by Otton SV *et al*^[8] and Ishida R *et al*^[9] indicated that multiple isozymes were involved in propranolol metabolism in rat liver microsomes. Nelson *et al*^[10] have observed that stereoselectivity of propranolol metabolism in 9000 g liver supernatant differs depending on the positions of metabolism. Although the metabolic fate of propranolol in rat has been studied extensively, the impact of PB and BNF induction on stereoselective propranolol metabolism in rat hepatic microsome was rarely reported. This experiment studied the stereoselective metabolism of propranolol in rat hepatic microsomes induced by BNF and PB and the enzymatic parameters were compared with that of the control.

MATERIALS AND METHODS

Chemicals and solutions

R(+) and S(-)-propranolol (hydrochloride), β -naphthoflavone (BNF), phenobarbital (PB) NADP and NADPH were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Tris-hydroxymethyl aminomethane (Gibco BRL) and bovine serum albumin (Serva) were purchased from Shanghai Reagent Station. All other chemicals were obtained from the common commercial sources.

Tris-HCl buffer (0.1 mol/L, pH 7.4): 1.21 g of Tris-hydroxymethyl aminomethane was dissolved in 60 mL of water. The solution was adjusted to pH 7.4 by concentrated hydrochloride acid and then diluted with water to the desired volume of 100 mL. This solution was used to prepare rat hepatic microsome.

Ammonium acetate buffer: 4.0 g of ammonium acetate was dissolved in 10 mL glacial acetic acid and then diluted with water to the desired volume of 1000 mL (pH 4.0). This solution was used to prepare mobile phase.

Preparation of hepatic microsomes

Sprague-Dawley rats (male, 160 g - 200 g) were divided into three groups. One group received i.p. injection of sodium PB dissolved in physiological saline (0.9% NaCl) (80 mg/kg-d) for 3 days, another group, BNF in cornoil (80 mg/kg-d) for 3 days and the last group received nothing 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-HCl buffer. Hepatic microsomes were prepared with the ultracentrifugation method described by Gibbson GG *et al*^[11]. All manipulations were carried out in a cold bath. Pellets were re-suspended in sucrose-Tris buffer (pH 7.4) (95:5) and immediately stored at -30 °C.

Protein concentrations of the microsomal preparations were measured by the method of Lowry *et al*^[12] using crystalline bovine serum albumin as the protein standard.

Incubation of propranolol and rat hepatic microsomes

0.5 mL incubation mixture containing 1 mg/mL microsomal protein per milliliter (85 mmol/L Tris-HCl buffer (pH 7.4), 50 mmol/L nicotinamide, 15 mmol/L MgCl₂, 3 mg/mL DL-isocitric acid tri-sodium salt, 0.4 units/mL isocitric dehydrogenase) was used. Phase I metabolism was performed with 0.5 mL of the mixture bubbled with oxygen for 1 min and R(+) or S(-)-propranolol enantiomer as the substrate. After 5 min pre-incubation under air at 37 °C, reaction was started by adding 10 μ L of NADPH regenerating system (10 mg NADP and 3 mg NADPH in 100 μ L of 1% NaHCO₃). The reaction was stopped after the indicated time by adding 0.5 mL of methanol and centrifuged at 4000 r/min for 10 min. 10 μ L of the supernatant was sampled into HPLC.

HPLC procedure for propranolol determination in rat hepatic microsomes

A HPLC procedure was established to assay propranolol enantiomers in rat hepatic microsomes. After the termination of the reaction with methanol, 10 μ L of the sample was applied to a reversed phase column (Shim-pack CLC-ODS 15 cm \times 0.6 cm id, 10 μ m particle size). Propranolol was monitored with a UV detector at 290 nm. The mobile phase was made up with ammonium acetate buffer (pH 4.0)-methanol (50:50). The flow rate was 1.0 mL/min. Figure 1 shows the typical elution of propranolol in incubation solution.

Statistical analysis of the data

The maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) values for propranolol were determined by regression analysis of Lineweaver-Burk plots. The $\bar{x} \pm s$ of three determinations of V_{max} and K_m was calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of V_{max}/K_m . The statistical difference between propranolol enantiomers was tested using an unpaired *t* test.

RESULTS

Validation of HPLC

Linearity Drug-free microsomes were spiked with increasing concentrations of propranolol enantiomers (10 μ mol/L - 620 μ mol/L). The solution was constituted according to "Incubation of propranolol with rat hepatic microsomes" with no occurrence of metabolism reaction. Propranolol enantiomers were assayed by HPLC precisely described. Standard calibration curves were

constructed by performing a linear regression analysis of the peak area (Y) of propranolol enantiomers versus their concentrations (X), i.e., R(+)-propranolol: $Y = 705.7 + 311.2X$, $r = 0.9987$; S(-)-propranolol: $Y = 697.2 + 311.4X$, $r = 0.9970$. The limit of detection (single-to-noise ratio = 3) for propranolol was $3 \mu\text{mol/L}$.

Precision and accuracy The spiked drug-free microsomes at 3 concentration levels ($60 \mu\text{mol/L}$, $120 \mu\text{mol/L}$ and $240 \mu\text{mol/L}$) were assayed following the procedure of 2.1.1. Results were listed in Table 1.

Table 1 Accuracy and precision to assay propranolol in rat liver microsome

Target concentrations ($\mu\text{mol/L}$)	Recovery (%)	Precisions (RSD, %)	
		Intra-assay ($n = 3$)	Inter-assay ($n = 3$)
60	98.8	5.1	5.6
120	99.5	3.5	4.8
240	101.0	3.2	5.3

Concentration-time curves and variation of the ratio of R(+)/S(-) propranolol concentration in microsomes after incubation of different time Phase I metabolism was performed with 0.5 mL of the mixture and $60 \mu\text{mol}$ of propranolol enantiomers as the substrate. The incubation procedure was carried out according to 1.3. and 1 mL of methanol was added to stop the reaction at 0, 40, 80, 160, 320 min respectively. The mixtures were then analyzed by HPLC. Results are shown in Figure 2 and Table 2.

Table 2 Ratio of R(+)/S(-) propranolol concentration in incubation media at different incubation time

Group	Ratio of R(+)/S(-) propranolol					
	0	5	10	15	20	30 (min)
Control	0.989	9.99 ± 0.07	1.01 ± 0.10	1.02 ± 0.02	1.04 ± 0.04	1.07 ± 0.02
BNF	0.989	0.94 ± 0.05	0.93 ± 0.06	0.93 ± 0.04^a	0.95 ± 0.05^c	0.91 ± 0.05^{bc}
PB	0.989	1.05 ± 0.06	1.04 ± 0.08	1.05 ± 0.10	1.09 ± 0.05	1.09 ± 0.06

Values were obtained from propranolol concentration at $120 \mu\text{mol/L}$ for each enantiomer, BNF and PB: microsomes from the rats induced with BNF (β -naphthoflavone) or (phenobarbital) $80 \text{ mg}/(\text{kg}\cdot\text{d})$, ip, 3 d, respectively. $\bar{x} \pm s$, $n = 3$. $^a P < 0.05$, $^b P < 0.01$, compared with control; $^c P < 0.05$, compared with PB by unpaired t test.

It was indicated that at the propranolol concentration level of $120 \mu\text{mol/L}$, propranolol enantiomers were metabolized in different rate in different microsomes. The ratio of R(+)/S(-) propranolol concentration in incubation media in control and PB group increased, whereas that in BNF group decreased. The ratio of R(+)/S(-) propranolol concentration in BNF group was significantly different with the corresponding ratio

in control group or PB group at 15, 20 and 30 min ($P < 0.05$, 0.01).

Enzymatic kinetic parameters for propranolol metabolism in liver microsomes from control, BNF and PB induced rats The enzymatic kinetic parameters of propranolol enantiomers were calculated by Lineweaver-Burk method with the substrate concentrations of $20 \mu\text{mol/L}$ - $600 \mu\text{mol/L}$ in three forms of rat hepatic microsomes after 10 min incubation (1.3). The results were listed in Table 3.

Table 3 Enzymatic kinetic parameters in propranolol enantiomer metabolism *in vitro* in rat hepatic microsomes induced by β -naphthoflavone or phenobarbital

Group	Enantiomer	Km	V_{max}	Clint	R(+)/Vmax:
		$\mu\text{mol/L}$	$\text{mmol/g}\cdot\text{min}$	$\text{L}/\text{min}/\text{g protein}$	S(-)/Vmax
Control	R(+)	30 ± 8	1.5 ± 0.2^b	60 ± 3^b	0.5
	S(-)	18 ± 5	2.9 ± 0.3	170 ± 30	
BNF	R(+)	34 ± 3	$3.8 \pm 0.3^{\text{th}}$	$111.0 \pm 1^{\text{th}}$	1.14
	S(-)	$39 \pm 7^{\text{d}}$	$3.3 \pm 0.5^{\text{g}}$	$84 \pm 5^{\text{h}}$	
PB	R(+)	38 ± 17	$0.07 \pm 0.03^{\text{ef}}$	$2.0 \pm 2^{\text{ef}}$	0.038
	S(-)	$36 \pm 10^{\text{d}}$	$1.94 \pm 0.07^{\text{e}}$	$56.0 \pm 1^{\text{e}}$	

Clint (intrinsic clearance) is the ratio of V_{max}/Km , mean $\pm s$, $n = 3$. $^a P < 0.05$, $^b P < 0.01$, $^c P < 0.001$, compared with S(-) propranolol; $^{\text{d}} P < 0.05$, $^{\text{e}} P < 0.01$, $^{\text{f}} P < 0.001$, compared with corresponding enantiomer in control group; $^{\text{g}} P < 0.01$, $^{\text{h}} P < 0.001$, compared with corresponding enantiomer in PB group with unpaired t test.

Km of propranolol enantiomers in control group had no stereoselectivity ($P > 0.05$), whereas V_{max} and Clint had stereoselectivity of S(-)-propranolol ($P < 0.01$). For BNF induced microsome, Km and V_{max} had no stereoselectivity between R(+), S(-)-propranolol ($P > 0.05$), and Clint had significant difference between the two enantiomers ($P < 0.05$). For PB group, Km had no stereoselectivity ($P > 0.05$), and V_{max} , Clint had stereoselectivity of S(-)-propranolol ($P < 0.001$).

Comparing the enzymatic parameters of R(+)-propranolol among three microsomes, Km had no statistical difference ($P > 0.05$), whereas V_{max} and Clint had statistical differences ($P < 0.05$, 0.01 or 0.001); compared with the control group, V_{max} for BNF group increased 2.5 times and that for PB group decreased 20 times; clint for BNF and PB group increased or decreased 1.8 and 30 times, respectively. With the same way to compare those parameters of S(-)-propranolol, Kms for BNF and PB group increased 2.2 and 2.1 times, respectively, but had no statistical difference with each other; V_{max} for PB group decreased about 1.5 times and that for BNF group nearly remained the same, in addition, no statistical difference was found between PB and BNF group; Clint for BNF and PB group decreased 2 times and 1.5 times respectively and there was significant difference between BNF and PB group.

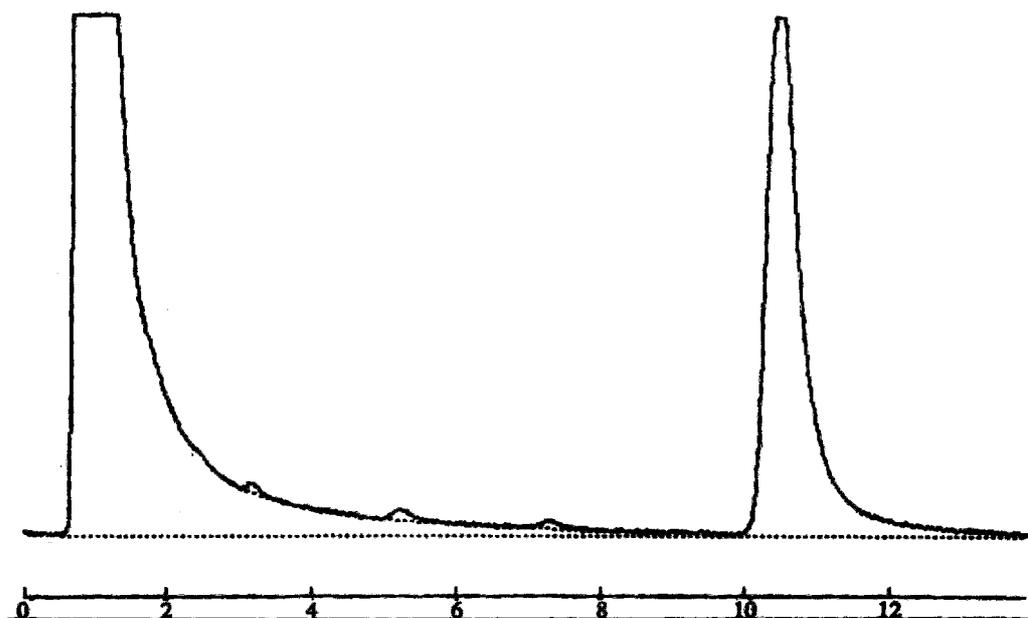


Figure 1 Chromatograms of propranolol after incubation with rat hepatic microsomes. A Shim-pack CLC-ODS column (15cm×0.6cm i.d.) was used. The mobile phase was constituted with ammonium acetate buffer (pH4.0)-methanol (50:50) with flow rate at 1.0mL/min. Propranolol was monitored at 290nm. Propranolol: $R=10.1$ min.

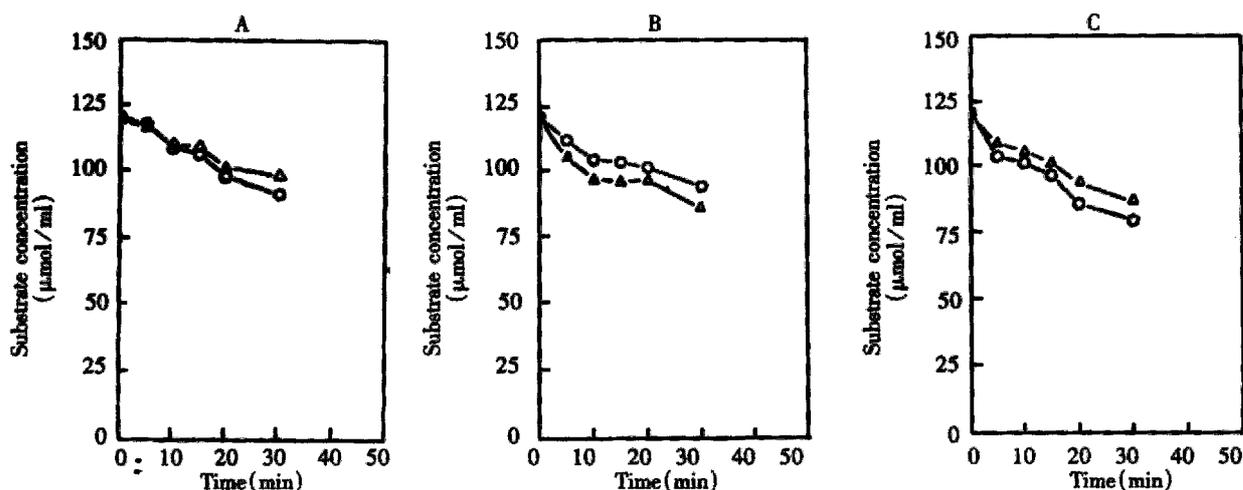


Figure 2 Concentration time curves for R(+) and S(-)-propranolol metabolism in rat hepatic microsomes. A. Microsome of control. B. Microsome induced by BNF. C. Microsome induced by PB. $\Delta-\Delta$: R(+)-propranolol. $\circ-\circ$: S(-)-propranolol.

DISCUSSION

In this *in vitro* study, stereoselectivity of propranolol occurred in catalyzing velocity and intrinsic clearance in control group, and no stereoselectivity was observed in enzyme affinity to the substrate. The introduction of BNF and PB caused changes in the composition of CYP subfamilies and therefore influenced the stereoselective catalyzing ability of microsome to propranolol metabolism, or even reversed the sequence of stereoselectivity, whereas the affinity of enzyme to substrate remained nearly the same and had no stereoselectivity. This phenomenon indicated that, regio-structure of binding site in the activity

center of enzyme was almost unchanged, and that of the catalyzing site was significantly changed in propranolol metabolism in rat hepatic microsomes after the introduction of PB and BNF, the influence of BNF and PB induction had reversed effect on the catalyzing stereoselectivity of microsome to propranolol.

BNF is an inducer of CYP-1A subfamily^[13-15] and PB is that of CYP-3A^[15], CYP-2B subfamily^[16,17] (IIB1 and IIB2^[18]). Different kinds of cytochrome P-450 may be involved in propranolol metabolism, depending on the metabolic positions^[10]. CYP-1A is suggested to catalyze 4, 5-

hydroxylation and *N*-desisopropylation stereoselectively^[19,20]. CYP-1A2 accounts for about 10% to 15% of the total CYP content of human liver and is the major enzyme involved in the metabolism of propranolol^[21]. Another subfamily CYP-2D6 mainly catalyzes 4, 5 and 7-hydroxylation stereoselectively^[22,23] and it has been confirmed that CYP-2D6 does not contribute to *N*-desisopropylation of propranolol^[8]. *N*-desisopropylation in propranolol enantiomer metabolism is mainly mediated by CYP-1A2^[24,25]. Masubuchi Y *et al*^[26] reported that there is competition between enantiomers of propranolol for the enzyme, probably the same enzyme, a cytochrome P450 isozyme in the CYP-2D subfamily. All of these showed that different cytochrome subfamilies have different functions in metabolism of propranolol enantiomers and the optical isomers of propranolol have different stereoselectivities in metabolism. Our results indicated that CYP-1A was involved in propranolol metabolism and showed the stereoselectivity of R(+)-enantiomer in general. CYP-3A, CYP-2B subfamily does not play a main role in propranolol metabolism *in vitro*, though it showed the stereoselectivity of S(-)-enantiomer.

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Protective effect of early enteral feeding on postburn impairment of liver function and its mechanism in rats

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Subject headings early enteral feeding; liver; postburn impairment

Abstract

AIM To study the protective effect of early enteral feeding (EEF) on the postburn impairment of liver function and its mechanism.

METHODS Wistar rats with 30% of total body surface area (TBSA) full-thickness burn were employed. The effects of EEF on the postburn changes of gastric intramucosal pH, endotoxin levels in portal vein, water contents of hepatic tissue, blood concentrations of tumor necrosis factor (TNF- α), plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as the blood contents of total (TB) and direct bilirubin (DB), total protein (TP) and albumin (ALB) were serially determined within 48h postburn.

RESULTS EEF could significantly improve gastric mucosal acidosis, reduce portal vein endotoxin level and water content of hepatic tissue, as well as plasma concentrations of TNF- α at all timepoints after severe burns ($P < 0.01$); postburn elevation of the plasma activities of ALT, AST and the contents of TB, DB were effectively prevented, whereas the plasma concentrations of TP and ALB were markedly increased 24 h and 48 h postburn in EEF group compared with that of the burn without EEF group ($P < 0.01$).

CONCLUSION EEF has significant beneficial effects on the improvement of hepatic function in

rats after severe burn, and is probably related with an increase in splanchnic blood flow, reduction of the absorption of gut-origin endotoxin and the consequent release of inflammatory mediators.

INTRODUCTION

Acute impairment of hepatic function is one of the most common serious complications after severe burns with an extremely high incidence^[1,2]; however, its prevention and treatment have not yet been effectively improved so far. In recent years, abundant researches have suggested that the posttrauma translocation of gut-origin endotoxin may lead to remote organ injury, and is also the major contributor to the hepatic dysfunction^[3-5]. Meanwhile, it has become increasingly apparent that early enteral feeding (EEF) in various pathological conditions may produce multiple beneficial effects, including the stimulation of splanchnic and hepatic circulation, maintenance of gut mucosal integrity, prevention of intramucosal acidosis and permeability disturbances, and alleviation of the translocation of gut-origin bacteria and endotoxin^[6-10]. We therefore presume that EEF might be possible to improve hepatic function in severe burns, which up to now has been seldom documented. Thus, the present study is designed to verify this hypothesis, and in an attempt to seek ways to improve further the treatment of severely injured patients. This is no doubt of both theoretical and practical importance.

MATERIALS AND METHODS

Animals

Healthy adult wistar rats of either sex, weighing 220 g \pm 30 g, were employed in the study. They were housed in individual metabolic cages in a temperature conditioned room (22 °C-24 °C) with a 12 h light-dark cycle, allowed access to standard rat chow (provided by Experimental Animal Center, Third Military Medical University) and water ad libitum, and acclimatized to the surroundings for 7 days prior to the experiments.

Operative procedure

All animals were weighed and anesthetized with 1%

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pentobarbital sodium (30 mg/kg, ip). After laparotomy, a polyethylene catheter (1.5 mm in diameter) for enteral feeding was inserted into duodenum on the anterior wall 1.5 cm from pylorus via a puncture hole made by a metal needle. The catheter was appropriately fixed, tunneled under the skin and exited through the nape skin. Animals were housed and fed as described above after operation.

Burn injury and resuscitation

After a recovery period of 24 h, the animals inserted with feeding tube were anesthetized, dorsal hair shaved and then placed in a wooden template designed to expose 30% of the total body surface area (TBSA), and then immersed in water at 92 °C for 20 seconds, which resulted in a clearly demarcated full-thickness burn. One hour after burn injury, the animals were resuscitated with 10 mL of warm 0.9% NaCl (normal saline solution, 37 °C) given by intraperitoneal injection. Control animals were similarly anesthetized and shaved but not burned.

Feeding and experimental protocol

Nutrient liquid for feeding was prepared before use as one with a caloric value of 2.1 KJ/mL by mixing nutritional powder (ENSURE, USA) with appropriate amount of warm boiled water. According to different feeding regimens, animals were randomly divided into three groups: ① EEF group. Enteral feeding was initiated 1h postburn in burned animals via feeding tube with a total caloric of 202 KJ·Kg⁻¹·24 h⁻¹; the nutrient liquid required for 24 hours was administered evenly at 6 timepoints. ② Burn group. The animals were treated exactly the same as EEF group, except that the nutrient liquid was substituted by equal amount of saline. ③ Control group. Only the feeding tube was inserted, whereas no tube feeding and burn were conducted. The animals in this group were allowed access to standard rat chow, nutrient liquid and water ad libitum. Timepoints for different measurements and assays in all groups were made at the 3rd, 6th, 12th, 24th and 48th h postburn, except for the determination of liver tissue water content, which was performed at the 12th after thermal injury. For plasma assays, rats were sacrificed by decapitation at each timepoint and heparinized blood was collected in separate tubes, spun at 3 000 g for 10 min, and the plasma frozen at -20 °C until analysis.

Measurements

The gastric intramucosal pH (pHi) was determined with an indirect method as previously described^[11] with minor modification. Briefly, animals were anesthetized and given cimetidine (15 mg) intraperitoneally 1h prior to each timepoint, and then a polyethylene catheter was inserted into

gastric lumen through pylorus via a puncture hole on the anterior wall of duodenum made by a metal needle after a midline laparotomy. An amount of 2.5 mL normal saline was injected into gastric lumen through the catheter and aspirated out to get rid of intragastric residues, and then 1.5 mL normal saline was injected and retained in the gastric lumen. After an equilibration interval of 60 min, 1 mL of saline solution were aspirated and Pco₂ determined using the blood gas analyzer. A simultaneously obtained arterial blood sample was used for determining the [HCO₃⁻]. pHi was then calculated as:

$$pHi=6.1+\log\left(\frac{[HCO_3^-]}{[Pco_2\times 0.03]}\right)$$

The multifunction-biochemical analyzer Beckman Synchron CX-7 was used for performing performing liver function tests. The plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), as well as the blood contents of total (TB) and direct bilirubin (DB), total protein (TP) and albumin (ALB) were determined at each timepoint.

Portal plasma endotoxin levels were assayed with the limulus-amoebocyte-lysate test (LAL)^[12]. In brief, plasma samples were diluted tenfold with pyrogen-free water and heated to 75 °C for 5 min to inactivate the plasma inhibitor. The samples were incubated with LAL at 37 °C for 33 min. The chromogenic substrate was added and the samples incubated for another 3 min. Acetic acid stopped the reaction. The optical density was read at 545 nm and endotoxin concentration was expressed as Eu/mL.

Radioimmunoassay of TNF-α levels in systemic circulation was conducted according to the instructions with kits from Dong Ya Research Institute of Immuno-technology.

Liver tissue water contents were determined with a method as reported in a previous study^[13] with minor modification. Eight Liver tissue samples for each group were harvested at 12 h postburn, weighed, put in oven at 90 °C for 24 h, and then weighed again. The liver tissue water contents were calculated as:

$$\text{Liver tissue water contents}=(\text{wet weight}-\text{dry weight}/\text{wet weight})\times 100\%$$

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

Postburn EEF has beneficial effects on the hepatic functions as demonstrated by the significantly reduced plasma activities of ALT, AST and the blood contents of TB and DB, whereas the plasma

concentrations of TP and ALB were markedly increased 24 h and 48 h postburn in the EEF group compared with that of the burn group without EEF as shown in Tables 1 and 2.

Gastric mucosal acidosis was significantly improved in EEF group animals as indicated by the elevation of gastric pHi at most of the postburn timepoints, however, gastric pHi in the burn group sustained in lower levels until 48 h postburn (Table 3).

Table 4 displays the changes in portal endotoxin levels after severe burns. Three hours postburn, endotoxin concentration significantly increased in the burn group and reached a peak in 6 h; another increase appeared after 24 h and persisted until 48 h postburn. However, the portal endotoxin levels in

animals that received EEF markedly decreased at nearly all timepoints postburn compared with that of the burn group.

The data for plasma TNF- α levels are shown in Table 5. In accordance with other observations, EEF could also significantly reduce TNF- α levels in the systemic circulation at most postburn timepoints as compared with that of burn animals.

The hepatic tissue water contents in the three experimental groups were 71.17 % \pm 0.60 %, 73.01 % \pm 0.52 % and 70.18 % \pm 0.52 % respectively. Evidently, the liver tissue water content in the EEF group was significantly lower than that in the burn group without EEF 12 h postburn ($P < 0.01$).

Table 1 The effects of EEF on the postburn changes of plasma ALT, AST activities and TB, DB contents ($\bar{x} \pm s$)

Group (samples)	Postburn hours				
	3	6	12	24	48
EEF (40)					
ALT (mmol·s ⁻¹ /L)	1.21 \pm 0.07 ^{b,d}	1.54 \pm 0.14 ^{b,d}	1.75 \pm 0.17 ^{b,d}	1.39 \pm 0.09 ^{b,d}	1.09 \pm 0.09 ^{b,d}
AST (mmol·s ⁻¹ /L)	8.58 \pm 0.64 ^{b,d}	11.47 \pm 0.81 ^{b,d}	14.30 \pm 1.04 ^{b,d}	9.75 \pm 0.80 ^{b,d}	7.24 \pm 0.65 ^{b,d}
TB (mmol/L)	16.85 \pm 2.01 ^{a,d}	14.97 \pm 2.36 ^d	12.90 \pm 2.01 ^{a,d}	10.82 \pm 1.71 ^{b,d}	6.59 \pm 1.61 ^b
DB (mmol/L)	7.72 \pm 1.90 ^d	4.68 \pm 1.46 ^{b,d}	2.42 \pm 0.78 ^b	1.72 \pm 0.36 ^b	1.74 \pm 1.09 ^b
Burn (40)					
ALT (mmol·s ⁻¹ /L)	2.06 \pm 0.13 ^d	2.90 \pm 0.19 ^d	3.19 \pm 0.23 ^d	2.99 \pm 0.17 ^d	2.21 \pm 0.14 ^d
AST (mmol·s ⁻¹ /L)	12.20 \pm 0.77 ^d	18.77 \pm 0.84 ^d	23.13 \pm 1.14 ^d	16.18 \pm 0.94 ^d	12.56 \pm 1.00 ^d
TB (mmol/L)	19.26 \pm 2.97 ^d	16.98 \pm 2.11 ^d	15.08 \pm 2.37 ^d	18.32 \pm 2.69 ^d	10.82 \pm 1.97 ^d
DB (mmol/L)	8.26 \pm 2.17 ^d	9.77 \pm 2.02 ^d	5.50 \pm 1.32 ^d	7.10 \pm 1.43 ^d	3.54 \pm 0.94 ^d
Control (40)					
ALT (mmol·s ⁻¹ /L)	0.61 \pm 0.09	0.57 \pm 0.07	0.63 \pm 0.08	0.58 \pm 0.07	0.64 \pm 0.10
AST (mmol·s ⁻¹ /L)	1.55 \pm 0.10	1.64 \pm 0.09	1.60 \pm 0.10	1.71 \pm 0.11	1.58 \pm 0.10
TB (mmol/L)	5.63 \pm 1.41	6.04 \pm 1.27	5.81 \pm 1.62	6.17 \pm 1.02	5.76 \pm 1.38
DB (mmol/L)	1.62 \pm 0.56	1.46 \pm 0.39	1.55 \pm 0.42	1.73 \pm 0.41	1.53 \pm 0.47

^a $P < 0.05$, ^b $P < 0.01$ vs burn group; ^d $P < 0.01$ vs control.

Table 2 The effects of EEF on the postburn changes of plasma total p protein and albumin levels (c/g·L⁻¹, $\bar{x} \pm s$)

Group (samples)	Postburn hours				
	3	6	12	24	48
EEF (40)					
Total protein	43.10 \pm 2.31 ^d	42.49 \pm 3.00 ^d	47.61 \pm 4.39 ^c	58.33 \pm 2.93 ^b	62.36 \pm 4.18 ^{b,d}
Albumin	19.32 \pm 1.34 ^d	19.49 \pm 1.63 ^{a,d}	22.76 \pm 2.19 ^d	25.70 \pm 2.40 ^b	26.77 \pm 1.25 ^b
Burn (40)					
Total protein	43.54 \pm 2.51 ^d	44.79 \pm 2.03 ^d	44.80 \pm 3.63 ^d	48.84 \pm 4.30 ^d	52.77 \pm 1.45
Albumin	19.78 \pm 2.11 ^d	20.99 \pm 1.23 ^d	21.54 \pm 1.72 ^d	21.84 \pm 1.84 ^c	22.50 \pm 0.83 ^d
Control (40)					
Total protein	53.67 \pm 2.43	57.41 \pm 1.83	52.55 \pm 2.62	55.76 \pm 3.18	53.92 \pm 2.88
Albumin	25.38 \pm 1.62	25.72 \pm 1.38	26.08 \pm 1.72	24.46 \pm 1.33	25.64 \pm 1.43

^a $P < 0.05$, ^b $P < 0.01$ vs burn group; ^c $P < 0.05$, ^d $P < 0.01$ vs control.

Table 3 The effects of EEF on the postburn changes of gastric intramucosal pH ($\bar{x} \pm s$)

Group	Samples	Postburn hours				
		3	6	12	24	48
EEF	50	7.119 \pm 0.078 ^{a,b}	6.943 \pm 0.089 ^{a,b}	7.074 \pm 0.037 ^{a,b}	7.285 \pm 0.098 ^a	7.257 \pm 0.077 ^{a,b}
Burn	50	7.017 \pm 0.037 ^b	6.826 \pm 0.049 ^b	6.802 \pm 0.080 ^b	6.949 \pm 0.082 ^b	7.074 \pm 0.041 ^b
Control	50	7.321 \pm 0.054	7.296 \pm 0.067	7.296 \pm 0.067	7.306 \pm 0.069	7.348 \pm 0.074

^a $P < 0.01$ vs burn group; ^b $P < 0.01$ vs control.

Table 4 The effects of EEF on the postburn changes of portal endotoxin in level (Eu/mL, $\bar{x}\pm s$)

Group	Samples	Postburn hours				
		3	6	12	24	48
EEF	40	0.683±0.072 ^{a,b}	0.797±0.085 ^{a,b}	0.542±0.078 ^{a,b}	0.725±0.061 ^{a,b}	0.461±0.049 ^{a,b}
Burn	40	1.394±0.126 ^b	1.518±0.173 ^b	1.124±0.133 ^b	1.627±0.215 ^b	1.168±0.188 ^b
Control	40	0.206±0.032	0.195±0.043	0.189±0.049	0.204±0.037	0.215±0.051

^aP<0.01 vs burn group; ^bP<0.01 vs control.

Table 5 The effects of EEF on the postburn changes of plasma TNF- α level (ng/mL, $\bar{x}\pm s$)

Group	Samples	Postburn hours				
		3	6	12	24	48
EEF	40	1.48±0.38 ^{a,b}	2.57±0.45 ^{a,b}	2.36±0.47 ^{a,b}	1.92±0.26 ^{a,b}	1.68±0.45 ^{a,b}
Burn	40	1.92±0.19 ^b	4.49±0.47 ^b	3.51±0.45 ^b	4.07±0.71 ^b	3.24±0.61 ^b
Control	40	0.83±0.08	0.78±0.11	0.83±0.12	0.81±0.09	0.81±0.09

^aP<0.01 vs burn group; ^bP<0.01 vs control.

DISCUSSION

Nutritional support plays an important role in the management of critically ill patients for preventing and treating multiple organ failure^[14]. However, the concept of the administration of enteral nutrition very early after injury is relatively new^[8]. More than a decade ago, Moore *et al*^[15] reported that immediate postoperative feeding by needle catheter jejunostomy was safe and feasible; and that early nutritional support could decrease the incidence of septic complications in the severely injured patient. In a subsequent study, Mochizuki *et al*^[16] showed that immediate enteral feeding in burned guinea pigs was associated with a decrease in the hypermetabolic state. They demonstrated that early enteral feeding could suppress the expected rise in glucagon, cortisol and norepinephrine after major burn injury, compared with delayed enteral feeding. Since then results of a number of clinical and animal studies were reported, showing that very early enteral feeding could preserve the gut barrier function, diminish hypermetabolic response, maintain caloric intake, reduce infective complications and significantly shorten hospital stay following injury^[6,7,16-18]. Unfortunately, most of these studies paid more attention merely to its trophic and metabolic effects, whereas the other benefits such as its role played in the protection of splanchnic functions were greatly neglected. In the present study, we showed that postburn EEF could result in a low level of plasma ALT, AST activities and TB, DB contents, as well as a rapid restoration of plasma TP and ALB level that have significantly decreased after severe burns. These clearly meant that early enteral feeding could effectively improve hepatic dysfunction caused by burn injury. A previous study showed that circulating levels of bile acids could be a sensitive and specific indicator of liver function, an elevation of serum bile acid levels indicating a deterioration in liver function^[19]. In a rat model of hemorrhagic shock, Zaloga *et*

al^[19] found enteral administration of a peptide-based diet early after hemorrhagic shock, could significantly prevent the elevation of circulating bile acid levels, whereas a 3.6 times of serum bile acid level above baseline was noted in animals with same amount of enteral saline therapy. In a similar rat model, Bortenschlager *et al*^[20] also observed that enteral nutrients significantly decreased liver injury. After hemorrhagic shock, AST in saline controls and enterally fed animals increased from 246 U/L±17 U/L to 1605 U/L±593 U/L and from 283 U/L±39 U/L to 551 U/L±94 U/L respectively; ALT increased from 60 U/L±4 U/L to 726 U/L±355 U/L in controls and 61 U/L±6 U/L to 161 U/L±38 U/L in enterally fed animals. These results further indicated that EEF could protect animals from liver injury in various forms of injury.

The mechanisms of EEF in improving postburn liver function so far have not been fully clarified yet. It has been noted that in severe trauma including burns, the loss of a large amount of body fluids and the release of stress hormones cause a sharp reduction of blood flow to many organs, especially the gastrointestinal tract. Reduced intestinal blood flow then leads to translocation of bacteria and/or their toxic products through the gut mucosa. Subsequent bacteria and/or toxin-induced persistent and excessive release of cytokines (i.e. tumor necrosis factor, interleukins) from hepatic macrophages and complement activation may initiate progressive multiple organ failure and even cause death^[21-23]. In accordance with this theory, many studies suggested that the hepatic ischemia and endotoxemia occurred in various pathological conditions and were the major contributors to liver dysfunction^[3-5,24]. However, Zaloga^[25] also proposed that deprivation of exogenous nutrients for a certain period of time, via a mechanism of substrate lack and tissue antioxidant system depletion, could also compromise organ function.

Postprandial gut hyperemia is a local vascular response to the presence of foodstuff in the lumen, an important physiological phenomenon for food digestion and absorption. Even though in some

pathological conditions, this phenomenon still exists. In burned guinea pigs, Inoue *et al*^[26] using radiolabeled microspheres demonstrated that during initial 24 h of enteral feeding, blood flow to the jejunum and cecum was higher in the fed group than in the control. Purcell *et al*^[27,28] studied oleic-acid-induced lung injury in dogs mechanically ventilated with positive end-expiratory pressure (PEEP) which limited hepatic blood flow and oxygen delivery, and found that in such dog receiving EEF there were a significant increase in hepatic blood flow and oxygen delivery, with a highest increase in portal blood flow. In a dog model of splanchnic ischemia induced with endotoxin, Eleftheriadis *et al*^[29,30] reported that after early enteral feeding, portal vein, hepatic and superior mesenteric artery blood flow; hepatic and intestinal microcirculation; hepatic tissue PO₂ and energy charge; and intestinal intramucosal pH, which were all reduced in the early septic condition, were significantly increased. In present study, we showed that postburn EEF could effectively restore reduced gastric intramucosal pH, decrease endotoxin concentrations in portal vein and TNF- α levels in systemic circulation, and alleviate liver tissue edema, as compared with saline feeding burn controls. All above indicate that in addition to provide nutrients, posttrauma EEF exerts its protective effect on liver function most likely via a mechanism of postprandial hyperemia to improve gut blood flow and splanchnic ischemic status, and to maintain gut mucosal integrity, which may block the vicious circle of mutual activation between the translocation of gut origin bacteria with their toxic products and the release of inflammatory mediators^[31], thereby reducing hypoxic and inflammatory tissue damage.

The fact that EEF may improve postburn hepatic function is of both theoretically and practically importance. Although the results from animal study can not be applied directly to humans, the data from this study might provide valuable clues to the further improvement of prevention and treatment of post-traumatic multiple organ dysfunction syndrome. Now, EEF should not be considered merely as a simple nutritional support. Further investigations are needed to demonstrate whether or not the results from this animal experiment can apply to clinical settings.

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Reduced gastric acid production in burn shock period and its significance in the prevention and treatment of acute gastric mucosal lesions

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Subject headings gastric mucosal lesions; gastric acid; burn shock

Abstract

AIM To investigate the changes of gastric acid production and its mechanism in shock period of severe burn in rats.

METHODS A rat model with 30% TBSA full-thickness burn injury was employed and the gastric acid production, together with gastric mucosal blood flow (GMBF) and energy charge (EC) were measured serially within 48h postburn.

RESULTS The gastric acid production in the acute shock period was markedly inhibited after severe burn injury. At the 3rd h postburn, the gastric juice volume, total acidity and acid output were already significantly decreased ($P < 0.01$), and reached the lowest point, $0.63 \text{ mL/L} \pm 0.20 \text{ mL/L}$, $10.81 \text{ mmol/L} \pm 2.58 \text{ mmol/L}$ and $2.23 \text{ mmol/h} \pm 0.73 \text{ mmol/h}$ respectively, at the 12th h postburn. Although restored to some degree 24 h after thermal injury, the variables above were still statistically lower, compared with those of control animals at the 48th h postburn. The GMBF and EC were also significantly reduced after severe burns, consistent with the trend of gastric acid production changes.

CONCLUSION Gastric acid production, as well as GMBF and EC was predominantly decreased in

the early postburn stage, suggesting that gastric mucosal ischemia and hypoxia with resultant disturbance in energy metabolism, but not gastric acid proper, might be the decisive factor in the pathogenesis of AGML after thermal injury, and that the preventive use of anti-acid drugs during burn shock period was unreasonable in some respects. Therefore, taking effective measures to improve gastric mucosal blood perfusion as early as possible postburn might be more preferable for the AGML prevention and treatment.

INTRODUCTION

Acute gastric mucosal lesion (AGML) is one of the most common visceral complications early after severe burns. In patients with thermal injury that involves 30% or more of the total body surface area (TBSA), there was a 14% to 25% incidence of clinically evident gastrointestinal complications and 83.5% of the patients had endoscopic evidence of gastrointestinal disease^[1]. Although it was reported recently that, burn-induced stress ulcer occurred less frequently with the advances of intensive care supports, AGML still caused a high mortality when complicated with severe bleeding^[2], which was recognized as a potentially life-threatening event in such critically ill patients^[3,4]. Therefore, such gastrointestinal complications after cutaneous thermal burn remain a problem of great interest and importance.

Several hypotheses have been proposed to explain the mechanism of burn-induced gastric mucosal injury, but no single factor appears to be invariably capable of producing lesions of the gastric mucosa^[5]. Traditionally, increased gastric acid production has been long considered as one part of the stress response and the main contributor to the pathogenesis of AGML after severe burns^[6]. Consequently, much attention has been paid to acid-neutralizing and/or inhibiting agents in the prevention and treatment of burn-induced gastrointestinal complications. In recent years, however, it is increasingly and widely assumed that

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tissue ischemia resulting from hypoperfusion is the initial and principal factor, which may trigger re-perfusion injury, for the AGML formation^[7]. Meanwhile, the necessity and rationality of AGML prophylaxis by using acid-neutralizing and/or inhibiting agents have also been challenged^[8,9].

Gastric acid secretion is an active metabolic process with energy consumption, which requires sustained and adequate blood supply^[8]. It has well been documented that the splanchnic circulation is the first to be reduced in critical illness and the gut is one of the first organs to have the adequacy of its tissue oxygenation compromised in shock^[10,11]. We therefore presumed that the gastric acid production in burn shock period might be reduced, which is contrary to what we have thought of before but remains lack of direct evidence.

With this background, the present study is conducted to serially determine the gastric acid production, and the changes of blood flow and energy charge of the gastric mucosa during burn shock period, in order to elucidate the characteristics of gastric acid production in early postburn stage and their mechanisms, as well as to provide useful information for the AGML prophylaxis at clinical settings.

MATERIALS AND METHODS

Animals

Healthy adult Wistar rats of either sex, weighing 220 g±30 g, were employed in the study. They were housed in individual metabolic cages in a temperature conditioned room (22 °C-24 °C) with a 12 h light-dark cycle, allowed access to standard rat chow (provided by experimental animal center, Third Military Medical University) and water ad libitum, and acclimatized to the surroundings for 7 days prior to the experiments.

Burn injury and resuscitation

Animals were fasted for 12 h before burn injury, and during 48 h postburn period they were allowed water ad libitum. After induction of anesthesia with 1% pentobarbital sodium (30 mg/kg, ip), dorsal hair was shaved, and animals were placed in a wooden template designed to expose 30% of the total body surface area (TBSA), and then immersed in water at 92 °C for 20 seconds, which results in a clearly demarcated full-thickness burn. One hour after burn injury, the animals were resuscitated with 10 mL of warm 0.9% NaCl (normal saline solution, 37 °C) given by intraperitoneal injection. Control animals were similarly anesthetized, shaved and resuscitated but not burned.

The animals burned were randomly divided into five groups for the different measurements and assays that were performed 3, 6, 12, 24 and 48h

postburn (PBH₃, PBH₆, PBH₁₂, PBH₂₄ and PBH₄₈).

Measurement of gastric acid production

Three hours prior to each timepoint, animals were anaesthetized, laparotomized, and then the pylorus were ligated. After sacrificed by decapitation at each timepoint, rats were re-laparotomized to obtain the gastric juice. The pH and volume of each collection were recorded and by using a microtitrator its hydrogen ion concentration was measured by titration with 0.02 mol/L sodium hydroxide to an endpoint indicated by phenolphthalein. The total acidity and total acid output of each gastric juice collection were calculated.

Determination of gastric mucosal blood flow

GMBF was determined as previously described^[12]. Radioactive biomicrospheres were prepared with toad red blood cells labeled with ^{99m}Tc. At each timepoint, anesthetized animals underwent cannulation of right carotid artery for the injection of radioactive microspheres with PE-50 polyethylene tubing (inside diameter 0.58 mm and outside 0.97 mm). The catheter was carefully advanced into the left ventricle, as confirmed by the ventricular pressure curves monitored with a four-channel physiological recorder. Another catheter for drawing a reference blood sample was introduced into the aorta abdominalis via left femoral artery. The prepared suspension of radioactive microspheres was mixed vigorously for at least 2min before each injection. Then 0.2mL of the suspension in an injection syringe (approximately 1.5-2.0×10⁵ microspheres) was counted for radioactivity by a γ -scintillation counter before being slowly and uniformly injected into the left ventricle during a 30sec period and the infusion tube was flushed with 0.2 mL heparinized saline solution. The injection syringe was rinsed five times with saline solution into a counting tube for measurement of residual radioactivity in the syringe. Thus net radioactivity injected into the animal was the original minus residual radioactivity. Withdrawal of the reference blood sample, having started 20sec before the microsphere injection, was performed by a syringe pump at a constant rate of 0.4 mL/min for 90sec. After withdrawal of the reference blood, animals were killed with an overdose of sodium pentobarbital. The gastric mucosa was sampled, weighed and then counted in γ -scintillation counter. The GMBL was calculated by the following equation and expressed as "mL/min·g tissue"^[13]:

$$\text{GMBL (mL/min}\cdot\text{g tissue)} = \frac{\text{Reference sample flow} \times \text{Tissue activity}}{\text{Reference sample activity} \times \text{Tissue weight}}$$

Biochemical assays of gastric mucosal energy charge

At each timepoint, the glandular mucosa of stomach was sampled by scraping with razor and stored in liquid nitrogen. On determination, adenine nucleotides were assayed as previously reported with some modifications^[14]. Briefly, the sample was powdered in a liquid nitrogen bath and then weighed and homogenized in 20 volume of 10% perchloric acid for deproteinization. The homogenate was centrifuged for 30 min at 12000×g. The pH of the resulting supernatant was adjusted to 7.0-7.6 with 5 mol K₂CO₃/L. Then another centrifugation was performed and the supernatant was used to assay for adenine nucleotides by using high-performance liquid chromatography with a reverse-phase column at a flow rate of 1 mL/min with a buffer of 0.1 mol PBS/L. The ATP, ADP and AMP concentrations in gastric mucosa were then obtained from the eluant fractions. The adenylate energy charge was calculated according to the following equation: Energy charge=(ATP+0.5×ADP)/(ATP+ADP+AMP)

Statistical analysis

Data are expressed as mean±SE. Experimental results were analyzed by analysis of variance and *t* tests for multiple comparisons. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

The gastric acid production in the acute shock period was markedly inhibited after severe burn injury. At the 3rd h postburn, the gastric juice volume, total acidity and acid output were already significantly decreased, on decreasing, to reach the lowest point at the 12th h postburn. Although restored to some degree and kept 24 h after thermal injury, the variables above were still statistically lower as compared with those of control animals 48 h postburn (Table 1). The GMBF and EC were also significantly reduced after severe burns, and were consistent with the trend of gastric acid production changes (Tables 2, 3).

Table 1 The postburn changes of gastric acid production in rats (x±s)

Groups	Animals	Volume (mL/3h)	Total acidity (mmol/L)	Total acid output (μmol/h)
Control	10	4.41±0.88	97.36±14.40	140.14±16.84
PBH ₃	10	1.65±0.24 ^b	60.28±10.46 ^b	32.84±6.14 ^b
PBH ₆	10	1.07±0.19 ^b	43.78±4.59 ^b	15.47±2.21 ^b
PBH ₁₂	10	0.63±0.20 ^b	10.81±2.58 ^b	2.23±0.73 ^b
PBH ₂₄	10	2.58±0.39 ^b	86.89±12.21 ^a	75.45±18.69 ^b
PBH ₄₈	10	2.52±0.20 ^b	82.34±12.82 ^b	69.28±12.92 ^b

^a*P*<0.05, ^b*P*<0.01 vs control.

Table 2 The postburn changes of gastric mucosal blood flow in rats (mL/min·g, x±s)

Groups	Animals	GMBF	Groups	Animals	GMBF
Control	6	0.89±0.25	PBH ₁₂	6	0.24±0.05 ^b
PBH ₃	6	0.36±0.12 ^b	PBH ₂₄	6	0.54±0.11 ^b
PBH ₆	6	0.31±0.05 ^b	PBH ₄₈	6	0.71±0.17 ^a

^a*P*<0.05, ^b*P*<0.01 vs control.

Table 3 The postburn changes of ATP, ADP, AMP and EC in gastric mucosa in rats (x±s)

Groups	Animals	ATP(μmol/g)	ADP(μmol/g)	AMP(μmol/g)	EC
Control	6	4.76±0.41	2.58±0.18	1.06±0.09	0.72±0.02
PBH ₃	6	3.53±0.24 ^b	2.37±0.17	1.39±0.15 ^a	0.65±0.02 ^b
PBH ₆	6	3.16±0.40 ^b	2.03±0.23 ^b	1.99±0.24 ^b	0.58±0.03 ^b
PBH ₁₂	6	2.38±0.34 ^b	1.56±0.17 ^b	2.64±0.31 ^b	0.48±0.03 ^b
PBH ₂₄	6	3.41±0.38 ^b	2.26±0.22 ^a	1.73±0.26 ^b	0.62±0.02 ^b
PBH ₄₈	6	3.96±0.37 ^b	2.34±0.26	1.69±0.15 ^b	0.64±0.01 ^b

^a*P*<0.05, ^b*P*<0.01 vs control.

DISCUSSION

It has been long considered that certain amount of hydrogenion existing in gastric lumen is the prerequisite for the AGML formation^[15,16]. However, the roles of gastric acid in the pathogenesis of AGML have not been fully elucidated so far. In a clinical study, Pruitt *et al*^[6] noted that the mean outputs of total gastric acid in burn patients with normal mucosa, AGML, and AGML with complications (such as bleeding and perforation) were 1.42, 3.32, and 5.37 mmol/h respectively. Lucas *et al*^[17] also found an increase in gastric acid production was positively related the severity of AGML in traumatic patients observed with endoscopy. Interventions designed to decrease gastric acid production, such as vagotomy or administration of aluminum hydroxide or anticholinergic drugs, have all been shown to decrease the incidence of ulcers. In the case of a burn injury or other severe injury, the presence of acid even at subnormal levels may be sufficient to produce gastrointestinal complications. However, in humans and experimental animals there has not been a consistent association of acute gastric ulcerations after burn injury with hypersecretion of gastric acid^[18].

The early phase after severe burn is the critical period for AGML formation. It was reported that the incidence of AMGL within 72h reached as high as 76%^[1,19]. Therefore, investigating the changes of gastric acid production in shock period is both theoretically and practically of importance for the further understanding of the pathogenesis of AGML and, on this basis, improving its treatment and prophylaxis. In present study, we showed that gastric juice volume, total acidity and acid output in burned rats during the early phase of severe burns

were significantly decreased compared with unburned controls, with the lowest at 12th h and persisting at lower levels until 48 h postburn. In particular, the total acid outputs at the 6th, 12th, and 24th h in burn animals corresponded only to 1/9, 1/63, and 1/2 of those in control rats. These results indicate that in burn shock period the gastric acid secretion is markedly inhibited.

The production of gastric acid is an active metabolic process with energy consumption, requiring sustained and adequate blood supply^[8]. It is well known that during the period of hypovolemic shock such as that induced by burn, blood is preferentially shunted to the "vital" organs, such as the brain and heart, at expense of the splanchnic circulation, causing a sharp reduce of blood flow to the gastrointestinal tract^[20]. Therefore, ischemia may be a major factor in the development of AGML after shock and injury. In a report by Horton, blood flow to the small intestine and stomach decreased significantly 5 h postburn, but flow returned to the normal level 24 h after burn only in the small intestine, but not in the stomach^[18]. In this murine burn model, we noted that there was a significantly decrease in GMBF and EC with a trend consistent with the serious inhibition of gastric acid production after severe burns. Thus, the decreased acid production might be resulted from ischemia and hypoxia of the gastric mucosa that are metabolically unable to produce normal quantities of acid. Furthermore, while being a causative factor for the AGML formation, gastric acid is also one of the most important barriers against invading pathogenic micro-organisms^[21]. Gastric pH<3.5 is usually bactericidal for most species^[22]. In this sense, the inhibition of acid secretion in early postburn period is the manifestations of both local impairment of gastric mucosal function, as well as the dysfunction of host defense mechanism as a whole.

Prophylaxis for stress ulceration continues to be an important part of the management of critically burned patients. However, controversy remains regarding the necessity and rationality of the regimen used^[23,24]. Available options at present include antacids, various H₂-receptor antagonists, prostaglandins, proton pump inhibitors, and sucralfate, nearly all of which exert their pharmacological actions, to more or less extent, through the acid-neutralizing and/or inhibiting mechanism^[25]. In fact, postburn acute mucosal lesions occurred not only in the gastric mucosa, but also in some organs that cannot produce acid such as small and large intestines, and even the gallbladder mucosa^[26]. In patients without titratable gastric acid, diffuse erosive gastritis remains occurring

within 72 h of burn injury^[18]. It was documented in some reports that the incidence of pathological changes in gastric mucosa was not alleviated by using antacids in clinical settings^[27], but could be prevented or reduced by satisfactory resuscitation and advanced intensive care support, even in a condition lack of anti acid prophylaxis^[28,29]. In burned rats, Skolleborg *et al*^[5] found with postburn fluid resuscitation sufficient to maintain aortic blood pressure, gastric mucosal erosions were prevented even when gastric pH was at 1.0. All of these, combined with our results, show in different aspects that gastric acid is not a leading and crucial, but an aggravating factor that functions on the basis of ischemic impairments, for the AGML formation. Whereas, the present AGML prophylaxis mainly by the use of anti-and neutralizing acid drugs is not only making the mucosa more susceptible to acid injury^[30], but also resulting in numerous side-effects including breaking the defensive barrier of gastric acid, which may lead to the colonization and translocation of gut organisms and thus increase the risk of nosocomial infections^[25,27,28,31]; therefore, taking effective measures to improve splanchnic blood perfusion as early as possible postburn may be more preferable than the mere blockade of gastric acid production for the AGML prevention and treatment.

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Tumor growth inhibition effect of hIL-6 on colon cancer cells transfected with the target gene by retroviral vector

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Subject headings interleukin-6 gene; gene transfection; gene therapy; colonic neoplasms

Abstract

AIM To observe the tumor inhibitory effects by transfecting IL-6 cDNA into colon cancer cell line HT-29 with retroviral vector pZIP cDNA.

METHODS Human IL-6 gene was reconstructed in retrovirus vector and transfected into incising cells PA317 by lipofectamine mediated method, the clones of the cells transferred with hIL-6 were selected by G418, and targeted HT-29 cells were infected with the virus granules secreted from PA317 and also selected by G418. Test gene transcription and expression level by hybridization, ELISA and MTT assay, etc. Analyze tumor inhibitory effects according to the cell growth curve, plating forming rate and tumorigenicity in nude mice.

RESULT Successfully constructed and transfected recombinant expressing vectors pZIPIL-6 cDNA and got positive transfected cell lines. The colon cancer cell line (HT-29 IL-6) transfected with the hIL-6 gene by retroviral vector was established. The log proliferation period and the doubling time of this cell line was between 4 to 7 days and 2.5 days according to the direct cell count, the cell proliferation was obviously inhibited with MTT assay, the plating inhibitory rate was 50% by plating efficiency test. When HT-29 IL-6 cells were inoculated into the nude mice subcutaneously, carcinogenic activity of the solid tumor was found superior to the control group and the size of tumor was not significantly enlarged. Injection of combination virus fluid containing IL-6 gene into transplantation tumors could inhibit the growth and development of the tumor.

CONCLUSION IL-6 could inhibit the growth and proliferation of colon cancer cells by retroviral vector-mediated transduction.

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INTRODUCTION

The incidence of colorectal carcinoma ranks top ten among the digestive system diseases. The clinical treatment so far is not satisfactory, which has been existed of the top ten malignancy threatening human life. The authors intended to use modern molecular biology technology to explore the new strategies in treatment of colorectal carcinoma. IL-6 is a multifunctional cytokine which has been confirmed to have anti-tumor effect^[1]. Here, we reported a colorectal carcinoma cell line transfected with IL-6 gene, which can secrete a large amount of IL-6 and study its tumor-inhibitory effect both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

The colorectal carcinoma cell line (HT-29 cell strain) was preserved in our laboratory, and PA317 package cell was generously provided by Professor Zhang Hong-Quan from the Academy of Military Medical Sciences of China.

Main reagents

Restrict enzyme, EcoRI, BamHI, Klenow enzyme and T4 DNA ligase were purchased from Sino-American Biotechnology Company (SABC).

Plasmid

pUCIL-6cDNA, pZIPcDNA was introduced Dr. Jiang Bo from the Academy of Military Medical Sciences of China.

Animals

Twenty BALB/c-nu/nu female nude mice, 3-4 weeks old, were purchased from Shanghai Tumor Institute.

Construction of hIL-6 expressing strain

By utilizing restrict enzyme digestion and DNA recombinant technology, we constructed a recombinant retroviral plasmid which was named pZIPIL-6cDNA. Then the plasmid was transfected into PA317 package cell by using liposome^[2]. After screening the positive (anti-drug) clones with G418, we collected supernatants of positive clones containing recombinant virus and transfected the adenocarcinoma HT-29 cell line with the supernatant and screening of drug-resistance clones by G418.

Cell growth and proliferation test

According to the direct count assay^[3], the log proliferation and the doubling time of HT-29 cells transfected with or without pZIPIL-6cDNA were calculated. The proliferation rate of the two groups was cultured in 96-well plate and observed by MTT assay at 48 h, 72 h, 96 h and 120 h, respectively^[4].

Plating efficiency test

Prepared double layer agar gel in 6-well plate (soft agar layer) containing 0.3% agar, 20% BSA; the lower layer containing 0.5% agar, 10% BSA, which was referred as hard agar layer. Then different amount of 2×10^3 HT-29/IL-6 cells or HT-29 cells was added per well to continue for 3-4 weeks, the colony formation efficiency was calculated, including the number of the average colonization unit/the number of cell added per well.

Tumor-inhibitory test in nude mice

Twenty mice were randomly divided into four groups and each five mice. The first two groups received inoculation of HT-29 cells. After tumor formation, one group was injected pZIPIL-6cDNA recombinant viral suppressant intratumorally, which was called IL-6 gene therapy group. The other served as control group. The other two groups were inoculated with pZIPcDNA or pZIPIL-6cDNA transfected HT-29 cells, respectively. 5×10^6 cells were injected at left front leg subcutaneously. Then observed after forty days and 6 items were recorded including the time of tumor formation (i.e. the time when tumor diameter was about 3 mm), the number of tumor-bearing mice, tumor growth rate, lesion size, and health condition of nude mice as well as pathologic characteristic of the tumor.

RESULTS

Screening for transfected cell clones

After recombinant vector (pZIPIL-6) was transfected into PA317 package cells, the positive clones were screened for 4-5 weeks and drug-resistant clones gradually formed, at the same time untransfected cells died completely after 1-2 weeks and no colonization was found. The recombinant viral suppressant titer was 5.1×10^8 cfu/L by using 3T3 cells as indicating system^[2].

The tumor inhibitory effect of IL-6 in vitro

By direct counting assay, the difference in growth between HT-29 cells and HT-29 IL-6 cells is shown in Figure 1, the doubling time of HT-29 and HT-29 pZIP was 1.5 and 1.6 days, respectively, and the log proliferation phase was 2 - 7 days and 3 - 8 days, respectively. But the doubling time of HT-29 IL-6 cells was 2.5 days, log proliferation phase was 4-7 days, with a maximal cell number of 2.5×10^5 ,

which was significantly lower than HT-29 cells. The results of MTT assay are shown in Figure 2, in which the cell proliferating curve of HT-29 IL-6 cells increased more slowly being more stabilized than other groups. These demonstrated that IL-6 inhibited the growth and proliferation of colon cancer cells (HT-29) significantly, and retroviral vector influenced neither the proliferation of HT-29 cells nor the biologic effect of IL-6.

The effect of IL-6 on colony formation of HT-29 cells on soft agar

The HT-29 cell and HT-29 IL-6 cell clones were formed gradually, between 12-14 days after inoculation on the soft agar, and a number of them only appeared 3-4 weeks after. A single cluster with more than 50 cells was regarded as a clone unit, and the efficiencies of colonization were calculated (Table 1). The efficiencies for HT-29 IL-6 cells and HT-29 cells were 2.21% and 4.29%, respectively, this demonstrated the clone inhibitory rate was 50% for transfected cells. Therefore, it was shown that transfected IL-6 gene could inhibit the capacity of HT-29 colonization. It might be of significance in the prevention of HT-29 tumor cell from proliferating and invasion.

The tumor-inhibitory effect of IL-6 in vivo

Local edema disappeared inoculation after 2-3 days. After 8-14 days there appeared tiny tumors which enlarged continuously (Table 2). The average tumor formation time of gene-transfected group was delayed 5.5 days as compared with the control group, and one mouse did not show any sign of tumor bearing. In IL-6 treated mice, the tumor grew slower, the health condition was much better, and no cachexia was seen, in particular in the intratumorously-injected mice. Pathology sections showed there was more distinct apoptosis (Figure 3).

Table 1 IL-6 effects on colony formation of HT-29 cells on soft agar

Cell	n	Number of colonies					χ	Colony formation
		1	2	3	4	5		
HT-29	5.0	86.0	89.0	92.0	80.0	97.0	84.2	4.21
HTIL-6	5.0	42.0	52.0	41.0	39.0	47.0	44.2	2.21 ^a

^a $P < 0.05$, rS.

Table 2 Growth sequel of transplantation tumors

Group	Nude mice number	Number of tumor-bearing mice	The time of tumor presentation (days)	Tumor size (mm)	Growth rate (mm/day)
HT-29	5	5	8	20.8	0.65
HT-29pZIP	5	5	8.5	18.2	0.57
HT-29IL-6	5	4	13.5	8.5 ^b	0.48
Recombinant virus	5	5	8	6.5 ^b	0.15 ^b

^b $P < 0.01$, in comparison with HT-29 cell group.

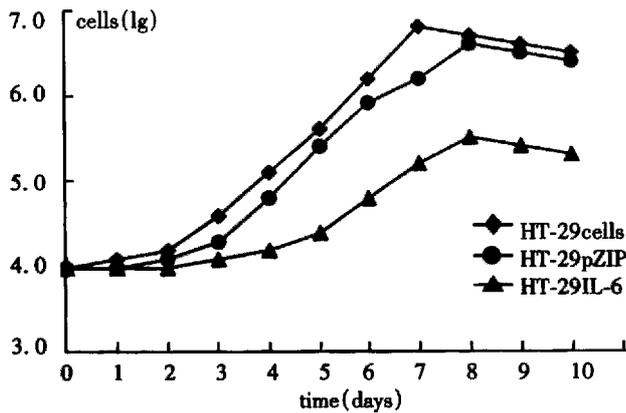


Figure 1 Growth curve of HT-29 IL-6 cells.

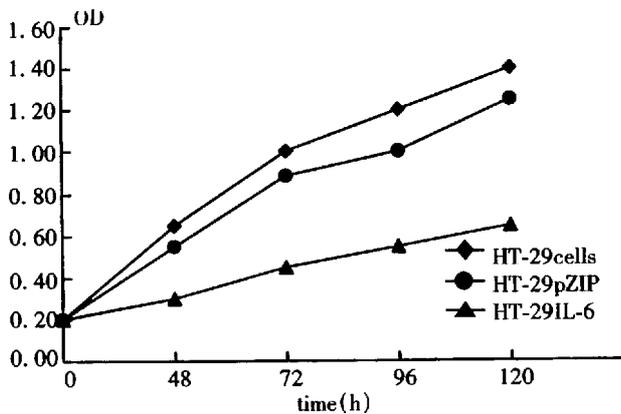


Figure 2 Cell proliferating curve by MTT.

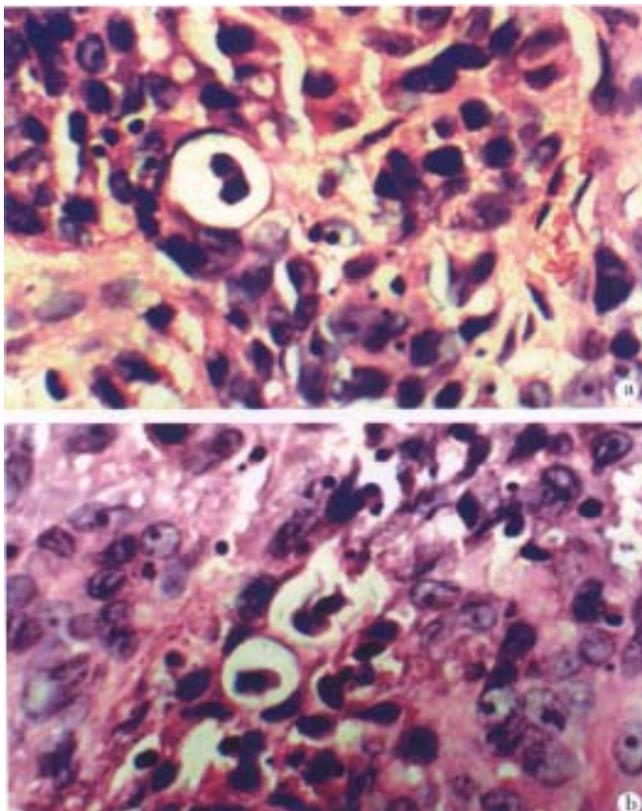


Figure 3 Histopathology of transplantation tumors of nude mice. $\times 400$

A. Injection of HT-29 IL-6 cells.

B. Injection of recombinant retrovirus in transplantation tumors.

DISCUSSION

Recently, pioneered studies showed that IL-6 could promote or inhibit the growth of tumor cell depending upon the cell type^[5]. For example, the IL-6 cDNA encoding sequences were inserted into the downstream of SV40 promoter in pH EBOSV plasmid, then the plasmid was transfected into ROHA-9MCEBV3-B cell. The observation showed that exogenous IL-6 gene expression could switch the cell to a tumor phenotype. 10-15 days after the injection of transfected ROHA-9MCEBV3 -B cell, tumor formations were observed, but the control group was not, which suggested that IL-6 secreted by the transfected cell promote cell proliferation in carcinogenesis. On the other hand, administration of IL-6 alone could inhibit the metastasis of tumor induced by xylene anthracene^[6]. Currently, the mechanism remained unknown, however, it may be associated with different IL-6R expression on tumor cell surface. Only when the latter reaches a certain level and binds to IL-6 with high affinity could it transmit IL-6 signals and showed significant inhibitory activity. IL-6 is a cytokine with multifunctions as stated above. For instance, when the IL-6 gene was transfected into D122 Lewis lung cancer cell line which has low immunity and high metastatic potential, growth of transfected cell was inhibited *in vitro*, and the density of MHC antigen expressed on cell surface was 2.5-4.5 times greater than that in the wild type of D122. Immunizing the mice with inactivated transfected cells, the mice showed an enhanced activity of CTL and macrophage, the immunization could prevent mice from challenge of wild type D122 cells. These phenomena demonstrated that IL-6 not only could inhibit tumor growth directly but also promote anti-tumor activity indirectly. Through activating host cellular immunity^[7]. Ullmann *et al*^[8] found that IL-6 could enhance CEA and HLA antigen expression on colon cancer cell surface and these antigens expression level correlated with IL-6 treatment dose and duration positively. Other reports showed that CEA expression level of IL-6 gene transfected into colon cancer cell line (HT-29 cells IL-6) was much higher than that of untransfected or transfected with unrelated gene cells^[9]. Apart from these studies, IL-6 could enhance the induction of LAK cell or NK cell, promote the maturity and differentiation process, and increase the tumor killing activity of LAK and NK cells in a dose-dependent manner. Overall, introducing IL-6 gene into cells may have wide biological effects on the regulation of immunological response by autocrine or paracrine pathways, thus inhibit or kill tumor cell directly or indirectly^[10,11]. Regulatory disturbance induced by apoptosis is closely associated with carcinogenesis, making

tumor apoptosis a new target for cancer therapy^[12]. Gene regulation and signal transduction are the key process of apoptosis. Tumor cells could escape surveillance of immune system, not only because of the downregulation of antigen expressed on cell surface and host immune response, but also the inhibition of apoptosis of abnormal cell. We reported the first time that many more apoptotic cells in the group injected with HT-29 IL-6 cells and IL-6 gene therapy group were induced than the control group. So far, we have not seen any report on apoptosis induced by IL-6, even some researchers reported IL-6 could inhibit the programmed cell death in M1 leukemia cell strain^[13]. IL-6 is a potent immunoregulatory factor, and participates in regulation of cell apoptosis. Thus, we conclude the apoptosis induction of tumor cell is the main mechanism of IL-6 for growth inhibition of tumor cells both *in vitro* and *in vivo*. Our laboratory studies provided the potential use of IL-6 gene therapy in colo-rectal cancer.

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Effect of cholecystokinin and secretin on contractile activity of isolated gastric muscle strips in guinea pigs

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Subject headings gastric muscle strips; cholecystokinin; secretin; guinea pigs

Abstract

AIM To study the effect of cholecystokinin-octapeptide (CCK-8) and secretin on contractile activity of isolated gastric muscle strips in guinea pigs.

METHODS Each isolated gastric muscle strip was suspended in a tissue chamber containing 5 mL Krebs solution constantly warmed by water jacked at 37 °C and supplied with a mixed gas of 95% O₂ and 5% CO₂. After incubating for 1h under 1g tension, varied concentrations of CCK-8 and secretin were added respectively in the tissue chamber and the contractile response was measured isometrically on ink-writing recorders. **RESULTS** CCK-8 could increase ① all regional circular and longitudinal muscular tension at rest (fundus LM 19.7%±2.1%, *P*<0.01; fundus CM 16.7%±2.2%, *P*<0.01; gastric body LM 16.8%±2.3%, *P*<0.01; body CM 12.7%±2.6%, *P*<0.01; antrum LM 12.3%±1.3%, *P*<0.01; antrum CM 16.7%±4.5%, *P*<0.01; pylorus CM 12.7%±5.0%, *P*<0.05); ② contractile frequencies of body LM, both LM and CM of antrum and pylorus CM (5.1/min±0.2/min to 5.6/min±0.2/min, 5.9/min±0.2/min to 6.6/min±0.1/min, 5.4/min±0.3/min to 6.3/min±0.4/min, 1.3/min±0.2/min to 2.3/min±0.3/min, respectively, *P*<0.05); ③ the mean contractile amplitude of antral circular muscle (58.6%±18.4%, *P*<0.05) and ④ the motility index of pylorus CM (145.0%±23.8%, *P*<0.01), but decrease the mean contractile amplitude of gastric body and antral LM (-10.3%±3.3%, -10.5%±4.6%, respectively, *P*<0.05). All the

CCK-8 effects were not blocked by atropine or indomethacin. Secretin had no effect on gastric smooth muscle activity.

CONCLUSION CCK-8 possessed both excitatory and inhibitory action on contractile activity of different regions of stomach in guinea pigs. Its action was not mediated via cholinergic M receptor and endogenous prostaglandin receptor.

INTRODUCTION

CCK and secretin have important actions on gastrointestinal motility *in vivo* and *in vitro*, but few reports have covered the gastric smooth muscle motility in guinea pigs. In this paper, we studied the action of CCK and secretin on isolated gastric smooth muscle in guinea pigs and compared it with those of the corresponding regions in rats^[1].

MATERIAL AND METHODS

Guinea pigs, weighing 250 g-350 g, male or female, were fasted but with free access to water for 24 h, hit the head to lose consciousness and the whole stomach was removed. The stomach was opened along the greater curvature and muscle strips (8 mm×2 mm) were cut, parallel to either the longitudinal or circular fibers [longitudinal muscle (LM) and circular muscle (CM) of fundus, body, antrum and CM of pylorus]. After removal of mucosa, each strip was suspended in tissue chamber containing 5 mL Krebs solution and constantly warmed by circulating water jacked at 37 °C and supplied with 95% O₂ and 5% CO₂. One end of the strip was fixed to hook on the bottom of the chamber while the other one was connected by thread to an external isometric force transducer (JZ-BK, BK) at the top. Preparations were subjected to 1 g load tension and washed with 5 mL Krebs solution every 20 min. The contractions of gastric strips in 7 tissue chamber were simultaneously recorded on ink-writing recorders (LMS-ZB, Chengdu)^[2]. After incubating for 1 h, CCK-8 (10⁻¹ μmol/L, 10⁻² μmol/L, 5×10⁻² μmol/L) or secretin (10⁻¹ μmol/L, 10⁻² μmol/L, 5×

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10^{-2} $\mu\text{mol/L}$) was added to the tissue chamber separately. CCK-8 and secretin were added together at the same time. To attain the final concentration $50 \mu\text{L}$ of reagent were added in a 5 mL bath.

We measured the resting tension of every strip, mean contractile amplitude of muscle strips of gastric body and antrum and the motility index [MI = Σ (amplitude \times duration) of CM strips of pylorus. Frequencies of contraction were calculated by counting the contraction waves per minute. The results were presented by the increased percentage (%) of spontaneous contraction ($\bar{x} \pm s_{\bar{x}}$ %). The data were analyzed by Student's *t* test, and *P* values < 0.05 were considered as significant^[3].

RESULTS

Effects of CCK-8 on spontaneous contraction of gastric muscle strips

CCK-8 increased the resting tension of muscle

strips in various regions of the stomach (Table 1), the mean contractile amplitude of antral CM and the motility index of pyloric CM, but decreased the mean contractile amplitude of body and a ntrum LM (Table 2), which were concentration-dependent. It also increased contractile frequencies of body LM, both LM and CM of antrum and pylorus CM ($5.1/\text{min} \pm 0.2/\text{min}$ to $5.6/\text{min} \pm 0.2/\text{min}$, $5.9/\text{min} \pm 0.2/\text{min}$ to $6.6/\text{min} \pm 0.1/\text{min}$, $5.4/\text{min} \pm 0.3/\text{min}$ to $6.3/\text{min} \pm 0.4/\text{min}$, $1.3/\text{min} \pm 0.2/\text{min}$ to $2.3/\text{min} \pm 0.3/\text{min}$, respectively, $P < 0.05$).

Atropine (10^{-2} mmol/L) decreased the tension of gastric muscle strips the mean contractile amplitude and the motility index of gastric strips in varyin g degrees but did not significantly affect the frequency of contractions. Atropine (10^{-2} mmol/L) given 3 min before administration of CCK-8 (10^{-2} $\mu\text{mol/L}$) did not affect the CCK-8 action on gastric strips (Tables 1, 2).

Table 1 Effects of CCK-8 on the resting tension of gastric strips in guinea pigs

Part of stomach		CCK-8	CCK-8+secretin	Atropine and CCK-8		Indomethacin and CCK-8	
Fundus	LM	19.7 ± 2.1^b (n = 19)	23.1 ± 2.1^b (n = 17)	-25.1 ± 4.0^b (n = 17)	47.1 ± 6.0^b (n = 17)	-34.5 ± 4.4^b (n = 17)	37.6 ± 5.6^b (n = 17)
	CM	16.7 ± 2.2^b (n = 16)	16.0 ± 1.8^b (n = 15)	-14.9 ± 2.5^b (n = 17)	35.2 ± 6.5^b (n = 17)	-14.1 ± 3.2^b (n = 17)	30.3 ± 7.0^b (n = 17)
Body	LM	16.8 ± 2.3^b (n = 19)	20.4 ± 1.9^b (n = 17)	-21.7 ± 3.4^b (n = 17)	18.3 ± 2.8^b (n = 17)	-8.3 ± 3.1^a (n = 16)	13.0 ± 1.7^b (n = 16)
	CM	12.7 ± 2.6^b (n = 11)	15.9 ± 3.3^b (n = 13)	-6.7 ± 1.8^b (n = 15)	18.3 ± 2.4^b (n = 15)	-5.7 ± 1.8^a (n = 14)	12.3 ± 2.1^b (n = 14)
Antrum	LM	12.3 ± 1.3^b (n = 13)	11.6 ± 2.0^b (n = 15)	-2.9 ± 1.2^a (n = 14)	8.6 ± 1.7^b (n = 14)	-1.9 ± 0.8^a (n = 14)	4.9 ± 0.9^b (n = 14)
	CM	16.7 ± 4.5^b (n = 11)	28.4 ± 4.1^b (n = 17)	0 (n = 14)	23.8 ± 4.1^b (n = 14)	5.0 ± 5.0 (n = 16)	30.0 ± 4.8^b (n = 16)
Pylorus	CM	12.7 ± 5.0^a (n = 10)	8.9 ± 3.1^a (n = 12)	0 (n = 13)	8.2 ± 2.4^a (n = 13)	0 (n = 10)	8.0 ± 3.4^a (n = 10)

L(C)M, longitudinal (circular) muscle; dose: CCK-8 10^{-2} $\mu\text{mol/L}$, secretin 10^{-2} $\mu\text{mol/L}$; atropine 10^{-2} mmol/L, indomethacin 10^{-2} mmol/L; ^a $P < 0.05$, ^b $P < 0.01$ vs control; 0, no effect; $\bar{x} \pm s_{\bar{x}}$ % increase.

Table 2 Effects of CCK-8 on the mean contractile amplitude of body and antrum, and the motility index of pylorus in guinea pigs

Part of stomach		CCK-8	CCK-8+secretin	Atropine and CCK-8		Indomethacin and CCK-8	
Body	LM	-10.3 ± 3.3^a (n = 19)	-8.4 ± 3.4^a (n = 17)	-17.1 ± 4.8^b (n = 17)	1.5 ± 2.1 (n = 17)	-8.6 ± 2.7^a (n = 16)	-9.7 ± 3.3^a (n = 16)
	CM	-2.3 ± 2.3 (n = 11)	0 (n = 13)	-23.3 ± 6.6^b (n = 15)	0 (n = 15)	0 (n = 14)	0 (n = 14)
Antrum	LM	-10.5 ± 4.6^a (n = 13)	-9.9 ± 4.3^a (n = 15)	-22.2 ± 5.9^b (n = 14)	-12.3 ± 4.8^a (n = 14)	-8.2 ± 2.6^a (n = 14)	-10.0 ± 2.9^a (n = 14)
	CM	58.6 ± 18.1^a (n = 11)	48.8 ± 10.8^b (n = 17)	-15.7 ± 5.4^a (n = 14)	49.8 ± 11.6^b (n = 14)	0 (n = 16)	45.6 ± 6.6^b (n = 16)
Pylorus	CM	145.0 ± 23.8^a (n = 10)	162.5 ± 31.7^a (n = 12)	-41.9 ± 12.2^a (n = 13)	161.5 ± 55.0^a (n = 13)	0 (n = 10)	140.0 ± 21.9^b (n = 10)

L(C)M, longitudinal (circular) muscle; dose: CCK-8 10^{-2} $\mu\text{mol/L}$, secretin 10^{-2} $\mu\text{mol/L}$; atropine 10^{-2} mmol/L, indomethacin 10^{-2} mmol/L; ^a $P < 0.05$, ^b $P < 0.01$ vs control; 0, no effect; $\bar{x} \pm s_{\bar{x}}$ % increase.

Indomethacin (10^{-2} mmol/L) also decreased the resting tension and the mean contractile amplitude, but did not significantly affect the frequency of contractions. Indomethacin (10^{-2} mmol/L) given 3 min before administration of CCK-8 (10^{-2} μ mol/L) similarly did not affect the CCK-8 action on gastric strips (Tables 1, 2).

Effects of secretin on the spontaneous contraction of gastric strips

Secretin (10^{-1} μ mol/L, 10^{-2} μ mol/L, 5×10^{-2} μ mol/L) did not significantly affect the resting tension, the mean contractile amplitude, the motility index and the contractile frequency on gastric strips.

Effects of combined CCK-8 and secretin on the spontaneous contraction of gastric strips

When CCK-8 (10^{-2} μ mol/L) and secretin (10^{-2} μ mol/L) were combined, the effect was similar to CCK-8 (10^{-2} μ mol/L) alone (Tables 1, 2).

DISCUSSION

CCK-8 could increase the mean contractile amplitude of antral CM and motility index of pyloric CM of guinea pigs which was similar to that seen in rats^[1]. CCK-8 increased the resting muscle tension, the contractile frequency of body LM, antral LM and CM and pyloric CM, it decreased the mean contractile amplitude of body and antral LM, but did not affect the gastric strips of the corresponding regions in rats. Meanwhile, the mechanism of action of CCK-8 on gastric smooth muscle has not been elucidated. Grider *et al.*^[4] considered CCK-8 could contract isolated CM of body in guinea pigs, but it was not blocked by TTX and atropine, therefore CCK-8 might have direct effect on smooth muscle cell. Gerner^[5] believed that CCK-8 could increase the resting tension of fundus, and contractile amplitude of antrum which could be partly mediated via cholinergic nerve pathway. Our results demonstrated that the CCK-8 effect was neither blocked by atropine nor blocked

by indomethacin, it suggests that action of CCK-8 is not mediated via prostaglandin. The fact that CCK-8 increased the contractile frequency of gastric strips in guinea pigs as well as in dogs required further study^[6]. Secretin did not affect gastric strips activity obviously in guinea pigs and when CCK-8 and secretin were combined the effect was similar to that of CCK-8 alone. It showed that secretin neither affect the isolated gastric strips in guinea pigs nor affect the action of CCK-8 on gastric strips, but secretin decreased the resting tension of LM and CM of fundus, and body, the mean contractile amplitude of LM and CM of antrum in rats. When CCK-8 and secretin were combined, the action the increase in mean contractile amplitude of LM and CM of antrum by CCK-8 was eliminated in rats. There was marked discrepancy in response to secretin in isolated gastric strips of guinea pigs and rats. It was reported that guinea pig had a special gastrointestinal pancreas system^[7], whether these are related to the above factor as yet could not be answered. CCK-8 and secretin both have physiological modulating function on gastric emptying in rats^[8], whether the same occurs in the guinea pigs, need further studies.

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Immunocytochemical identification and localization of APUD cells in the gut of seven stomachless teleost fishes

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Subject headings stomachless teleost fishes; APUD cells; intestinal mucosa; immunocytochemistry

Abstract

AIM To study the cell types, localization, distribution density and morphology of APUD cells in the intestinal mucosa of stomachless teleost fishes.

METHOD By using the peroxidase-antiperoxidase complex (PAP) immunocytochemical staining technique the identification, localization and morphology of immunoreactive (IR) endocrine cells scattered in the intestinal mucosa of grass carp (*Cyrenopharyngodon idellus*), black carp (*Mylopharyngodon piceus*) and common carp (*Cyprinus carpio*) were investigated with 20 kinds of antisera prepared against mammalian peptide hormones of APUD cells, and likewise by using avidin-biotin-peroxidase complex (ABC) method those of silver carp (*Hypophthalmichthys molitrix*), bighead (*Aristichthys nobilis*), silver crucian carp (*Carassius gibelio*) and bluntnose black bream (*Megalobrama amblycephala*) were also studied with 5 different antisera. The replacement of the first antiserum by phosphate buffered saline (PBS) was employed as a

control. IR endocrine cells were counted with a square-mesh ocular micrometer from 10 fields selected randomly in every section of each part of the intestine specimen. The average number of IR endocrine cells per mm² was counted to quantify their distribution density.

RESULT Gastrin (GAS), Gastric inhibitory peptide (GIP), glucagon (GLU), glucagon-like immunoreactants (GLI), bovine pancreatic polypeptide (BPP), leucine-enkephalin (ENK) and substance P (SP)-IR endocrine cells were found in the gut of grass carp, black carp and common carp, and somatostatin (SOM)-IR endocrine cells were only seen in common carp. GAS, GIP and GLU-IR endocrine cells were found in the intestinal mucosa of silver carp, bighead, silver crucian carp and bluntnose black bream. Most of IR endocrine cells had the higher distribution density in the foregut and midgut, and were longer in shape. They had a long apical cytoplasmic process extended to the gut lumen and a basal process extended to adjacent cells or basement membrane and touched with it. Sometimes, the basal cytoplasmic process formed an enlarged synapse-like structure in the contiguous part with basement membrane. This phenomenon provided new morphological evidence for neuroendocrine and paracrine secretory function of these enteroendocrine cells.

CONCLUSION At least 8 kinds of IR endocrine cells were found in the gut of stomachless teleost species for the first time in China. These IR endocrine cells scattering in the gut mucosa belong to the APUD system. Among them, the hormones secreted by SP-, ENK-, SOM- and GLU-IR endocrine cells belong to the peptides of dual distribution in the brain and gut. This provided new evidence for the concept of brain-gut peptide. According to the cell types, distribution density, morphological characteristics and variety in shape of APUD cells in the gut of stomachless teleost fishes, it is deemed that the digestive tract of fishes is also an endocrine organ of great importance and complexity.

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INTRODUCTION

Gastrointestinal endocrine cells are different from other cells of endocrine gland, are discrete cells spread among the gastrointestinal mucosal epithelial cells. Pearse first proposed that all endocrine cells which produced peptide hormones were called APUD (amine precursor uptake and decarboxylation) cells in 1968^[1,2]. APUD cells can be divided two groups: in one are those located in the nervous system, and in the other group are there distributed in the peripheral organs, mainly the digestive system. Many studies of the mammalian gastro-entero-pancreatic (GEP) endocrine system have been reported, which, to date, have resulted in the identification of about 18 endocrine cell types and at least as many hormones^[3-5]. Enteroendocrine cells of fishes are difficult to demonstrate. For example, most of the identification procedures, which were found to be positive for mammalian enteroendocrine cells, failed on adult fish species. There was only a faint reaction with argyrophilic stains and with lead haematoxylin^[6], and also could not identify the types or species of endocrine cells^[7]. Although endocrine cell types that exist in the fish gut may differ from those of mammals, the questions of a cross-species specificity of anti sera against mammalian hormones and whether these antisera will show immunoreactivity in the gut of fish are very important. Recent years immunocytochemical studies using antisera against mammalian hormones clearly showed the existence of endocrine cells in the digestive tract of some teleost fishes^[8-12]. Our series of studies also showed that antisera against mammalian hormones could be effectively used on teleost species, and identify the types of APUD cells in the GEP of fish species^[13-19]. This paper reports the cell types, localization, distribution density and morphology of APUD cells in the gut of 7 kinds of teleost fish species cultivated mainly in China. The studies will provide new informations about neuro-endocrinology (the concept of brain-gut peptide hormones) animals, physiology, pathology and gastroenterology of animals.

MATERIALS AND METHODS

Specimens

Seven kinds of teleost fishes, grass carp (*C. idellus*), black carp (*M. piceus*), common carp (*C. carpio*), silver carp (*H. molitrix*), bighead (*A. nobilis*), silver crucian carp (*C. gibelio*) and bluntnose black bream (*M. amblycephala*), 3-6 fish of each species, were used in this studies. All fishes were reared temporarily and dissected after 2 days fasting. The digestive tract was drawn out, and the gut was divided into five pieces: the anterior segment of foregut, posterior segment of foregut,

midgut, anterior segment of hindgut and rectum. All specimens were fixed by immersion in Bouin's fluid for 24 h, dehydrated and made transparent through ethanol-xylene serial procedures, embedded in paraffin (54 °C) and sectioned (5 µm). The sections were mounted on slide, treated with gelatin and dried 12 h at 45 °C.

Antisera and main reagents

The details of antisera and main reagents used are listed in Table 1.

Immunocytochemical staining steps and counting

① Put into 3% H₂O₂-methanol for 10min at 20 °C to block auto-peroxidase; ② to incubate with normal goat serum for 30min at 20 °C for preventing nonspecific reactivity; ③ drop in 20 different first antisera separately and incubate for 20 h at room temperature (for PAP method); ④ drop in 5 kinds of first antisera separately and incubate for 20 h at room temperature (for ABC method); ⑤ for PAP method to see reference 13, and for ABC method to consult reference 19; ⑥ the control was treated in step with adjacent continuous section of immunocytochemical staining slice except replacement of the first antisera with phosphate buffered saline (PBS). IR endocrine cells were counted and photographed with an Olympus photomicroscope (PM-10AD) from 10 fields selected randomly in every section of each part of the gut per specimen with a square-meshed ocular micrometer (0.5 mm). The average number of IR endocrine cells per mm² was counted to quantify their distribution density.

RESULT

Cell types and distribution characteristics of IR endocrine cells

GAS-, GIP-, GLU-, BPP-, ENK-, GLI-, SP- and SOM-IR endocrine cells were found in the intestinal mucosa of *C. carpio*; these cells, except SOM-IR endocrine cells, were seen in *C. idellus*, and *M. piceus*. Only GAS, GIP- And GLU-IR endocrine cells were found in the gut of *H. molitrix*, *A. nobilis*, *C. gibelio* and *M. amblycephala*. The location, distribution and density of IR endocrine cells in the gut of 7 teleost fishes are listed in Table 2 & Table 3. Most of IR endocrine cells distributed in the foregut of 7 kinds of fish species. Only SP and GLI-IR endocrine cells were not found in the foregut of *C. carpio*, but they were most abundant in the midgut. SOM-IR endocrine cells distributed only in the foregut and midgut of *C. carpio*. In most conditions, the distribution of cell types and density were decreasing along the gut in a distal direction after the foregut. GAS-, GIP- and GLU-

IR endocrine cells distributed in the gut of all 7 kinds of fishes. GLU-IR endocrine cells were the most, up to 126 cell s/mm², in the midgut of *H. molitrix*. All controls for every IR endocrine cell in the gut of 7 kinds of fish species were negative. Eight kinds of IR endocrine cells distributed mainly

among the epithelium of intestinal mucosa, and only a few of them existed in the lamina propria (Figure 1). Some of the cells were dispersed in the middle and bottom part of the gut fold (Figure 2), but most of them were scattered in the apical part of the gut fold (Figures 3-10).

Table 1 Details of antisera and main reagents used

Antisera against	Working dilution	Code	Specificity	Source
Synthetic human gastrin	1:5000	GP-1304	No cross reaction with cholecystokinin-8	Dr. N Yanaihara, Shizuoka
Leucine-enkephalin	1:80000	1671	—	UCB-Bioproducts Bruxelles
Bovine pancreatic polypeptide	1:12000	615-R-110	Cross-react s with human pancreatic polypeptide	Dr. RE Chance, Indianapolis
Substance P	1:2000	MAS 035B	—	Sera-Lab., Sussex
Gastric inhibitory polypeptide	1:10000	G/R/34-IIID	No cross reaction with glucagon	Dr. D Grube, Hannover
Porcine glucagon	1:1000	RPN1602	Wholly cross react with pancreatic and intestinal glucagon	Amersham International pl., Amersham
Synthetic human cyclic somatostatin	1:3000	—	—	Dr. S Ito Niigata
Glucagon-like immunoreactants	1:1000	RPN1604	Wholly cross react with pancreatic and intestinal glucagon	Amersham International pl., Amersham
Insulin	1:1000	47291	—	—
Avian pancreatic polypeptide	1:10000	Iance-10/5/81	No cross reaction with glucagon	Dr. JR Kimmel, Kansas City
5-Hydroxytryptamine	1:10000	Lot.16302	—	Immunonuclear Corp. Stillwater
Synthetic porcine motilin	1:1000	R-1104	Reacts against entire molecules	Dr. N Yanaihara, Shizuoka
Natural porcine cholecystokinin-33	1:3000	—	Reacts with cholecystokinin 11-20, no cross reaction with gastrin	Dr. J Yamada, Shizuoka
Synthetic porcine secretin	1:1000	R-801	Reacts with the C- and N-terminals	Dr. N Yanaihara, Shizuoka
Synthetic bovine neurotensin	1:1000	R-3501	—	Dr. N Yanaihara, Shizuoka
Synthetic porcine vasoactive intestinal polypeptide	1:2000	R-502	Reacts against entire molecules	Dr. N Yanaihara, Shizuoka
Bombesin	1:3000	27070	Cross reaction with GRP Immunonuclear	Corp. Stillwater
Neuron specific enolase	1:1000	—	—	Dako, Copenhagen
Prochymosin	1:2000	—	—	A. Andren, Uppsala
Pepsinogen	1:2000	—	—	A. Andren, Uppsala
Rabbit PAP	1:100	Z-113	—	Dako, Copenhagen
Guineapig PAP	1:50	24699	—	Dako, Copenhagen
PAP Kit	1:100	61-2003	—	Zymed Lab. Inc., South San Francisco, USA
ABC Kit	1:50	PK-4001	—	Vector Lab. Burlingame USA

Table 2 Distribution and density of 8 IR endocrine cells in the gut of three teleost species ($\bar{x}\pm S$)

Fish species	Gut parts	GAS	ENK	BPP	SP	GLI	GIP	GLU	SOM
Crass carp (<i>C. idellus</i>)	I	64±13	85±17	52±12	56±14	44±19	23±19	61±7	—
	II	26±6 ^b	57±11 ^a	31±16 ^c	76±20 ^a	45±22 ^c	11±7	44±4 ^b	—
	III	—	22±7 ^b	—	57±10 ^c	27±9 ^c	—	31±3 ^b	—
	IV	—	17±10 ^b	—	17±9 ^b	—	—	21±5 ^b	—
	V	—	1±2 ^b	—	18±7 ^b	—	—	9±5 ^b	—
Black carp (<i>M. piceus</i>)	I	77±8	57±6	72±6	20±5	68±17	59±13	2±3	—
	II	63±5 ^b	51±5 ^c	58±7 ^a	47±4 ^b	88±11 ^c	34±20 ^a	1±5 ^c	—
	III	32±4 ^b	38±4 ^b	22±4 ^b	38±3 ^b	92±30 ^c	31±19 ^a	—	—
	IV	23±5 ^b	57±9 ^c	—	60±7 ^b	16±12 ^b	17±11 ^b	—	—
	V	—	86±12 ^b	—	72±9 ^b	10±6 ^b	8±6 ^b	—	—
Common carp (<i>C. carpio</i>)	I	25±6	38±4	12±6	—	—	4±3	2±2	13±12
	II	20±5 ^c	39±5 ^c	9±8 ^c	18±4 ^b	6±1 ^b	2±2 ^c	1±5 ^c	8±5 ^b
	III	12±2 ^b	28±3 ^b	—	31±6 ^b	11±4 ^b	—	—	10±8 ^b
	IV	3±4 ^b	9±5 ^b	—	19±3 ^b	—	—	—	—
	V	—	—	—	—	—	—	—	—

I=Anterior segment of foregut; II=Posterior segment of foregut; III=Midgut; IV=Anterior segment of hindgut; V=Rectum; —: IR endocrine cell wasn't found, PAP method was used in the table; n=5, ^aP<0.05, ^bP<0.01, ^cP>0.05.

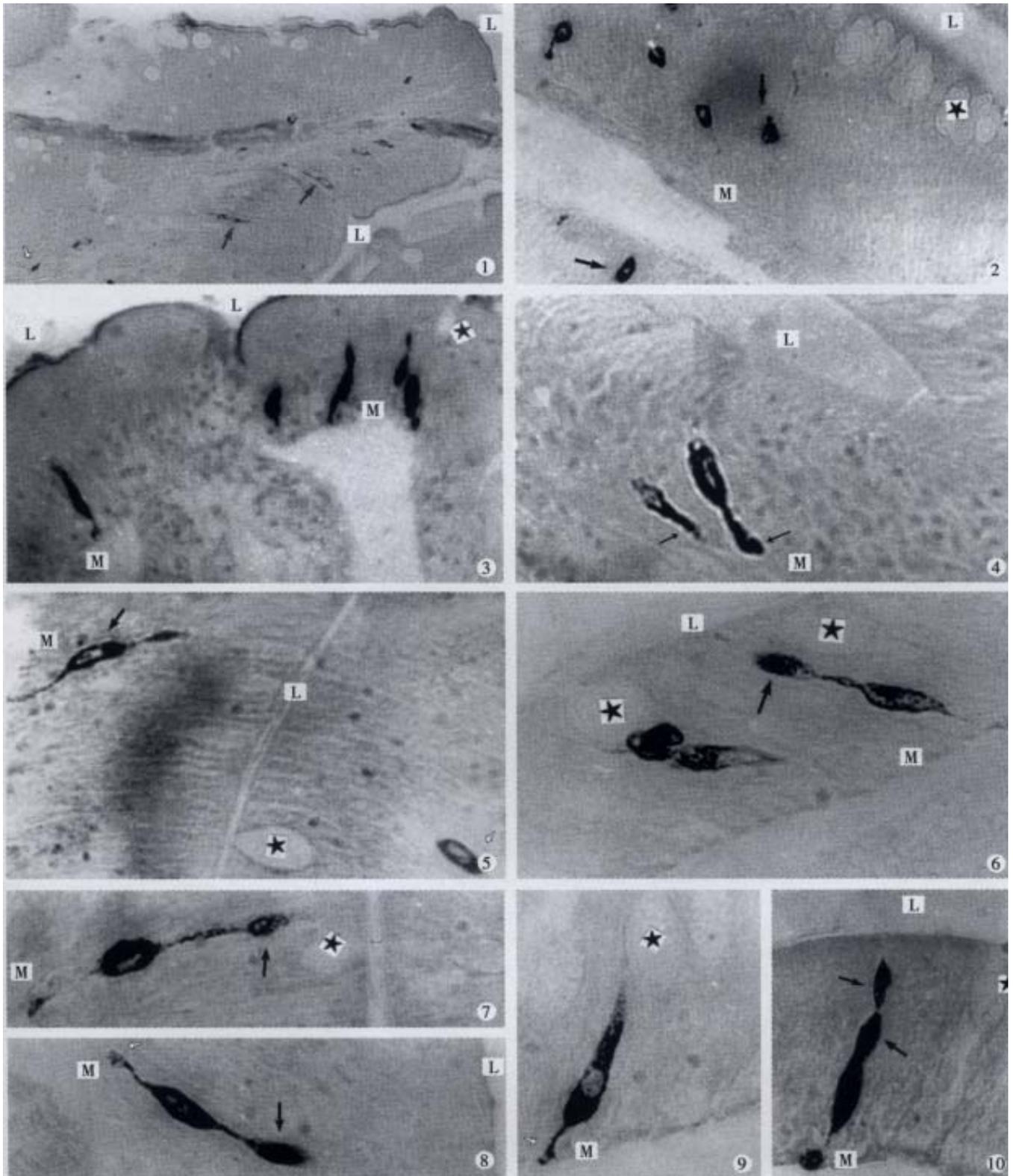


Figure 1 IR endocrine cells in epithelium (↑) and lamina propria (-), ×100

Figure 2 IR endocrine cells (↑) in bottom part of the gut fold, ×200

Figure 3 IR endocrine cells in apical part of the gut fold, ×400

Figure 4 Basal processes (↑) extended to basement membrane, ×600

Figure 5 IR endocrine cells of open type (↑) and close type (-), ×600

Figure 6 Enlarged process (↑) in sac-shaped, ×1000

Figure 7 & 8 Apical process (↑) as sac in shape, basal process (-) like synapse, ×1000

Figure 9 Basal process (-) extended to basement membrane, ×1000

Figure 10 Apical process (↑) as sac in shape, basal process (-) formed an enlarged synapse-like structure, ×1000-In figures, ★: goblet cell; L: gut lumen; M: basement membrane.

Table 3 Distribution and density of 3 IR endocrine cells in the gut of four teleost species ($\bar{x}\pm s$)

Fish species	Gut parts	GAS	GIP	GLU
Silver carp (<i>H. molitrix</i>)	I	17±5	2±2	43±10
	II	2±4 ^b	6±3 ^a	64±35 ^c
	III	—	—	126±36 ^b
	IV	—	—	61±26 ^c
	V	—	—	30±28 ^c
Bighead (<i>A. nobilis</i>)	I	35±10	13±3	29±16
	II	—	—	27±7 ^c
	III	—	—	79±7 ^b
	IV	—	—	60±62 ^c
	V	—	—	47±46 ^c
Silver crucian Carp (<i>C. gibelio</i>)	I	32±17	1±2	53±18
	II	3±3 ^b	—	20±11 ^b
	III	—	—	—
	IV	—	—	11±13 ^b
	V	—	—	—
Bluntnose black Bream (<i>M.</i> <i>Amblycephala</i>)	I	31±8	4±1	32±16
	II	—	—	24±6 ^c
	III	—	—	4±3 ^b
	IV	—	—	24±11 ^c
	V	—	—	6±8 ^b

I = Anterior segment of foregut; II = Posterior segment of foregut; III = Midgut; IV=Anterior segment of hindgut; V = Rectum; —: IR endocrine cell wasn't found, ABC method was used in the table; n = 5, ^aP<0.05, ^bP<0.01, ^cP>0.05.

Morphological feature of IR endocrine cells

All the IR endocrine cells showed a dark brown colour, and in the cell body and cytoplasmic processes, the secretory granules were seen clearly (Figures 6-9). The shape of IR endocrine cells was distinctive and variform, and quite different from the epithelial and goblet cells. Most of them had one or two long cytoplasmic processes, causing the apical part to be long and thin, the basal part narrow and the middle part of cell body to be broader. The cell nuclei were located in the middle part of body, and showed an empty bubble shape. Sometimes, the cytoplasmic processes showed one or several expanded sacs which were filled with secretory granules (Figures 6-9). The basal cytoplasmic processes emerged an enlarged synapse-like structure in the contiguous place with basement membrane or adjacent cells (Figures 7-10). A few of IR endocrine cells had only one cytoplasmic process or none (Figures 2, 5).

DISCUSSION

The results of the present study revealed that 3-8 kinds of enteroendocrine cells in the gut of 7 stomachless teleost fishes could produce immunoreactive response with antisera which were prepared against mammalian hormone. At the present time, because of the absence of isolates gastro entero pancreatic hormones in fishes antisera

against fish hormones are not available, and so the other 12 antisera against mammalian hormones used in the present study did not cause immunoreactive response with enteroendocrine cells of teleost fishes. Therefore, further investigations into the enteroendocrine cells of teleost fishes are necessary.

The description of GAS-, SP-, GLI-, BPP- and ENK-IR endocrine cells in the present study was generally similar to the results of Rombout's study^[6,20] on the enteroendocrine cells in the stomachless fish *Barbus conchonioides*. The result that the 8 kinds of IR endocrine cells which were more abundant in the first half of the gut of teleost fishes in our study was also similar to that of the study on *B. Conchonioides*^[20]. Rombout^[6] considered SOM-IR endocrine cells only existing in the gut of stomach-containing teleosts; this was different from our study in which SOM-IR endocrine cells were found in the gut of a stomachless teleost fish *C. carpio*.

The distribution density and location of various endocrine cells have some relation to their function. For example, GAS-IR endocrine cells in the stomach of human and other mammals stimulate gastric acid secretion, but these cells distribute mainly in the anterior segment of foregut of 7 stomachless fish species. The anterior segment of the foregut (gut bulb) of stomachless teleosts is like the stomach, and serve a dual function of storing and digesting food-stuff^[7,13]. There is a great amount of food in the anterior segment of foregut, and because of the stimulation of undigested particles of food, the activation of endocrine cells there is increased, resulting in the release of greater amount of hormones^[15,20]. Substance P can stimulate a contractile function of smooth muscle^[1], and SP endocrine cells are more numerous in the rectum; thus these endocrine cells may enhance the contraction of smooth muscle while fish excretes^[7]. The differences of cell types and amounts of IR endocrine cells in various fish species relate to their feeding behavior and feeding habits^[14-16,21]. Owing to the differences of chemical composition and pH value of the contents in the gut, the amount of hormones released by endocrine cells is also affected^[13,14]. Our studies verify that IR endocrine cells of stomachless teleost fishes belong to the open type^[1,21], that is, hormones are carried to the gut lumen (lumen-endocrine) by a long apical cytoplasmic process^[2,21]. The sac-shaped enlargement of cytoplasmic process has the function of storing. The close type IR endocrine cells lacking the cytoplasmic process seem to be caused by the angle during sectioning^[7]. In fact, almost all enteroendocrine cells seem to be the open type^[1]. In addition, some IR endocrine cells' basal

cytopoasmic processes extend to the basement membrane or adjacent cells, and form the synapse like structure in the contiguous portions, there by providing morphological evidence for the paracrine and neuroendocrine functions, and furthermore the peptide hormones they secrete may have neurotransmitter function^[1,2,21,22]. Previous studies propounded the peptide hormones SP, ENK, SOM and GLU to be present only in the brain^[1], but the present study confirmed their existence also in the gut mucosa of stomachless teleost fishes. Wang *et al.*^[1] thought that the area of gastrointestinal mucosa was very large; the sum total of gastrointestinal endocrine cells exceeded a lump sum of cells in all other endocrine gland; thus, the digestive tract mucosa was reputed to be the biggest and the most complex endocrine organ in body. Rombout^[6] concluded the endocrine system of digestive tract in teleost species was almost as complicated as in mammals. According to the present progress in the studies on the APUD cells in gastro-entero-pancreatic endocrine system of the fish species^[21], it is deemed that the digestive tract of fish species is also an endocrine organ of great importance and complexity.

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Biliary acute pancreatitis: a review

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Subject headings cholelithiasis/physiopathology; pancreatitis/physiopathology; bile reflux; common bile duct; sphincterotomy, endoscopic

Tiscornia OM, Hamamura S, de Lehmann ES, Otero G, Waisman H, Tiscornia Wasserman P, Bank S. Biliary acute pancreatitis: a review. *World J Gastroenterol*, 2000;6(2):157-168

INTRODUCTION

It is axiomatic that the most effective and soundly based plan of treatment of any disorder is one aimed at the mechanism or mechanisms responsible for its development^[1]. This basic notion, coupled with recent reports^[2-11] in which, surprisingly there is a total lack of reference to the probable involvement of autonomic-arc-reflexes in the physiopathogenesis of biliary acute pancreatitis have prompted this presentation. Undoubtedly, this disease entity has numerous causes, an obscure physiopathology, few effective remedies, and, often, an unpredictable outcome. At the turn of the century, Opie^[12,13] brought to light the association between gallstone migration and acute pancreatitis. When referring to the intimate evolving process, most authors steadily adhere to the Opie's theories: chemical and physical. The former is linked to the common-channel or biliary-reflux mechanism. The latter, to the stone-elicited Wirsung duct obstruction, with the resultant hypertension in the pancreatic ductal tree. What is indeed surprising is the lack of speculation on the subsequent steps that necessarily must follow the initial chemical and/or physical injuries that end up in an episode of acute pancreatitis. The core of our contention is that the pathophysiology of biliary acute pancreatitis is centered on two basic mechanisms: the activation of autonomic arc reflexes and the disruption of the

entero-pancreatic feedback loop. Superimposed on the aforementioned pivotal processes we must consider, in some cases, the aggravating role of alcoholism.

In this presentation we will also try to point out those features that justify to consider the Pfeffer method, or of the closed-duodenal-loop, as an experimental surgical model suitable to mimic the clinical condition of acute pancreatitis. Furthermore, we will analyze the properties of local anesthetics that, according to our contention, are valuable agents to either prevent and/or treat efficaciously an episode of biliary acute pancreatitis.

ACTIVATION OF AUTONOMIC-ARC-REFLEXES

The clinical or surgical circumstances that usually evoke an episode of biliary acute pancreatitis, e.g. a stone that migrates into the duodenum or gets impacted in the distal end of the common bile duct^[12,13], endoscopic maneuvers (sphincterotomy, sphinctero-manometry, retrograde cholangio-pancreatography), surgical manipulation, in or close to the duodeno-pancreas, percutaneous liver biopsy-associated hemobilia^[14], have as a main starting locus of activation of the autonomic-arc-reflexes the peri-Vaterian duodenum. This intestinal segment is the most sensitive area of what we have conceived as the trigger of an imaginary pancreatic revolver (Figure 1). Our choice of this pedagogical reference was suggested, on the one hand, by the morphologic resemblance with the gun that the pancreatic gland offers in its general anatomical outline, and, on the other, by its functional characteristics^[15,16]. The exquisite sensitivity of the trigger zone rests on its innervation density. This has been suggested, initially, by the macroscopic anatomical dissection findings in human cadavers and dogs^[17-19]. Recently, by those observed in rats and the opossum (Figure 2). Subsequently, histochemical studies^[20-23] and the utilization of very elaborate tracers^[21-23], allowed to fully ratify the initial gross anatomical observations. Some of the nerve fibres that jump the duodeno-pancreatic cleft belong to the vagal system. They arrive to the head segment of the pancreas following a pathway through the gastric and duodenal walls^[19,24-34]. Others contingents of nerve fibres are intrinsic to the duodeno-pancreas. They connect the enteric

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nervous system of the stomach and duodenum with the pancreatic gland (Figure 3). Some of them are cholinergic, and many are serotonergic. They end either in the pancreon units^[15,16,18,19] and/or the intrapancreatic ganglia^[17-19, 20-23, 25,26]. Following the same route, nerve fibres that belong to the afferent component of the autonomic nervous system jump the duodeno-pancreatic cleft in search of either the nodose ganglion of the vagus or the dorsal root ganglia of the spinalcord^[35-46]. These duodeno-pancreatic nerve fibres are the basis of duodeno-pancreatic reflexes.

They are short, medium, and long^[17-20,24-26,33]. The short duodeno-pancreatic reflex connect the enteric nervous system of the duodenum with the intrapancreatic ganglia and/or the bullets of the pancreatic islets: the pancreon units and the Langerhans islets^[21-23]. The medium duodeno-pancreatic reflex gets its integration in the celiac ganglia. The long duodeno-pancreatic reflex complete the arc reflex loop in the central nervous system, primarily in the hypothalamic-bulbar nuclei. A feature to emphasize is the presence of command neurons in the peri-Vaterian duodenum^[28-32]. They exert a pre-programmed control of the interdigestive motor complex. Concerning the source of the duodenal innervation, its proximal segment, at least in the rat, is under the control of the left or anterior vagal nerve. Contrariwise, the peri-Vaterian duodenum is primarily subjected to nervous influences coming from the celiac ganglia^[47-61].

The pancreatic gland innervation depends on the following complex systems: the vagal, the splanchnic-celiac, the entero-pancreatic and the sensory-afferent^[17-26, 40-46], (Figure 3). In the first two complexes, one should take into consideration the existence of three different kinds of impulses: cholinergic, adrenergic and peptidergic. The above description is valid for both the exocrine and the endocrine pancreas^[46]. Summarily, the vagal complex arrives to the pancreatic gland following the pathways of either the digestive tract wall (stomach-duodenum) or through the celiac ganglia. Its nerve fibres are essentially afferent. It also carries peptidergic influences and efferent impulses, primarily cholinergic. The splanchnic-celiac-ganglia-complex is also a nervous afferent pathway, namely of the pain sensation. From the efferent point of view, this complex is primarily of sympathetic impulses. Secondly, is a transmitter of peptidergic and cholinergic efferent impulses. The entero-pancreatic-complex is the result of neurons that project from the gut to the pancreas. According to the Gershon group^[21-23], the nerve cells are essentially cholinergic and serotonergic. They influence the activity of intrapancreatic neurons and

islet cells^[46]. The sensory-afferent of the autonomic nervous system, through the mediation of its fine, amyelinated, capsaicin-sensitive type C fibres, constitute the basis of the neurogenic-inflammation^[35-39,59-65].

As a preamble to the consideration of the autonomic-arc-reflexes, it seems appropriate to point out that the autonomic ganglia in the extrahepatic bile ducts and the pancreatic gland display similar histoarchitectural and physiologic features to those of the enteric nervous system. This is explainable by their common embryological origin^[25,26]. When deprived of all outside neuronal influences, the intrapancreatic ganglia can be shown to possess an intrinsic activity. The latter, coupled to outside cholinergic, sympathetic and peptidergic impulses give origin, within the gland, to what has been described, with tacit consensus, as cholinergic tone^[15-18, 25-27]. This is involved in an intricate interaction with the hormonal system. It has been ascertained on both pancreon segments: the acinar and the centroacinar-ductal, responsible, respectively, of the bicolic and the hydrolitic components of the exocrine pancreatic secretion^[15,16]. A feature to emphasize is the role of the celiac ganglia as an integration center of different kinds of autonomic arc reflexes^[17-19,21-25, 47-61]. The nerve fibres that arrive at the celiac ganglia come from different segments of the digestive tract, the extrahepatic bile ducts and the pancreas. In this autonomic nervous structure they establish an intricate interrelationship. The Faradic electrical stimulation of the celiac ganglia triggers an intrapancreatic ischemic process. This evolves as a result of the opening of arterio-venous shunts^[50-57]. We have delineated this autonomic-arc-reflex as sympatho-ischemic (Figure 4). It has been clearly observed subsequent to the instillation of bile or bile salts in the pancreatic ductal tree^[53]. The changes in the pancreatic vessels (spasms) and the gland's capillary circulation (ischemia) have been objectivized in plastic casts^[53]. The full development of the sympatho-ischemic autonomic-arc-reflex leads to a depression of the pancreon's secretory process. The exocrine pancreatic secretion inhibition can also be evoked through another type of autonomic-arc-reflex. We have pinpointed it as secretory-inhibitor. Its conception arose from our observation in canines equipped with duodenal Thomas cannula and studied in the conscious state (Pavlov frame). Indeed, when intemperate maneuvers are employed, especially if either liquid or air is injected abruptly into the main pancreatic duct (distention), a sudden arrest of exocrine pancreatic secretion is frequently observed. This secretory-inhibitor autonomic-arc-reflex can be elicited from other segments of the digestive tract. We have

observed it in rats when distending with a balloon either the antral-fundic junction of the stomach or the peri-Vaterian duodenum segment. A nervous reflex of this sort has been described by Warkentin *et al*^[49] on bile secretion when distending the colon or its nerve supply.

The pathway of this autonomic-arc-reflex is probably that of the afferent component of autonomic nervous system. At pancreas level, an antidromic mechanism (pseudo-axonic), and/or an arc reflex loop integrated either in the intrapancreatic or the celiac ganglia is a possibility. The involvement of a peptide like CGRP^[66], PP^[67] or some other agent, like serotonin could be a logical assumption to make. The intimate physiopathogenic mechanism is probably related to with the recent studies of Ohshio *et al*^[6] in rats. According to these authors, a short-term pancreatic duct obstruction interferes with the acinar cell secretory process down-stream of hormone-receptor binding, intracellular Ca²⁺ release and protein-kinase activation. The pseudo-axonic autonomic-arc-reflex, that leads to neurogenic inflammation, is evoked as a result of the irritation of the afferent component of the autonomic nervous system. Normally, through these nerve fibres a significant percentage of the information originated in the stomach, duodenum, extrahepatic bile ducts and pancreas reach the central nervous system. The impulses travel through special type of nerve fibres: thin, unmyelinated, capsaicin-sensitive (type C). In their course to the nodose ganglion of the vagus, or the dorsal roots ganglia of the spinal cord, they send collaterals where they exert a modulatory influence on the neural transmission (Figure 3 and 5). It is interesting that capsaicin (red-pepper agent), infused intraduodenally, evokes an exocrine pancreatic secretion equivalent to a 15 % of that obtained with a maximal dose of CCK^[63]. This pancreon's secretory response results from the activation of CCK-A receptors by the peptide CGRP released from the afferent nerve fibres. Others vasoactive peptides are involved, like SP, neurokinin-A, VIP, SOM and DYN. After synthesis in the somata of the afferent neurons, the bulk of the peptides is transported to their peripheral endings. The co-released peptides interact in the control of pancreatic function. Their antidromic release elicit vasodilatation and extravasation of plasma proteins and formation of edema (neurogenic inflammation)^[35-39,59-65,69-71]. A feature to take into account for its pathophysiologic implications is that SP, and other sensory peptides released in the pseudo-axonic reflex, are capable of activating mast-cells to release histamine and other factors. Furthermore, leukocytes, particularly neutrophils, granulocytes, monocytes and lymphocytes are stimulated to adhere

to the vascular endothelium and to emigrate to the surrounding tissue. Once neurogenic inflammation is triggered, monocytes release prostaglandins, thromboxane and cytokines^[36-39]. Some of the latter agents are vasoactive by themselves and, in addition, can activate afferent nerve endings and thereby provide a positive feedback loop which reinforces the initial stimulation of the afferent component of the autonomic nervous system (Figure 3 and 5). This whole process probably favors the absorption of endotoxin from the gut lumen into the blood stream. This agent is capable of inducing pancreatic lesions, depression of the exocrine pancreatic secretion and, in the liver, on the one hand, inhibition of albumin secretion, and, on the other, enhancing the secretion of both fibrinogen and C-reactive protein^[72]. In the afferent component of the autonomic nervous system it should be pointed out the presence of different types of receptors: chemoreceptors, chemonociceptors, polymodal nociceptors and warmth receptors^[60]. These nervous structures allow to detect those noxious stimuli that are potentially or actually harmful to the tissues. Besides, they also sense innocuous physiologic stimuli, such as pH, bile, distention. The afferent neurons represent a first line of defense against trauma, or, as in the stomach, to the injurious effects of ethanol^[62,63]. It is probable that the same happens in the pancreatic gland. That, normally, in this organ, the afferent component of the autonomic nervous system fulfill some sort of cytoprotective function against the deleterious effects of injurious agents, can be inferred by the fact that its permanent ablation, like the one that results from long-term surgical bilateral splanchnicectomy, aggravates the pancreatic lesions (necrosis) induced by 24h closed-duodenal-loop. This at least has been our experience in unpublished observations in rats^[73].

All the foregoing support the notion of autonomic-arc-reflexes involvement in the physiopathogenesis of biliary acute pancreatitis. Their degree of participation surely varies in each clinical case. This depending on the intrinsic characteristics of the injurious agent, its degree of persistence and the patient's neuroendocrine reactivity.

DISRUPTION OF THE ENTERO-PANCREATIC FEEDBACK LOOP

When bile and/or pancreatic juice cannot reach the duodenal lumen, the entero-pancreatic feedback loop gets interrupted. This triggers the rising of blood's CCK levels, and, through the induction of an increased cytosolic Ca²⁺ concentration, a supramaximal stimulation of the pancreon's acinar cells is elicited^[74-90]. The foregoing is linked to the

evoking of both positive and negative duodeno-pancreatic reflexes^[74]. Indeed, when in the duodenal lumen the influence normally exerted by bile, trypsin and chymotrypsin lessens (bile and/or pancreatic juice diversion bile and/or pancreatic duct obstruction) the releasing of CCK from the duodenal mucosa is markedly enhanced. This peptide through a paracrine mechanism in the duodenal wall, activates the autonomic nervous system. Thus, a positive duodeno-pancreatic-reflex (increased intrapancreatic cholinergic tone) is induced. This effect is potentiated by a concomitant annulment of a negative duodeno-pancreatic-reflex. The latter was postulated by us in the eighties^[74]. It might have as a pathway the entero-pancreatic nervous complex. A neurotransmitter (SOM ?, PP ?, serotonin ?) induced by the presence in the duodenal lumen of trypsin, chymotrypsin and/or bile, might be at the basis of restricting influences on the pancreas units (Figure 6).

Others features to take into account to better understand the entero-pancreatic feedback loop is that a monitor peptide in the pancreatic juice, and a CCK-releasing peptide, derived from the duodenal mucosa, exert a direct stimulating action on the CCK-releasing cells (I cells). These small peptides are normally inactivated by intraduodenal trypsin and chymotrypsin. Concerning bile, this secretion, besides activating the postulated negative duodeno-pancreatic-reflex, normally exerts an indirect feedback inhibition. The latter might result from the stabilization of pancreatic juice's protease by the calcium ions in it contained^[74,83-90]. A physiologic detail to emphasize is that unblocking of the bile obstruction allows the duodenal mucosa to release secretin^[91,92]. This hormone may help to dislodge, through an enhanced exocrine pancreatic secretion of water and bicarbonate, eventual intrapancreatic protein plugs. Besides, it may attenuate the noxious influences of CCK on the pancreas's acinar cells. The latter has been shown, initially by Kanno *et al*^[93], subsequently by Renner *et al*^[94], and, recently, through an agent (tetrafenyl actone) that has proved to possess a releasing capacity of this hormone from the intestine^[95]. A feature to be emphasized is that both secretin and VIP, through a second messenger (cAMP), either prevent or restore, in the acinar cells, the CCK-induced disrupted microtubules and microfilaments.

Aggravating role of chronic alcoholism

When the usual agents that elicit the biliary acute pancreatitis exert their actions superimposed on a background of chronic alcoholism, the degree and extension of the pancreatic acute inflammatory lesions are significantly enhanced^[96,97]. This notion is important because the incidence of a chronic

ethanol intoxication as a background of an episode of biliary acute pancreatitis is rampant. This assertion is confirmed by several recent papers^[96,97]. Experimentally, Gronr-os *et al*^[98-100] have ratified, in rats, our primary contention, described in dogs and rats^[101-118], that in the pathophysiology of ethanol-evoked pancreatic lesions two major factors play a crucial role: an elevated intrapancreatic acetylcholine level (high cholinergic tone) and an enhanced acinar cell response to CCK. At clinical level, Brugge *et al*^[119] have provided additional support to this postulation. Recently, we have suggested^[115,117,118] that the forementioned changes might be consequence of an alcohol-induced loss of a normal braking mechanism exerted by higher autonomic nervous centers. The braking on the pancreas units might depend on peptides like CGRP^[66], PP^[67] and/or SOM^[120,121].

As eventual aggravating influences associates to alcohol intoxication, one should consider the probable participation of an ethanol-evoked sphincter of Oddi dysfunction. Two reports give support to this assumption. The first one, performed in monkeys^[128], has disclosed that after the administration of a 130 mL/L ethanol solution there is a significant increase of the main pancreatic pressure. The second report^[121], has put in evidence, in humans, that acute intraduodenal ethanol induces an increase of the Vaterian resistance. This phenomenon is enhanced when performed in chronic alcoholics.

Others additional effects related to ethanol intoxication that merit their consideration are: the reduction of the capillary blood flow^[122] and the activation and migration of leukocytes in the pancreatic gland^[123].

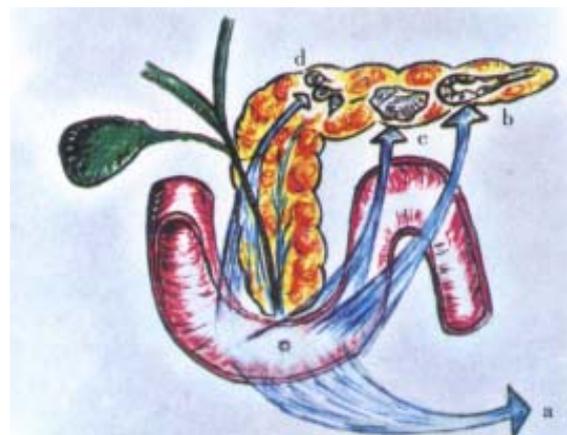


Figure 1 A schematic representation of the duodeno-pancreas. The image pedagogically resorted to is that of a revolver with its trigger represented by the peri-Vaterian duodenum (a) and the bullets by the pancreas units (b) and the Langerhans islets (c). The intrapancreatic ganglia (d) are also depicted. The latter integrate different nervous arc reflexes and are pivotal in the generation of the intrapancreatic cholinergic tone.

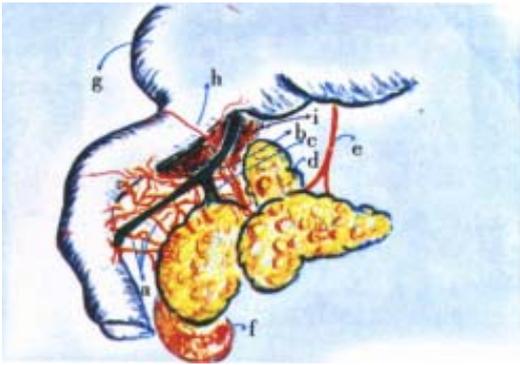


Figure 2 Some anatomical features of the opossum: rich density of nerve fibres jumping the duodeno-pancreatic cleft (a). The joining of the main pancreatic duct (c) with the bile duct (b) giving origin to a long common bile-pancreatic segment before reaching the outlet into the duodenum. The presence of an extension of the pancreatic gland into the hepatic hilum (d). Arriving to the pancreas of the vagus nerve (e). Right kidney (f). Stomach displaced upward and to the right (g). Duodenal and pyloric branches of the hepatic artery (h). Liver (i)

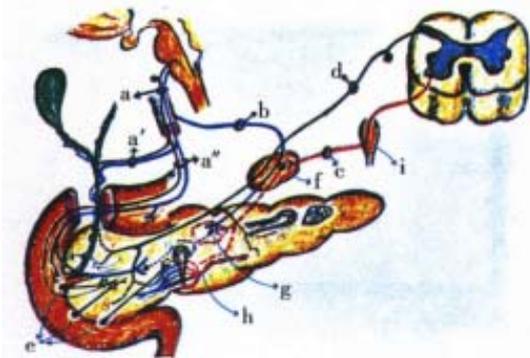


Figure 3 Complex nervous systems that innervate the extrahepatic bile ducts and the duodeno-pancreas. Vagal: Left or anterior vagus nerve (a). Hepatic branch (a'). Gastric branch (a''). Splanchnic-eliac: Pre-ganglionic fibre (c). Celiac ganglion with post-ganglionic neuron (f). Sensory-afferent: Afferent fibres and neurons of the dorsal roots ganglia of the spinal cord nerves (f). Enteropancreatic: Nerve fibres connecting the neurons of the enteric plexus with the pancreatic gland (e). Parasymphathetic synapses in the intrapancreatic ganglion (g). Arterio-venous shunts of the pancreas' microcirculation system (h). Ganglia of the sympathetic chain (i)

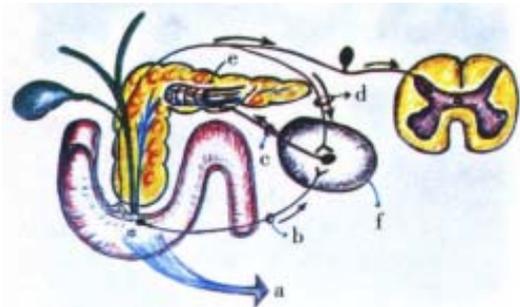


Figure 4 Physiopathogenesis of biliary acute pancreatitis. Activation of autonomic-arc-reflexes. A: Sympatho-ischemic reflexes. Trigger of the pancreatic revolver, the peri-Vaterian duodenum region (a). Entero-eliac reflex (b). Celiac-pancreatic reflex (c) giving the origin to the opening of the arterio-venous shunts in the pancreatic gland microcirculation. Celiac ganglia (f)

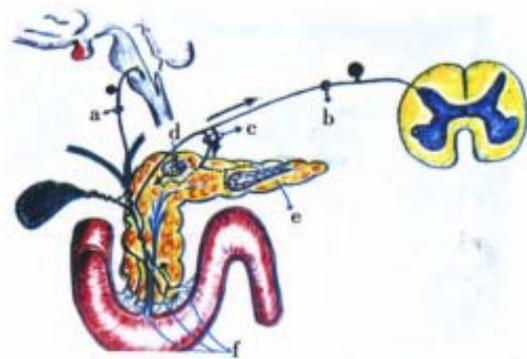


Figure 5 Activation of autonomic-arc-reflexes.

B: Pseudo-axonic reflex. Afferent nerve fibres of the vagal nervous complex (nodose ganglion) (a). Afferent nerve fibres of the splanchnic-eliac nervous complex (b). Antidromic discharge through a collateral nervous branch (c). Degranulation of mast cells (d). Pancreon units (e). Afferent nerve fibres of the peri-Vaterian duodenum, the trigger zone

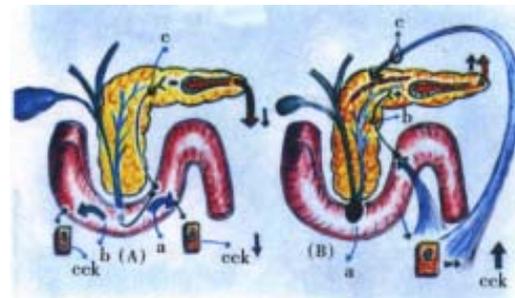


Figure 6 Disruption of the entero-pancreatic feedback loop.

A: Normal condition: Brake of CCK release.

Direct brake (trypsin, chymotrypsin) of the CCK-releasing peptide and of the monitor peptide (a). Indirect brake (bilis). Stabilization of trypsin and chymotrypsin (b). Bile-pancreatic secretion activation of a negative duodeno-pancreatic reflex (c).

B: Bile-pancreatic duct obstruction condition

Loss of the normal bile-pancreatic secretion-evoked brake of CCK release. Stone impactation in the Vaterian papilla (a). CCK-induced paracrine-neural duodenopancreatic reflex (b). CCK-elicited hormonal activation of pancreon units (c). Neural (b) and hormonal (c) mechanisms. Neural (b) and hormonal (c) pathway to the CCK-evoked supramaximal stimulation of the acinar component of the pancreon units.



Figure 7 Experimental mimicking of biliary acute pancreatitis in rats.

Modification of the classical Pfeffer method (closed duodenal loop), the temporary or short-term closed duodenal loop method. Filling of a duodenal loop (a) through the gastric antrum (b) with 7% sodium taurocholate and a few drops of methylene blue, at constant pressure. Height of liquid infusion column=110cm (c)

Experimental mimicking of biliary acute pancreatitis

At this stage, it seems relevant to analyze the data afforded by the experimental, surgical-induced acute pancreatitis of the Pfeffer method or of the closed-duodenal-loop^[124-131]. This procedure, according to our view, offers a suggestive approximation to those conditions which usually interplay, in a clinical setting, in an episode of biliary acute pancreatitis. Indeed, the closed-duodenal-loop model offers a series of conditions that somehow mimic those frequently seen in human cases of biliary acute pancreatitis, e.g. the distention and chemical injury of the peri-Vaterian duodenum, the bile-pancreatic hypertension, the eventual reflux of the duodenal content into the bile-pancreatic ducts, the disruption of the entero-pancreatic feedback loop due to the exclusion of both bile and pancreatic juice from the intestinal lumen and the bacterial aggression (endotoxemia).

In recent experiments in rats with a short-term closed-duodenal-loop method, that we have modified from Orda *et al*^[132] and De Rai *et al*^[133], we have observed changes of the pancreatic gland that, taking into account the brevity of the experiments, were unexpected^[134]. Indeed, after filling a duodenal loop with a 70 g/L taurocholate solution plus a few drops of blue-methylene, under a constant pressure of a 110 cm fluid column height, and keeping those conditions for only 3 min, the macroscopic evaluation of the pancreatic gland disclosed, 3 hours after, the presence of edema and spotty foci of acinar cell necrosis (Figure 7). Remarkably, the above changes were obtained under the total absence of any reflux of the duodenal content into the bile-pancreatic ducts. This crucial detail was verified by means of a constant visual observation of the duodeno-pancreas. Something we learned from the above test, is that it takes normally more than 20min, and sometimes even more than an hour, to appreciate the reflux of the duodenal content into the bile-pancreatic ducts. When this does indeed occur, a marked pancreatic edema is seen, and quite rapidly, a notorious hemorrhagic acute pancreatitis distinctly develops.

The above described results give further solid support to our contention that the irritation of the duodenum, at the level of the peri-Vaterian duodenum (trigger zone of the pancreatic revolver) is capable of activating autonomic-arc-reflexes. The latter, coupled with the changes evoked by the disruption of the entero-pancreatic feedback loop (bile-pancreatic obstruction) (Figure 6), probably explains, as we have already pointed out, the physiopathogenesis of the pancreatic lesions. This speculations of ours, that biliary acute pancreatitis is might pivot around the activation of autonomic-

arc-reflexes and a disruption of the entero-pancreatic feedback loop could perhaps be extended to explain, at least partially, the liver changes that recently have been pointed out by Iso gai *et al*^[135] in human patients with biliary acute pancreatitis. Indeed, in the reports of these authors, a feature to be emphasized is that both the liver's histopathologic changes and the biochemical abnormalities were of the same order of magnitude in patients with and without impacted stones in the Vaterian region. This set of circumstances allow to infer that besides bile duct hypertension other factors (autonomic reflexes) might be at the basis of the above findings. An anatomical detail that gives support to the precedent contention is that of the rich density of nerve fibres in the hepatic hilum that we have put in evidence in macroscopic dissection studies in human cadavers^[17-19].

Another observation that deserves to be emphasized because it affords additional indirect basis to presume an involvement of autonomic-arc-reflexes in the physiopathogenesis of biliary acute pancreatitis is the one associated to a pure distention of bile ducts without the intervention of any intemperate maneuver or the irritation of a chemical agent. This is what suggestively happens with the acute pancreatic inflammation that have been reported in cases of hemobilia, accidental or iatrogenic (post-percutaneous liver biopsy)^[14].

Preventive and therapeutic value of local anesthetics

All the above findings give coherent basis to consider an eventual beneficial effect of local anesthetics either as preventive and/or therapeutic agents of an episode of biliary acute pancreatitis. A long experience in conscious dogs with lidocaine spray in the peri-Vaterian duodenum^[19,24,28,30,33] has convinced us, on the one hand, of their efficacy to interrupt noxious autonomic-arc-reflexes and depress the intrapancreatic cholinergic tone, and, on the other, of their relaxing capacity on both the main pancreatic duct outlet and the duodenal motor activity. The above anesthetic-induced changes make easier the catheterization of the Vater papilla in man. Consequently, the changes of its traumatization are significantly reduced. A detail to be emphasized is that of the atropine-like effects evoked by local anesthetics (procaine, lidocaine) in the duodenum and the sphincter of Oddi described by Varela-López *et al*^[136,137], by Velasco Suarez^[138,139], Cottini^[24] and by our group^[24,28,30,33].

The forementioned authors have given convincing evidence of the local anesthetic value not treating clinical cases of sphincter of Oddi dysfunction or bouts of recurrent stone impaction in the distal common bile duct. The procedure most frequently used was of a duodenal infusion of

procaine clorhidrate (20 mL of a 10 g/L solution) or an oral ingestion of this anesthetic (0.2 g up to 1.2 g/24 h). With this latter approach, we succeeded in sparing a sphincterotomy in several patients with common bile duct residual stones following a laparoscopic cholecystectomy.

It is worth remembering that Albanese^[69], Longo and Sosa Gallardo^[64,65], Salazar^[59] and Ochsner^[70] have given solid accounts of the clinical therapeutic value of a temporary interruption of the autonomic-arc-reflexes by means of a local anesthetic of the celiac ganglia. This was experimentally ratified by the group of Waisman^[58]. Indeed, these authors have shown, in rats subjected to a 24 h closed-duodenal-loop procedure, that those animals in which a single infiltration with lidocaine was performed had a significantly longer survival than the controls.

Local anesthetic bathing of the duodenum, namely at the level of the peri-Vaterian duodenum, or the anesthetic infiltration of the duodeno-pancreatic cleft, and/or of the celiac plexus, during an eventual laparotomy, constitute, according to our postulation, an efficacious means to prevent, or attenuate, the intensity of autonomic-arc-reflexes. Furthermore, they surely contribute to depress the intrapancreatic cholinergic tone. This is important in cases of an episode of biliary acute pancreatitis superimposed on a background of chronic alcoholism. They might do this through the interruption, on the one hand, of cholinergic impulses that course through the gastro duodenal wall, and, on the other, by blocking the CCK release from the "I" endocrine cells and the subsequent evoking of duodeno-pancreatic reflexes^[18,19,25,26,63,140]. The latter is suggestively supported by the recent demonstration that vagal mucosal receptors are directly sensitive to CCK-8^[141,142]. Besides, as it has been pointed out by Bj-rck *et al*^[71] and Mc Cafferty *et al*^[143], many other properties of local anesthetics, in addition to those of inhibiting action potentials by blocking sodium channels, are surely involved. The foregoing might explain the therapeutic success reported with intrarectally infused lidocaine in the treatment of ulcerative colitis^[71]. In a recent report^[144], it has been shown that in patients subjected to ERCP and therapeutic endoscopy (e.g. sphincterotomy, stone removal, etc.) and randomly assigned to have ① 10 mL of 10 g/L lidocaine sprayed onto the ampulla before or after, or, ② saline, either pre or post ERCP, that local anesthesia applied to the ampulla before ERCP facilitates cannulation of the ampulla and appears to reduce hyperamylasemia whether given before or after ERCP. When considering further experimental evaluation with local anesthetics, related to either the prevention or treatment of

biliary acute pancreatitis, one should take into consideration the results recently reported in rats by Mc Cafferty *et al*^[143]. Indeed, it is remarkable that the intrarectal administration of 0.5 mL of 25 g/L carboxymethylcellulose, containing lidocaine hydrochloride, at dosis ranging from 5 mg/kg to 100 mg/kg had induced a significative reduction of the colitic score and of the myeloperoxidase activity.

In recent experiments in rats with our "short-term-closed-duodenal-loop" model^[134], we showed that previous bathing of the duodenum with lidocaine (20 mg/kg) reduced the histopathologic score of pancreatic necrosis observed in the control group. This was confirmed in a subsequent series^[145]. It is our postulation that through the interruption of autonomic-arc-reflexes one could interfere in the release of cytokines at pancreas level^[146-154]. This is also suggested by our recent findings in the opossum^[155].

Concerning the variable effects of a local anesthetic (lidocaine) on exocrine pancreatic secretion according to the route of administration, e.g. intraduodenal vs intravenous, we have acquired a long experience in dogs equipped with a duodenal fistula (Thomas cannula) and tested in the conscious state^[33]. From them, we have learned that spraying of the papillar zone (trigger of the pancreatic revolver) with lidocaine (50 mg each, 10 min for 2 h) induces a significant depression (60%) of the plateau levels of all secretin-induced parameters. Remarkably, and coherent with the anatomical details previously outlined, the above exocrine pancreatic secretion changes were not observed with lidocaine spraying outside the papillar zone. Other suggestive findings were, firstly, that the intravenous infusion of the same amount of lidocaine (500 mg dissolved in 200 mL physiological saline = 12.5 mg/kg), did not modify any of the exocrine pancreatic secretion parameters and secondly, that when the intraduodenal lidocaine testing was performed in alcohol-fed dogs (2-year), the degree of the anesthetic-induced depression of the exocrine pancreatic secretion was less notorious (35%) than when carried out in controls (65%). We have interpreted this difference as a reflection in the ethanol-fed animals of a higher duodeno-pancreatic cholinergic tone. It is interesting that at clinical level this assertion has been ratified by Brugge *et al*^[119]. Other considerations that seem relevant to point out is that of the extraneuronal effects of local anesthetics. In addition to our presumption that through the interruption of autonomic-arc-reflexes they interfere with the release of inflammatory mediators at pancreas level, they inhibit the phospholipase A2 enzyme and its interaction with its specific substrate: the cell membrane phospholipids. The latter was shown by

Aho *et al*^[151] following their experiments on acute pancreatitis treated with a procaine solution (40 mg/kg).

Finally, and in order to complete the attempt to interrupt the evolving of the mechanism that we have considered as pivotal in the physiopathogenesis of biliary acute pancreatitis, it would seem logical to add to local anesthetics the simultaneous administration of pancreatic enzymes^[75,83,86,87] and, eventually, of a calcium channel blocker^[89,152]. Concerning the former, the oral and/or the intragastric-intraduodenal administration of pancreatic enzymes, might accomplish, on the one hand, the depression of the CCK release from the intestinal mucosa, and, on the other, the evoking of our previously described neural mechanism of "pancreon" inhibition, the negative duodeno-pancreatic reflex. In relation with calcium channel blockade, it is interesting to point out the recent finding of Hughes *et al*^[153] in rats. Indeed, these authors have shown that the administration of diltiazem is associated with significant reduction in serum TNF-alfa levels as well as amelioration of pancreatitis by biochemical and pathological criteria. They emphasize that TNF-alfa mediates tissue injury through the activation of inflammatory cells, the up regulation of adhesion molecules, the production of nitric oxide and the release of other cytokines and mediators of inflammation.

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The omentum

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INTRODUCTION

The word omentum derives from the ancient Egyptians who, when embalming human bodies, used to assess their "omens" by looking at the variations in what we recognise today as the omentum^[1]. Galen (128-199 AD) thought that the role of the omentum was to warm the intestines. This was on the basis of a gladiator who had an omental resection after a stab injury and suffered greatly from cold for the rest of his life^[2]. A more conventional view of the omentum is that it plays a central role in peritoneal defence by adhering to sites of inflammation, absorbing bacteria and other contaminants, and providing leukocytes for a local immune response^[3]. This review details current knowledge on the origins, structure, and function of the omentum, and discusses its role in the peritoneal cavity during various disease states.

ORIGINS

The omentum appears to have evolved as a primitive effector organ in lower vertebrates. It develops as a loose mesothelial sheet of tissue from the yolk sac and is capable of basic immune functions such as allorecognition, natural cytotoxic reactions and the elaboration of cytokines. This area resides in lower vertebrates within a region delineated by the anterior limbs, foregut and mesonephros. That region is analogous to the boundaries of the developing omentum in mammals^[4]. The immune system in humans has evolved from these origins to a very sophisticated level, yet the omentum has retained an important role in immune defence within the peritoneal cavity.

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DEVELOPMENT AND STRUCTURE

The greater omentum develops in the eighth week of gestation from the dorsal mesogastrium^[5]. It is composed of two mesothelial sheets which enclose predominantly adipocytes embedded in a loose connective tissue, and also aggregates of mononuclear phagocytic cells. The omentum has a rich vascular supply with numerous characteristic capillary convolutions which are termed omental glomeruli due to their similarity to renal glomeruli. These capillary beds lie directly under the mesothelium^[6]. The size of the omentum varies from 300 gm to 2000 gm with a surface area of 300 cm² to 1500 cm².

In the omentum, the leukocytes aggregate in the perivascular area to form what is termed milky spots. These structures were first described by the French anatomist Ranvier in 1874^[7]. The cells derive their origin from the mononuclear phagocyte system^[8] and are arranged around the omental glomeruli that lie directly beneath the mesothelium^[9]. These structures are supported by delicate networks of reticular fibres which constitute the framework of the organ^[10]. In humans, milky spots comprise of macrophages (70%), B-lymphocytes (10%), T-lymphocytes (10%), mast cells, and stromal cells. On an ultrastructural level, it has been found that the macrophages are present in different stages of maturation, and that they can readily enter or leave the milky spots^[11]. The mean number of cells in one milky spot is approximately 600^[12] (Figure 1). Milky spots develop as specific structures in the greater omentum between the 20th and 35th week of gestation^[5]. The number of milky spots is highest in infancy and gradually decreases with age^[13].

Both the endothelium lining the omental capillaries and the mesothelium overlying the milky spots are specially adapted to facilitate transmigration of leukocytes^[14], and for rapid fluid shifts. The endothelial lining of the blood vessels in the milky spots is either discontinuous or contains fenestrations^[15]. Similarly, there are intercellular pores (the classical stomata of von Recklinghausen) between the mesothelial cells overlying milky spots, and there is an absence of the associated basal lamina in the submesothelial connective tissue^[16] (Figure 1).

The macrophages in the mature omentum are essentially scavengers. They appear to differentiate from monocytic precursors in the milky spots and are not dependent on precursors derived from the bone marrow^[17]. They are dendritic in shape and

have marked phagocytic abilities. They avidly phagocytose intraperitoneally injected carbon particles and bacteria. When activated, the macrophage precursors in the milky spots proliferate, migrate to the mesothelial surface, and transform into dendritic-shaped macrophages. This process in mice is dependent on macrophage colony stimulating factor (MCSF) being locally produced in the milky spots^[17]. Interestingly, the omental macrophages, despite their dendritic shape, lack many specific features of true dendritic cells.

The omentum contains large numbers of B and T lymphocytes which are usually located in the periarteriolar area. Following antigen challenge of the peritoneal cavity, the number of lymphocytes in the milky spots may increase up to 40-fold. Although it is unclear whether this increase represents local proliferation or an influx of cells. With such stimulation, the B and T-lymphocytes are found to segregate into distinct areas *in situ*, and the lymphocytes appear to be associated with stromal cells. Nonetheless, these aggregates do not represent secondary lymphoid organs, because they do not contain interdigitating cells or follicular dendritic cells^[11,18]. The omentum appears to be a primary site of B-lymphocyte development^[19,20]. In experimental animals, the omentum is a source of unique B-lymphocytes that demonstrate specific surface markers. These B-lymphocytes are predominantly CD5+(Ly1+), and are common in not only the omentum but also the peritoneum. However, they are rare in the blood, spleen and lymph nodes. Conventional B and T-lymphocytes are not found in the omentum. The CD5+B lymphocytes develop in the omental milky spots independently from the thymus or bone marrow^[19,20]. Hence, the fetal omentum, like the fetal liver and bone marrow, acts as a primary site of B-lymphocyte development^[21] and may be considered as a sort of intestinal thymus^[4,22]. The function of these CD5+ B lymphocytes remains obscure, nonetheless, they are most likely a remnant of a more primitive immune system which is in keeping with the evolutionary origins of the omentum.

Mesothelial cells lining the peritoneal cavity and endothelial cells lining blood vessels share the same mesodermal origin^[23]. Human omental microvascular endothelial (HOME) and mesothelial (MESO) cells share many phenotypic properties. In distinguishing between the two cell types, HOME and not MESO cells express a number of specific surface markers (i.e. E-selectin, P-selectin (CD62), and Le-y) and form tube-like structures when cultured on Matrigel. MESO cells differ from HOME cells based upon the expression of cytokeratins; their rapid proliferation in response to platelet-derived growth factor, and a change from an epitheloid to fibroblast-like morphology in response to tumour necrosis factor and epidermal

growth factor. Both HOME and MESO cells express tissue plasminogen activator and plasminogen activator inhibitor, form typical cobblestone monolayers, and are immunoreactive to von Willebrand Factor and Ulex europaeus I lectin^[23,24]. Urokinase activity is only expressed by MESO cells^[24].

OMENTAL MIGRATION

In 1896, Stichler placed snails into the peritoneal cavity of dogs and observed that they were walled off by omentum^[25]. A few years later (1910), in his text entitled "Introduction to Surgery", Rutherford Morrison referred to the omentum as "a special protective agency the abdominal policeman. It travels around the abdomen with considerable activity, and is attracted by some sort of inflammation in neighborhoods in which mischief is brewing". These observations lead to a study in dogs where it was revealed with fluoroscopy that there is no movement of the omentum following the insertion of enterobacteria into the peritoneal cavity^[25]. In 1926, Florey and others^[26] conducted a series of experiments and concluded that there was no intrinsic omental movement, but rather passive movement. This movement resulted from both the general activity and the position of the animal, and also from the peristalsis of the gut and the action of the diaphragm. This allowed the omentum to move about the abdominal cavity and adhere either foreign bodies or areas of inflammation. It was also noted that the omentum was limited in its ability to attach to foreign bodies in the pelvis and above the liver because it was unable to make physical contact with them^[27]. This led Sir Charles Sherrington^[28] to comment "that the doubled-up posture of the acute abdominal case is precisely that which will cause the omentum to be moved low down in the abdominal cavity".

OMENTAL FUNCTION

Foreign bodies

Most surgeons have observed the ability of the omentum to adhere to intra abdominal foreign bodies such as drains and catheters. In dogs it has been noted that following the placement of various types of drains into the peritoneal cavity, that within seven days all tubes are surrounded and occluded by omentum^[27]. This may lead to drainage problems in patients requiring long term catheters placed in the abdominal cavity, such as those for peritoneal dialysis. In such patients, removing the omentum has been found in a number of retrospective and uncontrolled studies to reduce the incidence of catheter blockage and to improve drainage^[28-32]. In addition, omentectomy does not appear to alter the peritoneal diffusion capacity^[33]. Because of this, it has been advocated that partial omentectomy is integral to the surgical technique of peritoneal catheter placement^[28-32]. In contrast,

Lewis *et al.*^[34] argued that the absolute risk reduction with omentectomy in preventing catheter blockage was only 0.18 in a series of 38 patients undergoing 66 catheter placements. It also remains to be clarified whether omentectomy has other deleterious side effects such as an increased incidence of peritonitis. None of the reported trials have adequately addressed this issue.

Peritonitis

The omentum performs a number of functions during episodes of peritonitis. The first of these is the rapid absorption and clearance of bacteria and foreign material from the peritoneal cavity. The omentum is the only site, other than the diaphragmatic stomata, that has a documented ability to absorb particles from the peritoneal cavity^[35]. But unlike the stomata, the omentum contains potent local effector mechanisms that are mediated by especially macrophages (and probably also B lymphocytes) contained within the milky spots. These macrophages appear to be the principal site for the phagocytosis of particles and bacteria from the peritoneal cavity^[13].

The second function of the omentum is to supply leukocytes to the peritoneal cavity. In experimental animals with peritonitis, the omentum appears to be the principal site by which firstly macrophages and then neutrophils migrate into the peritoneal cavity^[36,37]. The macrophages are derived from the milky spots which provide the correct microenvironment and growth factors for

macrophage proliferation and maturation. The correct microenvironment and growth factors for macrophage proliferation and maturation. The structure of the milky spots and their associated capillary structures aids this process (Figure 1). Because the mesothelium is absent over the milky spots, and the basement membrane is discontinuous, there is rapid exposure of the resident macrophages to intraperitoneal stimulants. This activates the macrophages which then demonstrate marked surface membrane activity and migrate through the stomata of the milky spots into the peritoneal cavity^[37].

The omentum also allows for the easy migration of neutrophils from the circulation^[7]. Due to the structure of the milky spots, there is direct exposure of the postcapillary venules to inflammatory stimuli from the peritoneal cavity^[38]. The neutrophils are then recruited from the circulation and extravasate via the post-capillary venules in the glomerular tufts into the milky spots and then via the mesothelial stomata into the peritoneal cavity. In one study on mice with peritonitis, the post capillary venules in the milky spots of the omentum were the only abdominal sites detected where plasma extravasation occurred, and the omental milky spots were the major route through which leukocytes migrated into the peritoneal cavity^[38]. In addition, the omentum was the only abdominal organ which showed an increase in blood flow during peritonitis^[38]. Milky spots do not seem to serve as a source of dendritic cells^[7].

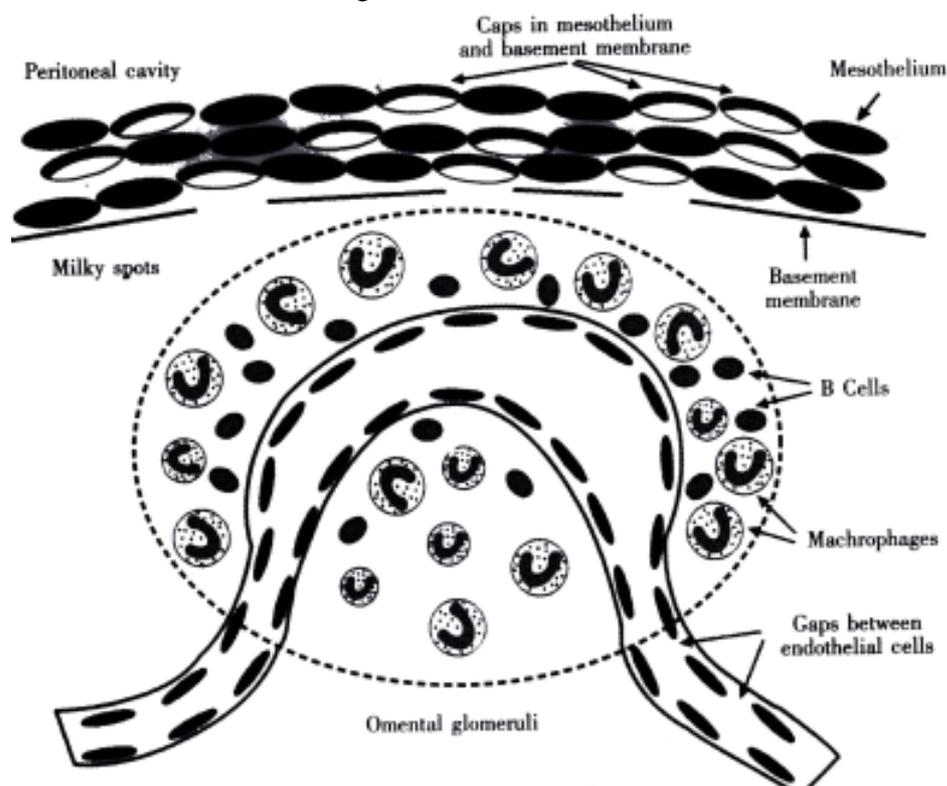


Figure 1 The basic structure of a milky spot in the omentum.

The third function of the omentum is to adhere to and attempt to seal off areas of contamination. The omentum can rapidly produce a layer of fibrin by which to adhere to the contaminated area at the point of contact. In the course of a few days, the fibrin begins to organise with the development of new blood vessels and fibroblasts. In the long term, if the host survives, the contaminated area will be walled off with collagen, and thereby forming dense adhesions^[46].

Removal of the omentum impairs peritoneal defence mechanisms^[3]. In experimental peritonitis, omentectomy has been found to reduce survival^[40], and influence a number of peritoneal defence mechanisms^[41]. In particular, there is a reduction in the total intra peritoneal cell counts, with macrophages being the most effected. There is also a reduction in neutrophil chemotaxis, although their phagocytic activity appears unchanged^[41]. In humans, omentectomy also appears to impair the peritoneal defence mechanisms^[42,43]. One retrospective analysis compared a group of 406 patients having omentectomy with proctocolectomy and ileoanal anastomosis with a group of 239 patients having a similar procedure without omentectomy^[42]. The patients were followed up for a mean of 4.3 years. The results showed that the omentectomy group had a significantly higher incidence of postoperative sepsis (4% vs 10%, $P < 0.01$) and sepsis requiring reoperation (3% vs 8%, $P < 0.01$) when compared with patients retaining their omentum. In addition, there was no difference in the incidence of partial or complete small bowel obstruction due to adhesions between the two groups.

Neovascularisation

It has long been recognised that the human omentum can promote angiogenic activity in adjacent structures to which it is applied. Indeed, lipid material obtained from the omentum has been found to induce angiogenesis in rabbit corneas after only a single injection. This angiogenic material obtained from the omentum is abundant in supply^[44]. Further evaluation of the factors involved in this process have found that the human omental microvascular endothelial cells (HOME cells) express the angiogenic peptide 'basic fibroblast growth factor'^[39]. This process of neovascularization allows the omentum to provide vascular support to adjacent tissues such as the gut and promote function and healing in ischaemic or inflamed tissue^[45,46]. Another example of the angiogenic activity of the omentum has been its ability to support splenic autotransplantations. Although the clinical practice of re implantation of splenic remnants following splenic injuries has largely been abandoned by surgeons, it is interesting

to note that such implants are supported by the omentum and function to a limited capacity^[47]. The omentum has also be found to be capable of supporting free structures such as the trachea, segments of intestine, sciatic nerve grafts. Such structure can then be used for reconstructive purposes^[48-50].

CLINICAL ISSUES

Primary pathology

In contrast to its numerous advantages, the omentum is rarely a site of primary pathologic change. There have been several case reports of primary omental torsion in both children and adults, and primary omental infarction in adults. Patients usually present with an acute abdomen and have localized signs of peritonism plus or minus a palpable mass. They are readily treated by partial omentectomy^[51-53]. In addition, both benign (lipoma, fibroma) and malignant (liposarcoma, fibrosarcoma, angiosarcoma) soft tissue tumours may occur in the omentum, although they are very rare.

Reconstruction

Surgeons have long exploited the unique structure and function of the omentum^[54]. In particular, its rich blood supply that supports a high absorptive capacity, its pronounced angiogenic activity which may support local tissues (and ischaemic tissues), its innate immune function, its ability to adhere to local structures, and finally its high concentration of 'tissue factor' which promotes haemostasis^[2,55]. In 1927, Perrotti^[56] used free and pedunculated flaps of omentum to enhance intestinal suture lines in dogs. Omentum has also been used: to close perforations in the gastro-intestinal tract; to reinforce gastro-intestinal anastomoses; to aid haemostasis during liver resections; to line the bed of hydatid cysts in the liver; as a pedicled graft to cover defects or to reconstruct areas from the chest wall to the perineum; to protect exposed carotid arteries; as free vascularized grafts in head and neck surgery; to repair bronchopleural fistula; and others^[54,57,58].

There are several reports of the use of an omental flap to reconstruct the mediastinum in patients with mediastinitis secondary to open heart surgery^[59-61]. These reports are retrospective and not adequately controlled. Nevertheless, they all comment that omental flaps are associated with fewer septic complications than pectoralis major flaps, and are associated with high rates of healing and lower mortality when compared with debridement. Similarly, there have been reports on the use of the omentum as a free transfer graft for the treatment of chronic ulcers, progressive hemifacial atrophies, and contused wounds. The transferred omentum appears to maintain its volume and nature under normal circumstances^[62].

Gastrointestinal anastomoses

There have been numerous studies evaluating the use of the omentum to support gastrointestinal anastomoses. In animals, there have been conflicting results as to whether reinforcing a compromised (i.e., ischaemic or technically inadequate) anastomosis with well vascularized omentum improves healing^[1,63,64]. However, the clinical relevance of studying anastomotic healing of grossly ischaemic segments of bowel is, I believe, questionable. In contrast, Carter *et al*^[65], evaluated the ability of omental wrapping to improve the healing of anastomoses using non compromised large intestine. They observed no improvement in fatal leak rates.

There has been one large clinical trial evaluating this issue in humans. This included 705 patients undergoing elective resections from the caecum to the midrectum with a mean age of 66 years. Patients were randomized after colectomy to undergo either omental reinforcing of the colonic anastomosis or no reinforcing. The intraoperative findings were similar between the two groups, except that there were significantly more septic operations performed in the control group. When comparing the omental reinforcement group with the controls, there was no significant difference in either anastomotic leakage (4.7% vs 5.2%) or deaths (4.9% vs 4.2%). The authors concluded that omental reinforcement of colorectal anastomosis was of no clear benefit *et al*^[66].

Neurosurgery

Placing the omentum on the brain surface by surgical transposition or transplantation has been found to result in the development of numerous neovascular connections between these two structures. This phenomenon occurs even in the absence of cerebral ischemia. In a series of 30 children with moyamoya disease, aged from 2 to 17 years, omental transplantation was used to improve vascularity in either the anterior or the posterior cerebral artery territory. All 19 patients treated with omental transplantation to the anterior cerebral artery and 11 (84.6%) of the 13 treated with omental transplantation to the posterior cerebral artery showed improvement in their neurological state^[67].

Vascular synthetic grafts

Synthetic vascular grafts lined with HOME cells appear to remain patent for longer periods^[23]. However, HOME cells remain difficult to extract and culture. In contrast, MESO cells can be readily harvested in large numbers from the omentum, and by culturing them in specific conditions their natural tendency to express tissue factor which is thrombogenic can be inhibited. Such cells are an

excellent alternative to HOME cells in seeding synthetic grafts^[68]. This technique has been used to line the luminal surface of small diameter prosthetic bypass grafts, thereby lowering the grafts thrombogenicity. These grafts were then implanted into the carotid artery of dogs and have been found not to develop neointimal hyperplasia or stenosis when compared with controls^[69].

Malignancy

The omentum has been observed to be a frequent site of metastatic disease for many malignancies. In animals, malignant cells inoculated into the peritoneal cavity preferentially infiltrate the milky spots in the omentum and grow into distinct metastatic^[70,71]. The omentum appears capable of supporting not only malignant cells in the milky spots but free intraperitoneal cells. It achieves this due to its intrinsic angiogenic properties. In animals, removing the omentum impacts on the survival of free intraperitoneal malignant cells and there by reduces the rate of local recurrence^[72,73]. Because of these observations, the omentum is frequently removed as part of resections for malignancies of various intra abdominal organs^[74].

Ovarian cancers, in particular, are characterized by their tendency to spread intraperitoneally and involve the omentum. Hence, there has evolved a general consensus that surgical management of ovarian cancer should include optimal cytoreduction^[75-78]. The minimum surgical requirements of this are to perform a total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. The apparent value of performing an omentectomy is that it provides staging information and selects patients for adjuvant chemotherapy^[79,80]. In addition, in patients with advanced disease, there appears to be a survival advantage in debulking tumour deposits^[81]. Nonetheless, with borderline ovarian tumours, omentectomy is also frequently advocated but the evidence to support this remains limited^[82].

The removal of the omentum in patients with ovarian cancer is not universal. In 1993, a United states national survey of the treatment of patients with ovarian cancer concluded that out of a total of 12, 316 patients with ovarian cancer, the requirements for what were defined as a minimum surgical resection were met in only around 60% of patients (bilateral salpingo-oophorectomy 67%; abdominally sterectomy 55%; and omentectomy, 59%)^[83]. Another analysis of omentectomy in patients with ovarian cancer found that the mean omentectomy size was only 203.5cm² (normal omentum = 792 cm²), and secondary ovarian cancer was present in 61%. Thus the optimal extent of omental resection and histological examination remains to be clearly defined^[80].

The omentum is intimately associated with the stomach and the gastric lymphatic drainage. Therefore, it is invariably removed as part of a curative resection for gastric cancer^[84,85]. Nonetheless, there is no clear evidence to provide guidance as to the extent of such a resection, and whether the entire omentum has to be removed^[86,87]. Pseudomyxoma peritonei is a rare neoplasm characterized by mucinous ascites and the mucinous involvement of peritoneal surfaces, omentum and bowel loops. Usually pseudomyxoma peritonei is associated with benign or malignant mucinous tumor of the appendix or ovary, and cytoreductive resections, including omentectomy, are advocated as the treatment of choice^[88,89].

CONCLUSION

Our concept of the omentum as an abdominal policeman has obviously evolved since the days of Rutherford Morrison. We now understand that it occupies a central position in the peritoneal defence mechanisms. It achieves this by virtue of its innate immune function, its high absorptive capacity, and its ability to adhere to adjacent structures to both seal off gastrointestinal defects and promote their healing with its pronounced angiogenic activity. Because of these attributes, surgeons have utilised the omentum in a variety of settings, from reconstructing soft tissue defects, to supporting tissues to promote healing. In managing patients with intra abdominal malignancies, the role of omentectomy requires further evaluation to determine whether it is associated with a clear survival advantage, and to evaluate how much needs to be removed. In conclusion, the omentum needs to be viewed as an important intra abdominal organ and hence careful consideration needs to be given before it is removed.

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Etiology and evaluation of diarrhea in AIDS: a global perspective at the millennium

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INTRODUCTION

It has now been almost 20 years since the initial descriptions of a heretofore unrecognized disorder afflicting homosexual men and manifesting as *Pneumocystis carinii* pneumonia and Kaposi's sarcoma. With the identification of the human immunodeficiency virus (HIV) as the etiology of this syndrome, there has been exponential growth in our understanding of this devastating immune disorder. During the first decade of the acquired immunodeficiency syndrome (AIDS), there was an explosion of cases in the United States and Africa. Subsequently, there have been successive waves of the epidemic throughout the world with cases now documented in essentially all countries. Elucidation of the infectious etiology and routes of transmission of HIV have provided opportunities for population-based preventative measures. Although these measures have been highly successful in many developed countries, the number of new cases worldwide still remains staggering. It is estimated that approximately 1 million people in the United States are HIV-infected. As of 1998 alone, more than 33 million people were infected with the AIDS virus worldwide with almost 6 million new cases occurring in 1998, representing approximately 16000 new cases each day, the majority of these occurring in developing countries^[1]. By the year 2000, it is estimated that there will be almost 40 million HIV-infected people throughout the world. The epidemic currently inflicts a heavy human and economic toll on sub-Saharan Africa, and although the number of cases is currently small, the Russian and China could potentially be poised for the next

wave of the epidemic. Given their present rate of infection, the Indian subcontinent and southeast Asia will likely bear the greatest burden of the disease early in the 21st century.

Since the initial descriptions, it was recognized that chronic diarrhea was one of the most common and debilitating complications of HIV infection, occurring in about 50% of patients in North America and in up to 100% of patients residing in developing countries^[2-4]. Chronic diarrhea is important in these patients because it results in significant morbidity and mortality, reduced quality of life, and higher health care costs^[5]. In the early stages of immunodeficiency, HIV-infected patients are susceptible to the same enteric pathogens that cause diarrhea in the immunocompetent host. However, as immunodeficiency progresses, these patients become susceptible to a wide variety of opportunistic infections (OI) many of which have a predilection for the gut^[1-3,5]. In general, most opportunistic disorders are not observed until the CD4 count falls below 200×10^6 cells/L, and usually $<100 \times 10^6$ cells/L^[4,6].

Across all continents, the introduction and rapid implementation of potent combination antiretroviral therapy, termed highly active antiretroviral therapy (HAART), has profoundly affected the incidence and complications of AIDS including diarrhea. Cohort studies have clearly shown a fall in AIDS-related OI, hospital admissions as well as mortality rate of HIV-infected patients which began several years prior to 1996, the time when protease inhibitors were released^[7-10]. These changes which occurred prior to protease inhibitors may be the result of combination therapy with reverse transcriptase inhibitors which have modest efficacy in reducing HIV RNA levels^[10]. Since 1996, however, there has been even more substantial improvement in these outcomes. Although expensive, HAART appears to be cost-effective given the reduction in the number of hospital admissions and AIDS-related complications^[11]. When effective, these drug regimens markedly and rapidly reduce HIV RNA while the CD4 lymphocyte count rises^[12]. Studies which examine viral burden and architecture of lymph nodes have also demonstrated salutary effects of HAART^[13]. Tangible evidence of immune reconstitution is that prophylaxis against *Pneumocystis carinii* pneumonia may be discontinued in patients who have an excellent

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virologic response to HAART and a sustained rise in CD4 count to greater than $200 \times 10^6/L$ ^[14]. However, patients who have received multiple antiretroviral agents but who have persistently high level HIV viremia will not have such profound immunologic and virological effects with these new regimens due to drug-resistance^[15].

As the immune status of HIV-infected patients improves with HAART, they become less prone to opportunistic disorders and the infectious etiologies of diarrhea parallel that observed in immunocompetent patients. Nevertheless, several antiretroviral agents, particularly the protease inhibitors, can themselves cause diarrhea^[16]. Thus because of HAART, the number of patients in whom OI are identified as causes of diarrhea has fallen while the frequency of unexplained and drug-induced diarrhea appears to be rising^[17].

The identification of enteric pathogens in patients with diarrhea is important since a number of efficacious antimicrobial therapies are available^[18]. Also, the use of HAART as primary "therapy" of OI represents a new paradigm for management; when effective in raising the CD4 count and lowering HIV RNA levels, OI may completely remit with HAART alone^[19-20]. To best identify these OI, various approaches for the work-up of diarrhea in AIDS have been proposed^[6,21-25]. However, the diagnostic methods often differ between studies making comparisons difficult. In addition, most of these diagnostic algorithms were derived in developed countries where endoscopy and mucosal biopsy is widely available, and are therefore not universally applicable.

As we enter the new millennium, it seems appropriate to highlight changing concepts related to the etiology and evaluation of diarrhea in HIV-infected patients. Given that the spectrum of pathogens differs geographically and the resources for diagnostic evaluation vary widely, contrasts will be drawn based on these epidemiological and economic differences where applicable and where data is available to draw meaningful distinctions.

ETIOLOGY

The infectious causes of diarrhea in AIDS are extensive (Table 1), and the most important etiologic agents will be briefly discussed individually focusing on geographic differences in prevalence. Worldwide, the most common causes of diarrhea in HIV-infected patients are enteric bacteria including *Shigella flexneri*, *Salmonella enteritidis*, and *Campylobacter jejuni*. In contrast to the normal host, these bacteria have been identified in stool from patients with chronic diarrhea^[23,26,29]. *Clostridium difficile* is a prevalent pathogen typically reported in developed countries, whereas tuberculosis is a common complication of AIDS in

developing countries^[27,28]. Cryptosporidia is the most frequently identified parasitic cause of diarrhea throughout the world^[23,26,29]. Cytomegalovirus (CMV), cryptosporidiosis, microsporidia and Mycobacterium avium complex (MAC) the classic gastrointestinal OI of AIDS become important pathogens when immunodeficiency is advanced. AIDS-related neoplasms such as Kaposi's sarcoma or lymphoma and fungi rarely cause diarrhea. As noted above, medications, usually protease inhibitors, have recently become important causes of diarrhea^[16].

Table 1 Enteric pathogens in AIDS

Viruses

Cytomegalovirus
Astrovirus
Picornavirus
Coronavirus
Rotavirus
Herpesvirus
Adenovirus
Small round virus
HIV

Bacteria and mycobacteria

Salmonella
Shigella
Campylobacter
Clostridium difficile
Treponema pallidum
Spirochaetes
Neisseria gonorrhoeae
Vibrio cholerae
Pseudomonas ?
Staphylococcus aureus
Mycobacterium avium-complex
Mycobacterium tuberculosis

Parasites

Giardia lamblia
Entamoeba histolytica
Microsporidia
-*Enterocytozoon bienersi*
-*Encephalocytozoon intestinalis*
Cyclospora
Cryptosporidium
Isospora belli
Blastocystis hominis?

Fungi

Histoplasma
Candida albicans

Viral

CMV is one of the most common OI in AIDS patients from developed countries presenting late in the course of HIV infection; the median CD4 count in most reports is $<50 \times 10^6$ cells/L and the CD4 count is $<100 \times 10^6$ cells/L in almost all patients. Among patients with AIDS and diarrhea from developed countries, CMV has been identified in gastrointestinal biopsies in as many as 45% of cases^[22,30]. In contrast, one study of rectal biopsies from 29 African patients with chronic diarrhea and abnormal appearing rectal mucosa did not identify any patients histologically with CMV^[31]. It is unclear whether CMV is less commonly reported

from developing countries due to the infrequent use of endoscopy or true differences in infection. Another explanation for geographic disparities in the prevalence of CMV disease is the fact that AIDS patients from developing countries often die before immunodeficiency becomes so advanced that CMV manifests. Other viruses have been identified in stool from AIDS patients with chronic diarrhea including adenovirus, rotavirus, astrovirus, picobirna virus and coronavirus^[32,33]; given the lack of specific therapy, the clinical relevance of these viruses is unclear (Table 2). There are also reports that HIV itself can be isolated from and identified in enterocytes and colonic cells, and that HIV may have a direct cytopathic effect on the intestinal mucosa causing an enteropathy.

Table 2 Laboratory tests used to investigate diarrhea in HIV -infected patients

Stool studies
Stool cultures (<i>Salmonella</i> , <i>Shigella</i> , <i>Campylobacter</i>)
Toxin (<i>Clostridium difficile</i>)
Stool for ova and parasites (<i>Giardia lamblia</i> , <i>Entamoeba histolytica</i>) (using saline, iodine, trichrome and acid-fast stains)
Stool stains:
a. Modified Kinyoun acid-fast (<i>Cryptosporidium</i> and <i>Isospora belli</i>)
b. Concentrated stool (Zinc sulfate, Shethersucrose flotation)
Fecal fat
Giardia antigen
Blood studies
CD4 cell count
White blood cell and differential
Creatinine, electrolytes
Liver function tests
Total albumin
Blood cultures (<i>Mycobacterium avium</i> -complex)
Tissue and fluids
Duodenal aspirate (<i>Giardia lamblia</i> , microsporidia)
Biopsy: duodenum, jejunum, ileum, colon, rectum
Biopsy stains:
a. Hematoxylin-eosin
b. Giemsa or methenamine silver (fungi)
c. Methylene blue-azure II-basic fuchsin (microsporidia)
d. Fite (mycobacteria)
Immunohistochemical stains (Cytomegalovirus)
In-situ hybridization (CMV)
DNA amplification (CMV)
Electron microscopy for cryptosporidia, adenovirus
Touch preparation
Culture of colonic mucosal biopsy
a. Cytomegalovirus
b. <i>Herpes simplex</i> virus
c. Bacteria

Bacterial and mycobacterial

The spectrum of bacterial pathogens causing diarrhea in HIV-infected patients is similar to the normal host. When comparing studies from throughout the world, no important differences, with the exception of *C. difficile*, in the spectrum of bacterial infections are apparent. The most frequently identified pathogens are *Campylobacter*, *Salmonella*, *Shigella*^[23,26,34]. *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Aeromonas hydrophila* have also been associated with severe enterocolitis in HIV-infected patients^[35]. *C. difficile* colitis is a frequent cause of diarrhea in

HIV-infected patients from developed countries likely because of these patient's frequent exposure to antimicrobials and requirement for hospitalization^[36]. Small bowel bacterial overgrowth does not appear to be common in AIDS patients with chronic diarrhea^[37].

Mycobacterium avium complex (MAC) is a common pathogen in AIDS patients with advanced immunosuppression; up to 39% of patients from the USA may develop this infection when the CD4 count remains $<10 \times 10^6$ cells/L^[38]. MAC infection has been less frequently reported in AIDS patients with diarrhea from developing countries^[27]. This apparent infrequency of MAC in third world countries is likely multifactorial and may relate to the fact that this infection is usually diagnosed by blood cultures or, like CMV, that patients from these countries die from other causes such as tuberculosis (TB) prior to developing such advanced immunosuppression required for MAC infection. The presentation of TB in AIDS is well recognized to be different and often extrapulmonary. Autopsy studies suggest that the gastrointestinal tract is commonly involved in disseminated TB and such involvement can result in gastrointestinal symptoms including diarrhea^[28]. In contrast to MAC, TB can manifest with less advanced immune deficiency, and CD4 cell counts may exceed 200×10^6 cells/L.

Parasites

Among the protozoa, *Cryptosporidium parvum* is the most frequently identified parasite causing chronic diarrhea in AIDS even in developed countries^[4,29,39]. Cryptosporidia is a very important cause of diarrhea in developing countries where prevalence rates of 20% are commonly reported^[40]. This high prevalence perhaps is due to the fact that contaminated water is a common source for this pathogen^[41]. Although a cause of acute diarrhea, cryptosporidiosis is typically identified in HIV-infected patients with chronic diarrhea. Microsporidia (*Enterocytozoon bienersi* and *Encephalitozoon intestinalis*) are now recognized as important causes of diarrhea in AIDS patients^[39-42]. In some studies of HIV-infected patients with chronic diarrhea, microsporidia are the most commonly identified pathogens. In a study from New York^[43], microsporidia were found in 39% of AIDS patients undergoing gastrointestinal evaluation for diarrhea; however, in other areas throughout the world and even the USA^[44], this pathogen is rarely identified even in patients undergoing small bowel biopsy. These geographic differences, both globally and in the same country, in the prevalence of microsporidiosis are unexplained. *Isospora belli* is a rare gastrointestinal pathogen in HIV-infected in North America, whereas it is endemic in some developing countries such as Haiti^[40]. The infrequency observed in

developed countries has been attributed to widespread *Pneumocystis carinii* prophylaxis with trimethoprim-sulfamethoxazole which is highly effective in the treatment of Isospora. Cyclospora have also been identified in HIV-infected patients with chronic diarrhea most often from developing countries^[45]. Despite a high frequency of stool carriage in asymptomatic homosexual men, amebic dysentery or invasive amebic disease (ameboma or liver abscess) have been rarely reported in HIV-infected patients, even from developing countries^[46]. Stool carriage of ameba in HIV-infected patients is not only limited to non-pathogenic strains of ameba such as *E. dispar*, *E. hartmanii* and *E. coli*, but also to non-pathogenic *E. histolytica*^[47]. Giardiasis is neither more common nor more severe among patients with AIDS than in the non-immunocompromised host and geographic differences in prevalence have not been reported^[4]. Helminths are rare causes of diarrheal disease but have been recognized most often in third world countries.

CLINICAL PRESENTATION

An overview

When evaluating the HIV-infected patient with diarrhea, one should first attempt to determine the site of origin of the diarrhea, i.e., small bowel ("enteritis") or colon ("colitis"), and the clinical history alone may be helpful^[48]. Not only will localizing the site of origin of diarrhea direct the diagnostic evaluation, but most pathogens have predilections for specific organs. For example, microsporidia and *Giardia* result in small bowel disease whereas bacteria cause colonic disease. Enteritis is classically manifested as large volume (often >2 L/d) watery stools, often associated with dehydration, electrolyte disturbances, malabsorption, and weight loss. Abdominal pain, when present, is usually crampy and periumbilical in location. Symptoms such as nausea, vomiting, bloating, distention, and borborygmi are also commonly associated with a small bowel diarrhea. In contrast, colitis is characterized by frequent, small volume stools, which may contain mucus, pus and/or blood, and is often accompanied by "proctitis" symptoms tenesmus, frequency, urgency, dyschezia and proctalgia. Abdominal pain is typical for colitis and tends to be localized to the lower abdomen. When colitis is severe, abdominal pain can be marked suggesting an acute abdomen. Fever is common with a systemic infection (e.g. MAC) or bacterial colitis. A fundoscopic examination may demonstrate retinitis consistent with CMV.

The value of clinical parameters for tailoring the work-up of diarrhea remains controversial. Wilcox *et al*^[48] demonstrated that the clinical presentation and severity of immunodeficiency were useful in predicting which patients with negative

stool tests may benefit from endoscopic examination. In this study^[48], patients with no identifiable pathogen after endoscopic examination had less systemic symptoms (weight loss) and a higher CD4 count. Indeed, diarrhea remained unexplained in patients who had not lost weight, and no OI was found in those patients with a CD4 > 50×10⁶ cells/L. Connolly *et al*^[49] also identified factors in a similar patient population which appeared to predict the presence of a gastrointestinal infection by endoscopic evaluation including weight loss^[49]. In a later study from the same group, however, Blanshard *et al*^[23] questioned the usefulness of clinical parameters. That study^[23] concluded that the presenting clinical features and hematological and biochemical parameters could predict neither the presence nor site of pathogens. Although there were significant differences in CD4 counts, stool volumes, and weight loss between the patients with and without an identified pathogen, there was such a large overlap that these clinical features could not be reliably used to identify those patients most likely to have a pathogen^[23]. The only presenting symptom and physical finding that was statistically significantly different between the two groups was the presence of abdominal pain, and this was most common in patients with CMV. Surprisingly, weight loss and malabsorption did not correlate with the presence of small bowel pathogens.

In contrast to the clinical presentation alone, however, all studies support the value of the CD4 count in stratifying the risk for opportunistic disorders particularly for patients with no prior AIDS-defining illness^[6,23,26,50]. When blood testing is not widely available, surrogate markers of severe immunocompromise may be helpful to stratify risk including the presence of thrush, oral hairy leukoplakia, or the wasting syndrome. In summary, although all studies have not come to similar conclusions, history and physical examination remain an important part of the assessment of the patient's general condition and hydration status and may help prioritize the evaluation.

CLINICAL MANIFESTATIONS BY ETIOLOGY

Viral

CMV infection of the colon characteristically presents with chronic watery diarrhea, abdominal pain, wasting, anorexia, weight loss^[51], and abdominal pain is an important clue to the diagnosis^[23]. Fever is not universal. Small bowel infection with CMV is far less common than colonic disease. The diarrheal stools may be semiformal or formed and may be accompanied by gross bleeding and fecal leukocytes. Lower gastrointestinal hemorrhage without diarrhea may be the initial manifestation and results from either severe colitis or isolated well circumscribed ulcers^[52]. When the

distal colorectum is involved, symptoms of proctitis are often reported. Toxic megacolon, intestinal perforation, mass lesions and colonic stricture have also been observed as complications of CMV enterocolitis^[53]. Localized or diffuse pain may be elicited on abdominal examination. Other physical findings are non-specific, and will primarily reflect CMV-related complications such as dehydration, perforation (acute abdomen), gas trointestinal bleeding, or altered vision (retinitis).

The most common colonic manifestation of HSV in HIV-infected patients is distal proctitis, which may be misinterpreted as diarrhea. If the disease extends to the proximal colon (very rare), proctocolitis with hematochezia and diarrhea may result^[53]. Other viruses such as astrovirus, adenovirus, picobirnavirus have been associated with chronic watery diarrhea^[32,33].

Bacteria

Although bacterial enterocolitis can occur at any stage of immunodeficiency, unusual presentations of these enteric bacteria became apparent early in the AIDS epidemic where *Salmonellasp.*^[54] and *Campylobacter sp. bacteremia*^[53] were reported as initial manifestations of AIDS. In general, however, the clinical presentation of these organisms in AIDS is similar to immunocompetent patients except that diarrhea may be chronic. Bacterial enterocolitis usually manifests as an acute diarrheal illness (less than 2 weeks duration). *Salmonella gastro enteritis* more commonly presents with watery diarrhea, abdominal pain, fever, nausea and vomiting but can manifest as an enteric fever. *Shigella* and *Campylobacter* usually present as a dysentery with the classic "colitis" symptoms mucopurulent bloody diarrhea, tenesmus and fever. Lower abdominal pain and fever may be prominent, while nausea and vomiting are infrequent. Physical findings include fever, tachycardia and abdominal pain which may be severe. Digital rectal examination may demonstrate frank blood or pus. Initial experience suggested that the clinical presentation of *C. difficile*-colitis was different in the HIV-infected patient^[55], but prospective studies have shown no differences as compared to non-immunocompromised patients^[56]. *C. difficile* can present fulminantly without diarrhea with clinical signs of peritonitis or even ascites^[53].

The most common manifestations of MAC infection are fever, wasting, chronic diarrhea, abdominal pain, night sweats and intestinal malabsorption^[4]. Frank colitis or hematochezia, which may be massive, are both rare presentations of gastrointestinal MAC infection^[53]. Although gastrointestinal tuberculosis may be symptomatic, the clinical picture is often dominated by fever and wasting and/or pulmonary disease^[27]. It is extraordinarily unusual for gastrointestinal TB to

present as chronic diarrheal illness. Most commonly, gastrointestinal TB is identified incidentally at the time of autopsy in patients with disseminated disease^[28].

Parasites

Cryptosporidia, microsporidia, cyclospora and *Isospora belli* are associated with chronic, watery diarrhea, dehydration, malabsorption and weight loss- features typical for a severe small bowel diarrhea. Cryptosporidiosis is more severe in patients with marked immunosuppression, but is generally self-limited if the CD4 count is $\geq 200 \times 10^6$ cells/L^[6]. The disease may be particularly devastating in developing countries. Overall, diarrhea due to microsporidiosis tends to be less severe than with cryptosporidia; weight loss is variable. Giardiasis characteristically presents with chronic diarrhea, abdominal bloating, borborygmi and intermittent abdominal pain.

TOOLS FOR INVESTIGATION

Perhaps the most important assessment in the evaluation of diarrhea in HIV-infected patients is the CD4 count, because the absolute value reflects the *patient's* stage of immunodeficiency thereby stratifying the risk for OI. As noted above, clinical surrogate markers, when present, can also be used to gauge the degree of immunodeficiency. Since most opportunistic disorders are not observed until the CD4 count falls below 200×10^6 cells/L, and usually $< 100 \times 10^6$ cells/L, the method and extent of evaluation will depend on the absolute CD4 count. For example, if the CD4 count is higher than 100×10^6 cells/L, evaluation for MAC is not necessary given that MAC infection occurs primarily when the CD4 count is $< 50 \times 10^6$ /L. Likewise, the yield of endoscopy for OI in a patient with a CD4 count $> 200 \times 10^6$ /L will be very low^[48,50].

Numerous stool culture media, *histopathological* stains of mucosal biopsies and molecular tools (e.g. polymerase chain reaction) have been employed to identify infectious agents as the cause of diarrhea in HIV-infected patients^[57-60]. The list of diagnostic tests to evaluate diarrhea in HIV-infected patients is extensive (Table 3); however, rarely will it be necessary to use more than a few of these tests. Limited resources will also play an important role in defining the extent of the diagnostic evaluation. Local epidemiology of infections should also dictate the approach.

Stool studies

Stool staining for fecal leukocytes with methylene blue is useful as their presence suggests an inflammatory origin of the diarrhea (colitis) and may help predict the presence of a colonic pathogen and yield of proctoscopy^[48]. Stool cultures for *Salmonella*, *Shigella* and *Campylobacter* should be

routinely submitted, even in patients with chronic diarrhea. A-C. difficile toxin screen is appropriate in developed countries and those at risk. Blanshard *et al*^[23] has shown that the yield of stool cultures increases with multiple stool samples. They found that the yield of one stool culture was 18%, 3 stool cultures 38.7%, and 6 stool cultures 46.7%^[23]. Culturing of colonic mucosal biopsies for enteric bacterial pathogens does not significantly increase the diagnostic yield^[61]. Additional stool tests should include ova and parasite examination, a modified acid fast stain to evaluate for cryptosporidia, Isospora and cyclospora and appropriate staining for microsporidia. Giardiasis may be difficult to detect on routine stool ova and parasite examination; a stool antigen test for Giardia is now available^[62]. As with any diagnostic strategy, the tests ordered should reflect the likely cause of infection, and in the HIV-infected patient, the history and immune status are most helpful.

Table 3 Drugs used in the therapy of diarrhea in AIDS

Condition	Treatment	Dosage	Duration
Cytomegalovirus	Ganciclovir	5mg/kg,2/d	2-4wk
	Foscarnet	90mg/kg,2/d	2-4wk
<i>Herpes simplex</i>	Acyclovir	400-800mg,14d (5/d)	
	Foscarnet	60-90mg/kg,2/d	14d
	Valacyclovir	500mg,3/d	14d
Cryptosporidia	Famciclovir	500mg,2/d	7-10d
	No definite therapy known		
	Paromomycin	25-35mg/kg,3/d	14-28d
	Spiramycin	1g,3/d	14-28d
	Azithromycin	900mg,1/d	14-28d
Microsporidia	Letrazuril	50-100mg,1/d	14-28d
	Bovine colostrum	50g,1/d	14-28d
	Albendazole	400mg,2/d	14-28d
<i>Septata intestinalis</i>	No definite therapy known		
<i>Enterocystozoon</i>	Albendazole	400mg,2/d	14-28d
	Metronidazole	500mg,3/d	14-28d
<i>Isospora belli</i>	TMP-SMX DS	1 tab,4/d	10-14d
Cyclospora	TMP-SMX DS	1 tab,4/d	10-14d
<i>Giardia lamblia</i>	Metronidazole	500mg,3/d	10-14d
<i>Entamoeba histolytica</i>	Metronidazole	750mg,3/d	10-14d
	Iodoquinol	650mg,3/d	10-14d
MAC	Clarithromycin	500mg,2/d	4wk
<i>Clostridium difficile</i>	EMB, Cipro, RFP		
	Metronidazole	500mg,3/d	10-14d
Salmonella	Vancomycin	250mg,4/d	10-14d
	Ciprofloxacin	500mg,2/d	10-14d
Shigella	TMP-SMX DS	1 tab,4/d	10-14d
	Ciprofloxacin	500mg,2/d	10-14d
<i>Campylobacter</i>	Ampicillin	500mg,4/d	10-14d
	Ciprofloxacin	500mg,2/d	10-14d
	Erythromycin	500mg,4/d	7-14d

RFP=Rifampicin, EMB=Etrhambutol, TMP-SMX=trimethoprim sulphamethoxazole, DS=double strength.

Blood tests and serology

Serologic studies for CMV antibodies are not diagnostically helpful in AIDS given the high positivity rate in these patients. The role of CMV

antigenemia as a diagnostic test or predictor of subsequent disease of the gut has been best studied in the transplantation setting where high level CMV antigenemia seems to be predictive of subsequent clinical disease^[63]. In addition, when disease is active, CMV antigenemia is also usually present. Although CMV antigenemia is common in HIV-infected patients, particularly in those with advanced immunodeficiency, the diagnostic value for the symptomatic patient as well as the predictive value for subsequent gastrointestinal disease remains unknown. Positive cultures of blood or bone marrow biopsy establish the diagnosis of disseminated MAC, but do not prove active gastrointestinal involvement. Entamoeba antibody titer (ELISA) is only useful for the evaluation of invasive amebiasis (e.g. liver abscess) and not for the diagnosis of amebic dysentery.

Radiographic studies

Plain abdominal X-rays are not generally helpful for the evaluation of chronic diarrhea. With severe colitis, however, colonic dilation and "thumbprinting" of the mucosa may be observed, and computed tomography (CT) may reveal circumferential thickening of the wall of the colon with inflammatory infiltration of the mesentery. Barium studies including small bowel follow-through or barium enema play no role in the evaluation of AIDS-related diarrhea, but may be useful if Crohn's disease is considered. If barium studies are clinically indicated, stool studies must be obtained first as barium interferes with the stool microscopic examination.

Histology

Histology plays a primary role in the diagnosis of most mycobacterial, viral and fungal etiologies of diarrhea. Parasitic infections may also be diagnosed histologically but are typically identified first on stool studies. Cytology, culture and *in situ* hybridization are also reliable diagnostic techniques. Immunohistochemical stains of mucosal biopsies to confirm various viral infections may be required, but in most cases, their use does not offer an advantage over conventional light microscopy when reviewed by an experienced pathologist^[58]. Viral culture of biopsy specimens are generally less sensitive and specific than multiple mucosal biopsies but when positive may help support the histologic findings^[64].

Although electron microscopy of small bowel biopsies is considered the gold standard for the diagnosis of microsporidiosis, recent studies have shown hematoxylin and eosin, Gram stain, Brown-Brenn, Giemsa, or modified trichrome staining of small bowel biopsies to have sensitivities of 77%-83% with specificities approaching 100%^[53,59,60]. In one study^[60], electron microscopy only resulted in the detection of an additional 4% of cases. These

results suggest that the value of electron microscopy over conventional staining techniques for the detection of parasitic pathogens is negligible especially microsporidia.

Endoscopy

A number of studies have investigated the yield and impact of upper and lower endoscopy for the evaluation of diarrhea in AIDS (Tables 4-6). An obvious advantage of endoscopy is that it permits direct visualization of the gut and opportunity for mucosal biopsy. Conversely, the procedure is invasive, expensive and not readily available worldwide. The diagnostic yield of colonoscopy in HIV-infected patients with chronic diarrhea and negative stool studies ranges from 27% to 37%, with CMV being the most common etiology identified (Table 4). Since CMV usually involves the distal colon, sigmoidoscopy with biopsy may represent a sufficient work-up^[23,25,48,52]. Nevertheless, in 13%-39% of patients, CMV infection can be limited to the right colon^[24,51]. Therefore, if CMV is suspected as the cause of diarrhea and the distal colon is normal at sigmoidoscopy, a full colonoscopy is warranted. It is still not clear whether colonoscopy has a higher yield than flexible sigmoidoscopy for the detection of clinically relevant pathogens in addition to CMV^[24]. This is an important issue worldwide as a limited evaluation, which includes a rectal biopsy obtained by proctoscopy or sigmoidoscopy, is easier to acquire and more readily available than colonoscopy.

The value of upper endoscopy (EGD) for the evaluation of chronic diarrhea has also been demonstrated (Table 5). As with colonoscopy, most studies focus on the diagnostic yield in patients with negative stool tests. The most common organisms detected by EGD and biopsy are cryptosporidia and microsporidia comprising > 90% of the identified pathogens. Wilcox *et al*^[48] obtained small bowel biopsies in 48 HIV-infected patients with chronic diarrhea and nondiagnostic stool tests. In this study^[48], a potential pathogen was detected in 21 patients (44%) with 7 patients (17%) having small bowel disease; microsporidia was most commonly found. In this study, ileal biopsies were not routinely obtained at colonoscopy. In another study patients with previous negative work-up by stool studies underwent duodenal, ileal, and colonic biopsies^[50]; cryptosporidia were detected in 53% of biopsies. In contrast to these studies^[48,50], Blanshard *et al*^[23] published their experience using endoscopy (EGD and flexible sigmoidoscopy) with biopsy in conjunction with stool testing as the initial evaluation in patients with chronic diarrhea. The usefulness of first-line endoscopy was demonstrated by the high yield (83%) of potential pathogens identified during the initial evaluation. Although this more aggressive

approach resulted in a high yield, it may not be applicable to most clinical practices. More recently, Kearney *et al*^[25] studied 79 patients with chronic diarrhea and negative stool studies. The authors examined the yield of an endoscopic work-up (EGD and colonoscopy which included biopsies of the terminal ileum) including comparisons of proximal and distal colon biopsies. An infection was diagnosed in 22 of 79 patients (28%); biopsy of the left colon yielded an enteric pathogen in 17 of these 22 patients and in 100% of patients (15 of 15) with CMV colitis. Combined colonic and terminal ileal biopsies missed no pathogens. Duodenal biopsies yielded no additional pathogens beyond those identified by colonoscopy and terminal ileal biopsy. The authors concluded that for patients with CD4 counts less than 100×10⁶ cells/L and negative stool studies, flexible sigmoidoscopy with biopsy was a sufficient initial endoscopic evaluation, and if colonoscopy was performed, biopsies of the terminal ileum should be obtained.

Table 4 Yield of colonoscopy with biopsy in HIV infected patients with chronic diarrhea

Ref	No. pts.	Diagnosis	Most common dx (No.pts)	Distal	Proximal
Connolly*	81	34 (42)	CMV (12)	32	+
Wilcox	48	13 (27)	CMV (11)	12	1
Kearney	79	22 (100) ^o	CMV (15)	17	1
Bini	317	116 (37)	CMV (75)	60 (55)	33 (30)

Diagnosis expressed as number of patients (%). Numbers under distal and proximal represent number of patients.

*patients with Cryptosporidium on stool testing were excluded, some patients had more than one diagnosis; ^oCMV and KS in one patient each; ^obiopsy of terminal ileum at time of colonoscopy; pts: patients; dx:disease.

Three patients had diagnosis with biopsy at terminal ileum

Table 5 Yield of upper Endoscopy in HIV-infected patients with chronic diarrhea

References	No. pts.	Diagnosis n(%)	Most common dxs
Connolly	33	2 (36)	Crypto 2 CMV 4
Wilcox	48	9 (19)	Micro 7, crypto 1 CMV 1
Bown	56	15 (26)	Crypto 6, micro 5
Kearney	79	9 (11)	Micro 5
Bini	442	123 (28)	Micro 54 (12%)

Crypto: cryptosporidium; Micro: microsporidium; Dxs:diseases

Table 6 Yield of extensive work up of HIV-infected patients with negative stool studies

Ref	No. pts.	Diagnosis n(%)	Most common diagnosis
Wilcox	48	21 (44)	CMV 9 Micro 7
Kearney	79	22 (28)	CMV 15 Micro 5
Connolly	33	12 (36)	Crypto 5 CMV 4

Crypto: cryptosporidium; Micro: microsporidium.

There remains some disagreement on the most cost-effective approach for the evaluation of diarrhea in AIDS, but the published data does provide the evidence to propose a reasonable diagnostic algorithm. Various authors^[2,4], and more recently the American Gastroenterology Association^[6], have proposed a stepwise approach for the evaluation of chronic diarrhea in AIDS. The first step includes at least three sets of stool samples to investigate for bacterial pathogens (*Salmonella*, *Campylobacter* and *Shigella*) and parasites (cryptosporidia and microsporidia). Testing for *C. difficile* was recommended for patients at risk for this infection (use of antibiotics, recent hospitalization). In HIV-infected patients with a CD4-count $< 100 \times 10^6$ cells/L and fever, blood cultures should be drawn to investigate for MAC. If these tests are negative, the second step is to perform flexible sigmoidoscopy. Colonoscopy was recommended only in selected patients (i.e. patients with marked immunosuppression and clinical manifestations of dehydration, wasting, diffuse abdominal pain)^[6]. If no etiology was found after steps one and two, EGD with biopsy of the duodenum was recommended to exclude microsporidia and cryptosporidia^[6].

Although the available literature demonstrates that both upper and lower endoscopy play a crucial role in the work-up of HIV-patients with chronic diarrhea, the main question is at what point during the work-up should endoscopy be undertaken and should both lower and upper endoscopy be performed in the same session. A stepwise approach seems reasonable when evaluating these patients, but it may not be ideal given the high diagnostic yield when performing stool studies combined with upper and lower endoscopy. Nevertheless, this aggressive approach^[23] may not be widely applicable given the fact that in routine clinical practice stool studies are relatively inexpensive, noninvasive, and often positive. We still believe stool studies should generally be obtained before endoscopic evaluation. The predictive value of quantitating fecal fat and measuring d-xylose absorption for intestinal disease in HIV-infected patients with chronic diarrhea has not been established.

When all tests are negative, symptomatic therapy with antidiarrheal agents is frequently successful in alleviating symptoms^[66]. Occasionally, despite antimicrobial treatment of an identified pathogen, the diarrhea may continue. Under this circumstance, further investigations are warranted as the patient may be co-infected with other pathogens or have another cause for the diarrhea. Discontinuation of all antiretroviral agents may help determine if a drug-induced diarrhea is causative.

CONCLUSIONS AND RECOMMENDATIONS

A thorough history and physical examination are essential to evaluate the patients' general condition and will help focus the work-up of diarrhea in

AIDS. In addition, routine blood tests are useful to evaluate objectively the severity of the diarrhea, such as hydration status and electrolyte disturbances. A markedly elevated white blood cell count may suggest bacterial colitis or a complication such as perforation or intra-abdominal abscess formation. A critical factor that will determine the diagnostic algorithm (s) is the immune status as reflected by the absolute CD4 count and/or surrogate markers. We believe that a stepwise approach is reasonable and the first step should include stool studies. Although the positivity rate increases with the number of submitted samples, obtaining three or more stool samples is often difficult. We recommend at least one complete set and additional stool samples to further exclude parasitic diseases in the patient who has no localizing symptoms. From the published evidence, it is clear that endoscopy with biopsy should be performed sooner rather than later in patients in whom noninvasive tests are nondiagnostic especially those with CD4 counts $< 100 \times 10^6$ cells/L. When either upper or lower gastrointestinal tract symptoms are present and stool studies are negative, endoscopy directed to the probable organ of involvement is appropriate. If the patient is undergoing colonoscopy and there is additional suspicion for small intestinal pathogens, an attempt should be made to intubate and biopsy the terminal ileum during colonoscopy, as the yield of detecting microsporidia may be equivalent to EGD with small bowel biopsies^[25]. If localizing symptoms are absent and stool studies are negative, the most appropriate test is sigmoidoscopy with biopsy.

Emphasis should be placed on the changing epidemiology of diarrhea in AIDS in this era of HAART^[67]. Combination antiretroviral therapy has been shown to lead to resolution of diarrhea caused by organisms such as microsporidia and cryptosporidia^[19] and thus represents a new paradigm for the management of OI. In addition, combination antiretroviral therapy improves the immune status of the HIV-infected patient making them less susceptible to opportunistic disorders. For patients receiving HAART, the infectious causes of diarrhea appears to be decreasing, while antiretroviral agents such as protease inhibitors (most commonly nelfinavir) are increasingly important as a cause of diarrhea^[17]. The implications of these etiologic changes on the overall management of HIV-infected patients with diarrhea require further study.

THERAPY

The objective of this article was to emphasize the investigation of diarrhea in AIDS, therefore the reader is referred to recent publications on the therapy of gastrointestinal infections in AIDS^[18,66]. A table with the current drugs and dosages and duration of therapy for the most common gastrointestinal pathogens is provided (Table 3).

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New development of biliary surgery in China

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CHARACTERISTICS OF BILIARY CALCULOUS DISEASES IN CHINA: THE CHANGING SCOPE

Diseases of the biliary tract in China is complicated with the prevalence of primary infection of the bile duct system. In the middle of the 20th century, biliary infection, biliary parasitic infestation, and biliary stones made up the three chief components of biliary diseases in China. As to the calculous diseases of the biliary tract, the relative incidence of primary bile duct stones accounted for 50% of the total cases. Therefore, calculous disease accounted for 60.1% among 228 surgical cases in the Chongqing Southwest Hospital, and 60 of the 80 common bile duct stones were primary bile duct origin (including primary intrahepatic duct stones)^[1,2].

Among 2390 autopsies in Chongqing area, 50 cases were found to harbor stones in the biliary tract, and intrahepatic stones accounted for 38% of the cases^[3]. Furthermore, among 2398 cases of biliary calculous disease reported in the Chinese Journal of Surgery and analyzed by Huang Chia-Su, 36% were with gal lbladder stone, 14.3% with secondary bile duct stones and 49.3% with primary bile duct stones (including intrahepatic stones)^[4]. These findings witnessed the importance of primary bile duct stones, especially the intrahepatic stones, in the surgical treatment for biliary calculous diseases in China.

In the years of 1983-1985, we conducted the first nationwide survey under the sponsorship of the Chinese Surgical Association, totally 11342 surgical cases of gallstones were collected and analysed^[5]. The results showed that 52.8% were gallbladder stones, while 20.1% were primary bile duct stones

and 16.1% intrahepatic stones. The second survey was conducted in 1992, 10 years after the first survey, the results showed a further increase of relative incidence of gallbladder stones (79.9%) with the decrease of relative incidence of primary bile duct and intrahepatic stones (6.1% and 4.7% respectively). This trend of changes was mostly marked in the metropolitan cities^[6].

This change of the spectrum of gall stone disease in China was explained by: the improvements of people's living standard and sanitary conditions; and the widespread use of ultrasound for diagnosis and screening purpose.

Revisit of clinicopathology and surgical treatment of intrahepatic lithiasis

In general, there is a trend of decline in prevalence of intrahepatic stones accompanied with the decrease of new patient number. However, it is not a disappearing disease in China. Intrahepatic lithiasis complicated with biliary obstruction and hepato-biliary infection is still the most frequent cause responsible for the death from benign biliary diseases.

Intrahepatic stone is a common disease in East Asian countries. The prevalence is 4.1% in Japan. In 1986, Nakayama reported a prevalence of 53.5% in Taiwan, which is obviously deviant because of the limitation of data source. Recently, Su *et al* reported a retrospective review of 17182 cases with biliary calculous surgery performed over the years from 1971 to 1990 in 28 Chinese hospitals in Taiwan and found the prevalence of 20.3% for intrahepatic stones which was higher than 16.1% in mainland China^[7-9].

The classical clinical picture of intrahepatic stones is often a debilitated patient with wide spread intrahepatic stones, and has received a number of operations. Patients usually come to the hospital for treatment of severe complications. Surgery was often limited to control biliary infection. Therefore, in the analysis of 4197 cases of intrahepatic lithiasis collected from 71 hospitals in 1988, 90% of them showed hypoproteinemia, one third showed marked anemia, 37% had previous operations, more than 30% complicated with postoperative residual stones and serious morbidity complicated the postoperative course in 13% of the cases^[10].

Scanning electronic microscopic observations of the microvasculature of resected liver specimens that

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harbored intrahepatic stones revealed that there were marked changes of portal venules i.e. distorsion, narrowing and obliteration of the lumen together with extensive fibrosis and disappearance of liver parenchymal tissue. These changes were strictly segmental, confined to that segment where intrahepatic stones located and causing mechanical obstruction. Furthermore, these changes leading to the atrophy of liver parenchyma were irreversible in the late stage of disease^[11]. However, in the recent 10-20 years, owing to the wide spread use of antibiotics in the treatment of biliary infections and the improvement and accessibility of modern imaging facilities (BUS, CT scan, etc.), intrahepatic stones at its earlier stage with minimal infectious complications or clinical symptoms can be diagnosed with certainty. In the early stage, intrahepatic stone was often found to be a very localized lesion, strictly confined to one segment or two subsegments (most frequently the II and VI segment of Couinaud's classification), while the remaining portion of the liver was found to be normal grossly^[12].

Since hepatic lobectomy for the treatment of intrahepatic stones was advocated by Huang in 1958^[13], liver resection has become widely accepted as a routine for management of intrahepatic stones, especially those in the left lateral segment of the liver^[14-19]. In a survey of 4197 surgical cases of intrahepatic stones^[10], lobectomy or segmentectomy was used in 728 cases (17.3%). However, in those hepato-biliary surgical centers, adoption of liver resection for the treatment of intrahepatic stones was around 50% of the total operations, this was also true in the reports from hospitals in Taiwan and Hong Kong^[9,18]. But, if those data were subjected to detailed analysis, one would find that more than 85% of the liver resections were done on the left side of the liver. This is in disaccordance with the distribution of stone inside the liver, in which right side of the liver is not excepted. The main reason of the less use of right liver resection in intrahepatic stones is technical. A complete removal of intrahepatic stones requires a regular lobectomy or segmentectomy, it differs from those irregular resections commonly practised in the treatment of liver cancer^[12].

In 1994, the author advocated the use of systemic regular segmentectomy or subsegmentectomy in the treatment localized intrahepatic stones especially in right side of the liver. The operations were technically feasible and theoretically correct.

The results of surgical treatment of intrahepatic stones has been much improved in the past decades. However, the aim of complete cure still awaits solution of some serious problems such as residual

stones, recurrence and progressive liver damages. The types of surgical operations have been variable, but they can be categorized into three main types: liver resection, hepaticojejunostomy and cholelithotomy. Clinically they were often used in combination. We have evaluated the long-term results of surgical treatment of 959 cases of intrahepatic stones in a period of 40 years in the Southwest Hospital, Chongqing, according to three different periods (1963-1975, 1975-1981, 1983-1992). Satisfactory results were obtained in 73.9%, 80.1%, and 87.1% respectively^[6]. Therefore, results appeared to be improving, but, only half of the patients could obtain a complete relief from symptoms.

Among the surgical modalities, resection was found to give the best outcome, as demonstrated by a 91.16% success rate among 439 cases. This result has been proved by many other reports in China. Liver resection usually combined with hepaticojejunostomy to solve the problem of bile stasis, however, our clinical experience tended to favor resection alone for localized intrahepatic stones which might be more suitable for those earlier cases, since it might give better long-term benefit than combined hepaticojejunostomy.

THE PROBLEMS OF HEPATIC DUCT STRICTURES

Strictures of the large hepatic ducts as the results of inflammation and cicatrization was responsible for more than 80% of the surgical failures of intrahepatic stone operations^[20,21]. However, stricture of the hepatic duct is a common complication of intrahepatic stones, it was seen in 25% to 65% of surgical intrahepatic stone cases. In a review of 3938 cases of intrahepatic stones, stricture of the hepatic duct occurred in 6.96% to 41.94%, being 24.28% on an average^[4,10,20]. The strictured segment of hepatic duct was usually circumscribed and accompanied with destruction of elastic fibers, ductal and periductal fibrosis. Stricture of the hepatic duct was found more frequently in repeated bile duct operations.

As to the treatment of accompanied hepatic duct strictures, a series of novel operative procedures have been developed by the author^[18,21,22]. The basic surgical principles of these procedures include wide incision of the strictured site, excision of the stenotic valve and approximation of posterior bile duct wall to create a wide hepaticojejunostomy stoma by using the incised stricture segment as its posterior wall and the Roux-en-Y limb anteriorly. Such operation may be combined with hepatic resection to ensure complete removal of the lesion. It was called combined procedure.

Plastic operation on hepatic duct strictures

made a better result on surgical treatment of intrahepatic stone. Therefore, in 107 cases of intrahepatic lithiasis complicated with hepatic stricture, good result was obtained in 87.9% of the cases. In some cases, the combination of wide hilar incision and partial liver resection enables larger exposure of the bile duct over hepatic hilum. This wide incision may especially benefit the cases with widespread intrahepatic stones and markedly dilated intrahepatic bile ducts, and good results, whether evaluated on short-term or long-term base, can be anticipated^[23,24].

CARCINOMA OF THE BILIARY TRACT

Primary carcinoma of the gallbladder and the bile duct appeared to be more frequently seen in recent years. However, it is not certain whether there was an actual increase in case instances or only more of these patients were diagnosed correctly as a result of more availability of modern imaging facilities. In a nationwide survey of surgical cases of biliary tract cancer in 1989, 1098 cases were analysed^[25]. Extrahepatic bile duct cancer was found in 75.2% of the total cases, and gallbladder cancer accounted for 24.4%, with preponderance of bile duct cancer. While Shi from the Xi'an Medical University Hospital reported^[26] a retrospective analysis of 830 cases covering the past 40 years in the 7th National Biliary Surgical Conference held in Xi'an in 1997 that carcinoma of the gallbladder occurred in 72.4%, in comparison with 27.6% of extrahepatic bile duct carcinoma. This discrepancy reflected the variations of the disease prevalence in different portions of China.

CARCINOMA OF GALLBLADDER

Carcinoma of gallbladder accounted for about 1%-2% of cholecystectomies as revealed through analysis of 31 series totally of 2300 cases reported in the 7th National Biliary Surgical Conference, 1997. Other clinical characteristics of gallbladder cancer in China were: the female to male ratio 2:1, the average age was 57 years, and 60% of the patients complicated with gallstone. Zou in a second gallbladder cancer survey in 1999^[27], analysed 3776 cases, the prevalence of carcinoma of gallbladder was 0.4%-3.8% of gallbladder operations, averaging 1.96%. This result corresponds well with that obtained in 1997.

The prognosis of surgical treatment of carcinoma of gallbladder is still grave except in those lesions confined to Nevin I, II grade. According to Zou's report that the 3 and 5-year survival rate of Nevin IV, V grade tumors in 211 patients treated by radical cholecystectomy was 2.8% and 0% respectively, while among 189 patients treated with extended radical resection was

5% and 1.3% respectively. The main problem in the treatment of gallbladder cancer was the lack of early diagnosis, for most of the patients, even at the present, were still admitted to the hospitals with clinical triads (abdominal pain, abdominal mass and jaundice). Cholecystectomy for carcinoma of gallbladder in situ and in those patients with malignant changes of adenoma of the gallbladder carried an excellent result for long-term survival.

Both clinical and pathological observations support the concept of gall adenoma-carcinoma sequence, but how many primary gallbladder carcinoma came from adenoma is still unclear. Adenoma of gallbladder is often diagnosed as "gallbladder polyps" by ultrasoundists. In a survey of 341 cases of gallbladder polyp in 1989 by the author, cholesterol polyp accounted for 57.7%, inflammatory polyp 12.0%, adenoma 17%, myoadenoma 1.7%, hyperplasia 5.5%, and carcinoma 5.8%. In a series of 235 cases of gallbladder carcinoma, 23 was proved to be of malignant change of adenoma, accounting for 10% of the total cases of gallbladder carcinoma.

BILE DUCT CARCINOMA

Clinically, prevalence of carcinoma of the bile duct was found to be increased in recent years. In a review of cancer of the biliary tract, we are of the opinion that the entire bile duct system, for simplicity, may be divided into three parts, that is the intrahepatic bile duct, the hilar bile duct, and the extrahepatic bile duct. Hilar bile duct is the most common site of extrahepatic bile duct carcinoma (exclude the ampullary cancer), it covered 58%-75% of the total cases. The diagnosis of hilar bile duct cancer was difficult and the resectability rate was low, about 10% of the surgical cases. However, marked improvement was noted both in the preoperative diagnosis and the surgical treatment since the middle of the 80s due to the accessibility of modern imaging facilities, the earlier diagnosis, the better selection of operative patients, and the improvement in surgical technique. The resectability rate, at present, of hilar bile duct carcinoma has been increased to about 50% with a mortality rate below 5%. From 1986 to 1990, Huang ZQ from The General Hospital of PLA, Beijing reported a series of 50 cases of hilar bile duct carcinoma in which resection was completed in 31, with a resectability rate of 62% and no postoperative 30-day mortality^[28,29]. In the Fifth National Biliary Surgical Conference held in Guangzhou, 1991, 139 resections of hilar bile duct carcinoma were reported with a mortality rate of 0%-22%, being 5% on average. Furthermore,

recent reports from the Beijing Surgical Week, 1999, that in a period of 12 years (1987-01/1999-01), the General Hospital of PLA, Beijing, treated 157 cases of hilar bile duct carcinoma, 106 cases completed the resection, with a resectability rate of 67.5%. Among those resections, 59 (37.6%) were considered to be radical (no macro-or microscopic tumor residuals in the resected margins)^[30]. Another report from the Southwest Hospital, Chongqing, showed that from 1978 to 1997 totally 181 cases of hilar bile duct carcinoma were treated, in which 97 were resected, the resection rate being raised to 66.3% in the second period after 1991, and the radical resection rate was also increased from 18% to 38% after the year 1991.

Carcinoma of the hepatic duct bifurcation often known as Klatskin tumor, was considered to be a slowly growing malignancy and might be with a better prognosis. However, from recent experience, this conclusion has been challenged. The long-term result of surgical resection of hilar bile duct carcinoma has been far from satisfactory. The 5-year survival rate of the radical resection group was from 13.2% to 17%^[30,31]. Patients often died of local recurrence and hepatobiliary failure. Therefore, researches on the mode of recurrence in hilar bile duct carcinoma was undertaken by the author and his collaborators.

Coordinated clinical and pathological studies showed that metastasis of hilar bile duct cancer occurred rather early in the clinical cases. Therefore, in 32 cases of resections, metastasis was evidenced in 26(83.9%) of the cases, but the mode of spread was particular, such as nerve invasion in 57.7%, direct liver invasion in 42.3%, soft tissue infiltration in 42.3%, liver metastasis in 7.7%, and lymph node metastasis was only found in one case (1.8%). Histologically, 21 (65.6%) of the 32 resected specimens were well differentiated adenocarcinoma, 6 papillary adenocarcinomas, 3 were of low differentiation, and 2 carcinoma simplex^[29].

In 40 resected cholangiocarcinoma specimens, perineural space infiltration index (PNI) by cancer cells in relation to the median survival time was investigated, the result showed a reverse correlation (Table 1).

Table 1 Neural infiltration index and median survival time

Differentiation of tumor cells	PNI*	Median survival time (m)
Papillary	0.31±0.12	32
High diff.	0.39±0.18	13.5
Moderate diff.	0.74±0.39	10.8
Low diff.	0.85±0.41	7.2

*PNI>7.0=severe nerve infiltration.

In 78 surgical cholangiocarcinoma resection specimens (collected between 1989-1996), the significance of neural cell adhesion molecule (ECAM) in relation to clinicopathological findings was investigated, 68 of the 78 specimens showed nerve infiltration, blood vessels infiltration in 72, and lymphatic infiltration was present in 68. In 68 cases with neural infiltration, NCAM expression was found positive in 51 cases. Furthermore, a reverse relationship was found between the positive NCAM expression and the degree of tumor cell differentiation (Table 2).

Table 2 Expression of NCAM and tumor differentiation

Cell type	NCAM expression	
	(-)	(+)
Papillary	5	3
High diff.	10	12
Moderate diff.	8	16
Low diff.	3	21

$P=0.0267, \chi^2=9.20$

For further demonstration of the spread of cholangiocarcinoma cell along the perineural space, observations of computer assisted 3 dimensional reconstruction of the pathological section was performed in 2 cases, totally 110-200 slides were selected for reconstruction using a SHOW 3D image analysis system. The results showed that a dense net-work of small vascular and lymphatic channels together with a branching net-work of tumor infiltrates are in close relationship along the nerve fiber. By using the "wire framing" technique to visualize the structures, it was demonstrated that the tumor cells stayed sporadically in perineural space, lymphatics, and small vessels far from the primary focus of carcinoma. Probably, carcinoma cells involving a nerve at a place far from the original site must have reached there via lymphatics, vascular vessels or by direct invasion. These facts might be important in explaining the high recurrence rate of hilar bile duct carcinoma after conventional radical resections.

In a clinical pathological study of 40 resected hilar bile duct cancer specimens, a research group from the Third Military Medical University reported that 72.5% had near-by lymphatic invasion by the tumor, 77.5% showed blood vessels invasion within the tumor, 82.5% had perineural space invasion; the density of blood vessels inside the tumor and the perineural space infiltration index are positively correlated with metastasis of the cancer. In the presence of metastasis, 100% recurred within 3 years after the resection with an average of 9.6 months, while in the cases without metastasis, 64.3% recurred 3 years after the

resection with an average 17.5 months^[32].

INTRAHEPATIC LITHIASIS COMPLICATED WITH HEPATIC DUCT CARCINOMA

Since Sanes and McCallum (1942) first reported 2 cases of bile duct cancer complicating intrahepatic stones, sporadic cases reports were found in the world medical literatures. But, however, prevalence of bile duct cancer on the background of intrahepatic stones seemed to be increased in recent decade. Chijiwa from Japan reported 8 (7.3%) cases of hepatic duct carcinoma among 109 intrahepatic stones in 1973-1992^[33]. Chen (1993) from the Cheng Gang Hospital, Taipei reported 35 cases of bile duct carcinoma in 255 cases of intrahepatic stones in a period of 3 years duration (1988-1990)^[34]. In Mainland and China, hepatic duct carcinoma was found in 1.46% to 1.5% of intrahepatic stones operations as was reported by Huang (1981) and Guo (1995) from Chongqing. In the recent 6 series, of 661 cases of lobectomies for intrahepatic stones, 16 hepatic bile duct cancers were found, with a prevalence rate of 2.4%. Transition of hyperplasia and atypical hyperplasia from hyperplastic mucosa to bile duct cancer may be seen during pathological investigations^[35,36]. Carcinoma usually developed on the background of chronic biliary infection. Patients may have 10-40 years of biliary disease history and often had multiple operations including biliary enterostomy.

Hepatic duct carcinoma complicating intrahepatic lithiasis offered a very poor prognosis because of delay in diagnosis, and this is the another reason of advocating early resectional treatment of intrahepatic stones.

IMPACT OF LAPAROSCOPY ON CONVENTIONAL BILIARY SURGERY

Laparoscopic cholecystectomy was first introduced to China in February 1992. Presently, trained personnels and facilities are available in most parts of China. The largest series of patients treated by laparoscopic cholecystectomy in a single institution has been well over 20000. Almost 90% of gallbladder stone disease can be treated by laparoscopic cholecystectomy with minimal risk. In a review of 3986 cases of laparoscopic cholecystectomy from 28 institutes in 1992, the average bile duct injury rate (the most serious complication of cholecystectomy) was 0.31%. In a review of collected series of 39238 cases from 1992 to 1995 by Huang *et al*^[38] showed that the incidence of bile duct injuries was 0.32% and a biliary complication rate of 0.6%. Data of 142 946 laparoscopic cholecystectomies from 222 institutes collected in 1998 by Liu GL showed the bile duct injury rate dropped to 0.19% and the biliary

complication rate lowered to 0.14%. At present, the rapid development of medical technology, the tendency of minimally invasive operation and the application of laparoscopic cholecystectomy changed the leading position of conventional cholecystectomy as the "gold standard" of treatment for gallbladder stone disease since Carl Langenbuch in 1882^[38-40].

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Current status of radiology in China

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Subject headings radiology/history; radiology/education; radiology/trends; China

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INTRODUCTION

Radiology has been greatly advanced in China since its founding in 1949 and has been developed faster and further more since China adopted the policy of socioeconomic reform in 1978. It plays an increasingly important role in the medical health care and treatment in the country and has reached the world's advanced level in certain fields. We now briefly review the history of China's radiology so as to give a clear picture of its development.

BRIEF REVIEW OF THE HISTORY OF CHINA'S RADIOLOGY

The first X-ray machine was imported and used in clinic by Kailuan Hospital of Hebei Province in 1911^[1], followed by a few missionary hospitals in Beijing and Shanghai. The first X-ray machine designed and produced by China was born in Shanghai in 1952^[2]. The early head and body CT sets were imported to Beijing, Shanghai and Tianjin, respectively from the beginning of the 1980's. It was a great event representing the commencement of CT imaging epoch in China. From then on, more and more scanners were imported and till now distributed in majorities of large and medium-sized hospitals. In the Chinese radiologic history, several eminent radiologists who made great contributions to the development of Chinese radiology in different generations are worthy to be mentioned and commemorated.

The pioneer of Chinese radiologists was Professor Zhi-Guang Xie (C.K. Hsieh) of Peking Union Medical College (PUMC). After graduation he worked in the Department of Radiology in PUMC Hospital in 1923, and studied under the instruction of a famous American radiologist, Paul. C. Hodges, and became the chairman of that

department in 1928 after completing his radiologic research career in Michigan University of America. He also was the first Chinese doctor who held the membership of American College of Radiology. In 1948, he returned back to his hometown Guangdong Province and continued his practice and research in some affiliated hospitals of Sun Yat-Sen University of Medical Sciences until he died in 1966.

Among many radiologists trained by Professor Xie, and Drs. Du-Shan Rong and Zhao-Xuan Wang who are the most famous and regarded as the pioneers of China's radiology just as Professor Xie. Both of them graduated from PUMC in 1929 and 1933, respectively and worked as research fellows in radiology in America in the 1930s. After the year of 1949, Professor Rong became the Director of the Department of Radiology, Zhongshan Hospital, Shanghai Medical University and Professor Wang was the Director of Radiological Department, the First Affiliated Hospital, Beijing Medical University. Both of them owned the honorable membership of American College of Radiology. The three professors were considered as the founders of China's radiology. In 1952, they established the National Committee of the Chinese Society of Radiology and Chinese Journal of Radiology in Beijing. They contributed to the progress of China's radiology in training radiologists and initiating the clinical and research work, e.g., studies on the relationship between early lung cancer discovered by conventional X-ray examination and pathology, and establishing the criteria for the X-ray diagnosis and staging of silicosis. In 1956, Professors Xie and Wang collaborated in creating the formulae (called Wang-Xie Formulae) to calculate the surface area of the heart of the Chinese people. Professor Wang passed away in 1986. Professor Rong had a very important book entitled "X-ray diagnosis" published with three volumes in 1978 as the chief editor. By now, the second and third edition of the book have been published and still considered as the classical and necessary reference for Chinese radiologists. Professor Rong died of renal cancer in 1988.

Among the 2nd or the 3rd generations of Chinese radiologists, there are also many distinguished radiologists who have made great contributions to the Chinese radiology, especially in the advanced modern radiologic techniques and researches. Among them, Professor Guo-Zhen Li, Yu-Qing Liu and En-Hui Wu are admitted as the most distinguished ones. Professor Li is now working at Beijing Hospital. She also graduated from PUMC in 1943 and spent three years in Billing's Hospital, Chicago University during 1948-1950. Recently she was awarded as the honorary

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Dr. Meng Su Zeng, graduated from Nanjing Medical University in 1986, and earned M.D. from Shanghai Medical University in 1995, now associated professor of radiology, and specialized in radiologic diagnosis and studies of abdominal diseases, having over 30 papers and two books published.

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membership in the committee of RSNA. Her book entitled "Clinical Body CT Diagnosis" published in 1986 and 1994, which helps a great deal in popularizing and enhancing CT knowledge throughout the country. Professor Liu is the major founder of cardiovascular radiology in China, now working in Fuwai Hospital of PUMC and has been appointed as the Honorary President of Chinese Society of Radiology since 1993. He is the academician of Chinese Academy of Engineering (Division of Medicine and Health), the only one working in the field of radiology. Professor Wu, presently working in the General Hospital of Tianjin Medical University is one of the founders of neuroradiology in China. He is the first Chinese radiologist ever elected to the honorary membership by the Committee of RNSA in 1988. These three radiologists have made great contributions to the development of Chinese radiology. Now many eminent radiologists of new generation have emerged in different fields of radiology.

RADIOLOGICAL EQUIPMENT AND DISTRIBUTION IN CHINA

There is great difference in the radiological equipment distribution between urban and rural areas in China because of the obvious difference in the medical care system and economic conditions. In general, the amount of radiological equipment in cities and rich areas (e.g. eastern coastal area) is larger than that in rural and poor areas. In general, large hospitals such as university hospitals, teaching hospitals and provincial hospitals have all been equipped with DSA, CT, MRI, ECT, color Doppler US, etc., some of them are in the state-of-the-art (e.g., spiral/helical CT, ultra-fast CT, PET and super magnetic strength MRI). Those hospitals represent the whole medical care level of our country. Medium-sized hospitals such as city central hospitals and county hospitals have also been supplied with conventional CT and Doppler US in the developed economic areas, and some of them even with the spiral CT, MRI, ECT and DSA. A few of district hospitals and community health centers have also purchased the conventional CT and Doppler US, but majority of city central hospitals and county hospitals still have not been installed with CT and Doppler US in the undeveloped economic areas, especially those hospitals of the impoverished rural areas possess only the conventional X-ray machines and ultrasounds (including GI X-ray machine and fluoroscopic X-ray machine). Basically all of hospitals (including urban community health station and rural community health central) have been equipped with conventional X-ray machines and ultrasounds around the whole nation. According to the statistics, there are approximately 100 thousand X-ray machines of all kinds, 3 thousand CT, 3 hundred MRI and 2 thousand Doppler US by the end of 1997 in China^[3]. Simultaneously great step

progress has been made in the design and home-made radiological equipment since China adopted the reform and open-up policy in 1978. So far, China can make a variety of conventional X-ray machines with the capacity below 800mA, and some of them have been exported to African and Asian countries. Through setting up the joint enterprises with GE and Siemens companies, the whole body CT scanners were produced in China beginning from 1987. The Chinese Academy of Sciences has produced the real time ultrasound of high quality in cooperation with some developed countries in 1988. Meanwhile the 0.15T MRI of permanent magnet model, was developed and produced successfully, with some imported electronic elements. The imaging quality of this MRI machine is high enough to display the neuro-system with recent improvement of both software and hardware. Now China has began to study and develop the 0.2T MRI of new type which already has been installed and put into use in some hospitals. All these indicated that China has the capability of producing some imaging equipment^[4-13] in some aspects.

MODES OF RADIOLOGICAL DEPARTMENT AND EDUCATION IN CHINA

In general, the mode of the department of radiology in Chinese hospital is quite different from that of western countries. The divisions of ultrasound and nuclear medicine are independent disciplines and separated from the department of radiology in most of the hospitals, with little involvement in the academic activities. Ultrasonographers are trained differently from radiologists. Only a few hospitals, have merged the US, nuclear medicine and radiology into a unified department in both organization and specialty. The Ministry of Public Health has pronounced in 1990 that the department of radiology should become a clinical department if it is able to carry out interventional therapy independently. This means that the staff of radiological department shared the same responsibility and right as those of clinical department. Since then, the interventional therapy in China has made a great progress, and many hospitals have established the division of interventional therapy within the department of radiology and trained a lot of doctors under this discipline.

Great importance has been attached to the medical education and the training of radiologists as well. Among the 126 medical universities (schools) or colleges with clinical medical discipline, 7 medical colleges had medical imaging discipline^[14]. A part of students enter the department of radiology as residents after 5-7 years of study at college. A proportion of young radiologists pursued graduate studies for 3-6 years if they passed the required examinations. They are promoted through several years of practice to a higher position according to their experiences, achievements of clinical work and

number of published papers (generally, become an associated professor after 2-5 years, and a professor after 5-7 years). The title and length of education for fellows are different from that of America. Ph.D. (namely medical fellowship in America) can be trained and obtained in only 8 authorized medical schools, including Beijing Medical University, Shanghai Medical University, Tianjin Medical University, Peking Union Medical College, West China Medical University, Sun Yat-Sen University of Medical Sciences, The Second Military Medical University and the Fourth Military Medical University^[15,16].

CURRENT RADIOLOGIC CLINICAL PRACTICE AND RESEARCH IN CHINA

During the past 15 years, Chinese radiologists have accumulated a lot of clinical experience and made some valuable researches in CT, MRI, interventional radiology, angiography, ultrasound, nuclear medicine, etc., Approximately 10 thousand papers have been published in all kinds of Chinese radiological journals and periodicals (Table 1), but relatively a small number of research articles are submitted to western journals, because of the English language barrier.

Table 1 Main Chinese journals of radiology

1. Chinese Journal of Radiology (Beijing), established 40 years ago
2. Journal of Clinical Radiology (Huangshi)
3. Journal of Practical Radiology (Xi'an)
4. Journal of Clinical Medical Imaging (Shenyang)
5. Chinese Journal of Medical Imaging Technology (Beijing)
6. Roentgenpraxis (Wuhan)
7. Journal of Foreign Medicine: Clinical Radiology (Tianjin)
8. Chinese Medical Abstract: Radiological Diagnosis (Wuhan)
9. Chinese Journal of Medical Imaging (Beijing)
10. Journal of Interventional Medicine (Shanghai)
11. Chinese Journal of Medical Computed Imaging (Shanghai)
12. Modern Medical Imaging (Xi'an)
13. Medical Imaging (Tianjin)
14. Journal of Diagnostic Imaging & Interventional Radiology (Guangzhou)

CT

Clinical applications of CT technique have gradually been popularized throughout the country. Before the invention of spiral CT, dynamic incremental CT enhancement scanning was performed and adopted in most of the hospitals, mainly for detection of focal hepatic lesions. In addition, CTA and CTAP were also used for discovering the small primary and secondary hepatic lesions in the selected cases. In recent years, imported spiral CT scanners were installed in most of the large hospitals, some new techniques and researches regarding spiral CT (such as CT angiography, multi-planner or 3D imaging of bronchus, bone, etc.) were developed, particularly in university hospitals. Dual phase or multiphase spiral CT enhancement procedures were studied primarily in small hepatocellular carcinoma as well as in pancreatic and renal cancers. It was proved to be very sensitive in detecting the small neoplastic

lesions, in particular the hypervascular masses in liver during the arterial phase or combined phases. HRCT technique was used routinely in pulmonary cancer, single pulmonary nodule and diffuse pulmonary diseases. Special CT procedures for digestive tract, including mainly recto-colonic cancer and gastric cancer by filling those organs with ordinary water were successfully investigated, and the protocol accompanied by pronounced enhancement improved greatly the accuracy of the tumor staging. All kinds of CT training programs for radiologists were offered annually by the senior radiologists in order to improve their clinical experiences, master the new techniques and overcome some pitfalls. In a couple of hospitals in Beijing and Shanghai, electronic beam CT (UFCT) was available and the comparative studies of the detection and quantitative measurement of the calcification on the coronary arterial wall with angiography were performed to estimate the risk of the coronary heart attack^[17-22].

MRI

Since 1990, more MRI equipment have been imported to our country, different magnetic strengths of MRI machines have been installed in lots of large hospitals as well as in a few of medium-sized hospitals. In many hospitals, most cases of MRI inspection are still devoted to neurological system and skeletal system. MRI examination is mainly carried out to find the early cerebral ischemia and neurological tumors in many hospitals. Some university hospitals have used MRI to investigate a series of changes about cerebral ischemia and hemorrhage and compare with pathology in order to guide the clinical treatment. Investigation of MRI in skeletal system includes mainly the tumor of bone and soft tissue, trauma and other diseases of the joints. Significant achievements and good experiences have been obtained in studies on cardiac ischemia, cardiac infarction and reperfusion after infarction with dynamic enhanced MRI scans in comparison with isotope examination and coronary angiography in some hospitals (main university hospitals). MR angiography (MRA) was done successfully in cerebral, carotid and limb arteries, but with unsatisfactory results in thoracic and abdominal aorta and their branches. Over the past two years, 3D dynamic contrast enhanced MRA has been developed with improvement of imaging quality, such enhanced MRA technique was also applied to venous system, particularly to the portal venous system which can be shown simultaneously with hepatic veins and inferior vena cava as well as their collateral in cirrhotic cases. The good results have been yielded in more than 100 cases by this exams from Zhongshan Hospital, Shanghai Medical University. Other new MRI techniques such as MR cholangiography (MRC), MR cholangiopancreatography (MRCP), MR urography (MRU) and

MR myelography (MRM) aroused great interest and were improved with introduction of single shot SE sequence. According to the report of Zhongshan Hospital of Shanghai Medical University, fast dynamic enhanced MRI was superior to SE sequence in identifying the early lesions and making differential diagnosis for abdominal organ diseases. For small hepatocellular carcinoma, it might be better than dual phase contrast spiral CT and should be considered as a supplementary method. Currently, some new techniques like MR spectroscopy, MR with specific liver contrast media, rectal endocoil for pelvic organs, diffusion and perfusion for early cerebral ischemia, functional MRI, etc. are being developed and will be applied in the near future^[23-30].

Interventional radiology and angiography

Interventional radiology as a new branch of radiology was introduced into China in the mid 80's, and since then it has made spectacular progress. Transcatheter management of neoplasm is a very active field in interventional radiology and has been applied to the treatment of tumors at various sites of the body, especially for tumors of lung and liver. According to the statistics of Zhongshan Hospital of Shanghai Medical University, interventional treatments for liver cancer have been given to approximately 8000 advanced cases. The 1-, 2-, 3- and 5-year survival reached 65.2%, 35.3%, 24.6% and 9.8% respectively. In Changhai Hospital of the Second Military Medical University, the same work was carried out with similar result. The Chinese People's Liberation Army General Hospital and China Medical University Hospital have reported that they used TIPS technique for the treatment of portal hypertension and massive bleeding in about 500 cases with fruitful result. Several hundred cases with mitral stenosis have been treated by non-operative methods with excellent results in Fuwai Hospital of Peking Union Medical College, Guangdong Provincial Hospital and Jiangxi Medical University Hospital. In addition, percutaneous transluminal angioplasty was also widely used to dilate pulmonary valve, coronary and renal arterial stenosis and Budd-Chiari syndrome. Vascular and nonvascular stent placement for vessels, bronchus, esophagus, bile duct, etc. have also been performed, and preliminary clinical experience has been achieved. Percutaneous aspiration biopsy guided by X-ray, CT and ultrasound is widely performed in diagnosis and treatment for the lesions of liver, lung, kidney, pancreas and bone. The interventional diagnosis and treatment, (e.g. thrombolytic therapy for cerebral ischemia) are not very common in China and MRI interventional therapy is still unavailable. Many large and medium-sized hospitals have the advanced DSA X-ray machines used for all kinds of angiography such as coronary angiography and cardioangiography, but

the application is gradually decreasing as noninvasive procedures (i.e., MRA, CTA and color Doppler US), have come into being^[31-35].

Ultrasound and nuclear medicine

Sonography is very popular in China, even being used for physical checkups because it is cheaper and safer and needs less time than other imaging modalities and easy to operate. The hospitalized patients basically received ultrasound examination on admission. Ultrasound (including color Doppler US and 3D US) is widely used for abdominal disease and heart disease and gynecologic and obstetrical diseases (including congenital and acquired heart diseases). The pregnant women undergo US examinations every 3 to 4 months in order to monitor the growth of the baby according to the physician's suggestion. In recent years, some new ultrasonic techniques have been put into clinical practice in a few hospitals such as contrast-enhanced US and endosonography.

Nuclear medicine departments and labs have been set up since the 1950s in many large hospitals and a few medium sized hospitals. Single photon emission computed tomography (SPECT) is mainly used for radiopharmaceutical studies and as a routine examination, it can provide diagnostic information in the setting of a nuclear medicine clinical services. Most of university hospitals have equipped this facility in China and has used it routinely and frequently in the investigation of the brain, the heart and the skeleton, especially in detecting the metastasis of bone. On the other hand, radioimmunological assay and analysis are also important parts of nuclear medicine. Positron emission tomography (PET) has been put into clinical practice in Shandong Province, Shanghai and Beijing, respectively. The Ministry of Public Health has approved Beijing and Shanghai to set up respectively clinical center of PET.

RADIOLOGICAL TRENDS OF CHINA IN FUTURE

In short, radiology of China has been greatly advanced over the past two decades and has played very important roles in the prevention and treatment of diseases, however, it is still in its developing stage. Some renowned radiologists in China have predicted that lots of hospitals will be supplied with spiral CT to replace conventional CT being used widely in clinical practice in 2-3 years. Some university hospitals will purchase the most advanced multislices spiral CT and multiple-functional MRI machine (e.g., possessing of EPI) in order to catch up the latest step of the world. Due to the need of clinical practice, development of PACS (picture archiving and communications system) continues to be a subject of intense interest and some university hospitals will be equipped with PACS, making the imaging transfer easier and quicker between radiological departments and clinical departments or among the divisions within the departments of radiology (including CT, MRI,

SPECT and US).

In regard to radiological research, the Chinese Society of Radiology recommended the improvement of early detection for small cancer such as small liver carcinoma, lung cancer and pancreatic cancer as the research objectives. In many hospitals, noninvasive procedures (i.e., MRA, CTA, MRCP and Dopple US) are being gradually substituted by the invasive procedures in radiological examination. Further studies are needed to get the optimal protocol of imaging modalities. Experimental researches in the field of radiology ought to be strengthened.

Lastly, radiology ultrasonography and nuclear medicine should be incorporated as a unified subject in term of administration and academic exchange in order to increase the accuracy of diagnosis and decrease unnecessary imaging examination. We are convinced that radiology of China will have a vigorous prospect and play more and more important roles in the care of people's health.

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Original Articles

Co-morbidity, not age predicts adverse outcome in *Clostridium difficile* colitis

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Subject headings clostridium difficile; colitis/old age; comorbidity/old age; diarrhea; colitis/therapy; physical examination

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Abstract

AIM To examine whether age alone or co-morbidity is a risk factor for death in older adults who developed *Clostridium difficile* (Cd) colitis during hospitalization.

METHODS A retrospective, observational study design was performed in our Lady of Mercy Medical Center, a 650-bed, urban, community-based, university-affiliated teaching hospital. 121 patients with a positive diagnosis of Cd colitis (aged 23-97 years) were studied, and data pertinent to demographic variables, medical history, co-morbidity, physical examination, and laboratory results were collected. Age was examined as a continuous variable and stratified into Age1 (<80 vs 80+); Age2 (<60, 60-69, 70-79 and 80+); or Age3 (<60, 60-69, 70-79, 80-89, 90+).

RESULTS Cd colitis occurs more frequently with advancing age (55% of cases >80 years). However, age, *per se*, had no effect on mortality. A history of cardiac disease ($P=0.036$), recurrent or refractory infection >4 weeks ($P=0.007$), low serum total protein ($P=0.034$), low serum albumin ($P=0.001$), antibiotic use >4 weeks ($P<0.010$), use of over

4 antibiotics ($P=0.026$), and use of certain classes of antibiotics ($P=0.035-0.004$) were predictive of death. Death was strongly predicted by the use of penicillin-like antibiotics plus clindamycin, in the presence of hypoalbuminemia, refractory sepsis, and cardiac disease ($P=0.00005$).

CONCLUSION Cd colitis is common in the very old. However, unlike co-morbidity, age alone does not affect the clinical outcome (survival vs death).

INTRODUCTION

Infection with the *Clostridium difficile* (Cd) bacterium is associated with a wide spectrum of gastrointestinal disorders that range from asymptomatic, to mild and sporadic diarrhea, to life threatening colitis^[1]. In older people, Cd colitis occurs frequently and is associated with high morbidity and mortality^[2]. In addition, epidemiological studies of -Cd-induced diarrhea have identified an almost exclusive relationship to both antibiotic use and advancing age^[3]. Those most commonly affected are older people, the immunocompromised, and patients who undergo gastrointestinal surgery^[4]. In this report, we examined whether age alone or co-morbid conditions are risk factors for an adverse clinical outcome (death) in a sample of 121 patients who developed Cd- colitis during hospitalization.

MATERIALS AND METHODS

This study was conducted at a 650-bed community teaching hospital in the Bronx, New York. We performed a retrospective review of the medical records of all patients who were admitted from the community or nursing homes during 2a (1995-02/1997-02). 121 patients with a positive diagnosis of Cd colitis were identified. Most Cd patients were older adults from the inner city. Demographic variables were collected, as were all data pertinent to their past and present medical history and their recent physical examinations. A positive diagnosis of Cd colitis was based on medical history,

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gastrointestinal symptoms or signs plus a positive enzyme-linked fluorescent immunoassay of *Cd* cytotoxin in A, and/or endoscopic biopsy-proven pseudomembranous colitis. Patient ages ranged from 23 to 97 years (78±14). Included were 13 patients below 60 years (10 females and 3 males), 16 patients from 60-69 years (7 females and 9 males), 25 patients from 70-79 years (14 females and 11 males), 52 patients from 80-89 years (35 females and 17 males), and 15 patients from 90-97 years (9 females and 6 males). Study variables included patient's age, sex, and length of hospital stay. Data referring to pre-existing medical conditions (diabetes, dementia, tube feeding, COPD, hypertension, cardiac disease), gastrointestinal symptoms, fever, and the number, class, and duration of all antibiotics used were collected. Sepsis or infection were defined as the presence of a body temperature >38°C, heart rate >90 beats/min, respiratory rate >20/min, and WBC count >12×10⁹/L. Sepsis not responsive to therapy after four weeks was defined as refractory. Laboratory results (total protein, albumin, BUN, creatinine, creatinine clearance, CBC) were noted.

The effect of age was examined using age both as a continuous variable and stratified into integer variables as follows: Age1 (2 groups; 23-79 years and over 80), Age2 (4 groups; 23-59, 60-69, 70-79, and over 80 years), Age3 (5 groups; 23-59, 60-69, 70-79, 80-89, and over 90 years). Study variables [low total protein (<70g/L), low albumin (<35g/L), elevated BUN (7.15mmol/L), high creatinine (132.6µmol/L), low creatinine clearance (<50mL/min)] were examined using Fisher's exact test, Student *t* test for unpaired data, and (simple, multiple, and logistic) regression analysis to determine whether age, co-morbidity, antibiotic use, or specific laboratory chemistries were associated with an adverse clinical outcome (death). Statistical analyses were performed on an IBM-compatible PC using STATA-TM (version 5.0, Stata Corp., College Station, TX, <http://www.stata.com>) and a two-tailed significance level of *P*<0.05 was considered to be significant.

RESULTS

In this sample of 121 *Cd* colitis patients ranging in age from 23-97 years, we observed that *Cd* colitis occurred more frequently in those with advanced age, (55% of our subjects were >80 years). Nevertheless, when age was examined as a continuous variable (23-97 years) or stratified into different integer variables (Age 1, Age 2, and Age 3) it was not associated with increased mortality (Table 1). We also found that a number of co-morbidity related variables were predictive of an adverse outcome (Table 2). For example, the

presence of cardiac disease (*P*=0.036), an elevated serum BUN (*P*=0.039), low serum total protein (*P*=0.034), and low serum albumin (*P*=0.001) predicted death. In addition, recurrent or refractory infection over 4 weeks (*P*=0.007), prolonged antibiotic use (*P*=0.010), use of 4 or more antibiotics (*P*=0.026), and use of different classes of antibiotics (*P*=0.035-0.004) were found to predict death. The strongest predictor of death was the combined use of penicillin-like-antibiotics plus clindamycin, in the presence of hypoalbuminemia and refractory sepsis >4 weeks, in a patient with a history of cardiac disease (*P*=0.00005).

Table 1 Patient age and sex vs outcome at discharge (n,%, $\bar{x}\pm s$)

Variable	Alive	Dead	<i>P</i> value
Patient age (yrs)	76.1±15.0	78.9±13.5	0.383(1,a)
Patient sex (M/F)	36/58	10/17	0.546(2)
Age 1 (yrs)			
23-79	45(83%)	9(17%)	
80-97	48(73%)	18(27%)	0.192(2)
Age 2 (yrs)			
23-59	12(92%)	1(8%)	
60-69	12(75%)	4(25%)	
70-79	21(84%)	4(16%)	
80-97	49(73%)	18(27%)	0.428(2)
Age 3 (yrs)			
23-59	12(92%)	1(8%)	
60-69	12(75%)	4(25%)	
70-79	21(84%)	4(16%)	
80-89	37(71%)	15(29%)	
90-97	12(80%)	3(20%)	0.577(2)

(1):Student *t* test; (2):Fisher's exact test; (a):Additional logistic regression analysis.

Age (as continuous variable) did not predict adverse outcome or death (*P*=0.382).

Table 2 Co-morbidity vs adverse outcome at discharge

Variable	Not predictive	Predictive	<i>P</i> value
Co-morbidity			
Cardiac disease (1)	—	×	0.036
COPD	×	—	0.200
Dementia	×	—	0.075
Diabetes	×	—	0.622
Hypertension	×	—	0.387
Elevated BUN	—	×	0.039
High creatinine	×	—	0.106
Low creatinine clearance	×	—	0.928
Nutrition related			
Tube-fed	×	—	0.241
Low serum total protein	—	×	0.034
Low serum albumin	—	×	0.001
Sepsis related			
Aminoglycosides	×	—	0.034
Clindamycin	—	×	0.004
Penicillin-like (2)	—	×	0.035
Antibiotic use >4wk	—	×	0.010
Use of >4 antibiotics	—	×	0.026
Fever (>38.3°C)	×	—	0.181
Infection >4wk	—	×	0.007

Results are given as predictive or not predictive of death as the adverse outcome and were determined using logistic regression analysis.

(1): 64 cardiac patients were identified with 19 deaths during hospitalization.(2): Excluding cephalosporins.

DISCUSSION

Our study revealed that the use of broad spectrum penicillins (ampicillin, amoxi cillin clavulanic acid, nafcillin, piperacillin, ticarcilin-clavulanate, ampicillin-sulbactam), cephalosporins (cefazolin, cefuroxime, cefoxitin, ceftaz idime, ceftriaxone), macrolides (erythromycin, clarithromycin, azithromycin), aminoglycosides (gentamicin, amikacin), vancomycin, clindamycin, ciprofloxacin, trimetroprim-sulfamethoxazole, and doxycycline was associated with an increased risk of *Cd* colitis. In particular, we found that clindamycin, aminoglycosides and penicillin-like antibiotics were positively related to an adverse outcome (death). Interestingly, although cephalosporins are among the most common agents associated with the development of *Cd* colitis, reflecting their widespread use, our study did not identify their use to correlate with increased mortality^[5].

Our patients ranged in age from 23 to 97 years with the majority (108/121) over the age of 60 years and advancing age is often referred to as a risk factor for developing *Cd* colitis^[2-4]. Other factors reported to be associated with an increased risk of *Cd* colitis are immunosuppression, hospitalization, surgical procedures involving the gastrointestinal tract and medications that alter intestinal motility or intestinal flora^[3-8]. Among medications, the key factor appears to be antibiotic therapy that can alter the normal equilibrium of colonic flora and create an environment that permits *Clostridium difficile* to flourish^[7]. Many antibiotics have been linked to *Cd* infection, but broad spectrum antibiotics with activity against enteric bacteria are the most frequently implicated agents^[7]. Clindamycin is notorious for its association with *Cd* colitis^[8]. The literature further suggests that the most common antibiotics associated with *Cd* colitis are the broad spectrum penicillins and cephalosporins which may simply reflect their widespread use^[5]. *Cd* colitis infection may be induced by oral, parenteral, or topical antibiotic therapy^[9].

Antibiotic associated *Cd* colitis has been linked to *Clostridium difficile* bacterium and to the cytotoxin produced by the organism^[9-11]. Antibiotic-associated diarrhea and colitis are important and increasingly frequent complications of antibiotic therapy that occur often in hospitals, nursing homes, and in the community^[12]. Population based studies in Sweden showed that the incidence of *Cd* toxin positive stools increases 20-100 fold when persons aged 10-20 yr are compared with those over 60^[13]. Multivariate analysis of hospitalized patients, colonized with the *Cd* bacterium, also show that increasing age correlates

positively with the clinical expression of the disease^[14]. This finding suggests that aging promotes susceptibility to *Cd* colonization, *Cd* toxin production, and the onset of *Cd* colitis^[15].

While most studies demonstrate that *Cd* colitis occurs commonly in the geriatric population, few comment on patient age as a variable that could affect an adverse outcome. We analyzed age as a continuous variable and as different integer variables and our analyses did not reveal that age was directly associated with an adverse outcome (death). As presented in this report, we believe that other factors associated with the aging process such as cardiac disease, refractory sepsis and/or low serum albumin were the more likely contributory factors linked to an increased risk of death. Among comorbid factors, coronary artery disease and congestive heart failure appeared to be the strongest predictor of death. Of our 64 cardiac patients, 19 died during hospitalization.

Other co-morbid factors studied such as diabetes mellitus, hypertension, COPD, and dementia did not affect mortality. In addition, the use of tube feeding, the presence of gastrointestinal symptoms, fever, and laboratory abnormalities such as high serum creatinine or low creatinine clearance were not predictive of increased mortality. On the other hand, we observed that an elevated BUN alone was predictive of increased mortality ($P=0.039$). Elevated BUN may have resulted (in these patients) from one or more of several pre-renal factors such as cardiac failure, volume depletion, catabolic states, and gastrointestinal bleeding as the basis. Also, factors predictive of death were refractory or recurrent infection lasting longer than 4 weeks ($P=0.007$), the use of penicillin-like antibiotics, ($P=0.035$), clindamycin ($P=0.004$) or aminoglycosides ($P=0.034$), and antibiotic use over 4 weeks ($P=0.01$). Obviously, the presence of recurrent or refractory infection usually requires prolonged use of antibiotics. Thus, it is conceivable that long-standing infection alone led to the increased mortality, while prolonged use of antibiotics was merely an association or a contributing factor to the adverse outcome. While we did not study treatment failure or relapse, previous work from our institution examined the factors influencing treatment failure and relapse, age, sex, co-morbid illnesses, and found that the type(s) of antibiotics that precipitate colitis were not associated with either the response to therapy or relapse^[16,17].

Nutritional status also appears to influence outcome in *Cd* colitis patients. In our study, hypoalbuminemia was strongly predictive of an adverse outcome ($P=0.001$). The literature states that serum albumin (<25 g/L) is a risk factor for

increased mortality, more significantly, a fall in serum albumin of greater than 11g/L at the onset of symptoms of *Cd* infection is positively associated with poor outcome^[17].

Hypoalbuminemia probably results from a loss of body protein in the stool caused by the inflammatory exudate, or by depressed hepatic protein synthesis in response to sepsis or malnutrition which is common among hospitalized elderly^[18]. Low serum albumin is a well-recognized marker for malnutrition^[19] and is strongly associated with poor clinical outcome in *Cd* colitis^[17].

While *Cd* colitis is more common in older individuals, our study suggests that age, per se, does not influence clinical outcome (survival/death). We observed that refractory sepsis, requiring use of antibiotics (in particular, certain classes of antibiotics) for over four weeks in the presence of hypoalbuminemia and coexisting cardiac disease were strongly predictive of adverse outcome (death) in *Cd* colitis in our hospitalized patients ($P < 0.00005$). Other factors such as COPD, dementia, hypertension, elevated creatinine or low creatinine clearance, and tube feeding were found not to affect the outcome in *Cd* colitis.

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Agglutination of *Helicobacter pylori* coccoids by lectins

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Abstract

AIM To study the agglutination pattern of *Helicobacter pylori* coccoid and spiral forms.

METHODS Assays of agglutination and agglutination inhibition were applied using fifteen commercial lectins.

RESULTS Strong agglutination was observed with mannose-specific *Concanavalin A* (Con A), fucose-specific *Tetragonolobus purpureas* (Lotus A) and N-acetyl glucosamine-specific *Triticum vulgare* (WGA) lectins. Mannose and fucose specific lectins were reactive with all strains of *H. pylori* coccoids as compared to the spirals. Specific carbohydrates, glycoproteins and mucin were shown to inhibit *H. pylori* lectin-agglutination reactions. Pre-treatment of the bacterial cells with formalin and sulphuric acid did not alter the agglutination patterns with lectins. However, sodium periodate treatment of bacterial cells were shown to inhibit agglutination reaction with Con A, Lotus A and WGA lectins. On the contrary, enzymatic treatment of coccoids and spirals did not show marked inhibition of *H. pylori* lectin agglutination. Interestingly, heating of *H. pylori* cells at 60°C for 1 hour was shown to augment the agglutination with all of the lectins tested.

CONCLUSION The considerable differences in lectin agglutination patterns seen among the two differentiated forms of *H. pylori* might be attributable to the structural changes during the events of morphological transformation, resulting in exposing or masking some of the

sugar residues on the cell surface. Possibility of various sugar residues on the cell wall of the coccoids may allow them to bind to different carbohydrate receptors on gastric mucus and epithelial cells. The coccoids with adherence characteristics like the spirals could aid in the pathogenic process of *Helicobacter* infection. This may probably lead to different clinical outcome of *H. pylori* associated gastroduodenal disease.

INTRODUCTION

Helicobacter pylori has established firmly as a human pathogen causing chronic active gastritis and peptic ulcer^[1-3]. In addition, there are epidemiological data that support association between *H. pylori* infection and the development of varieties of gastric cancers^[4-6]. However, the pathogenetic mechanisms of *H. pylori* induced gastroduodenal disease are not well established^[7-9].

The widespread prevalence of *H. pylori* infection indicates its infectivity. In general, the success of a pathogen depends on both its virulence and pathogenicity^[8-9]. Of these, adherence to surface receptors is an essential step in the pathogenesis for many bacteria^[10]. The attachment is mediated by adhesins or ligands which may be soluble or cell-associated and can be demonstrated by haemagglutination of various species of erythrocytes^[10-11]. Previously, it was reported that the surface of *H. pylori* contains lectins or adhesins which may influence its adherence to the membrane of surface mucous cells^[12]. Since lectins have the ability to bind to a wide variety of microbial substances containing simple or complex carbohydrates, they have been used to detect cell wall modifications, elucidate complex cell wall carbohydrates and detect intra-strain variations in cell wall carbohydrates or carbohydrate linkages^[13-14].

The specificity of lectin binding to bacterial surface carbohydrates has been reported^[15] and exploited as a tool for typing microorganisms, such as *Neisseria*^[16,17], *Staphylococcus*^[18], *Legionella*^[19], *Bacillus*^[20], *Campylobacter*^[21-22], *Helicobacter*^[23] and *Streptococcus* species^[24-25]. Many

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bacteria, including *H. pylori*, were found to have cell wall associated lectins which allow them to bind selectively to mucus and epithelial cells^[8,12]. Emody et al (1988)^[26] also showed that *H. pylori* lectins attach to red cells from various animal species. Moreover, it was suggested that bacterial surface haemagglutinins and lectins play a significant role in pathogenesis of many mucus-associated infections^[11]. However, lectins were studied only on spiral forms of *H. pylori*. In this study, we applied lectin agglutination to characterize the carbohydrate residues on the cell wall of *H. pylori* coccoids as compared to spiral and to examine the agglutination profiles of *H. pylori* coccoids and spirals.

MATERIALS AND METHODS

Bacterial strains and spiral forms of *H. pylori*

Two local strains, RH 54, V₂ and a standard strain, NCTC 11637 were grown on moist chocolate blood agar at 37°C for 3 days in a humidified, 5% CO₂ incubator (Forma Scientific) to provide homogeneous spiral forms as described in Khin and Ho (1994)^[27]. The spiral nature was observed under phase contrast microscope (Nikon Microphot-FXA, Japan).

Preparation of coccoid forms

Ageing coccoids were obtained as described in Khin et al (1996)^[28]. Additionally, induced coccoids were also prepared by culturing the 3-day-old spirals in brain heart infusion medium supplemented with 10% horse blood and 0.4% yeast extract and exposed to amoxicillin (Sigma) in a final concentration of 5mg/L. The broth containing coccoid forms when inoculated onto moist chocolate blood agar and incubated in a humidified incubator (Forma Scientific, USA) at 37°C with 5% carbon dioxide did not show any growth after 12 days of incubation.

Both spiral and coccoid forms were washed in PBS (pH 7.4) for 3 times. The bacterial cells were resuspended to a final concentration of 1×10^{10} cells/mL in PBS. Spirals and coccoids were enumerated in triplicates using a bacteria counting chamber under phase contrast microscope.

Lectins

The lectins used (Table 1) were selected on the basis of their reported specificities, to cover the widest possible range of sugars. All are commercially available from Sigma (St. Louis, USA). The lectins were resuspended at 1µg/L in PBS (pH 7.4) and stored at -20°C until use.

Agglutination assay

Agglutination assay was carried out according to

the method described by Ascencio et al (1990)^[23]. The assay was performed in triplicates. Binding of lectins to coccoids and spirals were assayed using glass slides by mixing 20µL of bacterial suspension with an equal volume of lectin solution. A negative control was included for each strain by adding 20µL of PBS instead of lectin solution. The agglutination reaction was scored as follows:

3=strong positive reaction with large clumps;
2=strong- positive reaction with moderate size clumps;
1=positive agglutination with fine clumps; w=weak agglutination; -=no agglutination.

Table 1 Major sugar specificities of lectins used in this study

Lectin	Abbreviation	Carbohydrate specificity
Arachis hypogaea	PNA	β-D-Gal(1-3)GalNAc
Bandeirae simplicifolia	BS-I	α-Gal, α-GalNAc
Concanavalin A	Con A	α-D-Man, α-D-Glc
Datura stramonium	DSA	(D-GlcNAc) ₂
Glycine max	SBA	D-GalNAc
Helix promatia	HPA	D-GalNAc
Lens culinaris	LcH	α-D-Man
Narcissus pseudonarcissus	NPA	α-D-Man
Pisum sativum	PEA	α-D-Man
Tetragonolobus purpureas	Lotus A	α-L-Fuc
Triticum vulgare	WGA	(D-GlcNAc) ₂ , NeuNAc
Vicia faba	VFA	D-Man, D-Glc
Vicia sativa	VSA	D-Glc, D-Man
Vicia villosa	VVA	D-GalNAc
Vigna radiata	MBA	α-Gal

Gal=D-Galactose; GalNAc=N-acetyl galactosamine; GlcNAc=N-acetyl glucosamine; NANA=N-acetyl neuraminic acid; Man=Mannose; Glc=Glucose; Malt=Maltose; Suc=Sucrose; Fuc=L-Fucose.

Agglutination Inhibition Assay

To test whether the agglutination was inhibited by specific glycoproteins or carbohydrates, 20µL of each lectin solution was incubated with an equal volume of the test substance at 20°C for 1 hour before adding 20µL of bacterial suspension. The agglutination reaction was scored accordingly.

Carbohydrates (Gal, GalNAc, GlcNAc, NANA, Man, Glc, Malt, Suc and Fuc) were dissolved at a final concentration of 0.1M in PBS (pH 7.4). Glycoproteins (bovine submaxillary mucin, porcine stomach mucin, fetuin, asialofetuin, bovine orosomucoid and gangliosides II) were dissolved at a final concentration of 0.1% in PBS. All these chemicals were purchased from Sigma.

Pre-treatment of bacterial cells

H. pylori RH 54 spirals and coccoids were treated with sodium periodate (0.075 M), sulfuric acid (0.025N), formalin (1% w/v) and glycine hydrochloride buffer (pH 2.2). Bacterial cells were also heated at 60°C for 1 hour. Alternatively, these spirals and coccoids were treated with neuraminidase, trypsin, pepsin, proteinase K and chymotrypsin each at 0.1µg/L for 1 hour at 37°C.

After treatment, bacterial cells were washed three times with PBS and resuspended to 10^{10} cells/mL in PBS and used for the agglutination assay.

Reproducibility

Reproducibility of lectin agglutination was confirmed by repeating the test three times.

RESULTS

The coccoids and spirals of all three strains of *H. pylori* tested showed different agglutination reactions to the 15 lectins. The sugars recognized by *H. pylori* coccoids and spirals were Man, Fuc, Glc, Gal, GalNAc and (GlcNAc)₂ (Table 2). Among the 15 lectins tested, strong agglutination patterns were observed with Man-specific Con A, Fuc-specific Lotus A and (GlcNAc)₂-specific WGA lectins (Table 2). All of the coccoids and spirals were agglutinated by the Man-specific Con A, PEA and VFA lectins, GalNAc-specific HPA and Glc-specific VSA lectins (Tables 2 and 3). RH 54 coccoids reacted with all the 15 lectins tested (Table 2) whereas NCTC 11637 coccoids were found to be non-reactive with WGA and VVA lectins. Similarly, SBA, VVA and MBA lectins were not reactive to V₂ coccoids (Table 2). The spirals were refractory to agglutination with Man-specific LcH and NPA lectins (Table 2). Interestingly, NCTC 11637 spirals were non-reactive with Fuc-specific Lotus A, Gal-specific PNA and BS-I and (GlcNAc)₂ specific DSA and WGA lectins.

α -D-Man/Con A, LcH, NPA, PEA and VFA binding

It is interesting to note that Man was recognized by all the coccoids as compared to the spirals, since all the coccoids were agglutinated by the Man-specific Con A, LcH, NPA, PEA and VFA lectins (Tables 2 and 3). In contrast, three strains of spirals were agglutinated by Con A, PEA and VFA, but none of the spirals was reactive to LcH or NPA lectins (Tables 2 and 3).

α -L-Fuc/lotus A affinity

Three strains of *H. pylori* coccoids were agglutinated by Fuc-specific Lotus A lectin (Tables 2 and 3). All the spirals except NCTC11637 spirals reacted with Lotus A (Table 2). Strong agglutination pattern was shown by RH 54 and HpV₂ coccoids as well as spirals (Table 2).

(D-GlcNAc)₂/DSA and WGA binding

Both RH 54 and V₂ coccoids as well as spirals had an affinity for GlcNAc residue as shown by agglutination with DSA and WGA lectins. NCTC 11637 spirals were non-reactive to both DSA and WGA but their corresponding coccoids showed agglutination with DSA (Table 2).

D-GalNAc/HPA, SBA and VVA binding

Among the three strains of coccoids, only RH 54 coccoids reacted with GalNAc-specific HPA, SBA and VVA lectins. Among these three lectins, HPA was the only lectin which agglutinated all the coccoids tested. Interestingly, V₂ coccoids lacked the affinity to both SBA and VVA lectins (Table 2). On the other hand, NCTC 11637 coccoids were agglutinated by SBA but not by VVA lectins (Table 2). All the spirals tested were found to be agglutinated by HPA, SBA and VVA lectins (Table 3).

D-Glc, D-Man/VSA binding

Three strains of *H. pylori* coccoids and their corresponding spirals were observed to be agglutinated by VSA lectin (Tables 2 and 3). Strong agglutination was given by NCTC 11637 and V₂ coccoids (Table 2).

β -D-Gal or α -Gal/PNA, BS-I and MBA binding

The coccoids of RH 54 and NCTC 11637 showed affinity to Gal-specific PNA BS-I and MBA lectins but V₂ coccoids were non-reactive to MBA lectin (Table 2). On the other hand, MBA lectin agglutinated with all the spirals tested (Tables 2 and 3). Among the spirals, only NCTC 11637 spirals were non-reactive with PNA and BS-I lectins (Table 2).

Table 2 Lectin agglutination patterns of *H. pylori* coccoids and spirals

Lectin	RH 54		NCTC 11637		V ₂	
	Coccoids	Spirals	Coccoids	Spirals	Coccoids	Spirals
Con A	2	1	3	2	2	1
LcH	2	0	2	0	1	0
NPA	1	0	1	0	1	0
PEA	2	1	2	W	1	1
VFA	1	1	2	1	1	1
Lotus A	3	3	1	0	3	3
DSA	1	2	1	0	1	2
WGA	2	3	0	0	2	3
HPA	1	2	1	1	1	1
SBA	1	1	1	1	0	3
VVA	1	1	0	1	0	1
VSA	1	1	2	1	2	1
PNA	1	1	1	0	1	2
BS-I	2	2	1	0	1	2
MBA	1	3	1	1	0	1

Sugar specificity/Lectins

Man- ConA=Concanavalin A; LcH=Lens culinaris;

NPA=Narcissus pseudonarcissus;

PEA=Pisum sativum;

VFA=Vicia faba

Fuc- Lotus A=Tetragonolobus purpureas;

(GlcNAc)₂- DSA=Datura stramonium;

WGA=Triticum vulgare

GalNAc- HPA=Helix pomatia;

SBA=Glycine max;

VVA=Vicia villosa.

Glc- VSA=Vicia sativa

Gal- PNA=Arachis hypogaea;

BS-I=Bandeirae simplicifolia

MBA=Vigna radiata

3=strong positive reaction with large clumps; 2=strong positive reaction with moderate size clumps; 1=positive agglutination with fine clumps; w=weak agglutination; -=no agglutination.

Table 3 Strains of *H. pylori* agglutinated by lectins of various specificities

Sugar-specificity/Lectin	No. of <i>H. pylori</i> strains agglutinated	
	Coccoids	Spirals
α -D-Man, α -D-Glc/Con A	3	3
α -D-Man/LcH	3	0
α -D-Man/NPA	3	0
α -D-Man/PEA	3	3
D-Man, D-Glc/VFA	3	3
α -L-Fuc/Lotus A	3	2
(D-GlcNAc) ₂ /DSA	3	2
(D-GlcNAc) ₂ /WGA	2	2
D-GalNAc/HPA	3	3
D-GalNAc/SBA	2	3
D-GalNAc/VVA	1	3
D-Glc, D-Man/VSA	3	3
β -D-Gal(1-3)GalNAc/PNA	3	2
α -Gal, α -GalNAc/BS-I	3	2
α -Gal/MBA	2	3

Reproducibility testing revealed that lectin patterns were not altered after subculture. Among the three *H. pylori* strains, RH 54 was chosen since it showed agglutination with at least 13 out of 15 lectins tested. Of the 15 lectins, Con A, Lotus A and WGA which gave strong agglutination were chosen for further studies on the specificity of these lectin agglutination (Tables 4 and 5). Specific sugars, glycoproteins and mucin altered the lectin-bacterial agglutination reactions. As shown in Table 4, bovine submaxillary mucin reduced RH 54-Con A agglutination but did not alter the Lotus A and WGA binding patterns. Of great interest was the porcine stomach mucin, which not only reduced the binding ability to Con A and Lotus A but also completely inhibited RH 54-WGA binding. Fetuin slightly reduced RH 54 coccoids-WGA binding and the spirals-Con A agglutination patterns. Other glycoproteins like asialofetuin, bovine orosomucoid and gangliosides II display a considerable inhibitory ability on binding of RH 54 by Con A and WGA. More importantly, *H. pylori* Lotus A agglutination patterns were strong and were not inhibited by various glycoproteins with the exception of porcine stomach mucin that slightly reduced the coccoids-Lotus A binding capacity.

In the same way, Gal, GalNAc, GlcNAc, NANA, Glc and Suc affected inhibition on *H. pylori* lectin agglutination reactions except Lotus A agglutination pattern (Table 4). It was interesting to note that Man and Malt completely inhibited coccoids and spirals-binding to Con A. On the other hand, the strong agglutination of Lotus A with coccoids and spirals was completely inhibited by fucose (Table 4). In general, agglutination to Con A was either weakened or completely inhibited by carbohydrates. In contrast, WGA agglutination remained unaffected with *H. pylori* spirals whereas the agglutination with coccoids reduced.

RH 54 spirals and coccoids exhibited different agglutination patterns according to their pre-treatment (Table 5). Pre-treatment of the bacterial cells with formalin (1.0%), glycine HCl buffer (pH 2.2) and sulphuric acid (0.025 N) did not alter the agglutination patterns with lectins. In contrast, sodium periodate treatment of bacterial cells was shown to entirely inhibit agglutination reaction with Lotus A and WGA lectins, but reduce the agglutination with Con A. Of interest was the heating of *H. pylori* cells at 60°C for 1 hour which was shown to augment the agglutination reaction with all three lectins (Con A, Lotus A and WGA) tested (Table 5).

Enzymatic treatment of coccoids and spirals did not show marked inhibition of helicobacter-lectin binding patterns (Table 5). Neuraminidase, trypsin, pepsin, proteinase K and chymotrypsin did not change bacterial cells-Lotus A or bacterial cells-WGA agglutination. However, prior treatment of RH 54 coccoids with enzymes decreased agglutination with Con A.

Table 4 *H. pylori* lectin-binding inhibition

Inhibitor	RH 54 coccoids			RH 54 spirals		
	Con A	Lotus A	WGA	Con A	Lotus A	WGA
Glycoproteins						
Bovine submaxillary mucin	1	3	2	0	3	3
Porcine stomach mucin	1	2	0	1	3	0
Fetuin	2	3	W	W	3	3
Asialofetuin	1	3	1	W	3	3
Bovine orosomucoid	1	3	W	1	3	2
Gangliosides II	1	3	W	0	3	2
Carbohydrates						
Gal	1	3	1	W	3	3
GalNAc	1	3	1	W	3	3
GlcNAc	W	3	1	0	3	3
NANA	1	2	1	1	3	3
Man	0	3	1	0	3	3
Glc	W	3	1	0	3	3
Malt	0	3	1	0	3	3
Suc	W	3	1	0	3	3
Fuc	1	0	2	0	0	3
Control	2	3	2	1	3	3

The grade score to assess the reaction corresponds to Table 2.

Table 5 Effect of chemical and enzymatic treatments on *H. pylori* lectin binding

Inhibitor	RH 54 coccoids			RH 54 spirals		
	Con A	Lotus A	WGA	Con A	Lotus A	WGA
NaIO ₄ (0.075M)	W	0	0	W	0	0
H ₂ SO ₄ (0.025N)	1	3	2	1	3	3
Formalin(1.0%)	2	3	2	1	3	3
Glycine HCl buffer(pH 2.2)						
60°C(1 hour)	1	3	2	1	3	3
Neuraminidase	1	3	2	1	3	3
Trypsin	1	3	2	1	3	3
Pepsin	1	3	2	1	3	3
Proteinase K	1	3	2	1	3	3
Chymotrypsin	1	3	2	1	3	3
Control	2	3	2	1	3	3

The grade score to assess the reaction corresponds to Table 2.

DISCUSSION

In this study the carbohydrate residues on the cell wall of *H. pylori* spirals and coccoids were characterized by lectin agglutination performed on the glass slides. The observations that the use of glass slides for helicobacter-lectin agglutination is simple, cost-effective and rapid, was supported by Davidson *et al* (1982)^[18] and Wong *et al* (1986)^[21] in their studies on *Staphylococcus* and *Campylobacter* species, respectively. The microtitre plates were also used to study the interaction between bacterial cells and lectins for testing large numbers of strains^[20,29-30]. However, slide assays have the advantages of yielding results in less than two minutes. This finding concurred with that of Wong *et al.* (1986)^[21] who reported that slide agglutination assay was the procedure of choice for determining lectin reaction patterns among *Campylobacter jejuni* and *Campylobacter coli*. More importantly, interference from auto-agglutination is less frequent because bacterial cells-lectin agglutination occurs rapidly on glass slides before non-specific agglutination in the controls become evident.

The fifteen lectins evaluated in this study were selected because their major specificities were for common saccharides present on the bacterial surface. Typing of bacterial strains in epidemiological studies using plant lectins was found to be a powerful method for differentiating strains of related species^[18,20-21]. In this study, RH 54, NCTC 11637 and V₂ spirals were refractory to agglutination with LcH and NPA lectins, specific for mannose residues, implying that the spirals might have less affinity for mannose (Table 2). On the other hand, NCTC 11637 spirals did not react with Fuc-specific Lotus A, Gal-specific PNA and BS-I and (GlcNAc)₂-specific DSA and WGA lectins. It indicates that different lectin agglutination pattern shown by NCTC 11637, may be useful for discriminating it from RH 54, V₂ and other *H. pylori* strains.

Moreover, among *H. pylori* coccoids, RH 54 reacted with all lectins while NCTC 11637 was non-reactive with GalNAc-specific VVA and (GlcNAc)₂-specific WGA lectins (Table 2). In contrast, V₂ coccoids did not show reactivity to Gal-specific MBA and GalNAc-specific SBA or VVA lectins. The possible reason of different lectin agglutination patterns seen among the spiral and coccoid forms of three *H. pylori* strains studied, might be attributable to the considerable differences among different strains as well as the two morphological forms of the same strain in the affinity for the carbohydrate receptors of the host. This may eventually influence the adherence capabilities of *H. pylori* in gastric colonization resulting in different gastroduodenal pathology. It is

suggested that lectins can also be used for characterization of *H. pylori* strains which will confer as a useful method for epidemiological studies to determine the natural history and mode of transmission of *H. pylori* as was also reported by Ascencio *et al* (1990)^[23].

Lectins have been used to identify and distinguish microorganisms based on different sugar components on their cell surfaces^[14]. All the coccoids tested were shown to recognize Man-specific lectins, in contrast to the spirals (Table 3), suggesting that the coccoid forms of *H. pylori* might have predilection for Man-receptors of the gastric tissues. This could possibly be due to acquisition of Man-residues which may become available for binding gastric tissues during transformation from spirals to coccoids. Baczako *et al.* (1995)^[31] reported the differences in lectin binding properties of the antral and body surface mucosa of the human stomach and questioned whether it may be relevant for *H. pylori* affinity. The lectins from the jack bean, Con A as well as fava bean, VFA known to have an affinity for Man>Glc residues and another mannose-specific lectin, PEA reacted with three strains of *H. pylori* coccoids and spirals (Table 4). It indicates that Man/Glc residues present on the cell surface may contribute to their binding. Lectin histochemistry studies showed that the staining for mannose and glucose (bound by succinylated Con A) was negative in normal mucosa but was positive in *H. pylori* infected mucosa^[31]. It is therefore possible that *H. pylori* coccoid form having stronger affinity for mannose residues (Tables 3 and 4) might mediate its adherence to the corresponding mannose receptors existing in the gastric mucous cells for its colonization. It is suggested that the two differentiated forms of *H. pylori* might have various forms of Man-associated lectins which allow them to bind selectively to the mucous and epithelial cells thereby facilitating gastric colonization.

The specificity of bacterial agglutination by lectins resides in the unique cell surface structures of the bacteria interacting with the carbohydrate-specific lectins^[18,32-33]. The lectin from asparagus pea, Lotus A shown to an affinity for Fuc residues were recognized by three strains of *H. pylori* coccoids and spirals with the exception of NCTC 11637 spirals (Table 2). It was reported that fucose was more likely to function as a receptor for *H. pylori* related adhesion^[34]. It is possible that both the coccoid and spiral forms might have different Fuc specificities on the surface of the cells, contributing to the various degrees of *H. pylori* adhesion. Such different affinities might mediate their binding to gastric mucosa resulting in various forms of gastroduodenal pathology.

H. pylori coccoid forms may have retained the

lectin binding sites for Fuc determinants, in an unmodified form or could have been the components of the surface of the coccoids. Support of this view comes from the observations that several *Bacillus cereus* expressed different lectin binding sites on spores and vegetative cells^[20]. In addition, the lectin-staining pattern of surface mucous cells demonstrates, that there are mainly neutral carbohydrates (fucose and galactosamine) localised within the mucous granules^[12]. Therefore, it is possible that cell wall of *H. pylori* coccoids and spirals bearing different affinity for Fuc residues might influence the binding to different gastric mucous cells receptors to obtain various pathogenic potentials in *Helicobacter* infection.

N-acetylglucosamine specific lectins from wheat germ, WGA and jimson weed, DSA showed agglutination with RH 54 and V₂ spirals and coccoids (Tables 2 and 3). On the other hand, DSA agglutinated only NCTC 11637 coccoids. Interestingly, the refractory of NCTC 11637 spirals and coccoids agglutination with WGA suggests either masking of GlcNAc-residues, absence of these units, or exposure of these units may differ between *H. pylori* strains. RH 54 and V₂ spirals showed stronger agglutination reaction with WGA lectins than the coccoids (Table 2). This could be due to the acquisition of thick polysaccharide capsule on the coccoidal cells^[35] resulting in weaker agglutination with WGA lectin. A similar mechanism was demonstrated on *Neisseria* species in which WGA was shown to specifically agglutinate *Neisseria gonorrhoeae* but not the encapsulated *N. meningitidis* suggesting that agglutination with WGA is due to the lack of a capsule on the gonococci^[16].

All the spirals studied were shown to recognize GalNAc-specific lectins from HPA, SBA and VVA (Table 3) as was shown earlier by Ascencio *et al* (1990)^[23]. *H. pylori* coccoids showed weaker affinity for GalNAc residues (Tables 2 and 3) suggesting that structural modifications might occur on the cell wall of the coccoids during morphological transformation from the spirals. It is proposed that *H. pylori* coccoids, a morphological variant of the spirals, might bind with different capacities to galactosamine receptors localised within gastric mucous granules, as was also observed for the spirals by Bode *et al.* (1988)^[12]. After binding to the host cells, it may then take part in various mechanism of adhesion resulting in the gastroduodenal disease. Adhesion is considered as one of the virulence factors that allow *H. pylori* spirals to survive in the hostile gastric environment. Its ability to adhere to gastric mucosal cells and mucus^[7,9,36] suggests that the coccoids may also use the same adherence mechanism for gastric colonization.

The ability of three strains of coccoids as compared to all spirals in agglutinating Glc-specific lectin (VSA) (Table 3) could be due to the retention of Glc residues on the cell surface of the coccoid forms during morphological conversion. Since the cell wall is an important virulence factor in many bacteria^[25], the cell wall of *H. pylori* coccoids bearing similar Glc residues like the spirals, may be attributable to the virulence.

The absence of agglutination with PNA, which detects β-D-Gal (1-3) GalNAc and with BS-I, which detects α-Gal and α-GalNAc, suggests that these carbohydrates are not present on the reactive surface of NCTC 11637 spirals (Tables 2 and 3). This agglutination pattern might aid in differentiating *H. pylori* spirals. It was also shown by Ascencio *et al.* (1990)^[23] who developed the lectin typing system of 50 *H. pylori* strains for epidemiological studies. On the other hand, Gal-specific MBA lectin did not agglutinate V₂ coccoids (Table 2) indicating the lack of this sugar on the cell wall of the coccoids.

Porcine stomach mucin completely inhibited RH 54-WGA binding (Table 4) verifying that the agglutination is specific and this glycoprotein has the highest affinity for that lectin. Inhibition studies with various carbohydrates suggest that Glc, Fuc, Man, Gal and GlcNAc are present on the surface of *H. pylori* coccoids and spirals indicating that this application may provide valuable information about the specific sugars present on the cell surface of the two differentiated forms of *H. pylori*. In an earlier study, Facinelli *et al.* (1994)^[30] stated that *Listeria*-lectin binding inhibition studies were useful for characterization of specific sugars present at the cell surface of an organism.

RH 54 coccoids and spirals were found to be non-reactive with Con A, Lotus A and WGA lectins after sodium periodate treatment (Table 5) suggesting that NaIO₃ probably had destroyed the surface carbohydrate structures of these cells. A similar observation was made by Ascencio *et al.* (1990)^[23]. However, the reactions were shown to be resistant to sulphuric acid (0.025N), glycine hydrochloride (pH 2.2) and formalin (1.0%) indicating that these chemicals were not effective to alter the *H. pylori* lectin binding.

Treatment of *H. pylori* cells at 60°C for 1 hour was shown to give stronger agglutination capacities with Con A, Lotus A and WGA lectins (Table 5). It could be possible that heating releases the concealed carbohydrate residues to be exposed onto the surface of *H. pylori* spirals and coccoids. Heating was found to eliminate interference of non-specific cell agglutination. A comparable finding was reported on *Campylobacter jejuni* and *C. coli* that bacterial interaction with lectins was greatly

enhanced by heating the cultures to 100°C and holding for 30 to 60 minutes^[21]. They found that heating had made it feasible to type most rough and auto-agglutinating strains as well as smooth cultures. Pre-treatment may therefore be a prerequisite for agglutination with lectins. Pre-treatment was shown to augment the agglutination reactions of *Streptococcus* species^[25] although it increases the time required to carry out the assay^[24,37].

RH 54 coccoids-Con A agglutination patterns changed after treatment with enzymes such as, neuraminidase, trypsin, pepsin, proteinase K and chymotrypsin (Table 5). It could be due to the partial destruction of the bacterial surface structures by enzymes resulting in weaker agglutination reactions. However, Lotus A and WGA lectins did not alter the agglutination patterns, indicating that pretreatment with enzymes could probably be necessary for the lectin typing assays. It agrees with the studies on streptococci that when trypsin or other proteolytic enzymes partially hydrolyse proteoglycans, some carbohydrate residues may become available for binding^[25,37]. At the same time, other carbohydrate residues may be destroyed or modified. This study shows that lectin-bacterial agglutination patterns may differ according to the pre-treatment of the bacteria. In addition, the bacterial surface structures might be partially destroyed by enzyme treatment as carbohydrate residues dissolve in the buffer while others, previously hidden, then become exposed on the surface^[25]. Enzymatic treatment may therefore be useful for typing *H. pylori* as was also reported by Ascencio *et al.* (1990)^[23].

Lectin reactions provide an attractive alternative method for typing microorganisms with minimum specialised facilities. In addition, lectin agglutination assay is rapid, inexpensive, reproducible, require no special equipment, simple to perform and a useful method for epidemiological studies. This study shows that lectins are stable and easy to store, commercially available and active at low concentration. However, lectin agglutination may be affected by culturing conditions, growth on solid or liquid media and is affected by specific treatment such as trypsin treatment or boiling before assay. More importantly, amoxicillin-induced coccoids tend to give auto-agglutination reaction. This could explain the divergence of structural changes on the cell surface of the coccoids due to the antibiotics or aging process.

It is apparent that the surface of *H. pylori* contains a variety of carbohydrates which may play a role in adherence phenomenon. Comparison of lectin agglutination reactions between the coccoids and spirals show that different kinds of sugar residues might be present on the two forms which might provide evidence for the complexity of the

adherence process. It is possible that *H. pylori* coccoids and spirals, bearing different affinities for carbohydrate receptors on the cell surface may account for the variation in the adhesive properties of the organism for antral mucous cells in the stomach. This may probably reflect different strategies used by the two differentiated forms of *H. pylori* in colonizing gastroduodenal tissues, resulting in different disease profiles such as acute and chronic gastritis or peptic ulcer.

With regard to bacterial surface lectins, which often play a role in the initial step of immune defence against phagocytosis, it is assumed that both spiral and coccoid forms may involve in the interaction of *H. pylori* host immune mechanisms. As a rule, *H. pylori* infection is almost always associated with inflammation^[9]. However, peptic ulcer disease and gastric carcinoma occur only in a subset of individuals infected chronically with *H. pylori*^[9]. Therefore, it is proposed that, like spirals, coccoid forms also play an important pathogenic role in *H. pylori* infection.

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Growth-inhibiting effects of taxol on human liver cancer *in vitro* and in nude mice

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Subject headings paclitaxel; liver neoplasms; apoptosis; mitosis; *in vitro*; DNA; RNA; microscopy, wection; mice, nude

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Abstract

AIM To investigate the effects of taxol on SMMC-7721 human hepatoma and its mechanisms.

METHODS *In vitro* cell growth was assessed by trypan blue exclusion method. Experimental hepatoma model was established by seeding SMMC-7721 cells subcutaneously into Balb/c (nu/nu) nude mice. *In vivo* tumor growth was determined by measurement of tumor diameter with Vernier calipers. The syntheses of DNA, RNA and protein were analyzed by incorporation of ³H-thymidine, ³H-uridine and ³H-leucine respectively. Using light and electron microscopes to observe the morphological changes of cells including mitosis and apoptosis.

RESULTS Taxol was effective against SMMC-7721 human hepatoma cell growth in the ranges of 2.5nmol/L-10nmol/L- with mitotic arrest and apoptosis *in vitro*. DNA, RNA and protein syntheses in cells were also obviously suppressed by *in vitro* treatment of taxol for 72h. Taxol at 2.5nmol/L reduced ³H-thymidine uptake to about 34% of the control value ($P < 0.05$). Increasing the dose of taxol to 20nmol/L resulted in a greater decrease in ³H-thymidine incorporation to 60% of the control value ($P < 0.01$). At a concentration of

20nmol/L, the ³H-uridine and ³H-leucine uptakes were reduced to 52% ($P < 0.05$) and 63% ($P < 0.01$), respectively. *In vivo*, taxol significantly inhibited SMMC-7721 tumor growth at 10mg/kg, i.p. once daily for 10d. A more than 90% decrease in tumor volume was observed by day 11 ($P < 0.01$) similarly with mitotic arrest and cell apoptosis.

CONCLUSION Taxol has a marked anticancer activity in SMMC-7721 human hepatoma both *in vitro* and in nude mice. Its mechanisms might be associated with mitotic arrest, subsequently, apoptosis of the hepatoma cells. No obvious toxicity was observed with *in vivo* administration of taxol.

INTRODUCTION

Taxol, a stimulator of tubulin polymerization and microtubule assembly, was initially considered promising because of its cytotoxic activity against several tumor cell lines *in vitro*^[1,2]. Subsequent *in vivo* studies demonstrated that taxol was effective against a variety of murine tumors and human xenografts^[3,4], especially advanced human carcinomas refractory to conventional chemotherapy^[5,6]. Now, taxol has emerged as one of the most active anticancer agents in clinic for the therapy of ovarian, breast, and non-small-cell lung cancer^[7,8]. Hepatocellular carcinoma (HCC) is among the most common malignancies in the world. The incidence rate is as high as 34 persons per 100000 per year in some high-incidence areas in Asia such as China. However, few studies show that taxol is effective against HCC, especially *in vivo*^[9-13]. Furthermore, HCC is usually insensitive to chemotherapeutic drugs used previously in clinic and there is thus an urgent need for the evaluation of new active drugs against HCC. Therefore, we systematically investigated the activities of taxol against HCC *in vitro* and in nude mice.

Apoptosis is an active cellular death process induced by physiological or pathological factors^[14]. Most anticancer agents cause cell death by apoptosis^[15]. Incubation of cells with taxol has been

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found to result in cell cycle arrest at G₂/M phase and cell apoptosis in many tumor cell lines^[16,17]. Our previous *in vitro* studies also show that SMMC-7721 human hepatoma cells treated with taxol exhibit significant G₂/M phase arrest and subsequently occurred apoptosis^[18]. However, Milross *et al.*^[11] reported that taxol caused mitotic arrest but could not induce apoptosis in hepatoma cells. The present studies indicate that taxol can induce mitotic arrest and apoptosis in SMMC-7721 cells not only *in vitro* but also *in vivo*.

MATERIALS AND METHODS

Drugs

Taxol was obtained from Shanghai Hualian Pharmaceutical Factory, Shanghai, China. For *in vitro* experiments, it was dissolved in dimethyl sulfoxide (DMSO) to make stock solution, which was then diluted to desired concentrations with the culture media. The DMSO concentration was kept under 0.001% in all the experiments that did not exert any detectable effect on cell growth or apoptosis. For *in vivo* experiments, taxol was dissolved in a Cremophor EL vehicle (Cremophor: alcohol=50:50, v/v) at a concentration of 6 µg/L. This stock solution was further diluted with normal saline (1:5.2, v/v) immediately before injection. 5-Fluorouracil was from Shanghai Puguang Pharmaceutical Factory, Shanghai, China and diluted with normal saline to desired concentration before injection.

Cell culture and cell viability assay

SMMC-7721 human liver carcinoma cell line was obtained from Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China). The cells were grown on monolayer cultures in 37°C, 5% CO₂ incubator in RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 15% heat-inactivated new-born calf serum with or without compounds as described above. At assay time, cells were collected, mixed with an equal volume of phosphate buffer salt solution containing 4g/L trypan blue dye, and manually counted. Actual cell numbers were calculated by multiplying diluted times compared with initial cell numbers.

Animal

Female Balb/c (nu/nu) nude mice (18 g - 20 g; 6wk-8wk of age) were purchased from Shanghai Institute of Materia Medica, Chinese Academy of Sciences and maintained in our own specific pathogen-free mouse colony. All experiments involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Measures of biomacromolecular synthesis

According to the previous literature^[19], the syntheses of DNA, RNA, and protein of cells were measured by quantitating ³H-thymidine, ³H-uridine, and ³H-leucine incorporation, respectively, in the presence and absence of taxol. Briefly, cells were seeded into each well of 96-well Costar flat-bottom culture plates (Costar, Charlotte, NC) at 5×10³ cells/well in 100 µL of culture medium. After 24h, taxol was added into each well according to the desired concentrations. Control samples contained culture medium and DMSO. After 48h, the cells were incubated with ³H-thymidine, ³H-uridine, or ³H-leucine (1 µCi/well) for 24h at 37°C respectively. At the end of the incubation, the plates were immediately placed on ice and frozen at -80°C. After thawing, ³H-labeled DNA, RNA, or protein were precipitated onto glass fiber discs (Whatman Ltd, Maidstone, UK) using a micro-harvester. For harvesting, the wells were extensively rinsed with distilled water, then, air-dried. The discs were then added to scintillation vials along with 1 mL of scintillant. Radioactivity was measured on a Beckman LS6000 TAβ counter (Beckman Instruments, Irvine, CA).

Human hepatoma implantation into nude mice

SMMC-7721 human solitary hepatoma was implanted into the right flank of female nude mice by subcutaneous injection of 2.5×10⁶ viable cells. Tumor size (mm³) was evaluated by measurement of two perpendicular diameters with Vernier calipers and using the formula 1/2LW², where L is the longest diameter and W is the diameter perpendicular to L^[20]. Palpable tumors were usually detected after 1wk and reached 5mm to 10mm in diameter by day 10. When tumor grew to 5mm-10mm in diameter, the mice were treated with taxol or vehicle by i.p. injection in a volume of 0.2 mL per mouse, once daily for 10 days. Body and internal organ weight of animals were also observed to assess the toxicity of taxol.

Microscopic observation

In vitro, cells were fixed with 25 g/L glutaraldehyde, and postfixed with 20g/L osmium tetroxide. After dehydration, the samples were embedded in Epon 812 epoxy resin, and then sectioned. The sections were routinely stained. *In vivo*, after the mice were killed by cervical dislocation, tumor tissue was immediately excised and placed in neutral-buffered formalin. The tissue was then imbedded in paraffin blocks from which 4 µm sections were cut and stained with hematoxylin and eosin. The morphologies of cells including mitosis and apoptosis were examined under electron and light microscopes.

Data analysis

The data of *in vitro* cell growth and the incorporation of ^3H -thymidine, ^3H -uridine and ^3H -leucine into cells were analyzed by Student's *t* test. Statistical comparisons of tumor volumes were performed by analysis of variance at different time points after initiating treatment.

RESULTS

In vitro studies

SMMC-7721 cells were treated with different concentrations of taxol for different times and the effect of taxol on the cell growth was examined by trypan blue exclusion method. As shown in Figure 1, the growth of SMMC-7721 cells was markedly inhibited by taxol in the ranges of 2.5nmol/L-10nmol/L. Moreover, taxol-induced cytotoxicity was found to be time-dependent when the cells were exposed to the agent for 48h-120h.

Taxol was significantly effective at inhibiting ^3H -thymidine incorporation with dose-dependent relationship when SMMC-7721 cells were continuously exposed to taxol for 72h. Taxol at 2.5nmol/L reduced ^3H -thymidine uptake to about 34% of the control value ($P<0.05$). Increasing the dose of taxol to 20nmol/L resulted in a greater decrease in ^3H -thymidine incorporation to 60% of the control value ($P<0.01$) (Figure 2). Taxol also displayed similar dose-dependent efficacy at suppressing ^3H -uridine and ^3H -leucine incorporation when the cells were constantly exposed to the drug for 72 h. At a concentration of 20 nmol/L, the ^3H -uridine and ^3H -leucine uptake was reduced to 52% ($P<0.05$) and 63% ($P<0.01$), respectively (Figure 2).

These results show that taxol has significant inhibiting effects on DNA, RNA and protein syntheses of SMMC-7721 cells. The results from light microscope observation indicated that taxol at 10nmol/L caused obvious morphologic changes of SMMC-7721 cells when compared with untreated cells (Figure 3A). After 24h treatment (Figure 3B), the cells got round, dipter-enhanced. As time went on for 48h, cells detached and blebbed (Figure 3C). By analysis with electron microscope, it was found that SMMC-7721 cells exposed to taxol 10nmol/L for 24h presented mitotic arrest (Figure 4B), and some cells appeared in pre-apoptotic sign as compared with untreated cells (Figure 4A). After cells were treated by taxol for 48 h, the nuclei were shrank, and the chromatin was condensed to form high density clumps, which were located along the nuclear envelope (Figure 4C), or formed regularly shaped crescents at the nuclear edges. It is suggested that taxol could induce typical characteristic morphological changes of mitotic arrest and apoptosis of cells.

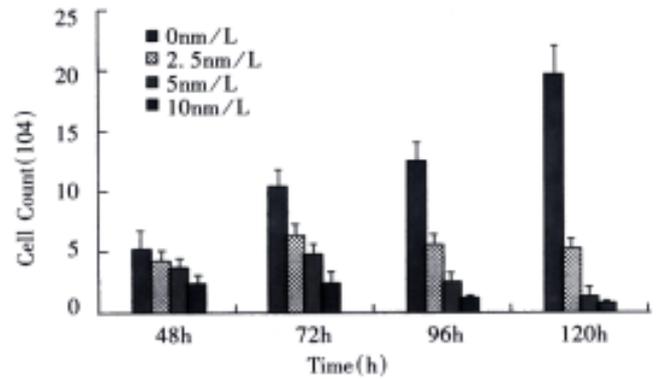


Figure 1 Effect of paclitaxel on SMMC-7721 cell growth.

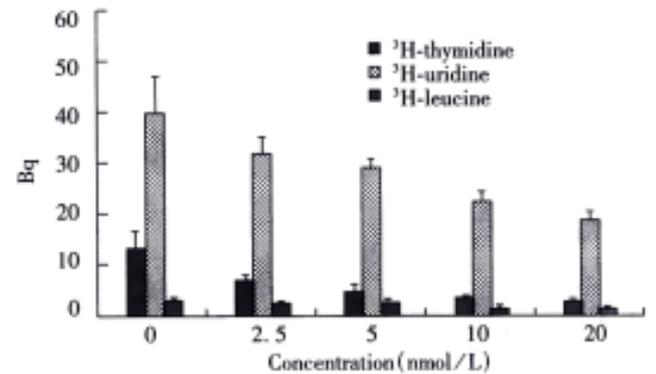


Figure 2 Effects of taxol on ^3H -thymidine, ^3H -uridine and ^3H -leucine incorporation.

In vivo studies

Nude mice bearing SMMC-7721 tumors were treated with different doses of taxol. The taxol response of SMMC-7721 human hepatoma depicted as tumor growth curves in Figure 5 showed that tumors were significantly responsive to taxol when treated at 10mg/kg, i.p. once daily for 10d, although treatment with 2 mg/kg proved less effective. Tumor regression was evident by day 3 after taxol treatment ($P<0.05$), and a greater than 90% decrease in tumor volume was observed by day 11 ($P<0.01$). In contrast, the tumors in animals that only received corresponding solvent continued exponential growth. Mice treated with low or high doses of taxol did not display a body weight loss by day 11 ($P>0.05$). No difference of internal organ weight was found between treated and control groups. No obvious other toxicity was observed in mice receiving either 2 mg/kg or 10 mg/kg taxol for 10 d.

Animals were sacrificed at various times among 24 h and 120h after administrating taxol. As seen in Figure 6, taxol treatment caused marked morphological changes in SMMC-7721 tumor. Compared with control (Figure 6A), slight mitotic arrest and apoptosis of hepatoma cells were observed in mice with 2mg/kg taxol treatment for 10 days (Figure 6B). However, most cells exhibited the characteristic appearance of agent-induced

apoptosis after treated with 10mg/kg taxol for 10 days (Figure 6C). These results suggested that taxol-induced mitotic arrest and apoptosis of human hepatoma cells might be dose-dependent. Time-effect analysis indicated that taxol at 10mg/kg, i.p. for 24h caused mitotic arrest of most tumor cells with slight apoptosis (Figure 6D). By 48h, apoptosis became the dominant morphological features (Figure 6E). This phenomenon was more significant after 120h treatment than ever before (Figure 6F). Apoptotic cells were characterized by overall shrinkage and homogeneous dark basophilia. Frequently, several small apoptotic fragments were encountered in close proximity. On the basis of size and clustering, such fragments were considered to represent the remains of a single cell or apoptotic body.

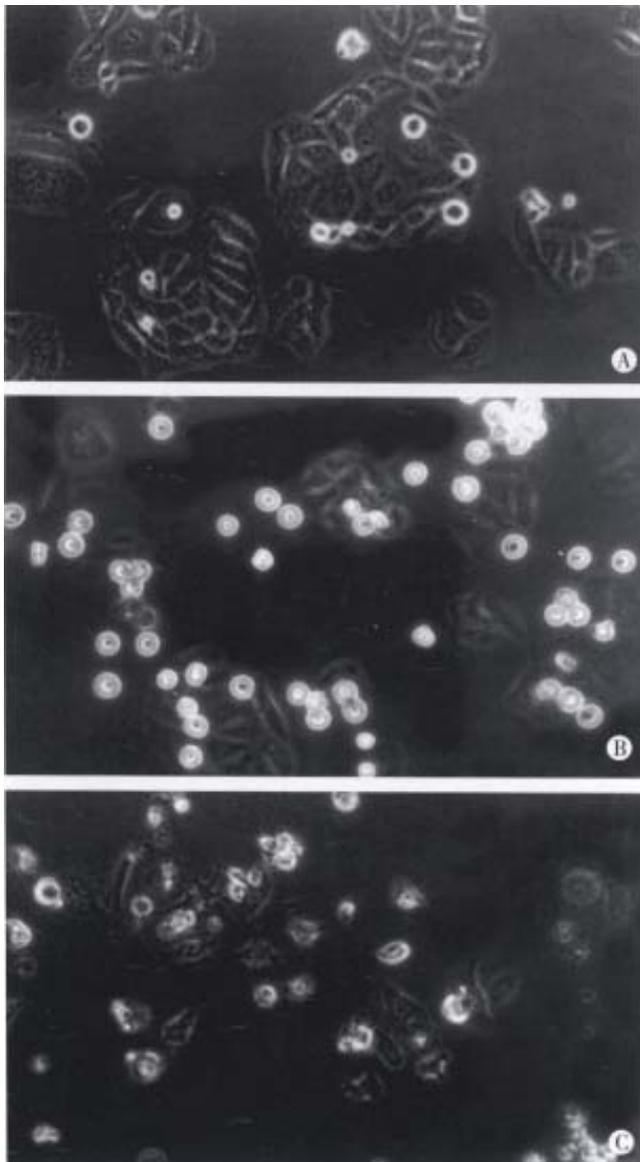


Figure 3 Morphological changes of SMMC-7721 cells observed by light microscope after treatment with taxol for different times. (A) Untreated cells; (B) SMMC-7721 cells got round, dipter-enhanced after exposure to 10 nmol/L taxol for 24 h; (C) SMMC-7721 cells detached and blebbed after exposure to 10nmol/L taxol for 48h. $\times 400$.

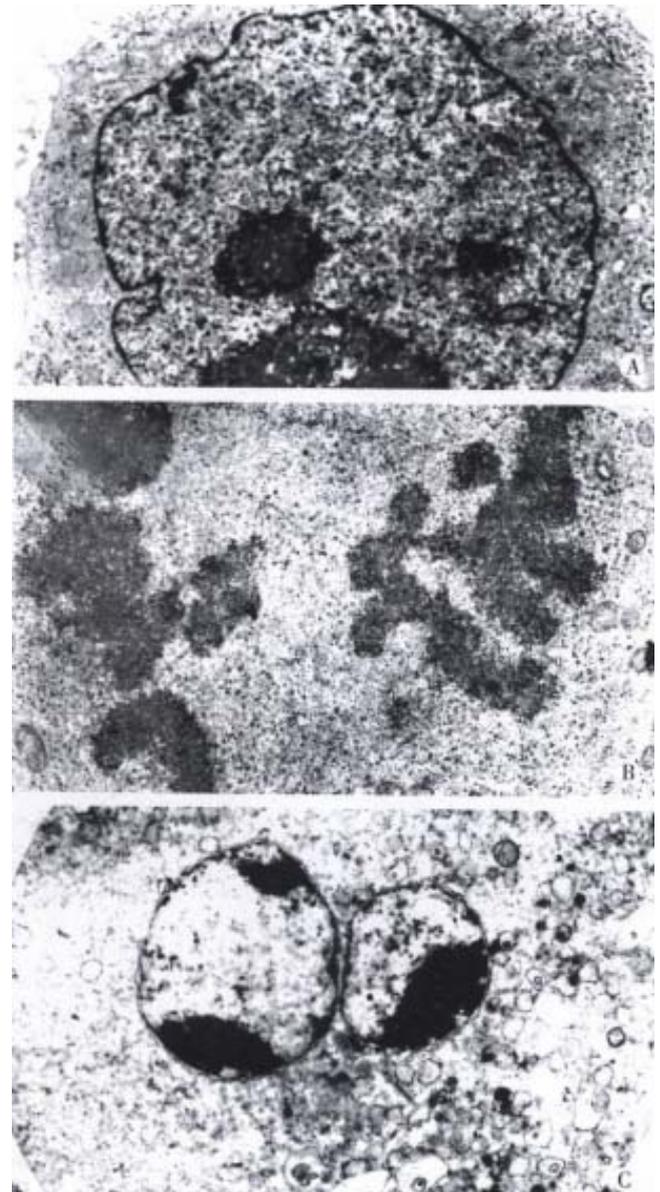


Figure 4 Ultrastructures of SMMC-7721 cells with or without treatment by taxol. Following 24h treatment of taxol, cells presented more mitotic Figure (B 7680 \times). For 48h, the cells appeared with regularly shaped crescents (C 7680 \times). The nuclei of untreated cells were very irregular in shape, with many gulfs and protrusions. (A 5760 \times)

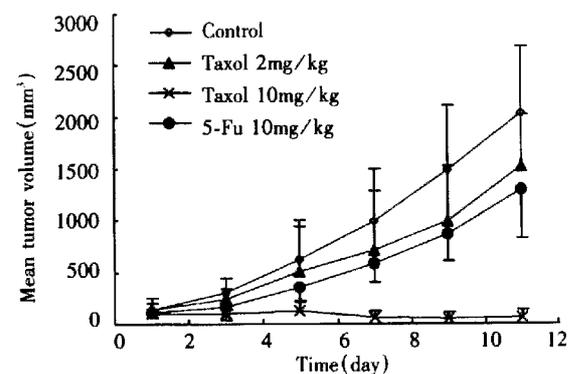


Figure 5 Effect of taxol on SMMC-7721 human hepatoma in nude mice.

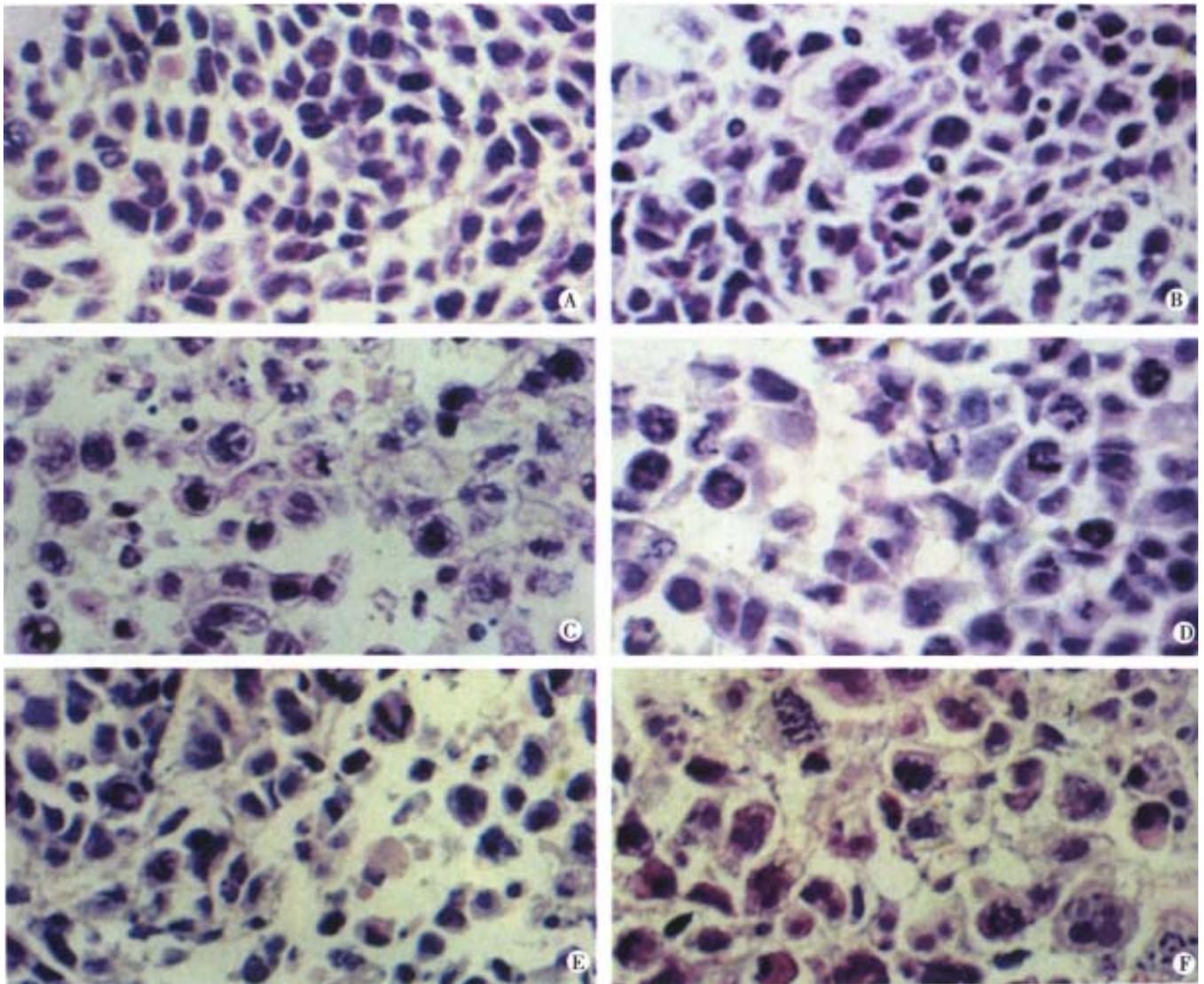


Figure 6 Histological changes of SMMC-7721 tumors after taxol treatment. (A) untreated control. (B) 2mg/kg taxol, i.p. once daily for 10d. Only slight morphological changes were presented. (C) 10mg/kg taxol, i.p. once daily for 10d. Most cells showed apoptotic changes and many apoptotic bodies were presented. Apoptosis were widespread. Apoptotic bodies were the small ovoid structures. (D) 24h after i.p. injection of 10mg/kg taxol. Mitotic arrest was marked. (E) 72h after i.p. injection of 10mg/kg taxol. Mitotic arrest could still be presented with more apoptotic cells. (F) 120h after i.p. injection of 10mg/kg taxol. Apoptosis was dominant phenomenon than ever before. Hematoxylin and eosin. $\times 400$

DISCUSSION

This study has demonstrated that taxol is significantly effective against SMMC-7721 human hepatoma growth both *in vitro* and *in vivo*. In most reports the arrest of cells in G₂/M phases and cell apoptosis caused by taxol are responsible for its anticancer activities^[21,22]. We have also found that taxol causes SMMC-7721 cell mitotic arrest and apoptosis *in vitro* and *in vivo*. It suggests that the anti-hepatoma effects of taxol are related to its mitotic arrest and cell apoptosis. Emphasis has been placed on the anti-hepatoma effects of taxol *in vivo*. Previous studies showed that taxol had no significant anticancer effect in HCC patients^[13]. Others reports also suggested no statistically significant hepatoma growth inhibition in mice

treated with taxol at 40 mg/kg, i.v.^[11]. The results presented herein indicate that taxol at 10 mg/kg, i.p. once daily for 10 days has significant inhibiting activity on HCC with more than 90% suppressive percentage and without any obvious toxicity. It is inferred that taxol may cause hepatoma growth inhibition at low dose and continuous administration. Our present studies also clearly demonstrate that taxol causes profound mitotic arrest and apoptosis. A time-course microscopic analysis of tumor cell mitosis and apoptosis in nude mice treated with taxol revealed that mitotic arrest occurred previously to apoptosis. Development of apoptosis lagged several hours behind mitotic arrest. Moreover, either the mitotic arrest or apoptosis of hepatoma cells induced by

taxol was clearly dose and time- dependent. These data have important therapeutic implications in HCC.

In conclusion, this is the first report of the anti-hepatoma effect of taxol, its mechanisms, and the relationship between the mitotic arrest and apoptosis both *in vitro* and *in vivo*.

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Inhibitory effects of *Curcuma aromatica* oil on proliferation of hepatoma in mice

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Subject headings *Curcuma aromatica*-oil; liver neoplasms; cell proliferation; mice

Wu WY, Xu Q, Shi LC, Zhang WB. Inhibitory effects of *Curcuma aromatica* oil on proliferation of hepatoma in mice. *World J Gastroentero*, 2000;6(2): 216-219

Abstract

AIM To reveal the inhibitory effects of *Curcuma aromatica* oil (CAO) on cell proliferation of hepatoma in mice.

METHODS Two tumor inhibitory experiments of CAO on hepatoma in mice were conducted. The inhibitory effects of CAO on proliferation of hepatoma in mice were evaluated by DNA image cytometry and immunohistochemical staining of proliferating cell nuclear antigen (PCNA).

RESULTS The tumor inhibitory rates of CAO were 52% and 51% in two experiments, respectively. Compared with those of the saline-treated control groups, both differences were statistically significant ($P < 0.01$). In the group of mice treated with CAO, the cellular nuclear DNA OD value (249 ± 70), are as ($623 \mu\text{m}^2 \pm 228 \mu\text{m}^2$) and DNA (2.38 ± 0.67) index of hepatic carcinomas were significantly lower than those of the control group (430 ± 160 , $1073 \mu\text{m}^2 \pm 101 \mu\text{m}^2$ and 4.48 ± 0.71). CAO also could increase diploidy cell rates ($29.00\% \pm 9.34\%$ vs $2.97\% \pm 5.69\%$, $P < 0.01$) and decrease pentaploidy cell exceeding rate ($30.04\% \pm 15.10\%$ vs $70.89\% \pm 14.94\%$, $P < 0.01$). In the group of mice treated with CAO, the labeling indexes of proliferating cell nuclear

antigen (PCNA-LI) were $30\% \pm 4\%$, which were significantly lower than $40\% \pm 6\%$ of the control group ($P < 0.01$).

CONCLUSION The inhibition of CAO on the growth of hepatoma in mice might be associated with its depression on cellular proliferative activity.

INTRODUCTION

Curcuma aromatica oil (CAO) is a volatile oil extracted from a traditional Chinese herb, *Curcuma aromatica* Salisb, which exerts various medical activities such as promoting blood circulation to remove blood stasis and treating cancers^[1]. It contains several major anti-tumor active ingredients: elemicin, curcuminol, curdione, etc^[2-5]. Our previous clinical and experimental studies revealed that CAO infused via hepatic artery had ideal therapeutic effects on both the patients with primary liver cancer and the rats with transplanted hepatoma^[6,7]. However, the anti-hepatoma mechanisms of CAO remain unresolved. The aim of the current study therefore is to determine the inhibitory effects of CAO on proliferation of hepatoma in mice.

MATERIALS AND METHODS

Materials

Ten g/L of CAO injections were prepared by the department of preparations in our hospital (Lot. 97041601). The 5-fluorouracilum (5-FU) was produced by Hebei Pharmaceutical Factory (Tianjin, Lot 970277). EMAIL-100 type automated Image Cytometry (ICM) was purchased from Yiming Company (Guangzhou). *Immunohistochemical* LSAB kit (K0679) and monoclonal antibody PC10 (M0879) to proliferative cell nuclear antigen (PCNA) were purchased from Dakopatts, Glostrup, Denmark. The statistics software package named Medical Statistics of China Medical Encyclopedia was provided by Department of Health Statistics of West China Medical University.

Animals

Kunming mice, male, weighing 18g-22g, were provided by the Laboratory Animal Center, the First Military Medical University. The ascites

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hepatoma carried by mouse was produced by the Cancer Institute of Sun Yat-Sen University of Medical Sciences.

Anti-hepatoma experiments

Two anti-tumor experiments were performed in the Grade II animal laboratory of our hospital. The hepatoma ascites carried by mouse was collected and was diluted with normal saline (NS) to 2×10^{10} cancer cells/L. The diluted hepatoma cells were put on ice during the implantation, and 0.2mL of hepatoma cells were subcutaneously transplanted to the right axilla in mice. All operations were performed under sterile conditions. After transplanted for 24h, the mice were numbered and randomly allocated into three groups: negative control group, receiving 0.2mL NS, ip; positive control group, given 20mg and 40mg/kg of 5-FU, ip; and treatment groups, given 100mg/kg of CAO, ip. The mice were housed in plastic cages under a 12-hour light/dark cycle and fed with a standard pellet diet and distilled water *ad libitum*. Some of the experimental details are shown in Table 1. At the following day of the termination of treatment, the mice were sacrificed with dislocation of neck. All tumors were peeled off, weighed and the tumor inhibitory rates were calculated. After the second experiment, all tumor specimens were fixed with 100mL/L neutral formalin for less than 48 hours, embedded in paraffin, and cut into 4 μ m thick series.

DNA image cytometry

The sections were dewaxed with xylene, rehydrated in decreasing concentrations of ethanol and the DNA staining was ordinarily performed with Foulgen (Figure 1). The cells in the selected visual fields were scanned in super-speed with a television monitor and the DNA histograms were obtained by calculating the integrated optical density with a computer (EMAIL-100). Several visual fields were randomly observed and 50-100 nuclei were detected for each section. The DNA in the normal mouse hepatic cells at G₀/G₁ phase was employed as the diploid (2C) control. The measurable cellular parameters and paraphrases by ICM were displayed as the follows. DNA optical density (DO) and nuclear area (NA) reflect respectively DNA quantity and nuclei size in hepatoma cells. DNA index (DI) represents the ratio of DNA quantities at G₀/G₁ phase between hepatoma cells and normal mouse liver cells, 5CER, 2C and 3-5C stand for pentaploidy cell exceeding rate, percentages of diploid cells and of the cells except 5CER and 3C-5C, respectively.

PCNA staining

Immunohistochemistry with monoclonal antibody against mouse PCNA was performed by the link

streptavidin-biotin peroxidase method (LSAB kit). Briefly, after sections were deparaffinized with xylene and rehydrated through graded alcohol, they were treated with 30mL/L H₂O₂ for 10 minutes to inactivate endogenous peroxidase activity. Following a 20-minute blocking step with normal horse serum, the sections were sequentially incubated with PC10 antibody diluted 1:100 for 30min, then in anti-rabbit IgG and in avidin-peroxidase complex for 30 minutes each. The immunoreaction was developed using 3',3'-diaminobenzidine in the presence of H₂O₂ to produce a brown precipitate. The sections were counterstained with hematoxylin. Positive and negative controls were included in each experiment. Specifically, for the latter the primary antibody was substituted with nonspecific rabbit IgG.

PCNA labeling index

All of the brown nuclei were regarded as positive for PCNA. The PCNA labeling index (PCNA-LI) was determined by observing more than 2000 nuclei in a few areas of the sections, and the percentage of PCNA-labeled nuclei was used for the analysis. To reduce any interobserver bias, the PCNA-LI of all sections was examined by two experienced pathologists who had no knowledge of the experimental grouping. The mean of the two counts of each observer was considered to be the PCNA-LI.

Statistical analysis

A statistical evaluation was performed using analysis of variance and *t* test. All statistical analyses were considered to be significant at a *P* value of 0.05 or less.

RESULTS

Inhibitory effects of CAO on hepatoma in mice

The results from the two experiments showed that the tumor inhibitory rates of CAO on hepatoma were 51.85% and 51.2%, respectively (*P*<0.01) (Table 1). Up to the termination of anti-tumor experiment, the body weight in the mice treated with 5-FU tended to decrease, while in the mice treated with CAO, it increased but lighter than that in the negative control group (*P*<0.05).

Effects of CAO on the DNA in the hepatoma cells of mice

As shown in Table 2, the DO, NA, DI and 5CER in the mice treated with CAO were lower than those in the control mice. CAO could increase the percentage of 2C hepatoma cells and had no significant effect on the percentage of 3.5C cells.

Effects of CAO on the expressions of hepatoma PCNA in mice

As shown in Table 3 and Figures 2 - 3, the percentages of PCNA positive nuclei in the mice treated with either CAO or 5-FU were statistically lower than those in the negative control mice (*P*<0.01).

Table 1 Inhibitory effects of CAO on growth of hepatoma in mice

Experiment	Drugs	Dose (kg ⁻¹ ·d ⁻¹)	Number	Body weight change (g)	Tumor weight (g)	Tumor inhibitory rate (%)
1	Ns	0.2mL	7	6.90	1.08±0.22	
	5-FU	40mg	7	-2.00	0.35±0.13	67.6 ^b
	CAO	100mg	7	4.35	0.52±0.16	51.8 ^b
2	Ns	0.2mL	10	5.55	1.72±0.45	
	5-FU	20mg	10	-1.85	0.45±0.15	73.8 ^b
	CAO	100mg	10	4.60	0.84±0.31	51.2 ^b

Results are expressed as $\bar{x}\pm s$, $n=10$, ^b $P<0.01$, vs negative control group.

Table 2 Effects of CAO on the hepatoma DNA parameters detected with ICM in mice

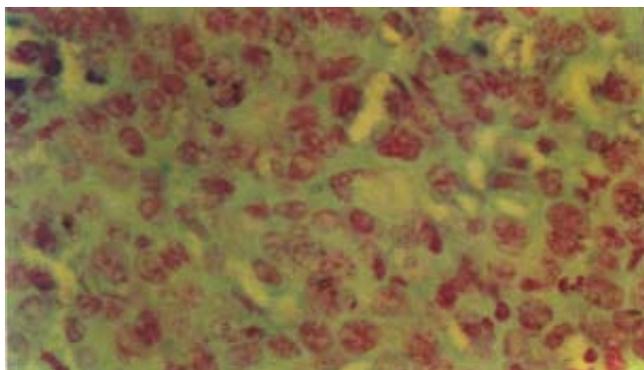
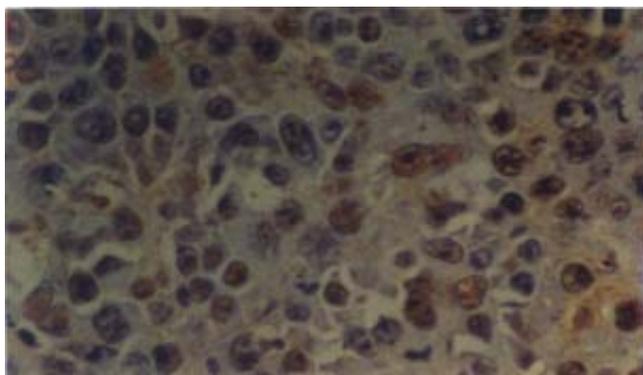
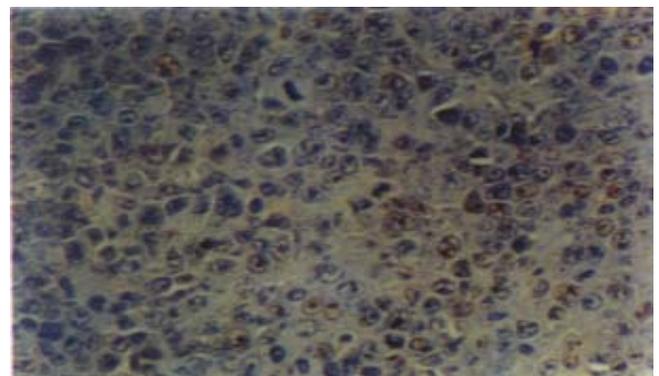
Group	DO	NA(μm ²)	DI	5CER(%)	2C(%)	3-5C(%)
Negative control	430±160	1073±101	4.48±0.71	70.9±14.9	2.97±5.69	28.2±16.0
CAO	249±70 ^b	623±228 ^a	2.38±0.67 ^b	30.0±15.1 ^b	29.00±9.34 ^b	41.0±17.4

Results are expressed as $\bar{x}\pm s$, $n=10$, ^a $P<0.05$, ^b $P<0.01$, vs negative control group.

Table 3 Effects of CAO on the expressions of hepatoma PCNA in mice

Group	PCNA-LI (%)
Negative control	40.0±5.7
Positive control	25.1±3.6 ^b
CAO	29.9±3.9 ^b

Results are expressed as $\bar{x}\pm s$, $n=10$, ^b $P<0.01$, vs negative control group.

**Figure 1** Feulgen's staining. ×250**Figure 2** PCNA staining for the tumor tissue in the untreated mice, many tumor cells stain positively. ×250**Figure 3** PCNA staining for the tumor tissue in the CAO-treated mice. The positively stained tumor cells were significantly fewer than those of the untreated mice. ×250

DISCUSSION

The early literature reported that CAO extracted from Chinese herb, *Curcuma aromatica* Salisb, could inhibit the growth of various cancer cells *in vitro* and *in vivo*^[1]. The recent studies showed that CAO was a compound consisting of many kinds of anti tumor ingredients such as β-elemene, curcuminol, curdione, etc^[2-5]. However, up to date, few researches on anti-hepatoma mechanisms of CAO compound have been conducted. In our previous clinical study, CAO infused via hepatic artery displayed a remarkable therapeutic effect on the patients with primary liver cancer^[7]. Our recent animal experimental results revealed that transcatheter infusion of CAO into hepatic artery could significantly inhibit the growth of the implanted hepatoma^[6]. The present *in vivo* study indicated that the tumor inhibitory rates of CAO were around 50%, which coincided with the results reported by the literature. The changes in body weight of the mice treated with CAO might result from their tumors being smaller than those carried

by the control group mice and the toxicity of CAO being much less than 5-FU.

Some studies have confirmed that ICM used for the DNA quantitative analysis of cancer cells have advantages of briefness, sensitivity and precision as compared with flow cytometry^[8,9]. In the various parameters obtained by ICM, the DO, NA and DI are positively correlated with cellular proliferation. The percentages of aneuploid cells reflect not only cellular malignancy but also the cellular proliferation. ICM has been employed for analyzing various biological features of cancer cell such as proliferation, differentiation, metastasis and prognosis. Zheng *et al*^[10] with ICM revealed that the invasiveness and the metastasis of the hepatic tumors enhanced with the increasing DNA synthesis of hepatocellular carcinoma in the patients with liver cancer. Our present study showed that CAO could significantly decrease the DNA quantity (DO, DI) in the hepatoma cells and shrink the nucleus area. In the untreated mice, the higher 5CER and the lower 2C reflected abnormal increasing proliferation. CAO could decrease the percentage of 5CER cells and increase the percentage of 2C, which showed its inhibition of cellular proliferation.

PCNA functions as a cofactor for DNA polymerase δ are associated with DNA repair in both the S phase and in DNA synthesis^[11,12]. Hino *et al*^[13,14] found that the expressions of PCNA were closely related to the malignancies of human hepatocellular carcinoma. With immunohistochemical methods, Irene *et al*^[15] and Suehiro *et al*^[16] affirmed that PCNA-LI was correlated with the metastasis and the prognosis of hepatocellular carcinomas. Therefore, PCNA is an important mark for evaluating the proliferation of hepatocellular carcinomas. In the present study, we used LSAB immunohistochemistry to stain the hepatoma nuclei of mice and the positive PCNA cells were at late G₁ and early S phases. The results indicated that CAO could remarkably decrease the hepatoma PCNA-LI in mice. Therefore, we assume that the inhibition of CAO on the growth of hepatoma in mice might be associated with its depressing PCNA protein, decreasing DNA-polymerase δ activity and interfering with DNA synthesis. Combining with the ICM results, we have drawn a preliminary conclusion that the depression of CAO on cellular proliferation could contribute to its inhibition on hepatoma growth in mice. The other possible mechanisms of the inhibitory effects of CAO on hepatomaremains further clarification.

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Cloning and expression of core gene cDNA of Chinese hepatitis C virus in cosmid pTM3

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Subject headings hepatitis C virus; gene, viral; cDNA; cosmid vector; gene expression

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Abstract

AIM To clone core gene cDNA of Chinese hepatitis C virus (HCV) into eukaryotic expression vector cosmid pTM3 and to express HCV core antigen in HepG2 cells.

METHODS Core gene cDNA of HCV was introduced into eukaryotic expression vector cosmid pTM3. Using vaccinia virus/bacteriophage T7 hybrid expression system, HepG2 cells were transfected with the recombinant plasmid pTM3-Q5 34 by lipofectin.

RESULTS From the transfected bacteria Top10F', 2 pTM3-Q534 clones containing the recombinant plasmid were identified from randomly selected 10 ampicillin-resistant colonies. By reverse transcription PCR and indirect immunofluorescence technique, HCV RNA and core protein was identified in HepG2 cells transfected with the recombinant plasmid.

CONCLUSION The construction of a recombinant plasmid and the expression of core gene cDNA of HCV in HepG2 was successful.

INTRODUCTION

Although the genome of several strains of hepatitis C virus (HCV) have been cloned and sequenced, the HCV particles have not been observed so far. Currently, because of very low infection efficiency, one of the major impediments to the structural analysis of HCV genome and genetic analysis of viral replication is the lack of a reliable cell culture system permissive for HCV replication. In this study, using recombinant DNA technique, we constructed a recombinant plasmid by subcloning core gene cDNA of Chinese HCV isolated into a eukaryotic expression vector pTM3 and expressed HCV core antigen in transfected HepG2 cells by using vaccinia virus/bacteriophage T7 hybrid expression system.

MATERIALS AND METHODS

Directional cloning of core gene of HCV in cosmid vector pTM3

Details of the plasmid construction are as follows. First, plasmid pQ534^[1] containing core gene cDNA of Chinese HCV (provided by Prof. QI Zhong-Tian, Second Military Medical University) was digested partially with *Pst*-I and *Sma*I, cosmid vector pTM3 (provided by St. Mary's Hospital, London University, England) was digested thoroughly with *Pst* I and *Sma*I, and the target gene and large fragment vector was purified by agarose gel electrophoresis. The 559 base pairs (bp) *Pst* I and *Sma* I target gene fragment was ligated with a 7418bp *Pst* I and *Sma* I linearized plasmid vector of pTM3 by bacteriophage T 4 DNA ligase overnight at 37°C, and stored at -20°C. The ligation product was routinely transformed into bacteria Top10F', and was incubated in an LB plate containing ampicillin overnight at 37°C. Ten bacterial colonies were individually transferred into 2mL of LB medium containing ampicillin in a loosely capped 15mL tube, and the culture was incubated overnight at 37°C with vigorous shaking. To confirm that the culture did contain the correct plasmid, we prepared a small amount of plasmid DNA and analyzed it by digestion with restriction enzymes. Then, we propagated the transformed positive bacterial colony, prepared and purified a large amount of plasmid DNA, and stored it at -20°C for transfections.

After the inoculum was removed, the cells were transfected with 1mg of pTM3-Q534 plus lipofectin (GIBCO-BRL) 1mg for 4 h at 37°C. For the

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control experiment, the infected cells were transfected with pTM3 instead of pTM3-Q534 for 4h. The cells were then incubated at 37°C for 4h in DMEM containing 2% fetal bovine serum.

Transient-expression experiment based on recombinant vaccinia virus

Briefly, HepG2 cells were seeded into 6-well plate and were approximately 80% confluent 24h later. The cells were infected with the recombinant vaccinia virus vvT-7-3 (provided by St. Mary's Hospital, London University, England) at the multiplicity of infection of 8 plaque-forming units (PFU)/cell for 1h at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing 2% fetal bovine serum.

Identification of HCV replication by reverse transcription PCR (RT-PCR)

Production of core region HCV-RNA in the transfected HepG2 cells was examined by RT-PCR using primers located at the core region of the HCV genome. (First PCR : sense 5'CCCAAACCTCAAA-GAAA3', antisense 5'AGCGGTATGTACCCCATG3'; second PCR: sense 5'CAGATCGTTGTGGAGTT3', antisense 5'GCAGCCCTCATTGC-CAT3'). RT-PCR was performed using a standard procedure described previously. In all experiments, RNA extracted from a liver specimen known to contain HCV was included as positive control and cloned HBV-DNA transfected HepG2 cells (HepG2 2.2.15 cell line) as negative control.

Identification of the transfected HepG2 cells by indirect immunofluorescent technique

After infection/transfection, the medium was removed and cells were washed once with PBS and were fixed with methanol/acetone (50:50) for 20min at -20°C. The methanol/acetone was removed and the cells were rinsed with PBS. For immunofluorescent assays, the fixed cells were incubated for 1h at 37°C with a 1:50 dilution of the clinical HCV positive human serum in PBS for 5min per wash. FITC-conjugated secondary antibody IgG (goat anti-human) were added at 1:5 dilution in PBS and incubated for 1h at 37°C. The cells were washed for 4-5 times as above. The stained cells were observed with a Meridian-ACAS 470.

RESULTS

Cloning of core gene cDNA of HCV in cosmid vector pTM3

In order to satisfy the directional cloning and the correct reading frame, plasmid pQ534 was double digested with *Sma*I and *Pst*I. Since there are 2 *Sma*I recognition sites in the core gene cDNA of HCV, partial digestion has to be carried out, so at first, pQ534 was digested with the restriction enzyme *Pst*I for 1h at 37°C, and then digested

with the second enzyme *Sma* I for 10min at 37°C. The 559bp segment containing core gene cDNA of HCV was purified by agarose gel electrophoresis (Figure 1).

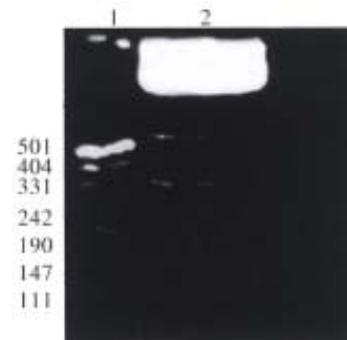


Figure 1 Incomplete digestion of plasmid pQ534 by restriction endonucleases *Sma*I and *Pst*I.

Lane 1: DNA molecular weight marker, lane 2: pQ534 digested with *Pst*I first, and then digested incompletely.

Identification of the recombinant plasmid pTM3-Q534 with restriction enzyme

From the recombinant plasmid transformed bacteria Top10F', 2 pTM3-Q534 clones were identified from 10 randomly selected ampicillin-resistant colonies. Plasmid DNA obtained by alkaline lysis was cleaved with restriction enzyme *Eco*RI/*Pst*I, a 534bp insertion segment was observed in 2 of the 10 colonies (Figure 2, lane 3, 4). The plasmid DNA of positive clones was digested with *Eco*RI, and actually only a 7970bp band was found after electrophoresis (Figure 2, lane 5). Because an *Eco*RI site also existed in the core gene cDNA of HCV (9 base pairs near the 5' terminus of core gene cDNA), if the target gene was reverse-inserted, the length between the site and the *Eco*RI site of the polyclonal site of plasmid pTM3 was 550bp, so a 550bp *Eco*RI fragment (7970bp) after electrophoresis. It was confirmed that construction of core gene cDNA of HCV in cosmid vector pTM3 was successful.

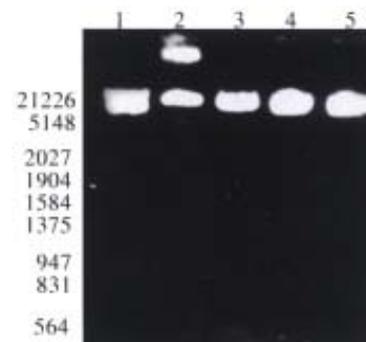


Figure 2 Identification of the recombinant plasmid pTM3-Q534 with restriction endonucleases.

Lane 1: 500ng Hind-III/*Eco*RI DNA marker; lane 2: pTM3 digested with *Sma*-I and *Pst*I; lanes 3 and 4: pTM3-Q534 digested with *Eco*-RI and *Pst*I; lane 5: pTM3-Q534 digested with *Eco*-RI.

RT-PRC

Positive-strand HCV RNA was detected in HepG2 cells over a 6-week period after transfection with pTM3-Q534. Negative-strand HCV RNA was detected over a 4-week period after transfection with pTM3-Q534. However, there was no HCV-RNA specific strand found in the control cells. It was demonstrated that there was HCV replication in the HepG2 cells transfected with pTM3-Q534.

Indirect immunofluorescence

In the experimental group, some of the HepG2 cells transfected with pTM3-Q534 were positive for HCV core protein. The other cells were negative. The negative control HepG2 cells transfected with pTM3 were all negative for HCV core protein (Figure 3), and the same results were obtained in the repeated experiments of transfection and indirect immunofluorescence.

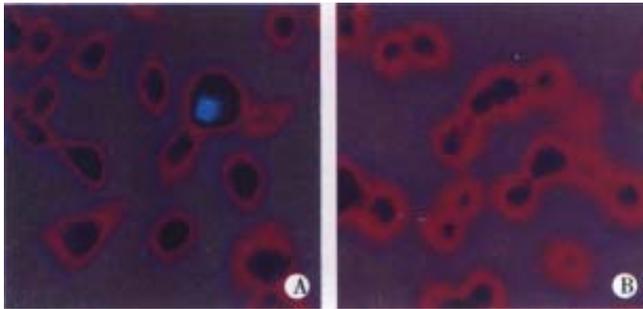


Figure 3 Indirect immunofluorescence staining of transfected HepG2 cells.

A: A part of the HepG2 cells infected with vvT-7.3 and transfected with pTM3-Q534 showing intracellular immunofluorescence; B: In control, no staining can be seen in the HepG2 cells infected with vvT-7.3 and transfected with pTM3.

DISCUSSION

More and more studies about the expression of HCV gene in mammalian cells have been reported in the world^[2,4,6,8,9], but no reports from China. In this study, we successfully cloned core gene cDNA of HCV into eukaryotic expression vector pTM3. To achieve transcription with high efficiency and sufficient translation and expression of the HCV genome, we used a vaccinia virus/bacteriophage T7 hybrid expression system^[3,5,7]. In this system, T7 RNA polymerase was expressed in the cells infected with a recombinant vaccinia virus vvT-7.3, and the

transfected target genes controlled under the T7 promoter sequence were transcribed with high efficiency. Thus, the core protein of HCV can be efficiently produced in the cells. Lipofectin reagent interacts spontaneously with DNA to form a lipid-DNA complex. The fusion of the complex with tissue culture cells could result in the efficient uptake and expression of the DNA. Compared to transfection methods employing calcium phosphate, a protocol using lipofectin reagent has been shown to be 50-100 fold more efficient. Cloning of core gene cDNA of HCV into eukaryotic expression vector pTM3 and expression of HCV core antigen in HepG2 cells confirmed that the recombinant plasmid pTM3-Q534 which contains core gene cDNA of HCV possesses the property of propagation in HepG2 cells. As all the results were reproducible, a solid foundation was laid for the further investigation in the expression of HCV genes in mammalian cell lines and the detection of the function of the various regions in HCV genome.

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FADD and TRADD expression and apoptosis in primary hepatocellular carcinoma

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Subject headings FADD;TRADD; apoptosis; carcinoma, hepatocellular; liver neoplasms

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Abstract

AIM To investigate the clinical features of FADD and TRADD expressions in primary hepatocellular carcinoma (HCC) and to determine their relationship with hepatic apoptosis.

METHODS FADD and TRADD expressions were detected by immunohistochemistry and hepatic apoptosis were determined by *in situ* end-labeling (ISEL).

RESULTS Ten (25.6%) cases of HCC were detected to express FADD protein. The positive rate in HCC is lower than that in non-cancerous adjacent liver tissues (62.5%) ($P < 0.05$). In those of grade I-II, 8 (38.1%) cases were FADD positive, while only 2/18 (11.1%) cases of grade III-IV had detectable FADD protein ($P < 0.05$). No relationship was found between FADD expression and other clinical features, such as gender, age, tumor size, differentiation or metastasis. ISEL positive cells can be seen in all cases of HCC. The hepatic apoptosis was associated with FADD expression as more apoptotic cells were detected in those cases which had moderately to strongly positive FADD, as compared with negative or weak positive FADD cases ($P < 0.05$). No relationship was found between FADD expression and hepatic apoptosis in non-cancerous adjacent liver tissues. Fifteen of 39 (38.5%) cases of HCC were found positive for TRADD protein, and similar positive rate (37.5%) in non-cancerous adjacent liver tissues ($P > 0.05$). The expression of TRADD is correlated with HCC differentiation,

as only 22.2% of moderately to highly differentiated HCC showed positive TRADD protein, while as high as 52.4% of poorly differentiated HCC had TRADD ($P < 0.05$). No relationship was found between TRADD expression and gender, age, tumor size or grade or metastasis, although 42.9% of HCC of grade I/II showed positive TRADD which was slightly higher than that of grade III/IV (33.3%, $P > 0.05$). Hepatic apoptosis was not related to TRADD expression in HCC or non-cancerous adjacent liver tissues.

CONCLUSION Loss of FADD expression plays an important role in HCC carcinogenesis, and expression of TRADD also contributes to HCC development. The cell apoptosis in HCC is associated with FADD expression. However, the expression of TRADD does not correlate well with hepatic apoptosis in HCC.

INTRODUCTION

Apoptosis is an area of intense scientific interest, which encompasses the study of and triggers mechanisms involved in mediating the cell biology of programmed cell death. Deregulation of apoptosis is generally considered as a critical reason for tumorigenesis. In addition to uncontrolled cell proliferation, decreased cell death is also involved in increase of tumor cell population^[1]. Fas, TNF α and TGF β play prominent roles in regulating liver cell apoptosis^[2]. Trimerization of the Fas receptor (CD95, APO-1), a membrane bound protein, after binding to Fas ligand, triggers cell death by apoptosis. The main death pathway activated by Fas receptor involves the Fas-associated death domain (FADD) adapter protein that connects Fas receptor to the caspase cascade. Transient expression of a dominant-negative mutant of FADD impairs Fas/Apo1-mediated apoptosis. TNFR-1 recruits and assembles, as a consequence of TNF α binding to TNFR-1, a signaling complex containing a number of death domain (DD)-containing proteins, including the TNFR-associated death domain (TRADD) adapter protein. The subcellular interactions of TNF-R1 and the TRADD adapter protein serving as anchor for the subsequent recruitment of other proteins into the signaling

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complex that directly lead to cell death or nuclear factor-kappaB (NF- κ B) induction^[3,4]. Silencer of death domains (SODD), a widely expressed approximately 60-kilodalton protein, was found to be associated with the death domain of TNF-R1 preventing constitutive TNF receptor 1 signaling^[5]. Hepatocellular carcinoma (HCC) is one of the tumors known to be resistant to Fas-mediated apoptosis. To elucidate the possible mechanisms of this resistance, we examined the FADD and TRADD protein expression in a series of primary HCC and observed the relationship between FADD and TRADD protein expression and hepatic apoptosis.

MATERIALS AND METHODS

Patients and samples

The surgically resected specimens employed in this study were obtained from consecutive patients with primary HCC who had undergone potentially curative tumor resection in the Department of General and Hepato-Biliary Surgery, Tongji Hospital during 1996-1997. A cohort of 39 cases was involved in this study. All cases were selected on the basis of availability of frozen material for study and on the absence of extensive chemotherapy-induced tumor necrosis. Materials were composed of 3 cases of grade I, 18 cases of grade II, 11 cases of grade III, the remaining 7 cases were of grade IV according to TNM system (1987). Twenty-one were poorly differentiated, 9 moderately and 9 well differentiated HCC. There were 34 males and 5 females aged from 24 to 71 years with an average of 46.1 ± 12.5 years. Eight cases of non-cancerous adjacent liver tissues were also included in the study. Routinely processed 4% paraformaldehyde-fixed, paraffin-embedded blocks of containing principal tumor were selected. Serial sections of 5 μ m were prepared from the cut surface of blocks at the maximum cross-section of the tumor.

Immunohistochemistry

Goat monoclonal antibody that recognizes the human FADD and TRADD protein was Santa Cruz product. StreptAvinin-Biotin-enzyme Complex (SABC) kit was purchased from Boster Biotechnology Inc. (Wuhan, China). Briefly, 5 μ m tissue sections were deparaffined, rehydrated through a graded series of ethanol, and heated in 0.01mol/L sodium citrate solution in microwave oven for 15min. The primary antibody was used at a dilution of 1:30 (for FADD) and 1:50 (for TRADD). After incubated overnight at 4°C, biotinylated anti-goat immunoglobulin and streptavidin conjugated to horseradish peroxidase were subsequently applied. 3,3'-diaminobenzidine was used for color development, and hematoxylin

was employed for counterstaining. Representative tissue sections were immunolabeled with normal goat serum as a negative control for the immunohistochemistry. The intensity of FADD and TRADD immunostaining was scored according to the percentage of positive cells: (-) no positive signal was found; (+) positive cell <25%; (++) 25%-50%; (+++) >50%.

Histochemical detection of apoptosis

Tumor cell apoptosis was identified by DNA fragmentation detection kit (QIA33-Kit, Calbiochem). Briefly, deparaffinized and rehydrated sections were permeabilized with proteinase K (20mg/L in 10mmol/L Tris, pH 8.0) for 20 min at room temperature and washed with 1 \times ATBS (20mmol/L Tris pH 7.6, 140mmol/L NaCl). After endogenous peroxidases were inactivated by using 30mL/L hydrogen peroxide for 5min and washed with 1 \times ATBS, equilibration buffer was added to each section and incubated at room temperature for 20min. Terminal deoxynucleotidyl transferase (TdT) enzyme in TdT labeling reaction mixture at a 1:20 dilution was pipetted onto the sections, followed by 1.5h incubation at 37°C. After terminating the reaction by immersing sections into stop solution and washing with blocking buffer for 10min at room temperature, the anti-digoxigenin-peroxidase was added to the sections. DAB solution was used for color development. Sections were counterstained by methyl green. A positive control was generated covering specimen with DNase I (1mg/L) for the first procedure. Specific positive tissue sections were used for negative control by substituting distilled water for the TdT in the reaction mixture. Positively stained tumor cells with morphological characteristics of apoptosis were identified using standard criteria, including chromatin condensation, nuclear disintegration and formation of crescentic caps of condensed chromatin at the nuclear periphery. According to, with small modification, Liang's report^[6], the positive ISEL was determined in least five areas at $\times 400$ magnification and assigned to one of the three following categories: (+) only sporadic positive cells were detected; (++) a cluster of apoptotic cells were observed; (+++) positive cells in a large scale or multi-cluster apoptotic cells were seen in representative tissue sections of each individual case.

Statistical analysis

The association between the variables was assessed using the Chi-square and Fisher exact tests. Differences in frequencies were considered statistically significant if *P* values less than 0.05.

RESULTS

Expression of FADD in HCC

Ten (25.6%) out of 39 cases had detectable FADD protein expression, the positive rate being lower than that of non-cancerous adjacent live tissues (62.5%). The positive signal was predominantly located at cytoplasm (Figure 1). In 18 cases of moderately-well differentiated HCC, the positive rate was slightly higher, however with no statistical significance, than that of poorly differentiated HCC (33.3% vs 19%, $P>0.05$). No relationship was found between FADD expression and other HCC clinical features including gender, age, tumor size and metastasis with exception for tumor grade, which showed that 38.1% were positive for FADD in cases of grade I/II and 11.1% in cases of grade III/IV ($P<0.05$, Table 1).

Expression of TRADD in HCC

Fifteen cases of HCC were immunolabeled by anti-TRADD antibody. The positive rate (38.5%) was similar to that of non-cancerous adjacent liver tissues (37.5%, $P>0.05$). The TRADD expression selectively restricted to cytoplasm (Figure 2). The intensity of TRADD staining was usually homogeneous within a case tested. Although TRADD was detected in 9 (42.9%) cases of grade I/II, the difference was not statistically significant as compared with that of grade III/IV which showed 33.3% of positive TRADD ($P>0.05$). Negative relationship with statistical significance was found between TRADD staining and HCC differentiation, because 52.4% of moderately/highly differentiated HCC were TRADD detected by IHC while only 22.2% of poorly differentiated HCC harbored TRADD protein ($P<0.05$). None of the other clinical parameters analyzed in TRADD-positive cases, including gender, age, metastasis and tumor size reached statistical significance ($P>0.05$, Table 1). TRADD and FADD coexpression was found in six cases of HCC, but without relationship between TRADD and FADD expression ($P=0.08$).

Relationship between tumor cell apoptosis and TRADD or FADD expressions

Apoptotic cells and bodies were found in all cases of HCCs examined by *in situ* end-labeling (Figure 3) according to the criteria described in Material and Methods. In the cases with moderately-strong positive FADD immunoreaction, more cells underwent apoptosis than those with weak or negative FADD expression ($P<0.05$). FADD protein expression was not related to hepatic apoptosis of non-cancerous adjacent liver tissues ($P>0.05$), and no significant differences were observed between TRADD expression and apoptosis in either HCC or adjacent liver sample ($P>0.05$, Table 2).

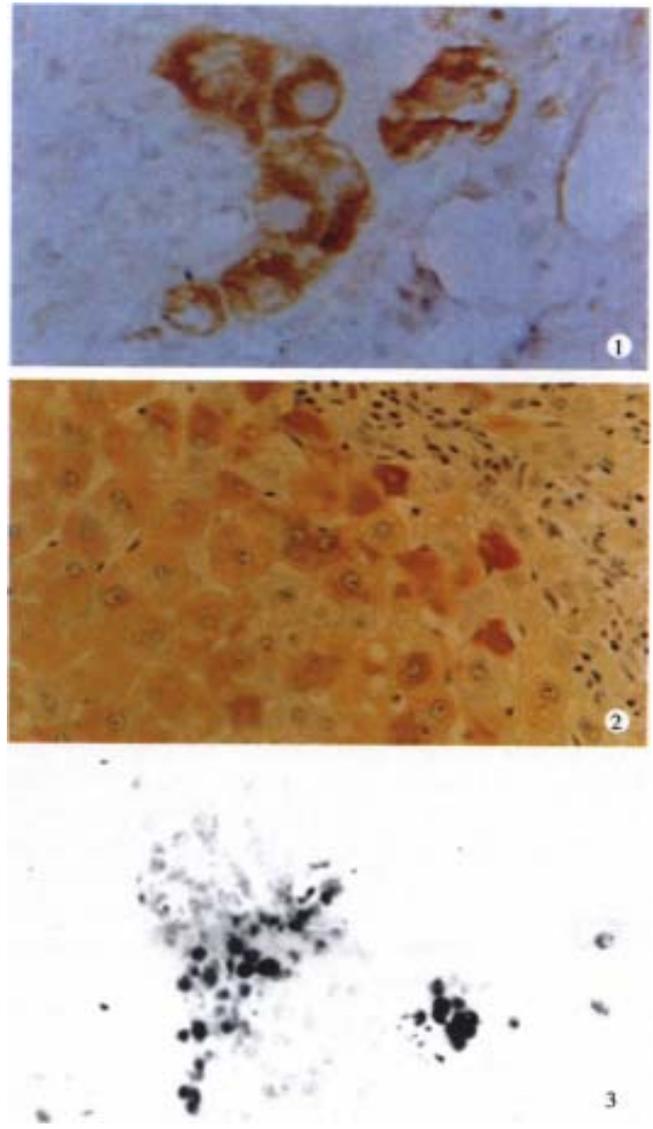


Figure 1 Expression of FADD in HCC. SABC×400

Figure 2 Immunolabeled TRADD in HCC. SABC×200

Figure 3 Apoptotic cells were determined by ISEL.×200.

Table 1 Expressions of FADD and TRADD in HCC

Variables	n	FADD		TRADD	
		Positive	%	Positive	%
Adjacent tissue	8	5	62.5 ^a	3	37.5
HCC tissue	39	10	25.6	15	38.5
Age(yr)					
≤60	28	6	21.4	9	32.6
>60	11	4	36.4	6	54.5
Sex					
Male	34	9	26.5	13	38.2
Female	5	1	20	2	40
Tumor size (cm)					
≥5	27	8	29.6	10	37
<5	12	2	16.7	5	41.7
Differentiation					
Moderately well	18	5	27.8	4	22.2 ^a
Poorly	21	5	23.8	11	52.4
TNM grade					
I/II	21	8	38.1 ^a	9	42.9
IV/IV	18	2	11.1	6	33.3
Metastasis					
yes	8	3	37.5	4	50
no	31	7	22.6	11	35.5

^a $P<0.05$

Table 2 Correlation between hepatic apoptosis and expressions of FADD and TRADD

Apoptosis	n	FADD expression				TRADD expression			
		-	+	++	+++	-	+	++	+++
HCC									
+	19	17	1	0	1 ^a	13	3	1	2
++	14	10	0	2	2	9	2	2	1
+++	6	2	1	2	1	2	1	2	1
Non-cancerous adjacent liver tissues									
+	6	3	2	1	0	4	2	0	0
++	2	0	1	1	0	1	1	0	0
+++	0	0	0	0	0	0	0	0	0

^aP<0.05 +/- vs ++/+ +++

DISCUSSION

Cancer results from excessive uncontrolled accumulation of cells. This may be the consequence of enhanced cell proliferation or of reduced cell death, or both. Inappropriate hepatic apoptosis is the critical link in liver injury and diseases. In hepatocarcinogenesis, the balance is disturbed between cell proliferation and death, which is precisely controlled in normal liver. Fas ligand and Fas receptor, tumor necrosis factor (TNF) and its receptor, and transforming growth factor β 1 (TGF- β 1) and its receptor are currently known as well established ligand/receptor interactions causing hepatocyte apoptosis^[2]. After binding to Fas ligand, Fas can interact with FADD protein via its death domain, leading to the activation of initiating cysteine proteases of the caspase family, e.g., caspase 8, and trigger apoptosis. TNF α -induced apoptosis need the presence of TRADD protein, which will transduce the signal from the binding of TNF α -with TNF-R1^[7]. Thus, loss of FADD and TRADD function will prevent apoptosis by failing to actively initiate caspase.

In our previous study, we found that the expression of Fas/FasL was not correlated with hepatic apoptosis in certain cases of HCC. So we detected the FADD protein expression in HCC. In this study, the FADD protein was expressed in HCC with a lower incidence than in positive cases in noncancerous adjacent liver tissues (25.6% vs 62.5%, $P<0.05$). A similar expression pattern of FADD was observed in a research consisting of 6 HBV-related HCC and 10 HCC cell lines by Shin *et al*^[8], FADD expression was dramatically reduced in both HCC specimens and cell lines, while FAP (for FAS associated protein) which pronounced antiapoptotic effect when overexpressed, increased in HCC. The result suggested that loss of FADD expression plays a critical role in HCC carcinogenesis and development. In addition, we found no significant correlation between FADD expression and HCC clinical parameters including age, gender, tumor size or metastasis, except for

tumor grade. The positive rate for FADD was 38.1% of grade I/II, a little higher than that of grade III/IV (11.1%), with statistical significance ($P<0.05$).

FADD alone can trigger apoptosis in a FAS-independent way. Kondo *et al*^[9] had used a selected group of malignant glioma cell lines containing negative or low-level Fas protein for study. The results indicated that about 85% of malignant glioma cells, regardless of Fas/APO-1 expression levels, underwent apoptosis after transient transfection with FADD expression vector. The retroviral transfer of FADD gene significantly enhanced the transduction efficiency and effectively inhibited both *in vitro* and *in vivo* survival of malignant glioma cells through induction of apoptosis. Chemotherapeutic drugs were reported to induce the accumulation of the FADD adapter molecule in several human cancer cells^[10]. It is proposed that FADD expression will contribute to HCC apoptosis. In this study, the apoptotic cells can be seen in all HCC samples and the intense is correlated to the FADD protein level. We found that more apoptotic cells were detected in HCC with moderate to strong FADD expression than those with weak positive or negative FADD. This relationship was not observed in non-cancerous adjacent liver tissues. The results indicate that FADD expression is associated with HCC hepatic apoptosis, but not in the noncancerous liver tissues.

TRADD protein was detected in 15 cases of HCC. The difference of TRADD expression in HCC and noncancerous adjacent liver tissues was not statistically significant. We proposed that the TRADD expression may be related to tumor differentiation of HCC because positive rate in moderately to well differentiated cases (52.4%) was higher than that in the poorly differentiated ones (22.2%). No relationship was observed between TRADD expression and tumor size, metastasis, age or gender of patients. Unlike the expression of FADD, TRADD expression was not significantly related to hepatic apoptosis. In fact, it is believed that in addition to FADD, TRADD can bind to RIP, preventing cell apoptosis via NF- κ B dependent and independent pathway^[2]. TRADD was also demonstrated to interact with death receptor 6 causing cell apoptosis^[11]. No relationship was found between FADD and TRADD expression, indicating that different mechanisms are involved in the regulation of FADD and TRADD expression.

There have been many studies on the mechanisms by which death receptor triggers apoptosis. Our knowledge of the intracellular signaling mechanism will enable us to improve the therapeutic strategies for the treatment of liver cancer^[1].

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Susceptibility to hepatocellular carcinoma associated with null genotypes of GSTM1 and GSTT1

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Subject headings liver neoplasms; carcinoma, hepatocellular; GSTM1; GSTT1; null genotypes

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Abstract

AIM In order to study the association between the null genotypes of GSTM1 and GSTT1 and the genetic susceptibility to hepatocellular carcinoma (HCC).

METHODS The genotypes of GSTM1 and GSTT1 of 63 cases of HCC and 88 controls were detected with the multiple PCR technique.

RESULTS The frequency of GSTM1 null genotype was 57.1% among the cases, and 42.0% among the controls, the difference being statistically significant ($\chi^2=3.35$, $P=0.067$), but χ^2 value approaching the significance level. The odds ratio was 1.84 (95% CI=0.91-3.37). The frequency of GSTT1 non-null genotype was 87.3% among the cases and 62.5% among the controls, the difference being statistically significant ($\chi^2=11.42$, $P=0.0007274$). The odds ratio was 4.13 (95% CI = 1.64-10.70). According to the cross analysis, the GSTT1 non-null genotype was more closely associated with HCC than GSTM1 null genotype, and these two factors play an approximate additive interaction in the occurrence of HCC.

CONCLUSION The persons with GSTM1 null genotype and GSTT1 non-null genotype have the increased risk to HCC.

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INTRODUCTION

Some previous studies have shown that glutathione s-transferase M1 (GSTM1) null genotype is a susceptible genotype to hepatocellular carcinoma (HCC)^[1]. A recent research has found that glutathione transferase T1 (GSTT1) also has the null genotype^[2], which is similar to GSTM1. GSTM1 and GSTT1 are the members of GST family, which can detoxify some extraneous chemicals^[3]. Persons with two null genotypes have no ability to do so^[2-4]. Few reports have been found on the association between GSTT1 null genotype and HCC as well as the interaction between GSTT1 and GSTM1 null genotype to HCC. We used the multiple PCR technique to detect GSTM1 and GSTT1 genotypes of 63 HCC cases and 88 healthy controls in an attempt to provide scientific ground on which the screening among high risk population is based.

MATERIALS AND METHODS

Sources of specimens

The specimens of non-cancerous liver tissue and peripheral blood of 63 HCC cases were provided by Qidong Institute for Liver Cancer, Zhongshan Hospital attached to Shanghai Medical University and the teaching hospital Nantong Medical College. The peripheral blood specimens of 88 healthy controls were provided by Qidong Institute for Liver Cancer, Haimen Municipal Anti-Epidemic and Health Station and Clinic of Shanghai Medical University. The cases were pathologically diagnosed as HCC. No consanguineous relationship existed among controls. The subjects mainly came from east China. There were 47 males and 16 females among the 63 cases, and 67 males and 21 females among the 88 controls.

Extraction of genomic DNA

The extraction of genomic DNA from liver tissues and peripheral blood was carried out according to the methods by Hoelzel^[5] and Tas^[6].

Multiple PCR

The corresponding fragments of GSTM1, GSTT1 and β -globulin genes were amplified with 3 pairs of primers synthesized according to the method recommended in the literature (Table 1)^[7-9]. The amplificate of β -globulin gene was designed as the internal control with the purpose to remove false negative outcome. Whether GSTM1 null genotype existed or not was judged by the fragment of

650bp, while GSTT1 null genotype by the fragment of 480bp. The total reaction volume was 50 μ L containing 100ng-600ng templates, 0.5 μ mol/L primers (3 pairs), 200 μ mol/L dNTP, 2.0mmol/L MgCl₂, 2.5U *Taq* DNA polymerase (Promega) and corresponding buffers. The reaction condition was: predenaturation at 95°C for 5 minutes; denaturation at 95°C for 1 minute; annealing at 58°C for 1min; extension at 72°C for 1.5min; and extension at 72°C for 5min after 30 cycles. The amplicates were separated with agarose gel electrophoresis for 50min at 120V, and inspected by ultraviolet reflector and pictured by ImageMaster VDS (Pharmacia) (Figure 1).

Table 1 The sequence of primers for PCR

Gene	Location	Sequence	Length of amplicates (bp)
GSTM1	5'	5'-CTC CTG ATT ATG ACA GAA GCC-3'	650
	3'	5'-CTG GAT TGT AGC AGA TCA TGC-3'	
GSTT1	5'	5'-TTC CTT ACT GGT CCT CAC ATC TC-3'	480
	3'	5'-TCA CCG GAT CAT GGC CAG CA-3'	
β -globulin	5'	5'-CAA CTT CAT CCA CGT TCA CC-3'	268
	3'	5'-GAA GAG CCA AGG ACA GGT AC-3'	



Figure 1 Agarose gel electrophoresis of amplicates from multiple PCR. Lanes 1 and 10 are PCR markers, lanes 2 and 3 indicate non-null genotypes of GSTM1 and GSTT1, lanes 4 and 5 indicate null genotypes of GSTM1 and GSTT1, lanes 6 and 7 indicate null genotype of GSTM1 and non-null genotypes of GSTT1, lanes 8 and 9 indicate non-null genotype of GSTM1 and null genotypes of GSTT1. Fragments of 268bp indicate internal controls.

RESULTS

The DNA templates used by the multiple PCR was extracted from the noncancerous liver tissues and the peripheral blood of HCC cases and the controls. The frequency of GSTM1 null genotype was 57.1% among cases and 42.0% among controls. The difference was not statistically significant, but χ^2 value approached the significance level ($\chi^2=3.35$, $P=0.067$). When GSTM1 null genotype was regarded as the exposure factor, odds ratio (OR) was 1.84 (95% CI=0.91-3.73). It was suggested that persons with GSTM1 null genotype had a 84% of increased risk to HCC as compared with persons with GSTM1 non-null genotype (Table 2).

Table 3 shows that the frequency of GSTT1 null genotype was 12.7% among cases and 37.5% among controls. The difference was statistically significant

($\chi^2=11.42$, $P=0.0007274$). The odds ratio was 4.13 (95% CI=1.64-10.70) when GSTT1 non-null genotype was regarded as the exposure factor, suggesting that in persons with GSTT1 non-null genotype the risk to HCC increased by 3.13 times as compared with persons with GSTT1 null genotype.

To study the interaction of GSTM1 and GSTT1 in the occurrence of HCC, the cross analysis was carried out. The results indicated that when GSTM1 null genotype and GSTT1 non-null genotype were regarded as exposure factors, and that GSTM1 non-null genotype and GSTT1 null genotype as non-exposure factors, for those only exposed to GSTM1 null genotype, only exposed to GSTT1 non-null genotype and exposed to both, their OR was 6.95, 11.8 and 23.00, respectively. It was suggested that GSTT1 non-null genotype was more closely associated with HCC than GSTM1 null genotype and these two factors exerted an additive interaction in the occurrence of HCC. According to trend χ^2 test, the rank association existed among these exposure factors.

Table 2 The association of GSTM1 null genotype and HCC

Genotypes	Cases		Controls		Total
	n	%	n	%	
Null	36	57.1	37	42.0	73
Non-null	27	42.9	51	58.0	78
Total	63	100.0	88	100.0	151

$\chi^2=3.35$, $P=0.067$ OR=1.84 (95% CI=0.91-3.73)

Table 3 The association of GSTT1 null genotype and HCC

Genotype	Cases		Controls		Total
	n	%	n	%	
Null genotype	55	87.3	55	62.5	110
Non-null genotype	8	12.7	33	37.5	41
Total	63	100.0	88	100.0	151

$\chi^2=11.42$, $P=0.0007274$

OR=4.13 (95% CI=1.64-10.70)

Table 4 The relationship between different exposure levels of GSTM1, GSTT1 and HCC

Exposure level		Cases		Control		OR
GSTM1	GSTT1	n	%	n	%	
-	-	1	1.6	16	18.2	1.00
+	-	7	11.1	17	19.3	6.59
-	+	26	41.3	35	39.8	11.89
+	+	29	46.0	20	22.7	23.20
Total		63	100.0	88	100.0	

$\chi^2_{trend}=16.413$, $P=0.000051$

GSTM1(+): GSTM1 null genotype, (-): non null genotype. GSTT1 (+): GSTT1 non null genotype, (-): null genotype

DISCUSSION

Numerous epidemiological studies have shown that HBV, aflatoxin and pollutants in drinking water are the main environmental hazard factors to HCC in China. The association between HBV and HCC has been universally accepted. But only 20%-25% of chronic HBV infected persons developed HCC. Therefore attention should be paid to the effect of

chemical carcinogen in the occurrence of hepatocarcinoma.

GST is a supergene family composed of 4 kinds of isoenzymes (α , μ , π , θ), which plays an important role in the second stage of biotransformation by conjugating extraneous chemicals with glutathione. Moreover, GST can combine itself with chemicals directly. After being absorbed, aflatoxin B₁ is transferred to the ultimate carcinogen, i.e. aflatoxin B₁-8, 9-epoxide, by the catalyzation of cytochrome P₄₅₀. GSTM1 can transfer aflatoxin B₁-8, 9-epoxide to be nontoxic metabolite which is highly-soluble and can be excreted out of body^[10]. Three alleles exist on GSTM1 gene locus, including a, band null. In persons with null genotype, GSTM1 can not be expressed in the liver and therefore has no ability to detoxify AFB₁ and other chemicals^[10].

With regard to the research into the association of GSTM1 null genotype and HCC, 4 of the 5 case-control studies showed significant difference^[1,11]. The result from one study is almost identical to ours. It is likely that the sample size plays a role in it. Summarizing these results, we believe that GSTM1 null genotype is a susceptible genotype to HCC.

GSTT1 is also a member of GST family. GSTT1 and GSTM1 are mutual isoenzymes and both have the null genotypes. There has been no report on whether GSTT1 participates in the metabolism of aflatoxin or not. Our research shows that GSTT1 null genotype frequency is 12.7% among cases, and 37.5% among controls, the difference being significant ($P=0.0007274$). The association between GSTT1 null genotype and HCC was reverse to that of GSTM1 null genotype and HCC. GSTT1 null genotype is a protective factor to HCC, thus lowering the risk to HCC. In other words, GSTT1 non-null genotype is a hazard factor to HCC. Its OR is 4.13 indicating that persons with GSTT1 non-null genotype increased the risk to HCC by 3.13 times as against persons with GSTT1 null genotype. The results imply that certain procarcinogens such as AFB₁ or other chemicals can be activated by GSTT1 and the metabolites are carcinogenic. It was reported that GSTT1 could activate dichloromethane into mutagen, inducing lung and liver cancer in mice^[2]. This report has provided evidence for our study.

We used cross analysis to study the interaction of GSTM1 and GSTT1 in the occurrence of HCC. When GSTM1 non-null genotype and GSTT1 null genotype were regarded as the non-exposure factors, for those only exposed to GSTM1 null genotype, only exposed to GSTT1 non-null genotype and exposed to both, their OR are 6.59, 11.89 and 23.20, respectively. GSTT1 non-null genotype is more closely associated with HCC than GSTM1 null

genotype and those two factors exert an additive interaction in the occurrence of HCC. According to the trend χ^2 test, the rank association existed among the different exposures.

In summary, we think that both GSTM1 and GSTT1 are the susceptible loci to HCC. The degree of accordance between their genotype and phenotype was as high as 98.5%-100%^[2,7]. Persons with GSTM1 null genotype have an increased susceptibility to HCC on account of lacking for GSTM1 activity in liver. Likewise, persons with GSTT1 non-null genotype, who possess GSTT1 activity in liver, have an increased susceptibility to HCC in which procarcinogen is activated by GSTT1.

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Changes of integrin expression in rat hepatocarcinogenesis induced by 3'-Me-DAB

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Subject headings integrins; 3'-Me-DAB; liver neoplasm/chemically induced; neoplasm metastasis; rats

Yuan ST, Hu XQ, Lu JP, KeiKi H, Zhai WR, Zhang YE. Changes of integrin expression in rat hepatocarcinogenesis induced by 3'-Me-DAB. *World J Gastroentero*, 2000;6(2):231-233

Abstract

AIM To investigate the expression of integrins in rats liver during 3'-Me-DAB induced hepatocarcinogenesis and to find out the relationship between integrins and liver cancer metastasis.

METHODS The expressions of integrins $\alpha_{1, \alpha_2, \alpha_3}$ and α_5 and epidermal keratin (EK) were observed by immunohistochemical PAP method.

RESULTS In the normal liver tissues, hepatocytes express integrins α_1 and α_5 and in the bile duct epithelium, EK. In liver cirrhosis, hepatocytes highly express integrins $\alpha_{1, \alpha_2, \alpha_3}$ and α_5 and in hyperplastic bile duct epithelium, integrins α_{1, α_5} and EK. Expression of integrins $\alpha_{1, \alpha_2, \alpha_3}$ and α_5 were obviously decreased in the preneoplastic nodules and primary carcinoma but expressions of integrins α_1 and α_5 in metas tasis in the lung and diaphragma were higher than those in primary carcinoma.

CONCLUSION Integrins α_1 and α_5 may play a major role in chemically induced hepatocarcinogenesis and metastasis in rats.

INTRODUCTION

The integrin superfamily of heterodimeric cell-surface receptors composed of distinct α and β subunits mediates the adhesion of cells to the extracellular matrix and in some instance, the intercellular adhesion. The integrins are thought to play important roles in differentiation and development, cell migration, and the complex process of tumor cell invasion and metastasis, as well as adhesion^[1]. Alterations in integrin expressions upon malignant transformation and in naturally occurring human malignances are now well established. However, the mechanisms that give rise to altered integrin protein expression following malignant transformation are poorly understood. We investigate the expression of integrins $\alpha_1, \alpha_2, \alpha_3$ and α_5 in rat liver during 3'-Me'-DAB induced hepatocarcinogenesis and expect to find out the relationship between integrins and liver cancer metastasis.

MATERIALS AND METHODS

Animal model^[3]

Male Wistar rats ($n=100$, provided by Experimental Animal Center, Shanghai Medical University), weighing 100g-150g, were divided into four groups: group A ($n=44$), fed with low choline maize powder and 0.03% 3'-Me-DAB for 12 weeks; group B ($n=28$), fed with low choline maize powder only for 12 weeks and group C ($n=28$), fed with standard diet only. After 12 weeks, the rats of group A and B were fed with standard diet. Four to rats were killed in each group at week 3, 6, 9, 12 and 16 and 8-12 at week 20.

Tissue preparation

Small pieces of liver samples (including tumor and metastasis) were divided in two parts, one embedded in O.C.T (Miles, USA), rapidly frozen and stored at -70°C , and the other was fixed with 10% neutral buffered formalin, embedded in paraffin and used for routine histological examination.

Immunohistochemistry

The antibodies against integrins $\alpha_1, \alpha_2, \alpha_3$ and α_5 were purchased from Chemicon International INC and those against epidermal keratin (EK) from Nichi Lei Co. Tokyo, Japan. The rabbit PAP kits were prepared by our department^[4]. Integrins $\alpha_1, \alpha_2, \alpha_3$ and α_5 and EK in normal liver, liver cirrhosis

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and liver tumor were detected by PAP method. Briefly, 5 μ m cryostat sections of the frozen tissues were fixed with cold acetone and 10% neutral buffered formalin (9:1) for 10min, and washed in 0.01mol/L phosphate- buffered saline (PBS, pH 7.4) for 5 times, each for 3min. The sections were treated with methanol (containing 0.2 mL/L H₂O₂) at 37°C for 30min and washed in PBS, and then incubated in PBS with 100mL/L normal goat serum at 37°C for 30min. The sections were incubated with rabbit anti-integrins α_1 (1:1200 dilution), α_2 (1:1200 dilution), α_3 (1:1200 dilution), α_5 (1:1500 dilution) and EK (1:1000 dilution) respectively at 37°C for 30min and the n at 4°C overnight. The sections were washed in PBS and incubated with goat anti-rabbit IgG (1:200 dilution) at 37°C for 40min. After washing in PBS, the sections were incubated with rabbit PAP complex (1:200 dilution). The color was developed with 0.5g/L 3, 3'-diaminobenzidine/0.5mL/L H₂O₂/0.05 mol/L PBS (pH 7.6) for 10min. Normal rabbit serum instead of the specific primary antibodies was used as negative control.

Semiquantitation of the results

Only cells with membranous staining were considered; cells with intense dotlike cytoplasm immunoreactivity caused by endogenous peroxidase were ignored. The intensity of staining was divided into +++ (strong), ++(moderate), + (weak), +/- (equivocal), - (negative). Variable patterns were indicated by combining the two extremes of staining intensity, e.g. +++/+/-.

RESULTS

Morphologic changes

Liver cancer model of the rats in group A was successfully induced by fed 3'-Me-DAB with low choline maize powder. In the early experiment (week 1-9), the main morphologic changes were steatosis and necrosis of hepatocytes, proliferation of oval cells and cholangiofibrosis. In the middle of the experiment (week 10-16), massively proliferated oval cells, and extensive areas of cholangiofibrosis were visible. In some livers, few hepatocytes remained; they were either entrapped in cholangiofibrotic structures or located at the peripheries of these lesions. Then, liver cirrhosis and preneoplastic nodules were obvious. At the end of the experiment, most of the rats in group A had liver tumor, mainly mixed hepatocarcinoma (HCC) and cholangiocarcinoma (CCC). Tumor metastasized to the lung, spleen and diaphragm in some rats (Figure 1). There were no obvious morphologic changes in the livers of rats in group B and C.

Expression of integrins and EK

The overall staining patterns are shown in Table 1.

Table 1 Pattern of integrins and EK in normal liver, liver cirrhosis and liver tumor

	α_1	α_2	α_3	α_5	EK
Normal liver tissue					
Hepatocytes	+	-	-	+	-
Sinusoidal endothelial cells	+	-	-	+	-
Bile duct epithelium	-	-	-	-	+++
Liver cirrhosis					
Hepatocytes	+++	++	++	+++	-
Sinusoidal endothelial cells	++	+	+	++	-
Oval cell	+/-	+/-	+/-	+/-	+++/-
Hyperplastic bile duct epithelium	++	-	-	++	+++
Preneoplastic nodules	+	+/-	+/-	+	-
Tumor					
Primary	-/+	-	-	-/+	+++/-
Metastasis	+++/-	-	-	+++/-	+++/-

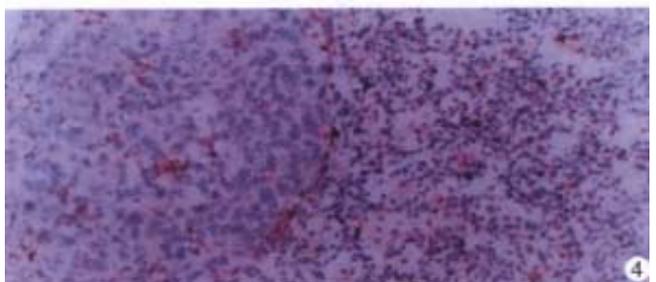
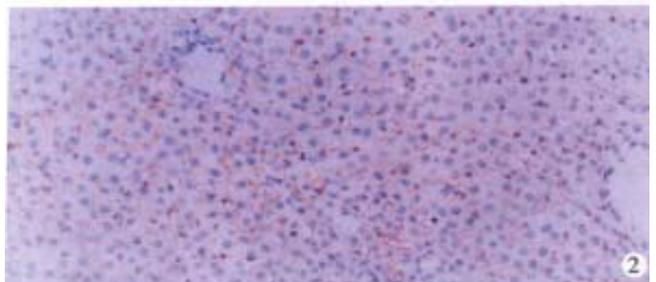
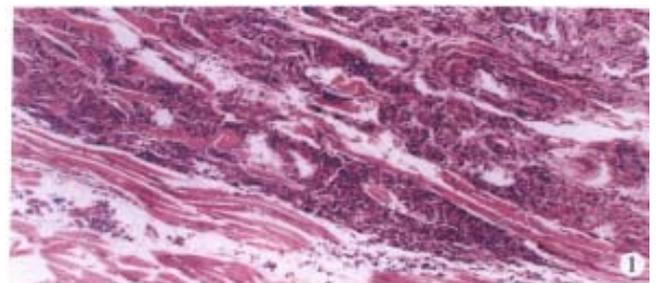


Figure 1 Tumor metastasis in diaphragm. HE \times 200

Figure 2 In liver cirrhosis, the expression of integrin α_1 was increased in hepatocytes and sinusoidal endothelial cells. PAP \times 200

Figure 3 Expression of α_1 was obviously decreased in the primary carcinoma(right)and in para-tumor liver cirrhosis(left), the expression of integrin α_1 was increased in hepatocytes and sinusoidal endothelial cells. PAP \times 100

Figure 4 Expression of α_1 in metastasis in the lung was higher than in primary carcinoma. PAP \times 200

In the normal liver tissues, integrins α_1 and α_5 were weakly expressed in hepatocytes and sinusoidal endothelial cells but not in bile duct epithelium. The predominant staining was located on the sinusoidal side plasma membranes of hepatocytes. EK was highly expressed in the bile duct epithelium but not in hepatocytes.

In liver cirrhosis, the expression of integrins α_1 , α_2 , α_3 and α_5 were increased in hepatocytes and sinusoidal endothelial cells but the distribution did not significantly change (Figure 2). However, hyperplastic bile duct epithelium only moderately expressed α_1 and α_5 . Oval cells were equivocal on expression of integrins but most of them highly expressed EK.

Expression of integrins α_1 , α_2 , α_3 and α_5 were obviously decreased in the preneoplastic nodules and primary carcinoma (Figure 3) but expression of integrins α_1 and α_5 in metastasis in the lung and diaphragm were higher than in primary carcinoma (Figure 4).

DISCUSSION

The β_1 subgroup of the integrin superfamily of cell-surface receptors (also known as VLA integrins) contains six heterodimers (VLA-1 to VLA-6), in which different α -subunits share a common β_1 subunit. They serve as receptor for extracellular matrix components as laminin (VLA-1, VLA-2, VLA-3, and VLA-6), collagen (VLA-1, VLA-2, and VLA-3), and fibronectin (VLA-3, VLA-4, and VLA-5) and are involved as counter-receptors for other cell-surface molecules (VLA-4 recognizes the vascular cell adhesion molecule-1 [VCAM-1]) in a cell-to-cell type of interaction^[2].

The integrins are thought to play important roles in differentiation and development, cell migration, and the complex process of tumor cell invasion and metastasis, as well as adhesion. The integrin β_1 and fibronectin were mainly distributed along sinusoids in normal liver, and the expression was weak in adult normal liver^[5]. The distribution did not significantly change during the regeneration and proliferation, but the expression was increased. They acquire some characteristics of fetal and neonatal hepatocytes, including alternations in cell surface properties, such as the composition of glycoconjugates, and the level of receptors for hormones and growth factors and of proteins involved in cell-cell contacts. In the early phase of liver carcinogenesis, proliferation of oval cells, cholangiofibrosis, proliferation of hepatocytes and the extracellular matrix (cirrhosis) were obvious. The high expression of integrins plays an important role in cirrhosis^[6].

Alterations in integrins receptor expression upon malignant transformation and naturally occurring human malignances are now well established^[7,8]. It was one of the properties of tumor cells that the expressions of integrins and fibronectin were decreased^[9]. Integrins such as fibronectin receptor transduce important growth inhibitory stimuli from the extracellular matrix. Integrins are involved not only in the adhesive, motile and invasive behavior of tumor cells but also in their growth regulation.

Expression of integrins α_1 , α_2 , α_3 and α_5 was obviously decreased in the preneoplastic nodules and primary carcinoma but expression of integrins α_1 and α_5 in metastasis in the lung and diaphragm was higher than in primary carcinoma. Integrins α_1 and α_5 may play a major role in chemically induced hepatocarcinogenesis and metastasis in rats, and it was necessary for tumor cells to adhere to the extracellular matrix in metastatic focus^[10,11].

EK is a mark of epidermal cells. EK was highly expressed by the bile duct epithelium but not by hepatocytes and might be a helpful tool in the differential diagnosis between HCC and CCC. Some oval cells expressed EK but some not. It suggested that oval cells are the progeny of hepatic stem cells, which might differentiate into preneoplastic parenchyma cells and might give rise to HCC.

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Immunohistochemical study on p53, H-rasp21, c-erbB-2 protein and PCNA expression in HCC tissues of Han and minority ethnic patients

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Subject headings liver neoplasms; p53; H-rasp21; c-erbB-2; proliferating cell nuclear antigen; immunohistochemistry

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Abstract

AIM To find out the difference of human primary liver carcinoma between Han and minority ethnic patients in Xinjiang.

METHODS Expression of p53, c-erbB-2, H-rasp21 protein and proliferating cell nuclear antigen (PCNA) in tumor tissues of 50 patients (Han 38, minority 12) with primary hepatic carcinoma was detected by immunohistochemistry (LSAB).

RESULTS The positive frequency of p53, c-erbB-2, H-rasp21 and PCNA expression was 46.0% (23/50), 70.0% (35/50), 68.0% (34/50) and 82.0% (41/50) in tumor tissues; 4.0% (2/50), 22.0% (11/50), 64.0% (32/50) and 52.0% (26/50) in peritumors respectively and a significant difference, except for H-rasp21, of oncogene alteration was found ($P < 0.05$) between tumor and non-tumorous tissues. Combined the three oncogenes alteration, 26% (13/50) tumor tissues had positive immunoreactivity, but in peritumor and normal livers it was negative. The positive rate of p53, c-erbB-2 and H-rasp21 protein expression was 39.5% (15/38), 60.5% (23/38) and 39.5% (15/38) in tumors of Han patients; 66.7% (8/12), 100% (12/12) and 75.0% (9/12) in minorities respectively, with statistical difference

($P < 0.05$).

CONCLUSION Overexpression of p53, c-erbB-2 and H-rasp21 in human primary liver carcinoma is an important biomarker of genetic alteration. The different frequency of these oncogenetic changes may reflect some environmental or/and ethnic hereditary factors affecting the liver carcinogenesis. The special life style of Han, Uygur, Kazak and Mongolia nationalities in Xinjiang may also be related to the etiopathogenesis of this disease.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major cancers with highest mortality in the world. There is a striking variety of HCC incidence rates between countries, with a highest to lowest of 112.5 for males and 54.7 for females. The high-risk populations are clustered in Sub-Sahara Africa and eastern Asia^[1]. Although many etiological factors, e.g., HBV, aflatoxin B1, heavy alcohol drinking, etc. have been established^[2-4], the exact mechanisms of HCC carcinogenesis is still poorly understood. The natural environment, multiple ethnic groups with different hereditary background, and special life style in Xinjiang are quite different from other areas in China, which may implicate the etiopathogenesis of HCC.

A great number of studies have demonstrated that human HCC hepatocarcinogenesis is a multistepwise and multigenetic alteration induced by various pathogens with the involvement of multifactorial etiology. Alterations of cytogenes, including mutation, amplification, allelic loss of oncogenes and tumor suppressor genes, are most common in many forms of human cancers, including HCC. Overexpression of p53 protein, c-erbB-2, c-myc and H-ras oncoprotein and proliferating cell nuclear antigen (PCNA) were observed in the tumor tissues of patients or experimental animals with HCC^[1-10]. In order to understand the possible mechanism of hepatocarcinogenesis and if there is any difference among various ethnic patients with HCC, we detected the expressions of p53 protein, c-erbB-2 and H-rasp21 oncoprotein, and PCNA in

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tumor, peritumor and normal liver tissues by immunohistochemistry.

MATERIALS AND METHODS

Patients

A series of 50 patients with HCC, including 38 Han, 12 minorities (Uygur 8, Kazak 2 and Mongolia 2); males 34, females 16; aged from 18 to 72 (average 50.1) years, was collected from the 1st Teaching Hospital of Xinjiang Medical University from 1991 to 1995.

Specimens

All the tumor and peritumor tissues of 50 cases and 10 specimens of normal liver tissue as a control were fixed in 100mL/L formalin and embedded in paraffin for routine histopathologic examination, and these paraffin blocks were obtained from the pathologic record. The blocks including 50 tumors, 50 peritumors and 10 non-tumors were cut into five sections of 4 μ m in thickness and underwent H.E. and immunohistochemical staining, respectively.

Immunohistochemical staining

Four- μ m thick sections were dewaxed, and rehydrated according to routine procedures. Nonspecific binding was blocked by incubation with bovine albumin for 10 minutes and endogenous peroxidase activity was blocked with 30mL/L hydrogen peroxide for 5 minutes. The sections were pretreated in a microwave oven at 750W for 10 minutes in citrate buffer (pH 6.0), and then incubated for 5 minutes in microwave oven with primary antibodies, including monoclonal anti-p53 protein (Do-7), anti-H-rasp21 oncoprotein, anti-PCNA (P-c 10) (1:40) and polyclonal anti-c-erbB-2 oncoprotein (1:200) (Dako), respectively. We used the LSAB kits (Dako) for immunostaining, the secondary antibodies and reagents were treated by the routine procedures in microwave oven. The colour was developed using 3-amino-9-ethylcarbazol (AEC). The sections were counterstained with Meyer's hematoxylin.

Assessment of staining reaction

Only tumor cells with distinct nuclear immunostaining in both p53 protein and PCNA were considered positive. Tumors were recorded as positive for c-erbB-2, H-rasp21 oncoprotein if more than 5% of tumors showed distinct membrane and cytoplasm staining. The immunoreactivity was registered semiquantitatively. The positive cells were scored as (-) negative; (+) 1-5 positive cells per high magnification (400 fold); (++) 6-20 positive cells; (+++) more than 21 positive cells found in p53 protein and PCNA immunostaining respectively.

Statistics

The immunoreactivity for p53, c-erbB-2, H-rasp21 and PCNA parameters were considered to represent discrete values in various lesions. The Chi square test (χ^2) was used and $P < 0.05$ was accepted as a statistically significant difference.

RESULTS

The immunostaining p53 protein and PCNA appeared red-brown in nucleus and tumor or peritumor cells with various densities were distributed unevenly (Figures 1, 2). The immunoreactivity of c-erbB-2 and H-rasp21 oncoprotein showed distinct membrane and cytoplasm staining (Figures 3, 4). Table 1 summarizes the different frequencies of immunostaining positive cells for p53 protein, H-rasp21, c-erbB-2 and PCNA in tumor and non-tumor tissues.

There was a significant difference among three oncogenes expression respectively except PCNA between Han and minorities ($P < 0.05$). The oncoprotein expression was lower in Han than in the three minorities. Table 2 summarizes the three oncogenes expression in tumor and non-tumor tissues and distribution of different nationalities.

Table 3 shows the combined expression of three oncogenes in HCC of different nationalities. The frequency was higher in minorities (41.7%) than in the Hans (21.1%), without statistical difference ($P > 0.05$).

Table 1 Expression of p53 protein, c-erbB-2, H-rasp21 and PCNA in various liver lesions

Histology	n	p53		c-erbB-2		H-ras		PCNA	
		n	%	n	%	n	%	n	%
Tumor (A)	50	23	46.0	35	70.0	34	68.0	41	82.0
Peritumor (B)	50	2	4.0 ^a	11	22.0 ^a	32	64.0 ^c	26	52.0 ^a
Cirrhosis	33	2	6.0	9	27.3	23	70.0	18	54.5
Non-cirrhosis	17	0	0	2	11.8	9	53.0	8	47.0
Normal liver (C)	10	0	0	0	0	3	30.0	0	0

^a $P < 0.05$, vs A, C; ^c $P < 0.05$, vs C; χ^2 test.

Table 2 Expression of p53, c-erbB-2, H-rasp21 and PCNA in HCC of different nationalities

Nationalities	n	p53		c-erbB-2		H-ras		PCNA	
		n	%	n	%	n	%	n	%
Han	36	15	39.5	23	60.0	15	39.5	32	84.2
Minority	12	8	66.7 ^a	12	100.0 ^a	9	75.0 ^a	9	75.0
Uygur	8	5	62.5	8	100.0	6	75.0	5	62.5
Kazak	2	1	50.0	2	100.0	2	100.0	2	100.0
Mongolia	2	2	100.0	2	100.0	1	50.0	2	100.0

^a $P < 0.05$, vs Han, χ^2 test.

Table 3 The nationality distribution of the combined expression of three oncogenes in HCC

Nationalities	n	p53+c-erbB-2+H-rasp21	
		n	%
Han	38	8	21.1
Minority	12	5	41.7

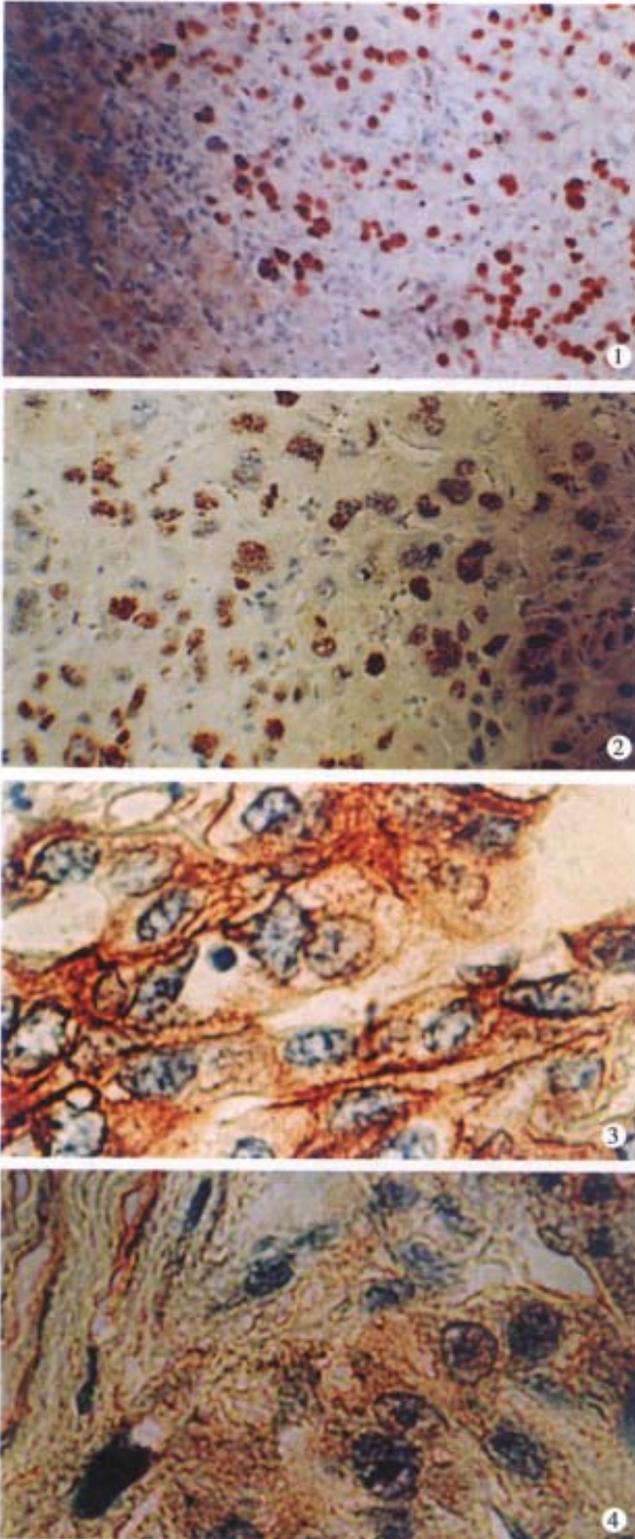


Figure 1 Strongly positive immunoperoxidase staining of the *p53* protein with the anti-*p53* mAb Do-7 in HCC, predominantly in nuclei. Absence of staining in nontumorous liver. LSAB method, $\times 100$

Figure 2 HCC with diffuse PCNA staining using mAb PC10. LSAB method, $\times 200$

Figure 3 Cholangiocellular carcinoma showing extensive strong gly membrane and plasma positivity for c-erbB-2 using poly-Ab code N. A485. LSAB method, $\times 400$

Figure 4 Diffuse H-rasp21 using mAb Ncc-Ras-001 staining in HCC and nontumorous tissues. LSAB method, $\times 400$

DISCUSSION

Alterations of oncogenes and tumor suppressor genes were critical events in hepatocarcinogenesis induced by HBV/HCV, chemical carcinogens, etc. have been demonstrated by biopathological and experimental researches^[7-10]. Cerutti^[11] and Tong^[12] reported that the mutagenesis of H-ras proto-oncogene and *p53* tumor suppressor gene may be the most important events change in hepatocellular carcinomas. Fujimoto *et al*^[13] observed some allelic loss of tumor suppressor genes in Chinese patients with hepatocarcinomas. Scor sone *et al*^[14] demonstrated that *p53* gene mutations clustered at codon 249 in HBV positive hepatocarcinoma patients in China. Recently, Ming *et al*^[15] also found that frequency of 249 codon mutation of *p53* gene was much higher in high prevalent area of hepatocellular carcinomas than that in the low-risk area in China. More than 95% (20/21) cancer specimens exhibited strong intranuclear accumulation of *p53* protein, which can be detected by immunohistology. However, Biersing *et al*^[16] and Vesey *et al*^[17] found little or no point mutations of *p53* gene in human hepatocarcinoma in Swedish and Aust ralian patients. Therefore, the overexpression of *p53* protein in hepatocarcinoma specimens can be used as the mutant *p53* biopathological mark in tumor tissues. Liu *et al*^[18] and Zu *et al*^[19] found that the frequency of intranuclear positive immunoreactivity of *p53* protein was significantly different between high-risk and low-risk HCC areas, and HBxAg can enhance *p53* protein accumulation, suggesting that HBxAg was capable of binding *p53* and formed a protein-protein complex which might reduce or inactivate the antiproliferative activity of *p53* and played an important role in the pathogenesis of HBV-associated hepatocarcinomas. In this study, the overexpression of *p53* protein revealed very significant difference between tumor (46%) and peritumor (4%) tissues, and negative in normal liver samples ($P < 0.05$), indicating that *p53* gene mutations played similar roles in hepatocarcinogenesis in Xin Jiang regardless of the etiological factors. Su *et al*^[20], Nakapoulou-*et al*^[21] and Yu *et al*^[22] have reported that c-erbB-2 oncogene was related to the dysregulation of proliferation and differentiation of the hepatocytes in hepatotumorigenesis, and overexpression in various pathological lesions had different frequencies. We observed that overexpression of c-erbB-2 oncoprotein in tumor (70%) and in peritumor (22%) and in normal liver tissues also showed very distinct difference ($P < 0.05$), suggesting that c-erbB-2 oncogene and tumor suppressor genes jointly participate in the occurrence and development of HCC, and related to

the abnormal regulation even malignant transformation of hepatocytes induced by various pathogenes. Ushijima *et al*^[23], Imai *et al*^[24], Tamano *et al*^[25] found that mutations of ras oncogene may be the early events, and the expression in tumor or non-tumor tissues can be detected with different rates. Lin *et al*^[7] found that the expression of N-ras in liver tumor was correlated to the differentiated status of altered liver cells. In this study, there was no statistical significance between tumor (68%) and peritumor (64%) tissues in the expression of H-ras oncoprotein. Positive cells were also found in 30% of normal liver specimens, reasonably which can not be explained. Proliferating cell nuclear antigen (PCNA), as an index of cellular proliferative status was determined in various lesions. Waga *et al*^[26], Maeda *et al*^[27] found that p53 tumor suppressor gene can control the cyclin-dependent kinases to regulate DNA replication involving PCNA interaction by p21 protein pathway. The overexpression of PCNA with high frequency was usually used as a reliable marker for assessment of tumor progress, premalignant evolution and clinical prognosis of patients with various malignancies. We found that the positive frequency of PCNA in tumor (82%) was significantly higher than in peritumor (52%) tissues ($P < 0.05$), indicating that the non-neoplastic tissues surrounding the hepatocarcinomas may have premalignant lesions including cellular hyperplasia, dysplasia, even *in situ* carcinoma. Therefore, these multiple molecular indexes in combination could be used as the biomolecular standard for diagnosis of malignant and benign lesions. The inactivation of tumor suppressor gene p53, Rb, p16, etc. was demonstrated in different forms, and implied the pathogenesis of human malignant diseases. Slagle *et al*^[28] and Shao *et al*^[29] found the loss of heterozygosity on chromosome 17p near the p53 gene or 1p in HBV-positive HCC in China.

It is noteworthy that the alteration of p53 tumor suppressor gene and c-erbB-2 and H-ras oncogenes in different ethnic patients with HCC revealed significant difference. The expression of p53 protein and c-erbB-2 and H-ras oncoprotein in carcinoma specimens was higher in the minority than in Han patients by immunohistochemical detection ($P < 0.05$). Moreover, the three combined abnormal expression of p53 protein and c-erbB-2 and H-rasoncoprotein in tumor tissues was higher in minority (41%) than in Han (21%) patients. This may reflect, to a certain extent, the differences of ethnic gene-susceptibility to various carcinogenes and effect on hepatocarcinogenesis. The pathogenesis of primary hepatic carcinoma is a multistage process with the involvement of multifactorial etiology, and the gene-environment

interactions are involved in the development of HCC in humans. In order to understand the exact difference concerning the etiopathogenesis of HCC among different ethnic groups, further studies with more samples are needed, especially in minority ethnic patients.

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DNA-based vaccination induces humoral and cellular immune responses against hepatitis B virus surface antigen in mice without activation of C-myc

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Abstract

AIM To develop a safe and effective DNA vaccine for inducing humoral and cellular immunological responses against hepatitis B virus surface antigen (HBsAg).

METHODS BALB/c mice were inoculated with NV-HB/s, a recombinant plasmid that had been inserted S gene of hepatitis B virus genome and could express HBsAg in eukaryotes. HBsAg expression was measured by ABC immunohistochemical assay, generation of anti-HBs by ELISA and cytotoxic T lymphocyte (CTL), by MTT method, existence of vaccine DNA by Southern blot hybridization and activation of oncogene C-myc by *in situ* hybridization.

RESULTS With NV-HB/s vaccination by intramuscular injection, anti-HBs was initially positive 2 weeks after inoculation while all mice tested were HBsAg positive in the muscles. The titers and seroconversion rate of anti-HBs were steadily increasing as time went on and were dose-dependent. All the mice inoculated with 100 μ g NV-HB/s were anti-HBs positive one month after inoculation, the titer was 1:1024 or more. The humoral immune response was similar induced by either intramuscular or intradermal injection. CTL activities were much stronger (45.26%) in NV-HB/s DNA immunized mice as

compared with those (only 6%) in plasma-derived HBsAg vaccine immunized mice. Two months after inoculation, all muscle samples were positive by Southern-blot hybridization for NV-HB/s DNA detection, but decreased to 25% and all were undetectable by *in situ* hybridization after 6 months. No oncogene C-myc activation was found in the muscle of inoculation site.

CONCLUSION NV-HB/s could generate humoral and cellular immunological responses against HBsAg that had been safely expressed *in situ* by NV-HB/s vaccination.

INTRODUCTION

Infection with hepatitis B virus (HBV) is the most important and most common cause of acute and chronic liver disease worldwide. Some of them eventually progress to cirrhosis or/and liver failure. Persistent HBV infection is associated with a high risk of primary hepatocellular carcinoma. Hepatitis B virus surface antigen (HBsAg) is the protein product of the S gene of the HBV genome and is the protective immunogen used for developing vaccine against HBV infection. The current commercial HBV vaccines which were divided into plasma-derived HBV vaccine and recombinant HBV vaccine by genetic techniques induce neutralizing antibody (anti-HBs) against HBV. Unfortunately, up to 5% of the adult population may not respond to the currently available HBV vaccines, so great efforts have been made to develop more successful vaccines to prevent and even treat chronic HBV infection^[1]. The newest approach is the use of naked DNA vaccine, the so-called genetic immunization, which has been shown to be effective at generating protective immune responses against a wide variety of diseases^[2-18]. This technique involves the transfer of a viral gene into muscle or skin cells of host by a plasmid vector with subsequent endogenous production and intracellular processing of the viral structural proteins into small antigenic peptide. Such peptides are expressed subsequently on the cell surface in the context of major

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histocompatibility complex (MHC) molecules, and may be secreted from the cells to stimulate a humoral and T-helper cell immune response. After genetic immunization, both humoral and cell-mediated immune responses developed^[19-24]. In this study, we evaluated both humoral and cellular immune responses against HBsAg, which were generated in a mice model by DNA-based immunization with naked DNA vaccine NV-HB/s.

MATERIALS AND METHODS

Preparation of nucleic acid vaccine NV-HB/s
S sequences encoding surface antigen of HBV were inserted into eukaryotic expression vector pRc/CMV (Invitrogen, San Diego, CA) under the transcription control of the cytomegalovirus early promoter. The reconstructed plasmid was then amplified in *E. Coli* DH5 α . After extraction and purification plasmid DNA was dissolved in PBS (pH 7.2) at the concentration of 1 μ g/L as the DNA vaccine NV-HB/s and stored at -70 $^{\circ}$ C until use for animal immunization.

DNA vaccine immunization in mice

Vaccine trials in mice were accomplished by the administration of NV-HB/s to 6-week-old BALB/c mice. Dosage of DNA vaccine inoculation was 100 μ g/100 μ L, 10 μ g/100 μ L, and 1 μ g/100 μ L. DNA vaccine was administrated by the following routes: ① intramuscular injection (I.M.) into both sides of tibialis anterior muscles, half dose for each side; ② intradermal injection (I.D.) at 1cm distal from the tail base; and ③ inoculated sites of tibialis anterior muscles were pretreated with the injection of bupivacaine (0.2 μ g per site) 7 days before the vaccination by I.M. at the same sites. The control groups included: pRc/CMV (as negative control), PBS (as blank control); commercial plasma-derived HBV vaccine which was the purified HBsAg (as positive control).

Serum samples were collected 3 days, 1 and 2 weeks, 1, 2, 4 and 6 months after inoculation through retrobulbar puncture and stored under -70 $^{\circ}$ C. Tibialis anterior muscle samples were collected from sacrificed mice 7 times with the same time schedule of the serum sample collection. Each muscle sample was divided into 2 pieces, one was stored at -70 $^{\circ}$ C, the other was fixed in formalin and embedded with paraffin for making into slides.

For detection of CTL activity, another group of mice was inoculated I.M. with 100 μ g NV-HB/s (commercial plasma-derived HBV vaccine as control) and boosted one month later with the same dosage. After one week of boosting, the mice were sacrificed to collect spleen cells for detection of CTL activity.

Detection of HBsAg and anti-HBs

By ABC immunohistochemistry assay, all muscle

tissue samples collected from the inoculation sites were tested for HBsAg expression; and by enzyme-linked immunosorbent assay (ELISA), all sera samples were detected for anti-HBs, which were immunologically induced by HBsAg.

Detection of CTL activity

Spleen cells were collected from the sacrificed mice, washed and suspended with HEPES solution at a concentration of 5 \times 10⁶ cells/mL as effect cells. SP2/0-HBs cells (myeloma cell line SP2/0 derived from BALB/c mice had been transfected with NV-HB/s DNA *in vitro*) were suspended with HEPES at a concentration of 5 \times 10⁴ cells/mL as target cells. The effect cells were pre-cultured with stimulating cells, which were SP2/0-HBs irradiated with 10000 rad, and mixed with target cells by the optimal ratio. After a 3h incubation at 37 $^{\circ}$ C, 5% CO₂ and 95% relevant humidity, CTL activities were detected according to the manual described for MTT test kit of CTL activity detection (Boehringer Mannheim Company). In brief, the mixtures were spun and the supernatant was collected, then LDH substrates were added to the supernatants. Half an hour later, O.D. value of the solution was measured. The percentage specific cytolysis was determined by the following formula: the specific cytolysis (%) = (experimental release - spontaneous release of effect cell - spontaneous release of target cells) / (maximum release of target cell - spontaneous release of target cells) \times 100%.

Existence of NV-HB/s plasmid in the muscles tissue

To find out how long the plasmid DNA could exist in the muscle tissue at the injection sites after inoculation, NV-HB/s was detected by *in situ* hybridization and Southern blot hybridization with Dig-pRc/CMV probe prepared in our laboratory with the protocol described by Zhao *et al*^[25,26]. For *in situ* hybridization, the fixed muscle samples slides were hybridized with Dig-pRc/CMV probe and for Southern blot hybridization, the frozen muscle samples at -70 $^{\circ}$ C were thawed, homogenized, digested with protease, and extracted with phenol/CH₂Cl₂, and the cell DNA recovered was precipitated with ethanol and dissolved in T.E. solution for Southern blot hybridization.

Pathological examination and detection for C-myc activation

Special attention was paid to the health condition of mice after inoculation. Routine pathological examination was made for all mice to know if there is any pathological change. The muscle slides were detected for C-myc mRNA by hybridization *in situ* with Dig-C-myc-cDNA probe with the protocol described by Zhao *et al*^[26,27].

RESULTS

HBsAg expression and antibody induction

After 3 days of inoculation, no HBsAg was detected in the muscle samples. However, 1/3 mice after 1 week of inoculation and 3/3 mice after 2 weeks, were HBsAg positive in the muscles collected from the mice vaccinated with NV-HB/s (Table 1). On the other hand, there was no detectable HBsAg in all sera samples.

Anti-HBs were initially positive 2 weeks later after inoculation while all mice were HBsAg positive in the muscles. The titers and seroconversion rate of anti-HBs were steadily increasing as time goes on. All the mice inoculated with 100 μ g NV-HB/s were anti-HBs positive one month later after inoculation, the titer could be 1:1024 or more. Titers and seroconversion rate were dosage-dependent. For the mice inoculated with 1 μ g or 10 μ g NV-HB/s, the positive rate of anti-HBs was only 3/6 or 5/6 respectively even 2 months after inoculation, while their titers of anti-HBs were also lower than that of mice inoculated with 100 μ g NV-HB/s ($P < 0.05$).

Humoral immune response induced with NV-HB/s vaccination were similar by either intramuscular or intradermal injection. The positive rates of anti-HBs were nearly the same although the titers induced by I.M. were higher than those by I.D. Pretreatment with bupivacaine before I.M. did not promote the humoral immune response in this experiment.

Table 1 HBsAg expression and anti-HBs induction in the mice inoculated with DNA vaccine against hepatitis B (NV-HB/s)

	Positive rate after inoculation						
	3d	1wk	2wks	1mo	2mos	4mos	6mos
HBsAg(%)	0 (0/4)	33.3 (1/3)	100 (3/3)	75 (3/4)	5 (3/4)	83.3 (5/6)	75 (6/8)
Anti-HBs(%)	0 (0/4)	0 (0/3)	66 \pm 7 (2/3)	100 (4/4)	100 (4/4)	100 (6/6)	100 (8/8)

HBsAg was detected from mice muscle tissue samples of injection sites by ABC immunohistochemistry. Anti-HBs was detected from sera samples by ELISA. The numbers in parenthesis indicate the number of mice with positive detection results/the total number of mice detected.

NV-HB/s vaccination induces HBsAg-specific CTLs

In our data, CTL activities were much stronger in NV-HB/s immunized mice (45.26%) as compared with those in plasma-derived HBsAg vaccine immunized mice (only 6%) ($P < 0.01$). Certainly there was no CTL activity detected from any negative control groups.

Persistence of NV-HB/s DNA in the muscles

Two months later after inoculation, all muscle samples were positive by Southern-blot hybridization for NV-HB/s DNA detection.

However, 6 months after inoculation, the positive rate decreased to 25%, and all the muscle samples were negative by *in situ* hybridization for NV-HB/s DNA detection. Considering that the DNA detection sensitivity by *in situ* hybridization is lower than by Southern-blot hybridization, the above-mentioned result implies that the amount of NV-HB/s existed in the muscle was very low and degenerated rapidly as time goes on.

Effect of NV-HB/s vaccination on oncogene C-myc

In mice sacrificed 3 days after injection, hyaline degeneration was observed, including swollen muscular fiber, disappearance of cross striation and red stain of myocytes plasma, which were slightly more apparent in mice inoculated with NV-HB/s or pRc/CMV than those inoculated with PBS or plasma-derived vaccines. In the former groups, some lymphocytes aggregated, and in one of them neutrophil clustering was found. These pathological changes disappeared 1 week later in the PBS-injected mice and 4 weeks later in NV-HB/s or pRc/CMV injected mice. During the experiment the mice looked healthy, and no C-myc mRNA was detected in all the muscle sample collected from the mice inoculated.

DISCUSSION

In this study, we evaluated the humoral and cellular immune response induced in BALB/c mice by DNA-mediated immunization with NV-HB/s, a recombinant plasmid which had been inserted S gene and could express HBsAg in eukaryotes. The results showed that even after a single intramuscular injection of DNA, a detectable antibody response could be induced and sustained which resembles that of natural HBV infection in terms of the fine specificity. There was no significant difference between the humoral immune response induced with NV-HB/s by intramuscular injection and those by intradermal injection^[28]. These data provided evidence that the envelope proteins encoded by the recombinant DNA has adopted a conformation similar to that of the proteins present during natural infection. This conclusion validates the use of DNA-based *in vivo* synthesis of the antigen for immunization purposes.

It is the important feature that DNA-based immunization is the *in situ* production of the expressed protein subsequent to the introduction of DNA carrying the protein coding sequences, mimicking in this respect a viral infection^[29]. In our data, the muscle samples had positive HBsAg expression in 1 week for 1/3 mice, and in 2 weeks after NV-HB/s inoculation for all the mice detected by ABC immunohistochemistry assay. However, no HBsAg was detected from any sera sample, which suggested that the quantity of HBsAg protein expression was high enough to induce immune

response in mice. Such endogenous protein synthesis could allow presentation of antigens by class I molecules of the major histocompatibility complex (MHC), thus resulting in the induction of CD8⁺ cytotoxic T lymphocytes (CTL). Therefore, the potential of DNA-mediated immunization in partially mimic viral infection promises the efficacy of live attenuated vaccines without the risk of inadvertent infection^[30-31].

DNA-based immunization was shown to induce a broad range of immune responses, including neutralizing antibodies, CTL, T-cell proliferation, and protection against challenge with the various pathogens. In this study, direct injection of NV-HB/s DNA encoding for HBsAg into the muscle of mice could induce humoral and, more important, cell-mediated immune responses.

Compared with immunizations with soluble recombinant proteins or peptides (e.g. the commercial plasma-derived HB vaccine), the advantage of DNA-mediated immunization is to induce a more Th1-like immune response with the production of inflammatory CD4⁺ T cell as well as cytotoxic T cell activity, presumably due to the intracellular processing of viral proteins into peptides and subsequent loading onto MHC class I molecules in transfected muscle cells and to be defined interactions of the complex with APCs. Immunization with soluble protein primarily leads to a humoral immune response due to processing through the MHC class II pathway. The disadvantage of the immunization with foreign peptides is that there is only a limited number of epitopes available for stimulation of the host immune response. In contrast, all naturally occurring B and T cell epitopes encoded for each protein by the DNA construct of interest are presumably preserved for recognition by TCRs and will consequently generate very broad humoral and cellular immune responses. In our data, NV-HB/s vaccination generated much stronger CTL activities (45.26%) than plasma-derived HBsAg vaccine inoculation could (6%) ($P < 0.01$).

Some types of local APCs will take up and process antigens to induce MHC class I and II restricted T-cell responses in DNA-mediated immunization. For intramuscular inoculation, myoblasts and myotubes express MHC class I molecules but not MHC class II and other co-stimulatory molecules^[32]. It was reported that pretreatment of the tissues with the anesthetic bupivacaine could dilate local vessels, thus enhancing DNA uptake by myocytes^[33]. Our data did not prove that the use of bupivacaine could improve responses to HNV-HB/s vaccination^[34]. As CTLs can recognize cells that are already infected, it might be desirable particularly in the prevention, and even treatment of chronic viral infection. Moreover, unlike antibody responses, which are usually type specific CTLs can crossreact

against different viral epitopes, thus potentially affording greater protection against disparate viral strains. During active viral replication, HBV has a very high mutation rate^[35]. In this approach, the vaccine escape mutants HBV strains could be conquered.

It is known that cytotoxic T lymphocyte (CTL) activity against HBV structural proteins is not detectable in peripheral blood lymphocytes derived from the individuals with persistent HBV infection. Some chronic HBV-infected individuals who had spontaneous clearance of HBV DNA from sera are often accompanied by increased CD4⁺ T-helper responses and acute exacerbation of liver disease. So an attractive hypothesis for the development of persistent viral infection is that HBV-specific CTLs are unable to clear virus from the liver because of substantially decreased intrahepatic levels or qualitative changes in CTLs activity^[36,37]. On the other hand, the observation of spontaneous HBV clearance in some individuals indicated that the suboptimal cellular immune response may be reversible. Therefore, strategies designed to boost the HBV-specific immune response or to alter the balance between the cytopathic and the regulatory component of the response may be able to terminate persistent infection. There is strong evidence that the efficacy of the CTL response to HBV structural proteins may be crucial for eradication of persistent viral infection. It has been shown that the adoptive transfer of HBsAg-specific CTLs into HBV transgenic mice was associated with HBV clearance from the liver by antiviral effects of secreted IFN- α and tumor necrosis factor α derived from sensitized cells, without killing hepatocytes^[38,39].

In this study, we presented evidence that DNA vaccine inoculation is capable of eliciting Ag-specific immune responses in both effector pathways of the immune system: the humoral and cellular immune responses. We have shown that NV-HB/s vaccination is able to induce humoral and CTL activity in mice using this approach. Our data may be beneficial for possible antiviral therapy of chronic HBV infection. Thus, NV-HB/s could be promising candidates as antiviral agents for persistent viral infection of the liver by inducing a strong cellular immune response after intramuscular immunization. However, it was noteworthy that the generation of such protective immune responses in humans remains to be established.

Finally, the safety of DNA vaccine remains theoretical concerns, for example, if the foreign DNA may integrate into the host genome with the possibility of disrupting normal genes and malignant transformation^[33]. Our study showed no proof of oncogene C-myc activation with specific immune response induced by NV-HB/s inoculation. The mice looked healthy except a brief trauma reaction at the inoculation site caused by injection.

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Role of P-selectin and anti-P-selectin monoclonal antibody in apoptosis during hepatic/renal ischemia reperfusion injury

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Subject headings hepatic ischemia reperfusion injury; renal ischemia reperfusion injury; P-selectin; antibody, monoclonal; apoptosis

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Abstract

AIM To evaluate the potential role of P-selectin and anti-P-selectin monoclonal antibody (mAb) in apoptosis during hepatic/renal ischemia-reperfusion injury.

METHODS Plasma P-selectin level, hepatic/renal P-selectin expression and cell apoptosis were detected in rat model of hepatic/renal ischemia-reperfusion injury. ELISA, immunohistochemistry and TUNEL were used. Some ischemia-reperfusion rats were treated with anti-P-selectin mAb.

RESULTS Hepatic/renal function insufficiency, up-regulated expression of P-selectin in plasma and hepatic/renal tissue, hepatic/renal histopathological damages and cell apoptosis were found in rats with hepatic/renal ischemia-reperfusion injury, while these changes became less conspicuous in animals treated with anti-P-selectin mAb.

CONCLUSION P-selectin might mediate neutrophil infiltration and cell apoptosis and contribute to hepatic/renal ischemia-reperfusion injury, anti-P-selectin mAb might be an efficient approach for the prevention and treatment of hepatic/renal ischemia-reperfusion injury.

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INTRODUCTION

Visceral ischemia-reperfusion injury is very common clinically. Up to now, there has been no effective measure for its prevention and treatment, so this pathological injury process has aroused great attention. Recently, it is proved that neutrophil infiltration, oxygen free radical, some cell factors (TNF- α and IL- β), adhesion molecules and cell apoptosis are related to ischemia-reperfusion injury^[1,2]. Depletion or functional inactivation of neutrophils has been demonstrated to prevent effectively reperfusion-induced tissue damage^[3,4]. P-selectin is expressed by activated platelets and endothelium, and contributes to the interactions of these activated cells with polymorphonuclear neutrophils (PMN) or monophages. It is also involved in the early stages of inflammation, thrombogenesis and tissue injury induced by these cells^[5,6]. In this article, the role of P-selectin in cell apoptosis in hepatic/renal ischemia-reperfusion injury, as well as the preventive and curative effect of P-selectin monoclonal antibody (mAb) were studied with the rat model of ischemia-reperfusion injury.

MATERIAL AND METHODS

Animal model

Ninety male Wistar rats (Shanghai Experimental Animal Center, Chinese Academy of Sciences), weighing 200g \pm 5g, were given free access to water for three days before experiment. The rats were anesthetized with 2.5% sodium pentobarbital intraperitoneally, and randomly divided into 2 groups. In one group of rats, the ligament linking liver, diaphragm and abdominal wall were separated, portal vein and liver artery that drain blood to left hepatic lobe were freed by blunt dissection and blocked with a microvascular clamp for 60 minutes, then the clamp was removed, and reperfusion was performed; while in another group, the left renal artery was freed, and blood flow was also blocked with a microvascular clamp for 60 minutes, then the clamp was removed and reperfusion was given, simultaneously, the right kidney was cut off. The two groups of rats were randomly divided into P-selectin monoclonal antibody treatment group ($n=20$) and non treatment group ($n=20$). They were divided into 4

subgroups according to the indicated time 1, 3, 6, and 24 hours after reperfusion. Five minutes before reperfusion, animals were randomly injected with P-selectin mAb (2mg/kg, Suzhou Medical College), or 0.9% NaCl solution intravenously. Sham-operated group ($n=5$) (anesthesia and opening celiac cavity were performed, not blocking hepatic or renal blood flow) served as control.

Collection and measurement methods of specimens

Blood and left hepatic lobe and renal tissues were harvested at the indicated time. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and blood urea nitrogen (BUN) and creatinine (sCr) were measured with a 747 automatic analyzer (Hitachi Boehringer Mannheim, Mannheim, Germany). Plasma P-selectin levels were estimated with ELISA method. Left hepatic lobe and renal tissues were fixed with 10% formalin, and embedded routinely in paraffin. Sections were cut 5 μ m thick and stained with hematoxylin and eosin for light microscope examination. Expression of P-selectin in hepatic and renal tissues was assayed by immunohistochemical method with labelled streptavidin biotin (LSAB) kit (Fujian Maixin Biotechnology Co, Products of Biotechnology Co. CA, USA)^[7]. Apoptotic cells in hepatic and renal tissues were detected by TUNEL kit (Boehringer Company). Ten HP visual fields ($\times 400$) were selected randomly within each section to count out apoptotic cells, cell with blue-purple nucleolus belonged to positive cell.

Statistical analysis

Data was presented with $\bar{x}\pm s$, and Student's *t* test was used to determine changes between different groups.

RESULTS

Hepatic and renal function evaluation

At 24 hours after hepatic ischemia-reperfusion, serum AST (667U/L \pm 142U/L) and ALT (491U/L \pm 94U/L) levels in saline-treated group were much higher than the sham group (74U/L \pm 19U/L and 55U/L \pm 12U/L, respectively), $P<0.05$. Treatment with P-selectin mAb resulted in significantly lower levels of AST (271U/L \pm 65U/L) and ALT (233U/L \pm 44U/L) compared with saline-treated group ($P<0.05$).

At 24 hours after renal ischemia-reperfusion, the levels of serum BUN (52.49 mmol/L \pm 8.91 mmol/L) and Cr (456.31 μ mol/L \pm 65.25 μ mol/L) in saline-treated group were much higher than the sham group (7.16 mmol/L \pm 1.42 mmol/L and 53.65 μ mol/L \pm 8.91 μ mol/L, respectively), $P<0.01$. Treatment with P-selectin mAb, significantly lowered the levels of BUN (20.28 mmol/L \pm 3.82 mmol/L) and Cr

(167.75 μ mol/L \pm 32.81 μ mol/L) as compared with saline-treated group ($P<0.01$).

Histopathologic changes of liver and kidney

After 1 hour of ischemia of left hepatic lobe, reperfusion was performed. With the observation of naked eye, the left hepatic lobe was more swollen than the right lobe while under light microscope, edema, degradation with different extent and necrosis of hepatic cells, interstitial congestion and infiltration of inflammatory cells were observed. After 1 hour of left renal blood flow blocking on rats, reperfusion followed, with the observation of naked eye, the renal cortex was paler, renal medulla displayed blood stagnation and dark colored; under light microscope, swell, degradation with different extent and necrosis of renal tubular epithelial cell were exhibited; simultaneously, interstitial congestion, edema and infiltration of inflammatory cells were also observed. While in the P-selectin mAb treatment group, the outward appearance of liver and kidney was similar to that of normal. Renal cells and tubular cells showed less swelling and no denaturalization or necrosis, and interstitial changes were not obvious.

Plasma P-selectin level changes

At 24 hours after hepatic/renal ischemia-reperfusion, plasma P-selectin levels of saline-treated group (5.16 μ g/L \pm 1.08 μ g/L and 8.92 μ g/L \pm 3.17 μ g/L, respectively) were much higher than the sham group (1.59 μ g/L \pm 0.25 μ g/L and 3.57 μ g/L \pm 0.89 μ g/L, respectively), $P<0.05$. Treatment with P-selectin mAb, resulted in significantly lower levels of P-selectin (2.89 μ g/L \pm 0.72 μ g/L and 6.02 μ g/L \pm 2.15 μ g/L, respectively) compared with saline-treated group ($P<0.05$).

The expression of P-selectin in hepatic and renal tissue

P-selectin expressed widely within hepatic and renal tissues in early stage of ischemia-reperfusion, which was distributed on small vessels of left hepatic lobe, part of hepatic cellular membrane, renal glomerulomesangium, capillary loops, tubules and interstitium, it was especially remarkable on hepatic small vessels and tubular epithelium. After the treatment with P-selectin mAb, the expression of P-selectin was not displayed in essence.

Cell apoptosis in hepatic and renal tissue

It was displayed with TUNEL method that there was few apoptotic bodies in hepatic and renal tissues in the control group. After 1 hour of hepatic and renal ischemia and reperfusion, a few apoptotic bodies emerged in hepatic tissues, renal tubules and interstitium; after 3-6 hours, a large quantity of apoptotic bodies were observed in the hepatic or renal tissues, especially in renal tubules; and after

24 hours apoptotic bodies gathered in flakes, and inclined to necrosis. The quantity of apoptotic bodies in the tissues of P-selectin mAb treated group was smaller than that in the non-treatment group, especially in renal tubules, there was much fewer apoptotic bodies. The examination results of cell apoptosis in hepatic and renal tissues of each group are shown in Table 1.

Table 1 The quantity of apoptotic bodies in hepatic and renal tissues of each group ($\bar{x}\pm s$, number/ $\times 400$)

	Renal ischemia -reperfusion group		Hepatic ischemia -reperfusion group	
	n	Quantity of apoptotic bodies	n	Quantity of apoptotic bodies
Control group	5	9.0 \pm 3.2	5	8.5 \pm 3.4
Non-treatment group				
1hr of reperfusion	5	23.4 \pm 2.9 ^{ab}	5	18.3 \pm 3.5 ^{ab}
3hrs of reperfusion	5	131.6 \pm 7.6 ^{ab}	5	74.9 \pm 6.8 ^{ab}
6hrs of reperfusion	5	209.6 \pm 8.4 ^{ab}	5	94.6 \pm 8.1 ^{ab}
24hrs of reperfusion	5	427.8 \pm 37.1 ^{ab}	5	397.4 \pm 40.2 ^{ab}
mAb treatment group	20	13.0 \pm 3.9 ^a	20	13.1 \pm 3.7 ^a

Compared with control group: ^aP<0.01; compared with mAb treatment group: ^bP<0.01.

DISCUSSION

Recently, the role of neutrophil and cell adhesion molecules in ischemia-reperfusion injury of multiple organs has aroused extensive attention^[5]. Investigators have used mAb directed against key adhesion domains^[8]. As a potential member of selectin family, P-selectin is mainly found in both weibel-plade body of epithelial cell of middle and small blood vessels and α -granule of platelet. It is expressed rapidly on the surface of these cells after their activation. In the earliest stage of inflammation, P-selectin initiates the adhesion, rolling of neutrophils, and then recruits them to injured site^[6]. Blockade of P-selectin expression or interaction with its ligands can attenuate leukocyte adherence and infiltration during ischemia/reperfusion injury or acute inflammation affording impressive functional and morphologic protection^[9-11]. Neutrophils participate in pathological injury through releasing oxygen free radicals and some enzymes. As a large number of neutrophils gather in the injured tissues of ischemia-reperfusion, reducing the infiltration of neutrophils can alleviate the ischemia-reperfusion injury evidently.

The expression of P-selectin and effect of P-selectin mAb on ischemia-reperfusion injury were observed in this study based on the established rat model of ischemia-reperfusion injury. Hepatic and renal tissue exhibited heavy histopathologic damages after hepatic/renal ischemia-reperfusion, while the serum AST and ALT levels as well as BUN and Cr were increased. It is indicated that ischemia-

reperfusion induced hepatic and renal injury which could be significantly attenuated when P-selectin mAb was administrated 5 minutes before reperfusion as shown by improved hepatic/renal function and less pathologic damage. The results suggested that P-selectin mAb has a protective effect on renal ischemia/reperfusion injury by means of inhibiting interaction of neutrophils and endothelium.

Up-regulated expression of P-selectin in plasma and hepatic/renal tissue after rat hepatic/renal ischemia and reperfusion indicated that P-selectin is associated with hepatic/renal ischemia-reperfusion injury. The leukocyte rolling and recruitment was delayed when P or E-selectin deficient mice were infected, suggesting that P-selectin is involved in the early events of inflammation mediated by leukocyte^[7,12]. Another study demonstrated that P-selectin might maintain chronic inflammatory state^[13]. Increased plasma P-selectin level indicates that: P-selectin was markedly released into blood circulation from activated platelets/endothelial cells by inflammatory mediators during acute hepatic/renal injury, and platelets or endothelial cells were activated *in vivo* to enhance hepatic/renal injury. P-selectin was widely expressed in hepatic/renal tissue in the early stage of injury, particularly in hepatic small vessels and tubular epithelium, suggesting that P-selectin might play a functional role in the early events of leukocyte adherence, activation and infiltration within the liver and kidney.

Results obtained in this study showed that P-selectin expression in hepatic/renal tissue was inhibited and plasma P-selectin level was significantly lowered in P-selectin mAb-treated group. It is consistent with down-regulated expression of sialyl Lewis X, a ligand for P-selectin located mainly in neutrophils, as with anti-P-selectin therapy (unpublished data). The experiment suggested that in the process of hepatic and renal ischemia-reperfusion injury, P-selectin mediated the infiltration of neutrophils in hepatic and renal tissues, neutrophils released a set of inflammatory media, which could aggravate the injury of tissues. When the effect of P-selectin was impeded, less neutrophils infiltration and pathological lesions could be observed in the tissue. This phenomenon showed that P-selectin mAb played a protective role in the injury of hepatic and renal tissues caused by ischemia-reperfusion.

Cell apoptosis, which is also named programmed cell death, is an active death process of cell, its imbalance or changes are relative to the occurrence of many kinds of diseases^[14]. Schumer et al^[15] studied the death form of cells at different times of renal ischemia-reperfusion in rats, and found that transient renal ischemia mainly led to cell apoptosis, permanent and severe renal ischemia

brought about necrosis of cells, so it was proved that cell apoptosis took part in cell injury process of acute renal ischemia. Besides this, Sasaki *et al*^[16] discovered that by 3 hours of reperfusion after hepatic ischemia in rats, the quantity of apoptotic cells increased markedly, and reached their peak. Therefore, study of the occurrence rule of cell apoptosis during hepatic and renal ischemia-reperfusion is helpful in the treatment of liver and kidney transplantation as well as the disturbance of renal function in acute renal tubular injury, moreover, it is also beneficial in seeking effective measures to promote the recovery of injured renal function.

We discovered that, after 1 hour of reperfusion following 1 hour of hepatic and renal ischemia, a few apoptotic bodies emerged, after 3-6 hours of reperfusion, large quantities of apoptotic bodies appeared and after 24 hours of reperfusion, the number of apoptotic bodies reached their peak and inclined to necrosis. This indicated that the time of reperfusion is closely related to the occurrence of cell apoptosis. After the treatment with P-selectin mAb, the situation of cell apoptosis in hepatic and renal tissues of rat became better than that of the control group, possibly due to the fact that P-selectin mAb impeded the adhesion and infiltration of neutrophils, and alleviated cell apoptosis in hepatic and renal tissues during reperfusion after ischemia.

It is considered currently that the death of renal tubular epithelial cells during reperfusion after transient renal ischemia is not caused by ischemia itself, but because the swollen cells can compress blood vessels during ischemia, which makes the corresponding tissue in a state of less blood flow during reperfusion, the insufficiency of blood flow can lead to a large scale of renal cell apoptosis^[17]. Our previous studies displayed that P-selectin expressed not only on the surface of platelet and endothelial cell, but also on that of renal tubular epithelial cells^[18]. So we speculate that ischemia may stimulate the up-regulation of the expression of P-selectin on the renal tubular epithelial cells. We discovered in this experiment that inhibiting the effect of P-selectin could reduce the apoptosis of renal tubular epithelial cells, so it was proved that P-selectin is an important factor causing renal tubular injury during the renal ischemia-reperfusion process.

It is suggested that P-selectin mediates the infiltration of neutrophils and the injury of renal tubular epithelial cells in tissues during hepatic and renal ischemia-reperfusion process in rats, inhibiting P-selectin can alleviate the accumulation of neutrophils and cell apoptosis in hepatic and renal tissues, and relieve hepatic and renal ischemia-

reperfusion injury. So research into the P-selectin and its inhibitor offers a new thought for the treatment of human liver and kidney transplantation as well as acute renal failure caused by renal tubular injury.

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Optic properties of bile liquid crystals in human body

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Subject headings cholelithiasis; liquid crystals; optic properties

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Abstract

AIM To further study the properties of bile liquid crystals, and probe into the relationship between bile liquid crystals and gallbladder stone formation, and provide evidence for the prevention and treatment of cholelithiasis.

METHODS The optic properties of bile liquid crystals in human body were determined by the method of crystal optics under polarizing microscope with plane polarized light and perpendicular polarized light.

RESULTS Under a polarizing microscope with plane polarized light, bile liquid crystals scattered in bile appeared round, oval or irregularly round. The color of bile liquid crystals was a little lighter than that of the bile around. When the stage was turned round, the color of bile liquid crystals or the darkness and lightness of the color did not change obviously. On the border between bile liquid crystals and the bile around, brighter Becke-Line could be observed. When the microscope tube is lifted, Becke-Line moved inward, and when lowered, Becke-Line moved outward. Under a perpendicular polarized light, bile liquid crystals showed some special interference patterns, called Malta cross. When the stage was turning round at an angle of 360°, the Malta cross

showed four times of extinction. In the vibrating direction of 45° angle of relative to upper and lower polarizing plate, gypsum test-board with optical path difference of 530nm was inserted, the first and the third quadrants of Malta cross appeared to be blue, and the second and the fourth quadrants appeared orange. When mica test-board with optical path difference of 147nm was inserted, the first and the third quadrants of Malta cross appeared yellow, and the second and the fourth quadrants appeared dark grey.

CONCLUSION The bile liquid crystals were distributed in bile in the form of global grains. Their polychroism and absorption were slight, but the edge and Becke*Line were very clear. Its refractive index was larger than that of the bile. These liquid crystals were uniaxial positive crystals. The interference colors were the first order grey-white. The double refractive index of the liquid crystals was $\Delta n=0.011-0.015$.

INTRODUCTION

Researches of recent years show that there are liquid crystals in the bile of the patients with gallbladder stone^[1-4]. Some scholars inferred the function of bile liquid crystals^[2]. In order to find out the properties of bile liquid crystals, probe into the relationship between bile liquid crystals and the formation of gallbladder stone and provide evidence for the prevention and treatment of cholelithiasis, we used the method of crystal optics, and determined the optic properties of bile liquid crystals in human under polarizing microscope with plane polarized light and perpendicular polarized light. No similar reports have been found so far.

MATERIALS AND METHODS

Materials

The bile was collected from the gallbladder extirpated surgically in the Second Affiliated Hospital of Kunming Medical College and kept at 4.0°C in the refrigerator for use.

Methods

Extraction of bile liquid crystals Firstly, the bile was centrifuged at 4000r/min for 30min with type

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800 centrifuge sedimentor made in China. Secondly, the supernatant was centrifuged at 30000r/min for 40min of equivalent time of centrifugation through 50g/L-150g/L linear concentration gradients of sucrose solution with Hitachi RPS 50-2 swing-out rotor and model SCP85H Hitachi automatic preparative ultracentrifuge at 25°C. After centrifugalization, the bile was taken out in different colors and put into different sample cups. After examination under a polarizing microscope, liquid crystals were selected for microslide preparation.

Microslide preparation Bile (6.0μL) was taken with China-made W-104 micropipet (0.2μL), and dropped to a slide and covered with a glass. It was stretched into a slice naturally for observation and measurement.

Measurement of bile refractive index With China-made 2WA871430 Abbe refractometer (0.0005), bile refractive index was measured after gradient centrifugation of sucrose density.

Measurement of bile liquid crystals thickness With China-made 0mm-25mm micrometer screw gauge (0.01mm) the area of cover glass was measured and the thickness of liquid crystals was worked out according to the volume taken out from the micropipet.

Polarizing microscopic observation Under a Japan-made BHSP Olympus advanced polarizing microscope, observations were made on the outside character of bile liquid crystals, the optical nature involved with light wave absorption and optical nature related with refraction rate. Under a perpendicular polarizing microscope, interference patterns of bile liquid crystals, disappearing phenomenon and mobility were examined. The size of Malta cross was measured with micrometer eyepiece (0.1mm) and micrometer objective (0.01mm). In the vibration direction at 45° angle relative to upper and lower polarizing plate, gypsum test-board with optical path difference of 530 nm and the mica test-board with optical path difference of 147nm were inserted to measure the optical symbol, interference color level and double refraction rate of the bile liquid crystals.

RESULTS

Under a polarizing and a perpendicular polarized light microscope, 100 pictures of different bile liquid crystals from 17 cases of cholecystolithiasis were observed and measured.

Outside character of bile liquid crystals

Under a polarizing microscope, bile liquid crystals were found scattered in the bile, appearing round, oval or irregularly round in shape (Figure 1a).

The optical nature of bile liquid crystals related with light wave absorption

Under a polarizing microscope, the color of bile liquid crystals was a little lighter than that of the bile being around. When the stage was turned round, no obvious changes occurred in the color of bile liquid crystals or the darkness and lightness of the color.

The optical nature of bile liquid crystals related with refractive index

Under a polarizing microscope, on the border between bile liquid crystals and bile being around, darker edge can be observed with a brighter line nearby, i.e., Becke-Line (Figure 1b). When the microscope tube was lifted, Becke-Line moved inward (to the liquid crystals), and when lowered, Becke-Line moved outwardly (to the bile). The refractive index of bile liquid crystals was bigger than that of the bile being around (1.3408-1.3494) (Table 1).

Table 1 The refractive index of the bile in human body

No.	Refractive index	No.	Refractive index	No.	Refractive index
001	1.3470	007	1.3441	013	1.3446
002	1.3494	008	1.3418	014	1.3443
003	1.3485	009	1.3408	015	1.3451
004	1.3486	010	1.3416	016	1.3454
005	1.3479	011	1.3435	017	1.3456
006	1.3473	012	1.3445	018	1.3457

Interference patterns and extinction of bile liquid crystals

Under a perpendicular polarized light, bile liquid crystals in human body presented some special interference patterns, called Malta cross. These Malta crosses had various kinds of shapes, which can be divided generally into three types. The first one was round in shape, with four symmetry, equal and broad bright double refraction areas, and the middle part was a narrow, dark cross; the second one was irregularly round in shape, with four symmetry bright double-refraction areas but not equal each other, in the middle, the narrow dark cross was lean towards different directions; and the third one shaped oval with four narrow bright double-refraction areas, in the middle there was a bigger dark cross (Figure 2a, b).

Brightness or darkness of Malta cross was changed regularly when the stage was rotated, which changed once for each 45° turning. If there was a turning of 360°, four times of brightness and darkness appeared, i.e., four extinction and four interference color phenomena occurred.

Mobility of bile liquid crystals

Little distilled water was added to the space between a slide and a cover glass containing bile liquid

crystals. Under a perpendicular polarized light, Malta cross was observed from bile liquid crystals, flowing swiftly accompanying with the mobile of the water. In the process of flowing, the shape of Malta cross changed incessantly with the flowing of liquid crystals. When it flew to the edge, Malta cross was changed into crystal grains of double refraction after drying.

Optical symbol and interference color level of bile liquid crystals

Under a perpendicular polarized light, in the relative direction of 45° angle of viration of upper and lower polarizing plate, a plaster test-board with optical path difference of 530nm was inserted, the first and the third quadrants of Malta cross was found present in bile liquid crystals being blue in color, and the second and the fourth quadrants being orange, and the black cross being purplish. When a mica test-board with optical path difference of 147nm was inserted, the first and the third quadrants of Malta cross appeared light yellow, and the second and the fourth quadrants appeared dark grey (Figures 3a, b, c). It is suggested that optical symbol of bile liquid crystals is uniaxial positive and interference color level is the first level grey^[5].

Size of Malta cross

Under a perpendicular polarized light microscope, the diameter (or long axis) of Malta cross was measured to be between 6.2×10^{-3} mm and 2.9×10^{-2} mm.

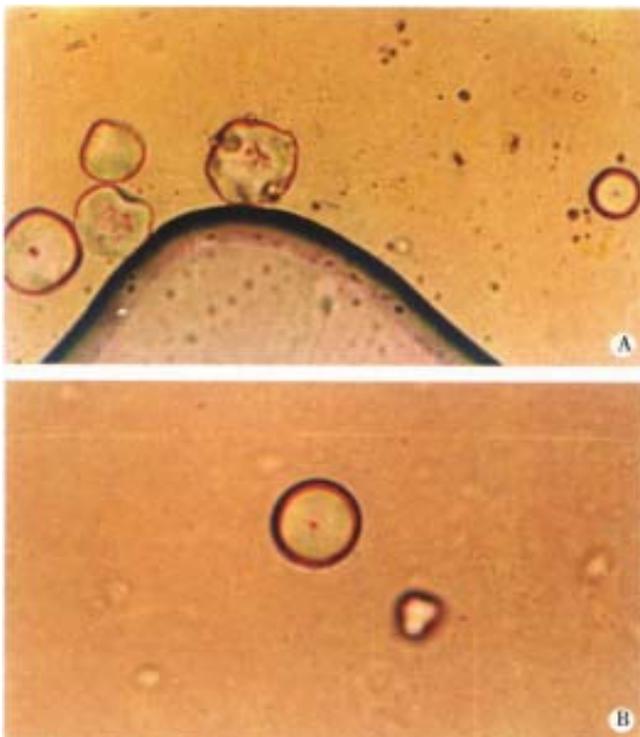


Figure 1 (a) The shape of bile liquid crystals under a polarizing microscope. $\times 400$
(b) The Becke-Line of bile liquid crystals. $\times 400$

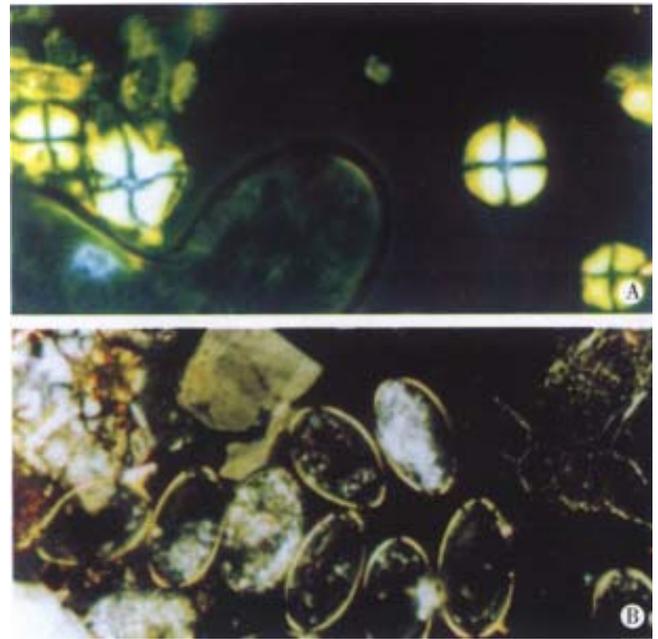


Figure 2 The interference pattern of bile liquid crystals under a perpendicular polarized light microscope-various shapes of Malta cross. $\times 400$

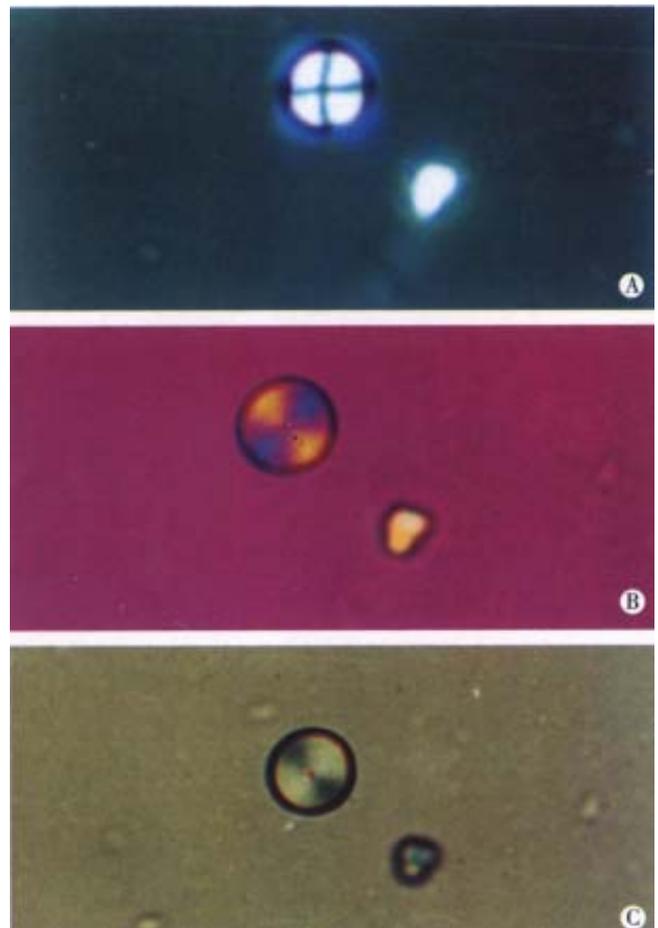


Figure 3 (a) Interference pattern of bile liquid crystals in human body before inserting test-board. $\times 400$
(b) Interference pattern of inserting plaster test-board with optical path difference of 530nm. $\times 400$
(c) Interference pattern of inserting mica test-board with optical path difference of 147nm. $\times 400$

Double refractive index of bile liquid crystals

It was measured that the area of bile was $328.3\text{mm}^2 \pm 0.7\text{mm}^2$, the thickness of liquid crystals $d = (1.8 \pm 0.1) \times 10^{-2}\text{mm}$, the interference color level was the first level grey-white, and optical path difference was $200\text{nm}-270\text{nm}$ ^[6]. According to the formula of that optical path difference: $R=d(N_e-N_o)=d \cdot \Delta n$, the double refractive index of bile liquid crystals was calculated as $\Delta n=0.011-0.015$.

DISCUSSION

Under plane polarized light, the outside character of bile liquid crystals was observed, which were not steric pattern, but a section contour of a certain direction formed by bile liquid crystals crossed with cover glass. Because the direction of section varies, different shapes can be observed, based on which its outside character can be determined. Under a polarizing microscope, the section of bile liquid crystals appeared totally round, oval or irregularly round, which only occurred in the section of different directions of a ball, indicating that bile liquid crystals existed in the bile in the form of ball-shaped grains.

Under a polarizing microscope, when the microscope stage is turned round, changes take place in the color of many crystals and the darkness or lightness of the color. This phenomenon which makes the color of crystals change by different vibrating directions of the light waves of the crystals is called polychromatophilia. The phenomenon which makes the darkness or lightness of the color change is called absorptivity^[5]. Under a polarizing microscope, when the stage was turned around, changes did not take place distinctively in the color of bile liquid crystals and the darkness or lightness of the color, indicating that polychromatophilia and absorptivity of bile liquid crystals were not prominent.

In the view of kinetic theory, in the surface layer of liquid, the direction of the absorptive force among the liquid elements points to the interior part of the liquid. If a slice of liquid is not acted upon by the outer force, it is bound to be in the shape of a ball under the action of element pressure, in fact, the little liquid drop is therefore, in the shape of a total ball^[7]. Liquid crystals in the bile existed in the bile in the form of ball grains, which confirmed that bile liquid crystals did have the character of liquid. Under the microscope with perpendicular polarized light, when little distilled water was added between a slide and a cover glass containing bile liquid crystals, the bile liquid crystals were found flowing swiftly along with water flow. In the process of flowing, the interference pattern shown by the bile liquid crystals-the shape of Malta cross, was changing continuously along with the flowing of bile liquid crystals, indicating that mobility of bile liquid crystals was very good.

Crystals are anisotropic substance, which is also called optical anisotropic substance. Its optical nature is different in directions. When light waves

enter anisotropic substance, double refraction occurs except the direction of optical axis, and they are separated into two kinds of polarizing light whose vibrating directions are vertical one another, with different travelling speeds, and unequal refractive indexes. When anisotropic substance is observed under a polarizing microscope with perpendicular polarized light, destructive interference pattern and four times extinction can be observed. Under a polarizing microscope with plane polarized light, on the boundary plane of two kinds of crystals, because of the reflection and refraction of light, darker edge and brighter Becke-Line could be observed^[5]. Under a polarizing microscope with perpendicular polarized light, bile liquid crystals in human body appeared in special interference pattern and four times extinction. The optic symbol was uniaxial positive. Under a polarizing microscope with plane polarized light, darker edge and brighter Becke-Line could be seen on the edge of bile liquid crystals contacting the bile around. It is shown that bile liquid crystals did have the character of crystals and the optical nature was similar to uniaxial positive crystals.

Bile in human body is a solution composed of water, bile pigment, bile salt, cholesterol, fatty acid, lecithin and inorganic salt. Under the physiological condition, the scale of bile salt, cholesterol, bile pigment lecithin, etc. in the bile was maintained in a proper scope, and the bile was in a liquid state, but when their scale was out of order, the cholesterol might produce crystals in the bile to form the concretion^[8]. In 1983, B.H. Brown *et al*^[2], an American scholar, for the first time put forward that bile liquid crystals were the preform of gallstone. Under polarizing microscope with plane polarized light and perpendicular polarized light, we have observed that bile liquid crystals had the character of both crystals and liquid. Malta cross shown by bile liquid crystals after drying turned into grains of double refraction. The results support the views mentioned above, which are of important value of reference to the research in the form of gallstone and the prevention and treatment of cholelithiasis.

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Effect of extracorporeal bioartificial liver support system on fulminant hepatic failure rabbits

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Subject headings artificial liver; liver support; fulminant hepatic failure; rabbit

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Abstract

AIM To evaluate the possibility of using cultured human hepatocytes as a bridge between bioartificial liver and liver transplantation.

METHODS In this experiment, the efficacy of extracorporeal bioartificial liver support system (EBLSS) consisting of spheroidal human liver cells and cultured hepatocytes supernatant was assessed *in vivo* using galactosamine induced rabbit model of fulminant hepatic failure.

RESULTS There was no difference of survival between the two groups of rabbits, but in the supported rabbits serum alanine aminotransferase, total bilirubin and creatinine were significantly lower and hepatocyte necrosis was markedly milder than those in control animals. In addition, a good viability of human liver cells was noted after the experiment.

CONCLUSION EBLSS plays a biologic role in maintaining and compensating the function of the liver.

INTRODUCTION

The recent development of bioartificial liver support system containing hepatocytes is an important event in the research of the artificial liver^[1]. This system possesses the potential metabolic and synthetic functions of the liver and may provide certain nutrients and cytokines necessary for the regeneration of liver tissues^[2,3]. Further development is expected in the research of the bioartificial liver to provide a new therapeutic procedure in the treatment of fulminant hepatic failure. Based on the studies of isolation and culture of human liver cells, we established an extracorporeal bioartificial liver support system (EBLSS) with cultured human hepatocytes and hollow fiber bioreactor. Its support efficiency was investigated preliminarily *in vivo* with animal model of hepatic failure.

MATERIALS AND METHODS

Animal

Male and female rabbits weighing between 2 kg and 3 kg were provided by the Experimental Animal Center of Third Military Medical University. All animals were allowed free access to food and water.

Induction of fulminant hepatic failure

A model of fulminant hepatic failure (FHF) in the rabbits was prepared by the method of Blitzer *et al*^[4] with slight modifications. D-Galactosamine (D-Gal, purchased from Chongqing University) at a lethality dose of 1.2 g/kg of body weight was dissolved in 9mL of 50g/L dextrose in water and pH was adjusted to 6.8 with sodium hydroxide. The solution was then given intravenously over 5min via ear vein.

Bioartificial liver system

The bioartificial liver system consisted of liver cells, hollow-fiber bioreactor and circulating unit. The cells were harvested from 4 month old human fetal liver by a two step collagenase perfusion technique modified from the method of Seglen^[5] and the hepatocytes and liver nonparenchymal cells were obtained by centrifugation at 50×g for 3min and 500×g for 3min respectively. Cell viability was initially >96% for all devices assessed by trypan blue exclusion and they were successfully cultured as multicellular spheroids with a synthetic technique. About 1×10⁸ viable cells were placed onto the outer space of the hollow fiber bioreactor. The hollow fibers (porous, 0.2μm) were polysulfone with a

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100kDa nominal molecular weight cutoff and a 1128 cm² surface area. Thirty mL anti-coagulate blood came from normal rabbit was perfused into the intracapillary space of hollow fiber bioreactor and the circulatory tube. A roller pump (Millipore ultrafiltration device) was used to circulate blood and a heater was used to maintain the animal blood and bioreactor temperature at 37°C-39°C. With this condition the system was ready for *in vitro* application.

Artificial liver support

The experimental animals were divided into two groups: group I animals ($n=5$) were treated with EBLSS inoculated with viable liver cells; group II, animals ($n=5$) were treated as control with EBLSS but without cells. Animals in both groups were anesthetized by pentobarbital (0.03g/kg, intravenously) and femoral artery and vein catheters were placed before experiment. Four hours after the induction of FHF, the femoral artery and vein was cannulated for EBLSS access. Hemoperfusion was through the EBLSS at a rate of 15 to 20mL/min. Heparin was administered at 150U/kg firstly and at 50U/kg every 30min. Perfusion was carried out for 4h. About 15mL supernatant of cultured hepatocyte and liver nonparenchymal cells was administered into the extracapillary space for an assisting treatment during the experiments.

Assay methodology

Blood samples were obtained at the initiation of the EBLSS hemoperfusion, during the treatment and at hourly intervals for 5h after liver support. Serum alanine aminotransferase (ALT), total bilirubin (TB) and creatinine (Cr) were determined in the clinical laboratory using a Beckman CX-7 autoanalyzer (Beckman Instruments, Inc., Fullerton, Calif.) by standard methods.

Liver biopsy specimens were obtained from each of the animal postmortem and fixed in 10% formalin. Histological analyses were carried out in the pathological laboratory using standard procedures. The liver cell spheroids loaded in EBLSS were recoll ected and dispersion by 0.01% pancreatin and 0.1 mmol/L EDTA. Their viability was determined again by trypan blue exclusion. The rate of adherence to dish coated collagen was obtained by phase contrast microscope after 24 h of normal culture.

RESULTS

Survival of FHF rabbits

The survival time of FHF rabbits in the control group was 9.2 h, 14.6 h, 15.7 h, 24.2 h and 34.1 h (19.6 ± 9.7 h). In EBLSS support group, besides one animal which died 10min after the initiation of artificial support, the survival time was 11.4 h, 14.9 h, 24.2 h and 25.1 h respectively (18.9 ± 6.8 h). There was no difference between the two groups ($P>0.05$).

Biochemical changes

In both groups of animals, there was a progressive increase in the concentration of serum ALT, TB and Cr in 10h after injection of D-Gal, but there was no statistical difference. Afterwards, a significant increase of serum ALT, TB and Cr was observed in control group. At the same time phase, serum ALT, TB and Cr were also increased in EBLSS support group, but the extent of increment was small relatively (Figures 1-3).

Pathological change

Histological examination of liver biopsy specimens obtained from the control rabbits demonstrated characteristic of classic acute hepatic necrosis, which contained multiple areas of lytic necrosis, disappearance of lobule construction, dissociation of hepatic cords and a large number of inflammatory cells infiltrated into the portal triads. In artificial liver support group, besides the large area of necrosis observed only in one animal, hepatocyte degeneration and focal necrosis were the main manifestation in the rest animals. The regeneration of hepatocytes was observed easily in this group (Figures 4, 5).

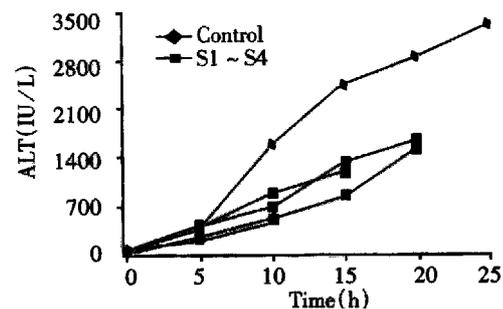


Figure 1 Serum ALT changes in FHF animals.

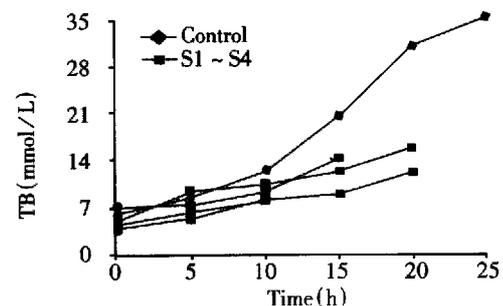


Figure 2 TB changes in FHF animals.

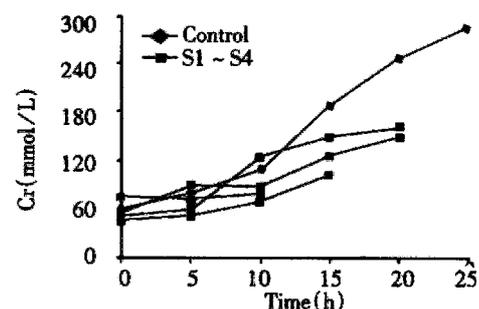


Figure 3 Serum Cr changes in FHF animals.

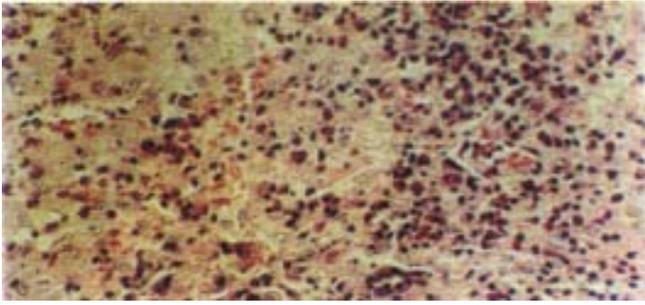


Figure 4 Liver biopsy specimens from control group. Large area of necrosis. LM,×200

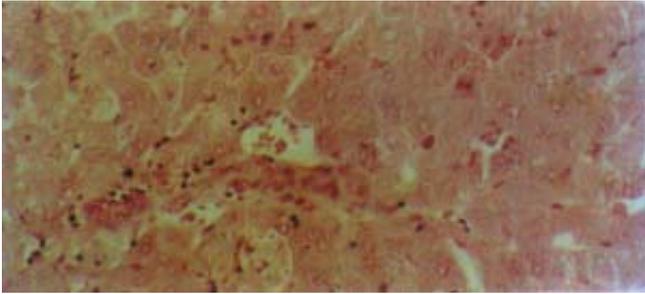


Figure 5 Hepatic histology of support animals: mild liver necrosis. LM,×200

Cell viability

Both liver cell viability and spheroids adherence rate were decreased by 13.1% and 26.2% respectively after the artificial liver support as compared with those before the experiment.

DISCUSSION

Medical treatment of fulminant hepatic failure is still not satisfactory, and the mortality is about 90%. It has been recognized that the liver transplantation is the only process in saving these patients. However, there is a shortage of donors and most patients have no sufficient time in waiting for the emergency transplantation. Therefore, many investigators devote their efforts to the development of artificial liver device as a liver support system to help maintain patients in alive and intact mentality until an organ donor becomes available. The liver is known as a complex metabolic organ with multi-functions essential for surviving, including gluconeogenesis, synthesis of blood proteins amino acid metabolism, urea synthesis, lipid metabolism, drug biotransformation, and removal of wastage. So the early artificial liver systems such as hemodialysis, hemadsorption and charcoal hemoperfusion that had limited purifying function can not bring a satisfactory result to these patients^[6]. A bioartificial liver comprising viable hepatocytes on a mechanical support has been considered to provide these essential functions more likely than a purely mechanical device^[7]. Up to now, a lot of data show that this new bioartificial liver system not only possesses the ability of specific detoxication and metabolism but also has synthetic functions^[8,9].

Bioartificial liver system could maintain a suitable internal environment for the FHF patients to wait for the chance of liver transplantation.

In this study, after the human liver cells were isolated by an alternative extracorporeal two step perfusion method with a good viability, we succeeded in carrying out the aggregate culture of hepatocytes and nonparenchymal cells. Subsequently, we place the multicellular spheroids and supernatant of cultured hepatocyte into a hollow fiber bioreactor to form a simple EBLSS and its liver support function was assessed *in vivo* by D-Gal-induced rabbit model with fulminant hepatic failure. The biochemical results show that the increase of serum ALT, TB and Cr was restrained effectively in support group animals. At the same time, we found the pathological change of supported animals was markedly improved and active regeneration of hepatocytes was easily seen in these animals. These data indicate that this EBLSS possesses the effect of liver support to the fulminant hepatic failure animal in certain extents. The results of EBLSS *in vitro* show that it protects the cultured hepatocyte from injury by the D-Gal induced toxic metabolites and promotes the regeneration of hepatocytes by the substances afforded from the hepatocytes and supernatant of cultural medium. Nevertheless, the effectiveness of EBLSS has not been satisfactorily demonstrated in *in vivo* animal experiment due to the difficulty in establishing extracorporeal circulation and maintaining a stable experimental condition, and because the animal is hard to endure the heavy hemodynamic burden.

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A study of preoperative methionine-depleting parenteral nutrition plus chemotherapy in gastric cancer patients

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Subject headings stomach neoplasms/drug therapy; methionine; parenteral nutrition

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Abstract

AIM To investigate the interference of methionine-free parenteral nutrition plus 5-Fu (MetTPN+5-Fu) in gastric cancer cell kinetics and the side effects of the regimen.

METHODS Fifteen patients with advanced gastric cancer were randomly divided into two groups, 7 patients were given preoperatively a seven-day course of standard parenteral nutrition in combination with a five-day course of chemotherapy (sTPN+5-Fu), while the other 8 patients were given methionine-deprived parenteral nutrition and 5-Fu (MetTPN+5-Fu). Cell cycles of gastric cancer and normal mucosa were studied by flow cytometry (FCM). Blood samples were taken to measure the serum protein, methionine (Met) and cysteine (Cys) levels, and liver and kidney functions.

RESULTS As compared with the results obtained before the treatment, the percentage of G₀/G₁ tumor cells increased and that of S phase decreased in the MetTPN+5-Fu group, while the contrary was observed in the sTPN+5-Fu group. Except that the ALT, AST and AKP levels were slightly increased in a few cases receiving -MetTPN+5-Fu, all the other biochemical parameters were within normal limits. Serum

Cys level decreased slightly after the treatment in both groups. Serum Met level of patients receiving sTPN+5-Fu was somewhat higher after treatment than that before treatment; however, no significant change occurred in the MetTPN+5-Fu group, nor operative complications in both groups.

CONCLUSION -MetTPN+5-Fu exerted a suppressive effect on cancer cell proliferation, probably through a double mechanism of creating a state of "Met starvation" adverse to the tumor cell cycle, and by allowing 5-Fu to kill specifically cells in S phase. Preoperative short-term administration of -MetTPN+5-Fu had little undesirable effect on host metabolism.

INTRODUCTION

Methionine (Met) has been reported to be an essential amino acid in tumor cell metabolism. Recent researches have demonstrated that proliferation was inhibited in some tumor cell lines when Met in the culture medium was replaced by its precursor homocysteine (Hcy), whereas normal cells grew well as controls; and once regaining Met, tumor cells recovered their usual activity of proliferation; these characteristics of tumor cells were referred to as Met dependency^[1,2]. In the Met depleted environment, the metabolism of nucleic acid, protein and biological membrane of the tumor cells were disturbed and their cell cycle deranged. If Met starvation was combined with the application of phase-specific chemotherapeutic agents, it would prohibit the proliferation and metastasis of tumor cells. On the basis of our previous experiments, further studies are designed to elucidate the influence of preoperative Met deprived parenteral nutrition plus 5-Fu on the cell cycle in the advanced gastric cancer (AGC) patients, and to explore whether any adverse effect to host metabolism could be produced, by monitoring the serum Met and cysteine (Cys) levels and blood biochemistry parameters.

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PATIENTS AND METHODS

Patients

From June to December 1996, fifteen AGC patients (8 males and 7 females) diagnosed by endoscopy and pathology, entered into the study. Their average age was 55 (39-71) years and the average body weight was 55 (33-78) kg. All the patients had normal liver and kidney functions and were free from metabolic diseases.

Groups

The patients were randomized to 2 groups, 7 patients of the control group were administered Met-containing standard parenteral nutrition plus chemotherapy (sTPN+5-Fu), and 8 of the study group were given Met-deprived parenteral nutrition plus chemotherapy (MetTPN+5-Fu).

Regimens of nutritional support and chemotherapy

All patients were permitted to take a small amount of soup and water, no blood products were used. The non-protein energy in the TPN was supplied by glucose and fat emulsion (20% Intralipid, SSPC). The amino acid solution used in the control group was HBC11S (Amino Acid Company, Tianjin) while that used in the study group was prepared cooperatively by Rui Jin Hospital and Shanghai Chang Zheng Pharmaceutical Plant (No. S 95-001 Rui Jin, Shanghai, China). The amount of fat-soluble and water-soluble vitamins, trace elements and electrolytes was same for both groups. The mean non-protein energy was 125 kJ/(kg·d) for both groups; the nitrogen intake was 0.165g/(kg·d) and 0.163g/(kg·d) respectively for the control and the study groups. The TPN was administered from d1 to d7, and 500mg/d of 5-Fu from d2 to d6. Operation was carried out on d8.

Laboratory tests

Cell kinetics Tissue samples of gastric cancer and normal gastric mucosa obtained through endoscopy at the beginning of the study and during gastrectomy were managed mechanically to produce monocellular suspension, and were then submitted to flow cytometry (FCM) study for cell kinetics.

Blood biochemistry Blood samples were collected before and after the treatment for biochemical studies: liver function tests: ALT, AST, γ -GT and AKP; kidney function tests: BUN and Cr; serum proteins: total protein (TP), albumin (Alb), transferrin (Tf) and prealbumin (PA); and serum Met and Cys.

Statistical analysis

Paired *t* test and Student *t* test were used.

RESULTS

Cell cycle study

In 2 patients of the control group, the number of tissue samples was incomplete because gastrectomy was not performed eventually, resulting in a total of 13 patients who entered the FCM analysis. In the study group, compared to the pre treatment data, the percentage of tumor cells in G₀/G₁ phases increased after the treatment, while the percentage of S phase cells decreased; there was no obvious change in the percentage of 2/M cells, but the percentage of S+G₂/M (representing phases of cell proliferation) decreased. In the control group, the percentage of G₀/G₁ of tumor cells decreased after the treatment while that of S phase increased. There were no obvious changes of G₂/M and S+G₂/M percentage (Table 1).

Table 1 Changes in tumor cell cycle before and after treatment ($\bar{x}\pm s$)

Phase		sTPN+5-Fu (n=5)		MetTPN+5-Fu (n=8)	
		Before	After	Before	After
G ₀ /G ₁	Before	67.10±27.68		78.30±11.01	
	After		63.35±28.72		83.45±9.26
S	Before	22.22±18.60		11.96±6.41	
	After		26.08±26.02		9.25±7.16
G ₂ /M	Before	10.66±9.87		8.49±3.89	
	After		8.56±3.76		8.50±4.17
S+G ₂ /M	Before	32.88±27.68		21.21±7.18	
	After		34.64±28.76		18.85±7.62

Regarding to the cell kinetics of normal mucosa, there was no significant change in both groups either before or after the treatment.

Liver and kidney function

Initially, all the patients of both groups had normal liver functions. After the treatment, blood biochemical parameters remained within normal limits in the majority of cases except that some of the parameters were slightly higher than normal in a few cases. All the patients had normal kidney functions during the study.

Serum proteins

Serum protein parameters of both groups rose after the treatment ($P>0.05$), except the Tf value in the control group. The increment was slightly higher in the control group than that in the study group, but there was no statistical significance (Table 2).

Table 2 Changes of serum protein levels before and after treatment ($\bar{x}\pm s$)

Serum protein	sTPN+5-Fu (n=7)		-MetTPN+5-Fu (n=8)	
	Before	After	Before	After
TP(g/L)	69.86±4.49	75.14±4.85	68.75±7.67	72.88±3.94
Alb(g/L)	38.89±5.95	42.29±5.22	35.57±4.98	37.85±6.09
Tf(g/L)	2.68±0.49	2.68±0.36	2.20±0.82	2.36±0.44
PA(mg/L)	225.83±44.36	312.43±96.14	181.57±49.85	219.88±70.17

Serum Met and Cys

The mean serum Met level was increased after the treatment in the control group ($P=0.03738$), while there was no obvious change in the study group. Serum Cys level had little changes after the treatment in both groups, with no statistical significance (Table 3).

Table 3 Changes of serum Met and Cys levels before and after treatment ($\bar{x}\pm s$)

	sTPN+5-Fu (n=7)		-MetTPN+5-Fu (n=8)	
	Before	After	Before	After
Met(mmol/L)	36.53±9.97	50.90±18.96 ^a	35.26±7.05	36.64±10.27
Cys(mmol/L)	70.13±22.79	56.23±17.70	54.83±14.12	57.21±23.47

^a $P<0.05$ vs before the treatment.

DISCUSSION

More than twenty years ago, Kreis *et al*^[3] explored by histoculture and animal experimentation, the possible role of Met deprivation in the diet or TPN in suppressing tumor growth and metastasis. By means of the FCM technique, Usami *et al*^[4] studied the effect of Met-deficiency on tumor cell growth and cell kinetics and found that the environment of "Met starvation", either *in vivo* or *in vitro*, could effectively interfere with cell recycling and block the tumor cells in G₁ phase. Hoffman *et al*^[5] noticed that by a method named the three-dimensional histoculture, when Met in the culture medium was replaced by Hcy, the proliferation of most Met-dependent tumor cells was inhibited in the latter S phase/G₂ phase. In spite of certain diversities in their experimental results, these authors shared the common view that the life cycle of tumor cells could be disturbed by "Met starvation". This concept favors the simultaneous use of phase-specific chemotherapeutic agents in an attempt to improve the overall therapeutic effects of neoplastic diseases.

Tumor tissues are composed of proliferating cells, non-proliferative and un-proliferating cell masses. The proliferating cells are those that are undergoing mitosis and are most sensitive to the phase-specific antitumor drugs, while those belonging to the non-proliferative or un-proliferating masses are not. By inference, more tumor cells are in the state of active proliferation, the greater effects would the phase-specific drugs exercise on the tumor cells. Maeta *et al*^[6] used preoperatively the MetTPN+5-Fu therapy to a group of AGC and colorectal cancer patients, found that the inhibition rate to thymidilate synthetase in the tumor cells was significantly higher than that in the control group, the name of "biochemical modulator" was thus coined to 5-Fu by these authors. Hoshiya, Goseki, Kitamura, Taguchi and

others proved the effectiveness in combining 5-Fu, cisplatin or adriamycin to -MetTPN in the treatment of gastric cancer and breast cancer. Kitamura *et al* and Taguchi *et al* further applied a new amino acid solution free of Met and Cys, named AO-90; this regimen was especially satisfactory in patients with distant metastasis and ascites, mounting the response rate to 45.5%^[7-10].

Our previous studies^[11-13] confirmed that the influence of sTPN on cell kinetics was to stimulate the tumor cells to enter into S phase; this would create an optimal condition for 5-Fu to exercise its greatest effect on tumor cells. In the present study, the effect of preoperative MetTPN+5-Fu on tumor cell kinetics in AGC patients was compared with that of sTPN+5-Fu. The results showed that, in the MetTPN+5-Fu group the percentage of tumor cells in G₀/G₁ phase increased and the percentage of cells in S and S+G₂/M phases decreased. The underlying rationale was not certified whether it was due to the decline in S phase cells caused by killing effect of 5-Fu, or an improved biochemical modulator effect of 5-Fu as described by Maeta *et al*^[6].

As a special therapeutic method which utilized the metabolic defect of tumor cells, there was still doubt that whether the -MetTPN+5-Fu regimen would bring about undesirable effects to the metabolism of normal cells remains to be clarified. Goseki *et al*^[14] studied the nutritional status of experimental rats after administration of a Met-free amino acid solution; no obvious side effects were found except slight decrement of a few nutritional parameters. Kurihara *et al*^[15] used the AO-90 amino acid solution plus chemotherapy in recurrent AGC patients, who manifested only a slight increase of nausea and anorexia. In rats with experimental gastric cancer submitted to the -MetTPN treatment, only a slight weight loss was observed as a side-effect; the serum protein levels and liver and kidney function underwent no significant change after a preoperative -MetTPN+5-Fu therapy for one week; serum Met and Cys levels were not obviously influenced; no operative complications occurred in any of the patients under study^[16].

In conclusion, a short-term preoperative -MetTPN+5-Fu in AGC patients did not cause any negative effect on normal host metabolism, while producing an evident interference with gastric cancer cell kinetics.

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Improved technique of vascular anastomosis for small intestinal transplantation in rats

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Subject headings rat; intestinal transplantation; vascular anastomosis; survival rate

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Abstract

AIM To establish a new improved vascular anastomotic technique to simplify the surgical technique and increase the survival rate of small intestinal transplantation in rats.

METHODS The graft removed en bloc consisted of entire small intestine, portal vein and aortic segment with superior mesenteric artery. The graft was perfused *in situ* and the gut lumen was irrigated during the operation. Heterotopic small bowel transplantation was performed by microvascular end-to-side anastomosis between the donor aortic segment with superior mesenteric artery and the recipient abdominal aorta, and by the formation of a "Cuff" anastomosis between the donor portal vein and the recipient left renal vein. Both ends of the grafts were exteriorized as stomas.

RESULTS A total of 189 intestinal transplantations were performed in rats, 33 of which were involved in the formal experimental group, with a survival rate of 84.8%. The average time for the donor surgery was 80min±10min; for graft repair 10min±3min; and for recipient surgery 95min±15min. The average time for the arterial anastomosis and the vein anastomosis was 18min±5min and 1min, respectively. The warm ischemic time and cold ischemic time were 22min±5min and less than 60min, respectively. The whole operation was completed by a single surgeon, the operative time being about 3 hours.

CONCLUSION The vascular anastomosis used

in this study could simplify surgical technique, reduce the operative time and elevate the survival rate of small intestinal transplantation in rats.

INTRODUCTION

Small intestinal transplantation may eventually become the most logical definitive treatment for small bowel syndrome. The small intestinal transplantation in rats has been the most frequently used animal model in the experimental investigation since it was first described by Monchik and Russel in 1971^[1]. However, the surgical technique of intestinal transplantation is rather complex and difficult, and the vascular anastomosis is a key technique for successful transplantation. We began to explore intestinal transplantation in rats in 1995, and have accumulated some experience and developed a stable and easy vascular anastomosis technique for small intestinal transplantation in rats.

MATERIALS AND METHODS

Animal and preoperative care

Male outbred Sprague-Dawley rats, inbred F344/N rats and inbred Wistar/A rats weighing between 150g and 400g were used as donors and recipients which were selected according to the protocol design. The rats received 5% glucose and 0.9% saline ad libitum for 24 hours (donors) and 12 hours (recipients) in the metabolic cage. They were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg) and atropine (0.05mg/kg). Both donors and recipients received lactated Ringer's solution at a rate of 4mL/h via the tail vein during surgery, the injection being controlled by a microdosage transfusion pump. Polyethylene (PE) catheter (4 mm in length) with an inner diameter of 1.5mm was used to make a cuff, two-thirds of the circumference diameter of the lower half part (2mm in length) of the PE catheter was cut off to form a holder in order to be grasped, and a shallow slot was cut out in the middle of outer tube wall of the upper half part (2mm in length) in order to be ligated.

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Donor operation

The abdomen was opened via a cross incision. The distal abdominal aorta was ligated beneath the level of renal artery to maintain adequate blood flow to the graft. Left renal vein, both sides of renal arteries and the origin of tuncus coeliacus were mobilized, ligated and divided. The origin of the superior mesenteric artery was mobilized, and the lumbar arteries from the aorta were meticulously ligated with 5-0 silk sutures. A segment of aorta with superior mesenteric artery was thoroughly liberated. The jejunum was divided just below the Treitz, sligament, the terminal of ileum was separated from the ileocecal valve, and the entire colon was removed. The portal vein and the superior mesenteric vein were met iculously separated from the pancreas and the surrounding conjunctive tissues, the pyloric and splenic veins which refluxed to portal vein were ligated and divided with 7-0 silk suture. Five mL 0.2% amikacin was slowly and gently infused into the intestine from the break-off of the jejunum to irrigate the gut lumen. The proximal end of the prepared aorta segment was ligated, the distal part of the aorta was cannulated with a fine polyethylene, and the graft was perfused *in situ* with 2mL-3mL cold heparinized (25U/mL) lactated Ringer, ssolution at a rate of 40mL/h controlled by a microdosage transfusion pump. At the same time, the portal vein was separated from the hepatic hilus, smashed ice crystal was put on the graft for rapid cooling. When the wall of intestine and mesentery turned white, and when a clear effluent from the portal vein appeared, the intestine and its vascular supply were removed en bloc (Figure 1) and stored in lactated Ringer, s solution at 4°C. Under condition of 4°C, the distal end of the aortal segment was ligated, the proximal of the aortal segment was made smooth, the holder of the Cuff was gently fixed with microsurgical vascular forceps, the portal vein was everted and covered onto the outer wall of the cuff, and fixed with ligation with 5-0 silk suture.

Recipient operation

The abdomen was opened via a midline and left abdominal wall horizontal incision was made to form a shape incision. The operative field was exposed with a self-retaining retractor. The left kidney and its surrounding tissues were mobilized. The origin of the left renal artery was ligated, and the tributaries of the left renal vein were ligated and divided. The left renal blood vessel was ligated at the level of renal hilus. The ligated suture remained counter-retracted, and the left kidney was then

removed. Segments (about 1cm-1.5cm long) of the recipient, sabdominal aorta was mobilized below the vessels to the left kidney. The small branches of this segment was carefully ligated. The mobilized segments were controlled proximally and distally with two microvascular clamps, and open ed via a longitudinal arteriotomy. The incision of arteriotomy was made to suit the anastomosis of the abdominal aorta of the donor. Heparinized (25U/mL) normal saline was used to wash away the residual blood clot in the vessel. The graft was placed on the lower left quarter of the abdominal cavity, and was ad justed to ensure that the vessels of donor, ssmall intestinal were not twisted and had no tension when they were anastomosed. The graft was surrounded by a tampon packed with ice crystals for cooling. The end-to-side anastomosis between the segment of aorta with superior mesenteric artery of the donor and the abdominal aorta of the recipient was performed with the aid of a binocular operating microscope. Two 9.0 nylon stay sutures were placed at both proximal and distal points of the arterial anastomosis to act as self-retaining retractors, and the anastomosis was performed using continuous 9-0 nylon suture. The essentials of the arterial anastomosis were that the interval of the sutures and the edge distance of the anastomosis should be homogenous, and the intimae of the arteries should be everted to form intima-to-intima anastomosis so as to prevent blood clotting. Eighteen to twenty sutures were needed for the anastomosis. When the venous vessels were anastomosed, a microvascular clamp was used to block the origin of the venous vessels of the left kidney. A hemostat was used to counter retract the ligated suture of left kidney hilum to fully unfold the renal venous vessel. A "T" shape incision was made on the anterior wall of the distal segment of left renal vein, and two angles of the incision was counter retracted with 9-0 nylon suture (Figure 2). Heparinized (25U/mL) normal saline was used to flush the venous vessel, the position of graft was adjusted. After ensuring that the venous vessel was not twisted, the Cuff of the portal vein of the donor was inserted in the left venous vessel of the recipient, and a ligation of 5-0 suture was used to fix the Cuff anastomosis (Figure 3). After the venous clamp was released, followed by the distal and proximal arterial clamps, pulsation of the superior mesentery artery of the graft turned to be distinguished, and the wall of the transplanted intestine appeared fresh red, and the ice-cream intestinal juice secreted from the intestinal lumen. Both ends of the graft were exteriorized as stomas. After flushing the abdominal lumen with normal saline containing cefazolin (10g/L) of 37°C, the abdominal wall was closed.

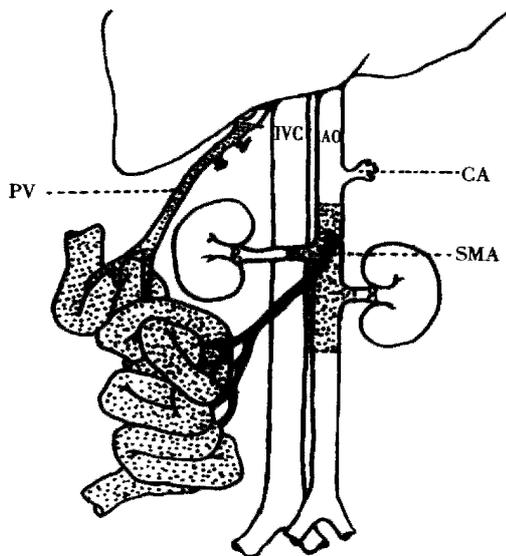


Figure 1 The en-bloc donor graft.
PV: portal vein; IVC: inferior vena cava; AO: aorta; CA: celiac artery; SMA: superior mesenteric artery.

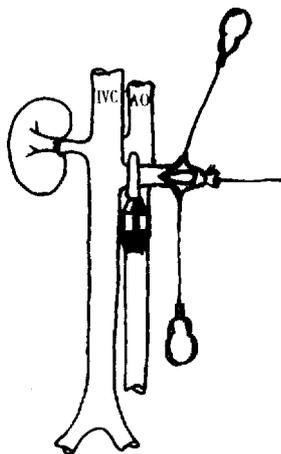


Figure 2 Exposure of the cavity of the left renal venous vessel of the recipient.
IVC: inferior vena cava; AO: aorta.

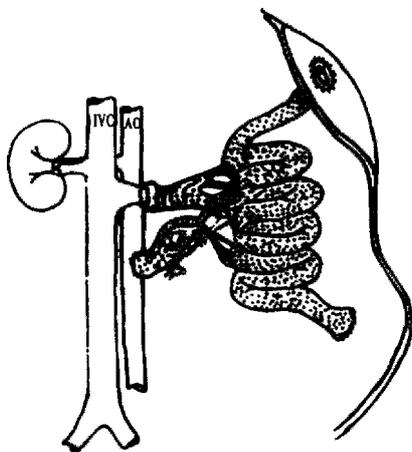


Figure 3 The vascular reconstruction for small intestinal transplantation.
IVC: inferior vena cava; AO: aorta.

Observation of surgical effect

The recipients were under meticulous observation after operation. Autopsy was performed if the recipient died, and the graft, the anastomosis of the vessels, heart, lung, liver, spleen, kidney and the native intestine of the dead recipient were harvested for pathological examination to find out the cause of death. Any death within 5 days was considered technical failure, and survival exceeding 5 days was considered technical success.

RESULTS

Our study was carried out in 3 stages and 189 operations were performed. Thirty-two operations were made to familiarize the anatomy of rats and train the surgical skill in the first stage. The second stage was the pre-experiment, 124 operations were performed to set up the surgical procedure, which was standardized and routinized, the longest survival of the recipient being longer than 10 months. The third stage was formal experiment, 28 cases of 33 operations succeeded. The cause of failure in the remaining 5 cases was anaesthesia accident (1), thrombosis of the arterial anastomosis (1), massive haemorrhage in abdominal lumen on postoperative day 3 (1), and hypovolemic shock followed by massive haemorrhage during operation (2). The success rate of the formal experiments was 84.8%.

The average time for the donor surgery was $80\text{min} \pm 10\text{min}$; the preparation time for the graft was $10\text{min} \pm 3\text{min}$; the operative time for the recipient was $95\text{min} \pm 15\text{min}$, and the time for arterial anastomosis and the venous anastomosis being $18\text{min} \pm 5\text{min}$ and 1min, respectively. The warm ischemic time of the graft (the interval when the graft was taken away from low temperature preservation) was $22\text{min} \pm 5\text{min}$, and the cold ischemic time was limited within 60min. The entire surgery was completed by a single surgeon, and the total operative time being about 3 hours.

DISCUSSION

There are five anastomosis patterns for small intestinal transplantation in rats: ① end-to-side arterial anastomosis between a segment of abdominal aorta/superior mesenteric artery of the donor and abdominal aorta of the recipient, and end-to-side venous anastomosis between the portal vein of the donor and inferior vena cava of the recipient^[2,3]. This is a classical pattern, and adopted by most investigators. ② End-to-side anastomosis between the carrel's patch of abdominal aorta with superior mesenteric artery of the donor and the abdominal aorta of the recipient, end-to-side anastomosis between the portal vein of the donor and the portal vein of the recipient^[4]. Compared with the cava-refluxing pattern, this portal-vein-refluxing pattern accords with physiology, and delays occurrence of rejection.

However, this surgical technique is more difficult, the incidence of complication is rather high, and a segment of the intestine of the recipient should be removed. ③ End-to-side vascular vessels a nastomosis between the superior mesenteric artery of the donor and the abdominal aorta of the recipient, and between the superior mesenteric vein of the donor and the inferior vena cava of the recipient^[5]. The main advantage of this model is that the donor operation is simplified by obviating the tedious dissection of portal vein and aorta which are very easily injured. However, a limitation of this pattern is that only a 10cm-25cm segment of jejunum can be used for transplantation rather than the total small bowel, and the small lumen of peripheral superior mesenteric artery and superior mesenteric vein may increase the vascular complication^[6]. Gordon compared pattern 1 with pattern 3 and discovered that there was no significant difference between these two patterns in the operation time, technic complexity and the experimental result^[7]. ④ The left native kidney is removed and revascularization of the graft is completed by two end-to-end anastomoses between the donor superior mesenteric artery and portal vein and the recipient renal vessels with the Cuff technique^[8]. The advantage of this pattern is that the technique for vascular anastomoses is simple, and has significantly reduced the graft warm ischemic time. However, the graft can only get 33% of the volume of blood supply that the native intestine can obtain, and the graft may lead to dysfunction because of chronic ischemia^[7]. ⑤ End-to-end anastomoses between the donor superior mesenteric vessels and the recipient superior mesenteric vessels, respectively. In this pattern, only a segment intestine is transplanted, and a segment of the native intestine should be removed. Furthermore, the incidence of vascular complication is very high^[9].

In our study, we combined the advantage of pattern 1 and pattern 4, and adopted arterial anastomosis by microvascular end-to-side anastomosis between the donor aortic segment with superior mesenteric artery and the recipient abdominal aorta, and performed the vein anastomosis by the formation of a "Cuff" anastomosis between the donor portal vein and the recipient left renal vein. This pattern can avoid hemodynamic instability of the recipient because there is no need to block the inferior vena cava of the recipient while there is a need in pattern 1. At the same time, it can avoid performing anastomoses of both the artery and vein vessels on the adjacent location of the abdominal aorta and the inferior vena cava of the recipient. Thus, exposure of arterial anastomosis is more clear, making the

arterial anastomosis very easy. In the arterial end-to-side anastomosis between a segment of the abdominal aorta of the donor and abdominal aorta of the recipient, the caliber of the anastomotic vessels is large enough to make the graft get sufficient blood supply. Therefore, this pattern can avoid chronic ischemia of the graft because of the end-to-end anastomosis between the superior mesenteric artery of the donor and the left renal artery of the recipient in pattern 4. The venous anastomosis with the Cuff technique can be accomplished within 1 minute, and the time for venous anastomosis is much shorter than that for traditional venous anastomosis. Thus, the warm ischemic and operation time is reduced, the surgical strike of the recipient is decreased, and the warm ischemic injury of the graft is reduced. The leakage of venous anastomosis is not taken into account after reperfusion because of the Cuff venous anastomosis technique, and no venous thrombus occurs after transplantation because the Cuff venous anastomosis is an intima-to-intima anastomosis of the vessel, and venous thrombus is one of the commonest causes of technique failure in traditional venous anastomosis. In summary, our improved vascular anastomosis technique, in which the arterial anastomosis is adopted by microvascular end-to-side anastomosis between the donor aortic segment with superior mesenteric artery and the recipient abdominal aorta, and the venous anastomosis is performed by the formation of a "Cuff" anastomosis between the donor portal vein and the recipient left renal vein, could simplify the surgical process, reduce the operation time and increase the survival rate of small intestinal transplantation in rats.

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Apoptosis induced by norcantharidin in human tumor cells

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Subject headings norcantharidin; oncoprotein Bcl-2; apoptosis; liver neoplasms; *immunohistochemistry*; Western blot analysis

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INTRODUCTION

The antitumor activity of norcantharidin (NCTD), the demethylated analogue of cantharidin, was studied in the early 1980s in China. NCTD has no side effects on urinary organs which cantharidin has shown and is easier to synthesize, and it can inhibit the proliferation of several tumor cell lines as well as transplanted tumors. Clinical trials with NCTD as a monotherapeutic agent indicated that NCTD had beneficial effects in patients with different kinds of digestive tract cancers, such as primary hepatoma, carcinomas of esophagus and gastric cancer, but no depressive effect on bone marrow cells. NCTD can increase the white blood cell count by stimulating the bone marrow and has some antagonistic effect against leukopenia caused by other agents. The exact cellular and molecular mechanisms of NCTD on tumor cells have not yet been elucidated to date^[1-3].

MATERIALS AND METHODS

Human tumor cell lines

Human hepatoma cell line BEL-7402, as monolayer cultures in RPMI-1640 medium supplemented with 20% FCS, was routinely grown at 37°C and 5% (V/V) CO₂. The response of tumor cells to NCTD

was studied during the logarithmic growth phase.

Reagents

NCTD was synthesized from furan and maleic anhydride via the Diels-Alder reaction. RNaseA and proteinase K were purchased from E. Merck, primary antibody (human Bcl-2 specific, rabbit polyclonal antibody) from Santa Cruz Biotechnology, In c, secondary antibody (biotinylated anti-rabbit IgG) and horseradish peroxidase streptavidin from Vector Laboratories, Inc, and NP-40 and DAB from Sigma.

Electron microscopy

Cell suspensions were centrifuged (200×g, 10min), and fixed as a pellet in 2.5% glutaraldehyde-1% osmium tetroxide buffered with PBS (pH 7.2). The cell samples were dehydrated in a graded ethanol series, embedded in Spury Resin and analyzed with standard procedures.

Isolation and analysis of apoptotic DNA fragments

Apoptotic fragments were isolated as described^[4]. After harvesting, the cell samples were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10s with lysis buffer (1% NP-40 in 20mmol/L EDTA, 50mmol/L Tris-HCl, pH 7.5; 10 μL-/10⁶ cells). After centrifugation for 5min at 1600×g, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2h with RNaseA (a final concentration of 5g/L) at 56°C followed by digestion with Proteinase K (a final concentration of 2.5g/L) for at least 2h at 37°C. After addition of 1/2 volume 10mol/L ammonium acetate, the DNA was precipitated with 2.5 volume ethanol, dissolved in gel loading buffer, and separated by electrophoresis in 1% agarose gels.

Immunocytochemical detection of Bcl-2

Apoptotic gene bcl-2 product was analyzed by standard SABC procedures. After washed with 0.01mol/L PBS (pH 7.4) three times for 5 min, cells were fixed with PLP (periodate-lysine-paraformaldehyde fixative) for 20 min at room temperature. Washed with PBS again, and cells covered with 3% H₂O₂ for 10min, 0.3% Triton x-100 for 30min and 3% sheep serum for 40min before

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primary antibody was added. Cells were covered with primary antibody (1:50) overnight at 4°C. Biotinylated secondary antibodies (1:200) were reacted for 30min at room temperature, then incubated cells with horseradish peroxidase streptavidin (1:400) for 30min at room temperature. Cells were washed in PBS three times for 5min between each step. Cells were incubated in DAB solution for 5min and examined under microscope. Cells were rinsed in distilled water and dehydrated through alcohol and xylene and mounted coverslip using a permanent mount medium for analysis by microspectrophotometer (LE ITZ DMRBE, Leica). Three to five sample slides in each group were chosen for analysis and 90 cells in each group were determined for mean light absorbance. The significance of difference between experimental data was validated using the *t* test.

Western blot

Approximately 5×10^6 cells were collected, and lysed in 100 μ L 2 \times electrophoresis loading buffer, boiled for 10min and electrophoresed through 12.5% SDS-polyacrylamide gels. Proteins were electrotransferred onto the nitrocellulose membrane. Filters were blocked overnight at 4°C with TBST [10 mmol/L Tris-HCl (pH 8.0), 0.15 mol/L NaCl, 0.05% Tween 20] containing 3% nonfat milk. All additional immunostaining steps were performed in TBST at room temperature. Filters were incubated with primary antibody (1:1000) for 2h. Biotinylated secondary antibodies (1:2000) were reacted for 30min. Immunoblots were reacted with horseradish peroxidase streptavidin (1:2000) for 30min. Filters were washed in TBST for 3 times for 20min between each step and were developed with DAB, and enhanced with H₂O₂ (0.03%).

RESULTS

Morphological changes of human hepatoma cells

The morphology of human hepatoma BEL-7402 cells changed significantly (Figures 1-3) after 24h-10mg/L NCTD treatment. Some cells condensed and some showed membrane bubbling aspect. Stained with Wright Giemsa, the cells showed M phase arrest and chromosome multipolar distribution. Nuclear chromatin compactions were observed under electron microscopy. At the same time, many membrane-enclosed bodies with cytoplasm and chromatin fragments were formed. These morphological changes are similar to the description of apoptosis^[5].

DNA agarose gel electrophoresis

A characteristic DNA "ladder" of apoptosis occurred in NCTD treated groups but did not appear in control groups (Figure 4).

Analysis of protein Bcl-2

Exist site of protein Bcl-2 presented yellow-brown reactant by immunocytochemical stain. Protein Bcl-2 was not expressed in the negative control group, and cells in control group were stained brown, showing that protein Bcl-2 expressed strongly in tumor cells. Treated with 10 mg/L NCTD for 24 h, the staining was weakened to light yellow-brown between negative control group and control group. The mean light absorbance of cells in the control group was 0.12 ± 0.01 ($n = 90$), while 0.08 ± 0.01 ($n = 90$) in treatment group. Compared with control cells, the expressions of Bcl-2 in treatment group were significantly decreased ($P < 0.001$). The result of Western blot analysis (Figure 5) showed that though protein Bcl-2 had a strong expression in BEL-7402 cells when treated with 10 mg/L NCTD for 24 h, 48 h and 72 h, the expression was weakened sharply.

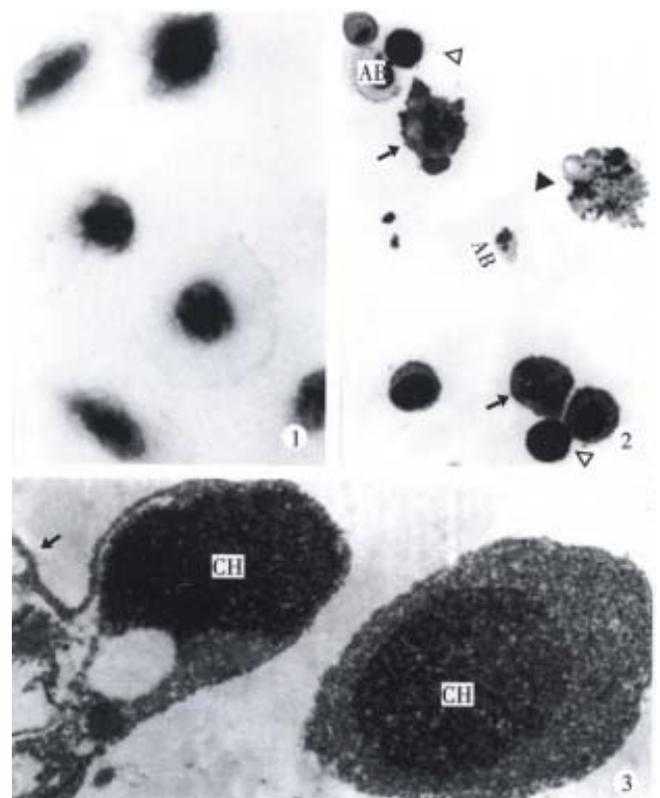


Figure 1 Control BEL-7402 cells, W-G staining. $\times 350$

Figure 2 BEL-7402 cells treated with 10mg/L NCTD for 24h. (†) indicates multipolar distribution of chromosomes in postmitotic cells. (▲) nuclear fragments in apoptotic cells, (▽) indicates condensed cells, (AB) apoptotic bodies containing one or two nuclear fragments. W-G staining, $\times 350$

Figure 3 Ultrastructural features of BEL-7402 cells treated with 10mg/L NCTD for 24h. Endoplasmic reticulum (ER) dilated in the apoptotic cell (†), the apoptotic bodies characterized by compaction of nuclear chromatin and condensation of cytoplasm. $\times 14000$

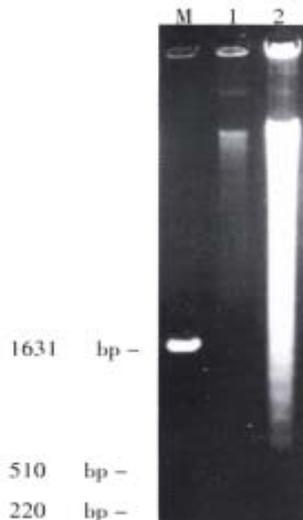


Figure 4 Agarose gel electrophoresis of DNA extracted from BEL-7402 cells. M: DNA marker; 1: control group; 2: group treated with 10 mg/L NCTD for 24 h.

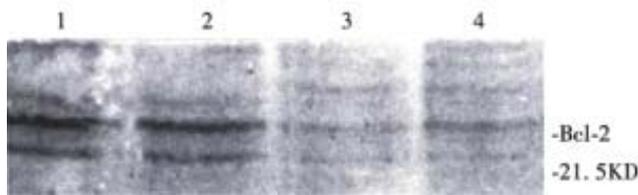


Figure 5 Western blotting analysis of Bcl-2 protein in BEL-7402 cells. 1: control; 2: treated with 10 mg/L NCTD for 24 h; 3: treated with 10 mg/L NCTD for 48 h; 4: treated with 10 mg/L NCTD for 72 h.

DISCUSSION

Apoptosis is an active, inherently programmed phenomenon. Morphologically, it involves rapid condensation and budding of the cell, with the formation of membrane-enclosed apoptotic bodies. A characteristic biochemical feature of the process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments^[6]. Obviously, NCTD can inhibit the growth of tumor cells mainly by inducing apoptosis.

Great progress has been made in researches on genetic regulation of apoptosis in recent years^[7]. Proto-oncogene *bcl-2*, might participate in the apoptotic process under some certain circumstances. The expression of Bcl-2 protein in BEL-7402 cells significantly changed in apoptotic process induced by NCTD. With the treatment time of NCTD prolonged, the expression of protein Bcl-2 decreased and paralleled with the apoptotic process. We believe that the decrease of protein Bcl-2 may play an important part in apoptosis of tumor cells induced by NCTD.

In a continuous observation, we found the treated BEL-7402 cells became round and separated from adjoining cells in the early stage, then bubbled, and membrane-enclosed bodies were formed. Stained with Wright-Giemsa, the round and bubbled cells were all presented with M phase arrest, and chromosome breaking, multipolar distribution, chromatin clumping and membrane-enclosed bodies with chromatin fragments forming were observed. All these indicated that the apoptosis induced by NCTD in BEL-7402 cells had a close relationship with tumor cell M phase arrest. More studies on the relationship between apoptosis and tumor cell M phase arrest are in progress.

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Inhibitory effect of IGF-II antisense RNA on malignant phenotype of hepatocellular carcinoma

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Subject headings carcinoma, hepatocellular; insulin-like growth factor II; genes, suppressor, tumor; RNA, antisense; liver neoplasms

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INTRODUCTION

According to the therapeutic effect and strategy of antisense RNA for hepatocellular carcinoma (HCC), we have specifically synthesized partial cDNA of human insulin-like growth factor II (IGF-II) and constructed IGF-II cDNA antisense eukaryotic expression vector. The constructed vector was introduced into hepatoma cell line SMMC-7721 to block the intrinsic IGF-II expression. The biological behavior changes of hepatoma cells were observed. All these would provide scientific basis for IGF-II antisense RNA in the treatment of HCC.

MATERIALS AND METHODS

Plasmid pIGF-II containing partial cDNA of IGF-II was constructed by ourselves^[1]. Eukaryotic expression vector pcDNA-3 was a gift from Professor Mao Ji-Fang in the Second Military Medical University. Hepatoma cell line SMMC-77 21 was provided by our own lab. Geneticin (G418), 1640 cultivation liquid and Li pofection were provided by Gibco/BRL (USA).

The construction and transduction of IGF-II antisense RNA expression vector: plasmid pIGF-II was doubly digested by Eco-RI/-Xba-I and the 106bp IGF-IIcDNA fragment was retrieved with

DNA purification kit (product of Huas hun Company). Plasmid pcDNA₃ was doubly digested by *Eco*-RI/*Xba*I and the large fragment was retrieved. The two fragments were co-incubated overnight with T₄ DNA ligase under 14°C, then transferred to *Escherichia coli* DH5α. The transformed colony was screened on LB agar containing amoxicillin. The transformed plasmid was extracted and then identified through dot blot (probes were made by ourselves) and PCR. The primer sequences of PCR were 5' CTAGA GCTTA CCGCC CCAGT GAGA 3', 5' AATTC TGCGG GCCTG CTGAA GTAG 3'. Because it belonged to the directional cloning, the posi tively transformed colony was reversely inserted into plasmid fragment, i.e., pIGF-IIAs. According to the method in reference^[2], 10 μg pIGF-IIAs was introduced into 5×10⁵ SMMC-7721 cells. The screenin g was performed in 1640 culture solution containing 400 mg/L G418, and pcDNA₃ was used as blank vector control.

The influence of pIGF-IIAs on hepatoma cell line SMMC-7721. ① cell growth curve: a total of 1×10⁴ logarithmic period growing cells were inoculated in 24-well culture plates. The cells were incubated at 37 °C with 5% CO₂ for 24 hrs, then the cells were collected, 3 wells once, the number of cells was counted after trypan blue staining, and the average value was calculated. ② Cell cycle analysis: 5×10⁶ logarithmic period growing cells were washed twice by PBS. One mL 70% ethanol was added to cell sediment. The cells were placed at 4 °C, were stained with PI 12 hours later and then measured with Elite Coulter flow cytometry. Cell cycle was analysed by Multicycle software. ③ Soft-agar colony formation expe riment was made as reference.

RESULTS

The construction of pIGF-IIAs (Figure 1). IGF-II cDNA was reversely inserted into pcDNA₃ vector, PCMV maintained high copies of the original vector in mammalian cells. There were multi-colony sites in BGHpa region, which were favorable for IGF-II cDNA insertion. The inserted sites located in *Eco*-RI and *Xba*I region. Neo and Amp were the selective marks of the gene in mammalian cells and bacterial cells. IGF-II cDNA was reversely inserted into pcDNA₃ vector, because it belonged to directional colony. The dot blot of the properly

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transformed plasmid showed that 106bp hybridization band was amplified by PCR. The positively identified band was pIGF-IIAs (Figure 1).

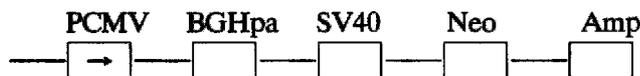


Figure 1 The constructional sketch map of pIGF-II.

pIGF-IIAs and pcDNA₃ were introduced into SMMC-7721 cells. After screening with G418 for 4 weeks, the number of transformed colonies was 26.00 ± 2.83 and 52.00 ± 4.66 (mean \pm SE), respectively.

After screening with G418, the growth curves of SMMC-7721 cells and transgenic cells are shown in Figure 2.

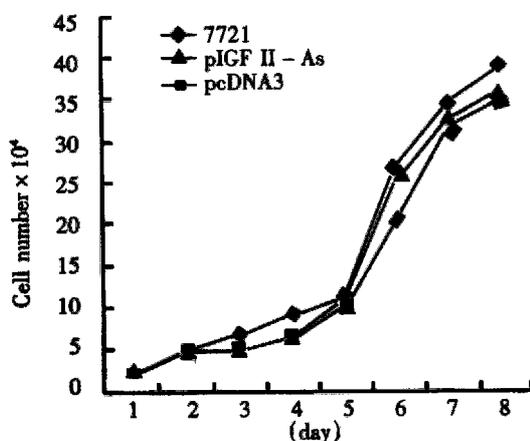


Figure 2 Growth curves of the three cell groups.

Cell cycle analysis was made with flow cytometry (FCM) (Table 1).

Table 1 Cell cycle analysis with FCM

Group	G ₀ /1	S	G ₂ /M
7721	72.40	21.50	6.10
pcDNA ₃	72.40	20.30	7.20
pIGF-IIAs	43.00	41.20	15.80

Colony formation in soft-agar cultivation. Cultivated on double layer soft-agar for 14 days, 10^4 cells were incubated in each culture dish. The cells in group 7721 and pcDNA₃ grew well and formed obvious clustered cell colonies. The number of colonies was 205.17 ± 72.02 and 146.50 ± 74.17 , respectively, while there was no colony formed in group pIGF-IIAs.

DISCUSSION

Antisense IGF-II gene was introduced into hepatoma cell line SMMC-7721 with antisense technique. The antisense RNA sequence matched with target mRNA, would selectively block the autocrine or paracrine growth stimulation mechanism of hepatoma cells, attaining to the purpose of antisense gene therapy. We found that the number of transformed colonies in pIGF-IIAs transfected SMMC-7721 was significantly less than that in pcDNA₃. This may be due to IGF-II antisense RNA suppressing the growth of transfected SMMC-7721. It may imply that IGF-II antisense RNA had the remarkable biological effect of suppressing the growth of hepatoma cells. The growth curves of the three group cells had no significant difference with G418 screening, which was consistent with Cajot's research on p53 gene transduced lung cancer cell line^[3].

The number of S-phase cells increased in pIGF-IIAs transfected group. However, there was no significant difference of the cell cycle in the other two groups. It was inferred that IGF-II antisense gene suppressed the overexpression of IGF-II and partially up-regulated IGF-II receptor and IGF-IR, which activated the kinases p34^{cdc2} related to DNA duplication^[4].

Cells of both pcDNA₃ and SMMC-7721 groups could form colonies on soft-agar, while cells in the antisense pIGF-IIAs group could not form colonies. It demonstrated that the malignant phenotype of hepatoma cells inclined to disappear. This was the phenomenon of tumor cells reversing gradually to normal cells.

This study demonstrated that the transduction of antisense IGF-II into hepatoma cells had the biological effect of suppressing the carcinogenesis of hepatoma cell *in vitro*.

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The prevalence of transfusion transmitted virus infection in blood donors

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Subject headings transfusion transmitted virus infection; blood donors; liver diseases; hemodialysis

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INTRODUCTION

A newly discovered DNA virus, transfusion transmitted virus (TTV), was reported as a cause of post-transfusion hepatitis of unknown etiology in Japan^[1]. In order to investigate TTV prevalence in southern China, a study was carried out among blood donors, patients with liver diseases and hemodialysis to determine the epidemiological characteristics.

MATERIALS AND METHODS

Samples

Sera or plasma samples (471) were obtained from volunteer blood donors from Shenzhen Baoan Blood Center and commercial blood donors from Dongguan Blood Center. Sera samples (117) from patients with liver disease and hemodialysis were collected from Shenzhen Baoan People's Hospital. All the sera or plasma samples were stored at -70°C for detection.

Reagents

Hepatitis B surface antigen (HBsAg), antibodies to hepatitis C virus (HCV), antibodies to human immunodeficiency virus (HIV) EIA kits were purchased from Abbott Laboratories (Abbott Park, IL). Syphilis TPHA kits were purchased from Randox Laboratories Ltd. IgM antibodies to hepatitis A virus, antibodies to hepatitis E virus ELISA reagent kits, HBV, HCV and HGV polymerase chain reaction (PCR) reagent kits were

purchased from the Institute of Hepatology of Beijing Medical University.

Laboratory tests

The serum specimens from blood donors were tested for alanine aminotransferase (ALT) levels (less than or equal to 40 IU/L), HBsAg, antibodies to HCV, antibodies to HIV and syphilis TPHA. The patients with liver disease were further detected for markers of HAV, HBV, HCV, HEV and HGV infection.

Detection of TTV DNA by nPCR^[2]

Nucleic acid was extracted from 100 µL serum with Acupure DNA/RNA extraction kit (Biotronics Tech. Corp.) following the manufacturer's recommended protocol. According to the sequence of TTV TA278 strain (GenBank accession number AB008394) two pair nested primers were designed for nested polymerase chain reaction (nPCR). The first-round PCR was performed with P1 (sense: 5'-CCAGGAGCATATACAGAC-3') and P2 (anti-sense: 5'-TACTTCTTGCTGGTGAAAT-3') for 30 cycles (pre-denaturing for 180s at 94 °C, denaturing for 40s at 94 °C, annealing for 40s at 55 °C, extension for 40s at 72 °C). The second-round PCR was carried out with P3 (sense: 5'-CAGACAGAGGAG-AAGGCAAC-3') and P4 (anti-sense: 5'-ACAGGCACATTACTACTACC-3') for 30 cycles for amplification of 309bp product. The second-round PCR was done in the same manner as the first-round. Amplified products were separated in 2% agarose gel electrophoresis, stained with ethidium bromide and observed under ultraviolet light.

Cloning and sequencing of TTV genome

The purified products of PCR were directly ligated into pBluescript-T vector (Invitrogen Company), and with recombinant plasmid obtained by introduction into *E. coli* XL1-blue, sequences were determined for both strands by the sanger dideoxy chain termination methods, and with sequencer (model ABI373; Applied Biosystems, Foster City, CA).

Data analysis

The sequences of TTV genome were analyzed by the computer program CLUSTALW version 1.1 and the computer program GOLDKEY version 1.1.

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Statistical analysis was made using statistical program SYSTAT version 3.0.

RESULTS

The results of TTV DNA determined by nPCR in blood donors

Four hundred and seventy-one sera samples from volunteer blood donors and commercial blood donors were all negative for markers of HBsAg, anti-HCV, anti-HIV and TPHA. Fifty-eight of 471 sera samples had an elevated ALT (mean 89 IU/ L±45 IU/L) and with no markers of hepatitis A-G virus infection. The results of TTV DNA detected by nPCR from blood donors are shown in Figure 1 and Table 1. Among volunteer blood donors with normal transaminase levels, 30 (14.7%) of 204 were positive for TTV DNA. In contrast, 48 (23.0%) of 209 commercial blood donors with normal transaminase levels, and 18 (31.0%) of 58 blood donors with elevated transaminase levels were positive for TTV DNA. The prevalence of TTV in blood donors with elevated transaminase levels was significantly higher than in volunteer blood donors ($P<0.01$) and commercial blood donors with normal transaminase levels ($P<0.05$). There was no significant difference between the commercial blood donors with normal transaminase levels and the blood donors with elevated transaminase levels.

Table 1 The results of TTV DNA detected by PCR in sera of blood donors

Blood donors	No. of sample	TTV DNA (+)	
		n	%
Commercial blood donors with normal ALT levels	209	48	23.1
Volunteer blood donors with normal ALT levels	204	30	14.4
Blood donors with elevated ALT levels	58	18	31.0

The results of TTV DNA detected by PCR in patients with liver diseases and hemodialysis

One hundred and five serum specimens from patients with liver diseases were detected for TTV

DNA by nPCR. The results are shown in Table 2. Among 36 non-A to E hepatitis patients, 15 (41.6%) were PCR positive for TTV DNA. One of 13 (7.7%) patients with hepatitis A virus infection was positive for TTV DNA, 3 (23.1%) of 13 patients with hepatitis B virus infection and 8 (18.6%) of 43 patients with hepatitis C virus infection were positive for TTV DNA. The prevalence of TTV in non-A to E hepatitis patients was significantly higher than in patients with HAV or HBV and HCV infection. Among 12 cases of hemodialysis, 5 (41.7%) were PCR positive for TTV DNA.

The sequencing results of TTV partial genome from blood donors

The anticipated DNA fragments amplified by PCR from 5 blood donors were cloned and sequenced for both strands. The similarity of nucleotide sequences of partial gene within TTV ORF1 region among 5 isolates from blood donors ranged from 98.4% to 99.4%. There was a 97% nucleotide identity among AB008394 (from Japan), TTVCHIN1 (from northern China) and our 5 isolates from blood donors (Figure 2 and Table 3).

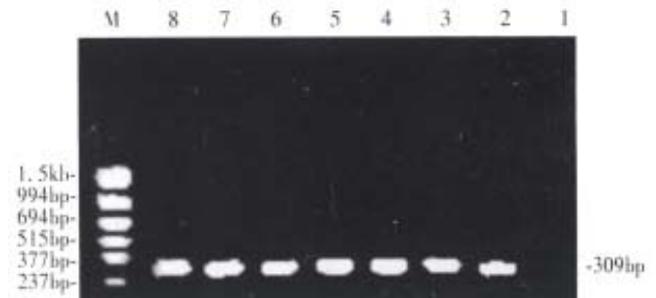


Figure 1 Agarose electrophoresis of TTV DNA PCR products. M: marker; 1: negative control; 2-8: positive

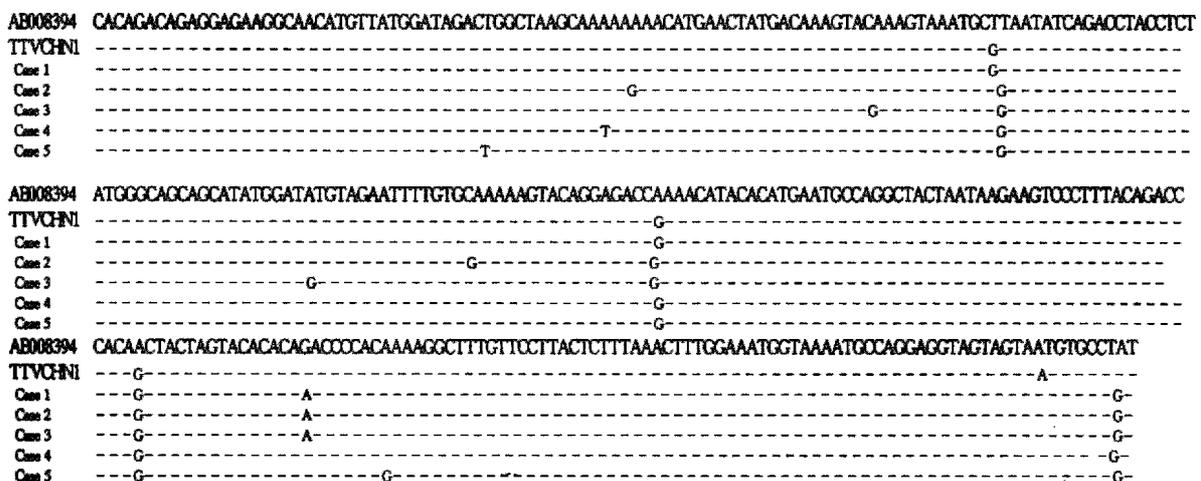


Figure 2 Comparison of isolates from blood donors (cases 1-5) with corresponding sequences from Japan (AB008394) and China (TTVCHN1).

Table 2 The results of TTV DNA detected by PCR in sera of the patients with liver diseases and hemodialysis

Patients	Cases tested	TTV DNA (+)	
		n	%
Non-A to E hepatitis patients	36	15	41.6
Hepatitis A patients	13	1	7.7
Hepatitis B patients	13	3	23.1
Hepatitis C patients	43	8	18.6
Hemodialysis	12	5	41.7

Table 3 Homologies of nucleotide sequences among isolates from blood donors of Japan and China (%)

TTV isolates	AB008394	TTVCHN1	Case 1	Case 2	Case 3	Case 4
TTVCHN1	98.7					
Case 1	98.4	99.7				
Case 2	97.7	99.1	99.4			
Case 3	97.7	99.1	99.4	98.7		
Case 4	98.4	99.7	99.4	98.7	98.7	
Case 5	98.1	99.4	99.1	98.4	98.4	99.1

DISCUSSION

Transfusion transmitted virus (TTV) is a newly discovered virus associated with the patients with post-transfusion hepatitis of unknown etiology in Japan. The preliminary studies showed that TTV was a single-stranded DNA virus, and the nucleotide sequence of TTV DNA genome was composed of approximately 3.7 kp, including two open reading frame (ORF) which encodes for 770 amino acids (aa) and 202 aa, respectively^[3]. TTV resembles to some known animal single-stranded DNA viruses, such as chicken anemia virus and human parvovirus B19. Some studies reported that TTV was associated with post-transfusion and acute and chronic hepatitis of unknown etiology. So far, no reliable serologic assay for antibodies against TTV infection has been developed. Thus, PCR was utilized to determine the prevalence of TTV infection in different populations.

Based on the conserved nucleotide sequence of TTV ORF1 gene, a nested-PCR for TTV DNA was established in our study. The specificity of amplified targets was documented by sequencing the PCR products selected from 5 blood donors positive for TTV DNA (Figure 2). The epidemiological investigation indicated that there was a high prevalence of TTV infection in volunteer blood donors and commercial blood donors. Simmonds *et al*^[4] reported that TTV viraemia was detected in 19 (1.9%) of 1000 non-remunerated regular blood donors from Scotland, 10% from England. Forty-four to 56% factor VIII and IX had a TTV contamination. In this study, nearly 15%-31% of blood donors from southern China were TTV DNA positive. Hemodialysis patients were considered at "high-risk" for exposure to parenterally transmitted viruses, such as HBV and HCV. TTV DNA positive rate was 41.7% in our data. The results suggested that TTV can be parenterally transmitted to recipients of blood or blood products.

Fifty-eight blood donors with elevated serum

ALT levels were tested without markers of hepatitis A to G viruses. The positive rate of TTV DNA was 31%, which was higher than in volunteer blood donors with normal ALT levels (14-7%). The results indicated that some blood donors with abnormal ALT was associated with TTV infection.

The pathogenicity of TTV is unclear. The clinical and epidemiological studies showed that the TTV infection was found among patients with fulminant hepatitis, acute and chronic liver disease of unknown etiology^[3,5]. In our study, the prevalence of TTV was higher in patients with non-A to G hepatitis (41.6%) than in patients with hepatitis A to C virus infection, indicating that TTV may be responsible for some cases of non-A to E hepatitis. Recent studies demonstrated that TTV may cause epidemic outbreak of hepatitis of unknown etiology by fecal-to-mouth way^[6]. The high prevalence of active TTV infection (14.7%) in the general population was found in our study, suggesting that TTV, similar to HBV, may have "symptom-free carriers". Further studies are urgently needed to determine the pathogenicity of TTV and the significance of "symptom-free carriers".

We amplified and sequenced the ORF1 partial gene of TTV genome from 5 blood donors and compared these sequences with those of isolates from Japan and northern China. Our preliminary results showed that there are less than 2% nucleotide divergence among 5 isolates from blood donors. Compared with the isolates from Japan and northern China, the similarities of nucleotide sequences were above 97%. The data suggest that the ORF1 region of TTV genome is conserved in different geographic areas.

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Effects of cold preservation and warm reperfusion on rat fatty liver

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Subject headings fatty liver; cold preservation; warm reperfusion; rats

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INTRODUCTION

Although liver transplantation for irreversible liver diseases is increasingly prevalent worldwide, patient die while waiting for donors because of organ short ages. One important problem commonly encountered is that fatty livers often affect the outcome of liver transplantation. It is reported that the incidence of abnormal fatty livers in autopsies after accidental death ranged from 15% to 24%. Since fatty livers may result in a primary nonfunction (PNF) liver graft, which contributes to an increased risk of mortality^[1], they are usually out of consideration in liver transplantation. However, some fatty livers can be successfully transplanted. Therefore, how to choose fatty livers as donor organs correctly is the crux of success in liver transplantation.

In this study, we preserved fatty livers of rats fed with a choline-deficient diet in cold Lactate Ringer's (LR) solution for various periods, and evaluated the effects of cold preservation on fatty liver in terms of portal perfusion pressure, endothelin-1, enzyme release in the effluent and mortality of sinusoid lining cell (SLC) using isolated perfused rat liver model.

MATERIALS AND METHODS

Animals and induction of fatty livers

Male Wistar rats, weighing 240 g - 260 g, were obtained from the Experimental Animal Center of Harbin Medical University. To induce fatty deposition in the livers, experimental rats were fed with a choline deficient diet (CDD) for 14, 28 or 42 days. The composition of this diet is shown in Table 1^[2].

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Table 1 Components of choline-deficient diet (CDD)

Components	Percentage
Casein	8.0
Lard	47.32
Sucrose	39.275
Minerals	4.0
Vitamins	0.65
L-cystine	0.625
Mg(OH) ₂ MgCO ₃	0.1
Vitamin D ₃	0.02
Vitamin E	0.01
Total	100.0

Surgical procedures and experimental groups

We used isolated nonrecirculating perfused rat liver as preservation-reperfusion model^[3]. Animals were anesthetized with pentobarbital (30mg/kg, ip). After cannulation of the bile duct and portal vein, the liver was flushed with 20 mL 0 °C LR solution via portal vein. The liver was removed immediately and stored in 0 °C LR solution. After cold storage for various periods, the liver was reperused for 30 min via the portal vein at 3 mL·g⁻¹·min⁻¹ with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37 °C) saturated with a 95% O₂:5% CO₂ mixture in a nonrecirculatory system. Animals were divided randomly into four groups: ① control group (n=21) fed with a standard diet including three subgroups containing 0 h (n=7), 6 h (n=7) and 12 h (n=7) cold storage; ② mildly fatty liver group (n=7) fed with a CDD for 14 days was preserved for 12 hours; ③ moderately fatty liver group (n=14) fed with a CDD for 28 days including two subgroups containing 6 h (n=7) and 12 h (n=7) cold storage; and ④ severely fatty liver group (n=14) fed with a CDD for 42 days consisting of two subgroups containing 0 h (n=7) and 6 h cold preservation.

Macroscopy and histology of livers before storage

The morphology of livers before cold storage was assessed by macroscopy and light microscopy. Liver biopsy specimens taken from the right lobes of the same site were stained with hematoxylin and eosin.

Portal perfusion pressure

The portal perfusion pressure was detected at the 30th min of reperfusion period when livers were reperused at 3 mL·g⁻¹·min⁻¹ constantly.

ET-1 assay

Two ml effluent was taken at the 30th min of reperfusion period for ET-1 assay. ET-1 values were detected by standard radioimmunoassay methods using a commercial radioimmunoassay kit (General Hospital of PLA)^[4]. Standard curves were obtained with known concentrations of ET-1.

Bile volume

The total bile volume was collected within 30 min reperfusion period. Bile volume was expressed as bile secretion $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\cdot\text{wet weight}$.

Enzymes in the effluent

Two ml effluent at the 30th min of reperfusion period was collected for detection of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) with an automated GEMSTAR Biochemistry Analyzer (USA).

Mortality of SLC

Trypan blue staining is indicative of loss of cell viability. After 30 min reperfusion, the liver was perfused with trypan blue (200 μm) for 5 min and fixed with a 2% paraformaldehyde: 2% glutaraldehyde solution in the perfusion buffer. Livers were sectioned at the same level in the left lobes and were paraffin-embedded and stained in two sets. One staining with hematoxylin and eosin allowed quantitating of SLC and the other staining with eosin alone permitted determination of the number of trypan blue positive (nonviable) SLC. Five pericentral and five periportal regions within a field measuring 325 $\mu\text{m}\times 325 \mu\text{m}$ were examined under high power ($\times 400$). Mortality of SLC was expressed as the ratio of the number of trypan blue positive SLC to the total amount of SLC.

Statistics

Statistical evaluation was done by Student's *t* test. The results were expressed as $\bar{x}\pm s$. Means were considered significantly different when $P<0.05$.

RESULTS

Macroscopy and histology of livers before storage

In the control group, the morphology of livers before cold storage was normal under macroscopy and light microscopy. The livers of the rats fed with a CDD for 14 days had no abnormality in the appearance, but their specimens contained fatty vacuoles in less than one third of the liver cells. They belonged to mildly fatty livers. The livers of rats fed with a CDD for 28 days were a little larger than normal and appeared slightly yellow. The specimens of these livers contained fatty vacuoles in more than one third of the liver cells but less than two thirds of the cells. They pertained to

moderately fatty livers. The livers of rats fed with a CDD for 42 days were obviously larger than normal and appeared grossly yellow. The specimens contained fatty vacuoles in two thirds or more of the liver cells and scattered hepatocyte necrosis occasionally, which were considered as severely fatty livers.

Portal perfusion pressure and ET-1 values

There was significant increase of portal perfusion pressures in each group in parallel with the duration of preservation (Table 2). No remarkable difference of portal perfusion pressures was found between mildly fatty liver group and control group after 12 h preservation, between moderately liver group and control group after 6 h preservation, between severely fatty liver group and control group without preservation. Portal perfusion pressures were significantly higher in moderately fatty liver group than in control group after 12 h preservation ($P<0.01$) and in severely fatty liver group than in control group after 6h preservation ($P<0.01$). The changes of ET-1 values in the effluent were consistent with those of portal perfusion pressures (Table 2).

Bile secretory volume

Bile secretory volume in each group decreased significantly as the preservation time prolonged (Table 2). There was no obvious difference between mildly fatty liver group and control group after 12 h preservation, between moderately liver group and control group after 6 h preservation, and between severely fatty liver group and control group without preservation. Bile production was markedly lower in moderately fatty liver group than in control group after 12 h preservation ($P<0.05$) and in severely fatty liver group than in control group after 6 h preservation ($P<0.01$).

Enzymes in the effluent and mortality of SLC

The changes of enzymatic levels (AST, ALT and LDH) in the effluent and mortality of SLC were consistent with those of portal perfusion pressures (Table 3).

Table 2 Portal perfusion pressure, ET-1 and bile production ($\bar{x}\pm s$, $n=7$)

Group	Preservation time (hours)	Portal perfusion pressure (cmH ₂ O)	ET-1 (ng/L)	Bile secretory volume ($\mu\text{L}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\cdot\text{wt}$)
Control	0	9.5 \pm 0.8	35.6 \pm 5.8	0.40 \pm 0.09
	6	12.8 \pm 1.3	60.2 \pm 8.4	0.28 \pm 0.07
	12	16.0 \pm 1.7	83.7 \pm 11.9	0.15 \pm 0.05
Mildly fatty	12	16.5 \pm 2.1	85.9 \pm 13.4	0.16 \pm 0.05
	6	13.9 \pm 1.7	65.8 \pm 10.1	0.26 \pm 0.08
Moderately fatty	12	20.1 \pm 2.3 ^b	124.5 \pm 27.6 ^b	0.11 \pm 0.03 ^a
	0	9.8 \pm 1.0	32.7 \pm 4.9	0.38 \pm 0.08
	6	18.5 \pm 2.1 ^c	90.4 \pm 15.9 ^c	0.12 \pm 0.03 ^c

^a $P<0.05$ vs control (12 h); ^b $P<0.01$ vs control (12 h);

^c $P<0.01$ vs control (6 h).

Table 3 AST, ALT, LDH in the effluent and mortality of SLC ($\bar{x}\pm s$, $n = 7$)

Group	Preservation time (hours)	AST (u.l ⁻¹ .g ⁻¹ .wt)	ALT (u.l ⁻¹ .g ⁻¹ .wt)	LDH (u.l ⁻¹ .g ⁻¹ .wt)	Mortality of SLE (%)
Control	0	2.42±0.08	8.75±1.40	23.64±4.52	<0.5
	6	2.58±0.12	8.90±1.51	25.72±4.79	5.9±1.4
	12	9.65±1.82	16.75±3.10	54.74±9.70	13.8±2.8
Mildly fatty	12	10.20±2.10	18.20±4.20	58.26±12.15	15.1±3.2
Moderately fatty	6	2.63±0.21	9.01±1.72	28.40±5.10	7.0±1.8
	12	13.50±2.74 ^b	24.10±5.86 ^b	74.48±19.5 ^b	18.7±4.3 ^a
Severely fatty	0	2.10±0.06	8.60±1.25	21.70±4.30	<0.5
	6	11.82±1.97 ^c	19.45±4.76 ^c	62.75±17.54 ^c	17.4±3.5 ^c

^a $P < 0.05$ vs control (12 h); ^b $P < 0.01$ vs control (12 h); ^c $P < 0.01$ vs control (6 h).

DISCUSSION

Although progress in organ retrieval, preservation, recipient implantation and the rarity of hyperacute rejection, has improved patient survival after orthotopic liver transplantation (OLT), PNF still occurs in 2%-23% of transplanted livers. Transplantation of a fatty liver may lead to PNF. Some researchers hold that fatty liver grafts are unsuitable for elective OLT, since clinical experience evidenced that such grafts may lead to PNF more frequently than nonfatty ones. But others disagree about this because of successful OLT cases with fatty liver grafts. In order to increase the usage of donor livers on the premise of the unaffected outcome of OLT, it is vital to decide whether to choose fatty livers as donor organs and how to choose them properly.

The CDD-induced fatty liver was produced according to methods described elsewhere. Free fatty acid (FAA) synthesized in the liver bind to phospholipid apoprote in B complex, which is excreted into the blood as very-low-density lipoprotein (VLDL). Choline is a precursor of phosphoryl choline and is important in lipoprotein production. Choline deficiency suppresses the synthesis of the phospholipid-apoprotein B complex, and inhibits VLDL-secretion from the liver. Furthermore, sucrose-rich diet elevates the triglyceride concentration in the liver. These factors may cause fatty deposition in the rat liver after 14 days of CDD.

In clinical transplantation, fatty livers are generally graded to three scales depending on the degree of fatty infiltration: mildly (<30%), moderately (30% to 60%) and severely (>60%)^[5]. According to these criteria, the liver of rats fed with a CDD for 14, 28 and 42 days should be classified as mildly, moderately and severely fatty livers, respectively. Anchony reported that the overall incidence of fatty infiltration in 124 liver donor biopsies was 24.4%, with 12.3% of biopsies exhibiting mild changes, 8.9% moderate changes, and 3.2% severe changes.

Fatty infiltration of the liver can occur in a variety of conditions. Common causes of fatty infiltration include alcohol intake, obesity, nutritional disorders (particularly malnutrition), drug therapy and diabetes although the reason diabetic patients may develop fatty deposition could be related to obesity.

Our experiment showed that there was no obvious difference in the preservative effects between mildly fatty liver group and control group after 12 h preservation and between moderately fatty liver group and control group after 6 h preservation. In view of this, if we gave up using all the fatty livers as donor organs, a lot of available donor livers would be wasted. Meanwhile, the present study demonstrated that preservation reperfusion injury was more severe in moderately fatty liver group than in control group after 12 h preservation and in severely fatty liver group than in control group after 6 h preservation. The increase in the preservation reperfusion injury was manifested as significantly higher portal perfusion pressure, higher ET-1 values, lower bile production, higher enzymatic levels in the effluent and increased mortality of SLC. Therefore, in order to lower the occurrence of PNF, some fatty livers, such as severely fatty livers, should be discarded resolutely although their function was normal or basically normal before storage.

The etiology of increased preservation reperfusion injury of fatty livers has not been clarified^[6,7]. To explain the loss of viability in fatty liver grafts after cold preservation, four underlying mechanisms are suggested: ① The solidification of triglycerides during cold storage causes the rupture of the hepacytes containing fat upon rewarming. The rupture of these cells results in the release of fat globules into the hepatic microcirculation with disruption of sinusoidal architecture, focal hemorrhage and hepatocellular necrosis. ② The increase in Kupffer cell activation was possibly caused by the increased number of Kupffer cells in fatty livers. Activated Kupffer cells can produce

many types of chemical substance and peptide mediators, which may play a significant role in microcirculatory disturbance and reperfusion injury. ③ FFA accumulation in the hepatic mitochondria cause inhibition electron transport in the respiratory chain, affects oxidative phosphorylation activity, reduces the production of ATP, leading to disturbance of energy metabolism. ④ Cellular disruption and release of triglycerides and free fatty acids activate phospholipases and lipid peroxidation, with free radical formation, thereby causing further cellular damage.

In summary, the present study confirms that moderately and severely fatty livers are highly susceptible to cold preservation reperfusion injury and are likely to lose their viability after cold storage more easily than the nonfatty livers, while no obvious difference of the preservative effects is found between mildly fatty livers and nonfatty livers. Fatty livers should not be discarded blindly only for its high incidence of PNF. However, fatty livers should not be used arbitrarily only for the shortage of donor organs either. To use only hepatic function test to assess donor liver is not enough, since the functions of fatty livers are always within normal range. The use of preoperative donor liver biopsies in many sites is considered the most valuable means for the assessment of abnormal hepatic pathology and the correct selection of donor livers. We proposed the following criteria for the

use of a fatty liver as a graft: ① a mildly fatty liver can be used in the same way as a nonfatty liver; ② a moderately fatty liver can be used depending on the time of preservation and the balance of the emergent needs of recipient and the donor organ supply; and ③ a severely fatty liver should be discarded without hesitation.

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Cloning and sequence analysis of human genomic DNA of augments of liver regeneration

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Subject headings augments; liver regeneration; cloning; genomic DNA; intron; exon

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INTRODUCTION

The liver is one of the organs, which have potential regenerative capability in mammalian animal^[1]. The study of the canine model indicated that the liver could regenerate to original size after 70% hepatectomy in only two weeks^[2]. So it is a hot research topic for the cellular and molecular mechanism of liver regeneration. Accumulated results demonstrated that the hepatocyte growth factor (HGF)^[3], insulin-like growth factor I and II (IGF-I, II)^[4], epidermal growth factor (EGF), transforming growth factor alpha (TGF alpha)^[5] and insulin^[6] are among the most important growth factors for liver regenerative regulation. In recent years, a heat-stable protein in the serum of the patients with various liver diseases has been noted for its potential stimulation effects on the liver regeneration, and this growth factor is called hepatocyte-stimulatory substance (HSS). Gradient purification and sequence analysis of HSS protein indicated that the HSS protein itself is the augments of liver regeneration (ALR)^[7], or called hepatopoietin (HPO)^[8]. The immunohistochemical staining indicated that the expression of the ALR mainly existed in platelets and the sperm cells in testes, and ALR also could be found in the liver and the spleen which contain many platelets^[9]. The analysis of the protein structure of the human and mouse ALR indicated that the primary protein structure of ALR does not contain a typical signal peptide sequence, and it is unknown if a specific

receptor is necessary for the effect of the ALR. Therefore, it is important to clone the genomic DNA sequence of the ALR and it is also very helpful for the analysis of the structure of the ALR genomic DNA and regulation at the transcriptional and post-transcriptional levels.

METHODS

Molecular cloning of human ALR genomic DNA
Using human ALR cDNA sequence as a reference, and BLAST search path as a tool, the GenBank established by National Center for Biological Information (NCBI), USA, has been searched for the homologous sequences.

Definition of the intron-exon structure of the human ALR genomic DNA

According to the Breathnath-Chambon rule and the human ALR cDNA coding sequence, the intron-exon structure of human genomic DNA was defined.

Homologous analysis of human and mouse ALR genomic DNA sequences

The homology of human and mouse ALR genomic DNA sequences was analyzed for their 5'-UTR, intron-exon structure and 3'-UTR sequences.

RESULTS

The retrieval results from the GenBank

Using human ALR cDNA (AF124604, human HPO2 mRNA, complete coding sequence) sequence as a reference, and BLAST path as a search tool, homologous DNA sequence was searched on GenBank. It was found that 5 cDNA and DNA fragments were homologous to human ALR cDNA sequence, including mouse ALR genomic DNA, rat ALR cDNA, human HPO1 cDNA partial sequence, human ERV1 cDNA and DNA sequence of human genomic DNA P1 clone derived from human chromosome 16 (Table 1).

Table 1 Homology sequences of human ALR cDNA searched from GenBank

GenBank No.	Name	Character
U31176	Human ERV1 mRNA	Complete coding sequence
AF124604	Human HPO2 mRNA	Complete coding sequence
AC005606	Human genomic DNA seucle	Chromosome 16
AF124603	Human HPO1 mRNA	Partial coding sequence
U40494	Mouse ALR genomic DNA	Complete coding sequence
D30735	Rat ALR mRNA	Complete coding sequence

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From Table 1, it is clear that human ALR cDNA has a high homology to P1 clone 109.8C (LANL) which is 16 (GenBank No. AC005606). Further analysis of human HPO2 cDNA complete sequence and HPO1 cDNA partial sequence showed that human ALR genomic DNA was between 44742-46554nt of P1 clone 109-8C (LANL). Human ALR genomic DNA consisted of 1813bp (Figure 1).

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1  cgacctggag accgacgoge ggggocpssg cggggggcgg aghahgcagg cggcctggg
61  etcgagccca gcccaagggc cgaactccga ttctctgttc ggcgagggag cctccggag
121  gggggcgtgc cgggactcgg tgaattccaa gacgtggatg cgggcgcagc agsaggcgca
181  gttctcctgc cpatittctc cagcccccgg cggcccctgt ccccgcoccc gcccaaggtac
241  cccgacagag ctteccaggg ttgctgtgoc etgnaecttg cccccgggt aggcocagoc
301  ttacagcctt catccpccgg tgggttgagat cgtctgcagg actttggcgg gattccagtg
361  gggccnccggc tgggcctgac agtggggcagc tttgggggoc tttgttcgga gnatgacac
421  actctcagtc ggcctccttc cgcagcggga caaccagttt agggagggact gccocgocgga
481  tgcgagggaa ctgggcccgc acagctgggac tgtctctcac accctggccg cactataccc
541  cgcactgcgc accccagcac agcagcaaga catggccagc ttatcacatt tattttctaa
601  gttttaccoc tgtgagagat gttcctgauga cctasgasa agttasatg tgtttgcag
661  cagcagagct ttgcactgga gccctgggact gggactctcg gctgaactta tagcggggaa
721  cgtgagagaa cggatcgaga ggtggcagaa gtttctctag gacgagggac ctocancgg
781  tggagactgg ggtatctga gccctctctc ctgctctcag agcccaagct gtcgggntct
841  gctgtggggt actgctctc cccagccac agggcttcca ggnagggatt ccttgccttg
901  tctgagcctg aaggcgttt cccgcnagtt agggagact cccctttgoc tgcctctag
961  agttaggnet ctggttttca sactttgga atocactgt tttgtccca agagccactc
1021  ctctctactc gaggatgcca agagccacca ctggcccctg cagctgttcc taggcagant
1081  gtatagggta gggccgcat tgtttctata aggettgcca gttgnaatga agtctcttgc
1141  tggactggcc cscacgggoc tgcctcagaa tgcctaugaa caagtccagt ttctcgctac
1201  aaggctgtgc ccagcccacc ccaggccaca ctctctcaga ggcacgggac actgagatgc
1261  gcttctcagg tgttagggag ttataaant gccaggtctg tgcctgastg tgttgccttg
1321  aggctcctgt ggtgtttgac auctgtcca ggtggctggt tgcagcgcoc tggagctcca
1381  tcaacccagg ggaactgacg gctcagcctt gttctgggag tgcctgtacc ttggagcata
1441  agggactcgc caggtgtagt tcaagcagat gccccagctc tcttctctg acscagaca
1501  gggactagtc agggagcagtg gggccctgic gtcctctcat tctttaccg ctctccctac
1561  acaggtctgt caggaaacc cagcagccc gcccccgggc atgtcttaca cagtggctgt
1621  gccactcaca caatgagctg aaccgcaagc tggccagacc tgactctgac tgcctcagag
1681  tggatgagcg ctggcgcgac ggtcggagag atgctctcgt tgactagagg gttgctagcc
1741  agactcctag ggcagcctag ccaggatagg ttggataggg gcaggagcact cattttagtg
1801  catcagacc aga
    
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Figure 1 Genomic DNA sequence of human ALR.

Structural analysis of human ALR genomic DNA sequence

Human genomic DNA consists of introns and exons. According to the Breathnath-Chambon rule of intron-exon junction structure, in conjunction with the coding sequence of human ALR cDNA, we found that human ALR genomic DNA has 3 exons and 2 introns. The 3 exons were located between 158nt-175nt, 446nt-642nt and 1565nt-1727nt of P1 clone 109-8C (LANL) of human chromosome 16, respectively.

Comparison of human and mouse ALR genomic DNA structures

To compare human and mouse ALR genomic DNA sequences, we found that the 3 exons were similar in length, but different in their 5'-UTR, introns and 3'-UTR regions in length. The 3 exons for both human and mouse ALR were 18nt, 197nt and 163nt, respectively. The comparative results are shown in Table 2.

Table 2 Comparison between human and mouse genomic DNA structure

	Human	Mouse
5'-UTR 1	57 nt	252 nt
Exon 1	18 nt	18 nt
Intron 1	270 nt	398 nt
Exon 2	197 nt	197 nt
Intron 2	922 nt	483 nt
Exon 3	163 nt	163 nt
3'-UTR	86 nt	535 nt

Chromosomal location of human ALR genomic DNA

The human ALR genomic DNA was homologous to a genomic DNA fragment derived from P1 clone 109-8C (LANL) of human chromosome 16p13.3, so human ALR genomic DNA should be assigned to human chromosome 16p13.3.

DISCUSSION

Augmenter of liver regeneration (ALR) plays a very important role in the regulation of liver regeneration. The expression sites were mainly located in platelets and sperm cells of testes. But the mechanism of triggering the expression, transportation and secretion of ALR from platelets and testes remained unknown. It is not clear if the secreted ALR function as a liver tropic factor via specific receptor on the hepatocyte membrane. Molecular cloning of human cDNA has been completed, but the transcription and post transcriptional regulation based genomic structure of ALR is still unclear. So it is very important to know the structure of human ALR genomic DNA. The regulation of human gene expression occurred at multiple levels, but there is no doubt that the transcription and post-transcriptional regulation is among the most important steps of their expressive regulations. In this study, we conducted DNA sequence homology search on the World Wide Web (WWW) in an attempt to find the homologous DNA sequence to human ALR cDNA in GenBank using BLAST as a tool, and found that human ALR genomic DNA consisted of 1813nt (GenBank accession number: AF146394). According to the Breathnath-Chambon rule and ALR cDNA coding sequence, we defined 3 exons and 2 introns in the genomic DNA sequence. Human ALR gene was also highly conserved, indicating that ALR plays a very important role in the whole evolution process.

Human genome project (HGP) has been planned to complete before the year of 2005. But in recent years, along with more scientists involved in this project and large investment into this project, there is strong evidence to predict that this HGP will be finished soon. The conduction of HGP will result in a big database of human genomic DNA

nucleotide sequence, and will define the final restriction map for human whole genome. The GenBank is a good and important information resource for both analysis and functional DNA cloning.

Wells *et al* used conserved motif sequence of chemokine as a reference, searched on the GenBank and obtained a gene coding for a new chemokine. This is the first example to clone a new gene only from GenBank database homology DNA sequence search^[10]. In the research of apoptosis, the CED-3 gene in *C. elegans* was demonstrated as a dead gene. Miura *et al* used this sequence as a reference to search homology DNA sequence to CED-3 gene in GenBank and found that interleukin-1 beta converting enzyme (ICE) is homologous gene to CED-3 gene in *C. elegans*. Later studies demonstrated that the gene transduction of ICE expressive vector could induce apoptosis in the NIH 3T3 murine fibroblast cell line^[11]. As a result, the gene homologous analysis is a good means to define the new functional gene. We also used the principle of gene homology and cloned a parasite surface protein amastin coding DNA for *Leishmania major* parasites^[12]. So the GenBank is not only a accumulated data bank of cloned nucleotide sequence, but a good channel to define new gene and new functional gene. After the HGP was completed, the post-HGP works will need the GenBank to identify new genes, and this will be a good alternative for the molecular biological studies.

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A preliminary study on ras protein expression in human esophageal cancer and precancerous lesions

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Subject headings esophageal neoplasms; ras protein; oncogene; gene expression; precancerous condition

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INTRODUCTION

The esophageal carcinoma is a common malignant tumor in Linzhou City (Linxian) of Henan Province in northern China. Although the etiology and natural history of esophageal carcinoma are not clear, a substantial amount of evidence has been provided to suggest that the development of human esophageal squamous cell carcinomas (SCC) is a multistage progressive process^[1-4]. An early indicator of abnormality in persons predisposed to esophageal SCC is an increased proliferation of esophageal epithelial cells, morphologically manifested as basal cell hyperplasia (BCH), and dysplasia (DYS), and carcinoma *in situ*, which could be considered precancerous lesions of esophageal SCC^[1-4]. Current molecular biology has suggested that many genes could participate in the different stages in esophageal carcinogenesis, and the synthetic effect of these different molecules might result in the malignant transformation of human esophageal mucosa^[5,6], e.g., the amplification and/or overexpression of certain gene such as *hst-1*, *int-2* and *c-myc*, have been observed in esophageal tumor s, moreover, the alteration of tumor suppressor gene *p53* is one of the important molecular changes in the early stage of esophageal carcinogenesis^[2-6].

Recent evidence has suggested that oncogene *ras* may play a role in cell growth and differentiation^[7,8]. The *p21* protein encoded by the *ras* gene family functions as G protein that

participates in membrane signal transduction pathways^[7]. Some sites of point mutation of *ras* oncogene, especially codons 12, 13, and 61, could be involved in malignant transformation cells^[7]. Several reports have suggested a close association between specific *ras* gene mutation patterns and suspected etiological factors in some human cancers, for example, N-*ras* mutations have been associated with melanomas induced by sunlight^[7]. *ras* gene mutations have been found in a significant percentage of human tumors, e.g. 75%-93% in pancreas adenocarcinoma, 7%-44% in colon cancer^[7]. No *ras* point mutations have been found in DNA extracted from primary human esophageal tumors in France and South Africa, this is in sharp contrast to some reports in which rat esophageal papillomas were induced by *methylbenzyl-nitrosamine*^[8]. This prompted us to re-evaluate the role of the *ras* family of oncogene in human esophageal carcinogenesis. In the present study, we analyzed the expression of *ras* protein in cancerous and precancerous tissues of the esophagus collected from the subjects in Linzhou, a high incidence area of esophageal carcinoma in China.

MATERIALS AND METHODS

Tissue collection and processing

Esophageal biopsies were taken from 54 symptom-free patients who volunteered to participate in a routine endoscopic screening for esophageal carcinoma in Linzhou City, China. Of the 54 subjects examined, there were 29 males (30-72 years of age, with a mean±SD of 47±15 years) and 25 females (32-70 years of age, with a mean±SD of 44±17 years). The surgically resected specimens were obtained from patients (36 patients, aged 40 to 74 years, with a mean±SD of 55±10 years) with primary esophageal carcinoma in Linzhou City, China. The patients had received no radiation therapy or chemotherapy before surgery. All of the tissues were fixed in 80% alcohol and embedded in paraffin. The section was 5 μm in thickness. Three or four adjacent ribbons were collected for histopathological analysis (hematoxylin and eosin stain), and for immunohistochemical staining.

Histopathological analysis

Histopathological diagnoses for esophageal epithelia were made according to the cellular morphologic

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changes and tissue architecture using previously established criteria^[1-4]. In brief, the normal esophageal epithelium contained one to three proliferating basal cell layers; the papillae were confined to the lower half of the epithelium. In BCH, the number of proliferating basal cells was increased to more than three cell layers; DYS was characterized by nuclear atypia (enlargement, pleomorphism, and hyperchromasia), loss of normal cell polarity, and abnormal tissue maturation. SCC was characterized by confluent and invasive sheets of cohesive, polymorphous cells with hyperchromatic nuclei.

Antibodies and reagents

The monoclonal antibodies (Pan-ras) Ab-3 (Oncogene Science Inc, USA) were mouse antibodies against human ras protein. Twenty mL/L crystalline bovine serum albumin (BSA), (Sigma Chemical Inc, USA) and ABC and DAB kits (Vector Laboratories Inc. USA) were used for immunohistochemical assay.

Immunohistochemical staining

The avidin-biotin-peroxidase complex (ABC) method was used for ras protein. Briefly, after dewaxing, inactivating endogeneous peroxidase activity, and blocking cross-reactivity with normal serum, the sections were incubated overnight at 4 °C with a diluted solution of the primary antibodies (1:100 for ras). Locations of the primary antibodies were achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin-complex conjugated to horseradish peroxidase, and diaminobenzidine. Normal serum blocking and omission of the primary antibody were used as negative controls.

The positive reaction was graded according to the number of brown-yellow particles appearing in cytoplasm. The diagnostic criteria for immunoreaction was based on previous reports^[2-4].

RESULTS

Histopathological analysis

Histopathologically, among 54 esophageal biopsies, 12 had normal esophageal epithelium (22%), 34 had BCH (63%), and 8 had DYS (15%). All of 36 surgically resected specimens were found to have esophageal invasive SCC.

Immunohistochemical analysis

By immunohistochemical analysis, immunostaining of ras protein was predominantly observed in cytoplasm. The positive immunostaining of ras protein was not found in the normal tissues and the tissues with different severity of lesions from esophageal precancerous biopsies. The

immunoreactivity of ras protein was observed in 9 (25%) of the 36 surgically resected esophageal cancer specimens (Table 1).

Table 1 Expression of ras protein in different severity of lesions from esophageal biopsies and carcinomas

Histological types	n	ras positive immunoreaction n(%)
Normal epithelium	12	0 (0)
Basal cell hyperplasia	34	0 (0)
Dysplasia	8	0 (0)
Esophageal carcinoma	36	9 (25)
Total	90	9 (10)

DISCUSSION

The unique observation in this study is that, the expression of ras protein was not observed in the normal esophageal epithelial and precancerous tissues, and the immunoreactivity of ras protein was observed in 9 (25%) of the 36 surgically resected esophageal cancer specimens. These results suggested that the expression of ras protein only occurs in the late stage of esophageal cancers, but not in early stage, and it might not be the important molecular change in the carcinogenesis of esophageal squamous epithelium.

The mutation of ras family of genes occurs extensively in human tumors, particularly tumors of the gastrointestinal tract and lung, the incidence being about 40% in colon cancers. Many researchers agree with the concept that the mutation of oncogene ras plays an important role in carcinogenesis of colon cancers^[7,8]. However, the conclusions of oncogene ras in esophageal carcinogenesis were not consistent. Hollstein *et al*^[9] found that tumor suppressor gene p53 had G-T transversion of mutation pattern in lesions of esophageal cancer in the areas where the tumor is closely associated with tobacco consumption, but the mutation of oncogene ras was not found in the same areas. No ras mutation was found with PCR technique in other areas of the world with high incidences of esophageal SCC, such as China^[10] or South Africa^[11], where N-nitrosamines and fungal toxins could play major roles. Experimentally, activating point mutation at codon 12 of H-ras was reported in rat esophageal tumor (papillomas) induced by the carcinogen methylbenzyl-nitrosamine^[12]. This was a typical example that the results of human molecular research was obviously unsimilar to animal studies. On the other hand, two recent studies reported that genomic amplification rates of K- and H-ras gene were up to 14% (7/51) and 40% (4/10) of esophageal cancer, respectively from France^[13] and Canada^[14], and one

immunohistochemical study of esophageal SCC from Italy and America showed that the positive staining of the ras protein was 88.5% (46/52)^[15]. Our results in the present study suggested that in the normal tissues and the tissues with different severity of lesions from esophageal biopsies, the expression of ras protein was not detected. Ras protein immunoreactivity was observed in 9 of the 36 surgically resected esophageal cancer specimens, accounting for 25%. The different results from the world suggested that the environmental factor might play an important role in esophageal carcinogenesis. Obviously, comparison with the molecular changes in the similar tumors in populations from different areas, is of great importance to reveal its carcinogenesis and further to understand its related etiology.

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Expression of VEGF₁₂₁ in gastric carcinoma MGC803 cell line

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Subject headings stomach neoplasms; vascular endothelial growth factor; monoclonal antibody; MGC803 cell lines

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INTRODUCTION

Vascular endothelial growth factor (VEGF) which is also known as vascular permeability factor (VPF) is a heparin-binding, dimeric polypeptide growth factor and a potent mitogen for endothelial cells. VEGF can stimulate the endothelial cell growth and enhance the motility through its two known receptors flt-1 and KDR^[1]. Acting through these receptors, VEGF may stimulate angiogenesis and promote tumor progression. VEGF₁₂₁, as one of the four VEGF protein isoforms containing the least number of amino acids, has all the biological function of VEGF and is the ideal isoforms for further studying VEGF at molecular levels^[2]. In this study, we cloned VEGF₁₂₁ cDNA from gastric carcinoma cell MGC803 and had it expressed in *E.coli* bacterial. Further identification of its expression products was carried out with anti-VEGF monoclonal antibodies. This gives new evidence to clarify the source of VEGF in tumor tissues.

MATERIALS AND METHODS

Materials

Balb/c mice, female, 6-8 weeks old, was purchased from the Animal Center of the Chinese Academy of Medical Sciences. HUVEC was obtained from the Academy of Preventive Medical Sciences and cultured in RPMI1640 with 15% fetal cow serum (full growth medium) at 37°C with 5% CO₂. MGC803 cell line was established from a

primary poorly differentiated mucoid adenocarcinoma of human stomach. Recombinant human VEGF₁₂₁ was obtained from the Department of Immunology, Beijing Medical University. ³H-thymidine was purchased from Shanghai Institute of Atomic Energy.

Preparation of VEGF₁₂₁ monoclonal antibodies

Balb/c mice were immunized subcutaneously with the purified fusion protein GST-VEGF₁₂₁ and hybridoma cell clones were obtained by traditional hybridoma technology. ELISA was used to screen hybridoma clones with recombinant fusion protein GST-VEGF and GST-P21 as antigen. The clones which reacted with GST-VEGF, but not with GST-P21, were subcloned. After three rounds of subcloning by limited dilution, VEGF₁₂₁, which had proliferation activity on HUVEC, was used as antigen to select the positive clones. The antibodies were purified through protein A-Sepharose CL-4B chromatography and their subclasses were measured.

Inhibition of the monoclonal antibody on HUVEC proliferation induced by VEGF₁₂₁

Assay of ³H-thymidine incorporation was used on HUVEC for neutralizing the activity of anti-VEGF₁₂₁ monoclonal antibody. HUVEC was seeded at a density of 2×10⁴ per well of 24 well plates and incubated with full growth medium for 48 hrs at 37°C. The cells were then incubated with serum free medium for 24 hrs, and the testing groups were added with VEGF₁₂₁ (10 µg/L) and anti-VEGF₁₂₁ monoclonal antibody at various concentrations. After 30 hrs culture, ³H-thymidine (37 KBq/mL) was added, and after 6 hrs, the cells were collected and measured in a liquid scintillation counter.

RT-PCR of VEGF₁₂₁ from MGC803 cells and HUVECs

Total RNA of both cell lines was extracted respectively by TRISOLV™ isolation of RNA kit (GIBCO BRL). First-strand cDNA was synthesized using the Superscript™-II Preamplification System for First Strand cDNA Synthesis Kit (GIBCO BRL) with 5 µg total RNA in a 20 µL reaction volume. Two µL cDNA was used as template in a 100 µL-PCR reaction volume. The primer for VEGF reverse transcription was oligodT. The cDNA

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encoding VEGF was amplified using forward primer (5'-GGG GGA TCC GCC TCC GAA ACC ATG AAC TT-3' containing Bam-HI restriction endonuclease site) and reverse primer (5'-CCC GAA TTC TCC TGG TGA GAG ATC TGG TT-3', containing Eco-RI restriction endonuclease site), and PCR was carried out in a DNA Engine™- Peltier Thermal Cycler Model PTC-200 (MT Research Inc, USA) at 95 °C for 5 min first, then at 94 °C for 45sec, 55 °C for 40sec, and 72 °C for 1min for 36 cycles, followed by 5 min at 72 °C.

Recombinant of VEGF on plasmid PGEX-2T vector

The PCR products were purified with DNA purified kit (QIAEGN), digested with Bam-HI/Eco-RI and ligated with fusion protein prokaryotic expression plasmid PGEX-2T. E. coli-XL-1 blue was transformed and the positive clones were selected by restriction endonuclease mapping.

Expression and identification of GST-VEGF₁₂₁ protein

Positive clones were selected and cultured with LB until the OD₆₀₀ reached to 1.0, then the 5 mmol/L IPTG was added for 4 h culture to induce the protein expression. The expression products were analysed by SDS-PAGE, and Western blot was carried out for further identification of the products.

RESULTS

Preparation and identification of anti-VEGF monoclonal antibodies

After three rounds of subcloning and selecting by ELISA with recombinant fusion protein GST-VEGF₁₂₁ and GST-P21, six clones stably secreting anti-VEGF₁₂₁ antibodies were obtained. Screened with purified VEGF₁₂₁, clone 5C₅, which showed more specific binding activity to VEGF, was selected for preparing ascites. The ascites were purified through chromatography with ProteinA- Sepharose CL-4B column. Its subclass is IgG2b.

Neutralization activity of anti-VEGF₁₂₁ monoclonal antibody

We measured the effects of anti-VEGF antibody 5C₅ at various concentrations on HUVEC proliferation induced with VEGF at a concentration of 2 µg/L. The results from analysis of ³H-thymidine incorporation showed that the VEGF antibody 5C₅ neutralized the activity of VEGF in a dose-dependent manner and blocked the VEGF-induced cell growth completely at a concentration of 10 mg/L (Figure 1).

Amplification of VEGF₁₂₁ cDNA

The VEGF₁₂₁- cDNA was amplified with its specific primers from MGC803 cells. Following 0.8% agarose gel analysis, a clear band about 550bp, which matched the predicated size, was generated (Figure 2, lane 2). No specific band was obtained from HUVEC (Figure 2, lane 3).

Recombinant of VEGF₁₂₁ and its expression

The recombinant PGEX2T-VEGF₁₂₁ digested with Eco-RI/Bam-HI released a fragment about 550 bp (Figure 2, lane 4). After induced by IPTG, the positive transformed E. coli XL-1 blue can stably express fusion protein GST-VEGF at the molecular weight about 40 KD. The proportion of expressed VEGF₁₂₁ to total bacterial protein was about 25% and it existed in the inclusion body (Figure 3).

Western blot analysis

Induced by IPTG, further identification of the expressed product was carried out by Western blot analysis. The results showed that 5C₅ can be specifically reacted with denatured GST-VEGF₁₂₁ (Figure 4).

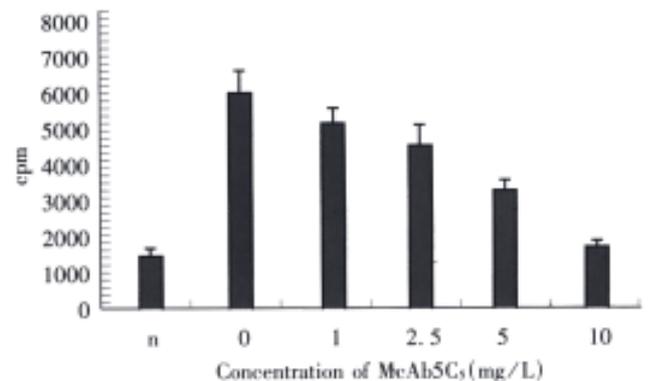


Figure 1 Neutralization of 5C₅ to the VEGF-induced HUVEC growth.

n: no VEGF or antibody added, 0.10: VEGF 2mg/L and antibody 5C₅ in various concentration were added.

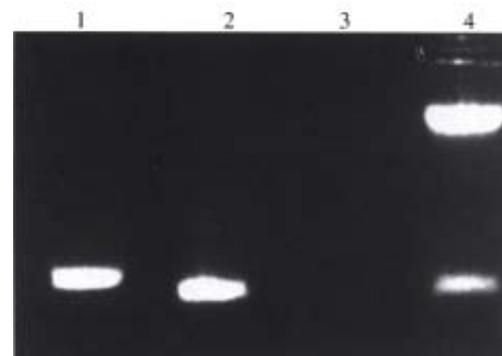


Figure 2 RT-PCR of VEGF₁₂₁ cDNA and identification of recombinant PGEX2T-VEGF.

1. 585/985bp DNA marker, 2. PCR product of VEGF from MGC803 cells, 3. PCR product of VEGF from HUVEC, 4. PGEX2T-VEGF digested with Bam-HI/Eco-RI.

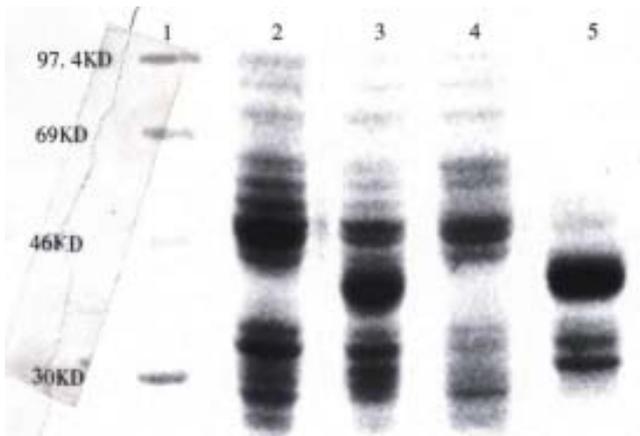


Figure 3 SDS-PAGE analysis of GST-VEGF expressed in *E. coli* XL-1 blue-1

Standards of protein molecular weight, 2. Total proteins from bacterial transformed with PGEX2T-VEGF₁₂₁ without induced IPTG, 3. Proteins from bacterial induced by IPTG, 4. Protein pellet of bacterial lysis without induced IPTG, 5. Protein pellet of bacterial lysis induced by IPTG.

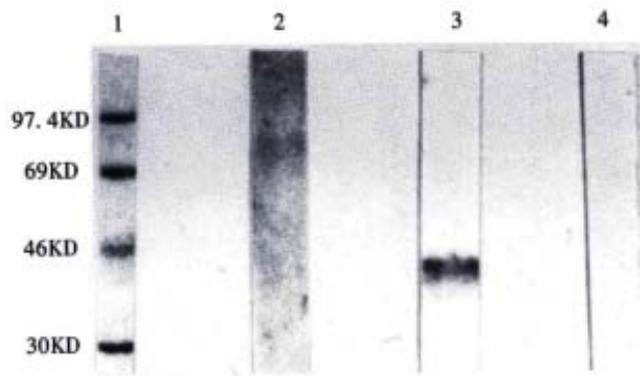


Figure 4 Western blot analysis of the bacterial expressed GST-VEGF₁₂₁ by 5C₅.

1. Standards of protein molecular weight, 2. Uninduced bacterial protein treated with 5C₅, 3. Induced bacterial protein treated with 5C₅, 4. Induced bacterial protein treated with normal mouse IgG.

DISCUSSION

Inhibition of tumor blood vessel growth is an important research area for tumor biotherapy in recent years. The process of angiogenesis involves stimulation of endothelial cell growth, motility and the release of proteases and the degradation of extracellular matrix. Blocking the overexpression of VEGF in tumor tissues and neutralizing its activities by monoclonal antibodies cast much light on VEGF related tumor therapy^[3]. In this study, by using

anti-VEGF monoclonal antibodies, we successfully neutralized the VEGF-induced HUVEC growth. These also clearly made the specificity of the prepared monoclonal antibodies.

There are different opinions on which kind of cells in tumor tissues can express VEGF. Wizigmann *et al*^[4] proved that VEGF is mainly expressed by tumor cells. It can bind to its receptors on HUVEC and stimulate cell growth by paracrine ways. However, Plate Hetal^[5] discovered that VEGF can be expressed by HUVEC in tumor tissues. Brown^[6] said that the HUVECs both in the tumor tissue and the normal tissue can express VEGF. In our study, we demonstrated, by RT-PCR, the expression of VEGF in gastric carcinoma MGC803 cells. We also found, by ³H-thymidine incorporation that the supernate of MGC803 cell s can promote the proliferation of HUVEC (data not shown). These suggested that MGC803 can express VEGF, but we failed to amplify VEGF cDNA from HUVEC.

To make clear about which kind of cells in tumor tissue can express VEGF is important for the blocking of its expression at genetic levels. We are preparing human anti-VEGF monoclonal antibodies by the phage display method to obtain the useful anti-VEGF antibodies for further research and its clinical application.

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A clinical and long-term follow-up study of perioperative sequential triple therapy for gastric cancer

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Subject headings stomach neoplasms/surgery; stomach neoplasms/drug therapy; intra-arterial chemotherapy; intra-peritoneal chemotherapy; curative resection; survival rate

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INTRODUCTION

Although the long-term postoperative survival rate of gastric cancer (GC) patients has been improved significantly since the local dissection of lymph node was widely used in China, yet the low curative resection rate and the high recurrence rate from peritoneal and hepatic metastases hinder it from further improvement. To alter the current unsatisfactory status of GC treatment, a sequential triple therapeutic scheme (STTS), consisting of pre-operative regional intra-arterial chemotherapy, curative resection of GC, and intra-operative or early postoperative intraperitoneal chemotherapy, was designed and adopted in this department since 1989. The follow-up data demonstrated that the therapeutic response of STTS is rather satisfactory. The results are reported as follows.

MATERIAL AND METHODS

General data

From February 1989 to October 1997, a total of 211 patients with GC were treated in this department, among them 167 were treated by surgical resection of GC, and follow-up data were obtained in 134 cases, with a rate of 80.24%, and a follow-up period of 5.144 months, averaging 34.6 months. Among the followed up patients, 112 had curative resection and 22 had palliative resection.

Sequential triple therapeutic scheme (STTS)

One or two times of selective intra-arterial chemotherapy with FAP or FMP scheme was done

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by DSA, and then surgical operation was performed in 2 weeks. The surgical operation was performed as follows: D1 resection for TNM staging I, D2+ or D3 resection (including combined organ resection) for TNM staging Ib to IIIb, D4 resection for only a few cases, palliative resection for TNM staging IV patients. Hyperthermic intraperitoneal chemotherapy was performed immediately after operation, MMC 40mg+ CDDP 200mg were dissolved in 4000mL NS, lasting 15 minutes at 42°C-45°C, the abdominal wall was sutured after the chemotherapy was completed. Among the 134 followed up cases, 81 received STTS treatment and 53 underwent only simple resection. Based on the new TNM staging system, the staging of two groups was essentially similar and comparable (Wilcoxon test, $P>0.05$).

Table 1 The TNM staging of STTS group and simple operation group

Stage	STTS group (n)	%	Simple operation group (n)	%
I	Ia	2	2	3.8
	Ib	6	4	7.5
II		15	11	20.7
III	IIIa	21	12	22.6
	IIIb	25	15	28.3
IV		12	9	17.0
Total	81		53	

Statistical analysis

Based on the data from the case with simple operation as a control, the treatment response of STTS was evaluated, the comparison between the two groups was analysed by Chi-square test, the cumulative survival rate was calculated by the life-table method. P value of less than 0.05 was considered as significant.

RESULTS

Effect of preoperative regional intra-arterial chemotherapy on the curative resection rate

Among the 211 patients, 101 were treated by preoperative regional intra-arterial chemotherapy, of whom 80 (79.2%) had curative resection. But among 110 cases treated by simple operation, only 68 (61.8%) had curative resection, hence the curative resection rate was significantly different between the two groups ($P<0.01$).

Comparison of survival rate between STTS and simple operation group

Among the 134 followed up cases, 43 died, the survival rates of 1, 3, 5 and 7 years were 87%, 64.6%, 60.9% and 55.2%, respectively. Statistical data demonstrated significant difference of survival rate between STTS and simple operative group except the 3-year survival rate. Among the 112 cases with curative resection, 71 cases received STTS treatment, and 41 received simple operation, the survival rate of the two groups was significantly different except for the 3 and 7-year survival rate (Table 2).

Table 2 Comparison of survival rate and curative survival rate between STTS group and simple operation group

Group	Survival rate (%)					Curative survival rate (%)				
	n	1	3	5	7 (yrs)	n	1	3	5	7 (yrs)
STTS	81	92.9 ^c	68.1 ^a	68.1 ¹	62.5 ^c	71	98.0 ^c	79.2 ^a	79.2 ^c	72.6 ^c
Simple operation	53	79.7	55.5	49.1	45.6	41	90.0	72.3	64.3	60.6

^a $P>0.05$, vs simple operation, ^c $P<0.05$, vs simple operation.

Effect of STTS on postoperative metastasis and recurrence rate

Of all 134 patients, 43 died from cancer metastasis and recurrence during follow-up period, 25 had peritoneal metastasis and 10 had hepatic metastasis. Statistical data showed that there was significant difference of the peritoneal and hepatic metastasis rate between STTS group and simple operation group (Table 3).

Table 3 Comparison of main recurrence and metastasis rate between STTS group and simple operation group

Recurrence (n)	Group (n)	n	Rate (%)	P value
Peritoneal (n=25)	STTS (n=81)	7	8.6 (7/81)	$\chi^2=13.53$
	Simple operation (n=53)	18	33.9 (18/51)	$P<0.005$
Hepatic (n=10)	STTS (n=81)	2	2.4 (2/81)	$\chi^2=7.39$
	Simple operation (n=41)	8	15.1 (8/53)	$P<0.01$

Relationship between free cancer cells in peritoneal cavity and TNM staging and intra-peritoneal chemotherapy

Of all 134 cases, peritoneal lavage was examined in 45 cases to detect the intra-peritoneal free cancer cells. It was positive in 11 (24.4%) cases and, 1, 1, 2, 3 and 4 cases were found at stage of I, II, IIIa and IV, respectively. In 5 followed up patients, 2 without intra-peritoneal chemotherapy died of peritoneal metastasis in 1 year and 2 years and 3 months, while in the 3 patients with intra-peritoneal chemotherapy, 1 died 5 years later and the remaining 2 were alive 5 and 8 years later respectively. Sixteen of the cases with negative free

cancer cells, were followed up, only 1 of them died from peritoneal metastasis 2 years after the operation.

Relationship between STTS and survival rate of lymph node positive cases

Among 112 cases with curative resection, lymph node metastasis was found in 66 cases, 47 of which were treated by STTS and 19 cases by simple operation. Statistical analysis showed that the survival rate of 1, 3, 5 and 7 years in STTS group were all higher than those in simple operation group, but they were not significant in log rank test.

DISCUSSION

Selection of the appropriate time for chemotherapy

The post-operative recurrence of GC arises mostly from the remnants of micro-cancerous foci. In the past, chemotherapy was done mostly post-operatively, when the vascular and lymphatic channels of the primary tumor had been cut and ligated in operation, and the cancer cells were embedded in the adhesion one week after operation. The remnant micro-cancerous foci cannot be exposed to chemotherapeutic drugs or the concentration of the drugs is insufficient, so the chemotherapy is rarely effective. Besides, during operation, free cancer cells are promoted to exfoliate and metastasis. The pre-operative chemotherapy can reduce the viability of cancer cells, reduce the tumor size, and decrease the chance of iatrogenic dissemination and hepatic metastasis so as to increase the opportunity for curative resection. Averbach *et al*^[1] and Ajani *et al*^[2] suggested that pre-operative, intra-operative and early post-operative period are the best time for chemotherapy.

Among the 211 cases, curative response of pre-operative regional intra-arterial chemotherapy was 79.2%, the 5-year survival rate of STTS group being 79.2%, but 61.8% and 64.3% respectively in the simple operation group ($P<0.05$). The results indicated that pre-operative regional intra-arterial chemotherapy, intra-operative and early post-operative intra-peritoneal chemotherapy can increase significantly the curative rate and the 5-year survival rate.

Indications for intra-operative and early post-operative intra-peritoneal chemotherapy

Peritoneal metastasis is the main cause of recurrence in GC, its rate was about 50%-60% in the literature^[3], and was 58% in this paper. The 3-year survival rate of cases with free cancer cells in peritoneal cavity was only 15.6%^[4]. Bonen Kamp reported that the median survival period was only

1.1 years^[5]. Recently, the positive rate of free cancer cells was reported to be 22.2%-32.5% in China^[6,7], and was 24.4% in this paper. According to Suzuki, free cancer cells were found in peritoneal lavage in 24% of stage Ib and 40% of stage II-III patients. False positive rate was less than 5%^[8]. The difference between Chinese and international reports may be due to the following factors: ① the results of examination are not exactly correct; ② the examination of lavage is usually performed after opening the abdomen before the operation, free cancer cells may turn from 'negative' to 'positive' at the end of the operation. In our series, one patient with negative free cancer cells and without intra-peritoneal chemotherapy died from peritoneal metastasis post-operatively. Among those with positive free cancer cells and without intra-peritoneal chemotherapy, 2 were of stage I and II respectively, and 3 survived more than 5 and 8 years. As a result, the stages more than Ib in GC patients are indications for intra-peritoneal chemotherapy.

About curative resection

Curative resection is the most important means to determine whether the GC patient is cured or not. The following standards should be achieved: ① No tumor invasion of the cut ends; ② the number of stations of regional lymph nodes dissected should exceed that of positive lymph nodes (D>R); ③ no remnant cancer tissue in adjacent organs and tissues. The results should be confirmed by pathological examination, but this is often too late. In other words, even the operation has achieved the requirements of curative resection, if there are some risk factors, such as sub-clinic cancer foci, invisible free cancer cells in peritoneal cavity, indistinct potential hepatic metastasis, etc., the so-called curative resection need pre-operative, intra-operative or early post-operative chemotherapy to compose a composite therapeutic approach so as to attain the real curative resection.

About lymph node metastasis

It is well-known that the prognosis of GC patients is

closely related to the lymph node metastasis. It is suggested in this paper that there was no significant difference in long-term survival rate between STTS lymph node positive group and simple operation group. This may be related to the insufficient number of cases accumulated, or due to the fact that the lymph node metastasis is an independent prognostic factor, and it depends mainly on the thoroughness of the dissection. It is impossible for various chemotherapeutic approaches to replace the curative resection at all.

CONCLUSION

We suggest that the STTS is a rational therapeutic scheme for GC. Follow-up results have demonstrated that STTS treatment can significantly increase the curative resection rate and the 5-year survival rate and decrease the post-operative recurrence rate as well. But this is still a preliminary report of the feasibility of STTS, more cases should be accumulated for further studies.

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Tumoricidal activation of murine resident peritoneal macrophages on pancreatic carcinoma by interleukin-2 and monoclonal antibodies

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INTRODUCTION

Macrophages play an important role in tumor lysis and growth inhibition. They can be activated to a tumoricidal state by a variety of agents such as IFN γ , TNF α or IL2. The killing mechanisms of activated macrophages have been extensively investigated^[1,2]. Recently, it has been proved that antibody dependent cellular cytotoxicity (ADCC) is one of the potent arms to lyse tumor cells resistant to cytotoxic macrophages, and that the antitumorous effect of a macrophage activator is significantly augmented by the combined use of mAbs capable of inducing ADCC to tumor cells^[3].

The present study was undertaken to investigate these possibilities using Capan-2 human pancreatic carcinoma cell line. IL2 and YPC3 mAb inducing ADCC of murine splenic lymphocytes served as activators. The anti-pancreatic carcinoma efficacy of activated macrophages derived from murine peritoneal effusion cells (PEC) was observed *in vitro*.

MATERIALS AND METHODS

Media and reagents

The medium used for all experiments and cell maintenance was RPMI 1640 (Gibco, USA). ABC kit and IL2 were purchased from Vactastain and

Ellite Co. Ltd, USA respectively. Na₂⁵¹CrO₄ was supplied by the Isotope Institute of Chinese Academy of Medical Sciences.

Animals and cell lines

Capan-2 cell line was obtained from the University of California, USA, and Balb/c mice from the Experimental Animal Center of Sun Yat-Sen University of Medical Sciences.

Monoclonal antibodies

YPC3 mAb was extracted from acites pretreated with YPC3 hybridoma cells in Balb/c mice and purified as described previously^[4,5]. 1-F/7 mAb against Dengue fever virus was provided by the Department of Microbiology of Sun Yat-Sen University of Medical Sciences. Both mAbs were dissolved in pH 7.4, 0.01M PBS (0.5g/L). Immunoreactivity was assessed by enzyme-linked immunosorbent assay (ELISA) on Capan-2 cells and avidin-biotin-peroxidase complex (ABC) immunohistochemistry of Capan-2 xenograft samples according to the methods described previously.

Effector cells (E)

One mL of incomplete RPMI 1640 medium was injected into the peritoneal cavity of Balb/c mice for 3 days in order to increase the number of PEC in mice. PEC was harvested by lavage of the peritoneal cavity of mice, washed, centrifuged twice and suspended in RPMI 1640 medium with 15% FBS at a concentration of 3-5 \times 10⁶/mL. PEC was further fractionated into non-adherent and adherent cells by 2h incubation in glass dish. Non-adherent cells were removed by washing three times with warm medium. Adherent cells (more than 85% of macrophages) were resuspended to a final density of 10⁶ cells/mL. PHA and IL2 (1000 U/mL) were added to adherent cell culture flasks. The flasks were incubated at 37 °C for 3 days at a humidified 5% CO₂ atmosphere. The cells were then collected, washed and resuspended in complete medium. Adherent PEC without r-IL2 served as control cells. Lymphocytes generated from murine splenic cells were used for experimental comparison.

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Cytotoxicity test^{6,7}

Four hours ⁵¹Cr-release assay was used to evaluate the cytotoxicity of peritoneal macrophages and lymphocytes activated by r-IL2. ⁵¹Cr labeled Capan-2 cells acted as target cells (T). E/T ratio was 25/1. The percentage of specific cytolysis in triplicate cultures was calculated by the formula:

$$\% \text{ specific cytolysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100$$

Maximum cpm was measured after incubation with 0.1N HCl. The spontaneous cpm, measured by incubation of the target cells alone, should be less than 30% of maximum cpm. To test ADCC, 25 mg/L of mAbs was incubated with the target cells for 30 min before addition of the effector cells. The percentage of specific cytolysis for ADCC was determined as described above.

RESULTS

mAb immunoreactivity assessment

Measured by ELISA on Capan-2 cells, the titer of YPC3 mAb (0.5 g/L) was more than 1:1000. YPC3 mAb was reacted with most of the pancreatic carcinoma cells determined by ABC immunohistochemistry on Capan-2 xenograft samples. No immunoreaction was found with control 1-F/7 mAb in ELISA and ABC immunohistochemistry. The results showed that YPC3 mAb might combine selectively with Capan-2 human pancreatic carcinoma cells.

The cytotoxicity of peritoneal macrophages activated by r-IL2 and YPC3 mAb

Table 1 shows that the cytolysis of peritoneal macrophages was evidently enhanced after activated by r-IL2 and YPC3 mAb. The percentage of cytolysis of peritoneal macrophages+Y-IL2+YPC3 mAb was 70.0%, 67.1% and 39.4% higher than the single peritoneal macrophages, peritoneal macrophages+Y-IL2 and peritoneal macrophages+YPC3 mAb, respectively. 1-F/7 mAb had no significant effect on cytotoxicity of peritoneal macrophages against Capan-2 cells.

Table 1 Cytolysis of peritoneal macrophages with different agents

Groups	n	Cytolysis (% , $\bar{x} \pm s$)
Macrophages	3	27.72±1.52 ^a
Macrophages+γ-IL2	3	22.47±4.32 ^a
Macrophages+YPC3 mAb	3	26.67±1.58 ^a
Macrophages+γ-IL2+1 F/7 mAb	3	27.02±2.78 ^a
Macrophages+γ-IL2+YPC3 mAb	3	37.06±10.78

^aP<0.05, vs macrophages+ Y-IL2+YPC3 mAb.

The comparison of ADCC between the effect of peritoneal macrophages and splenic lymphocytes activated by γ-IL2

Y-IL2 is a multifunctional protein which influences

numerous cell types and activates both macrophages and lymphocytes. Essentially similar cytotoxicity levels of activated macrophages and lymphocytes were obtained with 25 mg/L of YPC3 mAb (37.06±10.78 and 30.13±9.15 respectively, P>0.05). The results indicated that ADCC depended mainly on the reaction of antibodies with membrane antigens expressed on the target cells.

DISCUSSION

ADCC is a mechanism of immunologic lysis, in which cellular targets sensitized by specific antibodies are selectively lysed by FcR bearing nonsensitized effector cells⁶. Macrophages bearing Ig-FcR have ADCC effect on tumor cells mediated specific anti-tumor mAbs. This ADCC is one of the important killing mechanisms of macrophages³.

Murine YPC3 mAb, belonging to IgG1 isotype, was produced against the human pancreatic carcinoma cell line. It reacted with pancreatic carcinoma in 28/32 patients, did not react with 11 normal human pancreas tissues and only 9/78 non-pancreatic tumors showed weak cross-reaction by ABC immunohistochemistry⁴. Radioimmunolocalization of human pancreatic carcinoma by ^{99m}Tc labeled YPC3 mAb was achieved in Balb/c nude mice⁵. YPC3 mAb increased the anti pancreatic carcinoma efficacy of splenic LAK cells *in vitro* and the simultaneous injection of LAK cells and YPC3 mAb completely inhibited the growth of Capan-2 cell line in nude mice⁷. In this experiment, YPC3 mAb showed good affinity with Capan-2 cells and good synergism with Y-IL2. When combined with r-IL2, YPC3 mAb enhanced the cytolysis of peritoneal macrophages on Capan-2 cells, which was 70% more than peritoneal macrophages alone, and was higher than that of 1-F/7 mAb.

Some curative effects of lymphokine-activated killer cells and interleukin 2 alone on metastatic carcinoma of serosa cavity have been progressed. But the therapy needed a lot of effector cells and large doses of activators with obvious side-effect^{8,9}. Kawase *et al*¹³ reported that combined therapy of C3H/HeN mice bearing ascitic MH134 hepatoma with ip injection of γ-IL2 and tumor specific 11G2 mAb brought about potent suppression of the tumor growth, resulting in the significant increase in the number of tumor-free mice, whereas neither Y-IL2 nor the mAb could exhibit such a potent antitumor effect when used alone. The results provided possibility that antitumor mAbs capable of inducing ADCC could overcome a limitation of LAK therapy.

The prognosis of pancreatic carcinoma remains poor because of early peritoneal metastasis and lack of effective therapy in advanced patients^{10,11}. The combination of γ-IL2 and YPC3 mAb can increase

the effect of anti-pancreatic carcinoma *in vitro*. Ip injection of γ -IL2 and YPC3 mAb may provide a new way for local treatment of pancreatic carcinoma. But the research in the host bearing pancreatic carcinoma will be continued.

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Diagnostic value of amino acid consumption test on exocrine pancreatic insufficiency

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INTRODUCTION

Amino acid consumption test (AACT) has a high sensitivity and specificity in evaluating exocrine pancreatic insufficiency^[1,2], but its diagnostic value to exocrine pancreatic insufficiency in Chinese has not been well understood. In this study, the oral reagent stimulating pancreatic secretion (O-AACT) was used instead of cerulein (I-AACT) for amino acid consumption test and the diagnostic efficiency of O-AACT was evaluated and compared with I-AACT on the exocrine pancreatic insufficiency in Chinese.

MATERIALS AND METHODS

General data

A total of 176 volunteers and patients, divided into three groups, were studied after giving written informed consent.

Group 1. Normal controls consisting of 12 males and 8 females, aged 20-51 years. None of them had evidence of digestive diseases, and were alcoholics.

Group 2. Cholelithiasis group consisting of 31 males and 45 females, aged 22-60 years, including 44 cases with gallstone, 5 with common bile duct stone and 27 with intrahepatic lithiasis. Those who had any evidence of pancreatitis, acute cholangitis, or diabetes mellitus had been excluded.

Group 3. Pancreatic disease group consisting of 49 males and 31 females, aged 23-71 years, including 28 cases of pancreatic cancer, 19 of periampulla of Vater tumor, 12 of chronic pancreatitis (CP) complicated with pancreatic cysts, 12 with

common bile duct cyst complicated with CP, and 9 of CP. Those diagnoses were supported by the typical clinical manifestations and based on generally accepted morphologic alteration suggestive of pancreatic disease found in at least one of the following procedures: CT, surgery and/or pathology.

All patients received I-AACT and N-benzoyl-L-tyrosyl-para-aminobenzoic acid (purchased from Chongqing Medicine Industrial Laboratory) test (BT-PABA test). Among them, 12 in Group 1, 36 in Group 2 and 48 in Group 3, were also examined by O-AACT. Each test was made at an interval of two or three days. Gallbladder volume was determined by ultrasonography (US) at 0, 30, 45, 60, 90 and 120 min in 6 volunteers in Group 1 after drinking one of the three kinds of reagents stimulating pancreatic secretion at an interval of two days.

Methods

BT-PABA test This test was performed according to Imondi's method^[3]. The cut off limit for normal results was 45.0% of the orally administered dose.

I-AACT test According to Gullo's method^[2], the test was made in the morning after an overnight fast. Each of 176 subjects received a continuous intravenous infusion of cerulein (50ng/kg·h) (Purchased from Sigma Com., USA.) for an hour dissolved in 0.9% NaCl solution. During each study blood samples for amino acid determination were taken before the infusion was started at 0 min and during the infusion at 45 min, 60 min and 30 min after the infusion was stopped. Total plasma amino acid was estimated by the sodium-β-naphthoquinone-4-sulfonate salt colorimetric method. The individual basal amino acid level was taken as 100% standard for final calculations, the relative reduction rate of the amino acid levels at each time was calculated. Decrease by over 14.0% in plasma amino acid concentration was used as a normal limit index of pancreatic function.

O-AACT Oral reagent stimulating pancreatic secretion was used instead of infusion cerulein in AACT, the other procedure was similar to I-AACT. The oral reagent stimulating pancreatic secretion consists mainly of:

A. Salad oil 1 mL/kg, 0.1 mol/L HCl

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0.2 mL/kg, saccharose 0.5 g/kg added with water to 100 mL.

B. Salad oil 1.5 mL/kg, 0.1 mol/L HCl 0.2 mL/kg, saccharose 0.5 g/kg, added with water to 200 mL.-C. Salad oil 2.0 mL/kg, 0.1mol/L, HCl 0.2 mL/kg, saccharose 0.5 g/kg added with water to 200 mL.

Gallbladder volume Gallbladder volume (Ellipsoid formula $V=0.52 \times l \times b \times h$)-was measured by US each time before and after drinking the reagent stimulating pancreatic secretion.

Statistical analyses were made using Chi-square test, and analysis of variance.

RESULTS

The change of plasma amino acid level in I-AACT

Basal amino acid (AA) levels of all subjects varied between 1.12 mmol/L and 6.8 mmol/L. During intravenous infusion of cerulein, the levels of plasma AA in all subjects of three groups gradually decreased, the 13,45 and 5 cases had relative decline rate over 14.0% in Groups 1-3 at 45 min respectively, and 18, 68 and 11 cases at 60 min. Thereafter, plasma AA levels returned slowly toward initial AA values. The relative decline rate of plasma AA levels of 8 cases in Group 2 and 67 cases in Group 3 were still lower than 14.0% at 90 min.

The selection of oral reagent stimulating pancreatic secretion

When reagent A or B was selected, all volunteers, except two people who had slight flatulency, could drink all the reagent without discomfort. But after drinking a cup of reagent C, three volunteers had vomit and exited from the study, and gallbladder contraction occurred earlier in other three volunteers (Table 1). Reagent A was finally selected as the reagent stimulating pancreatic secretion in O-AACT because it caused steady gallbladder constriction.

Table 1 Gallbladder volume change after taking reagent ($\bar{x} \pm s$, ml)

Time	Reagent A n = 3	Reagent B n = 6	Reagent C n = 6
0 min	17.22±3.55	16.58±3.04	14.97±5.23
30 min	12.85±6.34	13.78±2.69	8.87±2.10
45 min	12.12±2.25 ^a	13.27±2.96	8.70±1.13
60 min	9.68±3.30 ^a	6.71±2.66 ^a	12.03±3.05
90 min	6.28±3.51 ^a	4.93±1.89 ^a	13.97±9.06
120 min	12.27±5.42	11.40±4.79	15.47±5.94

^aP<0.05 in comparison with 0 min in the same group. Analysis of variance: F_A=4.2799 (F₄₅=8.8133, F₆₀=14.4901, F₉₀=28.7727), F_B=12.1610 (F₆₀=35.8538, F₉₀=63.6581)

The change of plasma amino acid level in O-AACT During O-AACT, plasma AA levels gradually decreased, then returned slowly toward initial AA values. The change was similar to that in I-AACT (Table 2).

Table 2 The change of plasma amino acid level in O-AACT and I-AACT ($\bar{x} \pm s$, mmol/L)

AACT	Time (min)	Group 1 (n = 12)	Group 2 (n = 36)	Group 3 (n = 48)
I-AACT	0	4.23±0.78	4.64±1.49	3.91±1.32
	45	3.13±0.068 ^{a1}	3.53±1.49 ^{a6}	3.65±1.19
	60	2.68±0.85 ^{a2}	3.09±1.21 ^{a7}	3.49±1.19
	90	3.67±0.90	4.08±1.47	3.66±1.24
O-AACT	0	4.24±1.19	4.67±1.45	3.99±1.26
	45	2.73±0.99 ^{a3}	4.05±1.43	3.57±1.24 ^{b1}
	60	2.05±0.94 ^{a4}	3.01±1.17 ^{a8}	3.60±1.20 ^{b2}
	90	2.94±1.12 ^{a5}	3.84±1.49 ^{a9}	3.89±1.29

^aP<0.05, in comparison with 0min in the same group and the same method. Analysis of variance:

^{a1}F = 13.679, ^{a2}F = 21.45, ^{a3}F = 11.487, ^{a4}F = 25.095, ^{a5}F = 7.637, ^{a6}F = 9.938, ^{a7}F = 23.792, ^{a8}F = 28.622, ^{a9}F = 5.799.

^bP<0.05, in comparison with Group 1, analysis of variance:

^{b1}F = 4.746, ^{b2}F = 17.379.

Comparison of the diagnostic value of BT-PABA, O-AACT and I-AACT

Table 3 shows the number of cases of each group diagnosed as exocrine pancreatic insufficiency by BT-PABA, O-AACT or I-AACT. The diagnostic value of BT-PABA, O-AACT and I-AACT to exocrine pancreatic insufficiency is summarized in Table 4.

Table 3 The number of cases in each group diagnosed as exocrine pancreatic insufficiency

%	Group 1	Group 2	Group 3
O-AACT	12	36	48
>14.0%	11	30	13
14.0%	1	6	35 ^a
I-AACT	20	76	80
>14.0%	20	68	13
>14.0%	0	8	67 ^b
BT-PABA	20	76	80
>45.0%	20	62	16
>45.0%	0	14	64 ^c

P<0.05 in comparison with groups 1 and 2. Chi-square test (χ^2): a=33.439, b=102.208, c=77.848.

Table 4 The diagnostic value of BT-PABA, O-AACT and I-AACT in exocrine pancreatic insufficiency

	Sensitivity (%)	Specificity (%)	Accuracy (%)
O-AACT	72.9	85.4	79.2
I-AACT	83.8	91.7	88.1
BT-PABA	80.0	85.4	83.0

Chi-square test: P>0.05.

DISCUSSION

Exocrine pancreatic function test is generally divided into direct test (e.g., pancreozymin-secretin test, cerulein test, etc.) and indirect test (pancreolauryl test, BT-PABA test, etc.). Although it is more sensitive and accurate in diagnosis of exocrine pancreatic insufficiency, the direct test is not widely used in clinical practice because it is complex, time consuming, and bringing more sufferings to patients. Indirect test is simple, but with low specificity and accuracy in diagnosis of exocrine pancreatic insufficiency. Domschke detected the change of plasma AA level before and after intravenous infusion of cholecystikinin and secretin to diagnose exocrine pancreatic insufficiency, and the accuracy being 91.0%, which was confirmed further by Gullo who used cerulein instead of cholecystikinin and secretin stimulating pancreatic secretion.

The diagnostic value of AACT in exocrine pancreatic insufficiency in Chinese has not been well understood because of the difference in race, living custom, geography, and the high fat and protein food and higher incidence of chronic pancreatitis in Europe and America, but more carbohydrate food and lower incidence of chronic pancreatitis in this country. Our study shows that the diagnostic accuracy of AACT for exocrine pancreatic insufficiency in Chinese was similar to that in European and American people. But attention should be paid to the following points: ① The relative decline rate over 14.0% of plasma AA was found only in 13 and 45 cases of Groups 1 and 2 during intravenous infusion of cerulein at 45 min, and in 18 and 68 cases at 60 min, the maximum decrease of plasma AA occurred later than that of

Gullo's report. Therefore, the results should be analyzed in combination at 45 min, 60 min and 90 min. ② Venous blood should be collected gently to avoid hemolysis. Incorrect deproteinization or not done in time will lead to bias in results.

To further reduce the test cost and illumed by the idea of Lundh test, we used oral reagent free from amino acid instead of intravenous infusion of cerulein in AACT. Because the volunteers would not like intubation in duodenum, gallbladder contraction function was observed after drinking of reagent A to view indirectly the effect of the oral reagent stimulating pancreatic secretion, based on the theory that hydrochloric acid and fat stimulate bile and pancreatic fluid secretion, and gallbladder contraction.

In this study, we found that O-AACT, based on the same design theory of I-AACT, is simple, inexpensive and easy to accept and plays a good screening role in evaluating severe exocrine pancreatic insufficiency. But whether it is applicable to diagnosis for slight and moderate exocrine pancreatic insufficiency should be further studied.

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Effect of emodin and sandostatin on metabolism of eicosanoids in acute necrotizing pancreatitis

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INTRODUCTION

In order to study the therapeutic mechanisms of emodin, an extract of Rhubarb (*Rhizoma et Radix Rhei*, a traditional Chinese herbal medicine), and sandostatin in the treatment of acute necrotizing pancreatitis (ANP), we used the two drugs in rat models of the disease and observed the changes of plasma thromboxane -2 (TXB₂), 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α} and prostaglandin E₂ (PEG₂).

MATERIAL AND METHODS

Animals and reagents

One hundred and sixty male Sprague-Dawley (SD) rats (Shanghai Birth Control Institute, Shanghai) weighing 220g-280g were used. Emodin (Natural Medicine Institute, Pharmacology School of Shanghai Medical University, Shanghai); sandostatin (Sandoz Co.); sodium taurocholate (Sigma); radioimmunoassay kits of PGE₂, 6-keto-PGF_{1α} and TXB₂ (Institute of Thrombosis and Coagulation, Suzhou Medical College).

Methods

The rats were divided randomly into 4 groups. After fasted (water allowed over 12 hours, the rats were anesthetized with 2.5% pentobarbital (0.1 mL/100g, i.p.), and a constant venous infusion route (1 mL/h) was established via *vena femoralis*. The peritoneum cavity was then cut open and the pancreatocolangioduct was cramped at both the duodenal and hepatoportal ends before 5% sodium taurocholate solution (0.1 mL/100 g)

was injected into the duct for 1 minute followed by a pause of 4 minutes. Then, the following drugs were infused intravenously: emodin, 0.25 mg/100 g, every 6 hours in emodin group; or sandostatin, 0.2 μg/100 g in sandostatin group, or normal saline in sham-operation group in which no taurocholate was used or in controls. The rats were killed 3, 6 and 12 hours after onset of the disease and the survival number was recorded. Samples of blood, ascites and pancreas were collected for detection of PGE₂, TXB₂, 6-keto-PGF_{1α} (RIA), serum amylase, lipase (Bech man Biochem System and Kits) and pathological observation under light microscope or transmission electronmicroscope (TEM). Pancreatic pathological scoring was made by Schmidt method^[1] double-blindedly.

Statistics

Test of homogeneity of variance, analysis of variance, Student-Newman-Keuls (SNK) test, *t* or *t'* test and χ^2 test.

RESULTS

Survival rate of the rats

The 12 hour survival rate in emodin group was 56.3% (9/16) and 62.5% (10/16) in sandostatin group; both were significantly higher than that of control group (23.8%, 5/21; *P*<0.05, *P*<0.01). However, no significant difference was found between emodin and sandostatin groups.

Metabolites of eicosanoids

TXB₂ detected at 3, 6, and 12 hours after ANP in control group was significantly higher than that of sham-operation group, the highest value, 4.5 times was at 6 hours (*P*<0.01, Table 1), while 6-keto-PGF_{1α} in each detection was lower as compared with that of sham. operation group, but no significant difference was found. TXB₂ was decreased obviously in each detection in both emodin, and sandostatin groups in comparison with that of control group (*P*<0.01). But at 12 hours after ANP, TXB₂ was lower in emodin group than that of sandostatin group. PGE₂ or 6-keto-PGF_{1α} was higher in the 2 drug-given groups than that of control group, but with no statistical significance.

Enzyme activities, ascites volume and pathological findings

Serum amylase, lipase and ascites were significantly

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lower in two drug-given groups than that in control group ($P<0.05$). In sandostatin group, two enzyme parameters were lower than that in emodin group, but only amylase showed significant difference ($P<0.01$). Compared with that in control group, the pathological scores of necrosis of acinar cells were markedly lower either in emodin or sandostatin group ($P<0.05$; $P<0.01$, Table 2), but there was no significant difference between the

2 drug-given groups. Obvious karyopyknosis, nuclear degeneration, cytoclasis, and lots of microthrombi in capillaries were found under TEM in control group, while in 2 drug-given groups, there were much less acinar necrosis and microthrombosis, and the major changes were pachynesis, autophagocytic vacuoles or bodies, swollen mitochondria and distorted endoplasmic reticulum.

Table 1 Results of detection of metabolites of eicosanoids in 4 groups of rats with ANP (ng/L, $\bar{x}\pm s$)

Groups	TXB ₂			6-keto-PGF _{1α}		
	3 h	6 h	12 h	3 h	6 h	12 h
Emodin	290.16 \pm 145.62(6) ^{ad}	335.63 \pm 191.69(5) ^b	67.71 \pm 38.54(6) ^b	111.70 \pm 16.76(6)	127.68 \pm 12.35(6) ^{bc}	79.09 \pm 30.17(6)
Sandostatin	82.40 \pm 21.59(7) ^b	193.98 \pm 131.13(6) ^b	94.19 \pm 19.29(6) ^a	117.62 \pm 36.03(7)	111.96 \pm 26.75(7)	72.04 \pm 37.80(6)
Control	341.34 \pm 230.26(6)	746.65 \pm 141.88(6)	256.52 \pm 124.97(6)	67.63 \pm 29.25(7)	64.35 \pm 21.80(6)	65.45 \pm 20.54(6)
Sham-operat.	154.58 \pm 38.73(5) ^a	165.35 \pm 39.93(4)	148.63 \pm 50.78(6) ^b	95.65 \pm 28.59(5)	90.51 \pm 13.97(4)	95.99 \pm 34.66(6)

Notes: compared with in control group at the same time, ^a $P<0.05$, ^b $P<0.01$; compared with that in sham-operation group, ^c $P<0.05$, ^d $P<0.01$; (), the number of rats; PGE₂ is not listed because of no significant difference.

Table 2 Comparison of pathologic scores in SD rats with ANP 12hrs after onset ($\bar{x}\pm s$)

Groups	<i>n</i>	Edema	Inflammation	Necrosis	Bleeding
Emodin	8	2.62 \pm 0.53	2.94 \pm 0.32	1.19 \pm 1.13 ^a	0.25 \pm 0.46
Sandostatin	7	2.57 \pm 0.34	2.71 \pm 1.38	1.28 \pm 0.45 ^b	0.42 \pm 0.53
Control	8	2.56 \pm 0.42	3.75 \pm 0.53	3.43 \pm 0.62	0.63 \pm 0.50

Notes: compared with control group, ^a $P<0.05$, ^b $P<0.01$.

DISCUSSION

Rats with taurocholate-induced ANP might simulate human biliary (bile reflux) pancreatitis. The predominant pathophysiological change is severe disturbance of pancreatic microcirculation accompanied by abnormal metabolism of eicosanoids which precedes pancreatic bleeding and necrosis. Pancreas is the main site of the abnormal metabolism of eicosanoids in ANP; after that is the blood (platelet)^[1]. TXB₂ and 6-keto-PGF_{1 α} are stable metabolites of TXA₂ and PGI₂, respectively. The former is a strong microvascular constrictor and an agonist of platelet aggregation as well. It can induce deformation, release and secretion of platelets, resulting in local or systemic disorders of coagulation and bleeding. Pancreatic ischemia became worse with complete destruction of pancreatic cytoprotection^[2]. Some authors employed selective inhibitors of synthesis of TXB₂, or exotic PGE₂ in rats with ANP, and discovered that the mortality was reduced^[3]. These results, together with ours, suggested the role of abnormal metabolites of eicosanoids in the pathogenesis of ANP and possible therapeutic strategies to be adopted. Meanwhile, our study demonstrated that,

besides traditional viewpoints, inhibition of abnormal metabolism of eicosanoids, promotion of pancreatic cytoprotection, prevention of coagulation and microthrombosis and improvement of pancreatic microcirculation should also undoubtedly be included in the mechanism of the therapeutic roles of emodin as well as sandostatin in the treatment of ANP.

In conclusion, it can be suggested that, the mechanisms of emodin or sandostatin in the treatment of ANP should include modulation of abnormal eicosanoid metabolism and restoration or promotion of pancreatic cytoprotection which might be more important than the well-known "anti-enzyme" or "anti-secretion" speculation.

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Inhibitor of fatty acid synthase induced apoptosis in human colonic cancer cells

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Subject Headings colonic carcinoma; apoptosis; fatty acid synthase; cerulenin

Huang PL, Zhu SN, Lu SL, Dai SZ, Jin YL. Inhibitor of fatty acid synthase induced apoptosis in human colonic cancer cells. *World J Gastroenterol*, 2000;6(2):295-297

INTRODUCTION

The treatment of human epithelial malignancies is limited by drug resistance and toxic and side effects, which results in the failure in the treatment of majority of advanced cancer victims. To seek for a new, and specific antineoplastic therapy will provide hope for tumor treatment. Although disordered intermediary metabolism in cancer cells has been known for many years, much of the work focused on abnormal glucose catabolism. At the same time, little attention has been paid to fatty acid synthesis in tumor tissues, despite of the significance of fatty acid synthase (FAS) in some clinical human ovarian^[1], breast^[2], colorectal^[3], and prostatic cancers^[4,5]. Tumor cells which express high levels of fatty acid synthesizing enzymes use endogeneously synthesized fatty acids for membrane biosynthesis and appear to export large amounts of lipid. In contrast, normal cells preferentially utilize dietary lipid.

The mechanism by which inhibition of fatty acid synthesis produces its antitumor effects remains unexplained. In this study, we studied the effect of fatty acid synthase inhibitor-cerulenin, on the proliferation and apoptosis of colonic cancer cells.

MATERIAL AND METHODS

Cell lines and culture condition LoVo cells were maintained in RPMI 1640 with 10% fetal bovine serum. Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. A 5 g/L stock

solution of cerulenin (Sigma Chemical Co.) in DMSO was diluted to a final concentration of 5 mg/L or 10 mg/L. Human fibroblasts (collected from clinical operation) were cultured in RPMI 1640 for six generations, and then were maintained in the same growth medium as LoVo cells.

Morphological evaluation Exponentially growing LoVo cells were incubated with or without cerulenin in RPMI1640 for 24h and collected after trypsin was digested. The cells were spun onto glass slides, fixed in methanol, and stained by Wright-Giemsa's method. The slides were observed under Olympus light microscope.

Clonogenic assay Using limiting dilution method, LoVo cells were inoculated at 50 colony-forming cells/well into 96-well plates and incubated for 10 days, with triplicate cultures plated at each drug concentration (1.0×10⁻⁵ mol/L-1.0⁻⁹ mol/L). Colonies of greater than 50 cells were counted for all clonogenic assays. The rate of colony forming = (average colony number/planted single cell number)×100%. The rate of colony forming inhibition = (control colony forming rate-experimental colony forming rate)/control colony forming rate×100%.

Cell proliferation inhibition experiment (MTT) LoVo cells were inoculated at 8×10⁴ cell/mL into 96-well plates and incubated 24 h. Cerulenin of 1.13×10⁻⁵ mol/L-1.13×10⁻⁹ mol/L in concentration was given to the experimental group. Each well was added with 20 μL (5 g/L) MTT and incubated for 4 h. The plates were read on Dynatech microplate reader MR600 at 570 nm.

DNA fragmentation assay LoVo cells were incubated with or without cerulenin in RPMI1640 for 12h and collected after trypsin was digested. At harvest, medium and trypsinized cells were combined, and DNA was extracted from pelleted cells and subjected to 1.5% agarose gel. DNA was electrophoresed for 3 hours at 5 v/cm. The results were observed and photographed by UV light.

RESULTS

Inhibition of cell proliferation in human colonic cancer cells by cerulenin

The effects of fatty acid synthase inhibitor on cell

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growth were examined by MTT method. The inhibitory effects on proliferation are shown in Figure 1. Cerulenin can inhibit significantly the proliferation of LoVo cells and showed dose-response relationship. Therefore, FAS inhibitor-cerulenin might suppress the proliferation of human colonic cancer cells.

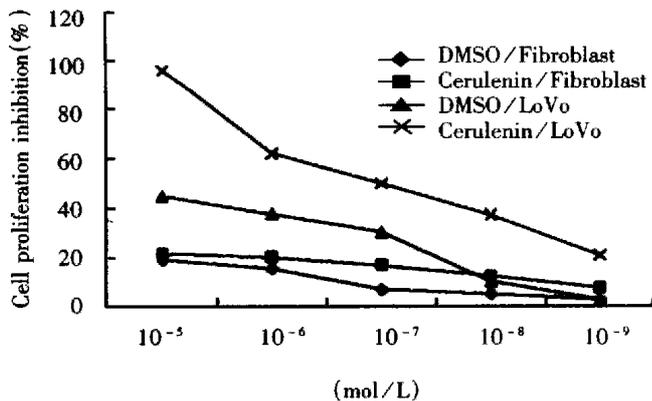


Figure 1 Cell proliferation inhibition experiment (MTT).

Reduction of clonogenic potential of human colonic cancer cells after cerulenin treatment

To assess the cellular consequences of fatty acid synthesis inhibition, the clonogenicity of LoVo cell was determined after a 10-day exposure to cerulenin (Table 1). LoVo cell line exhibited dose-dependent reduction in clonogenic potential.

Induction of apoptosis of human colonic cancer cells by cerulenin

The cytotoxic effects of cerulenin must occur within the context of cellular response. A wide variety of cellular injuries in cell death by initiating a cellular program for autodestruction, or apoptosis. Fragmentation of genomic DNA to high molecular weight (180 kb-200 kb) fragments is a characteristic early event in apoptosis and may represent the committed step of the process. Agarose gel electrophoresis was used to evaluate whether high molecular DNA fragmentation was a feature of the cellular response to cerulenin. LoVo cells generated detectable high molecular weight DNA fragments within 12 h after exposure to cerulenin. So, internucleosomal DNA can be detected on agarose gel as a ladder of DNA fragment (Figure 2). To confirm that the cytotoxic effects of cerulenin were mediated via apoptosis, and the treated cells were examined for morphological changes of apoptosis.

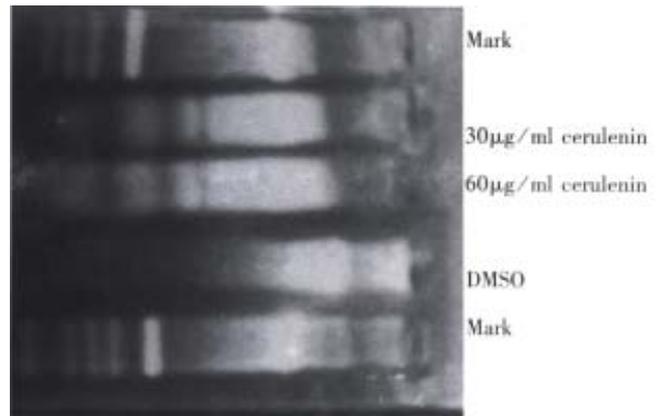


Figure 2 DNA fragmentation assay by agarose gel electrophoresis.

DISCUSSION

Fatty acid synthase played an important role in de novo biosynthesis of fatty acid and consists of two identical subunits each with M 250000 in cancer cells. Pizer *et al* reported that OVCAR-3 cells showed a range of elevation fatty acid synthesis in the carcinoma lines, which elevated approximately 10-fold above the level of control skin fibroblasts^[1]. The fungal metabolite, cerulenin, (2S-3R)-2-3-epoxy-4-oxo-7E, 10Edodecadienamidel], a specific, non-competitive inhibitor of fatty acid synthase, binds covalently to the condensing enzyme site on fatty acid synthase, causing complete inactivation^[6,7]. The condensing enzyme site on fatty acid synthase utilizes a biochemically distinctive catalytic mechanism which is not shared by other vital cellular enzymes and is thus well suited to be a therapeutic target. Our data showed that fatty acid synthase inhibitor-cerulenin could suppress the proliferation of LoVo cells *in vitro*, at the same time, it showed a dose-response relationship. We also found that cerulenin is indeed cytotoxic to cancer cells by clonogenic assay.

It is emphasized that there was a close relationship between apoptosis and proliferation in malignant neoplasm cells^[8,9]. In this study, we found that cerulenin could induce LoVo cells to die via apoptosis. Apoptotic cells demonstrated cell shrinkage, condensation and fragmentation of chromosomes. Nuclear DNA of apoptotic cells displayed ladder bands characteristic of internucleosomal DNA fragmentation. The data reported here demonstrated that inhibition of fatty acid synthesis inflicted rapid, lethal injury to carcinoma cells via activation of the cell death program. It is similar to that both FAS inhibitors produced rapid, profound inhibition of DNA replication and S phase progression in human cancer cells, meanwhile, apoptosis changes were detected^[10].

Table 1 The effects of cerulenin on proliferation of LoVo cells by means of clonogenic assay ($\bar{x} \pm s$)

	1×10^{-5} mol/L	1×10^{-6} mol/L	1×10^{-6} mol/L	1×10^{-6} mol/L	1×10^{-6} mol/L
DMSO control	46.16 \pm 3.74	25.18 \pm 1.66	19.21 \pm 7.32	10.62 \pm 3.28	8.93 \pm 5.05
Cerulenin	100 ^b	96.58 \pm 4.14 ^a	51.19 \pm 4.38 ^b	39.52 \pm 2.93	33.53 \pm 3.98 ^a

^a $P < 0.05$, vs DMSO control; ^b $P < 0.01$, vs DMSO control.

The strategy of cancer chemotherapy by FAS inhibition was based on the therapeutic index provided by elevated fatty acid synthesis in tumor cells and the apparent tumor preference for the endogenous pathway. Our data proved in principle that the metabolic pathway leading to *de novo* fatty acid synthesis was selective target for antimetabolic chemotherapy of cancer. Selectivity was conferred by two major factors ① LoVo cells, like some other human cancers, appear to be dependent on *de novo* fatty acid biosynthesis and therefore sensitive to FAS inhibition; and ② normal tissues (e.g. fibroblasts) seem predominantly to utilize dietary lipid and therefore to be resistant to the cytotoxic effects of FAS inhibition^[8]. Possible functional roles for endogenously synthesized fatty acid in cell growth include synthesis of structural lipids for membrane biosynthesis and lipid mediators of intracellular, autocrine or paracrine signals, as well as acylation of other macromolecules. It does appear, however, that for unclear reasons, certain tumors have an apparently obligatory requirement for endogenous fatty acid biosynthesis whereas normal cells do not, suggesting that inhibition of fatty acid biosynthesis may be a fruitful target for chemotherapy development.

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Effects of endotoxin on endothelin receptor in hepatic and intestinal tissues after endotoxemia in rats

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Subject headings endotoxin; endothelin receptor; liver; intestines; rats

Liu BH, Chen HS, Zhou JH, Xiao N. Effects of endotoxin on endothelin receptor in hepatic and intestinal tissues after endotoxemia in rats. *World J Gastroentero*, 2000;6(2):298-300

INTRODUCTION

Endothelins (ETs) has a potent and sustained vasoconstrictive effect on a variety of blood vessels. The vascular smooth muscle cell (VSMC) is the target for ETs. VSMC of the whole body contains endothelin receptor (ETR)^[1]. A great number of experiments have shown that three distinct complementary DNAs of ETR have been identified i.e., endothelin A receptor (ET-A receptor), endothelin B receptor (ET-B receptor) and endothelin C receptor (ET-C receptor). ET-A receptor was expressed in VSMC responsible for the contraction^[2]. The aim of this study is to confirm the effects of endotoxin on the activity of ETR, and the transcription and expression of ET-A receptor mRNA in hepatic and intestinal tissues.

MATERIALS AND METHODS

Materials

Endotoxin (O111:B4) and porcine endothelin-1 (ET-1) were purchased from Sigma Chemical Co. ¹²⁵I-ET-1 was supplied by Isotope Institute of Chinese Academy of Sciences. Digoxigenin DNA labeling and Detection Kit was provided by Boehringer Mannheim Co.

Methods

Thirty male and female Wistar rats weighing 210 g-215 g were fasted with access to water and libitum for 24 hours before experiment. Six rats were used as the control group. The others were given endotoxin at a dose of 10 mg/kg as an endotoxin-

treated group. All rats were killed at the 3rd, 6th, 12th, and 24th hour after administration of endotoxin and saline, with 6 rats in each group at each time point. After sacrifice by decapitation at the respective time points, the right hepatic and intestinal tissues were immediately removed and preserved in liquid nitrogen for use.

The hepatic and intestinal plasma membrane was prepared by the method of Koseki and Imari^[3] with minor modifications. The hepatic and intestinal tissue was homogenized in 10 vol (wt/vol) of 0.25 mol sucrose including 1mmol EDTA, and the homogenate was centrifuged at 10000×g for 10 minutes. The supernatant was centrifuged at 100000×g for 1 hour. The entire procedure was performed at 4 °C. The resulting pellet was resuspended in 200 μL of 0.25 mol/L sucrose including 1mmol EDTA, and kept in the deep freezer for assay. Twenty μL aliquot of the microsomal fraction, containing 20 μg- protein in 0.25 mol sucrose including 1mmol EDTA, was kept at 4 °C for 24 hours, with various concentrations of ¹²⁵I-ET-1 in 20 μL of the incubation solution. Twenty μL of ice-cold incubation solution was added and then centrifuged at 80000×g at 4 °C for 30 min. The supernatant was rapidly aspirated. The radioactivity was determined with an FJ-2108 liquid scintillation counter. Specific binding was determined by the total binding minus the nonspecific binding. The maximal binding capacity (Bmax) and affinity (Kd) of ETR were obtained using Scatchard plot analysis.

Total cellular RNA was isolated from the hepatic and intestinal tissue with guanidinium thiocyanate-phenol-chloroform method^[4]. Dot blot was used to identify and quantify ET-A receptor mRNA. Results were scanned in the computer to determine their quantity.

Statistical analysis

The data were expressed as mean±SD. The difference between the groups was analyzed with Student's *t* test. The difference was considered significant at *P*<0.05.

RESULTS

The change of activity of hepatic and intestinal ETR

The values of the Bmax and Kd of ETR of the

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normal hepatic and intestinal tissue are shown in Figures 1, 2. The K_d of ETR was 0.0328 in normal hepatic tissue, and 0.1263 in normal intestinal tissue. The B_{max} of ETR was 1.0288 in normal hepatic tissue, and 1.2303 in normal intestinal tissue. The B_{max} of ETR of the hepatic and intestinal tissue decreased gradually during endotoxemia. The B_{max} of ETR in the endotoxin-treated group decreased more significantly than that in the control group at the 3rd hour after endotoxin administration ($P<0.01$). The decrease of B_{max} lasted 24 hours. But the K_d of the hepatic and intestinal tissue did not change (Table 1).

The change of the relative quantity of ET_A receptor mRNA of hepatic and intestinal tissue
The relative quantity of ET_A receptor mRNA of hepatic tissue in the endotoxin-treated group increased more significantly than that in the control group at 3-24 hour ($P<0.01$). It increased most obviously at the 6th and the 12th hour after endotoxin administration (Table 2). Compared with the control group, the relative quantity of ET_A receptor mRNA of intestinal tissue in endotoxin-treated group also increased significantly between the 3rd and 12th hour after endotoxin administration ($P<0.01$) (Table 2).

Table 1 Effects of endotoxin on ETR of hepatic and intestinal tissue

	Control	Endotoxin-treated group			
		3rd	6th	12th	24th(h)
Liver					
$B_{max}(b_B/\text{pmol}\cdot\text{mg}^{-1}\text{p})$	0.9969±0.0155	0.7476±0.0356 ^b	0.5590±0.0219 ^b	0.4258±0.0156 ^b	0.5826±0.0586 ^b
$K_d(C_B/\text{nmol}\cdot\text{L}^{-1})$	0.0328±0.0018	0.0338±0.0016	0.0332±0.0014	0.0324±0.0010	0.0333±0.0017
Intestine					
$B_{max}(b_B/\text{pmol}\cdot\text{mg}^{-1}\text{p})$	1.2950±0.2070	0.8810±0.1026 ^a	0.6340±0.2109 ^b	0.5199±0.2260 ^b	0.6226±0.2120 ^b
$K_d(C_B/\text{nmol}\cdot\text{L}^{-1})$	0.1309±0.0183	0.1344±0.0140	0.1181±0.0173	0.1281±0.0173	0.1260±0.0169

^a $P<0.05$, ^b $P<0.01$ vs control group.

Table 2 Effect of endotoxin on relative quantity of hepatic and intestinal ET_A receptor nRNA ($\mu\text{g DNA/g RNA}$)

	Control	Endotoxin-treated group			
		3rd	6th	12th	24th(h)
Liver	17.13±2.11	57.96±11.51 ^b	86.35±16.01 ^b	95.50±13.24 ^b	27.93±3.05 ^b
Intestine	38.43±3.86	48.32±4.31 ^b	69.42±8.21 ^b	68.61±10.24 ^b	42.41±3.73

^b $P<0.01$ vs control group.

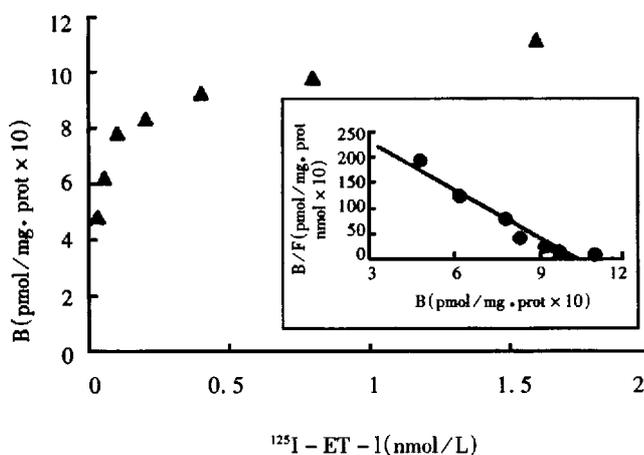


Figure 1 Specific binding of ^{125}I -ET-1 on liver hemogenates and corresponding Scatchard plot for normal rats.

$KD=0.0328$ nM, $B_{max} = 1.0288$ pmol/mg

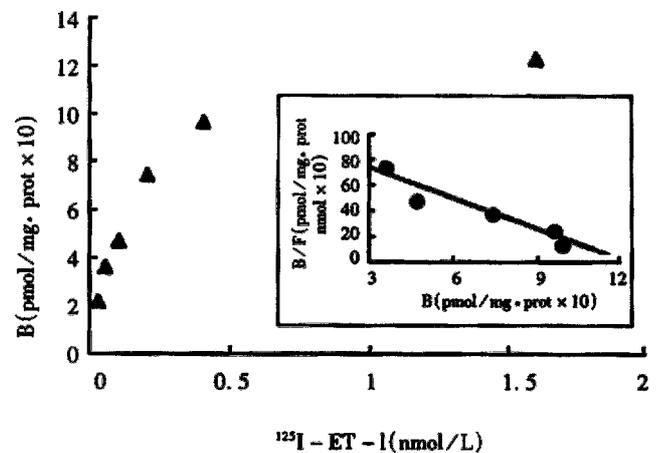


Figure 2 Specific binding of ^{125}I ET-1 on intestine hemogenates and corresponding Scatchard plot for normal rats.

$KD=0.1263$ nM, $B_{max} = 1.2303$ pmol/mg

DISCUSSION

ETs have been shown to be one of the most potent vasoconstrictors, which are produced not only by vascular endothelial cells, but also by a variety of non-endothelial cells, and have many biological functions^[5]. ETs should be combined with ETR of target cell, and activate a variety of signal transduction pathways so as to produce specific cellular responses^[6]. This study showed that the Kd of ETR of hepatic and intestinal tissue did not change during endotoxemia. The Bmax of ETR of hepatic and intestinal tissue in endotoxin-treated group decreased more significantly in early stage than that in control group. Decrease of the Bmax of ETR lasted 24 hours. The Bmax of ETR number of hepatic and intestinal tissue was the lowest at the 12th hour. After giving endotoxin, decrease of the ETR number of the hepatic and intestinal cell membrane was correlated with increase of ET-1 concentration (unpublished observation). Roubert *et al*^[7] reported that the 50% receptors of VSMC in culture were induced by 10^{-9} mol ET-1. During endotoxemia, the higher level of ET-1 was, the more ET-1 combined with the receptor, the lower number of receptor of cell membrane was, because the receptors of cell membrane combining with ET-1 squeezed into cells.

The relative quantity of ET_A receptor mRNA of hepatic and intestinal tissue increased obviously after endotoxin administration. This study showed that endotoxin could regulate transcription and translation of ET_A receptor mRNA. The results of ETR binding assay and ET_A receptor Dot blot

analysis showed that the ETR number of hepatic and intestinal cell membrane decreased, but the relative quantity of ET_A receptor mRNA of hepatic and intestinal tissue increased obviously in endotoxemia. It was likely that a mechanism of auto-regulation existed in the body. Down-regulation of ETR may be helpful in preventing endothelin from hypercontraction and reducing damage of tissues and organs. So it suggested that ETR may play an important role in the hepatic and intestinal pathophysiologic process following endotoxemia.

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TEffects of rhubarb and the active ingredients of rhubarb on the cytoplasmic free calcium in INT-MNC of rabbits

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Subject headings Fura-2/Am; rhubarb; emodin; sennosides; intestinal mononuclear cells; calcium

Lin XZ, Ma DL, Cui ZQ, Kang Y. Effects of rhubarb and the active ingredients of rhubarb on the cytoplasmic free calcium in INT-MNC of rabbits. *World J Gastroenterol*, 2000; 6(2):301-303

INTRODUCTION

The recent studies have shown that rhubarb has not only the effect of removing stasis by purgation, but also intestinal barrier effects^[1,2]. In order to further clarify the intestinal barrier mechanism of rhubarb, we studied the effects of rhubarb decoction and the active ingredients of rhubarb on the cytoplasmic free calcium in isolated intestinal mononuclear cells (INT-MNC).

MATERIALS AND METHODS

Materials

Fura-2/Am was purchased from the Shanghai Institute of Physiology, the Chinese Academy of Sciences. Eagle's MEM was purchased from Nissui Pharmaceutical Company, Japan. Collagenase (type IV) and emodin (EMD) were products of Sigma Chemical Co. Sennosides (SEN) was provided by the Department of Plant Chemistry of the Tianjin Institute of Materia Medica. Rhubarb decoction (equal to 1 g/mL of protophyte) was provided by the Department of Pharmacology of the Tianjin Acute Abdominal Institute of Integrated Traditional Chinese and Western Medicine. Dithiothreitol (DTT) was purchased from Feibiochemi.

Animals

Rabbits weighing 2.2 kg±1.2 kg, in either sex, supplied by the Animal Department of Tianjin Medical University. They were randomly divided into control, rhubarb, EMD and SEN groups.

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Preparation of INT-MNC suspension and Fura-2/Am loading

The rabbits were killed by a blow on the head and the abdomen was opened. Take 30 cm-40 cm of the ileum 10cm from the ileocecal part, wash out the stool and mucoid on the surface of the mucosa with Hank's solution (NaCl 137, KCl 5, Glucose 5.6 and Hepes 10 mmol/L, pH 7.4) which is Ca²⁺ and Mg²⁺ free. Take the intestinal mucosa gently with coverglass and put it into a conical flask containing Dithiothreitol (DTT, 1mmol/L) solution, swing and wash for 20 min at 37 °C, then put the mucosa into 5 mmol/L EDTA solution and swing for 30 min at 37 °C, and wash out the free epithelial cells using Hank's solution. Repeat the procedure of EDTA for 3 times. Then put the intestinal mucosa into Hank's solution containing collagenase. To digest it, swing for 3 h at 37 °C, filter 3 times with stainless sieve of 100 mesh. Put the cells into the lymphocyte separate solution and centrifuged at 500 rpm for 5 min. Take the cells of the middle layer (INT-MNC), wash with ice-cold Ca²⁺ and Mg²⁺ free Hank's solution and resuspend in Eagle's MEM solution and dilute to a concentration of 1-2×10⁸ mL/L.

Two mL cell suspension was incubated at super-thermostat water bath vibrator for 30 min in the presence of 5 μmol/L Fura-2/AM dissolved in dimethyl sulfoxide (DMSO) and 0.1% bovine serum albumin (37 °C, 40 rev/min, 95% O₂ + 5% CO₂). The Fura-2/Am-loaded INT-MNC was washed with Ca²⁺ and Mg²⁺ free Hank's solution and centrifuged at 1000 rpm for 10 min for three times. The INT-MNC was resuspended in 2 mL Ca²⁺ and Mg²⁺ free Hank's solution until use.

Fluorescence measurement and [Ca²⁺]_i calculated

The 2 mL Fura-2 loaded INT-MNC was incubated for 10 min at 37 °C for measurement. After the drugs were added and before measurement, the cell suspensions were blown and inspired 5-6 times with pipet to make the cell well distributed. Fura2/AM fluorescence was measured with RF-510 spectrofluorophotometer (Shimadzu, with thermostat). Emission wavelength (EM) was fixed at 490 nm. The excitation wavelength (EX) at 380 or 350 nm, the grating was 10 nm. The scanning speed was 100 nm/min. The changes of 350/380 nm fluorescence ratio were recorded by

alternating rapidly the EX of 350 nm and 380 nm manually (the rotating was finished within 4s-6s). The $[Ca^{2+}]_i$ was calculated using the general formula^[3] with K_d of 224 nmol/L: $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times S$. Before calculation, the auto-fluorescence unloaded with Fura-2 should be subtracted.

Statistical analysis

The results were expressed as mean values of groups $\bar{x} \pm s$. The Student *t* test was used for statistical comparison of difference of mean values between groups.

RESULTS

The effects of $CaCl_2$ on $[Ca^{2+}]_i$

In the resting status, the INT-MNC $[Ca^{2+}]_i$ was $252.55 \text{ nmol/L} \pm 42.54 \text{ nmol/L}$ ($n = 10$) in Ca^{2+} free Hank's solution containing ethyleneglycol-bis (β -aminoethyl ether)- N,N',N',N' tetraacetic acid (EGTA) 0.5 mmol/L. After adding $CaCl_2$ (0.25 mmol/L, 0.75 mmol/L, 1.5 mmol/L and 2.5 mmol/L) to INT-MNC suspension sequentially, the $[Ca^{2+}]_i$ levels were obviously elevated as compared with that at resting state ($P < 0.01$, Figure 1). This indicated that $[Ca^{2+}]_i$ was increased with the extracellular calcium level.

The effects of rhubarb decoction and SEN on $[Ca^{2+}]_i$

The INT-MNC was pretreated with rhubarb decoction (1 mg/L, 2 mg/L) or SEN (0.021 mmol/L, 0.084 mmol/L) for 15 min, in the resting state or after adding the above doses of $CaCl_2$, the INT-MNC $[Ca^{2+}]_i$ was more obviously lowered than the control groups ($P < 0.01$, Figures 1,2). The results showed that both rhubarb and SEN decreased the INT-MNC $[Ca^{2+}]_i$.

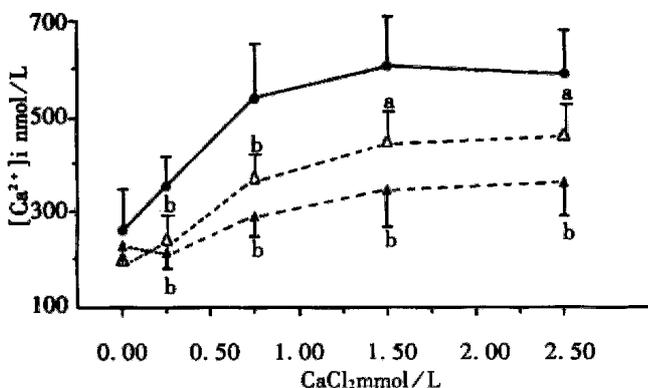


Figure 1 Effects of rhubarb decoction on the increase of $[Ca^{2+}]_i$ induced by $CaCl_2$ in isolated rabbits intestinal mucosal INT-MNC. ^a $P < 0.05$, ^b $P < 0.01$ vs control. ● Control ($n = 8$); △ Rhubarb decoction 1mg/L ($n = 5$); ▲ Rhubarb decoction 2mg/L ($n = 5$).

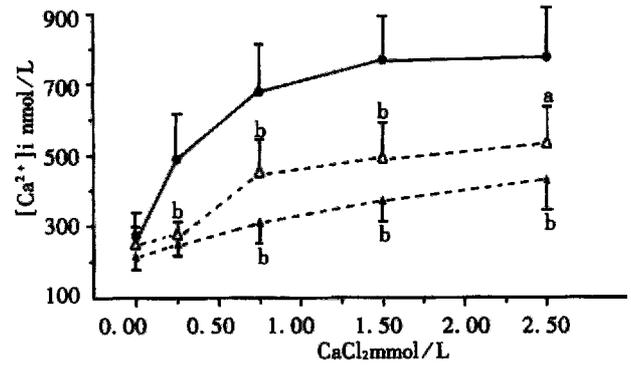


Figure 2 Effects of SEN on the increase of $[Ca^{2+}]_i$ induced by $CaCl_2$ in isolated rabbits intestinal mucosal INT-MNC. ^a $P < 0.05$, ^b $P < 0.01$ vs control. ● Control ($n = 6$); △ SEN 0.021 mmol/L ($n = 5$); ▲ SEN 0.084 mmol/L ($n = 5$).

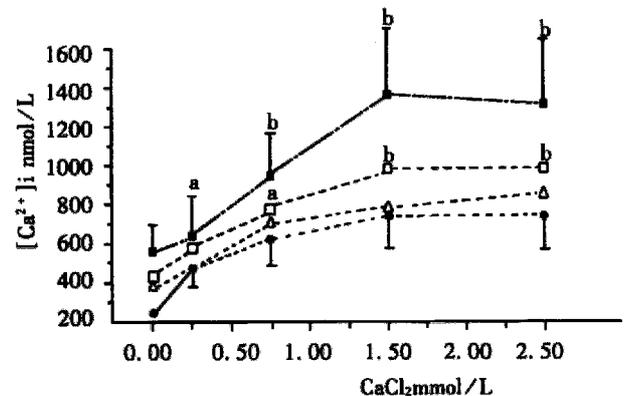


Figure 3 Effects of EMD on the increase of $[Ca^{2+}]_i$ induced by $CaCl_2$ in isolated rabbits intestinal mucosal INT-MNC. ^a $P < 0.05$, ^b $P < 0.01$ vs control. ● Control ($n = 10$); △ EMD 9.2 $\mu\text{mol/L}$ ($n = 10$); □ EMD 18.5 $\mu\text{mol/L}$ ($n = 8$); ■ EMD 37 $\mu\text{mol/L}$ ($n = 7$).

The effects of EMD on $[Ca^{2+}]_i$

After pretreated with EMD (9.2 $\mu\text{mol/L}$, 18.5 $\mu\text{mol/L}$, and 37 $\mu\text{mol/L}$) for 15 min, in the resting status or adding the above doses of $CaCl_2$, the INT-MNC $[Ca^{2+}]_i$ was significantly increased as compared with that of the control groups ($P < 0.01$, Figure 3). The results showed that EMD promoted not only the release of intracellular Ca^{2+} but also the extracellular influx.

DISCUSSION

Intestine is one of the largest immune organs. Intestinal mucosa immune defence system is made up of correlative lymphoid tissues. INT-MNC mainly consists of macrophage and lymphocytes. The separated INT-MNC produces immunoglobulin (Ig) spontaneously *in vitro*^[4]. It has an important effect on the immune regulation. Intracellular Ca^{2+} can regulate the endocrine system. When the Ca^{2+} level increases, Ca^{2+} produces physiologic effect as a

stimulant. But when intracellular Ca^{2+} has overloaded, the function of cell arrests, eventually initiated the cascade of deleterious events leading to cell death. This study indicates that the INT-MNC pretreated with rhubarb decoction or SEN, decreased the $[Ca^{2+}]_i$ dose-dependently as compared with the control groups ($P < 0.01$) when it is resting or is added with an equivalent amount of $CaCl_2$. This is consistent with the previous results that the rhubarb has a Ca^{2+} channel blocking activity^[5-7]. The antagonizing Ca^{2+} effects of rhubarb or SEN may be responsible for the intestinal barrier protective effect. On the contrary, the EMD promotes not only the release of intracellular Ca^{2+} , but the extracellular influx. This shows that the EMD can improve body immune function. Both SEN and EMD are active ingredients of rhubarb. The different effects of rhubarb and the different active ingredients of rhubarb on the Ca^{2+} level suggest that rhubarb has many kinds of immunological regulatory effects on INT-MNC.

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Effect of gastrin on protein kinase C and its subtype in human colon cancer cell line SW480

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Subject headings gastrin; protein kinase C; colon neoplasms; cell line

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INTRODUCTION

Gastrin is a trophic gastrointestinal hormone which is secreted by G cell. Gastrin has long been considered a growth stimulatory hormone for mucosa of the gastrointestinal tract^[1]. The growth responses of certain colorectal cancer cells, and xenografts, can be stimulated by endogenous gastrin^[2]. Protein kinase C (PKC) is a family of isozymes that plays a crucial role in transducing signals of many hormones, growth peptides, neurotransmitters, and its activation is crucial in tumor promotion^[3]. PKC is also involved in regulating cellular proliferation^[4]. On the basis of gastrin's effect on the growth of human colon cancer cell, we further studied its effect on PKC and its subtype so as to elucidate the molecular basis in signal transduction mechanisms regulating SW480 cell proliferation and provide an experimental evidence of antesignaling therapy for patients with colon cancer.

MATERIALS AND METHODS

Materials

Pentagastrin (PG) was purchased from Shanghai Li Zhu Biochemical Co., proglumide (PGL) from Shanghai No.11 Pharmaceutical Factory, PS, Histone III-s from Sigma Chemical Co., and γ -³²P-ATP from Beijing Yahui Co. Human colon cancer line SW480 was obtained from Silon-kittelng Cancer Center (American), and PKC- α , β , γ cDNA probes from Biological Department of Beijing Teacher's University. Dig-labelling and detection kit was

purchased from Boehringer Mannheim Co.

Methods

Cell in culture Cells were maintained in culture medium (RPMI 1640), containing 100 mL/L fetal calf serum in a humidified atmosphere of 950 mL/L air and 50 mL/L CO₂ at 37 °C.

Extraction of PKC from SW480 Cells were incubated with or without stimulating agents at the desired time intervals (1 min, 2 min and 5 min), and the reaction was terminated by the rapid addition of an ice cold homogenizing buffer. The preparations were centrifuged at 200×g for 5 min at 4 °C. The supernatants were discarded and the pellets were suspended in chilled buffer and were sonicated on ice for 45 min at 4 °C. The resulting supernatants (cytosolic fraction) were removed and kept on ice. The pellets were resuspended and centrifuged at 100 000×g for 45 min at 4 °C, and the supernatant represented the membrane fractions. The cytosolic or membrane fractions were used directly.

Measurement of PKC activity PKC was assayed by measuring the incorporation of ³²P from [γ -³²P] ATP into a specific peptide substrate (Sigma). The reaction was then terminated, and aliquots from each sample were spotted on specific binding papers. The papers were allowed to soak, and then were washed twice individually and processed for counting as described previously^[5]. Protein content of each sample was measured by the Bradford method, using bovine serum albumin as a standard^[6]. The assays were constantly made for linear range of enzyme activity with regard to protein concentration and incubation condition. Assays were performed in duplicate and the results were expressed as picomoles of ³²P incorporated in the peptide substrate/minute/mg protein.

Preparation of in situ hybridization samples Cells (approximately 1×10⁶/mL), which were plated and cultured in 24-well tissue culture plates (each well had a coverslip on the bottom) for 48 hours, and were incubated in the presence of PG (25 mg/L) for 24 hours. Control group cells were incubated with RPMI1640 containing 10% FCS instead of PG. Cells were incubated for 24 h, then the supernatants were discarded. The remains were washed with D-Hanks buffer for three times, fixed

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with formaldehyde for 5 min and stored in 4 °C ice-box.

Dig-labelling and detection of probe The probe was labelled by random hexade primer (RHP) method using Dig-labelling and detection kit-(Boehringer Mannheim Co.)

In situ hybridization of samples The procedures with some modifications were mainly as follows^[7]: the samples were baked at 85 °C for 5 min, deparaffinized and rehydrated in graded alcohol, then put into 200 mL/L cold acetic acid and at 4 °C for 20 min. Hybridization buffer with the concentration of the probe (1mg/L) was added to the slides and incubated at 42 °C for 20 h. After washed thoroughly, the slides were darkly developed with NBT-BCIP at room temperature for 0.5 h-1 h, and then slightly stained with 0.5% methyl green, and mounted by glycerine-gelatin.

Controls The slides were treated with 20 mg/L RNase at 37 °C for 30 min before hybridization. No probe was put into the prehybridization buffer.

Statistical analysis

A one-way analysis of variance (ANOVA, Excel 6.0 band) was used to test for statistical significance between paired or unpaired data. All values were expressed as mean±SEM and $P<0.05$ was considered statistically significant and $P<0.01$ was considered as very significant difference.

RESULTS

Effect of gastrin on PKC activity

We examined the effect of gastrin on PKC activity (including cytosol and membrane) in SW480 cells for the desired time intervals. The PKC activity of membrane was increased obviously and the PKC activity of cytosol was decreased when cells were treated by PG for 1 min. After 2 min, the PKC activity of membrane and cytosol was still higher than in control group, and after 5 min it turned basically to the previous level (Table 1).

Effect of proglumide on PKC activity

To investigate whether PKC activity is part of a receptor-mediated pathway, cells were incubated with the gastrin receptor antagonist PGL. The result shows that PGL (32 mg/L) almost abolished the effect of PG (25 mg/L) on PKC activity in SW480 cell (Table 2).

Effect of gastrin on expression of PKC- α , β , γ mRNA

Expression of PKC- α , β , γ -mRNA all existed in SW480 cells at some extent, but after PG treatment, the expression of PKC- β mRNA was increased more than that of α , γ mRNA (Figures 1-4).

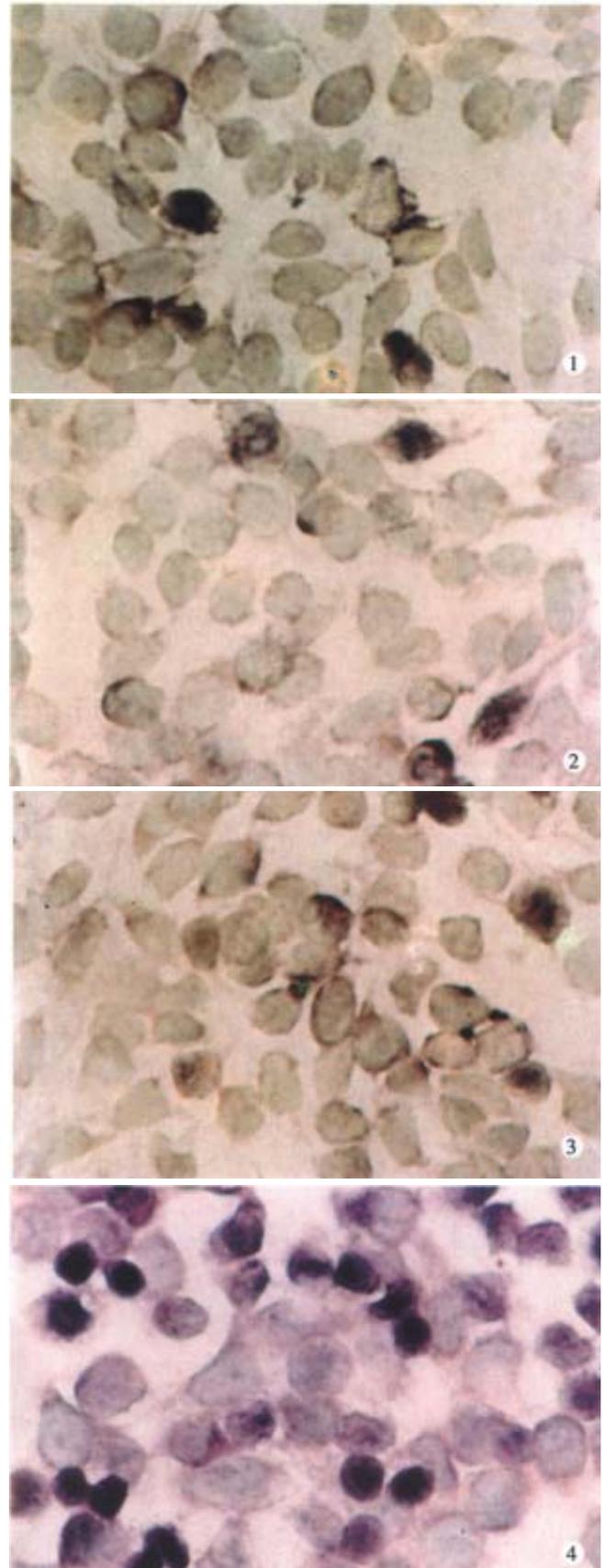


Figure 1 Expression of α -mRNA in PG group. ISH×200
Figure 2 Expression of γ -mRNA in PG group. ISH×200
Figure 3 Expression of β -mRNA in control group. ISH×200
Figure 4 Expression of β -mRNA in PG group. ISH×200

Table 1 Effect of gastrin on protein kinase C activation ($\bar{x}\pm s$)

	Protein kinase C (pmol·min·mg protein)	
	Cytosol	Membrane
Control	2.39±1.30	1.07±0.28
Gastrin (25 mg/L)	0.91±0.34 ^a	2.65±1.21 ^a
Gastrin (25 mg/L) +proglumide (32 mg/L)	2.30±0.61	1.05±0.20

Note: ^a $P < 0.05$ vs control ($n = 5$).

Table 2 Time-dependent effect of gastrin on protein kinase C activation

	Protein kinase C (pmol·min·mg protein)	
	Cytosol	Membrane
Control	2.39±1.30	1.07±0.28
PG (25 mg/L)		
1 min	0.91±0.34 ^a	2.65±1.21 ^a
2 min	1.44±0.34	1.87±0.57 ^a
5 min	2.28±0.49	0.98±0.18

Note: ^a $P < 0.05$ vs control ($n = 5$).

DISCUSSION

It is well known that the stimulation of PKC is a critical step in signal transduction pathways, and the translocation of PKC from cytosol to membrane was recognized as a symbol of PKC activation. Gastrin can increase the translocation effect^[8,9]. Differences in the distribution of PKC activities were reported in certain cultured cell lines, such as human stomach, colon and breast cancers due to the expression of various PKC subtypes^[10]. It is clear that cell growth is regulated by various growth factors through their specific receptors and receptor-linked signal transduction pathways^[11], such as the cAMP pathway, the tyrosine kinase pathway, PI hydrolysis, or mobilization of intracellular calcium. It has been observed that gastrin exerted a growth-promoting effect on colonic epithelial cells *in vitro*^[5], and the effect was mediated by the activation of PKC. But the mechanisms of gastrin as to how to regulate growth have not been clarified.

Our data showed that PG decreased the activity of PKC in the cytosol of cancer cells, while increased the activity of PKC in the membrane, and that the effect of PG on PKC possessed time-dependent character. In this experiment, we have also demonstrated that PGL, a gastrin receptor antagonist, blocked the effect of PG on PKC activation. The result indicated that PG stimulated PKC through a specific receptor-mediated pathway. It is clear that the signal pathway through PKC appears to be absolutely necessary for the control of gene expression and the cell cycle in the nucleus^[12]. Persons *et al*^[13] reported that transfected NIH3T3 cells carrying high-level expression vectors of rat *bra* in PKC-1cDNA displayed enhanced tumorigenicity when inoculated into nude mice. Yang XH *et al*^[14] reported that overexpression of PKC- β resulted in a high rate of cell growth. To further elucidate the role of specific PKC subtype in growth control and tumor promotion, we have

observed the effect of gastrin on expression of PKC- α , β , γ mRNA. The result showed that the differences among expression of PKC- α , β , γ mRNA originally existed in SW480 cells, but after gastrin treatment, the expression of PKC- β mRNA was increased more than that of PKC- α , γ mRNA. These results indicated that PKC- β may play a critical role in cell proliferation.

Chemo-endocrine chemotherapy with gastrin receptor antagonist, such as proglumide, can prevent the recurrence after resection of liver metastasis in colorectal cancer^[15]. In accordance with the role of PKC in gastrin stimulating cancer cells proliferation, PKC antagonists, such as staurosporine and bryostatin, have been used to treat colon cancer. So we can design and choose "signal drugs" for antisignaling therapy for patients with colon cancer.

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Endoscopic appearance of esophageal hematomas

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Subject headings esophagus; hematoma/etiology; endoscopy; gastrointestinal hemorrhage/diagnosis; hematoma/classification; wounds; injuries

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INTRODUCTION

Esophageal hematomas develop from the dissection of the mucosa from the muscular layers of the esophageal wall and represent an uncommon condition affecting all ages^[1-3]. Although the most common cause of esophageal hematomas is iatrogenic mechanical injury-induced by prolonged nasogastric intubation, difficult or forceful endoscopic intubation, or the result of variceal injection sclerotherapy—some may be spontaneous, particularly in patients receiving anticoagulants^[3-6]. Presenting symptoms most commonly include dysphagia, hematemesis, and sub-sternal or epigastric pain^[5,9].

In this report, we present four cases of esophageal hematomas diagnosed endoscopically, describe their clinical and endoscopic characteristics and propose a classification. As our cases exemplify, therapy is conservative and a favorable prognosis is the rule. Although the diagnosis has traditionally been made by barium esophagography^[1] or CAT scanning^[1,10], the increased use of endoscopy will allow increased recognition of esophageal hematomas even at a subclinical (asymptomatic) stage and ultimately lead to a better understanding of their etiopathogenesis and their prevention.

CASE REPORTS

Case 1 A 90-year-old man with a history of

hypertension, congestive heart failure, and mild aortic stenosis was referred for upper endoscopy to determine the cause of iron deficiency anemia. A colonoscopy had previously revealed diverticular disease. Laboratory values included a hematocrit of 30%, iron of 90 mg/L and a TIBC of 358. Coagulation studies were normal. Endoscopy revealed salmon-colored mucosa extending from 28 cm-34 cm from the incisors, consistent with Barrett's esophagus. A densely red, elevated lesion, consistent with a hematoma was noted at 19 cm (Figure 1a). No therapy was given. A follow-up endoscopy, performed one week later, showed complete resolution of the hematoma. Because of its location, the hematoma was attributed to a difficult, possibly traumatic, endoscopic intubation.

Case 2 A 59-year-old man was referred for upper endoscopy because of chronic reflux symptoms. His medications included omeprazole 20 mg po-daily, and aspirin 325 mg po-daily. Endoscopy revealed confluent esophageal erosions and ulcers consistent with Savary stage III esophagitis from 36 cm-33 cm. Upon biopsy of the esophagus, a purple-red, elevated lesion, consistent with a hematoma was noted at 35 cm (Figure 1b). Histologic examination of the biopsy sample revealed changes consistent with reflux esophagitis. The hematoma was attributed to the mucosal biopsy obtained in the context of aspirin intake.

Case 3 A 90-year-old man with solid food dysphagia was referred for esophageal dilation. He had a history of metastatic (stage 4) squamous esophageal cancer treated with radiation therapy and esophageal dilation. He had mild anemia, but no thrombocytopenia or coagulation abnormalities. Endoscopy revealed a mid-esophageal stricture at 5 cm-29 cm which was dilated with minimal resistance using Savary dilators over a guide wire. This resulted in a 1 cm-long submucosal hematoma at 26 cm (Figure 1c). No biopsy was obtained. Because the patient's dysphagia resolved, there was no follow-up endoscopy.

Case 4 A 51-year-old male with a history of rheumatic heart disease, status post aortic valve replacement, was hospitalized for mitral valve replacement. Because his postoperative course was complicated by enterococcal sepsis, a transesophageal echocardiogram was performed which excluded the presence of endocarditis. The

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following day, hematemesis was noted. Coagulation parameters were within the expected therapeutic range for prosthetic valves. An emergency upper endoscopy revealed a 3 cm-long, dark red esophageal mass at the GE junction, completely obliterating the esophageal lumen (Figure 1d). Biopsies obtained from the surface of the mass revealed organized clot and acute esophageal mucosal inflammation (not shown). Broad spectrum antibiotics and H₂ receptor antagonists were administered intravenously; effective anticoagulation was also continued. Although there was no more evidence of gastrointestinal hemorrhage, the patient eventually succumbed to sepsis ten days later. Autopsy revealed severe herpetic tracheobronchitis without any evidence of esophageal injury.

DISCUSSION

Esophageal hematomas appear as raised purplish-red lesions, mostly sub-mucosal in location, but occasionally obliterating the esophageal lumen. Depending on their etiology, they may be classified as spontaneous or traumatic. Based on the above observations, we propose that esophageal hematomas can be further classified according to the degree of involvement of the lumen in four stages (Table 1). Esophageal hematoma formation^[11,12], is an uncommon condition in which an intramural hemorrhage leads to a variable degree of submucosal dissection of the esophageal wall ranging from single or multiple localized hematomas to complete dissection of the esophagus^[1]. Esophageal hematomas may be spontaneous, associated with sudden changes in the transmural wall pressures due to a variety of causes including coughing, retching or protracted vomiting^[1,3], or may result from iatrogenic instrumentation, such as esophageal variceal sclerotherapy^[3,14,15] or transesophageal ultrasonography^[6] (Table 2). Hematomas may also be associated with direct trauma from abrasive foodstuffs such as taco shells^[16], pill-induced esophageal injury^[17], or with a coagulopathy, with little or no history of esophageal barotrauma^[5]. Other probable causes include esophageal stricture, diverticulum, esophageal arteriovenous malformation, and aspirin use^[18]. The majority of patients with esophageal hematomas are older than 70 yr, although isolated reports of patients as young as 21 yr have been made. In some studies, there seems to be a predilection for the female gender^[1].

Table 1 Stages of esophageal hematomas

I: Hematoma without surrounding tissue edema
II: Hematoma with surrounding tissue edema
III: Hematoma with edema plus compression of esophageal lumen
IV: Complete obliteration of the lumen with hematoma, edema, and organized clot formation

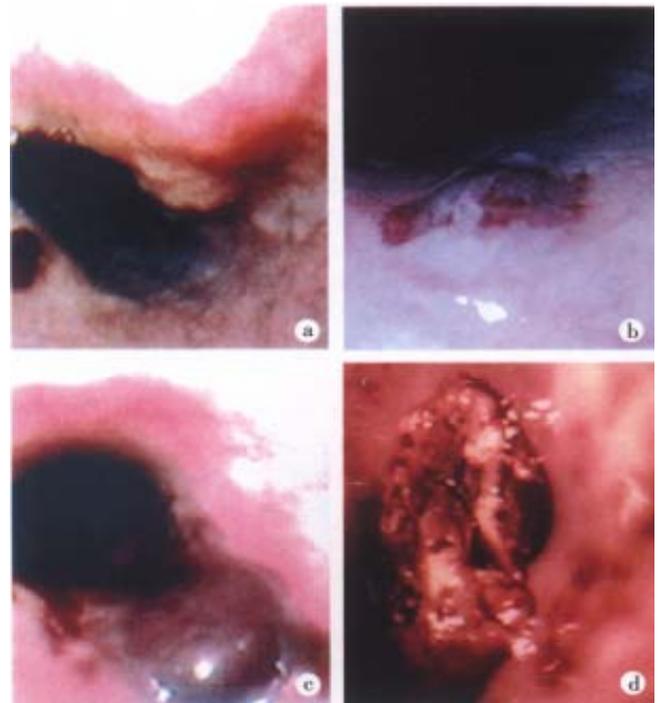


Figure 1 Endoscopic appearance of esophageal hematoma as. a: Stage I esophageal hematoma. There is no surrounding edema. Patient was asymptomatic. b: Stage II esophageal hematoma after an esophageal biopsy. Note the surrounding tissue edema. c: Stage III hematoma induced by esophageal dilation of a stricture. There is edema and separation of the surface mucosal layer that is partially obliterating the lumen. Patient was asymptomatic. d: Stage IV esophageal hematoma related to recent transesophageal echocardiography. Edema and clot formation led to complete obliteration of the esophageal lumen and hematemesis.

Table 2 Causes of esophageal hematomas

Spontaneous	Associated with coughing, retching, protracted vomiting Esophageal barotrauma Pill-induced esophageal injury Use of anticoagulants or aspirin Esophageal diverticulum A-V malformation
Traumatic	Endoscopic intubation, biopsy, dilation Variceal sclerotherapy Transesophageal echocardiography Foreign body ingestion

The clinical presentation is variable. Although mostly asymptomatic, esophageal hematomas may present with dramatic chest pain, severe dysphagia, and hematemesis. In such instances, they should be differentiated from Mallory-Weiss tear and Boorhaave's syndrome, conditions which they may closely mimic^[19-21]. Typically barium swallow or CAT scan have been used for the diagnosis, showing intraluminal filling defects or a double-barrelled appearance of the esophagus^[22]. More recently, endoscopy is the preferred investigation, especially when hematemesis is the presenting symptom^[23]. Because spontaneous resolution is the rule, the treatment of esophageal hematomas is conservative

and consists of a regimen of nil by mouth, intravenous alimentation and antibiotics in severe cases^[1]. Surgery is reserved only for rare instances, to drain a hematoma and close an esophageal mucosal tear^[24].

In our study, all four patients were male, the hematoma was visualized endoscopically, and was traumatic either from insertion of the scope (cases 1 and 4), or from endoscopic manipulation (cases 2 and 3). In 3 out of 4 of our patients the hematomas were asymptomatic. Because of the increased utilization of endoscopy for diagnosis and therapy, it is expected that esophageal hematomas will be encountered more frequently. This is particularly true in cases of esophageal dilation of strictures, repetitive multiple biopsies for surveillance of Barrett's esophagus, esophageal variceal sclerotherapy, and transesophageal echocardiography.

Hematomas related sclerotherapy seem to occur 2 d-4 d after injection, when tissue necrosis that extends into the submucosa reaches its maximum. Contributing factors include volume of sclerosant per injection, interval between treatments, paravariceal versus intra-variceal injection, bleeding diathesis, and occurrence of retching or prolonged Valsalva during or shortly after the treatment^[3-5]. Early endoscopy is useful in establishing the diagnosis and reveals an intraluminal bulge of the mucosa with dark blue discoloration.

The blind insertion of a transesophageal endosonographic probe for diagnosis of valvular heart disease^[25], and its increased utilization during cardiothoracic surgery^[6] represents an additional risk for esophageal hematoma formation, as exemplified in case 4 of this report. A recent review of the literature on the association of transesophageal echocardiography during cardiac surgery and gastrointestinal bleeding revealed that the overall percentage of postoperative gastrointestinal complaints following the procedure was 11%, and frank upper gastrointestinal bleeding was 2.1%^[6]. Another multicenter survey of 10419 examinations on the safety of transesophageal echocardiography revealed only 2 patients who developed bleeding complications which necessitated interruption of the examination^[7]. Realising that the risk of gastrointestinal bleeding is small but present, most authors recommend careful review of upper digestive symptoms to rule out preexisting esophageal pathology, ample lubrication of the endosonographic tip, and avoidance of fixation of the probe in a flexed position for prolonged periods in order to avoid contact pressure and esophageal mucosal damage^[8].

In conclusion, our study reviews the endoscopic features of esophageal hematomas and classifies

them in terms of etiology and appearance. With the widespread use of esophageal instrumentation, these lesions will be increasingly recognized endoscopically. Depending on their severity and endoscopic stage, esophageal hematomas may or may not be associated with symptoms. Treatment is generally conservative and favorable prognosis is the rule.

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Gastroesophageal reflux and *Helicobacter pylori*: a review

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Subject headings gastroesophageal reflux/therapy; *Helicobacter pylori*; epidemiology; peptic ulcer/therapy; stomach neoplasms/therapy; *Helicobacter* infections

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INTRODUCTION

Since the observation by Labenz *et al* that eradication of *Helicobacter pylori* (*Hp*) infection may be followed by development of reflux esophagitis in a relevant proportion of duodenal ulcer patients previously not affected by gastroesophageal reflux disease (GERD)^[1], a growing attention has been given to the potential interactions between *Hp* and GERD. Epidemiological studies have now demonstrated that the prevalence of GERD is steadily increasing in the developed countries^[2], as is the incidence of adenocarcinoma of the esophagus^[3], its most dangerous complication, while the prevalence of peptic ulcer and gastric cancer is falling^[4], in parallel with a falling prevalence of *Hp* infection in the western countries^[5]. It is therefore tempting to causally relate these phenomena. Despite the number of original papers and of reviews dealing with this topic, at least 3 issues are still debated: ① Does *Hp* infection interfere with the pathogenesis of GERD? ② Is the anti-secretory effect of *Hp* infection of any clinical relevance in the management of GERD patients? ③ Does long-term proton pump inhibitors (PPI) therapy accelerate development of atrophic changes in *Hp* +ve GERD patients? Finally, the relationship(s) between *Hp* and Barrett's esophagus may deserve some importance.

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The present review will focus on these 4 issues. The interested reader may also refer to some recent papers, dealing with the same subject^[6-9].

Hp AND PATHOGENESIS OF GERD

Several studies have now convincingly shown that the prevalence of *Hp* infection in patients with reflux esophagitis is somewhat lower than in normal subjects; in a careful review of 26 papers on this topic, O'Connor summarizes the data existing as follows: the overall prevalence of *Hp* infection in 2182 adult GERD patients is 40.3%, as compared with 50.2% in the 2010 controls^[9]. He concludes that this "difference in prevalence (is) intimating that the pathogenesis of GERD might be related in some way to the absence of *Hp*". In our view, more simplistically, the only link between *Hp* infection and GERD lies on the degree of gastric acid secretion, and through this, on esophageal acid exposure. In patients with a predisposition to GERD but without a clinical manifestation of GERD (symptoms and/or esophageal lesions), eradication of *Hp* may trigger it, disclosing the clinical picture. On the contrary, patients harboring the infection, may be protected if the infection involves the corpus (i.e. the acid-producing part of the gastric mucosa), because the amount of acid secretion and hence the esophageal acid exposure is reduced. In fact, no single paper has ever been published so far focusing on *Hp* infection as a pathogenetic (aggressive or defensive) factor of GERD-perse. On the contrary, El-Serag *et al* have now clearly demonstrated that, for the above reasons, corpus gastritis is protective against reflux esophagitis^[10]. They have investigated 302 subjects, 154 of whom with endoscopic signs of esophagitis; there was no difference between patients with and controls without esophagitis in the overall infection rates with *Hp* infection. Compared with controls, corpus gastritis was less frequent and less severe in patients with esophagitis. Finally, in a multivariate logistic analysis, age, sex smoking status, and the presence of chronic corpus gastritis exerted a significant influence on the presence of reflux esophagitis. This latter variable, however, showed an odds ratio of 0.46% only (95% confidence interval of 0.27-0.79), a value which is, albeit statistically significant, of doubtful clinical relevance.

In summary, the pathogenetic relationship

between *Hp* infection and GERD are probably weak and of indirect nature, being related to the amount of gastric acid secretion, a factor which is necessary but not indispensable for inducing GERD. The most relevant GERD pathogenetic factor is, as universally known, the occurrence of transient relaxation of the lower esophageal sphincter^[11], a factor which has not, to the best of our knowledge, been observed to be influenced by *Hp* gastric infection.

Is the antisecretory effect of Hp infection of any clinical relevance in the management of GERD patients?

A profound inhibition of acid secretion is the mainstay of treatment for reflux esophagitis, in particular in cases of moderate to severe RE^[12]. Therefore, the influence of *Hp* on the efficacy of acid-lowering treatment may be important for patients with RE. Verdu *et al*^[13] showed that omeprazole produces a greater decrease in gastric acidity in subjects with *Hp* infection than in those who are *Hp* negative, and that omeprazole produces a smaller decrease in gastric acidity after *Hp* infection has been cured^[14]. Similar findings have been obtained by Labenz *et al*^[15], who showed that in 17 FU patients, *Hp* eradication resulted in a marked decrease of the pH-increasing effect of omeprazole (24h median gastric pH: 5.5 vs 3.0, $P < 0.002$) that was most pronounced during night time. Base line intragastric pH remained unchanged after eradication (median gastric pH: 1.0 vs 1.1, $P = 0.05$). The same authors have also shown that this effect persisted for at least 1 year after *Hp* eradication^[16], whereas others have been shown that it is shared by other PPIs, such as lansoprazole^[17].

Despite this *Hp* mediated exaggeration of the effect of acid-suppressive drugs on intragastric pH is clearly proven, there is little evidence that this effect has any clinical relevance for the treatment of GERD patients with PPI. One reason is that the effect, due to the logarithmic scale of pH, a variation of one pH unit from 5 to 6 is 10 000 times less important than a variation from 1 to 2. The small variation in acid secretory capacity due to *Hp* colonisation is only "visible" when the acid secretion is already potentially reduced by PPI, but is otherwise unimportant.

Direct evidence shows in fact that, during acid suppressive therapy with ranitidine or omeprazole, *Hp* +ve or -ve GERD patients show a similar reduction of esophageal acid exposure, the entity of which is only influenced by the type of drug received^[18]. Furthermore, both groups of GERD patients require the same dose of omeprazole during long-term maintenance treatment to prevent symptomatic and endoscopic relapse^[19], and *Hp* status seems not to be an important prognostic

factor during long-term maintenance therapy with PPI; in a study conducted on 103 patients with RE grade 1 or 2, randomized to maintenance therapy with lansoprazole 15 or 30 mg daily for 12 months, it was observed that *Hp* infected patients relapsed as early as patients who were not infected^[20].

The only discordant piece of evidence comes from the very large study of Holtman *et al*^[21], who claims of a significantly better acute response of *Hp* +ve GERD patients treated with the PPI pantoprazole in comparison to *Hp* -ve; however, the difference of healing rates between the two groups after 8wk of 40mg daily was quite small (96.4% vs 91.8%, $P < 0.05$) and no difference at all was observed in GERD symptoms between infected and noninfected patients. There is therefore enough evidence to say, at least, that PPI maintenance therapy does not need to be titrated upon *Hp* status^[19]. It is therefore to be fully agreed upon the recommendation that "testing for *Hp* infection is not indicated in patients on long term treatment or in those considered for treatment with a proton pump inhibitor for GERD", as stated by the recent guidelines of the American College of Gastroenterology^[22].

Does long-term proton pump inhibitors (PPI) therapy accelerate development of atrophic in Hp +ve GERD patients?

Several studies have shown that treatment with PPI is associated with the worsening of gastritis (increase in severity score, spreading from the antrum to corpus and fundus)^[23-25]. Because superficial corpus gastritis may lead to atrophic gastritis, the increased body inflammation in *Hp* positive patients observed during short term PPI therapy may lead to atrophic gastritis during long term PPI treatment. This has been observed so far after omeprazole administration^[26], but the study was criticized in particular for the incorporation of an inappropriate control group^[27]. Moreover, the findings have not been confirmed by a randomized Swedish study comparing the efficacy of omeprazole maintenance treatment and antireflux surgery over a 3-years follow-up^[28]. Thus, on the basis of available evidence, long-term treatment with PPI up to 10 years appears to be a perfectly safe therapy^[29].

Hp INFECTION AND BARRETT'S ESOPHAGUS

The interest in BE is still growing since the early description of this entity in 1950^[30] for two main reasons: ① BE is associated with GERD, and also with an increased risk of adenocarcinoma^[31], thus representing a link between a common benign condition and a rare very malignant disease; ② The incidence of adenocarcinoma of the esophagus and cardia is increasing at the fastest rate among gastrointestinal (and also non GI) human

cancers^[3]. Since *Hp* exhibits a special affinity for gastric-type epithelium, and since *Barrett's* metaplasia contains columnar-lined epithelium, it is to be expected that *Hp* will also be able to attach the *Barrett's* epithelium, at least of the gastric type, independently from any involvement of *Hp* infection in the pathogenesis of esophageal mucosal inflammation.

It seems that the prevalence of *Hp* infection of the stomach in BE patients is not different from that exhibited by controls, roughly one third of the subjects^[9]. The colonization of metaplastic epithelium by the bacterium has been tested only in a minority of studies, but appears to be marginally lower^[9]. It seems therefore that the stomach represents the primary site of infection, with secondary colonization of columnar mucosa in the esophagus. Furthermore, most *Hp* positive patients show a very low bacterial load in their metaplastic epithelium, and no significant difference has been found in the severity of inflammatory changes between *Hp* +ve and *Hp* -ve *Barrett's* esophagus patients^[32]. Finally, recent work has confirmed that within the esophagus, *Hp* adheres only to gastric type metaplasia, which is not considered premalignant for adenocarcinoma^[33]. In conclusion, it is most probable that *Hp* has no etiologic role on the development of *Barrett's* esophagus, nor in the esophagitis associated with this metaplastic change; the colonization of *Barrett's* epithelium probably reflects only a shift from gastric antrum.

Another intriguing point is the prevalence of *Hp* infection and the intestinal metaplasia of the gastric cardia. It is in fact at present not known whether inflammation of the cardia indicates GERD and/or is a manifestation of gastritis caused by *Hp*. Recently two studies have shed some light on this issue^[34,35]: in the first, biopsies were obtained from the antrum, corpus and cardia from 135 *Hp*-infected patients with gastritis, ulcer disease, or RE. One hundred and thirty-two (97.7%) of them showed active carditis, resembling antral gastritis in most patients, but with less marked bacterial density and inflammatory process^[34]. The authors conclude that *Hp* gastritis commonly involves the cardia, that intestinal metaplasia in the cardia is a common finding in *Hp* gastritis, but that the cardia lower histologic density of the bacteria and inflammatory responses in comparison to the antrum are not clear. In the second work^[35], 22 GERD patients and 11 controls were compared in relationship to endoscopic and bioptic evaluation of inflammation, *Hp* infection and intestinal metaplasia in distal esophagus, cardia, fundus and antrum. It turned out that neither the prevalence of *Hp* infection (controls 48%; GERD 41%) nor cardia inflammation (controls 41%; GERD 40%) differed between the two groups. All 11 controls and 22 of

23 (96%) patients with GERD and cardia inflammation had *HP* infection. Cardia intestinal metaplasia was more common among controls (22%) than among GERD patients (3%, $P \leq 0.01$); all patients with cardia intestinal metaplasia had cardia inflammation, 7 had *Hp* infection, and 6 had metaplasia elsewhere in the stomach. The authors conclude that the prevalence of cardia inflammation is similar in patients with and without GERD, and is associated with *Hp* infection. Also, in this study, cardia intestinal metaplasia is associated with *Hp* related cardia inflammation ($P = 0.01$) and intestinal metaplasia elsewhere in the stomach, indicating that it is distinct from *Barrett's* esophagus.

The final point is the association, if any, between *Hp* infection and *Barrett's* associated adenocarcinoma. Again, two recent works have contributed to the improvement of our knowledge on this previously uninvestigated issue^[36,37]. Quddus *et al* report on 19 cases of adenocarcinoma arising in BE, who were examined for the presence of *Hp* after staining with three different techniques: all sections of BE, with or without dysplasia, adenocarcinoma and stomach (when available) were uniformly negative for the presence of *Hp*. The authors conclude that neither gastric nor esophageal infection with *Hp* is a requisite for the development of adenocarcinoma in BE^[36].

The second study aimed at comparing the prevalence of *Hp* and increasing grades of dysplasia. Biopsies from 19 malignant and 94 benign cases of BE were analysed histologically for *Hp*; 34% of non-dysplastic *Barrett's* epithelium was colonized with *Hp* compared with only 17% of dysplastic/malignant-cases ($P = 0.04$). No relationship was found between *Hp* status and ① length of BE; ② the presence of strictures or ulcers; ③ previous anti-reflux surgery. The authors therefore confirmed that *Hp* colonization of BE is not particularly common, and that a negative correlation exists with increasing severity of dysplasia^[37].

To summarize, from both studies it appears that it is unlikely that a causal relationship exists between *Hp* infection and *Barrett's* associated adenocarcinoma.

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Management of difficult inflammatory bowel disease: where are we now?

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Subject headings inflammatory bowel diseases/therapy; colitis/therapy; Crohn disease/therapy; endoscopy, gastrointestinal; social support; azathioprine

Rampton DS. Management of difficult inflammatory bowel disease: where are we now? *World J Gastroentero*, 2000;6(3):315-323

INTRODUCTION

Medical care of patients with inflammatory bowel disease (IBD) comprises general measures and specific pharmacological, nutritional, endoscopic and surgical therapies (Table 1)^[1-3]. In this paper, current management options for patients with two commonly difficult presentations of IBD, acute severe ulcerative colitis (UC) and steroid-refractory or dependent ileocaecal Crohn's disease (CD), are discussed. Practical considerations and newer developments are emphasized.

MANAGEMENT OF ACUTE SEVERE ULCERATIVE COLITIS

These patients should be admitted immediately to a gastroenterology ward for close joint medical, surgical and nursing care. The nutrition team and a stoma therapist in patients likely to need surgery should be involved promptly. Patients undergoing an acute attack of UC need to be made aware from the outset that they have a one in four chance of failing to respond to the primary treatment (intravenous steroids), and thus need either cyclosporin or colectomy during their admission (Table 1).

Establishing the diagnosis, extent and severity of disease

A carefully targeted history and appropriate investigations can help establish the diagnosis (Table 2) in patients presenting for the first time and, in those with established UC, to exclude infection and to assess disease extent (if not already known) and severity.

Blood and stool tests Stool should be sent to look for

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pathogens, and serology checked for amoebiasis, strongyloidiasis and schistosomiasis. Blood tests are better for establishing the activity of UC than making the diagnosis or identifying its extent. However, a raised platelet count is more common in UC than in infective colitis. The best measures of disease activity are haemoglobin, platelet count, ESR, C-reactive protein^[4] and serum albumin.

Sigmoidoscopy and rectal biopsy Cautious rigid or flexible sigmoidoscopy in the unprepared patient, and without excessive air insufflation, provides immediate confirmation of active colitis. Sigmoidoscopy also allows biopsy for histology: to minimise the risks of bleeding and perforation a small superficial biopsy should be taken from the posterior rectal wall less than 10 cm from the anal margin using small-cupped forceps. Anecdotally, colonoscopy may cause colonic perforation and dilatation in acute severe UC, and although some authorities have reported that it is both safe and useful for decision-making^[5], most patients can be managed satisfactorily without it. In patients with established UC, rectal biopsy is not routinely necessary. However, in those presenting for the first time, infective colitis may be suggested by an acute, focal and superficial inflammatory infiltrate with minimal goblet cell depletion and preservation of crypt architecture^[6]. Although colitis due to *Clostridium difficile*, cytomegalovirus, amoebiasis and *Crohn's* disease often has characteristic macroscopic appearances, histology may confirm these diagnoses.

Plain abdominal X-ray A plain film at presentation can be used to assess disease extent, since faecal residual visible on X-ray usually indicates sites of uninfamed colonic mucosa. Plain abdominal X-ray is also used to assess disease severity and in particular to exclude colonic dilatation (diameter > 5.5 cm) in sick patients, however, the gas pattern on a plain film may be misleading if there has been excessive air insufflation during a sigmoidoscopy or colonoscopy done shortly beforehand. In patients with suspected colonic perforation, the diagnosis can be confirmed by erect chest X-ray or a lateral decubitus abdominal film.

Radiolabelled leucocyte scans The intensity and extent of colonic uptake one hour after injection of autologous ⁹⁹Tc-HMPAO or ¹¹¹Indium-labelled leucocytes provides information about disease

activity and particularly extent, respectively, where doubt exists in patients with UC. Colonic uptake of leucocytes is not of course specific for UC and positive results are obtained in other inflammatory colonic diseases.

Table 1 Principles of management of acute severe ulcerative colitis

GENERAL MEASURES	
Explanation, psychosocial support	- patient support groups
Specialist multidisciplinary care	- physicians, surgeons, nutrition team, nurses, stoma therapist, counsellor
ESTABLISHING THE DIAGNOSIS, EXTENT/SITE AND SEVERITY	
	- clinical evaluation
	- FBC, ESR, C-reactive protein, albumin, LFTs, amoebic serology
	- stool microscopy, culture, C. difficile toxin
	- limited sigmoidoscopy and biopsy
	- plain abdominal X-ray
	- consider radiolabelled leucocyte scan
MONITORING PROGRESS	
	- daily clinical assessment
	- stool chart
	- 4-hrly temperature, pulse
	- daily FBC, ESR, C-reactive protein, urea and electrolytes, albumin
	- daily plain abdominal X-ray
SUPPORTIVE TREATMENT	
	- i.v. fluids, electrolytes (Na, K), blood transfusion
	- nutritional supplementation
	- heparin s.c.
	- haematinics (folate)
	- avoid antidiarrhoeals (codeine, loperamide, diphenoxylate), opiates, NSAIDs
	- rolling manoeuvre (if colon dilating)
SPECIFIC TREATMENT	
Medical	- corticosteroids i.v. (hydrocortisone or methylprednisolone) then p.o. (prednisolone)
	- continue 5-ASA p.o. in patients already taking it; otherwise start when improvement begins
	- antibiotics for very sick febrile patients, or when infection suspected
	- consider cyclosporin i.v. then p.o.) for steroid non-responders at 4-7 days
Surgical (for non-responders at 5-7 days, toxic megacolon, perforation, massive haemorrhage)	- panproctocolectomy with ileoanal pouch or permanent ileostomy
	- subtotal colectomy with ileorectal anastomosis (rarely)

Table 2 Management of active ileocaecal Crohn's disease. General measures, monitoring progress and supportive treatment are essentially as for ulcerative colitis

ESTABLISHING THE DIAGNOSIS, EXTENT/SITE AND SEVERITY	
	- clinical evaluation
	- FBC, ESR, C-reactive protein, ferritin, folate, B12, albumin, LFTs, Ca, Mg, Zn
	- stool microscopy, culture, C difficile toxin
	- plain abdominal X-ray
	- consider colonoscopy and biopsy, small bowel barium radiology, ultrasound, CT, MRI, leucocyte scan
SPECIFIC TREATMENT (separately or in combination)	
Medical	- corticosteroids i.v. (hydrocortisone or methylprednisolone) then p.o. (prednisolone or budesonide CR)
	- continue high dose mesalazine (Pentasa or Asacol) in patients already taking it; otherwise start when improvement begins
	- consider metronidazole, ciprofloxacin; also broad spectrum antibiotics for very sick febrile patients, or when infection/collection suspected
	- consider azathioprine/6-mercaptopurine (slow response) or anti TNF antibodies (infliximab) for steroid non-responders
Nutritional	- liquid formula diet
Endoscopic	- balloon dilatation
Surgical	- resection or stricturoplasty

Monitoring progress

Progress is monitored by twice daily clinical assessment, stool chart and 4-hourly measurement of temperature and pulse. Blood count, ESR, C-reactive protein, routine biochemistry and plain abdominal X-ray should be done daily in sick patients. The two most useful variables in predicting the outcome of the acute attack are stool frequency and C-reactive protein at three days: patients with values above 8 stools/day or 45 mg/L, respectively, have an 85% chance of failing to respond to intravenous steroids and needing cyclosporin or surgery during their admission^[4] (Table 1).

Supportive treatment

Intravenous fluids and blood Most patients require intravenous fluids and electrolytes, particularly potassium, to replace diarrhoeal losses. Serum potassium concentration should be maintained at or above 4 mmol/L, since hypokalaemia may predispose to colonic dilatation. Blood transfusion is recommended if the haemoglobin falls below 100g/L.

Nutritional support Patients can usually eat normally, with liquid protein and calorie supplements if necessary. Very sick patients may need total parenteral nutrition.

Anticoagulation Because active UC is associated with a high risk of venous and arterial thrombo-embolism^[7], patients should be given prophylactic subcutaneous heparin (e.g. low molecular mass heparin 3000-5000 U daily). Heparin does not appear to increase rectal blood loss even when given intravenously^[8].

Drugs to avoid Antidiarrhoeal drugs (loperamide, codeine phosphate, diphenoxylate), opioid analgesics, antispasmodics and anticholinergic drugs should not be prescribed in active UC since they may provoke acute colonic dilatation^[9]. Patients should also avoid NSAIDs in view of their adverse effects on the clinical course of IBD^[10]. If relief of mild pain is needed, oral paracetamol appears to be safe, while severe pain suggests colonic dilatation or perforation needing urgent intervention.

Rolling manoeuvre In very sick patients, particularly those with clinical and/or radiological evidence of incipient colonic dilatation, rolling into the prone or knee-elbow position for 15 minutes every two hours may aid in the evacuation of gas per rectum, particularly from the transverse colon^[11].

Specific medical treatment

The cornerstone of specific medical treatment of acute severe UC remains corticosteroids^[1,12]. Aminosalicylates and antibiotics have minor roles. Cyclosporin has become a useful option, but oral

azathioprine and 6-mercapto purine are too slow to work in patients with acute steroid-refractory attacks (Table 1).

Corticosteroids Hydrocortisone (300 mg/d-400 mg/d) or methyl prednisolone (40 mg/d-60 mg/d) are given intravenously. There is no advantage in giving higher doses, although continuous infusion may be more effective than once or twice daily boluses^[12]. On this treatment, about 70% patients improve substantially in 5 d-7 d. They are then switched to oral prednisolone (40 mg/d-60 mg/d), the dose being tapered to zero over 2-3 months. Conventionally, failure to respond to intravenous steroids after 7 d indicates urgent colectomy, but introduction of intravenous cyclosporin can now be considered as an alternative.

Aminosalicylates Aminosalicylates in full dose are continued in patients already taking them at the time of admission, and well enough to take oral medication, but do not have a primary therapeutic in acute severe UC. In case patients given aminosalicylates for the first time prove to be allergic to, or intolerant of them, initiation of these drugs is best delayed until the patient shows sufficient improvement on intravenous steroids to switch to oral treatment.

Antibiotics Although one study has suggested a role for adjunctive oral tobramycin^[13], the use of antibiotics is usually restricted now to very sick febrile patients, or to those in whom an infective component to their colitis is strongly suspected. Under such circumstances, a combination of antibiotics, for example ciprofloxacin or a cephalosporin with metronidazole, is often given.

Cyclosporin The only current evidence-based indication for cyclosporin in IBD is steroid-refractory acute severe UC. In a single small controlled trial^[14], the results of which have been largely confirmed by subsequent experience^[15,16], intravenous (4 mg·kg⁻¹·d⁻¹) for about 5 d) followed by oral (5 mg·kg⁻¹·d⁻¹-8 mg·kg⁻¹·d⁻¹) cyclosporin, given with continued corticosteroids, averted colectomy in the acute phase in 80% of patients failing to respond to 5 d-7 d of intravenous steroids alone. Enthusiasm for this approach has to be tempered by the frequency of relapse necessitating colectomy (up to 50%) that follows withdrawal of cyclosporin, and by its serious adverse effects which in turn demand frequent monitoring of cyclosporin blood levels and serum biochemistry in treated patients. The therapeutic range for monoclonal radioimmunoassay is 250 µg/L⁻¹-400 µg/L⁻¹ during intravenous treatment, and 150 µg/L⁻¹-300 µg/L⁻¹ as the trough level on oral treatment. Biochemical disturbances induced by cyclosporin include

hyperkalaemia, hypomagnesaemia and *hyperuricaemia*, as well as renal dysfunction. The most serious side effects of cyclosporin are opportunistic infections (20% patients) including pneumocystis carinii pneumonia, on account of which co-administration of prophylactic trimethoprim/sulphamethoxazole may be advisable; renal impairment, including a small reduction in glomerular filtration rate in most patients and, sometimes, an interstitial nephritis which is not always reversible on stopping cyclosporin; hypertension (30% patients); hepatotoxicity (up to 20%); and epileptic fits (3%), due to penetration of the blood-brain barrier by a vehicle, cremophor, in cyclosporin and essentially confined to patients with low serum cholesterol and/or magnesium concentration. Less serious side-effects include nausea, headache, paraesthesiae and hypertrichosis.

Further studies are needed to determine optimal usage of cyclosporin in UC. For example, precisely when should patients be given the drug, will a lower dose (2 mg·kg⁻¹·d⁻¹ iv) be as effective but safer, should trimethoprim/sulphamethoxazole be coprescribed as prophylaxis against pneumocystis carinii infection, and should oral cyclosporin or azathioprine be prescribed after the intravenous treatment? It is clear, however, that intravenous cyclosporin can be invaluable in patients with steroid-refractory acute severe UC, not least for buying time for improving their nutrition prior to, and/or preparing them psychologically for surgery.

Azathioprine and 6-mercaptopurine Oral azathioprine and 6-mercaptopurine are very effective in inducing and maintaining remission in patients with steroid-refractory or dependent IBD. Unfortunately, however, they take up to 4 months to exert their effect and are thus inappropriate for acute severe UC.

Possible new treatments The possible roles of anti-TNF-alpha antibody^[17], antibodies and antisense oligonucleotides to leucocyte/endothelial cellular adhesion molecules^[18], and intravenous heparin^[8] require further evaluation in controlled clinical trials.

Surgery

A colorectal surgeon should be involved in the care of patients with acute severe UC throughout their admission. Indications for urgent colectomy, which is required in about 25% of patients with acute severe colitis, include toxic colonic dilatation which does not respond within 24 h to intensification of medical treatment with rolling^[11,12], antibiotics and nasogastric suction, and deterioration or failure to improve on medical therapy in 5 d-7 d. Emergency surgery, after immediate resuscitation, is required in the rare patients, who develop colonic perforation or massive colonic haemorrhage.

Details of the surgical options available (pan-proctocolectomy with ileoanal pouch or permanent ileostomy, or, rarely, sub-total colectomy with ileorectal anastomosis) and their elective indications, are beyond the scope of this review.

MANAGEMENT OF ACTIVE CROHN'S DISEASE

Treatment of CD depends not only on disease activity and site, as in UC, but also needs to be tailored according to the patient's clinical presentation^[2,3]. Inflammation (Table 2), obstruction, abscess and fistula require different therapeutic approaches, and need to be distinguished by appropriate investigation before specific treatment is begun.

Assessment of disease activity

Its heterogeneous presentation makes assessment of disease activity in CD more complicated than in UC. For clinical trials, a large number of multifactorial clinical and/or laboratory-based scoring systems, such as the Crohn's Disease Activity Index (CDAI), has been devised, but none is suitable for ordinary clinical use^[2]. The working definitions of the American College of Gastroenterology^[2] are more practicable. Many patients with active CD can be looked after as outpatients, but those with moderate-severe and severe-fulminant disease need prompt, and in the latter instance immediate, hospital admission.

General measures

As for UC, patients with active CD should be looked after by a multi-disciplinary team with special expertise in IBD in a gastroenterology clinic or ward. Options for treatment (medical, nutritional, surgical) are wider than in UC, and it is essential that the patient is kept fully informed about his/her illness, and takes a place at the centre of the therapeutic decision-making process^[2,3].

Establishing the diagnosis and clinicopathological problem

In many patients, the diagnosis of CD and identification of its principal site will have been made before the current relapse. Investigations, therefore, are directed primarily to clarifying the dominant clinicopathological process so as to optimise subsequent treatment. In those individuals presenting acutely for the first time, the diagnosis needs to be established (Table 2).

Clinical evaluation Terminal ileal and ileocaecal CD usually present with pain, diarrhoea and/or a tender mass in the right iliac fossa. Inflammation and abscess tend to cause constant pain, often with fever; in patients with small bowel obstruction, the pain is more generalised, intermittent, colicky and associated with borborygmi, abdominal distension and vomiting. Where the diagnosis of CD has not yet been made, an appendix mass, caecal

carcinoma, lymphoma and, in some ethnic groups, ileocaecal tuberculosis require careful consideration.

Blood tests As in UC, the main value of blood tests is in assessing and monitoring disease activity, which is related directly to the platelet count, ESR and C-reactive protein and inversely to serum haemoglobin and albumin. However, in very sick patients, particularly with extensive small bowel disease and steatorrhoea, there may be laboratory evidence of malnutrition and malabsorption (anaemia, low serum iron, folate, Vit.B12, albumin, calcium, magnesium, zinc, essential fatty acids).

Endoscopy and biopsy In patients with right iliac fossa pain where the diagnosis of CD is in doubt, colonoscopy to the terminal ileum, with biopsies, is helpful. It can also be used to balloon-dilate short strictures. In established Crohn's colitis, colonoscopy during acute relapse is not routinely necessary and may be unsafe. In previously undiagnosed patients, digital rectal examination and sigmoidoscopy may show rectal induration or ulceration, or the presence of perianal disease. Furthermore, biopsy of macroscopically normal rectal mucosa may reveal epithelioid granulomata in a minority of patients with overt CD more proximally.

Plain abdominal X-ray A plain film is essential if intestinal obstruction is suspected. It may also hint at a mass in the right iliac fossa, and is often helpful, as in UC, in estimating extent or severity of Crohn's colitis.

Barium radiology Because it may exacerbate obstructive symptoms and pre-existing perforation, conventional barium follow through and small bowel enema should be avoided in severely ill patients with small bowel disease. In many centres, colonoscopy, because it allows biopsy and when necessary balloon dilatation of strictures, is used in preference to barium enema in patients with suspected large bowel and terminal ileal disease. Contrast fistulography is useful for the clarification of anatomical connections in patients with abdominal sinuses or fistulae.

Radiolabelled leucocyte scans ⁹⁹Tc-HMPAO or ¹¹¹Indium-leucocyte scanning can be helpful to identify, non-invasively, not only sites of large bowel inflammation, as in UC, but also in the small intestine. Delayed scanning can also be helpful in identifying intra-abdominal abscesses.

Ultrasound, CT scan and magnetic resonance imaging (MRI) Abdominal ultrasound and CT scan can be very useful in active CD, allowing not only the evaluation but also the percutaneous drainage of localised collections. CT also plays a central role in

defining abdominal fistulous tracks and sinuses, while endoluminal ultra sound and MRI are particularly useful for the anatomical delineation of perianal abscesses and fistulae.

Supportive treatment

Patients with active CD, like those with acute severe UC, need meticulous supportive treatment, including, as necessary, intravenous fluids and electrolytes, blood transfusion and prophylactic subcutaneous heparin^[7](Table 2).

Dietary advice and nutritional support All patients should be carefully assessed in relation to their nutritional intake and status, the latter clinically by measurement of body mass index [mass (kg)/height (m)²; (normal >20)]. Patients with stricturing small bowel CD should avoid high residue foods (e.g. citrus fruit segments, nuts, sweetcorn, uncooked vegetables) which might cause bolus obstruction. Special dietary and nutritional modifications are needed for patients with extensive small bowel CD or short bowel syndrome. Sick inpatients may need enteral or parenteral nutrition to restore nutritional deficits, while liquid formula diets offer effective primary therapy for some patients with active small bowel CD.

Smoking Patients with CD who smoke should be strongly advised to stop, since this habit has a major adverse effect on the long-term natural history of the disease, particularly in women^[19].

Drugs Codeine phosphate and loperamide are useful for the control of diarrhoea in patients with small bowel CD or resection; they should, as in UC, be avoided in active Crohn's colitis in case they provoke colonic dilatation. Cholestyramine sachets (4 g one to three times daily) reduce watery diarrhoea due to bile salt malabsorption induced by extensive terminal ileal disease or resection. Haematinics (Fe, folate, Vit.B12), calcium, magnesium, zinc and fat soluble Vit. (A,D,E,K) may be needed for the replacement of particular deficiencies, as may appropriate drugs for incipient or established osteoporosis.

Drugs to avoid NSAIDs may precipitate relapse of CD, as of UC^[10], and should be avoided. Likewise, in patients with small bowel stricturing due to CD, delayed release drugs should not be prescribed in case they cause bolus obstruction.

SPECIFIC TREATMENT OF ACTIVE ILEOCAECAECAL CROHN'S DISEASE

Therapeutic options include drugs, liquid formula diet and surgery, as separate alternatives or in combination, depending on the individual patient's age, presentation and personal preference (Table 2)^[2,3].

Drug therapy

Corticosteroids In active disease, oral steroids provide the quickest and most reliable response, 60%-80% patients improving in 3 wk-4 wk. Conventionally, prednisolone (40 mg·d⁻¹-60 mg·d⁻¹) is used, the dose being tapered by 5 mg every 7 d-10 d once improvement has begun. Very sick patients, or those needing to be fasted because of intestinal obstruction, need intravenous corticosteroids at least initially (e.g. hydrocortisone 300 mg·d⁻¹-400 mg·d⁻¹, methyl prednisolone 40 mg·d⁻¹-60 mg·d⁻¹). In patients able to take oral treatment in whom systemic steroid side effects are a major problem, a useful recent advance is the introduction of an oral controlled ileal release formulation of budesonide (Entocort CR, Budenofalk) (9 mg·d⁻¹). This steroid approaches prednisolone in efficacy, but because of first-pass metabolism, has fewer systemic side-effects and causes much less a drenal suppression, albeit at greater financial cost^[20]. Up to 20% of patients with CD may be difficult to wean off steroids after relapse. Of these, many will be able partially or totally to discontinue steroid therapy on introduction of an aminosalicylate or immunomodulatory agent.

Aminosalicylates Patients with only moderately active ileocaecal disease, most of whom can be treated as outpatients, can be tried on high dose oral mesalazine (e.g. Pentasa 2 g b.d., Asacol 1.2 g t.d.s.)^[21,22]; about 40% will go into remission in 2-3 months on such treatment, which may be preferred by individuals reluctant to use prednisolone.

Metronidazole and other antibiotics Metronidazole alone^[23] or in combination with ciprofloxacin^[24] is moderately effective in mild moderately active CD, but is insufficiently potent for use as sole therapy in patients ill enough to need hospital admission. Treatment needs to be given for up to 3 months, but may be confounded by nausea, vomiting, an unpleasant taste and/or patients' unwillingness to abstain from alcohol during this time. More seriously, metronidazole taken long-term may cause a peripheral neuropathy not always reversible on its discontinuation. The place of other antibiotics such as clarithromycin, clofazimine and rifabutin has not yet been adequately established in controlled trials. Conventional antituberculous therapy was not beneficial in a controlled trial in CD^[25]. Antibiotics such as amoxicillin, trimethoprim, ciprofloxacin and metronidazole are sometimes useful for the treatment of diarrhoea or steatorrhoea due to bacterial overgrowth in patients with small bowel CD.

Azathioprine and 6-mercaptopurine Patients not

responding to or dependent on corticosteroids who, because of extensive disease or previous resection, need to avoid operative treatment, can be treated with adjunctive oral azathioprine (2-2.5 mg·kg⁻¹·d⁻¹) or 6-mercaptopurine (1-1.5 mg·kg⁻¹·d⁻¹); the dose of steroids is reduced as improvement occurs^[26-28]. Such patients must be well enough to wait for up to four months for this to become apparent. Hopes that intravenous azathioprine could be used to accelerate response in active Crohn's have not been confirmed in a controlled trial^[29]. Up to 20% of patients cannot tolerate azathioprine because of nausea, rash, fever, arthralgia, upper abdominal pain and headache; in a minority of these patients, a switch to 6MP may avert these problems. More seriously, both drugs may cause acute pancreatitis in about 3% of patients, particularly in the first few weeks of treatment. Their other potentially serious side effects, bone marrow depression (which occurs in 2% patients) and cholestatic hepatitis, necessitate blood tests every two weeks for the first two months of therapy: thereafter, white cell count, platelet count and liver function tests should be monitored every 2 months^[30]. Opportunistic infections and a serious form of glandular fever have been reported in patients on azathioprine or 6MP. Although existing data in IBD is reassuring^[31], very long-term use, as in transplant patients, may yet prove to increase the risk of malignancy. Indeed, the risk of skin cancer makes it advisable to recommend to white patients on azathioprine or 6MP that they avoid excessive exposure to sunlight. Homozygous deficiency of 6-thiopurine methyl transferase (6TPMT), the enzyme responsible for the safe metabolic breakdown of azathioprine and 6-MP, occurs in about 0.2% of the population and may contribute to the occasionally serious side-effects of both drugs and its routine assay is not yet available. Allopurinol, by inhibiting xanthine oxidase, reduces metabolism of azathioprine. Patients on this drug should not be given either thiopurine. Usage of azathioprine and 6-mercaptopurine in CD is long term. However, in patients maintained in remission on azathioprine or 6MP, the risk of relapse after four years of treatment appears to be similar whether the drug is continued or stopped^[32]. In view of the potential toxicity of the long-term use of these drugs, their withdrawal should be considered in patients still in remission after four years treatment.

Methotrexate Methotrexate, given weekly as a 25 mg intramuscular injection, improves symptoms and reduces steroid requirements in chronically active steroid-dependent CD^[33], but its potential side effects (bone marrow depression, hepatic fibrosis, pneumonitis, opportunistic infections) restrict its use to the very small number of patients

with difficult CD refractory to safer treatments. Although a lower dose (12.5 mg weekly), given orally, may also prove beneficial in CD^[34], all patients given methotrexate need careful blood monitoring.

Mycophenolate mofetil In an unblinded trial in complicated CD, this newer immunomodulatory drug appeared to act quicker and produce fewer side-effects than azathioprine^[35] and double-blind controlled trials are needed to confirm these results.

Cyclosporin Has not been confirmed as useful in active ileocaecal CD^[38-40].

Anti-TNF-alpha antibody The first specific cytokine-related therapy to reach the bedside in CD is infliximab, a mouse-human chimeric (cA2) antibody to-TNF-alpha^[36-38]; this drug was launched in the USA in 1998 and in Europe in 1999. In patients with CD refractory to steroids and/or conventional immunosuppressive drugs, a single infusion of infliximab produced, at 4 weeks, some improvement in 64% patients, compared with 17% after placebo; remission occurred in 33% patients treated with infliximab but only 4% of those given placebo^[39]. Relapse tends to recur in the ensuing months: repeated infusions every 4 wk-8 wk may produce more lasting remissions^[40]. Infliximab is administered as a single^[40] or, to obtain a more prolonged response, multiple intravenous infusions^[40,41] at 4 wk-8 wk intervals, each given over 2 hours. The dose is 5 mg·kg⁻¹ per infusion, and the cost about £1000 (US \$1600) per infusion. Common minor side-effects include headache, nausea and upper respiratory tract infections. Serious, but not opportunistic, infections including salmonella enterocolitis, pneumonia and cellulitis have been reported. Infusion reactions occur in up to 20% patients, are usually mild and respond to antihistamines: however, adrenaline and corticosteroids should also be available when infusions are given. The development of human antichimeric antibodies (HACA) in up to 15% patients may cause a serum sickness reaction and diminished clinical response to repeated infusions. A lupus syndrome has been associated with anti-double-stranded DNA antibodies and cardiolipin antibodies in rheumatoid patients given infliximab. Rapid healing and fibrosis may precipitate bowel obstruction in patients with small intestinal strictures. Lastly, there are several reports of lymphoma in rheumatoid and Crohn's patients given infliximab, although whether these are due to the drug or the underlying disease is not yet clear. The benefits, or otherwise, of coprescription of azathioprine or 6-mercaptopurine in patients given anti-TNF antibody are not yet established. By analogy with the effects

of methotrexate in infliximab-treated patients with rheumatoid arthritis, conventional immunosuppressive drugs may have a synergistic effect and reduce the incidence of the development of autoantibodies and other adverse effects. It is conceivable, however, that immunosuppressive agents could increase the risk of lymphoma in patients on infliximab^[42]. In the future, selection of patients to be treated with anti-TNF antibody may depend not only on the ir disease phenotype (eg fistulating disease), but also their genotype. Preliminary evidence suggests that CD patients who are pANCA positive, and have particular TNF microsatellite haplotypes, for instance, show a poor response to infliximab.

Dietary therapy

In patients with a poor response to, or preference for avoiding corticosteroids, in those with extensive small bowel disease, and in children, an alternative primary therapy is a liquid formula diet. This can be either elemental (aminoacid-based), protein hydrolysate (peptide-containing) or polymeric (containing whole protein and not therefore hypoallergenic), and is given for 4–6 weeks as the sole nutritional source^[43]. This approach is probably as effective as corticosteroid therapy in the short term, about 60% patients achieving remission. Unfortunately, after the resumption of a normal diet, many patients relapse (50% at six months). Whether this can be prevented by selective and gradual reintroduction of particular foods to which individual patients are not intolerant^[44], or by the intermittent use of further enteral feeding for short periods, remains to be proven. The success of enteral nutrition as a primary therapy for CD is also limited by its cost, the unpleasant taste of some of the available preparations and the need often to give the feed by nasogastric tube or percutaneous gastrostomy. Such therapy does, nevertheless, offer a valuable alternative in the compliant minority of adults for whom it is appropriate.

Surgery

In patients whose ileocaecal disease fails to respond to drug or dietary therapy, particularly if they have short segment (less than 20 cm) rather than extensive disease, surgery is indicated. Indeed, some patients prefer surgery at presentation to the prospect of pharmacological or nutritional treatment of uncertain duration; there is no controlled data to confirm which approach is best. After surgery, there is a 50% chance of recurrent symptoms at 5 years and of further surgery at 10 years.

SPECIFIC TREATMENT OF OTHER PRESENTATIONS OF ACTIVE CROHN'S DISEASE

Obstructive small bowel Crohn's disease In patients presenting with obstructive symptoms and signs,

with appropriate abnormalities on plain abdominal X-ray, the principal difficulty lies in deciding whether stricturing is due to active inflammation, fibrosis with scarring or even adhesions. Sometimes laboratory markers (e.g. raised platelet count, ESR, C-reactive protein) and/or radiolabelled leucocyte scan can help to identify individuals with active inflammatory Crohn's, but in most instances a short trial of intravenous corticosteroids is given in addition to intravenous fluids and, if necessary, nasogastric suction. Parenteral nutrition is required if resumption of an oral diet is not likely in 5 d–7 d. If the stricture is in the upper jejunum, terminal ileum or colon, enteroscopic or colonoscopic balloon dilatation can be undertaken^[45]; the value of concomitant local injection of triamcinolone around the stricture is as yet unclear. In patients not settling after 48 h–72 h of conservative treatment, surgery is needed, options being local resection or, for short and/or multiple strictures, stricturoplasty. Patients responding to conservative therapy should be advised to take a low residue diet to reduce the chance of recurrent symptoms.

Intra-abdominal abscess Ultra sound, CT scan and/or radiolabelled leucocyte scan are usually used to confirm suspected intra-abdominal abscess in patients with Crohn's. Broad spectrum antibiotics are given and the abscess drained percutaneously under radiological control, and/or surgically. Subsequent treatment is usually of the underlying pathological process, for example, ileocaecal inflammation.

Intestinal fistula The relevant anatomical connections are clarified using contrast radiology, CT, endoluminal ultra sound and/or MRI. Restitution of nutritional well being is required using enteral or parenteral nutrition. Where there is no obstruction distal to the site of intestinal fistulae, medical therapy with oral, rectal or intravenous metronidazole and/or oral azathioprine or 6-mercaptopurine^[26] cause some fistulae to heal. Uncontrolled reports suggest that intravenous cyclosporin may heal fistulous Crohn's, while a controlled trial shows anti-TNF-alpha antibody (infliximab) infusions to be a promising option^[41]. Most patients with enterocutaneous, vesical or vaginal fistulae, however, require surgical resection of the fistula and local resection of involved intestine and/or other viscera.

Perianal disease Non-suppurative perianal CD may respond to oral metronidazole^[46] and/or ciprofloxacin given for up to three months, and to azathioprine or 6-mercaptopurine in the long term^[26]. Successful healing of >50% perianal (and other) fistulae was reported in 62% patients treated with three intravenous infusions of anti-TNF-alpha

antibody (infliximab) compared with 26% of those given placebo^[41]. Although in this study it is not clear whether the fistulous tracks, rather than simply their openings on to the skin, healed, and reopening of fistulae was common in the 6 months after treatment was stopped, infliximab may prove a useful advance in therapy. Patients with suppurating perianal CD need surgery, minimised as far as possible and abscesses should be drained and loose (seton) sutures inserted to facilitate the continued drainage of chronic fistulae. Defunctioning ileostomy or colostomy is of uncertain benefit.

Crohn's colitis The treatment of active Crohn's colitis closely resembles that of active UC (Table 1). In contrast to UC, oral metronidazole (400 mg b.d. for up to three months), if tolerated, can be used in patients with only moderately active disease who wish to avoid corticosteroids or aminosaliclates: the response rate is up to 50%^[23]. There is no data to support the use of cyclosporin. Meta-analysis data suggest that Crohn's colitis, like ileocaecal disease, responds to a liquid formula diet^[43]. In patients who require total colectomy, permanent ileostomy is usually preferred to an ileoanal pouch because of the high incidence of pouch breakdown and sepsis in CD. Ileorectal anastomosis is an option in patients with rectal sparing, though recurrence requiring further surgery is far more common than after ileostomy. In rare individuals with refractory segmental colitis, local resections of short diseased segments can be performed. Toxic megacolon is even more rare in acute severe Crohn's than it has become in UC.

Oral and upper gastrointestinal Crohn's disease

Treatment of oral and upper gastrointestinal CD follows the usual principles outlined above. Patients with oral Crohn's are best managed in close conjunction with specialists in oral medicine: controlled trial data are lacking, but options include topical, intral esional and oral steroids as well as oral thiopurines and liquid formula diet. Duodenal Crohn's may respond to omeprazole^[47]; endoscopic balloon dilatation of strictures can be helpful, but surgery other than stricturoplasty may be technically demanding and complicated by fistulation.

MEDICAL TREATMENT OF IBD—THE FUTURE

Improvements in future medical treatments are likely to take several directions. First, conventional therapies, such as steroids and aminosaliclates, are likely to be made available in formulations which focus delivery more accurately on the site of disease and thereby further reduce systemic side effects. More excitingly, the increase in our knowledge of the aetiology and pathogenesis of IBD will inevitably lead to the development of more selectively targeted pharmacological agents, of

which the first to reach clinical application has been anti-TNF-alpha antibody. Gene therapy, for example applied topically to involved gut mucosa, may prove an important step forward in UC and Crohn's as in other chronic inflammatory diseases outside the gut. The choice of treatment in individual patients with IBD will depend not only on the phenotypic expression of their disease, but also on their genotype.

Whatever therapeutic advances are made in the coming years, the management of patients with IBD, whether apparently straightforward or difficult, will continue to depend on close collaboration between physicians, surgeons, specialist nurses, dieticians, radiologists, pathologists and counsellor, and a clinical geneticist may need to join this team. Most importantly, the patient with IBD must be looked upon as a person rather than a case. As treatment becomes more complex, and the options more varied, it is essential that the patient remains at the centre of the decision-making process, and the individual with IBD must be the final arbiter of the type of treatment he or she is to be given.

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Treatment of *Helicobacter pylori* infection: analysis of Chinese clinical trials

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Subject headings *Helicobacter* infection/therapy; clinical trials; evaluating studies, *Helicobacter pylori*

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INTRODUCTION

Eradication of *Helicobacter pylori* (*Hp*) infection is generally not easy. Various clinical regimens have been recommended in the literature. With the experience from the other countries and the practice in China, Chinese doctors have tried many regimens. In this study, we collected and pooled the data from Chinese literature to evaluate the effect of different regimens in Chinese patients infected with *Hp*.

MATERIALS AND METHODS

Papers published from 1990 to 1997 were reviewed. The papers were cited from the index "Chinese Literature of Science and Technology, (Medicine)", Published by the Medical Information Institute of China, Beijing, and from the Chinese bio medical disks (CBMDISC). Papers were selected according to the following criteria: ① the papers must be published in full text; ② data must be from original studies from author's own unit; ③ *Hp* status must be determined using histology, microbiology and urea breath test; and ④ the studies should be appropriately designed and reported. If several papers were published from the same data source, the one with the best data was included.

RESULTS

Monotherapy Monotherapy has been fully proved to be not effective in *Hp* eradication, with a eradication rate between 10%-45%.

Dual therapy Proton pump inhibitor (PPI) dual therapy was introduced from western countries to China, whereas furazolidone was developed in China. The data are shown in Tables 1,2.

Triple therapy PPI and bismuth triplies were main regimens recommended. Furazolidone was fully practiced in China. Their results are shown in Tables 3-5.

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Quadruple therapy Only two studies were available using 1 week course of bismuth, PPI and two antibiotics. The eradication rates were 91% and 93%, and the occurrence rate of side effect being 33%^[19,20].

Table 1 PPI dual therapy

Authors	Ome	Amo	Eradication(%)	Healing(%)	Side effect
Zhou YH ^[11]	20 bid×14	500 qid×14	30/33 (91)	31/33 (94)	12%
Zhou YH ^[11]	20 qd×14	500 qid×14	31/35 (89)	32/35 (91)	
Nie YQ ^[2]	20 bid×14	500 qid×14	10/13 (77)	12/13 (92)	15%
Li YY ^[3]	20 bid×14	500 qid×14	8/11 (73)	9/11 (82)	
Hu FL ^[4]	20 bid×14	750 bid×14	13/22 (59)	18/22 (82)	
Zhou Y ^[5]	20 bid×14	750 qid×14	30/36 (83)	36/36 (100)	2.7%

Ome: omeprazole Amo: amoxicillin

Table 2 Furazolidone dual therapy

Authors	Furazolidone	Antibiotics	Eradication(%)	Side effect
Xiao SD ^[6]	100 qid×14	CBS 120 qid×14	66/90 (73)	
Mao PJ ^[7]	100 tid×28	Ran 150 bid×28	10/17 (59)	8.9%
Mao PJ ^[7]	100 tid×28	Ome 20 qd×28	15/18 (83)	
Li YN ^[8]	200 tid×7 100 qid×7	CBS 110 qid×28	34/34 (100)	
Li YN ^[8]	100 tid×14	CBS 110 qid×28	21/23 (91)	
Li YN ^[8]	50 tid×14	CBS 110 qid×28	13/21 (62)	
Xi BC ^[9]	200 tid×14	CBS 110 qid×28	24/24 (100)	

CBS: bismuth Ran: ranitidine Ome: omeprazole

Table 3 Triple therapy with Furazolidone

Authors	Fu	Antibiotics	Antibiotics	Eradication(%)	Side effect
Liu WZ ^[10]	200 bid×7	CBS 240 bid×7	Cl 500 bid×7	12/12(100)	57%
Liu WZ ^[10]	100 bid×7	CBS 240 bid×7	Cl 250 bid×7	25/27(93)	7.4%
Huang YS ^[11]	100 tid×5	Met 400 tid×5	Genta 40 tid×5	25/26(96)	
Xiao SD ^[6]	100 qid×10	CBS 120 qid×10	Met 200 qid×10	74/75(78)	
Chen JP ^[12]	100 qid×28	CBS 120 qid×28	Tetra 250 qid×28	35/54(65)	

Cl: clarithromycin Genta: gentamycin Tetra: tetracyclin

Table 4 Hp eradication with CBS triple therapy

Authors	CBS	Amo	Met	Eradication(%)	Side effect
Jia BQ ^[13]	120 qid×14	250 qid×14	200 qid×14	328/440(87)	7.8%
Jia BQ ^[13]	240 bid×14	500 bid×14	400 bid×14	139/156(89)	7.8%
Chen SP ^[14]	240 bid×14	1000 bid×14	400 bid×14	33/46(71)	
Li QN ^[15]	240 bid×14	500 bid×14	400 bid×14	13/16(81)	37.5%
Geng Z ^[16]	110 qid×14	500 qid×14	200 qid×14	64/76(84)	
Zhou LY ^[17]	120 qid×14	250 qid×14	200 qid×14	56/73(77)	
Li YY ^[18]	120 qid×14	250 qid×14	200 qid×14	20/25(80)	28%

Table 5 PPI triple therapy

Authors	PPI	Antibiotics	Cl	Eradication(%)	Side effect
Chen SP ^[14]	Ome 20 bid×7	Amo 1000 bid×7	500 bid×7	43/48(90)	21.1%
Chen SP ^[14]	Ome 20 bid×14	Amo 1000 bid×14	500 bid×14	45/47(96)	
Liu WZ ^[10]	Lan 30 bid×7	Fu 200 bid×7	500 bid×7	11/12(92)	10%
Liu WZ ^[10]	Lan 30 qd×7	Fu 100 bid×7	250 bid×7	27/30(90)	
Li QN ^[15]	Lan 30 bid×7	Met 400 bid×7	250 bid×7	14/16(86)	18.8%
Li QN ^[15]	Lan 30 bid×7	Met 400 bid×7	500 bid×7	15/16(94)	25%

DISCUSSION

Eradication of *Hp* is considered to be confirmed when the tests of *Hp* continue to be negative for at least 4 weeks after the discontinuation of treatment^[21]. Great efforts have been made to establish regimens with good efficacy and safety. It is recognized in western countries that a good regimen should reach the eradication rate of intention to treat (ITT) > 80% and per protocol (PP) > 90%^[22]. In most Chinese papers only the rate of PP was available, therefore used in this review. In consideration of high resistant rates to antibiotic and the high prevalence of *Hp* in the country, a regimen with a PP eradication rate > 85% should be accepted in our practice. With this standard we found that both PPI triple therapy and bismuth triple therapy with a two-week course were good for Chinese patients. The former had high adverse events and the latter was more expensive. In most treatment, the dosage of antibiotics was cut down in order to reduce the side effects. It was shown that the low-dose triple therapies could yield high eradication rates in Chinese patients because they had lower body weight. The limited data with one-week quadruple therapy showed that it could be a good alternative especially for the patients who failed to other regimens. Monotherapy and dual therapy were not suitable in practice because of their poor efficacy. These findings agree with the data from western literatures^[23].

The presence of resistant *Hp* strains is a severe problem in China and influences the efficacy of treatments. The resistant rates to metronidazole were reported to be between 28%-80%, and clarithromycin, <5%. However, there has been no reported resistance to bismuth, amoxicillin, furazolidone and tetracycline. These antibiotics should be used to replace metronidazole if the resistance exists. Some studies recommended furazolidone, which was less expensive, with low resistance but more adverse events.

Recently the consensus reports from European, American and Asia Pacific areas recommended the following regimens^[22,24,25]: ① PPI (ome 20 mg or lan 30 mg) + Cla 500 mg + Amo 1000mg; ② PPI + Cla 250/500 mg + Met 400 mg; ③ RBC/Bis + Cla 500 mg + Met 400 mg/Amo; and ④ PPI + Bis + Met + Tetra.

All were used twice daily for the one-week course. These regimens had a good efficacy in western countries, but have not been extensively examined in China. RBC is still unavailable in China. The preliminary results from our group showed that Lan30 + Cla500 + Met400 regimen reached a 93% eradication rate, but Lan30 + Cla250 + Met400 only 77%^[26]. More studies are needed before their establishment in this country.

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Protective effects of prostaglandin E1 on hepatocytes

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INTRODUCTION

Numerous studies have demonstrated the protective action of prostaglandin E1 (PGE1) on experimental animal models of liver injury and on patients with fulminant viral hepatitis. It could act on PGE1 receptor of the diseased vessels to dilate them and increase portal venous flow, improve the microcirculation of the liver, clear the metabolites of the liver cells, and increase oxygen supply to the liver tissues. PGE1 could also accumulate at the inflammatory portion, inhibit the release of lethal factors, stabilize the membrane of liver cells and lysosome, inhibit the active oxygen, and promote the proliferation of the liver cells. It is now used to treat fulminant hepatic failure.

PROTECTIVE ACTION OF PGE1 ON EXPERIMENTAL ANIMAL MODELS OF LIVER INJURY

The protective action of PGE1 has been shown on both experimental animal models of liver injury and patients with fulminant viral hepatitis. Beck *et al*^[1] examined the effects of long-term PGE treatment on liver and stomach in cirrhotic rats. Cirrhosis was induced by bile duct ligation. Sham-operation was performed as controls. Half of the rats received a PGE1 analogue, misoprostol (PGE1) (10 µg orally, daily) on d 1-d 29 postoperation, and the others received placebo only. Liver chemistry, portal pressures, and levels of prostaglandin E2, leukotriene B4, myeloperoxidase, and collagen in hepatic and gastric tissue of all rats on d 31 were determined. PGE1-treated cirrhotic rats had less hepatosplenomegaly, lower serum alanine aminotransferase levels, and portal pressures and higher arterial pressure than placebo-treated

cirrhotic rats. Hepatic and gastric leukotriene B4, myeloperoxidase and collagen levels were significantly lower in the PGE1-treated compared with placebo-treated cirrhotic rats. Placebo-treated cirrhotic rats had greater spontaneous and ethanol-induced gastric damage and failed to show gastric hyperemic response to ethanol, whereas PGE1-pretreated rats did. PGE1 did not significantly affect sham-operated rats. Beck suggested that long-term PGE1 administration was cytoprotective for both the liver and gastric mucosa in cirrhotic rats.

An animal model of hepatocytic necrosis was established by Gu^[2] with injection of D-galactosamine into peritoneal cavity. Examination at regular intervals after injection of PGE showed that the level of increased serum TB, ALT and GST and the degree of histological changes in the liver were less marked in PGE-treated animals ($n = 34$) than those in PGE-untreated animals ($n = 29$), suggesting that PGE has definite protective effect on experimental hepatocytic necrosis.

The effects of PGE1.CD on dimethylnitrosamine (DMN)-induced acute liver damage with intravascular coagulation in rats were biochemically and histopathologically investigated by Suzuki^[3]. PGE1.CD was administered i.v. 30 min before and 24 h after DMN-intoxication (pretreatment) and 30 min or 4 h to 24 h after DMN-intoxication (post-treatment). Pretreatment with PGE1.CD ($0.2-2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) dose-dependently suppressed the decrease of platelet counts and the elevation of blood biochemical parameters (PT, HPT, GOT, GPT, LDH, LAP, T-Bil) caused by DMN-intoxication. PGE1.CD ($0.5 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ or over) significantly suppressed the DMN-induced histopathological changes (occurrence of hemorrhage and necrosis). Post-treatment with PGE1.CD ($2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) also suppressed the liver damage. Furthermore, pretreatment with PGE1.CD ($2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) not only suppressed the disruption of hepatocytes, but also prevented the damages of sinusoidal endothelial cells and lysosomal membrane, and it reduced the increase of lipid peroxidation. PGE1.CD ($1 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ or over) significantly suppressed the decrease of hepatic tissue blood flow caused by DMN-intoxication. These results demonstrate that PGE1.CD has therapeutic efficacy against DMN-induced acute liver damage in rats, therefore, it will be clinically useful for the treatment of severe hepatitis such as fulminant hepatitis with intravascular coagulation in the sinusoid.

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PGE1 AND SEVERE HEPATITIS

The effect of PG on patients with fulminant and subfulminant viral hepatitis (FHF) was studied by Sinclair^[4]. Seventeen patients presented with FHF secondary to hepatitis A ($n = 3$), hepatitis B ($n = 6$), and non-A, non-B (NANB) hepatitis ($n = 8$). Fourteen of the 17 patients had stage III or IV hepatic encephalopathy (HE). The mean aspartate transaminase (AST) was $1844\text{U/L} \pm 1246\text{U/L}$, bilirubin $232\ \mu\text{mol/L} \pm 135\ \mu\text{mol/L}$, prothrombin time (PT) $34\text{s} \pm 18\text{s}$, partial thromboplastin time (PTT) $73\text{s} \pm 26\text{s}$, and coagulation Factors V and VII $8\% \pm 4\%$ and $9\% \pm 5\%$, respectively. Intravenous PGE1 was initiated 24 h-48 h later after a rise in AST ($2195\text{U/L} \pm 1810\text{U/L}$), bilirubin ($341\ \mu\text{mol/L} \pm 148\ \mu\text{mol/L}$), PT ($36\text{s} \pm 15\text{s}$), and PTT ($75\text{s} \pm 18\text{s}$). Twelve of 17 responded rapidly with a decrease in AST from $1540\text{U/L} \pm 833\text{U/L}$ to $188\text{U/L} \pm 324\text{U/L}$. Improvement in hepatic synthetic function was indicated by a decrease in PT from $27\text{s} \pm 7\text{s}$ to $12\text{s} \pm 1\text{s}$ and PTT from $61\text{s} \pm 10\text{s}$ to $31\text{s} \pm 2\text{s}$, and an increase in Factor V from $9\% \pm 4\%$ to $69\% \pm 18\%$ and Factor VII from $11\% \pm 5\%$ to $71\% \pm 20\%$. Five responders with NANB hepatitis relapsed upon discontinuation of therapy, with recurrence of HE and increases in AST and PT, and improvement was observed upon retreatment. After 4 wk of intravenous therapy oral PGE2 was substituted. Two patients with NANB hepatitis recovered completely and remained in remission 6 mos and 12 mos after cessation of therapy. Two additional patients maintained in remission after 2 mos and 6 mos of PGE2. No relapses were seen in the patients with hepatitis A virus and hepatitis B virus infection. Liver biopsies in all 12 surviving patients restored to normal. In the five non-responders an improvement in hepatic function was indicated by a fall in AST ($3767\text{U/L} \pm 2611\text{U/L}$ to $2142\text{U/L} \pm 2040\text{U/L}$), PT ($52\text{s} \pm 25\text{s}$ to $33\text{s} \pm 18\text{s}$), and PTT ($103\text{s} \pm 29\text{s}$ to $77\text{s} \pm 44\text{s}$), but all deteriorated and died of cerebral edema ($n = 3$) or liver transplantation ($n = 2$). These results suggest that PGE has beneficial effect on FHF.

According to the severity, hepatic failure was divided into early stage, typical symptom stage and late stage. A treatment group of 55 cases received PGE1 therapy and a control group received basic support therapy only. The results showed that difference of the total effective rate was not significant between these two groups, but in the early stage of hepatic failure, the effective rate in the treatment group was markedly higher than that in the control group. In addition, incidence of hepato-renal syndrome was lower in the treatment group^[2]. This study indicates that division of severe viral hepatitis into three stages for evaluation of therapeutic effect is rational and useful and early use of PGE1 may certainly show some efficacy.

While orthotopic liver transplantation (OLT) has become the treatment of choice for most

irreversible end-stage liver diseases, its role in patients with hepatitis B (HBV) infection is controversial. A high risk of reinfection of the transplanted graft, associated with significant morbidity and mortality, has been reported. Although passive and active immunization can delay the reappearance of virus in the allograft, there is not yet an effective therapy for recurrent HBV infection in liver transplant recipients. Twenty-eight OLT in 25 patients with acute and chronic HBV infections were performed^[5]. Twelve of the patients were HBV DNA-negative, six were HBV DNA-positive, and seven were not tested prior to transplantation. Only 19 patients surviving more than 100 days after transplantation were considered to have sufficient duration of follow-up (mean 734 days) to include in the analysis of recurrence. Five (26%) were free of recurrent disease at the time of last follow-up (mean 1031 days). Recurrent HBV in the allograft, as defined by positive immunoperoxidase stains of biopsy sections for viral antigens, was detected in 74% at a mean of 134 d posttransplantation. Histological changes of viral hepatitis were evident in 13 of 14 with positive immunostaining. Twelve of the 14 patients were treated, on an open trial basis, with intravenous and oral prostaglandin E (PGE) because of deteriorating clinical condition. Eleven of the twelve responded to PGE with an initial drop in serum transaminases, improvement in coagulopathy and resolution of encephalopathy. One patient failed to respond and died of myocardial infarction within 9 d of institution of therapy. Three of the eleven patients with an initial response relapsed and died of liver failure as a direct result of recurrent HBV after 13 d, 16 d and 37 d of treatment in association with generalized sepsis. Eight of the 12 patients (67%) had a sustained favorable response to PGE therapy (mean follow-up 737 days). All patients with a sustained response had accompanying improvement in histology and reduction in viral antigen staining in hepatocytes. Treatment with PGE appeared to be of benefit in recurrent HBV infection of the transplanted liver with an initial response rate of 92% and a sustained response rate of 67%.

The efficacy of PGE1 was demonstrated in the treatment of another 4 patients with subfulminant hepatitis of viral hepatitis B^[6]. Three patients suffered from hepatic encephalopathy of the first degree, and the remaining one of the second degree. In three patients the clinical and biochemical improvement came relatively quickly, followed by recovery. In one patient, due to drug intolerance, the treatment was discontinued on the third day. The recurrence of illness was noted with the moderate increase of serum aminotransferase activities without clinical deterioration, necessitating no further use of prostaglandin E1.

Bojic suggested that prostaglandin E1, applied in the treatment of patients with subfulminant form of hepatitis, has favorable effect on the course of illness.

In a rare case of severe acute hepatitis A complicated by pure red cell aplasia (PRCA), plasma exchange transfusion and glucagon-insulin (GI) therapy improved the consciousness, but bilirubin, transaminase levels, and IgM anti-HAV titer remained high. Intravenous administration of lipophilized PGE1 (lipo-PGE1) was added to the GI therapy. Bilirubin and transaminase levels were normalized in the wk 8 after the initiation of this combination therapy (17 wk after admission). The combined use of lipo-PGE1 with plasma exchange and GI therapy appeared to be useful for the severe hepatitis in this patient^[7].

Two patients with HIV infection developed acute hepatitis B with liver insufficiency and hepatic encephalopathy. After alprostadil infusion was begun, they improved quickly and got a full recovery^[8].

MECHANISMS OF HEPATIC CYTOPROTECTION OF PGE1

Indocyanine green disappearance enhanced by PGE1

Indocyanine green (ICG) is a reliable indicator reflecting hepatocyte function and hepatic blood flow. PGE1 has been indicated to increase hepatic blood flow and protect the hepatocyte. Tsukada^[9] found that PGE1 administration increased ICG-K in the liver cirrhosis (LC) and chronic hepatitis (CH) groups with normal liver function, and the ICG-K response was dose dependent when the dosage of PGE1 ranged from 0.01 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ to 0.05 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$.

Inhibitory effects of PGE1 on T-cell mediated cytotoxicity

The effects of PGE1 on cell-mediated cytotoxicity against hepatocytes were investigated using *in vitro* cytotoxic assay system by Ogawa^[10]. Isolated liver cells from normal C57BL/6 mice were used as the target cells, and effector cells were obtained from spleens of C57BL/6 mice in which experimental hepatitis had been induced by immunization with syngeneic liver antigens. In this assay system, spleen T cells adhering to nylon wool demonstrated a high cytotoxic activity against target liver cells. The cytotoxicity was markedly reduced by PGE1 at concentrations greater than 10^{-7} mol/L. Maximum suppressive activity was obtained when PGE1 was continuously present during the assay period. By contrast, indomethacin, a specific inhibitor of prostaglandin synthesis, enhanced the cytotoxic activity of effector cells. These data seem to indicate that exogenously added PGE1 has an inhibitory effect on cell-mediated cytotoxicity of effector spleen cells against target hepatocytes.

PGE1 enhance DNA synthesis of injured liver after partial hepatectomy by stimulating cyclic AMP production and increasing ATP level in hepatic tissue

D-galactosamine (D-gal) damaged rats were infused with PGE1 through peripheral vein for 40 min. before and after partial hepatectomy. DNA synthesis following 68% partial hepatectomy was severely inhibited by the pretreatment of D-galactosamine. PGE1 infusion (0.5 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, 1.0 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) enhanced the DNA synthesis inhibited by D-gal 600 mg/kg significantly ($P < 0.01$). After 20 min of PGE1 infusion cyclic AMP levels of liver tissue was increased as compared with saline infusion in D-gal (600 mg/kg) damaged rat ($P < 0.05$). Twenty min and 3 h after partial hepatectomy, ATP levels of liver tissue was enhanced in PGE1 treated group ($P < 0.05$). These results suggest that PGE1 enhances DNA synthesis of injured liver after partial hepatectomy by the mechanism of stimulating cyclic AMP production and increasing ATP level in hepatic tissue^[11].

PGE1 could accelerate the recovery of mitochondrial respiratory function after reperfusion, stabilization of membrane microviscosity

PGE1 has been indicated to increase hepatic blood flow and protect the hepatocyte. Kurokawa *et al*^[12] found that PGE1 could accelerate the recovery of mitochondrial respiratory function after reperfusion. When PGE1 was continuously administered to rats from 24 h before giving a dose of carbon tetrachloride, deranged serum glutamic pyruvic transaminase levels and prothrombin time were significantly reduced 12 h after intoxication compared with controls. A similar effect of PGE1 was seen at 24 h in D-galactosamine-intoxicated rats. Liver histology showed a comparable attenuation of injury in these rats. These results were consistent with reported effects of PGE2, suggesting that both prostaglandins may share a common pathway in protection against liver injury. When PGE1 or 16,16'-dimethyl PGE2 was added to the medium of primary cultured rat hepatocytes, lipid peroxidation-dependent killing of the cells by tert-butyl hydroperoxide was significantly attenuated without affecting the extent of malondialdehyde accumulation compared with controls. Both prostaglandins significantly reduced the extent of increased plasma membrane microviscosity of these cells. Masaki *et al*^[13] concluded that PGE1 and PGE2 may possess cytoprotective effects on liver parenchymal cells through stabilization of membrane microviscosity, which may contribute to protection against liver injury.

While the mechanisms of prostaglandin on protecting liver injury are not well understood, it has been demonstrated that dimethyl PGE2

abrogates the induction of tumour necrosis factor, leukotriene B4 (LTB4) and procoagulant activity by macrophages as well as attenuating the expression of major histocompatibility class antigens on the surface of hepatocytes, and may inhibit viral replication. From the present research, we came to a conclusion that increasing hepatic blood flow, accelerating the recovery of mitochondrial respiratory function after reperfusion, stabilizing the membrane microviscosity, decreasing the cell-mediated cytotoxicity against hepatocytes, enhancing DNA synthesis by stimulating cyclic AMP production and increasing ATP level in hepatic tissue made PGE1 as a hepatic cytoprotective agent.

TOXIC EFFECTS OF INTRAVENOUS AND ORAL PGE THERAPY ON PATIENTS WITH LIVER DISEASE

Prostaglandins are cytoprotective agents that have been shown to benefit patients with a variety of acute and chronic liver diseases. Few data exist on the frequency of adverse effects of prostaglandins in these patients. Cattral *et al.*^[14] retrospectively studied 105 patients with liver disease who were treated with either i.v. or oral PGE. Forty-four patients with primary nonfunction after liver transplantation and 36 patients with fulminant hepatic failure received i.v. PGE1 for 4.5 d \pm 2.6 d and 12.6 d \pm 10.9 d, respectively. Twenty-five patients with recurrent hepatitis B viral infection after liver transplantation received oral PGE1 for 105 d \pm 94 d or PGE2 for 464 d \pm 399 d. Twenty-six of 80 patients (33%) receiving i.v. PGE1 developed gastrointestinal and/or cardiovascular side effects and 8% developed arthritis. Twenty-three (92%) of 25 patients who received high-dose oral PGE1 or PGE2 incurred arthritis and/or gastrointestinal adverse effects. Twenty-five patients received prolonged PGE therapy (oral >60d; i.v. >28d). Of this group, 23 (92%) developed clubbing and cortical hyperostosis resembling hypertrophic osteoarthropathy. All adverse effects were dose related and resolved with reduction or cessation of therapy. PGE therapy resulted in a wide spectrum of multisystem adverse effects which were reversible with reduction or cessation of therapy. Although the administration of PGE was safe and generally well tolerated, close medical supervision is necessary to avoid serious side effects.

RECENT ADVANCES IN LIPID MICROSPHERE TECHNOLOGY FOR TARGETING PROSTAGLANDIN DELIVERY

Although PGE1 exhibits pharmacological activities in free form, it has been hypothesized and experimentally verified that carrier can target them more effectively at lower doses, thus causing fewer

side effects. Lipid microspheres (LM) with a diameter of 0.2 μ m are drug carriers prepared from soybean oil and lecithin, and the drug is incorporated within the LM. Lipo-PGE1 is LM preparations of PGE1 that are designed to accumulate at the vascular lesions. The newly developed lipo-PGE1 (lipo-AS013) could overcome the disadvantages of the preparation currently available^[15]. Lipo-AS013, a precursor of PGE1, is considered superior to free PGE1 in terms of its chemical stability and the retention ratio in LM in the body.

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Original Articles

Zonula occludin toxin, a microtubule binding protein

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Subject headings microtubules; microtubule proteins; microtubule-associated proteins; intercellular junctions; vibrio cholerae; cholera toxin; zonula occluding toxin

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Abstract

AIM To investigate the interaction of Zot with microtubule.

METHODS Zot affinity column was applied to purify Zot-binding protein(s) from crude intestinal cell lysates. After incubation at room temperature, the column was washed and the proteins bound to the Zot affinity column were eluted by step gradient with NaCl ($0.3 \text{ mol}\cdot\text{L}^{-1}$ - $0.5 \text{ mol}\cdot\text{L}^{-1}$). The fractions were subjected to 6.0%-15.0% (w/v) gradient SDS-PAGE and then transferred to PVDF membrane for N-terminal sequencing. Purified Zot and tau protein were blotted by using anti-Zot or anti-tau antibodies. Finally, purified Zot was tested in an *in vitro* tubulin binding assay.

RESULTS Fractions from Zot affinity column yielded two protein bands with a M_r of 60kU and 45kU respectively. The N-terminal sequence of the 60kU band resulted identical to β -tubulin. Zot also cross-reacts with anti-tau antibodies. In the *in vitro* tubulin binding assay, Zot co-precipitate with Mt, further suggesting that Zot possesses tubulin-binding properties.

CONCLUSION Taken together, these results suggest that Zot regulates the permeability of intestinal tight junctions by binding to intracellular Mt, with the subsequent activation of the intracellular signaling leading to the permeabilization of intercellular tight junctions.

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INTRODUCTION

Vibrio cholerae, the human intestinal pathogen responsible for the diarrhoeal disease cholera, elaborates a large number of extracellular proteins, including several virulence factors. The severe dehydrating diarrhoea characteristic of cholera is induced by cholera toxin (CT). A number of epidemiological studies^[1-4] have shown a concurrent occurrence of the CT genes (ctx-A and ctx-B) and the genes for two other virulence factors elaborated by *V. cholerae*, zonula occludens toxin (Zot)^[5] and accessory cholera toxin (Ace)^[6]. Zot increases the intestinal permeability by rearranging the intestinal cell cytoskeleton strategically located to modulate intercellular tight junctions^[7]. However, the first step of Zot signaling after the protein internalization remains to be established.

Microtubules (MT) are intracellular structures functionally and anatomically related to the cell cytoskeleton. MTs are composed of α -tubulin and β -tubulin. Factors known to regulate microtubule dynamic include microtubule-associated proteins (MAPs). Neuronal MAPs, the most abundant of which are MAP2 and tau, stimulate MT assembly^[8,9]. The best characterized function of MT network polymers is the bi-directional movement of membrane vesicles driven by the MT-based motor proteins, kinesin and cytoplasmic dynein. Different cargoes can be transported via MT-dependent vesicles, including various types of endocytic and exocytic vesicles^[10,11]. Connection of actin filament network has been found^[12]. In eukaryotic organisms, various cell functions, including cell shape and mobility require coordinational interaction between actin and MT cytoskeleton^[13].

In our study, we found that when cell lysates from mammalian tissues passed through a Zot affinity column, two proteins bound to Zot, the N-terminal sequence of one of these two proteins revealed that it corresponded to tubulin; Zot cross-reacted with antibody against Tau, another well described MAP isolated from mammalian brain; by using a microtubule binding assay, Zot was found co-precipitated with MT. Taken together, these results suggest that Zot is a new member of MAPs family. This Zot property may be involved in the Zot signaling leading to the regulation of intercellular TJ.

MATERIAL AND METHODS

Purification of 6-his Zot

Plasmid pSU111, containing the clone Zot gene in a pQE-30 vector with a 6-histidine tag at its N-terminal, was grown in LB medium with 20 g/L glucose, 25 µg/L kanamycin and 200 µg/L ampicillin at 37°C with vigorous mixing until the A_{600} reached 0.7-0.9. Cultures were then induced with 2 mmol·L⁻¹ isopropylthio-β-D-galactoside (IPTG, Fisher), followed by an additional 2 h culture period at 37°C with vigorous shaking. The cells were harvested by centrifugation at 4 000×g for 20 min and resuspended in buffer A (6 mol·L⁻¹ Guanidine-HCl, 0.1 mol·L⁻¹ Na-phosphate, 0.01 mol·L⁻¹ Tris-HCl, pH 8.0; 5 mL/g wet cell weight). After stirring for 1 h at room temperature, the mixture was centrifuged at 10 000×g for 30 min at 4°C. A 50% slurry of Superflow (QIAGEN, 1 mL/g wet cell) was added to the supernatant and stirred for 1 h at room temperature. The mixture was loaded onto a 5 cm×1.5 cm column and washed sequentially with buffer A, buffer B (8 mol·L⁻¹ urea, 0.1 mol·L⁻¹ Na-phosphate, 0.01 mol·L⁻¹ Tris-HCl, pH 8.0) and buffer C (8 mol·L⁻¹ urea, 0.1 mol·L⁻¹ Na-phosphate, 0.01 mol·L⁻¹ Tris-HCl, pH 6.3). Each wash step was continued until the A_{280} of the flow-through was less than 0.01. His-Zot was eluted by addition of 250 mmol·L⁻¹ imidazole (1,3-diaza-2,4-cyclopentadiene) to buffer C. After dialysis against urea, the eluate was diluted 200-500 times in PBS, stirred with 50% slurry Superflow (1 mL/g wet cell weight) for 2 h at room temperature, loaded onto another 5 cm×1.5 cm column, washed with phosphate-buffered saline (PBS) and eluted with 250 mmol·L⁻¹ imidazole in PBS. Purity of the His-Zot protein was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blot using polyclonal anti-Zot antibodies.

Analytical procedures

SDS-PAGE It was carried out on a 50 g·L⁻¹-150 g·L⁻¹ gradient gel, stained with Coomassie brilliant blue dye, destained by 75 mL·L⁻¹ acetic acid with 100 mL·L⁻¹ methanol and dried with Gel Drying Film (Promega).

Western blot analysis Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (MILLIPORE). Non-specific binding sites were blocked by PBS with 50 mL·L⁻¹ milk plus 1 g·L⁻¹ Tween-20. Primary and secondary antibodies were rabbit polyclonal anti-Zot antibody and anti-rabbit IgG (peroxidase conjugate, Sigma), respectively. Films were exposed with ECL detection reagent (Amersham) for 1 min, and developed by Konica SRX-101 developer.

Immobilization of his-Zot to AminoLink plus column

One mg of his-Zot in 4 mL coupling buffer (0.1 mol·L⁻¹ sodium phosphate, 0.15 mol·L⁻¹ NaCl, pH 7.2) and 40 µL of 5 mol·L⁻¹ sodium cyanoborohydride, were added to an equilibrated AminoLink plus column (Pierce) and gently mixed overnight at 4°C. After washing with coupling buffer, 4 mL of 1 mol·L⁻¹ Tris-HCl (pH 7.4) and 40 µL of 5 mol·L⁻¹ sodium cyanoborohydride were added to the column followed by gently mixing for 30 minutes at room temperature to block the remaining active sites. The column was washed with 1 mol·L⁻¹ NaCl and stored in PBS containing 0.5 g·L⁻¹ sodium azide.

Preparation of human tissue plasma membranes

Adult human brain tissues was obtained from the Brain and Tissue Banks for Developmental Disorders at the University of Maryland and used under the approval of the University's Institutional Review Board. Adult human heart and intestinal tissues were utilized for comparative analysis. Tissues were washed with buffer D (20 mmol·L⁻¹ Tris-HCl, 20 mmol·L⁻¹ EDTA, 250 mmol·L⁻¹ sucrose, pH 7.5), homogenized in buffer E (buffer D containing 5 mg·L⁻¹ leupeptin, 2 mg·L⁻¹ aprotine, 1 mg·L⁻¹ pepstatin, 10 mg·L⁻¹ phenylmethanesulfonyl fluoride (PMSF), and centrifuged at 5 000×g, 4°C for 10 min. Supernatants were centrifuged at 12 000×g, 4°C for 45 min. Precipitates were discarded and supernatants were centrifuged at 30 000×g, 4°C for an additional 90 min. Precipitates were dissolved in buffer E with 5 g·L⁻¹-3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sitting on ice for 60 min with gentle mixing every five minutes.

Affinity purification of his-Zot binding proteins

Membrane preparations obtained from human brain was loaded on an AminoLink plus -Zot affinity column, washed, and equilibrated with PBS at room temperature containing 1 g·L⁻¹ Triton X-100. The columns were incubated for 90 min at room temperature, washed with 8 volumes of PBS containing 1 g·L⁻¹ Triton X-100, and eluted with PBS containing 1 g·L⁻¹ Triton X-100 with 0.1 mol·L⁻¹ NaCl, 0.3 mol·L⁻¹ NaCl, 0.5 mol·L⁻¹ NaCl, 0.8 mol·L⁻¹ NaCl and 1.0 mol·L⁻¹ NaCl, respectively. Fractions were collected and subjected to SDS-PAGE. N-terminal amino acid sequence analysis.

N-terminal amino acid sequence analysis

The fractions of human tissue lysates containing Zot binding proteins were resolved by 50 g·L⁻¹

150 g·L⁻¹ gradient SDS-PAGE and transferred onto PVDF membranes using CAPS buffer [10mmol·L⁻¹ 3-(cyclohexylamino)-1 propanesulfonic acid and 100 mL·L⁻¹ methanol]. The protein bands were excised and subjected to N-terminal sequencing using a Perkin-Elmer Applied Biosystems Apparatus Model 494.

Microtubule binding assay

Spin-Down assay kit (Cytoskeleton) was used in the experiments according to the *manufacturer's* recommendations. MT(20 nmol·L⁻¹ final concentration) were obtained by mixing an aliquate of tubulin (20 μL, 5 g/L) and 2 μL, 200 mmol·L⁻¹ taxol in G-PEM buffer (80mmol·L⁻¹ Pipes pH 6.8, 1mmol·L⁻¹ MgCl₂, 1mmol·L⁻¹ EGTA and 1mmol·L⁻¹ GTP). One ug of Zot was incubated with 20 nmol·L⁻¹ microtubule at room temperature for 20 min, while MT-associated protein MAP2 and bovine serum albumin (BSA) were used as positive and negative control, respectively. Proteins attached to MT and unbound proteins were separated by centrifugation. Each reaction product was carefully placed on the top of a cushion (G-PEM buffer plus 400 g·L⁻¹ glycerol) in Ultraclear TM ultracentrifuge tube. Following centrifugation, supernants and pellets were carefully removed and supernants were mixed with 1/20th volum of 500 g·L⁻¹ TCA solution for precipitation protein using centrifuge. Both supernants and pellets of the preparations containing the tested proteins were analyzed by either SDS-PAGE (MAP2 and BSA) or western blotting (Zot).

RESULTS

Isolation of Zot binding proteins from human brain

His-Zot was successfully immobilized to AminoLink Plus gel with immobilization yields of 89%-95%, as established by the protein assay (Bio-Rad detergent-compatible protein assay). Plasma membrane preparations from human brain loaded on the Zot affinity column contained two major Zot-binding proteins with apparent molecular masses of approximately 45kU and 55kU, respectively (Figure 1, lane 2).

N-terminal sequencing of the Zot binding proteins from human brain

The N-terminal sequences of the two Zot/zonulin binding proteins are shown in Table 1. The two proteins were also compared to other protein sequences by Blast search analysis. The N-terminal sequence of the 55kU protein was 100% identical to the N-terminal sequence of tubulin (Table 1) whereas the -45kU protein band resulted 72% identical to the N-terminus of calprotectin, a

calcium binding protein associated to chronic inflammatory processes^[14] and the cystic fibrosis antigen (CFA)^[15]. This second protein resulted to be the Zot/zonulin brain receptor^[16].

Western immunoblotting experiments

To investigate whether Zot and tau (a well characterized MAP) are immunologically related, cross immunoscreening experiments were performed. As shown in Figure 2, both proteins were recognized by either anti-Zot antibodies (left panel) or anti-tau antibodies (right panel). These results suggest that Zot and tau are immunologically related.

Microtubule binding assay

To confirm that Zot possesses MT binding properties, an *in vitro* binding assay was performed. As shown in Figure 3, we observed that Zot co-precipitated with MT as shown by Western immunoblotting analysis, while no Zot was found unbound. BSA lacking MT-binding properties was present only in the supernant. MAP2, a well defined MT associated protein, co-precipitated with MT and was not present in the supernant. These results confirmed the Zot ability to bind to MT.

Table 1 N-terminal amino acid sequences of Zot binding protein (55kU), β-tubulin, Zot binding protein (45kU), calprotectin (MRP-8), and cystic fibrosis antigen

Sample	N-terminus	Identity(%)
Zot binding protein-55kU	MREIVHIQAGQAGNQIGAKF	
β-tubulin	MREIVHIQAGQAGNQIGAKF	100
Zot binding protein-45kU	LTELEKALNXGGVGHKY	
Calprotectin (MRP-8)	LTELEKALNSIIDVYHKY	77
Cystic fibrosis antigen	LTELEKALNSIIDVYHKY	77

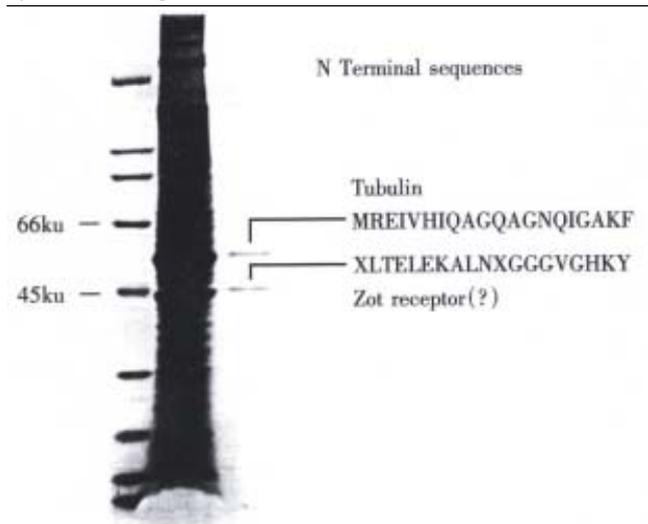


Figure 1 SDS-PAGE of Zot binding proteins isolated by affinity column chromatography from human brain cortex plasma membrane preparations. Lane 1, molecular mass standards; Lane 2, whole-plasma membrane lysate; lane 3, eluate with 0.5 mol·L⁻¹ NaCl in PBS containing 1 g·L⁻¹ Triton X-100.

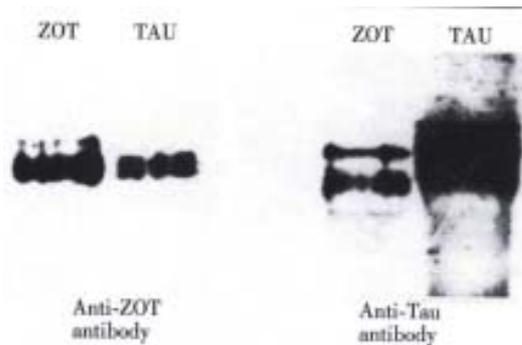


Figure 2 Western immunoblotting of Zot and tau using either anti-Zot antibodies (left panel) or anti-tau antibodies (right panel). Zot and tau were recognized by both antibodies.

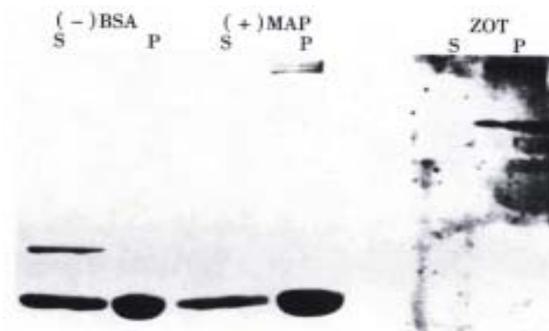


Figure 3 Microtubule binding assay. BSA (negative control) and MAP2 (positive control) were visualized by Coomassie staining (left panel) while Zot was visualized by immunoblotting (right panel). The bottom protein bands in the left panel are tubulins. As anticipated, BSA remained in the supernatant (S), while MAP2 was entirely precipitated in the pellet (P). Zot also appeared confined in the pellet, confirming that it bound to MT.

DISCUSSION

Tj is the hallmark of absorptive and secretory epithelia. As a barrier between apical and basolateral compartments, the tj selectively controls the paracellular passage of water, solutes and immune cells between epithelial and endothelial cells. Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway, since the resistances of eukaryotic cells plasma membrane are relatively high^[17]. Tj represent the major barrier in this paracellular pathway and the electrical resistance of epithelial and endothelial tissues seems to depend on the number of transmembrane protein strands and their complexity as observed by freeze-fracture electron microscopy^[18]. It has become abundantly clear that, in the presence of Ca²⁺, assembly of the tj is the result of cellular interactions that trigger a complex cascade of biochemical events that ultimately lead to the formation and modulation of an organized network of tj elements, the composition of which has been only partially characterized^[19].

Identification and characterization of Zot, a toxin produced by *Vibrio cholerae*, has provided

new information on the regulation of intercellular tj^[5,7,20-22]. After binding to its surface receptor, Zot is internalized^[23], and subsequently triggers a series of intracellular events including phospholipase C and PKC α -dependent actin polymerization which leads to the opening of tj^[7]. However, the complete cascade of the intracellular events activated by Zot, particularly concerning the early steps, remains undefined. There is now a large body of evidence that protein phosphorylation plays a major role in tj development^[24] and cytoskeleton rearrangement^[25]. In eukaryotic cells, junctional complex proteins, actin filaments, microtubules, and intermediate filaments interact to form the cytoskeleton network involved in determination of cell architecture, intracellular transport, modulation of surface receptors, paracellular permeability, mitosis, cell motility, and differentiation^[26]. We have previously demonstrated that there are two Zot binding proteins in the cell lysates of Zot-sensitive tissues^[16]. One has been characterized as the Zot/zonulin receptor. With this paper we showed that tubulin is the second Zot-binding protein. Based on these results, it is possible to hypothesize that Zot affects the actin filament network by binding to MT. The association of Zot to MT could be responsible of the effects of Zot on cell uptake and intracellular trafficking of molecules^[10,11] as well as the changes of tj structure and permeability. Alterations of intestinal tj occur in a variety of clinical conditions affecting the gastrointestinal system, including food allergies, malabsorption syndromes, and inflammatory bowel diseases. The knowledge that can eventually be acquired by studying the regulation of tj may have a tremendous impact on our understanding of the pathogenesis of these disease. It would not be surprising if the modification of tj structure and function by these pathological conditions would be an extension of normal physiologic regulation of tj.

However, several questions remain unanswered: what is the role of Zot-MT interaction on rearrangement of actin filament? Does this interaction affect the permeability of tj? Are MT-dependent cell functions, such as redistribution of organelles and the polarized distribution of membrane proteins, influenced by the MT-Zot binding? Experiments aimed at addressing these questions are presently in progress in our laboratory.

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Anticipation phenomenon in familial adenomatous polyposis: an analysis of its origin

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Subject headings familial adenomatous apolyposis (FAP); anticipation phenomenon; intergenerational bias; child generations; hereditary disorder; mortality

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Abstract

AIM To analyze the origin of the anticipation phenomenon, which means earlier death in successive generation in familial adenomatous polyposis.

METHODS The study subjects were 2161 patients with familial adenomatous polyposis and their 7465 first-degree relatives who were members of 750 families registered at our Polyposis Registry. The ages at death and cumulative mortality rates in the parent, the proband, and the child generations were compared for both all subjects and the patients alone.

RESULTS In the patients over 5 years of age, the mean age at death was 50.9 years for the parent, 42.3 years for the proband, and 33.3 years for the child generations, respectively ($P < 0.001$). The deceased rates in the three generations were 90.7%, 51.3% and 23.1% of the patients, respectively, and this difference was the main cause of the anticipation measured by parent-child pairing method. The cumulative mortality rates for all subjects failed to show anticipation, however the cumulative mortality rates for the patients showed the anticipation. The anticipation phenomenon was shown by any parent-child pairing methods for the deceased patients. Other important causes of the anticipation were different proportion of causes of death between generations ($P < 0.001$), and a low proportion of detected or deceased patients

($P < 0.001$) in the child generation.

CONCLUSION Anticipation in familial adenomatous polyposis may be caused by parent-child pairing methods as well as several intergenerational biases.

INTRODUCTION

The earlier onset of a hereditary disorder in successive generations, often with increased severity or early death, is known as anticipation^[1,2]. Despite the development of medical care over generations, this phenomenon is commonly encountered in human dominant type hereditary disorders including familial adenomatous polyposis (FAP) clinically. The anticipation was evaluated by Penrose^[1] in myotonic dystrophy and by Veale^[2] in FAP using parent-child pairs. They attributed the apparent anticipation to ascertainment biases and the general variability in age of onset in the parent-child pairs. They assumed that the modifying allelic gene might be the cause of the lack of the parent-child correlation. Of these two conditions, the cause of the anticipation in myotonic dystrophy was proved to expand trinucleotide repeats in the causative gene^[3-9].

However, a mutation of the APC gene is stable, and the site of mutation determines the severity or associated features of FAP with strong parent-child correlation^[10-13]. If the anticipation is the biological phenomenon in FAP, not only we need to investigate the cause of the anticipation in the APC gene, but also it has a considerable clinical meaning because colorectal cancer must be prevented by an early detection and treatment. Consequently, it has aroused our interest to study whether the anticipation phenomenon in FAP is a biological fact.

MATERIALS AND METHODS

Between January 1975 and December 1995, data were collected from 750 families from 1198 FAP patients registered at our Polyposis Registry^[14]. These FAP cases fulfilled the diagnostic criteria by Bussey^[15]. Turcot syndrome was excluded. Histories of their family members were obtained from their doctors who registered patients, death

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certificates and the National Family Registry^[16]. All first-degree relatives of proved FAP patients were recruited and no selection was made in these collections. Among the families of 9626 members (4991 men, and 4635 women), 2161 were FAP patients with colorectal cancer, and 7465 were their first-degree family members. Their births and deaths were certified by the National Family Registry or from the documents of the registry, and causes of death were ascertained by death certificates or by inquiring the doctors. The 9626 members were divided into three generations. They were 2958 individuals in the generation of the index patients (the proband generation), 4273 in the generation of their parents (the parent generation), and 2395 in the child generation of the indicated patients (the child generation). The ages at death, causes of death, and cumulative mortality rates of the three generations were compared to evaluate the features of anticipation. The cumulative mortality rates were calculated for both the entire group and the FAP patients by the Kaplan-Meier method. Chi-square test was used for comparison of occurrence rate in pairs of groups, and *t* test was used for the comparison of age.

RESULTS

There were a total of 3891 deceased individuals in the entire group. The proportion of deceased individuals in each generation was 66.0% in the parent generation, 39.5% in the proband generation, and only 10.5% in the child generation ($P < 0.001$, Table 1). The mean age at death was 41.6 ± 26.2 years in the parent generation, 26.3 ± 22.3 years in the proband generation, and 15.8 ± 16.8 years in the child generation. These low age and large standard deviation in the age at death were the result of early childhood death occurring before five years of age, accounting for 18.4% of all deaths in the parent generation, 33.4% in the proband generation, and 47.2% in the child generation (Table 1). We examined the causes of death in each generation and their age at death (Table 2). The death from unknown causes in the parent generation (0.84 to FAP deaths) was almost five times higher than that in the other two generations ($P < 0.001$). In this generation, the mean age at death (36.7 ± 19.8 years) from unknown causes was significantly lower than that at death from other causes ($P < 0.05$).

The number of FAP patients was 508 (17.2%) in the parent generation, 1316 (30.8%) in the proband generation, and 337 (14.1%) in the child generation (Table 3). Among them, the number of deceased FAP patients was 460 (90.6% of 508) in the parent generation, 675 (51.3% of 1316) in the proband generation, and 78 (23.1% of 337) in the

child generation. There were significant differences among the groups in the FAP death rate ($P < 0.001$). The age at death of FAP patients in each generation was 50.9 ± 12.4 years, 42.3 ± 12.3 years, and 33.3 ± 8.8 years, respectively, and the differences among the generations were significant ($P < 0.001$). These results showed that any random selection of deceased intergenerational pairing in FAP patients would produce anticipation caused by these differences between the generations.

The cumulative mortality rates of the FAP patients gave us a few clues to the anticipation (Figure 1). The generation included less young FAP patients, and the start of death was delayed as compared to other generations. It may be said pseudo-anticipation. Between the child and proband generations, the acceleration of death was observed in the age between 22 and 40 years. Both a low detection rate of FAP patients (14.1%, Table 3) in the child generation, and a low portion of deceased FAP patients (23.1%) among them may cause this situation because many FAP patients were remained undiagnosed. This suggests a comparatively short observation period in the child generation. The incidence of colorectal cancer increased rapidly between 20 to 40 years of age, and this period might be overestimated in this generation.

In order to minimize these intergenerational biases in FAP patients, we calculated the cumulative mortality rates for the entire members as well as for the FAP patients to evaluate the anticipation. The cumulative mortality rates for the all subjects were plotted on Weible's probability paper according to each generation (Figure 2). If the mortality curve of a descendant shifts to the left, it means earlier death or anticipation, and if a mortality curve shifts downside, it means the improvement of the general health condition of the group. The cumulative mortalities for both the parent and the proband generations were almost the same and parallel, while there was a slight vertical shift in these two curves, no horizontal shift was observed. This calculation method revealed no anticipation between the parent and the proband generations. The mortality rate of the child generation differed from those of the other generations; the overall cumulative mortality rate was low because of the lower infantile mortality in this generation. Although the mortality curve for the child generation showed a steeper slope line than those for the other two generations, the starting point of the steep slope, just after 20 years of age, was common to all the generations.

Table 1 Deceased subjects in each generation, *n* (%)

Generation	Total	Deceased	Age at death ($\bar{x} \pm s$, years)	Early childhood death [*] (% of the deceased cases)
Parent	2958	1952(66.0) ^b	41.6 ± 26.2	359(18.4)
Proband	4273	1687(39.5) ^b	26.3 ± 22.3	563(33.4)
Child	2395	252(10.5) ^b	15.8 ± 16.8	119(47.2)

^b $P < 0.001$, among the generations; ^{*}Death before the age of five years.

Table 2 The proportion of causes of death in each generation and their mean age at death (each number of deaths is shown as a proportion to the number of FAP deaths)

Generation	FAP	Extra colonic malignancies	Other diseases	Unknown cause	Death in childhood
Parent n = 1952	1.00 [*] (50.9 ± 12.4)	0.37 (57.6 ± 14.4)	1.24 (58.2 ± 19.6)	0.84 ^b (36.7 ± 19.8) ^a	0.78
Proband n = 1687	1.00 (42.3 ± 12.3)	0.11 (46.2 ± 15.8)	0.37 (36.7 ± 19.0)	0.19 (22.7 ± 16.7)	0.84
Child n = 252	1.00 (33.3 ± 8.8)	0.13 (26.2 ± 16.3)	0.44 (22.9 ± 14.2)	0.14 (21.9 ± 14.2)	1.53

FAP: Death from familial adenomatous polyposis and colorectal cancer. n: Total number of deaths.

^{*}: Deaths from FAP were calculated as 1.00. ($\bar{x} \pm s$, years): Age at death.

^aP<0.05, vs other causes of death; ^bP<0.001, vs other two generations.

Table 3 Age at death in the FAP patients, n(%)

Generation	Total number of subjects	Patients with FAP [*]	Deceased FAP patients	Age at death ($\bar{x} \pm s$, years)
Parent	2958	508(17.2)	460(90.6)	50.9 ± 12.4 ^b
Proband	4273	1316(30.8)	675(51.3)	42.3 ± 12.3 ^b
Child	2395	337(14.1)	78(23.1)	33.3 ± 8.8 ^b

^bP<0.001, among the generations

FAP: familial adenomatous polyposis.

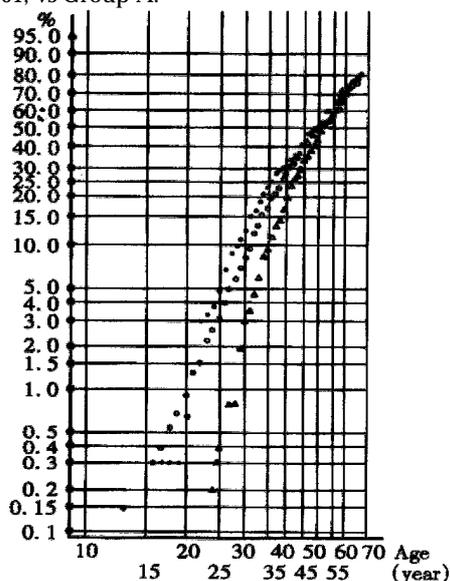
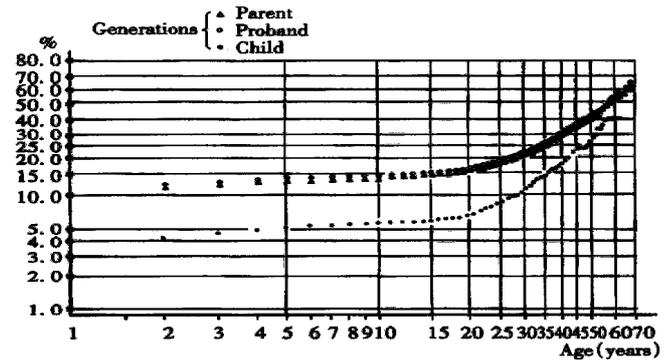
^{*}FAP patients and patients with colorectal cancer.

Table 4 Deceased parent-child pairs of FAP patients classified by the parental status of the child, and the anticipation

Age at death	Group A	Group B ^b
Parent>child	184	93
Parent≤child	57	5

Group A: Parent-child pairs in which the child had become a parent.
Group B: Parent-child pairs in which the child had not had any child.

^bP<0.01, vs Group A.

**Figure 1** Age specific cumulative mortalities of FAP patients. Circles: the proband generation; Triangles: the parent generation; Black dots: the child generation**Figure 2** Age specific cumulative mortalities of all subjects, including FAP patients and their first-degree relatives.

DISCUSSION

The anticipation phenomenon is so powerful that it surmounts the improvement of medical standards over years, and it is easily detected in hereditary diseases^[1,2,17-19]. Comparison of the age at death or onset in affected parent-child pairs is a conventional method in the study of the anticipation phenomenon^[1,2,17,18]. McInnis *et al*^[18] and Imamura *et al*^[19] applied the life-table analysis or random pairs method only to diagnosed patients and excluded other family members. If this method was applied to our FAP patients, the mean age at death in the proband generation was 8.5 years lower than that in the parent generation, and that in the child generation was 9 years lower than that in the proband generation. These figures exceed a standard period of 5 years to calculate postoperative survival of patients with colorectal cancer. This apparent anticipation was well correlated to the proportion of deceased cases in these three generations. This indicates that any fair pairing method will produce the anticipation. Veale^[2] estimated that a lack of parent-child correlation in onset of FAP was a major cause of sampling bias in his study over a limited period of time. He also concluded that the lack of correlation was due to the allele of the APC gene. Although his suggestions have not been denied as a contributor to the anticipation, some recent reports^[10-12] have suggested an apparent relationship between the site of the APC gene mutation and colorectal polyp density and retinal pigments in FAP patients. In addition, the comparison of the age at onset or death in parent-child pairs inevitably has two selection biases besides the intergenerational biases. One is that the parent has already been selected because he or she has already had at least one child, and this child must necessarily have already developed a symptom or died in order to make a parent-child pair. The other one is that a patient with a severe condition is difficult to gain a child, and this patient cannot be treated as a parent. We

confirmed the effect of selection bias in analysis of our 339 deceased parent-child pairs with FAP (Table 4). When the pairs were classified according to whether the child of the pair had become a parent or not, the childless group showed a significantly higher incidence of the anticipation phenomenon.

Recent reports have revealed that the elongation of trinucleotide repeat sizes is correlated with the increased severity of several hereditary neurological diseases such as myotonic dystrophy^[4], fragile X syndrome^[5], Huntington's disease^[6,7], and spinal and bulbar muscular atrophy^[8]. Although it is proved that the meiotic elongation of trinucleotide repeats accelerates these diseases, it does not necessarily mean that the elongation is inevitable in the meiosis. If we take parent-child pairs, it may seem as if the meiosis in these diseases constantly increases the length of trinucleotide repeats as the cause of the anticipation. The anticipation is such a powerful phenomenon as Ashizawa *et al*^[20] noted in 48% of 56 parent-child pairs that showed contractions of CTG repeat. Because anticipation is commonly encountered in clinical practice, its mechanism must be rather general. It is necessary to approach the elucidation of this phenomenon using some methods other than parent-child pair comparisons^[21]. We studied not only the FAP patients but also their first-degree relatives in our calculations of life tables. In this kind of studies, the change of medical environment among generations may influence the pattern of mortality. Besides its difficulty in taking the recent improvement of medical care in count, we have several reasons for not calculating this influence. ① parent-child pairing method in this series showed an apparent anticipation phenomenon; ② overall improvement of mortality in colorectal cancer was not so substantial as the anticipation phenomenon was diminished; and ③ proportion of early detection and preventive treatment in FAP patients was comparably small^[14]. Bias was minimized by our method because the effect of deaths of undiagnosed FAP patients in the parent generation was taken into account. In the parent generation, inherent bias was present in the form of deceased but undiagnosed young FAP patients. In the child generation, the bias was present mainly in the form of premature observation period and unidentified FAP patients, picking up only young deaths.

As the anticipation phenomenon is observed only in the conditions that threaten their life and reproductive ability, one of the methods to avoid these biases is to examine the anticipation phenomenon on the FAP specific phenotypes that do not affect the life of FAP patients. For example, congenital hypertrophy of the retinal pigment epithelium is specific to FAP, and it has no influence on optical function. Our experience did not show apparent anticipation phenomenon in number of the pigment areas^[22]. Despite these

results it will be wise to watch FAP patients for colorectal cancer before the age at which their parents had cancer.

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Review of 336 patients with hepatocellular carcinoma at Songklanagarind Hospital

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Subject headings hepatoma/therapy; biopsy; neoplasm staging; survival analysis; neoplasms metastasis; prognosis; drug therapy

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Abstract

AIM To determine the clinical presentations, survival and prognostic factors of hepatocellular carcinoma (HCC) in Southern Thailand.

METHODS Retrospective analysis was performed on the 336 hepatocellular carcinoma patients treated at Songklanagarind hospital between 1 January 1991 and 31 January 1999.

RESULTS Of these 336 patients, 276 were males and 60 were females. The mean age was 54.4 years. The common symptoms and signs were abdominal pain and hepatomegaly. The most common presentation of tumor was a dominant mass with daughter nodules. Portal vein involvement was found in 50% of total. Extra hepatic metastasis was found in 13%, and the lung was the most common site. There were 65.4% with evidence of cirrhosis and half of them were in *Child's* class B. HBsAg was positive in 72.6%. Regarding *Okuda's* tumor staging, 15%, 61% and 24% were stage I, II and III, respectively. Overall median survival was 2.1 months (11.5, 2.6 and 0.7 months for stage I, II and III respectively). Treatments of HCC improved patient survival (5.5 months vs 1.6 months for untreated patients). Most common causes of death were hepatic failure. Using multivariate analysis, the prognostic

factors identified were tumor staging, alpha-fetoprotein level above 10 000 $\mu\text{g}\cdot\text{L}^{-1}$, extrahepatic metastasis, portal vein thrombosis and treatment.

CONCLUSION HCC in Thailand is a fatal disease with poor outcome due to late presentation and high prevalence of liver cirrhosis. Early detection and proper management may improve outcome.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and is the leading cause of cancer death especially among males in South-East Asia including Thailand^[1,2]. This may be related to high prevalence of chronic hepatitis B infection (8%-15% in Asia and Africa, and 8%-12% in Thailand)^[3-7]. Though many types of treatment have been tried, HCC is still a fatal disease possibly associated with the advanced stage at which the disease is usually diagnosed^[2,8-11]. Thus, it remains a serious medical problem in this part of the world. There were many reports of the natural history of HCC in Japan, Mainland China, Southern Africa, Alaskan Eskimos, Taiwan, Italy, Spain and North America, but little information has been published from South-East Asia^[9-24]. Therefore, we reviewed 336 HCC patients at Songklanagarind Hospital to describe the clinical presentations and history of known risk factors and determine the survival rate, prognostic factors and the benefit of treatments.

MATERIALS AND METHODS

Patients

The medical records of 336 HCC patients admitted at Songklanagarind hospital between January 1, 1991 and January 31, 1999 were reviewed retrospectively. The diagnosis of HCC was made by liver biopsy or elevated serum alpha-fetoprotein level above 500 $\mu\text{g}\cdot\text{L}^{-1}$ with radiologic findings suggestive of HCC in patients whose liver biopsy was not available^[25]. Data from medical records, including patient demographic, known risk factors, clinical manifestation, abnormal physical findings, laboratory data (complete blood count, coagulogram, renal function test, liver function

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tests, viral hepatitis serology, serum alpha-fetoprotein level, chest X-ray, ultrasonography, CT scan, liver biopsy and other tissue biopsy if suggested metastasis), survival and treatments modality, were used for analysis. The known risk factors include alcohol drinking, history of blood transfusion and history of jaundice or viral hepatitis infection or known cases of cirrhosis. Patients were classified into 6 groups based on their clinical presentation: group 1, mass-related symptoms (abdominal pain or fullness, dyspepsia, palpable mass); group 2, cirrhosis-related symptoms (jaundice, GI bleeding, edema, abdominal enlargement, hepatic encephalopathy); group 3, liver abscess-like symptoms (high fever with abdominal pain and tenderness); group 4, non-specific symptoms (anorexia, nausea, vomiting, malaise, weight loss and anemia); group 5, metastasis symptoms (dyspnea, cough, bone pain and palpable lymph node); and group 6, asymptomatic cases (accidental finding by routine check-up or complain of other unrelated disease). The abnormal physical findings included anemia, jaundice, fever, hepatomegaly, splenomegaly, ascites and sign of chronic liver stigmata such as palmar erythema, spider nevi, gynecomastia and superficial dilated vein. A test of viral marker for hepatitis B (HBsAg) was done in most of patients but that for hepatitis C (anti-HCV) was not available until 1996. Tumor volume was calculated from ultrasonography or CT scan of the liver by a radiologist. Tumor volume or sum of tumors in instances multiple nodules were expressed as fraction of total liver and subsequently classified into two groups (tumor size $\leq 50\%$, $>50\%$ of the whole liver). Staging of HCC was made according to Okuda's^[9]. Cirrhosis was confirmed by liver biopsy or ultrasonography or CT scan and classified by Child-Pugh's (Class A, B or C)^[26]. The extrahepatic metastasis was confirmed by histology (incisional biopsy, excisional biopsy, necropsy or autopsy).

Therapy

The treatment of HCC ranged from no treatment, transhepatic artery oily chemoembolization (TOCE), percutaneous ethanol intralesional injection (PEI), hepatectomy, systemic chemotherapy and multimodality combination chemotherapy.

For systemic chemotherapy before 1995, we used intravenous adriamycin and/or 5-FU injection. Later this was changed to PIAF regimen (cisplatin $80 \text{ mg} \cdot \text{m}^{-2}$ body surface area (BSA) and adriamycin $40 \text{ mg} \cdot \text{m}^{-2}$ BSA at d1, followed by 5-FU $500 \text{ mg} \cdot \text{m}^{-2}$ infusion over 24 h for the following 3 d, with alpha-Interferon $5 \text{ mU} \cdot \text{m}^{-2}$, iv, 3 h after cisplatin and 5-FU every day).

TOCE was performed monthly by super-selective insertion of catheter to the right or left hepatic artery branch feeding the tumor then injection adriamycin 50 mg, lipiodol 8 mL and gelfoam. PEI was performed by using 10 mL of absolute ethanol injected percutaneously under CT-scan guide.

Multimodality combination therapy comprised a combination of several treatments depending on the tumor staging and complication, such as the patients who presented with advanced HCC and had portal vein involvement with lung metastasis, the treatment was started with systemic chemotherapy (PIAF regimen) until no evidence of extrahepatic metastasis remained and was then followed by TOCE and/or intralesional ethanol injection (PEI). Because of the limitation of retrospective study, we were unable to determine the exact outcome or improvement of general condition after treatments, so we determined the outcome by survival analysis.

In patients who were lost to follow-up, we determined the date and cause of death from the population register and personal contact with the family. Patient status was unable to determine in 20% of the patients. For statistical analysis these patients were considered as censored at the date of last contact.

Survival profiles were constructed using the Kaplan-Meier method. Prognostic factors were identified using Cox proportional hazards regression. *P* value <0.05 was considered statistically significant.

RESULTS

Of the 336 patients, 276 (82%) were male with a male to female ratio of 4.6:1. The mean age was 54.4 (a range of 20-89) years (54.3 years in male and 55 years in female) (Table 1). Diagnosis was confirmed histologically in 273 (72.3%) cases and 63 (18.7%) cases were diagnosed by a combination of elevated serum alpha-fetoprotein level above $500 \mu\text{g} \cdot \text{L}^{-1}$ and imaging such as ultrasound or CT scan showing a lesion compatible with HCC.

The most common symptom was mass-related such as abdominal pain, abdominal discomfort, dyspepsia and palpable mass (Table 2). Mean duration of symptoms was 49 d (range $<1 \text{ day} - 1 \text{ year}$). Among the abnormal physical findings hepatomegaly was the most common, followed by fever and jaundice (Table 2).

Elevation of alkaline phosphatase and serum aspartate aminotransferase (AST) was the most common abnormal finding in the liver function test. Serum bilirubin above $51.3 \mu\text{mol} \cdot \text{L}^{-1}$ was found in 30%. The most common radiologic finding was a solitary mass with or without daughter nodules (73%, Table 1). The tumor was located most frequently in the right lobe (53%) followed by both

lobes (37%) and left lobe (10%). Tumor volume larger than half of the total liver was found in 71% and portal vein thrombosis or portal vein involvement in 50%. Portal vein thrombosis or involvement was significantly associated with increased tumor volume ($P = 0.0005$). The mean serum alpha-fetoprotein level was 145 110 (range 2-7 990 000 $\mu\text{g}\cdot\text{L}^{-1}$) and values above 500 $\mu\text{g}\cdot\text{L}^{-1}$ (the cut point for diagnosing HCC) occurred in 64% (Table 1).

Table 1 HCC patient characteristics

Patient characters		n	%	Mean	Range
Age(years)	All			54.4	20-89
	Male			54.3	20-89
	Female			55.0	20-81
Sex	Male	276	82.0		
	Female	60	18.0		
Risk factors	Alcohol drinking	126	38.0		
	HBsAg positive (299 sample)	217	72.6		
	anti-HCV positive (135 sample)	10	7.4		
	Cirrhosis	219	65.2		
Liver function test	Total bilirubin ($\mu\text{mol}\cdot\text{L}^{-1}$)			63.8	3.4-752.4
	Direct bilirubin ($\mu\text{mol}\cdot\text{L}^{-1}$)			38.8	0.5-581.4
	Aspartate aminotransferase (U·L ⁻¹)			225	17-3890
	Alanine aminotransferase (U·L ⁻¹)			97	4-3370
	Alkaline phosphatase (U·L ⁻¹)			304	8-2080
	Albumin (g·L ⁻¹)			35.6	20-52
Radiologic finding	Globulin (g·L ⁻¹)			38.2	18-78
	Solitary type (total)	240	73.0		
(n = 329)	with daughter nodules	144	43.8		
	without daughter nodules	96	29.2		
	Multinodular type	47	14.3		
	Diffuse or infiltrative type	42	12.7		
Alpha fetoprotein ($\mu\text{g}\cdot\text{L}^{-1}$) (n = 295)				145 110	2-790 000
	<10	38	13.0		
	10-99	36	12.0		
	100-499	33	11.0		
	≥500	188	64.0		
Okuda's staging	Stage I	51	15.0		
	Stage II	205	61.0		
	Stage III	80	24.0		

The most common risk factor was chronic hepatitis B infection (72.6%), and 65.2% showed evidences of liver cirrhosis (Table 1). Patients with cirrhosis were classified into Child A, B and C in 20%, 55.3% and 24.7% respectively. Cirrhosis was found in 76.2% of alcoholic patients, 66.4% of HBV infected patients and 90% of HCV infected patients. Okuda's staging distribution was 15%, 61% and 24% for stage I, II and III respectively (Table 1). Spontaneous rupture of HCC was found 11% (4%, 9.3% and 20% in stage I, II and III). At the time of diagnosis, extrahepatic metastasis occurred in 43 cases (13.1%), 13.7%, 10.7% and 18.8% of stage I, II and III respectively. The metastatic sites were lung (76%), lymph node (16%) and bone (7%).

Overall median survival was 2.1 months (Stage I, 11.5 months; Stage II, 2.6 months; and Stage III, 0.7 months) (Table 3, Figure 1). The 1 and 2-year survival rates were 15% and 8% respectively. Treatment of HCC was associated with improvement of patient survival (5.5 vs 1.6 months

in non-treated group; $P = 0.011$) (Table 3, Figure 2). In the non-treated group ($n = 245$) median survival was 1.6 months (7.7, 1.8 and 0.6 months for stages I, II and III respectively, Table 3, Figure 3). Regarding treatment, patients treated with TOCE, intravenous chemotherapy, multimodality combination therapy and Tamoxifen administration had median survival times of 6.3, 5.33, 17.1 and 3 months, respectively (Table 3). Compared to the non-treated group, patients treated with TOCE, intravenous chemotherapy or combination therapy had significantly better survival ($P = 0.0005$, 0.011 and 0.007 respectively) whereas survival of the patients treated with Tamoxifen was not significantly different from non-treated ($P = 0.86$) patients.

Table 2 Presenting symptoms and abnormal physical findings of patients

Presenting symptoms	n	%
Mass-related symptoms (Abdominal pain or fullness, dyspepsia, palpable mass)	188	56
Cirrhosis-related symptoms (Jaundice, GI bleeding, edema, abdominal enlargement, encephalopathy)	59	17.6
Liver abscess-like symptoms (High fever with acute abdominal pain and tenderness)	45	13.4
Non-specific symptoms (Anorexia, nausea, vomiting, malaise, weight loss, chronic anemia)	26	7.7
Metastasis symptoms (Dyspnea, cough, bone pain, palpable lymph node)	11	3.2
Asymptomatic (Routine checked up or other unrelated disease)	7	2.1
Abnormal physical findings		
Hepatomegaly	282	83.9
Fever	185	50.5
Jaundice	143	42.6
Anemia	138	41.1
Ascites	123	36.6
Cachexia	86	25.6
Chronic liver stigmata	86	25.6
Edema	59	17.6
Splenomegaly	42	12.5

Table 3 Median survival

Group of patients	n	Median survival (months)
All	336	2.1
Stage I	51	11.5
Stage II	205	2.6
Stage III	80	0.73
Untreated	245	1.6
Stage I	26	7.7
Stage II	146	1.8
Stage III	73	0.63
Treated	91	5.5
Stage I	25	13.7
Stage II	59	4.2
Stage III	7	1.7
TOCE	44	6.3
Stage I	11	24.3
Stage II	29	5.5
Stage III	4	1.4
Chemotherapy (adriamycin and/or 5-FU)	16	5.2
Multimodality therapy (PIAF/iv, chemotherapy±TOCE±PEI)	12	17.1
Tamoxifen	9	3.0
PIAF regimen chemotherapy	5	.
Hepatectomy	4	§
PEI	1	§

*Data of PIAF regimen chemotherapy is not completely finished. §It were too small number of patients to evaluated.

The most common causes of hospital death ($n = 54$) were hepatic failure, GI bleeding and rupture of tumor (59.2%, 20.4% and 20.4% respectively). Among cases with hepatic failure, sepsis was the most common complication leading to death. Cox proportional hazards model reviewed the following, prognostic factors: Okuda's stage II ($P = 0.014$, hazard ratio 2.05), stage III ($P = 0.001$, hazard ratio 3.79), AFP level above $10\,000\ \mu\text{g}\cdot\text{L}^{-1}$ ($P = 0.001$, hazard ratio 2.03), lung metastasis ($P = 0.01$, hazard ratio 1.93), lymph node metastasis ($P = 0.015$, hazard ratio 3.76), portal vein involvement or thrombosis ($P = 0.0005$, hazard ratio 1.79) and treatment ($P = 0.011$, hazard ratio 0.91).

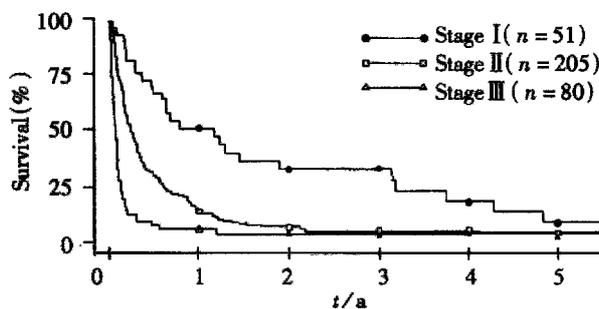


Figure 1 Kaplan-Meier survival curve for overall patients. Total ($n = 336$) median survival of 2.1 months; Okuda's stage I ($n = 51$), median survival of 11.5 months; Okuda's stage II ($n = 205$), median survival of 2.6 months; Okuda's stage III ($n = 80$), median survival of 0.7 months ($P = 0.014$ and 0.001 respectively).

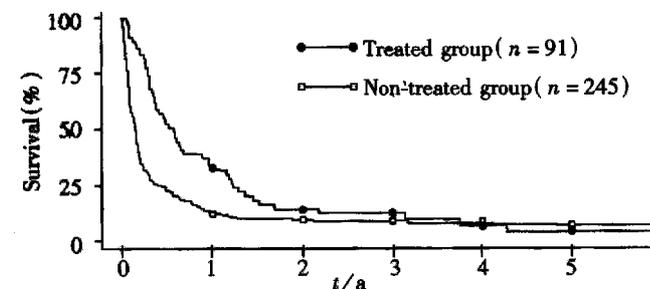


Figure 2 Kaplan-Meier survival curve in relation to treatment or non-treatment. Treated group ($n = 91$), median survival of 5.5 months; non-treated group ($n = 245$), median survival of 1.6 months ($P = 0.011$).

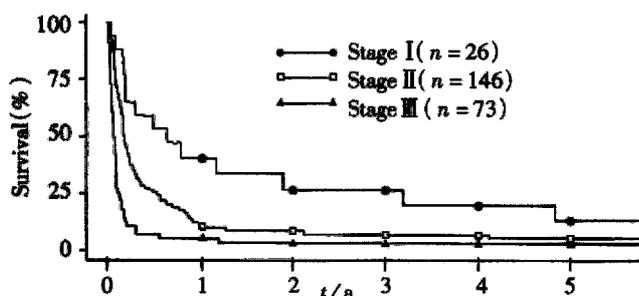


Figure 3 Kaplan-Meier survival curve in non-treated group. Total ($n = 245$), median survival of 1.6 months, Okuda's stage I ($n = 26$), median survival of 7.7 months; Okuda's stage II ($n = 146$), median survival of 1.8 months; Okuda's stage III ($n = 73$), median survival of 0.6 months.

DISCUSSION

Similar to other studies, we found that HCC was more common in males (a male to female ratio of 4.6:1) because the risk factors such as cirrhosis, chronic HBV infection and alcoholic are more frequently seen in males than females^[2,8,12-19,27,28]. In our study, the most common risk factor was chronic HBV infection (72.6%) as HBV is endemic in South-East Asia^[5]. The common symptoms were non-specific and included abdominal pain, dyspepsia, jaundice, hepatomegaly, anorexia and weight loss. Clinical jaundice was found in 42.6% and mainly caused by failure of hepatic function due to cirrhosis. The most common type of tumor in our patients was dominant mass with daughter nodules whereas multiple nodules or diffuse lesion are common in Western patients^[15,17,18]. The difference in risk factors may explain the variation in tumor characters as chronic HBV infection is the most important risk factors in our region while chronic HCV infection and alcohol drinking are the largest risk factors in Japan and Western countries^[3,8,13-18,27,29]. Cirrhosis was found in 65% of our patients and 80% of them were classified as Child's B or C. These may be associated with poor prognosis. In our study, hepatectomy and TOCE were not suitable in more than half of the patients due to portal vein involvement (50%) and advanced liver cirrhosis (24.7%). Extrahepatic metastasis was found in 13% and most of them were located in the lung, probably because of direct drainage into the right heart via the hepatic vein. Elevated serum alpha-fetoprotein above $500\ \mu\text{g}\cdot\text{L}^{-1}$ was found in only 64% of patients, so this tumor marker was not very sensitive for diagnosis of HCC in our country. Most our patients had advanced HCC (61% and 24% were stage II and III respectively). The overall median survival in our patients was 2.1 months because majority of our patients had advanced disease with significant liver cirrhosis. In treatment of HCC, hepatectomy and TOCE could improve survival^[9,13], but could not be performed because of liver cirrhosis and portal vein involvement. Systemic chemotherapy, arterial infusion and Tamoxifen administration could not improve survival^[9,20,24,30-32], whereas data of PIAF regimen chemotherapy showed complete pathological remission, but survival analysis did not^[33]. In our study, TOCE and multimodality therapy could improve survival, particularly in patients with stage I and stage II disease as compared with the non-treatment group (24.3% vs 7.7 months in stage I and 5.5 vs 1.8 months in stage II receiving TOCE and 17.1 months vs 0.63 months in patients receiving multimodality therapy). However, our study is only a retrospective study that had its limitation in comparing survival rate between different groups. The most of our patients died of hepatic failure (60%) as a majority of them had

advanced HCC and liver cirrhosis. By multivariate analysis, poor prognosis was associated with advanced stage of the tumor, serum alpha-fetoprotein level >10 000 µg·L⁻¹, extrahepatic metastasis and portal vein involvement.

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Three-dimensional image of hepatocellular carcinoma under confocal laser scanning microscope

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Subject headings HCC; nucleus, three-dimensional reconstruction; microscopy, confocal laser scanning

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Abstract

AIM To investigate the application of confocal laser scanning microscopy (CLSM) in tumor pathology and three-dimensional (3-D) reconstruction by CLSM in pathologic specimens of hepatocellular carcinoma (HCC).

METHODS The 30 µm thick sections were cut from the paraffin-embedded tissues of HCC, hyperplasia and normal liver, stained with DNA fluorescent probe YOYO-1 iodide and examined by CLSM to collect optical sections of nuclei and 3-D images reconstructed.

RESULTS HCC displayed chaotic arrangement of carcinoma cell nuclei, marked pleomorphism, indented and irregular nuclear surface, and irregular and coarse chromatin texture.

CONCLUSION The serial optical tomograms of CLSM can be used to create 3-D reconstruction of cancer cell nuclei. Such 3-D impressions might be helpful or even essential in making an accurate diagnosis.

INTRODUCTION

Under conventional light microscope, *histopathologists* often use plane image to evaluate the three-dimensional (3-D) cellular characteristics. Three-D configuration may be reconstructed by using serial mechanical sectioning, but its axis definition is not good, image is blur and tiny structure can't be shown clearly, and the specimen might be damaged. Using the serial optical tomograms and 3-D reconstruction function, confocal laser scanning microscopy (CLSM) can provide a much better quality 3-D image than conventional light microscope, and lead the observer into a brand-new 3-D world. Although CLSM has been used extensively in cell biology^[1], few applications were reported in routine clinical pathology such as three-dimensional DNA image cytometry by CLSM in thick tissue blocks of prostatic lesions and 3-D reconstruction by CLSM in routine pathologic specimens of benign and malignant lesions of human breast^[2-4]. In this study, 3-D reconstruction was performed on routine formalin-fixed, paraffin-embedded tissues of normal, and hyperplastic tissues of liver and hepatocellular carcinoma by using computer-assisted CLSM together with 3-D reconstruction. The goals of our study were to present 3-D morphologic characteristics of benign and malignant specimens of the liver and to attempt to demonstrate the usefulness of CLSM in routinely obtained surgical pathologic tissues.

MATERIALS AND METHODS

Two cases of normal liver tissue were selected from autopsy specimens collected in the Department of Pathology of Shanghai Medical University, six cases of hepatocellular carcinoma including adjacent liver tissue were routine clinical specimens collected in 1996 from the Liver Cancer Institute of Zhongshan Hospital affiliated to Shanghai Medical University.

All tissues were fixed in 10% formalin, embedded in paraffin, serial sections were cut at 5 µm and 30 µm. The 5 µm slices were stained with hematoxylin and eosin for conventional light microscopic observation. The 30 µm slices were stained with DNA fluorescent probe, YOYO-1 iodide (Molecular Probes, Eugene, Ore., USA).

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The sections were deparaffinized with xylene (10 min \times 2) and dehydrated with 100%, 95%, and 70% ethanol (5 min \times 2) and rinsed in distilled water for 2 min \times 5. The specimens were then fixed with 10% neutral buffered formalin for 30 min and washed with tap water. After rinsing with distilled water and 0.01M phosphate buffer 5 min \times 2, nuclear RNA was removed by incubating the sections for 30min at 37°C in 200 μ L of ribonuclease A (RNAase; Sigma, USA) at a concentration of 160 g/L in PBS. DNA was next hydrolyzed with 2N HCl for 25 min at 27.5°C. After rinsing with distilled water for 2 min \times 5, the sections were covered with 200 μ L of YOYO-1 iodide diluted into 1:2000 with PBS. The PBS was diluted 1:5 with distilled water to reduce the salt concentration. To this 200 μ L working solution of YOYO-1 iodide, 20 μ L of 0.1N HCl was added and the final solution was stored in the dark at 4°C for use. Homogeneous fluorescence intensity of nuclei at different depths of the confocal slices was obtained by agitating the YOYO-1 iodide for 1 h in the dark. Afterwards, the sections were rinsed with distilled water, covered with buffered glycerol, and the glass cover slip were sealed with finger nail polish. Sections were stored at 4°C in the dark until CLSM examination.

A Leica TCS-NT confocal laser scanning microscope equipped with epifluorescence optics and an appropriate combination of filters to visualize and digitize the images of the different specimens. An argon laser with an excitation wavelength of 488 nm was used to activate the green fluorescence of the YOYO-1 iodide-stained nuclear DNA (maximal absorption 491 nm and emission 509 nm). A 16 \times objective (numerical aperture of 1.30) was used to observe the specificity of the staining, and a 100 \times water objective (numerical aperture of 1.30) was used to study the details of chromatin pattern. Optical sections were collected throughout the entire stained thickness of the paraffin sections with a Z-step interval of 0.3 μ m or 0.6 μ m. The 3-D image processing was performed on a Leica computer with the original 3-D interactive visualization software. The collected confocal optical sections and the 3-D reconstructed images were printed with a Panasonic color video dye-sublimation copy processor.

RESULTS

Three-D morphologic features of normal liver cells

A microscopic field of view was selected from the normal liver specimen and comparison of the images taken under transmission conventional light microscope and CLSM is illustrated in Figure 1. The transmission conventional light microscopic image

was shown on the right side and the out-of-focus signals were visible. The out-of-focus blur reduced the contrast and sharpness of the final image. In the confocal image (left) the out-of-focus signals were cut off and only signals in focus were clearly visible. Optical sections (planes 1-60) were taken from the surface to the bottom of normal liver specimen with a Z-step interval of 0.5 μ m. Figure 2 shows the 10th, 20th, 30th, 40th, 50th and 60th plane digital images of Z-series. The confocal images were taken at 5, 10, 15, 20, 25 and 30 μ m depths, respectively. In the confocal images some nuclei appeared or disappeared depending on their orientation in space. The 60 2-D optical sections were computer focused on a plane (deep-focusing) to analyze the fine structure of chromatin patterns inside the nucleus and reconstructed 3-D images to display the 3-D detailed arrangement of nucleus. Figure 3 shows normal liver cells with similar round or ovoid nuclei, homogeneous intensity of YOYO-1 iodide fluorescence as well. Three-dimensional view was shown in Figure 4. The nuclear surface appeared smooth with homogeneous fluorescence intensity.

Three-D morphologic features of atypical hyperplasia of liver cells

Optical sections (planes 1-50) were taken from the surface to the bottom of atypical hyperplasia of liver specimen with a Z-step interval of 0.6 μ m. The structure of chromatin patterns inside the nucleus with homogeneous fluorescence intensity is shown with deep-focusing in Figure 5. Three-dimensional view is shown in Figure 6. The nuclear surface appeared smooth with homogeneous fluorescence intensity. But the volume of nucleus of atypical hyperplasia liver cell was bigger than that of normal liver cell.

Three-D morphologic features of hepatocellular carcinoma cells

Optical sections (planes 1-50) were taken from the surface to the bottom of hepatocellular carcinoma specimen with a Z-step interval of 0.6 μ m. The deep-focusing images were shown in Figure 7 and Figure 8. The structure of chromatin patterns inside the nucleus with heterogeneous karyotheca thickness, irregular and coarse chromatin texture, chromatin underside the karyotheca mainly. Three-dimensional view is shown in Figure 9 and Figure 10. HCC displayed remarkably different features in 3-D morphology, including: indented, molding, and irregular nuclear surface; marked pleomorphism; chaotic arrangement of tumor cell nuclei.

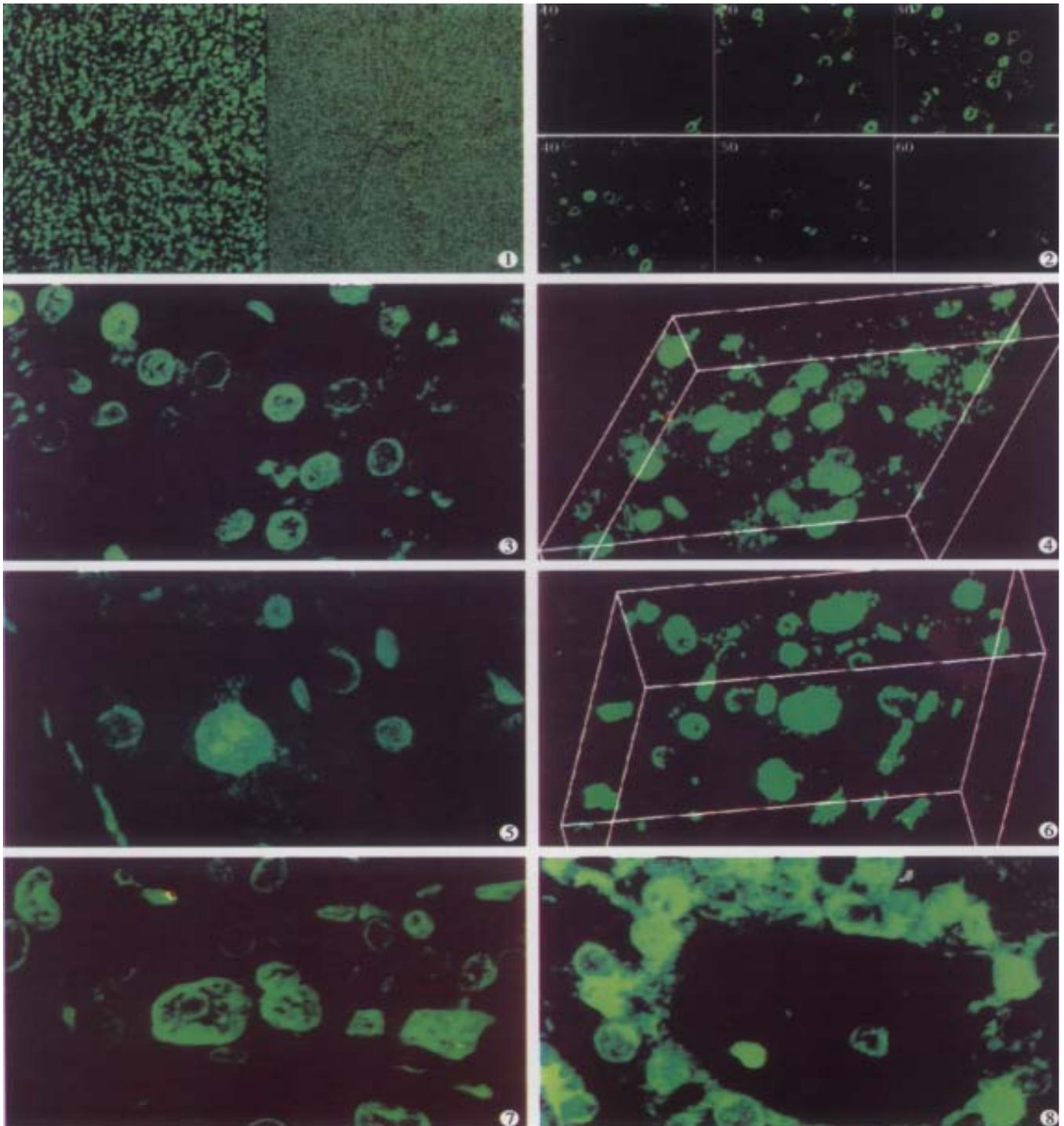


Figure 1 The transmission conventional light microscopic image was shown on the right side. The out-of-focus blur reduced the contrast and sharpness of the image. In the confocal image (left) the out-of-focus signals were cut off and only signals in focus were clearly visible. $\times 160$

Figure 2 The 10th, 20th, 30th, 40th, 50th and 60th plane digital images of Z-series. In the normal liver tissues of the confocal images some nuclei appeared or disappeared depending on their orientation in space. $\times 1000$

Figure 3 Deep-focusing image showed the normal liver cells with similar round or ovoid nuclei, similar in size and homogeneous intensity of YOYO-1 iodide fluorescence as well. $\times 1000$

Figure 4 Three-dimensional view of normal liver cells, the nuclear surface appeared smooth with homogeneous fluorescence intensity. $\times 1000$

Figure 5 Deep-focusing image of atypical hyperplasia liver cells, the structure of chromatin patterns inside the nucleus with homogeneous fluorescence intensity. $\times 1000$

Figure 6 Three-dimensional view of atypical hyperplasia liver cells. $\times 1000$

Figure 7 Deep-focusing image of HCC cells, the structure of chromatin patterns inside the nucleus with heterogeneous karyotheca thickness, irregular and coarse chromatin texture, chromatin underside the karyotheca mainly. $\times 1000$

Figure 8 Deep-focusing image of highly differentiated HCC chromatin texture. $\times 1000$

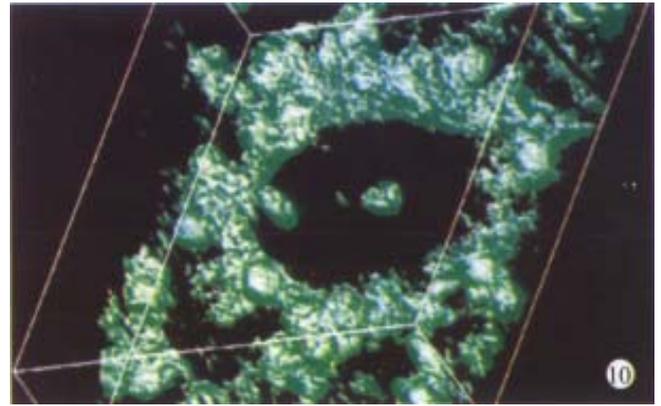
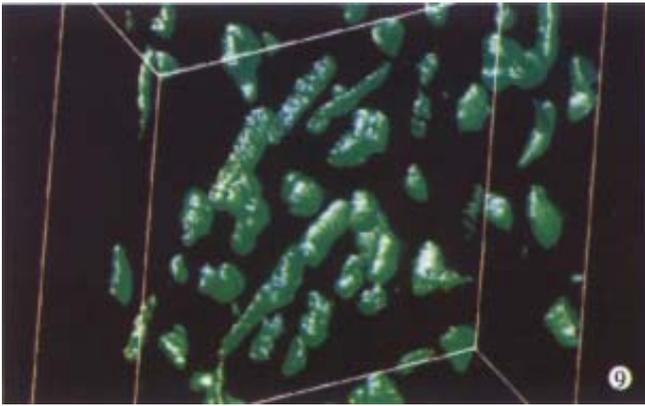


Figure 9 Three-dimensional view of spindle cell HCC. $\times 1000$

Figure 10 Three-dimensional view of tubular adenocarcinoma of HCC. $\times 1000$

DISCUSSION

The area, volume, shape, DNA content, and chromatin pattern of nuclei may be important for the diagnosis and prognosis of cancer. *Histopathologists* often use $4\ \mu\text{m}$ to $6\ \mu\text{m}$ thick paraffin sections to obtain representative and diagnostically relevant images. Due to the very limited section thickness in comparison with the size of the tissue, and the images are nearly two-dimensional, focusing up and down at high magnification provides a rough idea of the 3-D cellular characteristics, and such 3-D impression may be helpful or even essential in arriving at a certain diagnosis, especially for borderline lesions or tumors. However, in spite of the usefulness of such 3-D information about nuclei, conventional light microscopy is not always the ideal tool for 3D evaluation due to the interference of out-of-focus structures with the images of the focus plane studied. Much of the light emitted from the regions of specimen above and below the focal plane contributes to the out-of-focus blur, which seriously reduces the contrast and sharpness of the final image. Confocal laser scanning microscope allows the acquisition of optical sections from a thick specimen and out-of-focus blur can be reduced considerably and thus much sharper and clearer images will be obtained. CLSM has become an exciting new instrument in biomedical research because of its increased resolution over conventional light microscope and its utility for subsequent 3-D-reconstruction analysis^[5,6].

In this paper, the 3-D reconstruction have demonstrated 3-D contour of representative characteristics of normal liver cells, atypical hyperplasia liver cells, and HCC cells and the spatial relationship of nuclei, as well as the subtle structure of chromatin texture inside nuclei. This

paper emphasized the practical feasibility of CLSM and 3-D reconstruction from routine surgical histopathologic materials. To obtain desirable quality 3-D image from formalin-fixed, paraffin-embedded specimens, YOYO-1 iodide, a highly specific and sensitive (picogram sensitivity)^[7] DNA probe was utilized. Intense and homogeneous fluorescence was obtained by incubating the YOYO-1 iodide for 1h with agitation in the dark. To reveal subtle details of nuclear structure, RNA was removed by RNase predigestion, since nuclear RNA was stained by YOYO-1 iodide as well.

CLSM combines the three most advanced and important elements of our era, the microscope, the laser and the computer in one, moreover it is non-invasive and can be used on archival paraffin blocks. We anticipate that, in the future, pathologists may utilize these new techniques to make more precise diagnosis.

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Direct technetium-99m labeling of anti-hepatoma monoclonal antibody fragment: a radioimmunoconjugate for hepatocellular carcinoma imaging

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Subject headings antibody, monoclonal; antibody fragments; technetium-99m; hepatocellular carcinoma; liver neoplasms; radioimmunoimaging

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Abstract

AIM To directly radiolabel an anti-hepatoma mAb fragment HAB18 F(ab')₂ with ^{99m}Tc by stannous-reduced method, and assess the stability, biodistribution and radioimmunoimaging (R II). **METHODS** Immunoreactive fraction was determined according to Lindmo's method. Ellman's reagent was used to determine the number of thiols in the reduced F(ab')₂. Labeling efficiency and homogeneity were measured by paper chromatography, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Challenge assay involved the incubation of aliquots of labeled antibody in ethylenediaminetetraacetate (EDTA) and L-cysteine (L-cys) solutions with different molar ratio at 37°C for 1 h, respectively. Investigations *in vivo* utilized nude mice bearing human hepatocellular carcinoma (HHCC) xenografts with gamma camera imaging and tissue biodistribution studies at regular intervals. **RESULTS** The labeling procedure was finished within 1.5 h compared with the "pretinning" method which would take at least 21 h. *In vitro* studies demonstrated that the radiolabeled mAb fragment was homogeneous and retained its immunoreactivity. Challenge studies indicated that ^{99m}Tc-labeled HAB18 F(ab')₂ in EDTA is more stable than in L-cys. Imaging and biodistribution

showed a significant tumor uptake at 24 h post-injection of ^{99m}Tc-labeled HAB18 F(ab')₂. The blood, kidney, liver and tumor uptakes at 24 h were 0.56 ± 0.09, 56.45 ± 11.36, 1.43 ± 0.27 and 6.57 ± 3.01 (%ID/g), respectively. **CONCLUSION** ^{99m}Tc-HAB18 F(ab')₂ conjugate prepared by this direct method appears to be an effective way to detect hepatoma in nude mice model.

INTRODUCTION

The introduction of mAbs as targeting devices in nuclear medicine is well developed and many different antibodies which labeled with a variety of isotopes have been reported in cancer diagnosis. It seemed that ^{99m}Tc is the most popular radionuclide for nuclear medicine imaging because of its favorable physical characteristics, low cost, and ready availability. ^{99m}Tc labeled mAb fragments should be superior to other big molecule radioimmunoconjugates for use in tumor R II. A number of methods have been proposed for ^{99m}Tc labeling proteins, and mAbs in particular. In general, these methodologies can be divided into two categories: indirect and direct methods^[1]. In indirect method the protein was modified with a technetium binding ligand and then reacted with a technetium complex. Several bifunctional chelating agents have been synthesized and used, such as diethylenetriaminepentaacetic acid (DTPA)^[2], diamide dimercaptide N₂S₂ ligands, and hydrazino nicotinamide analog^[3]. Although it is said that the indirect method can lead to loss of immunoreactivity. Joiris *et al.* have tested that the derivatization of antibody or fragment by iminothiolane does not split the protein and keeps the immunoreactivity^[4]. By direct method, ^{99m}Tc metal ion binds directly to endogenous donor groups on the antibody. The method is simple to perform and compatible with practical clinical use. However, direct labeling of mAbs with ^{99m}Tc was reported to be unstable due to non-specific binding (low and high-affinity)^[5,6], but some reports suggest an improved labeling of proteins with ^{99m}Tc.

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In the Schwarz and Steinstrasser procedure, as modified by Mather and Ellison^[7], disulfide bridges in the mAb are reduced with 2-mercaptoethanol (2-ME). After purification, the resulting reduced antibody can be stored frozen until required for use. Labeling is accomplished by addition of stannous ion from a bone-scanning kit and pertechnetate. In addition to using regular reducing agents, such as 2-ME, stannous ions^[8], borohydride^[9], ascorbic acid^[10], dithionite^[11], or glutathione^[12] to generate sulphhydryl groups, other peculiar approaches also appeared recently. Direct ^{99m}Tc labeling of mAbs were finished by reduction of antibodies using photoactivation and insoluble macromolecular Sn (II) complex^[13,14]. With the development of direct method, there have been a few reports of successful use of this technique in colorectal, breast, and ovarian cancer imaging^[15-17].

In this report, we describe a direct method for radiolabeling anti-hepatoma monoclonal antibody fragment HAb 18F (ab')₂ with ^{99m}Tc . The stability and homogeneity of ^{99m}Tc -HAb18 F(ab')₂ were evaluated. The biodistribution and tumor localization in nude mice bearing a HHCC xenograft were studied.

MATERIALS AND METHODS

Monoclonal antibody

The mAb HAb18 is of murine IgG₁ isotype and was developed by our laboratory^[18]. F(ab')₂ fragment of HAb18 was generated by papain digestion with a molecular weight of 96 000 dalton^[19].

Tumors

Hepatocellular carcinoma grown in Balb/c mice was used as a prototype tumor model. Approximately 10⁷ HHCC cells obtained from Shanghai Cell Institute of Chinese Academy of Sciences were implanted in the left thigh of the animals and the tumors were allowed to grow for 8-10 days to approximately 1cm in diameter.

Antibody reduction

The antibody concentrated to 8 g/L in neutral PBS was reduced by reaction with a molar excess of stannous/glucoheptonate (Sn/GH) ranging from 10:1 to 50:1 (Sn/GH: MAb) at 37°C for 15 min-30 min. The Sn/GH with a mass ratio of 1:100 was dissolved in 50mM acetate-buffered saline (ABS), pH 5.3 purged with nitrogen. The reduced antibody was isolated from reductant through a PD-10 column (Pharmacia) equilibrated with 0.05 mol/L ABS. The number of resulting free sulphhydryl groups was assayed with Ellman's reagent 5, 5'-dithio-bis (2-nitrobenzoic acid), (DTNB, Sigma Chemical Co., USA)^[20]. One hundred μL of sample was mixed with 20 μL of 0.01 mol/L-DTNB and diluted to

3 mL with 0.05 mol/L Tris-HCl buffer pH 8.4. The mixture was incubated at room temperature for 15 min and coloration measured with an UV/VIS spectrophotometer at 412 nm. The number of thiols was obtained by comparison with a series of L-cysteine (L-cys) standards ranging from 0.312 mg/L to 10 mg/L.

The integrity of the reduced F(ab')₂ was determined by non-reduced SDS-PAGE with 100 g/L gel using Vertical Gel Electrophoresis System (Bio-Rad). The gel was stained with Coomassie brilliant blue R250. Control experiments were run using unreduced mAb F(ab')₂.

Radiolabeling

For labeling, 160 μg of reduced HAb18 F(ab')₂ was mixed with a 10 μL -20 μL of diluted Sn/GH solution (0.2 g/L), and pertechnetium solution (0.2 mL, 74MBq), (Chinese Academy of Atomic Energy) was injected into the mixture. The Sn/GH solution was freshly prepared each time by dissolving 100 mg GH and 1mg SnCl₂·2H₂O in 5 mL of saline purged with nitrogen. The reaction mixture was incubated for 0.5 h-1 h at 37°C before it was analyzed by Whatman 3MM paper chromatography which was then developed in acetone or 100 g/L trichloroacetic acid (TCA). R-f values for acetone are: mAb 0.0, ^{99m}Tc -GH 0.0, and $^{99m}\text{TcO}_4$ -0.9-1.0. R-f values for 100 g/L TCA are: mAb 0.0, ^{99m}Tc GH 0, and $^{99m}\text{TcO}_4$ - 0.7. Labeled mAb was differentiated from ^{99m}Tc colloid by the method of Thrall *et al*^[21]. The same strips impregnated with 10g/L-20g/L human serum albumin before development with 5:2:1, water: ethanol: 5N NH₄OH. Colloid remained on the bottom of the strip while mAb-bound isotope migrated with the solvent front.

The integrity of the labeled F(ab')₂ was assayed using the same non-reduced SDS-PAGE as described above. The gel was autoradiographed on x-ray film before stained with Coomassie brilliant blue R250.

Immunoreactivity assessment

The *in vitro* immunoreactivity of the radiolabeled HAb18 F(ab')₂ was evaluated by a live cell assay^[22]. Briefly, HHCC cells 5 × 10⁹/L were centrifuged (1 000r/min) for 5 min and washed twice with 1% bovine serum albumin (BSA) in PBS, then 5 serial 1:2 dilutions were made in 10 g/L BSA in Eppendorf tubes precoated with BSA. Radiolabeled HAb18 F(ab')₂ at a concentration of 40 ng/mL in 10 g/L BSA was added using a volume equal to half the volume of cell suspension. The total volume of cell-binding assay solution was 0.3 mL. After incubation for 2 h at 37°C, the total as well as the cell-bound radioactivity were counted in a gamma counter.

In Vitro stability studies

The stability was analyzed by using two different challenging agents, EDTA and L-cys. An aliquot of 50 μL $^{99\text{m}}\text{Tc}$ -HAb 18 F(ab')₂ solution was incubated with EDTA or L-cys at 37°C for 1 h. The molar ratio of mAb to challenging agent was at a maximum of 10 000:1. Dissociation ratio was analyzed on paper chromatography.

Biodistribution and imaging

Balb/c mice bearing HHCC were divided into three groups. Each group consisted of three animals and each animal received approximately 15 μg antibody with about 7.4MBq through a lateral tail vein. At time intervals of 4, 10 and 24 h postinjection, three groups of mice were killed, and imaged on a SPECT (Starcam 3 000, UK). Data were collected 100 000 counts per image and peak energy settings at the 140 keV (20%) window for $^{99\text{m}}\text{Tc}$. The blood and other organs of interest were collected. Tissues were washed, blotted, weighed and counted in a gamma counter. For each mouse, data are expressed as percent of injected dose per gram of tissue (%ID/g) after physical decay corrected.

RESULTS

Figure 1 represents the calibration curve for the determination of sulphhydryl groups using L-cys standards over a range of 0.312 to 10 mg/L, by plotting optical density at 412 nm versus L-cys standard concentrations after subtraction of the background due to Ellman's reagent. Linear regression was used and correlation coefficient 0.999 was obtained. Table 1 shows the influence of the reduction conditions on the number of free sulphhydryl groups detected by this thiol assay. As expected, increasing the molar ratio of Sn/GH to antibody in the reaction mixture does increase the number of apparent -SH groups per antibody, and increase the labeling efficiency correspondingly, which results in the labeling efficiency at a maximum of 84.2%. The free $^{99\text{m}}\text{TcO}_4^-$ and colloid amounts determined by Whatman 3MM paper using different developing systems were also showed in Table 1. In control experiments, labeling efficiency was 2% when unreduced HAb18 F(ab')₂ was used. SDS-PAGE by both staining and autoradiography showed that the radioactivity co-migrated with the proteins and that there were almost no protein fragments present within the 60:1 of molar ratio of Sn/GH to mAb (Figure 2). However, another SDS-PAGE in Figure 3 illustrates that fragmentation occurred during the reduction procedure when the molar ratio of Sn/GH to mAb was at 500:1.

As shown in Figure 4, the immunoreactive fraction, 0.84 was determined by plotting the inverse of the bound fraction compared with the inverse of the cell concentration, which is based on

the assumption that the total antigen concentration (cell concentration) is a good enough approximation for the free antigen concentration.

Challenging with EDTA did not remove $^{99\text{m}}\text{Tc}$ from the labeling conjugate remarkably, while L-cys at a molar ratio of 625:1 remove approximately one-tenth of the label (Figure 5).

Biodistribution of radioactivity in blood and excised tissues are displayed in Table 2. The preparation localized at the tumors was more than at any organ examined at both 10 h and 24 h after injection, except the kidneys. The lower radioactivity in blood at 24 h suggested fast blood clearance. The imaging results in Figure 6 showed significant tumor uptake at 24 h post-injection.

Table 1 Effect of molar ratio (Sn/GH: mAb) on quantity of-SH, free $^{99\text{m}}\text{TcO}_4^-$ and colloid and labeling efficiency (%), $n = 3$

Molar ratio (Sn/GH: mAb)	SH groups /mAb	$^{99\text{m}}\text{TcO}_4^-$	colloid	labeling efficiency
Control	0 \pm 0	62.1 \pm 4.5	1.1 \pm 1.2	2.0 \pm 0.9
10:1	0.43 \pm 0.04	3.0 \pm 1.4	1.4 \pm 1.14	4.6 \pm 3.8
20:1	1.25 \pm 0.10	2.9 \pm 1.1	1.4 \pm 0.77	2.8 \pm 5.1
30:1	2.46 \pm 0.08	2.0 \pm 0.9	3.2 \pm 1.47	8.6 \pm 3.2
40:1	3.34 \pm 0.09	1.8 \pm 1.2	2.8 \pm 1.38	4.2 \pm 2.8
50:1	3.61 \pm 0.12	2.1 \pm 0.8	3.6 \pm 1.58	4.4 \pm 3.4

Table 2 Biodistribution of $^{99\text{m}}\text{Tc}$ -HAb 18F(ab')₂ in nude mice bearing hepatoma ($\bar{x} \pm s$, %ID/g)

Organ	Time after injection (h)		
	4	10	24
Blood	2.21 \pm 0.24	1.45 \pm 0.15	0.56 \pm 0.09
Kidney	72.38 \pm 4.37	70.47 \pm 15.23	56.45 \pm 11.36
Liver	1.82 \pm 0.48	1.59 \pm 0.31	1.43 \pm 0.27
Lung	1.62 \pm 0.34	1.40 \pm 0.17	0.75 \pm 0.21
Stomach	1.37 \pm 0.39	1.05 \pm 0.28	0.50 \pm 0.29
Spleen	2.35 \pm 0.81	2.11 \pm 0.75	1.82 \pm 0.85
Large intestine	1.16 \pm 0.34	1.42 \pm 0.39	0.94 \pm 0.32
Small intestine	0.97 \pm 0.31	0.95 \pm 0.18	0.62 \pm 0.24
Heart	2.04 \pm 0.55	1.83 \pm 0.48	1.17 \pm 0.42
Muscle	1.15 \pm 0.20	0.77 \pm 0.28	0.51 \pm 0.25
Brain	0.18 \pm 0.02	0.07 \pm 0.04	0.02 \pm 0.01
Tumor	5.14 \pm 2.26	5.84 \pm 2.98	6.57 \pm 3.01

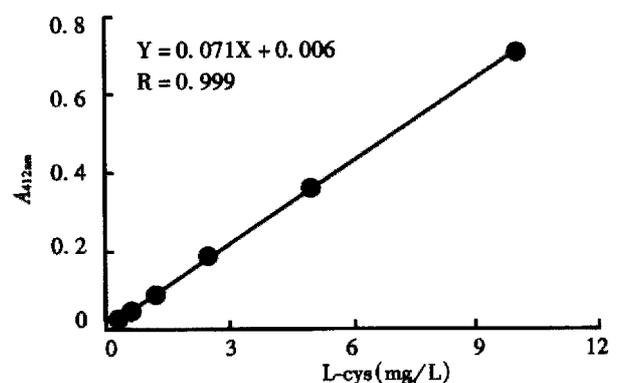


Figure 1 L-cysteine standard curve for sulphhydryl determination using Ellman reaction.

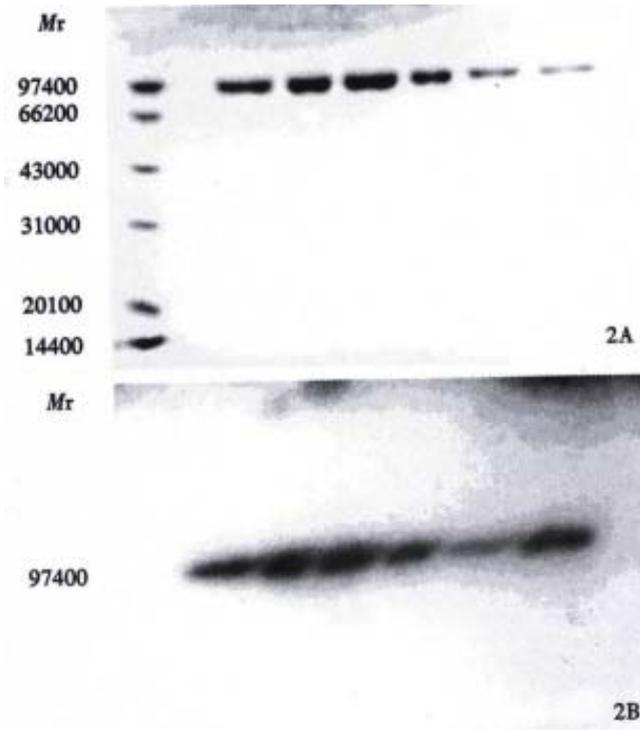


Figure 2 Effect of reduction on integrity of ^{99m}Tc-labeled HAb18 F(ab')₂ as monitored by SDS-PAGE. Vertical lanes represent molar ratios of Sn/GH to HAb18 F(ab')₂: 1, 10:1; 2, 20:1; 3, 30:1; 4, 40:1; 5, 50:1; 6, 60:1. (A) Coomassie brilliant blue R250 staining. Molecular weight (kD) is indicated at the left. (B) autoradiography.



Figure 3 Effect of reduction on integrity of HAb18 F(ab')₂ as monitored by SDS-PAGE. Molecular weights (kD) are indicated at the left. Vertical lanes represent molar ratios of Sn/GH to HAb18 F(ab')₂: 1, 1000:1; 2, 500:1; 3, 50:1; 4, 10:1; 5, unreduced F(ab')₂.

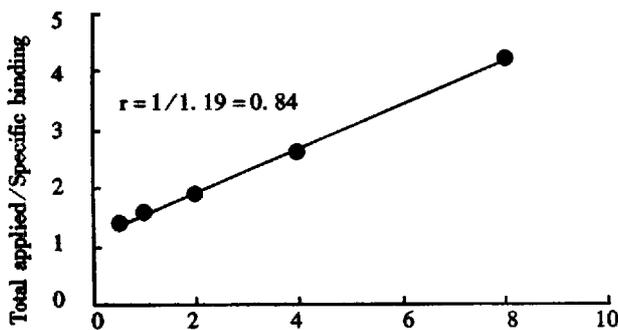


Figure 4 Binding assay for the determination of the immunoreactive fraction of ^{99m}Tc-labeled HAb18 F(ab')₂.

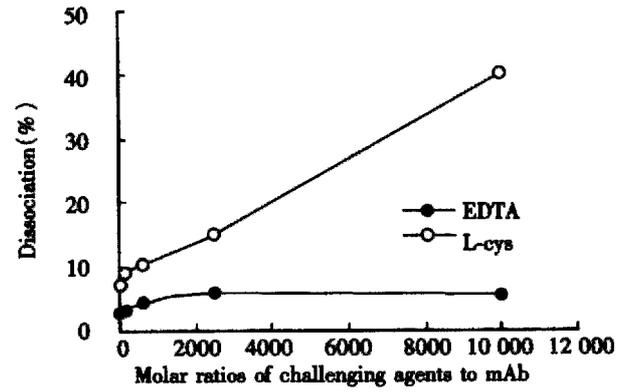


Figure 5 Dissociation of ^{99m}Tc-labeled HAb18 F(ab')₂ with increasing molar ratio of EDTA to mAb (●) and L-cys to mAb (○).

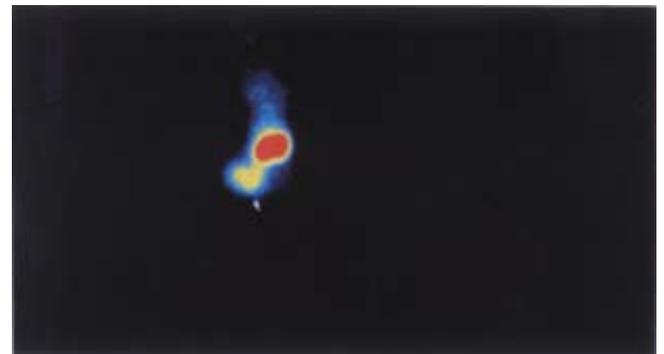


Figure 6 Images of nude mice bearing human hepatocellular carcinoma with ^{99m}Tc-HAb18 F(ab')₂ at 24 h.

DISCUSSION

Great efforts have been made to develop a method that can be used for the direct labeling of mAbs with ^{99m}Tc^[16]. Earlier studies involved the incubation of mAbs with stannous phthalate tartrate solution for up to 21 h at room temperature, which was named “pretinning” method. Clinical success with this method has been claimed by the author^[23].

One aim of our study was to further evaluate the role of stannous as a reducing agent in the direct labeling of mAb F(ab')₂ with ^{99m}Tc. The difference between the “pretinning” method and this method is that we use GH instead of phthalate-tartrate as transfer ligand and stabilizer to avoid Sn or Tc-colloid formation. To do this, we investigated the effect of the quantity of Sn/GH on the labeling time and efficiency. When the molar ratio of Sn/GH to mAb F(ab')₂ was constant, we found that there was no obvious difference on the number of-SH between the reduction time of 20 min and 30 min or even longer^[24]. The whole labeling process can be accomplished within 1.5h. Hnatowich *et al.* reported that labeling efficiency in the case of the stannous ion-reduced antibodies was generally in excess of 70%^[12], however, in our method molar ratio of Sn/GH to mAb was an important parameter to obtain good labeling results, and molar ratio of 40:1 or higher were needed to get labeling

efficiency of more than 80% (Table 1). The low percentage of free $^{99m}\text{TcO}_4$ and radiocolloid in each sample implied that pH 5.3 and GH are the optimal pH value and transfer ligand. Under this condition, the labeled mAb HAb18 F(ab')₂ keeps its immunoreactivity. Autoradiography of SDS-PAGE had only one migration of component identical to that of native HAb18 F(ab')₂ determination by staining with Coomassie brilliant blue R250 (Figure 2), which demonstrated that Sn/GH reduction is mild and does not destroy interchain bridges in mAbs. Labeling efficiency of 2% in control experiments using unreduced HAb18 F(ab')₂ indicated that there was no exchange with the low affinity sites and also demonstrated that reduction of disulfides is a necessary initial step in ^{99m}Tc direct labeling of antibodies. The bond between $^{99m}\text{Tc-SH}$ and Tc is stronger than that of N-Tc or O-Tc which was verified by the challenge assay of $^{99m}\text{Tc-HAb18 F(ab')}_2$ in the presence of EDTA. We found that EDTA even at a molar ratio of 10000:1 failed to remove a significant amount of ^{99m}Tc , this is in agreement with the results of Rhodes *et al*^[8]. But L-cys at 625;Å1 remove one-tenth of the label (Figure 5). Despite such instability of the label, there was no *in vivo* evidence of release of pertechnetate due to no thyroid imaging observed in the whole imaging process (Figure 7). Tumor localization of $^{99m}\text{Tc-HAb18 F(ab')}_2$ was successfully demonstrated in a human tumor/nude mouse xenograft model. Biodistribution and imaging results showed the highest tumor uptake at 24 h post-injection. Whereas kidney levels were found to be higher in the whole process. Accumulation of radioactivity in the kidney may be the result of retention of this metallic radionuclide by the kidney proximal tubule^[25], the possible release of ^{99m}Tc -labeled cysteine and glutathione^[26] stemming from the radioimmunoconjugate catabolism, and the relative amount of $^{99m}\text{Tc-GH}$. A technique has been used in patients to block renal tubule uptake of ^{99m}Tc -anti-CEA Fab' fragments by amino acid infusion^[27].

In conclusion, a radioimmunoimaging conjugate for hepatoma detection was prepared by direct labeling mAb HAb18 F(ab')₂-with ^{99m}Tc using stannous/glucoheptonate as reducing agent. Although the labeling efficiency is not satisfactory to some degree, it has several advantages: simple, easy and quick, besides, the labeled mAb fragment retains its immunoreactivity. Biodistribution and imaging studies reveal that this conjugate is useful for the detection of hepatoma.

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Effect of intestinal ischemia-reperfusion on expressions of endogenous basic fibroblast growth factor and transforming growth factor β in lung and its relation with lung repair

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Subject headings lung; intestinal ischemia-reperfusion injury; basic fibroblast growth factor; transforming growth factor β

Fu XB, Yang YH, Sun TZ, Gu XM, Jiang LX, Sun XQ, Sheng ZY. Effect of intestinal ischemia-reperfusion on expressions of endogenous basic fibroblast growth factor and transforming growth factor β in lung and its relation with lung repair. *World J Gastroentero*, 2000;6(3):353-355

Abstract

AIM To study the changes of endogenous transforming growth factor β (TGF β) and basic fibroblast growth factor (bFGF) in lung following intestinal ischemia and reperfusion injury and their effects on lung injury and repair.

METHODS Sixty Wistar rats were divided into five groups, which underwent sham-operation, ischemia (45 minutes), and reperfusion (6, 24 and 48 hours, respectively) after ischemia (45 minutes). Immunohistochemical method was used to observe the localization and amounts of both growth factors.

RESULTS Positive signals of both growth factors could be found in normal lung, mainly in alveolar cells and endothelial cells of vein. After ischemia and reperfusion insult, expressions of both growth factors were increased and their amounts at 6 hours were larger than those of normal control or of 24 and 48 hours after insult.

CONCLUSION The endogenous bFGF and TGF β expression appears to be up-regulated in the lung following intestinal ischemia and reperfusion, suggesting that both growth factors may be involved in the process of lung injury and repair.

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INTRODUCTION

Our previous investigations have shown that basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF β) play important roles in organ injury and repair after ischemia and reperfusion insult, and that there was a significant relationship between gene expression of bFGF or TGF β and lung repair^[1,2]. Because many growth factors are involved in wound repair by their mitogenic and non-mitogenic effects, we have further investigated the alteration of endogenous bFGF and TGF β in the lung tissue following intestinal ischemia-reperfusion injury and explored their effects on lung repair as well.

MATERIALS AND METHODS

Animal model and tissue preparation

Sixty male, pathogen-free Wistar rats, weighing 250 g \pm 10 g were used in this study. They were divided into 5 groups, which underwent sham-operation, ischemia for 45 minutes, and reperfusion for 6 hours, 24 hours and 48 hours after ischemia for 45 minutes, respectively. Anesthesia was induced by administration of 30 mg/kg of pentobarbital sodium. Following midline laparotomy, intestinal ischemia was achieved by complete occlusion of the superior mesenteric artery (SMA) with a non-crushing microvascular clip. Reperfusion was performed by removal of the microvascular clip after 45 minutes SMA occlusion. All animals were killed by exsanguination at designated times. The samples from right base of lung were obtained immediately and fixed in 10% formalin for analysis. All procedures but SMA clip were done in animals of sham-operated control group.

Immunohistochemical detection for bFGF and TGF β

Immunohistochemical detection was made using polyclonal anti-bFGF or TGF β antibody (Santa Cruz Co. and Zymed Co., respectively) by an indirect streptavidin/peroxidase (SP) technique. Experiments were performed following the manufacturer's recommendation. Paraffin-embedded sections were incubated with polyclonal anti-rat bFGF or TGF β antibody for 12 hours at 4°C

after antigen repair. Biotinylated IgG was added as second antibody. Horseradish peroxidase labeled streptomycin-avidin complex was used to detect second antibody. Slides were stained with diaminobenzidine, and examined under light microscope. The brown or dark brown stained cytoplasm and/or cell membrane was considered as positive. The phosphate-buffered saline (PBS) solution was used as negative control.

Statistical analysis

The slides from 5 animals in each group were used for observation and statistical analysis. One visual field in each slide was randomly selected and observed under light microscope with 400-fold magnification. The percentage of positive immunohistochemical staining cells was expressed as mean \pm SD. Statistical analyses were performed using paired *Student's t* test. $P < 0.05$ was considered significant.

RESULTS

Pathological alternations of lung tissue

The histological structure of alveolar and mesenchymal cells was normal in healthy lungs, while the lung tissues from ischemia and reperfusion rats were significantly damaged, with pulmonary edema and inflammatory cell infiltration.

Expression of bFGF and TGF β

Both bFGF and TGF β were expressed in alveolar epithelial cells and microvascular endothelial cells of normal lung tissues. The positive signals were of immunohistochemical staining in brown or dark brown color and localized in cytoplasm and/or membrane when observed under light microscopy (Figure 1A and B). After ischemia, the expressions of both bFGF and TGF β were increased, especially in the area of alveolar epithelial cells and capillary endothelial cells (Figure 2A and B). At 6 hours postinjury, the expression of bFGF was the same as that in the early injury, while that of TGF β was increased significantly. Many positive cells were type I alveolar cells (Figure 3A and B). Up to 24 hours and 48 hours postinjury, the expression of both growth factors returned to basal levels. By quantitative analysis, the expressions of both bFGF and TGF β were quite different in the early injury when compared with those of control group ($P < 0.01$, Table 1).

Table 1 Expression of bFGF and TGF β in lung following ischemia-reperfusion injury ($\bar{x} \pm s$)

Groups	Animals	bFGF	TGF β
Sham-operated	5	15.4 \pm 3.4	20.0 \pm 5.1
Ischemia 45min	5	61.8 \pm 7.5 ^b	63.4 \pm 7.0 ^b
Reperfusion 6h	5	42.4 \pm 10.1 ^b	50.6 \pm 7.1 ^b
Reperfusion 24h	5	29.0 \pm 5.5 ^b	32.8 \pm 8.7 ^a
Reperfusion 48h	5	15.6 \pm 3.3	19.4 \pm 7.1

^a $P < 0.05$, ^b $P < 0.01$, vs sham-operated.

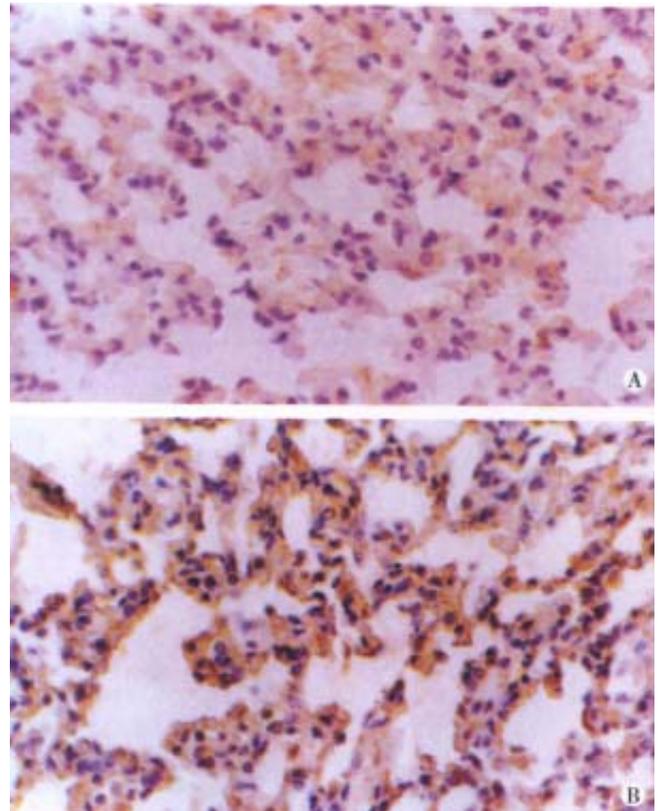


Figure 1 The expressions of bFGF and TGF β in normal lung. The weakly positive signal could be found in alveolar epithelial cells and microvascular endothelial cells. SP stain $\times 400$

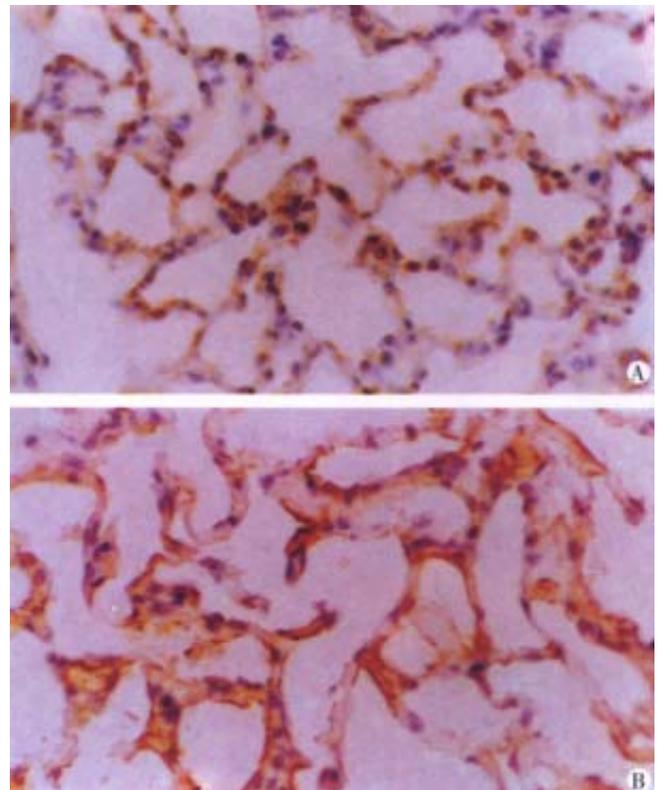


Figure 2 The expressions of bFGF and TGF β in damaged lung following ischemia (45 minutes) and reperfusion (6 hours). Deep staining of both growth factors could be found in alveolar epithelial cells and microvascular endothelial cells. SP stain $\times 400$

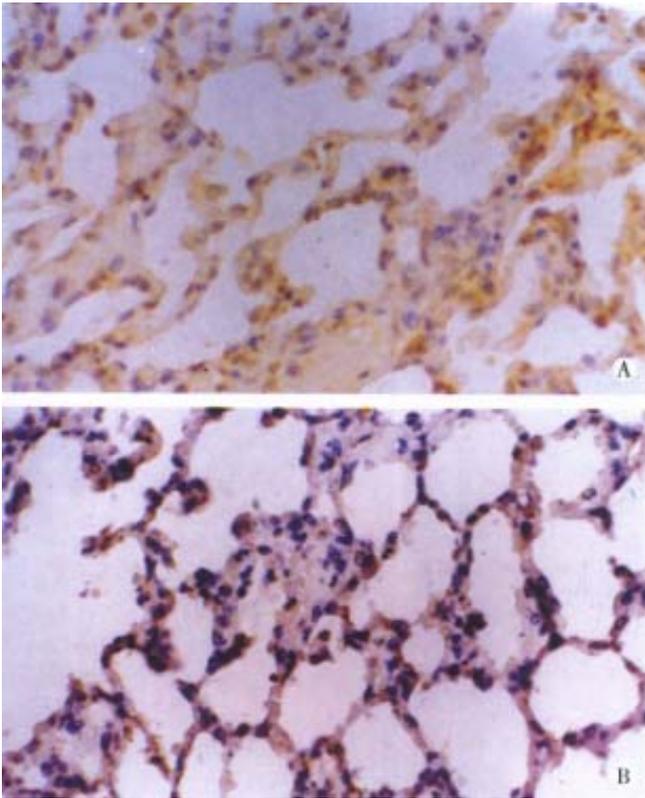


Figure 3 The expressions of bFGF and TGF β in damaged lung following ischemia (45 minutes) and reperfusion (24 hours). The staining of both growth factors could be found in alveolar epithelial cells and microvascular endothelial cells and the expression returned to normal levels. SP stain \times 400

DISCUSSION

The lung is one of the very important target organs in multiple organ dysfunction syndrome (MODS) or multiple system organ failure (MOSF) caused by severe injury^[3]. It has been found that in addition to the direct trauma, the lung could also be damaged by indirect injury such as shock, gut ischemia, reperfusion insult, etc. Under the condition of an inadequate mucosal blood flow, the gut barrier function can be progressively impaired and invaded by bacteria or endogenous endotoxin. This process is associated with activation of systemic inflammatory mediators including bacteriotoxin, inflammatory mediators, such as tumor necrosis factor (TNF) and interleukin (IL) and immunocytokines. The tissue damage was manifested as increased inflammatory reaction, low content of ATP in tissue, alveolar endothelial cell damage, enhanced permeability of microcirculation, etc^[4]. In severe cases, the animal would die of pulmonary failure. But most commonly, these changes are maintained brief because the lung has the ability of self-repair. Recent studies demonstrated that one of the important mechanisms of self-protection and self-repair was the effect of endogenous growth factor and/or nitric oxide synthetase^[2,5]. Therefore, the

localization and quantitation of endogenous growth factors play important roles in lung repair.

Both bFGF and TGF β are important growth factors involved in tissue repair. They are involved in dermal and epidermal wound healing via their chemotactic effects for inflammatory cells and mitogenic effects for tissue cells, such as epidermal cells, fibroblasts and endothelial cells. Normally, TGF β is stored and released from platelets and macrophages, while bFGF combined with heparin is stored in endothelial cells in an inactive form. Both the growth factors are involved in the process of capillary reconstruction and tissue regeneration by their mitogenic and non-mitogenic effects. At the same time, they can also be relieved from injured tissues^[6,7]. Our previous researches have indicated that severe trauma results in histological damage, further decreasing the endogenous growth factors. Thus, it is necessary to supply exogenous growth factors to promote internal organ repair. We have also found that slight ischemia can induce the expression of endogenous factors, and these growth factors participate in the process of wound healing^[8,9]. We also investigated the gene expression of both growth factors in the same animal model, and found that the changes of these gene expressions were consistent with the changes of their proteins. On the basis of these studies, we came to a conclusion that there is a positive relationship between growth factors and tissue repair, and induction of endogenous bFGF and TGF β by ischemia is necessary for tissue repair. By the end of tissue repair, they are restored in tissue again. This result demonstrates that growth factors are involved in organ repair by their increased synthesis or released from damage cells after ischemia-reperfusion insult.

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Analysis of *in vivo* patterns of caspase 3 gene expression in primary hepatocellular carcinoma and its relationship to p21^{WAF1} expression and hepatic apoptosis

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Subject headings carcinoma, hepatocellular; caspase 3; apoptosis; liver neoplasms; gene expression

Sun BH, Zhang J, Wang BJ, Zhao XP, Wang YK, Yu ZQ, Yang DL, Hao LJ. Analysis of *in vivo* patterns of caspase 3 gene expression in primary hepatocellular carcinoma and its relationship to p21^{WAF1} expression and hepatic apoptosis. *World J Gastroentero*, 2000;6(3): 356-360

Abstract

AIM To detect the expression of caspase 3 gene in primary human hepatocellular carcinoma (HCC) and investigate its relationship to p21^{WAF1} gene expression and HCC apoptosis.

METHODS *In situ* hybridization was employed to determine caspase 3 and p21^{WAF1} expression in HCC. *In situ* end-labeling was used to detect hepatocytic apoptosis in HCC.

RESULTS Twenty-one of 39 (53.8%) cases of HCC were found to express caspase 3 transcripts, while 46.2% of HCC failed to express caspase 3. Non-cancerous adjacent liver tissues showed more positive caspase 3 (87.5%, 7/8) as compared with HCC ($P < 0.05$). The expression of caspase 3 is correlated with HCC differentiation, 72.2% (13/18) of moderately to highly differentiated HCC showed caspase 3 transcripts positive, while only 38.1% of poorly differentiated HCC harbored caspase 3 transcripts ($P < 0.05$). No relationship was found between caspase 3 expression and tumor size or grade or metastasis, although 62.5% (5/8) of HCC with metastasis were caspase 3 positive and a little higher than that with no metastasis (51.6%, $P > 0.05$). Expression of

caspase 3 alone did not affect the apoptosis index (AI) of HCC. The AI was 7.12‰ in caspase 3-positive tumors ($n = 21$), while in caspase 3-negative cases ($n = 18$) 6.59‰ ($P > 0.05$). Expression of caspase 3 clearly segregated with p21^{WAF1} positive tumors as compared with p21^{WAF1} negative cases (16 of 23, 69.6% versus 5 of 16, 31.3%) with statistical significance ($P = 0.017$). In the cases with positive caspase 3 and negative p21^{WAF1}, the AI was found slightly higher, but with no statistical significance, than that with expression of p21^{WAF1} and caspase 3 (7.21‰ vs 6.98‰, $P > 0.05$).

CONCLUSION Loss of caspase 3 expression may contribute to HCC carcinogenesis, although the expression of caspase 3 does not correlate well with cell apoptosis in HCC. p21^{WAF1} may be merely one of the inhibitors which can reduce caspase 3 mediated cell apoptosis in HCCs.

INTRODUCTION

Hepatocellular carcinoma (HCC), representing 80%-90% of the primary liver cancer, is one of the leading causes of cancer morbidity and mortality on a global scale. More than 80% of liver cancer cases occur in the developing world, especially in China, where HCC is the second cause of cancer death and responsible for 130 000 deaths every year^[1]. Despite dramatic advances in basic and clinical research in the past decades, the exact molecular mechanism for hepatocarcinogenesis is unclear. Gene expression changes have been demonstrated in accordance with cell growth, differentiation and carcinogenesis^[2]. Tumor formation can result from a decrease in cell death, as well as an increase in cell proliferation. In addition to altered expression of cell cycle-related gene, dysregulation of apoptosis (programmed cell death) is thought to contribute to cancer by aberrantly extending cell viability and favoring the accumulation of transforming mutations^[3].

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Caspase is a large family which contains at least 14 members. It has been shown that caspase play an important role in regulating cancer cell death both induced by activated lymphocytes through Fas/FasL pathway and by chemotherapy agents^[4]. Caspase 3 or cyp32 is the key member of effector caspases. Caspase 3 had overexpression in B-cell chronic lymphocytic leukemia (B-CLL)^[5], acute myelogenous leukemia (AML)^[6], follicular small cleaved cell non-Hodgkin's B-cell lymphomas^[7,8], human breast cancer cell lines and primary breast tumors^[9] and neuroblastomas^[10]. Caspase 3 is a potent protease which can cleave a large scale of substrate, including cell-cycle related genes such as p21^{WAF1}^[4]. p21^{WAF1} is a cyclin-dependent kinase inhibitor and plays an important role in DNA damage-induced growth arrest. p21^{WAF1} overexpression can cause G₁ cell cycle arrest and further interrupt the apoptotic process at a point upstream from caspase 3 activation^[11]. In this study, we investigated the expression of caspase 3 in primary human HCC and its potential impact on tumor cell apoptosis and its relationship to p21^{WAF1} expression.

MATERIALS AND METHODS

Patients and samples

The surgically resected specimens employed in this study were obtained from consecutive patients with primary HCC who had undergone potentially curative tumor resection at the Department of General and Hepato-Biliary Surgery, Tongji Hospital during 1996-1997. A cohort of 39 cases was involved in this study. All cases were selected on the basis of availability of frozen material for study and on the absence of extensive chemotherapy-induced tumor necrosis. Materials were composed of 3 cases of grade I, 18 cases of grade II, 11 cases of grade III, the remaining 7 cases were grade IV according to TNM system (1987). The tumor lesions analyzed here included 21 poor, 9 moderate and 9 well differentiations. There were 34 males and 5 females, and the age ranged from 24 to 71 years with an average of 46.1 (SD, 12.5). Eight cases of non-cancerous adjacent liver tissues were also included in the study. Routinely processed 40 g/L paraformaldehyde-fixed, paraffin-embedded blocks containing principal tumor were selected. Serial sections of 5 μ m were prepared from the cut surface of blocks at the maximum cross-section of the tumor.

In situ hybridization staining for caspase 3 and p21^{WAF1} and scoring methods for its expression

The plasmid pET21b-cyp32 containing caspase 3 (cyp32) cDNA probe was kindly provided by Dr. JC. Reed (La Jolla, USA). After digestion with *Xho*I and *Nde*I, the fragment was separated by

electrophoresis through an agarose gel and recovered by QIA quick gel extraction kit (QIAGEN) using a micro-centrifuge according to the manufacturer's protocol. The p21^{WAF1} cDNA probe was kindly provided by Dr. SJ Elledge (Houston, USA). Preparation of p21^{WAF1} probe was described previously^[12]. The probes were labeled and detected using a Dig DNA labeling and detection kit (Boehringer Mannheim Biochemica, Germany). Briefly, 40g/L paraformaldehyde-fixed paraffin embedded samples were cut at 5 μ m and adhered to APES-treated slides. After deparaffinized and rehydrated through a graded series of ethanol, the sections were immersed in a 0.01 mol/L DEPC-treated PBS (pH 7.4) two times each for 5min, and then, in PBS containing 100 mmol/L glycine and PBS containing 3 mL/L Triton X-100 for 5 min in turns. Sections were permeabilized for 30 min at 37°C with TE buffer (100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0) containing 10 mg/L RNase-free proteinase K and washed with DEPC-treated PBS, then incubated at 42°C for 2 h with pre-hybridization buffer. Hybridization solution (400 mL/L deionized formamide, 500 g/L dextra sulfate, 1 \times Dehardt's reagent, 4 \times SSC, 10 mmol/L DTT, 1 g/L yeast tRNA, 1 g/L denatured salmon sperm DNA) containing 2 mg/L probe overlay each section after deprive prehybridization buffer from slides and hybridize at 42°C for 36 h in a humid chamber. The sections were washed in a shaking water bath at 37°C in 2 \times SSC, 1 \times SSC, 0.1 \times SSC for 15 min each, then washed with buffer I (100mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) for 20 min and with blocking solution (buffer I containing 20 mL/L normal sheep serum) for 30 min, and added sheep anti-Dig-alkaline phosphates (diluted at 1:800 in buffer I) and incubated for another 1h before development by NBT at 37°C for 3 h in the dark. Hybridization buffer containing no probe was used for negative control for each staining. Scoring method for caspase 3 and p21^{WAF1} expression was described by Kawasaki^[13]. Positive tumor cells were quantified by two independent observers, and the average percentage of positive tumor cells was determined in at least 5 areas at \times 400 and assigned to one of five categories: (a) 0, <1%; (b) 1, 1%-25%; (c) 2, 25%-50%; (d) 3, 50%-75% and (e) 4, >75%. The ISH staining intensity was scored as (a) weak 1+; (b) moderate, 2+; and intense, 3+. For tumors showing heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores <1 were defined as negative, otherwise were defined as positive.

Histochemical detection of apoptosis and

determination of the AI

Tumor cell apoptosis was identified by DNA fragmentation detection kit (QIA33-kit, Calbiochem). Briefly, deparaffinized and rehydrated sections were permeated with proteinase K (20 mg/L in 10 mmol/L Tris, pH 8.0) for 20 min at room temperature and washed with 1 × TBS (20 mmol/L Tris pH 7.6, 140 mmol/L NaCl). After endogenous peroxidases were inactivated by using 30 mL/L hydrogen peroxide for 5 min and washed with 1 × TBS, equilibration buffer was added to each section and incubated at room temperature for 20 min. Terminal deoxynucleotidyl transferase (TDT) enzyme in TDT labeling reaction mix at a 1:20 dilution was pipetted onto the sections, followed by 1.5 h incubation at 37°C. After the reaction was terminated by immersing sections into stop solution and washed with blocking buffer for 10 min at room temperature, the anti-digoxigenin-peroxidase was added to the sections. DAB solution was used for color development. Sections were counterstained by methyl green. A positive control generated covering specimen with DNase I (1 mg/L) for the first procedure. Specific positive tissue sections were used for negative control by substituting distilled water for the TDT in the reaction mixture. The AI was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells, given a percentage for each case. A minimum of 1 000 cells was counted under a 400-fold magnification. Positively staining tumor cells with morphological characteristics of apoptosis were identified using standard criteria, including chromatin condensation, nuclear disintegration and formation of crescentic caps of condensed chromatin at the nuclear periphery.

Statistical analysis

Variables associated with caspase 3 expression as well as the relationship between caspase 3 and p21^{WAF1} were analyzed by χ^2 test. Differences in the tumor cell AI for groups dichotomized according to caspase 3 expression were checked by independent *t* test.

RESULTS

Expression of caspase 3 gene in HCCs

By ISH staining, caspase 3 transcripts was detected predominantly in cytoplasm (Figure 1). Consistent with the presence of caspase 3 protein in human biopsy liver samples, expression of caspase 3 in non-cancerous adjacent liver tissue was also observed in 87.5% (7/8) of cases. The intensity of caspase 3 staining was heterogeneous within a case detected. The tumor cells positively stained by ISH range from 10% to 90%, depending on the cases

examined. After multiplying the weighted caspase 3 score, 21 cases of HCC in the present study were defined as positive (53.8%), with weighted caspase 3 score from 1 to 12.

The expression of caspase 3 and its association with clinicopathological variables

A clinicopathological analysis of caspase 3 positive cases is shown in Table 1. No statistical significance was observed in the prognostic parameters, including tumor size, metastasis, TNM grade, analyzed in the present study except for differentiation. The expression of caspase 3 is correlated with HCC differentiation. It was found that as high as 72.2% (13/18) of moderately to highly differentiated HCC showed caspase 3 transcripts positive, while only 38.1% of poorly differentiated HCC harbored caspase 3 transcripts ($P < 0.05$).

Relationship between caspase 3 and p21^{WAF1}

Twenty-three cases were detected expression p21^{WAF1} transcripts. Positive signal was predominantly located in cytoplasm with a heterogeneous distribution of positive tumor cells. The significance of p21^{WAF1} gene expression was discussed in our previous study^[12]. Expression of caspase 3 clearly segregated with p21^{WAF1} positive tumors as compared with p21^{WAF1} negative cases (16 of 23, 69.6% vs 5 of 16, 31.3%) with statistical significance ($P < 0.05$).

Table 1 Correlation between clinicopathological parameters and expression of caspase 3 in HCCs

	No. Caspase 3 expression (%)	P
Samples		
Non-cancerous adjacent liver	8 7(87.5)	<0.05
HCC	39 21(53.8)	
Age(year)		
<60	28 15(53.6)	NS
>60	11 6(54.5)	
Sex		
Male	34 18(52.9)	NS
Female	5 3(60.0)	
Tumor size (cm)		
>5.0	27 15(55.6)	NS
<5.0	12 6(50.0)	
Differentiation		
Well-moderate	18 13(72.2)	<0.05
Poor	21 8(38.1)	
TNM grade		
I-II	21 12(57.1)	NS
III-IV	18 9(50.0)	
Metastasis		
Negative	31 16(51.6)	NS
Positive	8 5(62.5)	
p21 ^{WAF1}		
Positive	23 16(69.6)	<0.05
Negative	16 5(31.3)	

NS: no statistic significance

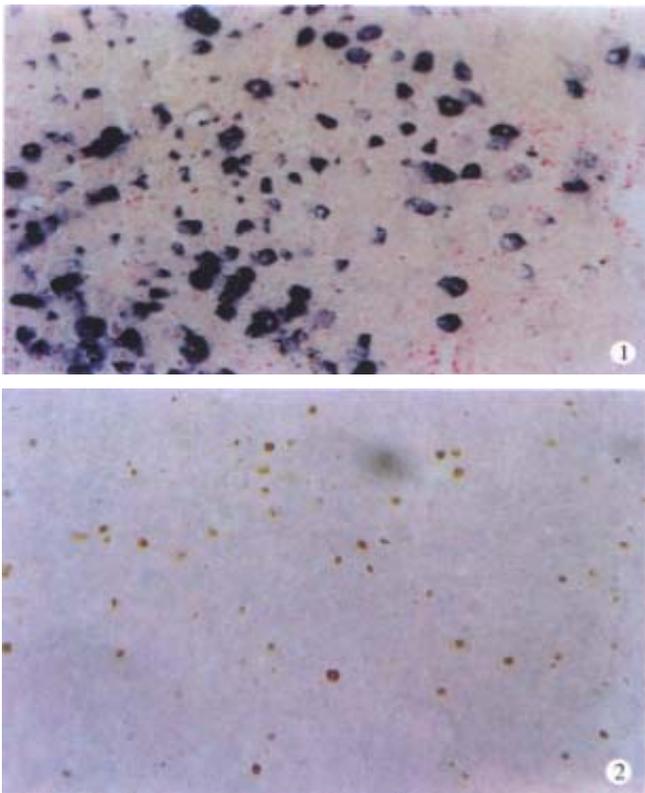


Figure 1 Caspase 3 transcripts were detected predominantly in cytoplasm by ISH ($\times 200$)

Figure 2 Apoptotic cells were determined by criteria as described in materials and methods. Arrow shows fragmented nucleus ($\times 200$)

Relationship between tumor cell apoptosis and caspase 3 expression

Apoptotic cells and apoptotic bodies were found in all cases of HCCs examined by *in situ* end-labeling (Figure 2). The mean AI of all tumors cases was 6.82% (s, 3.36%; range 0.87%-17.3%). No significant association was observed between AI and tumor stage. The mean AI for caspase 3-positive tumors ($n = 21$) was 7.12% (s, 3.75%), while in caspase 3-negative cases ($n = 18$) the AI was 6.59% (s, 2.98%), no statistical significance was found between the two groups ($P > 0.05$). In the cases with co-expression of p21^{WAF1} and caspase 3, the AI was found lower, but with no significance, than that of cases with positive caspase 3 and negative p21^{WAF1} (6.98% vs 7.21%).

DISCUSSION

In this study, we have shown that caspase 3 was expressed in most of HCC cases. In our opinion, we are the first to describe the cyp32 expression in human primary HCCs. Like the result of human biopsy and autopsy liver materials^[14], the non-cancerous adjacent liver tissue showed strong caspase 3 expression. As high as 46.2% of human primary HCCs failed to show caspase 3 expression.

In the 21 cases which express caspase 3, the expression showed heterogeneous pattern with a weighted score from 1 to 12. Because caspase 3 is the effector caspase in the apoptosis pathways, we think that loss of caspase 3 expression may play an important role in HCC carcinogenesis. Caspase 3 expression had no relationship with clinicopathological features except for tumor differentiation (Table 1). The result suggests that the expression of caspase 3 is correlated with tumor differentiation in HCC. It was reported that 33.1% cases of gastric carcinoma were caspase 3 positive^[7]. Two of 3 breast carcinoma tissues expressed caspase 3, the immunointensity was generally higher in invasive cancers^[8]. It raised the possibility that expression of caspase 3 in tumors showed tissue specificity. It was observed in this study that the apoptosis index (AI) was not associated with the expression of caspase 3 in HCC (7.12% in cyp32-positive cases versus 6.59% in cyp32-negative group). In the non-cancerous adjacent liver tissues more than 50% of the cells showed positive caspase 3, the AI was not increased as compared with HCC, no matter caspase 3 was positive or negative. This suggests that other factor(s) may exist in regulating normal cell apoptosis.

p21^{WAF1} was first reported as a universal inhibitor of cyclin-dependent kinase, which is required for G₁ to S transition^[15]. Previous studies demonstrated that p21^{WAF1} can interrupt the apoptotic process at a point upstream from caspase-3 activation, because serum starvation, which also synchronized cells in G₁ but did not induce p21^{WAF1}, did not protect cells from apoptosis, while restoring a late G₁ checkpoint by inducing p21^{WAF1} expression can protect cells from DNA damage induced apoptosis^[16]. p21^{WAF1} can bind procaspase-3 but not activate caspase 3. On the other hand, activated caspase 3 can cleave p21^{WAF1}. P21 cleavage by the activated cyp32 specifically abolished its interaction with PCNA and may interfere with normal PCNA-dependent repair^[15]. The presence of p21^{WAF1} in human HCCs was reported previously in our paper^[12]. In this context, the expression of p21^{WAF1} was also determined together with caspase 3 in an attempt to find whether there is relationship between them. We found that expression of caspase 3 was strongly associated with p21^{WAF1} in 16 cases of HCC. There was no significant difference in AI between p21^{WAF1}(+)/cyp32(+) and p21^{WAF1}(-) / cyp32(+) (6.98% vs 7.21%). The results indicated that p21^{WAF1} may be merely one of the inhibitors which can reduce caspase 3 mediated cell apoptosis in HCCs. In fact, some other caspase 3 inhibitors, for example survivin, were reported to have overexpression in human tumors, including gastric and colorectal cancer^[17,18]. Survivin is

believed to bind activated caspase 3 and further inhibit cell apoptosis. It was reported that XIAP can interrupt caspase 3 mediated apoptosis via the same way as p21^{WAF1}. So, further investigation on other caspase 3 regulating protein is needed to find the regulation mechanism of caspase 3 mediated apoptosis in human HCCs.

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Effects of salvianolic acid-A on NIH/3T3 fibroblast proliferation, collagen synthesis and gene expression

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Subject headings salvianolic acid-A, NIH/3T3 fibroblast, cell viability, cell proliferation, collagen, gene expression

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Abstract

AIM To investigate the mechanisms of salvianolic acid A (SA-A) against liver fibrosis *in vitro*.

METHODS NIH/3T3 fibroblasts were cultured routinely, and incubated with 10^{-4} mol/L- 10^{-7} mol/L SA-A for 22 h. The cell viability was assayed by [3 H]proline incorporation, cell proliferation by [3 H]TdR incorporation, cell collagen synthetic rate was measured with [3 H]proline impulse and collagenase digestion method. The total RNA was prepared from the control cells and the drug treated cells respectively, and α (1) I pro-collagen mRNA expression was semi-quantitatively analyzed with RT-PCR.

RESULTS 10^{-4} mol/L SA-A decreased cell viability and exerted some cytotoxicity, while 10^{-5} mol/L- 10^{-7} mol/L SA-A did not affect cell viability, but inhibited cell proliferation significantly, and 10^{-6} mol/L SA-A had the best effect on cell viability among these concentrations of drugs. 10^{-5} mol/L- 10^{-6} mol/L SA-A inhibited intracellular collagen synthetic rate, but no significant influence on extracellular collagen secretion. Both 10^{-5} mol/L and 10^{-6} mol/L SA-A could decrease α (1) I pro-collagen mRNA expression remarkably.

CONCLUSION SA-A had potent action against liver fibrosis. It inhibited NIH/3T3 fibroblast proliferation, intracellular collagen synthetic rate and type I pro-collagen gene expression, which may be one of the main mechanisms of the drug.

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INTRODUCTION

Radix salviae miltiorrhizae, one of the most frequently used Chinese herbs, is regarded to have effects on both blood production and circulation by traditional Chinese medicine, and is widely applied in clinical therapy for liver diseases, such as chronic hepatitis, hepatic cirrhosis, etc. Salvianolic Acid-A is one of the water soluble components from *Radix salviae miltiorrhizae*. It was reported to have good actions on peroxidation^[1]. Lipid peroxidation could stimulate hepatic stellate cell (HSC) transformed into myofibroblast like cell (MFBC) and collagen gene expression *in vivo* and *in vitro*, and played an important role in liver fibrogenesis^[2]. In our previous work^[3], it was found that SA-A could protect hepatic lipid peroxidation, and had marked effects against liver injury and fibrosis in carbon tetrachloride induced fibrotic rats. In order to investigate the mechanism by which SA-A protects against liver fibrosis, we observed the effects of SA-A on NIH/3T3 fibroblast proliferation, collagen protein production and procollagen gene expression.

MATERIALS AND METHODS

Drug

SA-A, molecular formula as $C_{26}H_{22}O_{10}$, molecular structure as shown in Figure 1, molecular weight 494, was extracted and identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

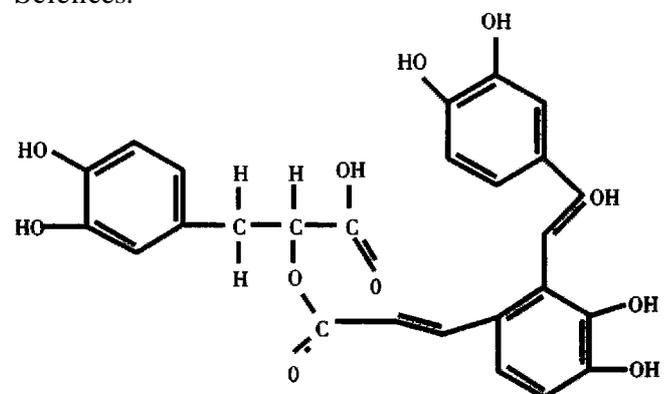


Figure 1 SA-A molecular structure.

Main reagents and solutions

PRMI-1640 Medium and Dubocal modified Eagle Medium (DMEM) were purchased from Gibco BRL

Co., new brown serum (NBS) from Shanghai Sino-American Co., purified type III collagenase (specific activity, 960U/mg), N-ethylmaleimide (NEM) and β -aminopropionitrile from Sigma Co. [^3H]proline ([^3H]Pro) from Amersham Co. methyl- [^3H] thymidine (TdR) from Shanghai Institute of Atomic Energy, guanidium thiocyanate from Serva Co. Access RT-PCR System Kit, PCR marker from Promega Co., Diethylpyrocarbonate, saturated phenol/chloroform mix and agarose from Shanghai Sangon Biotech Co. Other reagents all were of analytical grade.

The non-homogeneous scintillation liquid was dimethylbenzene solution containing 5 g/L 2,5-diphenyloxazol (PPO) and 0.5 g/L 1,4-bis[5-phenyloxazol-2]benzene (POPOP), the homogeneous scintillation liquid was dimethylbenzene solution containing 7 g/L PPO, 0.5 g/L POPOP, 100 g/L naphthalene and 400 mL/L₂-ethoxy-ethanol.

Cell line

Mouse NIH/3T3 fibroblasts were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and cultured with PRMI-1640 medium containing 100 g/L NBS, 100KU/L penicillin and 100 mg/L streptomycin. After the cell growth became confluent, they were digested with trypsin-EDTA and subcultured.

PCR Primers

The PCR primers for pro-collagen α 2(I) and β -actin were designed according to the published sequences and references in Table 1^[4], and were synthesized by Gibco BRL Co.

Table 1 PCR primer sequences and expected size of amplified products

Primers	Sequence	Size
α 2(I) collagen upstream	5'TGT TCG TGG TTC TCA GGG TAG3'	
α 2(I) collagen downstream	5'TTG TCG TAG CAG GGT TCT TTC3'	254 bp
β -actin upstream	5'ACA TCT GCT GGA AGG TGG AC3'	
β -actin downstream	5'GGT ACC ACC ATG TAC CCA GG3'	163 bp

Cell proliferation assay

Confluent NIH/3T3 fibroblasts in 24 well plates were incubated with 10^{-4} mol/L- 10^{-7} mol/L SA-A diluted in PRMI-1640 medium containing 100 mL/L NBS for 22 h, and [^3H]TdR (55.5KBq/well) was impu lused in the last 16 h. Then cells were harvested with trypsin digestion and collected on the filtration membrane, then sample radioactivity (cpm) in the non-homogeneous scintillation liquid was measured by Backman Wallac 1410 Scintillator. All tests were repeated 3 times.

Cell viability assay

According to *Mallat's* method^[5], confluent

NIH/3T3 fibroblast s in 24-well plates were incubated with 10^{-4} mol/L- 10^{-7} mol/L SA-A resolved in PRMI-1640 medium without NBS for 22 h, and [^3H]Pro (55.5KB q/well) was impu lused in the last 16 h. Then cells were collected and the cpm was measured as above.

Assay of cell collagen synthetic rate

According to Greets' method^[6], confluent NIH/3T3 fibroblasts in 6 well plates were incubated with 10^{-5} mol/L- 10^{-6} mol/L SA-A diluted in PRMI-1640 without NBS for 22 h, during the later 16h the culture media were changed to DMEM containing 185 KB q/mL [^3H]Pro, 100 mg/L- β -aminopropionitrile, 50 mg/L asc orbic acid as well as the same drugs. Then the culture media and cell layer extract were collected respec tively, dialyzed thoroughly and reacted with collagenase, etc. The total radioa ctivity in the samples (cpm_t), radioactivity in the samples treated with colla genase (cpm_c) and not treated with collagenase (cpm_b) were counted in the ho mogeneous scintillation liquid by Backman Wallac 1410 Scintillator. The new col lagen that cell produced, i.e. the fraction of collagenous protein expressed as percentage of total radiolabeled protein, was calculated using the formula:

$$\% \text{ of collagen} = 100 \div \left(5.4 \times \frac{\text{cpm}_t - \text{cpm}_c}{\text{cpm}_c - \text{cpm}_b} + 1 \right)$$

RNA extraction and RT-PCR (reverse transcription and polymerase chain reaction)

The total RNA was extracted from the control cells and SA-A incubated cells by the acid guanidium thiocyanate-phenol-chloroform method^[7]. The RNA quantity was determined by absorption at 260 nm, its purity was confirmed with A_{260}/A_{280} specrophoto meter readings that ranged from 1.6 to 1.9, and its integrity was checked by 9 g/L agarose gel ele ctrophoresis with ethidium bromide (EB) staining of 18S and 28S ribosomal RNA (Figure 2). With Access RT-PCR system kit, the cDNA synthesis and amplification was done in one tube following the manufacturer's instructions. In brief, 1 μg RNA, 50 pmol/L primers for α (1) I pro-collagen or β -actin were added to each reaction mixture respectively, which included 10 mmol/L dNTPs 1 μL , 25 mmol/L MgSO_4 2 μL , AMV reverse transcriptase 5U, Tfl DNA polymerase 5U, AMV/Tfl-5 \times buffer 10 μL . The reaction final volume was 50 μL and was covered with 20 μL mineraloil. Then with PCR Touchdown thermal cyler (Hybaid, England), RT-PCR reaction was run in the following procedures: ① 48 $^{\circ}\text{C}$ for 45 min, 1 circle. ② 94 $^{\circ}\text{C}$ for 2 min, 1 circle. ③ 94 $^{\circ}\text{C}$ for 30s, 60 $^{\circ}\text{C}$ for 1 min, 38 $^{\circ}\text{C}$ for 2 min, 30 circles. ④ 68 $^{\circ}\text{C}$ for 7 min, 1 circle. Five μL PCR product was run on 15 g/L agarose gel and observed by EB

staining under UV light, the electrophoresis is photo was transformed into computer, and $\alpha 1$ (I) pro-collagen intensity was analyzed with MPIAS500 image system, while the β -actin band intensity was subtracted as an internal standard.

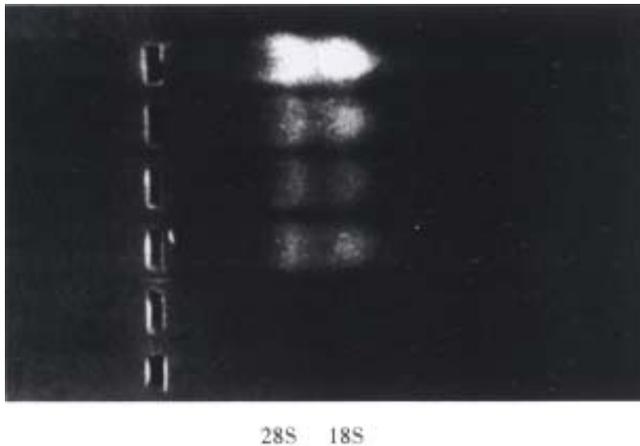


Figure 2 Total RNA gel electrophoresis photograph. 28S and 18S of total RNA run on 9 g/L agarose gel stained with EB.

Statistical analysis

Data were analyzed by *Student's t* test.

RESULTS

Effects on cell morphology and viability

10^{-5} mol/L- 10^{-7} mol/L SA-A had no marked effects on cell morphology, but 10^{-4} mol/L SA-A led to shrinkage and detachment of some cells, showing cytotoxicity to some degree. 10^{-4} mol/L- 10^{-7} mol/L SA-A did not decrease intercellular [3 H] Pro incorporation, while 10^{-6} mol/L SA-A could increase [3 H]Pro impulse ($P < 0.05$) and enhance cell viability (Table 2).

Effects on cell proliferation

10^{-4} mol/L- 10^{-6} mol/L SA-A remarkably decreased intercellular [3 H]TdR incorporation and inhibited cell proliferation ($P < 0.05$), 10^{-4} mol/L SA-A showed more significant effect ($P < 0.01$), but it induced some cell death, which may be associated with its cytotoxic action. 10^{-7} mol/L SA-A had no obvious effect on cell [3 H] TdR incorporation (Table 2).

Table 2 Effects of SA-A on cell intracellular [3 H]TdR and [3 H] Pro incorporation (cpm/well, $\bar{x} \pm s$, $n = 4$)

Group	[3 H]TdR	[3 H]Pro
Control	1482 \pm 486	21018 \pm 5473
10^{-4} mol/L SA-A	675 \pm 201 ^b	18659 \pm 2363
10^{-5} mol/L SA-A	969 \pm 183 ^a	23761 \pm 5430
10^{-6} mol/L SA-A	868 \pm 183 ^a	31408 \pm 4981 ^a
10^{-7} mol/L SA-A	1056 \pm 187	26080 \pm 4504

^a $P < 0.05$, ^b $P < 0.01$, vs control.

Effects on cell collagen synthetic rates

10^{-5} mol/L- 10^{-6} mol/L SA-A could inhibit intracellular collagen synthetic rate significantly ($P < 0.01$), but did not influence extracellular synthetic rate (Table 3).

Table 3 Effects of SA-A on NIH/3T3 fibroblast collagen synthetic rates (% , $\bar{x} \pm s$, $n = 4$)

Group	Intracellular	Extracellular
Control	0.78 \pm 0.03	2.57 \pm 0.37
10^{-5} mol/L	0.48 \pm 0.24 ^b	2.54 \pm 0.91
10^{-6} mol/L	0.43 \pm 0.26 ^b	3.02 \pm 0.69

^b $P < 0.01$, vs control.

Effects on procollagen $\alpha 2$ (I) mRNA expression

Both 10^{-5} mol/L and 10^{-6} mol/L SA-A decreased procollagen $\alpha 1$ (I) mRNA expression significantly ($P < 0.05$), but there was no difference between the two different concentration groups (Table 4, Figure 3).

Table 4 The relative expression amount of $\alpha 2$ (I) procollagen mRNA ($\bar{x} \pm s$, % of β -actin)

Group	<i>n</i>	Col $\alpha 1$ (I) mRNA
Control	3	98.71 \pm 9.96
10^{-5} mol/L SA-A	3	76.23 \pm 12.02 ^a
10^{-6} mol/L SA-A	3	68.44 \pm 8.06 ^a

^a $P < 0.05$, vs control.

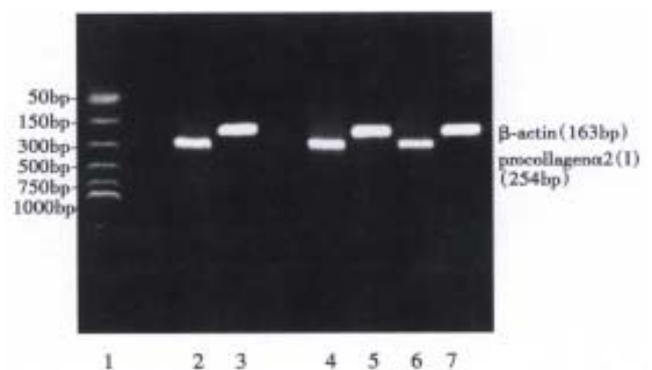


Figure 3 RT-PCR product gel electrophoresis photograph. Five μ L RT-PCR products of procollagen $\alpha 2$ (I) and β -actin run on 1.5% agarose gel stained with EB. Lane 1 as PCR marker, lane 2 and 3 as the control for procollagen $\alpha 2$ (I) and β -actin respectively, lane 4 and 5 SA-A 10^{-6} mol/L for procollagen $\alpha 2$ (I) and β -actin respectively, lane 6 and 7 as SA-A 10^{-5} mol/L for procollagen $\alpha 2$ (I) and β -actin respectively.

DISCUSSION

Hepatic fibrosis, a precursor of cirrhosis, is a common and important pathological feature of chronic liver diseases, which involves the abnormal accumulation of extracellular matrix (ECM)

proteins, particularly collagen^[8]. In fibrotic liver, ECM components are mainly produced by HSC and fibroblasts. It is known that during fibrogenesis, HSC undergoes a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation and ECM production, especially type I collagen synthesis. The mouse NIH/3T3 fibroblast also shared the features that active HSC (MFBC) presented, such as remarkable proliferation and substantial production of collagen, and stable cell line. In practice, NIH/3T3 fibroblast is often used as a desirable cell model for investigation of antifibrotic drugs.

In order to rule out the possibility of SA-A cytotoxic influence *in vitro*, the intracellular [³H] Pro incorporation was measured, and inverted microscopic observation was done. It was found that only 10⁻⁴ mol/L SA-A caused some cell detachment, decreased [³H] Pro incorporation, and showed cytotoxicity to some extents. 10⁻⁵ mol/L-10⁻⁷ mol/L SA-A did not influence cell morphology or inhibit cell viability. However, 10⁻⁶ mol/L SA-A enhanced cell viability. Both 10⁻⁵ mol/L-10⁻⁶ mol/L SA-A could inhibit intracellular [³H]TdR impulse that NBS stimulated. It is suggested that SA-A had an effective action against NIH/3T3 fibroblast proliferation.

Type I collagen is the predominant component of ECM during liver fibrosis. Its production involves two processes: the first is intracellular synthesis, including gene transcription, translation and modification to form procollagen, then procollagen alpha chains are secreted to the outside of the cell to form helix collagen by sorting and alignment etc. In the study, it was found that SA-A downregulated procollagen α 2(I) steady-state mRNA expression, and intracellular collagen synthetic rate, but exerted no effect on extracellular synthetic rate. It is suggested that SA-A influence on collagen production through the intracellular synthetic

process. The fibrogenic cells have two predominant features: one is active in cell proliferation, which led to increase in cell number, another is strong fibrogenic ability per cell, which led to accumulation of ECM. In the study, SA-A not only inhibited NIH/3T3 fibroblast proliferation, but also decreased collagen synthesis, showing a good action against liver fibrosis.

Salvianolic radix is widely used as an important component in Chinese herbal formulas for the treatment of chronic liver diseases. Salvianolic Acid-A, one of water-soluble ingredients from Salvianolic radix, had effective actions on hepatic peroxidation and fibrosis *in vivo*^[3]. In the paper, it is for the first time found that SA-A has the potential action against hepatic fibrosis *in vitro*, and its main mechanisms of antifibrotic action perhaps was associated with the inhibition of fibrogenic cell proliferation, collagen gene expression and protein synthesis.

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Primary porcine hepatocytes with portal vein serum cultured on microcarriers or in spheroidal aggregates

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Subject headings porcine hepatocytes; microcarriers; cell culture; spheroidal aggregate culture; portal vein serum

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Abstract

AIM To develop a culture mode providing durable biomaterials with high yields and activities used in bioartificial liver.

METHODS Hepatocytes were isolated from a whole pig liver by Seglen's method of orthotopic perfusion with collagenase. In culture on microcarriers, primary porcine hepatocytes were inoculated at a concentration of 5×10^7 /mL into the static culture systems containing 2 g/L Cytodex-3, then supplemented with 100 mL/L fetal calf serum (FCS) or 100 mL/L porcine portal vein serum (PPVS) respectively. In spheroidal aggregate culture hepatocytes were inoculated into 100 mL siliconized flasks at a concentration of 5.0×10^6 /mL.

RESULTS In culture on microcarriers hepatocytes tended to aggregate on Cytodex-3 obviously after being inoculated. Typical multicellular aggregated spheroids could be found in the two systems 24 h-48 h after hepatocytes were cultured. The morphological characteristics and synthetic functions were maintained for 5 wk in FCS culture system and 8 wk in PPVS culture system. In spheroidal aggregate culture about 80%-90% isolated hepatocytes became aggregated spheroids 24 h after cultured in suspension and mean diameter of the spheroids was 100 μ m. The relationship among the hepatocytes resembled that in the liver *in vivo*. Synthetic functions of albumin and urea of the

spheroids were twice those of hepatocytes cultured on monolayers.

CONCLUSION As high-yields and high-activity modes of culture on microcarriers or in spheroidal aggregate culture with portal vein serum are promising to provide biomaterials for bioartificial liver (BAL) efficiently.

INTRODUCTION

Great attention was paid world-wide to the bioartificial liver (BAL) aiming to release the clinical symptoms of severe acute liver diseases or liver injuries and act as a transitional bridge for liver transplantation. Modern BAL is mainly characterized by its multiple biofunctions resembling those of liver *in vivo* and such functions may be completely derived from the biomaterials in BAL. Years of researches at home and abroad showed that primary hepatocytes were the most ideal biomaterial of BAL. But for the morphological and functional changes of primary hepatocytes cultivated *in vitro* occurred obviously in short time, it was deadly restrained to use them as the biomaterial in BAL. To probe into this question, together with our proceeding researches on this subject^[1], we took the method of two-step orthotopic collagenase perfusion to isolate hepatocytes and cultured them on microcarriers Cytodex-3 statically in flasks in the systems supplemented with FCS or PPVS respectively, and also cultured them in the matrix DMEM containing porcine portal vein serum at a concentration of 100 mL/L through the way of spheroidal aggregating, then estimated the possibility of such hepatocytes acting as the biomaterial in BAL in view of their morphology and- biofunctions.

MATERIALS AND METHODS

Materials

The experimental animals were five native sex-unlimited pigs aged 1 mo-2 mo and weighed 10 kg-15 kg from the experiment animal center of SUN Yat-Sen Medical University. Insulin, glucagon, transferrin, collagenase IV, tyran and microcarrier Cytodex-3 were purchased from Sigma (USA). Solution of 50 g/L methyl silicon resin ethyl acetate

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was conducted by ourselves. Ten g/L osmic acid, 20 g/L pentanal and gradient acetone solutions were provided by the Department of Electron microscope of First Military Medical University. S-450 electron scanning microscope was from Japan. PPVS was collected in the experiment. The liver perfusion solution was prepared according to Table 1.

Methods

Isolation of porcine hepatocytes The experimental pig was kept fasting for 12 h preoperatively and anaesthetized with 30 g/L sodium barbiturate ip. After the abdominal skin being prepared and disinfected, celiotomy was performed, then portal vein and inferior caval vein below the liver exposed and 0.25 mg (125IU) heparin injected into the inferior caval vein. After being dissected, the portal vein and the inferior caval vein were ligated at the distal end and intubated at the proximal end respectively. When the inferior caval vein above the liver ligated at the superior end of liver vein, successive perfusing solutions I and II pre-warmed in 37°C water bath were perfused along the circuit via portal vein to inferior caval vein. Perfusing speed was about 50 mL/min-60 mL/min for 20 min. Perfusion was ended until the liver became pallor or yellow in color. Then the well digested liver were taken out, tore off the liver sheath and the isolated hepatocytes delivered into solution III pre-warmed in 37°C water bath. After being filtered by 150 µm, 100 µm and 80 µm sieve, the hepatocytes were suspended in solution and centrifuged at a speed of 1 000r/min for 3 min-5 min repeated three times. The deposited hepatocytes were added into solution IV to prepare the hepatocyte suspension for use. Portal vein blood was gathered from the distal end of the ligated portal vein and in a total amount of 1 300mL from 5 pigs. It was centrifuged at a 2 000r/min to isolate the portal vein serum, and the very serum centrifuged three times more to prepare the refined serum which finally amounted to 714 mL. The refined serum was filtered to be excluded from bacteria and preserved separately for use. Sample from the suspension was observed under light microscope to calculate the yielding rate of hepatocytes and stained by typran for determination of their activity.

Static culture of hepatocytes on microcarriers in flasks

Porcine hepatocyte suspension was divided randomly into two halves, then one half was cultured on microcarriers in FCS system and the other in PPVS system. Five pigs were done separately and by the same method. ① Culture of hepatocytes in FCS system: five flasks were prepared and each one added with 2 mL-3 mL 50 g/L sterile methyl silicon resin ethyl acetate solution. After the flasks being shaken mildly and equably, the remained

solution was discarded. The flasks were parched in 60°C electric oven for use. 20 mg-30 mg Cytodex-3 was deposited into the siliconized flasks and rinsed with 40 mL Ca²⁺ and Mg²⁺ free phosphate buffer solution (PBS) for three times, then dipped in PBS over night. After being sterilized at 15 pounds for 30 min, the microcarriers were dipped in DMEM matrix over 10 h for use. Hepatocytes were inoculated into the flasks at the concentration of 5 × 10⁶/mL and DMEM matrix containing 100 mL/L FCS, 100 µg/L glucagon, 100 µg/L transferrin, 200 µg/L hydrocortisone, 100 µg/L insulin and 200 µg/L fortum added up to the total volume, 50 mL. Then the flasks were deposited into an incubator with 100% humidified atmosphere containing 5% CO₂ at 37°C and shaken reelingly once every 15 min-30 min at the first 4 h, once every 2 h after 4 h and laid quietly after 8 h without further shaking. Ten mL supernatant fluid of the culture matrix was changed after 24 h and once every 2 d from then on. The supernatant fluid was centrifuged at 1 000r/min for discarding the cell fragments. Synthesized albumin and urea were determined. ② Culture of hepatocytes in PPVS system: 100 mL/L refined PPVS was added into the culture system instead of FCS, and the other demands were the same as those of the FCS culture.

Spheroidal aggregate culture of primary porcine hepatocytes

Five flasks were prepared and each one added with 2 mL-3 mL 50 g/L sterile methyl silicon resin ethyl acetate solution. After the flasks being shaken mildly and equably, the residual solution was discarded. The flasks were parched in 60°C electric oven for use. Hepatocytes were inoculated into the flasks at the concentration of 5 × 10⁶/mL and DMEM matrix containing 100 mL/L porcine portal vein serum, 100 µg/L glucagon, 100 µg/L transferrin, 200 µg/L hydrocortisone, 100 µg/L insulin and 200 µg/L fortum added up to a total volume, 50 mL. Then the flasks were deposited into the incubator with 100% humidified atmosphere containing 5% CO₂ at 37°C and shaken reelingly once every 15 min-30 min at the first 4 h, once every 2 h after 4 h and laid quietly after 8 h without further shaking. 10 mL supernatant fluid of the culture matrix was changed after 24 h and once every 2 d from then on. The supernatant fluid was centrifuged at 1 000r/min for discarding the cell fragments. Syntheses of albumin and urea were determined.

Morphological observation and counting of hepatocytes

The porcine hepatocytes cultured on microcarriers and those cultured as aggregated spheroids were observed under phase-contrast light microscope and photographed on an automatic microscopic photographing device successively (Olympus, Japan). In culture on microcarriers 1 mL samples

were collected every day to calculate the amount of cells in the culture system by means of trypan blau. On d7, cell sample was taken at a well-distributed state, culture matrix discarded, rinsed with PBS, then fixed for 0.5 h with 2 mL 20 g/L pentanal, rinsed with PBS again, fixed for 0.5 h with 10 g/L osmic acid, and next dehydrated gradientially for 10 min each stage, exchanged with acetic isopental ester for 4 hours, dried by CO₂ drier (Hitachi HCP₂, Japan), and finally splashed with ionic platinum in vacuum. Growth of the cells was observed on S-450 electron scanning microscope (Japan). In spheroidal aggregate culture, 1 mL samples were taken well-distributedly from the matrix for cell calculation on the d7 and d12.

Determination of albumin and urea syntheses

When the culture matrix was changed, samples of supernatant fluid were obtained from the culture on microcarriers and spheroidal aggregate culture to determine the contents of albumin and urea through an automatic biochemical analyzer (Beckman, USA).

RESULTS

Isolation of porcine hepatocytes

We successfully took the improved *Seglen's* two-step orthotopic perfusion method with collagenase to isolate the porcine hepatocytes. About 2.82×10^{10} hepatocytes were obtained and the survival rate 91.2%. Each gram pig liver offered 7.01×10^7 hepatocytes.

Cultivation of porcine hepatocytes

In culture on microcarriers, the amount of hepatocytes on the d7 in the two systems is shown in Table 2. Hepatocytes growth curve (Figure 1) was drawn on the basis of Table 2. On d18 hepatocytes in FCS culture system amounted to $(1.1 \pm 0.53) \times 10^7$ and those in PPVS system $(3.1 \pm 0.71) \times 10^8$. In spheroidal aggregate culture the amount of hepatocytes on d7 was $(3.12 \pm 0.26) \times 10^7$ and that on d10 $(1.39 \pm 0.51) \times 10^8$.

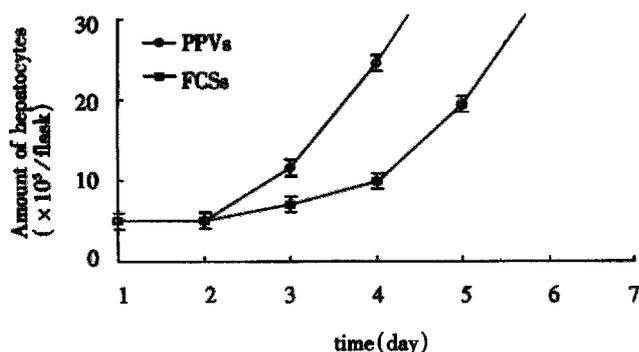


Figure 1 Hepatocytes growth curve in different culture systems on microcarriers.

Morphological observation of hepatocytes cultured on microcarriers

Observation under phase-contrast light microscope

Four hours after inoculation microcarriers were observed to be adhered with hepatocytes and the hepatocytes were beginning to expand. The phenomenon of bridge-link could be observed between microcarriers (Figure 2), i. e. the microcarriers were linked with each other through hepatocytes and arranged as slices or a string of beads (Figure 3). There was "snowball" effect between some microcarriers and hepatocytes. Twenty-four hours after culture over 1/3 microcarriers were covered with cells. The hepatocytes adhering to microcarriers was in irregular polygonal shape and thereafter these cells were observed to expand and grow. Forty-eight hours after culture most vivid hepatocytes were attached to microcarriers directly or indirectly. Growth of hepatocytes on microcarriers resembled that of monolayer growth in flasks. In FCS system hepatocytes began to adhere to microcarriers and expanded 2 h after inoculation. Double-nucleus hepatocytes could be observed and some linked to others in insular configuration. Twenty-four hours later about 60%-70% hepatocytes were attached. Three days after inoculation they began to proliferate and there were anile hepatocytes 6 days later. About 22 days later microcarriers were covered fully with hepatocytes. Five weeks after inoculation hepatocytes tended to become rough and blurry-edged. Granules and vacuoles could be observed in most of them. About 2 days later hepatocytes began to detach from microcarriers and died. Morphology of hepatocytes in PPVS system resembled that in FCS system at the first 24 h except that there were more double-nucleus hepatocytes in PPVS system. Three days after inoculation most microcarriers were covered with neonatal hepatocytes and they linked to others in spheroidal or trabecular conformation. Eighteen days later microcarriers were covered fully with hepatocytes and such condition could be maintained to the d52. Fifty-four days later hepatocytes could be observed to begin falling off from microcarriers and completed on the d56.

Observation under electron microscope

On d18 of culture, the electron microscope showed that the hepatocytes adhering firmly to the microcarriers in semi-spherical form. The microvilli on the surfaces of hepatocytes could be observed clearly. The amount of hepatocytes adhering to the microcarriers varied and they were distributed unevenly. Some microcarriers aggregated with others through cell-bridge as shown in Figure 4.

Morphological observation of spheroidal aggregated hepatocytes

When successively observing the normal primary porcine hepatocytes cultured in spheroidal-aggregates under the phase-contrast microscope, we found that: about 80%-90% hepatocytes began to aggregate spheroidally 24 h after being inoculated and diameters of the spheroids ranged from 62 μm to 134 μm . Each spheroid contained 10-20 hepatocytes. The hepatocytes at this time linked with each other loosely and were likely to be

dispersed. When observed under light microscope, they looked like "cell flowers" (Figure 5). On the d7 they were hyperplastic and linked with others tightly and looked like "cell spheroids" (Figure 6). On d10 they took the shapes of spheroid and irregular column as "algoid plant" (Figure 7). On the d20, the algoid hepatocytes began to atrophy and vacuolous granules appeared in them, the nuclei began to lyse and the hepatocytes ended in falling off and dying. On the d23, all of the spheroidal aggregating hepatocytes died.

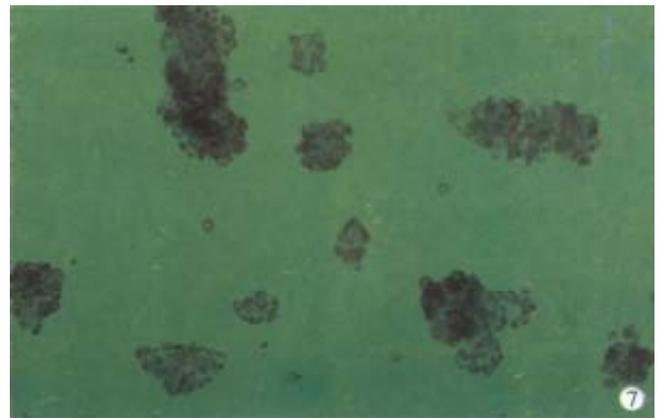
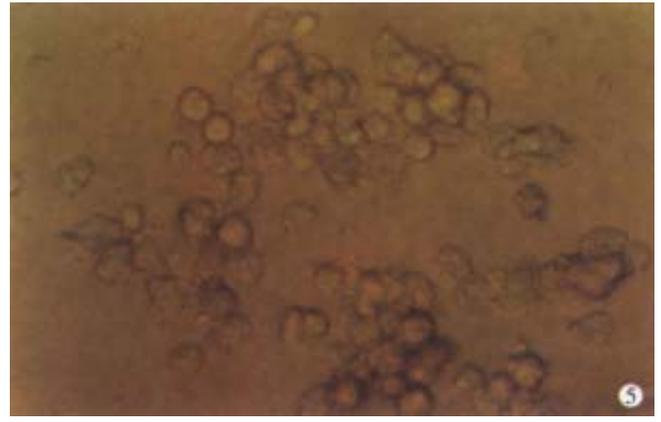
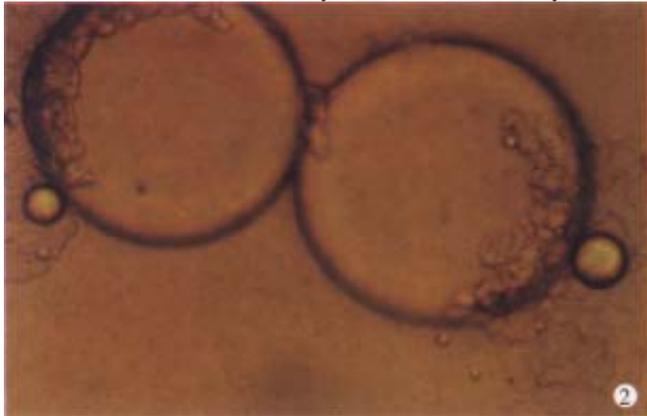


Figure 2 Four hours after inoculation cell-bridge could be observed between microcarriers (under phase-contrast microscope $\times 200$).

Figure 3 Microcarriers were arranged as a string of beads by adhered hepatocytes (under phase-contrast microscope $\times 400$).

Figure 4 Stringed microcarriers with adhered hepatocytes (under phase-contrast microscope $\times 730$).

Figure 5 Hepatocytes looked like "cell flowers" 24 h after being cultivated in suspension and aggregated spheroids (under phase-contrast microscope $\times 400$).

Figure 6 On the d7 hepatocytes aggregated into "cell spheroids" (under phase-contrast microscope $\times 400$).

Figure 7 On the d10 hepatocytes in the forms of algoid plants (under phase-contrast microscope $\times 200$).

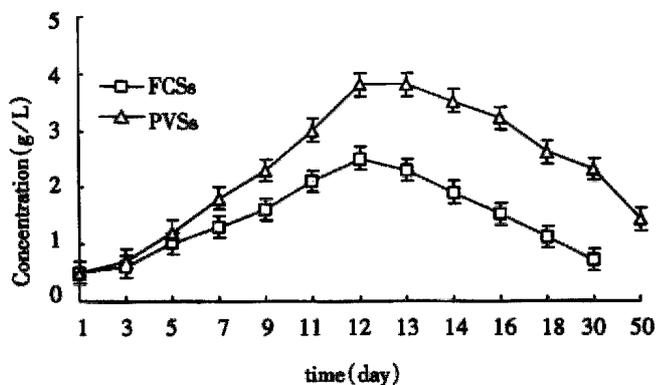
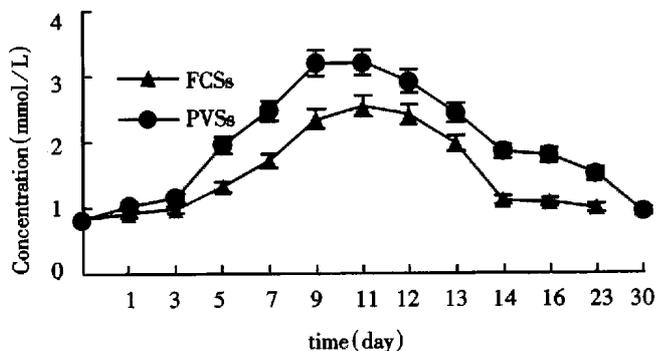
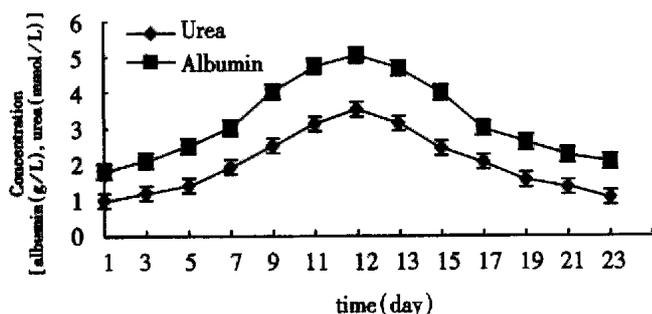
Table 1 Ingredients of different liver-perfusing solutions (g/L)

	NaCl	KCl	NaOH	CaCl ₂	MgCl ₂	Na ₂ SO ₄	HEPES	Collagen ase(IV)	Tricine	Tes	pH
I	8.3	0.5	0.22				2.4				7.4
II	3.9	0.5	2.6	0.53			24	0.5			7.4-7.6
III	4.0	0.4	2.1	0.136	0.13	0.1	7.2		6.5	6.9	7.4
IV	8.3	0.5	0.11	0.136			2.4				7.4

Table 2 Amounts of hepatocytes at various time in different culture systems on microcarriers ($\bar{x} \pm s, \times 10^5/\text{pore}$)

	1st	2nd	3rd	4th	5th	6th	7th(day)
FCSs	5.0±0.4	5.0±0.1	6.9±0.2	9.8±0.6	19.3±0.2	34.3±0.3	58.6±0.7
PPVSs	5.0±1.2	5.0±0.4	11.4±0.5 ^a	24.3±0.1 ^a	40.4±0.4 ^a	70.7±0.7 ^a	136.1±0.1 ^a

^aP<0.05 (compared with FCSs group)

**Figure 8** Changes of albumin synthesis in hepatocytes from different culture systems on microcarriers.**Figure 9** Changes of urea secretion in hepatocytes from different culture systems on microcarriers.**Figure 10** Dynamic changes of albumin and urea in the supernatant fluid.

Determination of albumin and urea syntheses

By using the automatic biochemical analyzer (Beckman, U.S.A.) we determined the contents of albumin and urea in the supernatant fluid at different times successively. In the two culture systems on microcarriers the values were kept in high levels from the d7 to d14 and got the summit on the d11 to d12 (Figures 8 and 9). As Figures 8 and 9 showed, functions of hepatocytes in PPVS system 3-5 days after inoculation were obviously higher than those in FCS system. Hepatocytes in PPVS can maintain their activity for about 50 days. In spheroidal aggregate culture the values were kept in high levels from d7 to d14 and got the summit on d10. From then on, the values degraded gradually and finally the hepatocytes lost their biofunctions on d23 (Figure 10).

DISCUSSION

Years of researches at home and abroad showed that primary hepatocytes were the most ideal biomaterial for BAL. But for the morphological and functional changes of primary hepatocytes cultivated *in vitro* occurred obviously in short time, it was deadly restrained to use them as biomaterial in BAL. The specific functions of hepatocytes lost in *in vitro* culture accounted for the lack of some *in vivo* elements. Then it was proposed that syntheses of albumin and urea could be promoted by adding some nutritional factors and hormones into the monolayer culture system. By now some improvements has been achieved in searching for a new effective system to cultivate primary hepatocytes *in vitro* by the following methods: ① adding hormonal agents such as insulin, glucagon, transferrin and hydrocortisone into the basal culture medium^[1]; ② adding extracellular biological matrix into the culture system^[2-4]; ③ enriching the culture system with hepatocytes growth stimulating factors such as hepatocytes growth factor and epithelial cell growth factor etc^[5,6]; ④ co-culturing with other endodermal and mesodermal cells; ⑤ three-

dimensional cultivation such as microcapsular and macrocapsular culture, spheroid aggregate, microcarrier culture and hollow fiber culture^[7,8]; ⑥ adding several amino acids and nutritional substances into the culture system; and ⑦ adding penetrating-inducer such as pentobarbiturate to the culture system^[9]. The above measures all aimed to make a circumstance mimic to the *in vivo* environment for the hepatocytes.

It is well-known that about 70% blood supply of liver comes from portal vein system. The growth of liver and maintenance of its functions depend mainly on the nutritional circumstance provided by the portal vein system, that is, hepatocytes *in vivo* are nourished by the contents of portal vein blood. The later provides various amino acids, energy substances and several growth factors. Our experiments were based on this very point and PPVS was added into the culture matrix. The results showed that hepatocytes in this system could durably survive and maintain their biofunctions. This fluid was the ideal circumstance to cultivate the biomaterial for BAL.

Microcarriers Cytodex-3 were used to provide three-dimensional space for adhering and suspending hepatocytes so as to bring benefit from fully utilizing of the nutritional components in the culture system. Our proceeding studies had proved that Cytodex-3 was the best microcarrier for primary hepatocytes^[3]. Hepatocytes on Cytodex-3 were readily to shape the microstructure similar to that of liver so they could grow and function well.

Landry^[10] *et al* found that when no adhering agent in the spheroidal-aggregate culture system the isolated hepatocytes tended to be changed less in morphology and could survive and maintain their activities for 4 wk-6 wk due to the resemblance of the structure between the aggregating spheroids and normal liver. Clayton^[11,12] *et al* found that the specific transcription gene of hepatocyte in little masses of liver could be kept well than that of isolated hepatocytes and obviously the three-dimension structure of hepatocytes is of great importance in maintaining their specific functions. Relationship between cell skeleton and specific proteins reflected the influences of morphology to functions, that is, cell functions directly related with levels of mRNA and specific proteins, and inversely related with levels of actin and canalicular protein. Sakai^[13] *et al* found that the inoculation concentration was of great influence to the formation of hepatocyte spheroids in the

suspending spheroidal aggregate culture system. When hepatocytes being inoculated at a high concentration it often occurred that pH of culture matrix declined, cell-to-cell and spheroid to spheroid relationships changed and multiple necrotic foci could be observed in hepatocyte spheroids. When hepatocytes being inoculated at a low concentration they were difficult to aggregate into spheroids. Sakai *et al* considered 5.0×10^6 /mL to be the optimal inoculation concentration in suspending spheroid aggregate culture system of hepatocytes.

On view of the above factors, we concluded that primary porcine hepatocytes cultured on Cytodex-3 in PPVS system or in spheroidal-aggregate were promising to act as the biomaterial of BAL. Still there is much to be desired to promote the density, function and surviving time of hepatocytes for clinical use.

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Relationship between insulin A chain regions and insulin biological activities

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Subject headings insulin/chemistry; biological activities; A chain analogues

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Abstract

AIM To study the relationship between insulin A chain regions and insulin biological activities, we designed a series of insulin analogues with changes at A21, A12-18 of C-terminal helical region and A8-10 located in the region of A6-A11 intra-chain disulphide bond. **METHODS** Insulin A-chain analogues were prepared by stepwise Fmoc solid-phase manual synthesis and then combined with natural B-chain of porcine insulin to yield corresponding insulin analogues. Their biological activities were tested by receptor binding, mouse convulsion and immunological assay.

RESULTS [A21Ala]Ins retains 70.3% receptor binding capacity and 60% *in vivo* biological activity. [DesA13-14, A21Ala]Ins and [DesA12-13-14-15, A21Ala]Ins still have definite biological activity, 7.9% and 4.0% receptor binding, and 6.2% and 3.3% *in vivo* biological activity respectively. [A15Asn, A17Pro, A21Ala]Ins maintains 10.4% receptor binding and 10% *in vivo* biological activity. [A8His, A9Arg, A10Pro, A21Ala]Ins, [A8His, A9Lys, A10Pro, A21Ala]Ins and [A8His, A9Lys, A10Arg, A21Ala]Ins have 51.9%, 44.3% and 32.1% receptor binding respectively, 50%, 40% and 30% *in vivo* biological activity respectively, and 28.8%, 29.6% and 15.4% immunological activity respectively.

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CONCLUSION A21Asn can be replaced by simple amino acid residues. The A chains with gradually damaged structural integrity in A12-18 helical region and the demolition of the A12-18 helical region by the substitution of Pro and Asn for A17Glu and A15Gln respectively can combine with the B chain and the combination products show definite biological activity, the helical structure of A12-18 is essential for biological activities of insulin. A8-10 is not much concerned with biological activities, but is much more important antigenically in binding to its antibodies, these results may help us design a new type of insulin analogue molecule.

INTRODUCTION

To elucidate the multi-functions of insulin molecule, hundreds of insulin analogues with changes involving 90% of the constituting 51 amino acid residues at various parts of insulin molecule have been prepared by either chemical modification or synthesis during the past two decades. In this communication we report the influences of three different regions of insulin A chain, especially the secondary structure region, i.e. A12-18 helical region, on insulin biological activities. Recently, thorough studies of insulin analogues with deletion of fragments of the molecule were also undertaken, but restricted mostly to changes in the C-terminal part of the B chain^[1,2], presumably due to its location outside the disulfide linkage and therefore ease of splitting off a tryptic or peptic fragment and recombining with a modified or shortened fragment by chemical or enzymatic semi-synthesis. No such enzymatic site was found in A chain, and most of its amino acid residues are confined inside disulfide linkages. Chemical synthesis might be a better choice for making analogues with modification or deletion in part of the A chain, which constitutes the main feature of this communication.

To obtain insulin analogues, we first set up the semisynthesis strategy of insulin. Insulin A chain was synthesized by the Fmoc solid phase synthesis strategy, and then synthetic A chain S-sulphonate was combined with porcine insulin B chain S-sulphonate forming crystalline insulin^[3]. Based on these experiences, the following insulin analogues

were semisynthesized. They are [A21Ala]Ins^[4], where A21Asn is replaced by Ala, and its derivatives with deletions in the A12-18 helical region, [DesA13-14, A21Ala] Ins^[4] and [DesA12-13-14-15, A21Ala]Ins^[4], and [A15Asn, A17Pro, A21Ala]Ins, where the helical structure of A12-18 was also demolished by the substitutions of Pro for A17Glu and Asn for A15Gln predicted by Chou-Fasman method, and [A8His, A9Arg, A10Pro, A21Ala]Ins^[5], and [A8His, A9Lys, A10Pro, A21Ala]Ins^[5], and [A8His, A9Lys, A10Arg, A21Ala]Ins^[5], in which His reminiscent A8 of chicken insulin with high biological activity took the place of A8Thr and Arg, Lys, Pro replaced A9Ser and A10Ile to examine the influences of these alterations on receptor binding and antigenicity of insulin. The sequences of insulin A chain analogues are as follows (Figure 1).

	1	5	10	15	20
Insulin A Chain	G.I.V.E.Q.C.C.T.S.I.C.S.L.Y.Q.L.E.N.Y.C.N				
1	G.I.V.E.Q.C.C.T.S.I.C.S.L.Y.Q.L.E.N.Y.C.A				
2	G.I.V.E.Q.C.C.T.S.I.C.S.....Q.L.E.N.Y.C.A				
3	G.I.V.E.Q.C.C.T.S.I.C.....L.E.N.Y.C.A				
4	G.I.V.E.Q.C.C.T.S.I.C.S.L.Y.N.L.P.N.Y.C.A				
5	G.I.V.E.Q.C.C.H.R.P.C.S.L.Y.Q.L.E.N.Y.C.A				
6	G.I.V.E.Q.C.C.H.K.P.C.S.L.Y.Q.L.E.N.Y.C.A				
7	G.I.V.E.Q.C.C.H.K.R.C.S.L.Y.Q.L.E.N.Y.C.A				

Figure 1 Insulin A chain analogue sequence.

MATERIALS AND METHODS

Materials

Porcine insulin (26.4U/mg), Shanghai Biochem-ical Plant; Sephadex G-15, G-50, Sepharose CL-6B, Pharmacia; Fmoc amino acid, prepared in the laboratory; DCC, acetonitrile, trimethylsilyl trifluoromethane sulfonate (TMSOTf), thioanisole, Fluka Co. trifluoro- acetic acid (TFA) Merck Co.; DTT, Serva Co.

Methods

Peptide synthesis Base labile N-Fmoc protected amino acid was coupled by DCC-HOBt to the 2% cross linked β -alkoxybenzyl alcohol resin. The functional side chain groups were protected by tBu for Cys, Glu, Tyr and Ser. Scheme of manipulation was the same as reported^[6]. The peptide chain was detached from the resin support with simultaneous removal of all tBu protecting groups by TFA except S-tBu of cysteine. S-tBu was deprotected by M TMSOTf-t thioanisole-TFA system^[5] and transformed to S-sulphonate by tetrathionate and sulfite with the procedure as reported^[7].

Isolation and purification of ASSO₃⁻ Crude ASSO₃⁻ fractions were purified by HPLC with gradient elution (A: 0.1% TFA, B: 60% CH₃CN, 0.125%

TFA).

Recombination with the natural (porcine) BSSO₃⁻ and purification This was carried out according to modified Chance's procedure^[4]. The crude product was purified by HPLC using the same procedure as that for ASSO₃⁻.

Receptor binding, *in vivo* activities and RIA The receptor-binding of insulin and analogues was determined by the displacement of labelled insulin from the insulin receptor on the human placental membrane according to Feng's^[8] procedure. The *in vivo* activity was estimated semiquantitatively by mouse convulsion assay. RIA kit produced by the Shanghai Biological Product Institute was used for RIA of insulin and analogues.

RESULTS

Peptide synthesis

Insulin A chain analogues were prepared starting from 300 mg-500 mg each of Fmoc-alanyl resin and products obtained from each step and the final overall yield is given in Table 1.

Table 1 Data on the synthesis of A chain analogues

A Chain analogues	FmocAla-resin mg(mmol)	Target peptide-resin(mg)	After HPLC(mg)	Overall yield(%)
1	500(0.19)	769	62.1	12.3
2	500(0.19)	749	54.6	12.0
3	500(0.19)	724	45.4	12.4
4	500(0.32)	865	20.0	4.5
5	300(0.135)	657	39.6	10.6
6	300(0.135)	648	40.1	11.2
7	300(0.135)	660	41.3	11.1

Reconstitution of A and B chain and purification of the products

According to the modified Chance's procedure, insulin analogues were obtained by purification of the crude reconstituted products. The identifications of amino acid analysis and HPLC indicated homogeneous products of insulin analogues.

Biological activities

The receptor-binding capacities calculated from the receptor binding curve, *in vivo* activities and immunological activities are shown in Table 2.

Table 2 Biological activities of insulin analogues (%)

Sample	Receptor binding	Mouse convulsion	Immunological activity
Ins	100	100	100
1B	70.3	60	
2B	7.9	6.2	
3B	4.0	3.3	
4B	10.4	10	
5B	51.9	50	28.8
6B	44.3	40	29.6
7B	33.1	30	15.4

DISCUSSION

A21Asn was considered to be a key amino acid of the insulin molecule in regard to its receptor binding. It has been very conservative in the evolutionary process, and when replaced the analogues showed remarkable diminution of biological activity. Our results showed that the analogue with the A21 Asn replaced by Ala exhibited substantial activity *in vivo* and especially higher level of receptor binding, indicating the non-essentiality of A21Asn in receptor binding and biological activity as well. It could well be replaced by other amino acid residues with good retention of biological activities. Better results were observed when the side chain of the A21 amino acid is smaller or not present (Gly)^[9]. However, deletion of the A21 residue is fatal as the activity of desA21 insulin showed only less than 1% of the activity. Apparently, like some non-essential amino acid residues in the insulin molecule, A21 is important in maintaining the specified spatial configuration of insulin required for its biological activities.

It is generally accepted that during the reconstitution of A and B chain to insulin A6-11 disulfide bond formed first and this led to the formation of right natural conformation by self adjustment. When the B chain was getting in touch with the intrachain disulfide, A6-11, a hydrophobic nucleus was formed to enhance the proper pairing of two Cys in both ends of the B chain to corresponding Cys of the A chain^[10]. The success of our reconstitution with deleted A chain supports to a certain extent this suggestion. It showed that the C-terminal helical region in the A chain of insulin was not as important as the N-terminal helical region in reconstitution with the B chain. The biological activities of insulin analogues with gradually damaged structural integrity in A12-18 helical region were similar to that of insulin analogue with the demolition of the A12-18 helical region by the substitution of Pro and Asn for A17Glu and A15Gln respectively. The observations suggested that the helical structure of A12-18 is essential for biological activities of insulin.

It was generally suggested that the enhanced activities of [A8His]Ins^[11], chicken and turkey insulins were ascribed to the His at A8^[12-14], which should account for the higher affinity for insulin receptor. Our results showed that the presence of His at A8 apparently did not enhance the receptor binding. The receptor binding of [A8His] Ins should lie at the range of about 50%. The enhanced

potency of these insulins containing His at A8 may not result solely from an apparent and direct effect due to the presence of A8His. A8His and A10Arg signify a higher influence on insulin antigenicity. A8His will enhance the binding activity to the anti-insulin serum^[11], while A10Arg will decrease this binding, but amino acid at A9 had very little to do with antigenic activity. In addition to studies on how to elucidate the action of an insulin molecule to its receptor and express the physiological activity that follows such action, the purpose of studies on the structure activity relationship of insulin will rely more on how to get a better analogue with high potency but low antigenicity. We hope that the information provided in this study will be beneficial to the development of a new type insulin analogue with those qualities through design of an appropriate architecture of this important hormone.

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Helicobacter pylori infection and risk of gastric cancer in Changle County, Fujian Province, China

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Subject headings Helicobacter pylori; stomach neoplasm; risk factors; case control studies

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Abstract

AIM To evaluate the effects of *Helicobacter pylori* infection on and other environmental factors on the development of gastric cancer.

METHODS A population-based case-control study was conducted in Changle County, Fujian Province. The primary gastric cancer cases were histologically confirmed or diagnosed by surgery between January 1996 and March 1998. Healthy controls were randomly selected and matched by age, sex, and neighborhood of residence. A total of 101 pairs were included in the study. Specially trained interviewers conducted face-to-face interviews with the subjects according to a standardized questionnaire. *Helicobacter pylori* infections were measured by serum IgG antibody to *Helicobacter pylori*. Conditional Logistic Regression analysis was used.

RESULTS The presence of IgG antibody to *Helicobacter pylori* was 63.7% in study subjects, 56.0% in patients with cardiac cancer, and 60.5% in patients with non-cardiac gastric cancer. The risk factors of gastric cancer in Changle County were identified such as low educational level [OR=3.864; 95% confidence interval (95% CI) 1.604-9.311], low consumption of fresh vegetables (OR=4.925; 95% CI 1.356-17.885), high intake of fish sauce (OR = 10.587; 95% CI 2.821-39.738), unscheduled meals (OR = 4.254; 95% CI 1.445-

12.552), and *Helicobacter pylori* infection (OR = 3.453; 95% CI 0.901-13.224).

CONCLUSION *Helicobacter pylori* infection may be important in the etiology of gastric cancer, but major risk factors other than *Helicobacter pylori* are responsible for the high gastric morbidity in Changle County.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is associated with gastric cancer^[1-4]. However, only a small proportion of individuals developed gastric cancer in comparison with the relatively high prevalence of *H. pylori* infection in the general population. In this study we evaluated the effects of *H. pylori* infection and other environmental risk factors on the development of gastric cancer in Changle County, one of the areas with highest morbidity of gastric cancer in the world.

MATERIALS AND METHODS

Study subjects

One hundred and one gastric cancer patients (87 males and 14 females) and the same number of normal controls, individually matched with region, sex and age (± 3 years) were included in the study. The studied subjects must have resided in Changle County for more than 20 years. The primary gastric cancer patients were histologically confirmed or diagnosed by operation during January 1996 - March 1998. Those who have ever been diagnosed as having gastric diseases in the past 3 years were not eligible as controls.

Data collection

Trained interviewers interviewed the patients and controls. A standardized questionnaire was used to obtain information on basic demographic characteristics (gender, race, year and place of birth, and education), dietary habits, personal habits (smoking and drinking), medical history and so on.

H. pylori assay

The presence of anti-*H. pylori* - IgG was assessed using the commercially available high-molecular-weight cell-associated protein *H. pylori* -immuno-

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assay (Enteric Products Inc, Westbury, New York). The assay was done in 96-well microtiter plates. Sera diluted 1:101 were added, and peroxidase conjugated anti-human IgG was used as the detector. The absorbance of the solution was measured at 450 nm.

Statistical analysis

Data were handled by Epi-info. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Conditional Logistic Regression using STATA software

RESULTS

A total of 101 pairs were included in the study. They were all Han nationality. The age of the patients ranged from 32 years to 78 years, averaging 58.93 years. The educational level was higher in controls than in the patients. The serum samples of 101 patients and 100 controls were tested for antibodies against *H. pylori*. Among them, 128(63.68%) were *H. pylori* positive. The prevalence of *H. pylori* infection was not significantly different in different age (above 30 years) and sex groups. The demographic characteristics as well as the distributions of prevalence of *H. pylori* infection in different sex and age groups are presented in Table 1 and Table 2.

Table 1 Demographic characteristics as well as Helicobacter pylori infection in patients and controls

	Patients		Controls		P value
	n	%	n	%	
Age (years)					
30-39	4	(4.0)	5	(5.0)	
40-49	18	(17.8)	18	(17.8)	
50-59	25	(24.8)	22	(21.8)	
60-69	34	(33.7)	38	(37.6)	
≥70	20	(19.8)	18	(17.8)	0.9597
Gender					
Males	87	(86.1)	87	(86.1)	
Females	14	(13.9)	14	(13.9)	1.0 000
Education					
College	1	(1.0)	1	(1.0)	
High school	17	(16.8)	68	(67.3)	
Elementary school	65	(64.4)	24	(23.8)	
Illiterate	18	(17.8)	8	(7.9)	0.0 000
<i>H. pylori</i>					
Positive	60	(59.4)	68	(68.0)	
Negative	41	(40.6)	32	(32.0)	0.2 064

Table 2 Helicobacter pylori infection in study subjects

Age group (years)	Men			Women		
	n	Hp(+)	%	n	Hp(+)	%
30-39	7	7	(100.0)	2	2	(100.0)
40-49	29	18	(62.1)	7	5	(71.4)
50-59	46	28	(60.9)	1	1	(100.0)
60-69	58	39	(67.2)	13	6	(46.2)
≥70	33	18	(54.6)	5	4	(80.0)
Total	173	110	(63.6)	28	18	(64.3)

$\chi^2 = 5.68, P = 0.2241; \chi^2 = 4.22, P = 0.3769$

No statistical difference was found in respect to presence of IgG antibody to *H. pylori* between patients and controls (Table 3). No significant difference was observed in anatomic distribution, although the prevalence of *H. pylori* infection was higher in non-cardiac gastric cancer (60.5%) than in cardiac cancer (56.0%), (Table 4).

Table 3 Helicobacter pylori infection and gastric cancer

Controls	Patients		Pairs
	Hp(+)	Hp(-)	
Hp(+)	39	29	68
Hp(-)	20	12	3
Pairs	59	41	100

$c^2 = 1.31, P = 0.25$

Table 4 Helicobacter pylori infection in gastric cancer cases

Gastric cancer	n	<i>H. pylori</i> infection	
		n	%
Cardiac	25	14	56.0
Non-cardiac	76	46	60.5

$\chi^2 = 0.04, P = 0.8395$

Further analysis was conducted using Conditional Logistic Regression. The results showed the risk factors of gastric cancer in Changle County were fish sauce intake, low consumption of fresh vegetables, unscheduled meals, low educational level and *H. pylori* infection (Table 5).

Table 5 The results of multivariate Conditional Logistic Regression analysis

Factors	β	S_x	OR	95%CI
Low educational level	1.3 517	0.4 487	3.864	1.604-9.311
Low consumption of vegetables	1.5944	0.6580	4.925	1.356-17.885
High intake of fish sauce	2.3596	0.6748	10.587	2.821-39.738
Unscheduled meals	1.4479	0.5508	4.254	1.445-12.552
<i>H. pylori</i> infection	1.2392	0.6852	3.453	0.901-13.224

DISCUSSION

Changle County is a hyperendemic area of gastric cancer^[5]. Chronic *H. pylori* infection has been identified as the most important risk factor of gastric cancer^[6]. The results of our study showed that residents in Changle County had a high prevalence of *H. pylori* infection. This coincided the fact the concept of high prevalence of *H. pylori* infection in the high risk area of gastric cancer. However, no statistical significant difference was found in respect to presence of *H. pylori* infection between patients and controls. The lack of association in this study may be due to the high prevalence of *H. pylori* in Changle County. *H. pylori* infection can initiate a sequence of histological alterations in the mucosa that may finally result in the development of gastric cancer,

but not all infected subjects will eventually develop gastric cancer. *H. pylori* alone cannot account for development of gastric cancer. Apart from *H. pylori* infection, other factors may play important roles in carcinogenesis.

The development of gastric cancer is believed to be a multistep and multifactorial process. In the Correa model of gastric carcinogenesis, environmental factors are related to the evolution from normal gastric tissue through superficial gastritis, atrophic gastritis, intestinal metaplasia and dysplasia to carcinoma^[7]. Environmental co-factors other than microbial agents may play roles in initiation, promotion or progression of gastric cancer.

The results of this study suggest that dietary factors, such as high intake of fish sauce and low consumption of fresh vegetables possibly increase the risk of gastric cancer. Fish sauce is a kind of traditional sauce consumed daily by Changle residents. It is usually produced from several kinds of sea fishes after long fermentation processes. The mutagenicity of fish sauce has been reported by several experimental studies^[8,9]. There was a large amount of important precursors of N-nitrosamines detected in fish sauce. High intake of fish sauce was an important risk factor involved in the etiology of gastric cancer. However, consumption of diets high in vegetables is the most effective means in preventing gastric cancer. Vegetables contain many biologically active compounds that may be responsible for an anticarcinogenic effect against gastric cancer^[10]. Additionally, unscheduled meals may cause injuries of gastric mucosa and potentiate the effects of carcinogens. Our study indicated that unscheduled meals might be one of the etiological factors for gastric cancer. In this study the educational level was higher in controls than in patients.

This supported the results of previous studies that socioeconomic status is a risk factor for gastric cancer, which in turn is related to diet and education.

These findings indicate that primary prevention should be focused in Changle County on reducing the etiological factors: fish sauce intake, deficiency in fresh vegetables, and *H. pylori* infection. Further elucidation of these risk factors for stomach cancer in Changle and assessment of the interaction between these risk factors and *H. pylori* infection are important for investigating the possible mechanisms of gastric carcinogenesis.

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Construction of HBV-specific ribozyme and its recombinant with HDV and their cleavage activity *in vitro*

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Subject headings hepatitis B virus; hepatitis D virus; ribozyme gene; recombinant DVRZ

Wen SJ, Xiang KJ, Huang ZH, Zhou R, Qi XZ. Construction of HBV-specific ribozyme and its recombinant with HDV and their cleavage activity *in vitro*. *World J Gastroentero*, 2000;6(3):377-380

Abstract

AIM To construct the recombinant of HDV cDNA and HBV-specific ribozyme gene by recombinant PCR in order to use HDV as a transporting vector carrying HBV-specific ribozyme into liver cells for inhibiting the replication of HBV.

METHODS We separately cloned the ribozyme (RZ) gene and recombinant DVRZ (comprising HDV cDNA and HBV-specific ribozyme gene) into the downstream of T7 promoter of pTAdv-T vector and studied the *in vitro* cleavage activity of their transcripts (rRZ, rDVRZ) on target RNA (rBVCF) from *in vitro* transcription of HBV C gene fragment (BVCF).

RESULTS Both the simple (rRZ) and the recombinant ribozyme rDVRZ could efficiently catalyze the cleavage of target RNA (rBVCF) under different temperatures (37°C, 42°C and 55°C) and Mg²⁺ concentrations (10 mmol/L, 15 mmol/L and 20 mmol/L) and their catalytic activity tended to increase as the temperature was rising. But the activity of rRZ was evidently higher than that of rDVRZ.

CONCLUSION The recombinant of HDV cDNA and ribozyme gene had the potential of being further explored and used in gene therapy of HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) can cause acute and chronic B-type hepatitis in man. The conventional ways available for curing this disease have not been very efficient. This promotes people to explore novel genetic therapeutical ways. Hammerhead ribozyme is a kind of antisense RNA which can specifically cleave the target RNA^[1,2]. In the light of this, people have developed many effective genetic vectors containing ribozyme genes, the transcripts of which showed catalytic activity *in vitro* and *in vivo*^[3-5]. But how to improve the stability and efficiency of ribozyme and specifically carry ribozyme gene into only target cells or tissues has been a tackling problem. HDV, a human hepatitis agent, is a defective RNA virus, the replication cycle of which relies on the infection of HBV^[6]. So HDV can be developed as a specific transporting and replicating vector *in vivo* for ribozyme to reach liver^[7]. In this study, we constructed HBV-specific hammerhead ribozyme gene (RZ) and the recombinant (DVRZ) of HDV cDNA (DV) and ribozyme gene (RZ) and made a careful investigation of their *in vitro* catalytic activity under various conditions. The positive results encouraged us to further explore the feasibility of using HDV as a vector carrying ribozyme for inhibiting the replication of HBV *in vivo*.

MATERIALS AND METHODS

Plasmids

The plasmid pSVC-D3 (containing HDV cDNA) as one of two templates of recombinant PCR was kindly offered by Prof. Taylor of American. pTAdv-T vector used for cloning was purchased from Clontech Corporation.

Major reagents

RiboMax transcription kit, acrylamide, bisactylamide, dNTP, rNTP and Taq polymerase were purchased from Promega. [α -³²P]UTP from Beijing Yuhui Corporation. Advantage-TM PCR pure kit (gel purification kit) from Clontech. X-film from Kodak. DNA polymerase I from Biolabs. T7/Sp6 sequencing kit from Pharmacia.

PCR primers

Primers P1 and P2 covered the whole sequence of designed HBV-specific hammerhead ribozyme gene (RZ) and were used to amplify ribozyme gene (53bp) because of 9nt base-pairing of their 3' ends.

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The sequence of HBV-specific hammerhead ribozyme was designed according to the requirement of domains of ribozyme^[1] and the sequence of HBV C gene fragment.

The ribozyme gene (RZ: 53bp) was also used as one of two templates for recombinant PCR to construct the recombinant (DVRZ) of HDV cDNA and ribozyme gene (mentioned below).

P3, P4 were both recombinant primers, each of which was composed of partial sequences of ribozyme and HDV in order to replace the sequence (17-67) of near 5'-end of HDV with ribozyme by recombinant PCR. P5 was the 3'-end sequence of HDV cDNA.

P3, P4, P5 were used to construct the recombinant (DVRZ) of HDV cDNA (DV) and HBV-specific ribozyme gene (RZ) by one-tube recombinant PCR^[8].

P6, P7 were used to amplify HBV C gene fragment (BVCF), which was the transcription template of target RNA (rBVCF). T7 was partial sequence of T7 promoter region of pTAdV-T vector and was used for sequencing (with Sp6) and identifying (with P2, P5, P7) whether 5'-end of foreign fragment forwardly inserted the downstream of T7 promoter.

The sequencing of all gene fragments was performed on ABI391 automatic sequencer (Pharmacia). The sequences and positions of these primers are listed in Table 1.

Table 1 The sequences and positions of primers

Primer name	Sequences	Positions
P1	5'-AACATTGACATAGCTCTGATGAGTCCGTGAG-3'	RZ:1-31
P2	5'-TCCAGGGAATTAGTACTTTGTCTCCACGGAC-3'	RZ:53-23
P3	5'-AGCAAGCTTGAGCCAAAACATGACATAGCTCT-3'	HDV:1-16,RZ:53-37
P4	5'-CTCCGACGTTCCAATGCTCCAGGGAATTAGTAGT-3'	HDV:84-68,RZ:53-37
P5	5'-GTGCAATTCGGGCTCGGGCGGCGATCCAGCAGTC-3'	HDV:1680-1647
P6	5'-GATAAGCTTTTACATAGAGGACTCTTGG-3'	HBV:1650-1677
P7	5'-CTGGAATTCGGCGAGGGAGTTCTTCTCTAG-3'	HBV:2480-2450

Construction and cloning of HBV-specific ribozyme gene(RZ)

Because of 9nt base-pairing between 3'ends of P1 and P2, direct PCR could produce complete 53bp ribozyme gene (Figure 1). After gel-purification (according to Advantage-TM PCR pure kit, Clontech Corporation), the PCR product (RZ) was directly cloned into the downstream of T7 promoter of pTAdV-T vector and so the resulting recombinant plasmid pTA-RZ was obtained. PCR with T7/P2 as primers and pTA-RZ as template could produce about 100 bp DNA fragment if 5'-end of ribozyme gene was forwardly inserted in to the downstream of T7 promoter of pTAdV-T vector and so could be used to identify the recombinant plasmid pTA-RZ of forward insert. The preparation of competent DH5 α cells and transformation of plasmids were performed according to the reference^[9]. The cloned ribozyme gene was finally verified by sequencing with T7/Sp6 primers.

Construction, cloning and sequencing of the recombinant (DVRZ) of HDV cDNA and HBV-specific ribozyme gene

Thirty μ L PCR reaction system was established^[8]: 20 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl₂, each dNTP 200 μ mol/L; Primers P3, P4, P5 were separately 0.05 μ mol/L, 0.005 μ mol/L, 0.05 μ mol/L. The two templates were recombinant plasmids pTA-RZ (containing ribozyme gene) and pSVC-D3 (containing HDV cDNA), each 10 ng. Taq polymerase 2 μ L, denaturation at 92 $^{\circ}$ C-50s; annealing at 53 $^{\circ}$ C-50s; elongation at 70 $^{\circ}$ C-120s; 33cycles; the final elongation at 70 $^{\circ}$ C lasted 5 min. PCR product (DVRZ, 1.7kb) was identified by agarose-gel (1.5%) electrophoresis and then purified with Advantage-TM PCR pure kit. The obtained DNA fragment (DVRZ) was directly cloned into the downstream of T7 promoter of pTAdV-T vector. The resulting recombinant plasmid pTA-DVRZ (positive clones on Ampr & white-blue plate) was verified by PCR with primers T7/P5 and sequencing with Sp6/T7 primers. PCR with primers T7/P5 could produce about 1.7kb-1.8kb DNA fragment and was used to identify the forward insertion of 5'-end of DVRZ. Sequencing was performed on ABI 391 automatic sequencer (Pharmacia).

Cloning and isolation of target gene fragment (HBV C gene fragment-BVCF)

PCR with primers P6, P7 was performed to amplify HBV C gene fragment from serum of HBV. Extraction of sample DNA and PCR reaction were performed according to the reference^[10]. PCR product (BVCF) was directly cloned into the downstream of T7 promoter of pTAdV-T vector and the resulting recombinant plasmid pTA-BVCF (positive clones on Ampr & blue-white plate) was verified by PCR with T7/P7 and bidirectional sequencing with T7/Sp6 primers. PCR with primers T7/P7 was utilized to identify the forward-direction insertion of 5'-end of BVCF.

Linearization of recombinant plasmids pTA-RZ, pTA-DVRZ and pTA-BVCF

These plasmids were separately digested with BamHI and then filled with Klenow fragment for *in vitro* transcription of inserted gene fragments (RZ, DVRZ, BVCF).

In vitro transcription of linearized plasmids pTA-RZ and pTA-DVRZ

³²P-UTP-labeled transcription of the two plasmids was first carried out in order to test the effect of transcription. But the plasmids' transcripts (rRZ, rDVRZ) used for catalyzing the cleavage of target RNA (rBVCF-transcript of plasmid pTA-BVCF containing HBV C gene fragment-BVCF) were not ³²P-labeled. *In vitro* transcription was performed with T7 RNA polymerase according to RiboMax Transcription kit and the reference^[11]. The

transcripts (rRZ, rDVRZ) would additionally contain 100nt partial sequence of pTAdV-T vector at their both ends. rRZ and rDVRZ were extracted by phenol/chloroform/iso-propyl alcohol (25:24:1), precipitated with ethanol and resuspended with H₂O (RNase-free). The latter was the recombinant of HDV and ribozyme.

Preparation of ³²P-UTP-labeled target RNA (rBVCF)

Linearized plasmid pTA-BVCF (containing HBV C gene fragment) was *in vitro* transcribed and the target RNA (rBVCF) was produced. 20 μL transcription system was so established: ATP, CTP, GTP each 2.5 mmol/L and UTP 0.1 μmol/L, 0.5 μCi/μL[α-³²P]UTP, 2 μg linearized plasmid pTA-BVCF, T7 RNA polymerase 20U, RNasin 10U, 37°C-60 min. The product was loaded for 5% PAGE-7M urea autoradiographed electrophoresis. Gel-band of 1cm length and 0.5 cm breadth was cutted off from the position of target RNA and soaked overnight with NES buffer (0.5 mol/L NH₄Ac, 1 mmol/L EDTA, 0.1% SDS) and extracted with phenol/chloroform/iso-propyl alcohol (25:24:1). The supernate was precipitated with ethanol and then the pellet was harvested and resuspended with H₂O (RNase-free). rBVCF(831+100nt) contained also 100nt partial sequence of pTAdV-T vector at its two ends.

Cleavage reaction

Ten μL reaction system was established: 0.1 mol/L Tris-HCl (pH 8.0), 20 mmol/L MgCl₂, rRZ or rDVRZ and their target RNA (rBVCF) each 2 μL, mixed and incubated under different temperatures (37°C, 42°C, 55°C) for 1h. Negative control with ³²P-labeled rBVCF incubated at 55°C without rRZ & rDVRZ was performed. In the meantime, rRZ and rDVRZ were separately incubated with target RNA (rBVCF) at 37°C under different Mg²⁺ concentrations (10mmol/L, 15mmol/L). In the end, 10 μL ion-free formamide and 1 μL loading buffer (50% glycerol, 1mmol/L EDTA, 0.04% bromophenol blue) were added to terminate the reaction. Then after incubated at 65°C for 10 min, 5 μL reaction sample was loaded for 5% PAGE-7M urea electrophoresis.

RESULTS

Construction and cloning of ribozyme gene

53bp DNA fragment was obtained by PCR with primers P1/P2 (Figure 1). This was in accordance with the size of anticipated ribozyme gene. PCR using T7/P2 as primers and plasmid pTA-RZ as the template amplified a DNA fragment of about 100 bp, which identified forward-direction insertion of ribozyme gene into the downstream of T7 promoter of pTAdV-T vector. Sequencing verified the HBV-specific ribozyme gene. The sequence of ribozyme gene was as follows: 5'-AACATTGACATAGCTCTGATGAGTCCG-TGAGGACAACTACTAATTCCTGGA-3'

Construction, cloning and sequencing of the recombinant (DVRZ) of HDV cDNA(DV) and HBV-specific ribozyme gene(RZ)

About 1.7kb DNA was amplified by recombinant PCR, which indicated the anticipated recombinant DNA molecule DVRZ (Figure 1). After DVRZ's cloning into pTAdV-T vector and transforming into DH5α on Ampr & white-blue plate, PCR with primers T7/P5 produced about 1.8 kb-DNA fragment and identified the positive recombinant plasmid pTA-DVRZ of correct DVRZ's insertion direction. Sequencing of DVRZ with primers T7/Sp6 (pTA-DVRZ as template) confirmed the construct DVRZ.

Cloning and isolation of target gene fragment (HBV C gene fragment-BVCF)

Anticipated 831bp target DNA fragment was amplified by PCR from the serum of HBV (Figure 1). After its cloning into pTAdV-T vector and then transformed into DH5α, PCR with primers T7/P7 produced an about 0.9 kb DNA fragment and so identified the positive recombinant plasmid pTA-BVCF of BVCF's correct insertion direction. Finally, sequencing with primers T7/Sp6 confirmed the inserted BVCF.

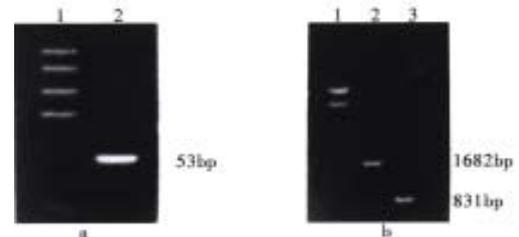


Figure 1 Construction of HBV-specific ribozyme gene (RZ), its recombinant with HDV cDNA (DVRZ) and target DNA (BVCF) by PCR. a: Lane 1-PCR marker, Lane 2-ribozyme gene RZ (53bp). b: Lane 1-λ DNA/-Hind III+Eco RI marker, Lane 2-recombinant DVRZ (1682bp) Lane 3-target gene BVCF (831bp).

In vitro transcription of linearized recombinant plasmids pTA-BVCF, pTA-RZ and pTA-DVRZ

Target RNA (rBVCF) of 931nt was transcribed from pTA-BVCF. HBV-specific ribozyme (rRZ) of 153nt was transcribed from pTA-RZ.

HBV-specific recombinant ribozyme (rDVRZ, containing HDV) of 1782nt was transcribed from pTA-DVRZ (Figure 2). All these transcripts (rBVCF, rRZ, rDVRZ) contained the same partial sequence of pTAdV-T vector at their both ends after transcription with T7 RNA polymerase.

Cleavage reaction

Ribozymes rRZ and rDVRZ without ³²P-label were separately incubated with target RNA (³²P-labeled rBVCF) under different temperatures (37°C, 42°C, 55°C) and Mg²⁺ concentrations. The results from autoradiographed electrophoresis showed that under these temperatures and Mg²⁺ concentrations, both

rRZ and rDVRZ could catalyze the cleavage of target rBVCF into two RNA fragments (721nt, 210nt) and their catalytic activity tended to increase with the rising of temperature. Comparatively, the catalytic activity of rRZ was higher than that of rDVRZ. But it seemed that Mg^{2+} from 10 mmol/L to 20 mmol/L had no obvious effect on their cleavage activity (Figure 3).

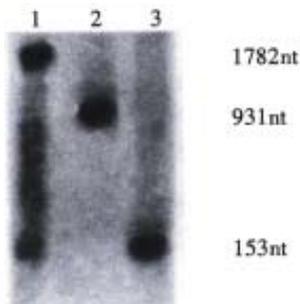


Figure 2 *In vitro* transcription of linearized pTA-RZ, pTA-DVRZ and pTA-BVCF (Lane 1-rDVRZ,1782nt. Lane 2-rBVCF,931nt. Lane 3-rRZ,153nt).

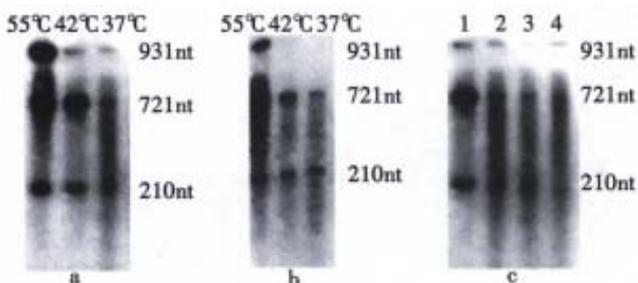


Figure 3 Cleavage of target RNA (rBVCF) catalyzed by rRZ and rDVRZ. a: Cleavage of target RNA (rBVCF) by rRZ under 37°C, 42°C and 55°C with 20 mmol/L Mg^{2+} . b: Cleavage of target RNA (rBVCF) by rDVRZ under 37°C, 42°C and 55°C with 20 mmol/L Mg^{2+} . c: Cleavage of target RNA (rBVCF) separately by rRZ and rDVRZ at 37°C under different Mg^{2+} concentrations (10 mmol/L, 15 mmol/L) (Lane 1,2-cleavage by rRZ separately under 10 and 15 mmol/L Mg^{2+} ; Lane 3,4-cleavage by rDVRZ separately under 10 and 15 mmol/L Mg^{2+})

DISCUSSION

Hammerhead ribozyme is a kind of antisense RNA with specific catalytic activity, which can catalyze the specific cleavage of target RNA^[1-3]. So far, many specific ribozyme constructs have demonstrated their catalytic activity both *in vitro* and *in vivo*^[12-15]. HDV, is a defective RNA virus, the replication cycle of which must be dependent on the infection of HBV^[6]. In view of the characteristics of hammerhead ribozyme and HDV, we constructed the recombinant (rDVRZ) of HDV and ribozyme by one-tube recombinant PCR with 3 primers and intended to have HBV-specific ribozyme carried into liver cells by using HDV as a transporting vector. As the first step, we studied the *in vitro* cleavage activity of HBV-specific ribozymes-rRZ and rDVRZ. The results showed that both rRZ and rDVRZ had the ability of catalyzing the specific cleavage of target RNA (rBVCF-*in vitro* transcript of HBV C gene fragment) into two RNA fragments (721nt, 210nt).

The activity of ribozyme rRZ (without containing HDV) was much higher than that of recombinant rDVRZ, which in part cleaved the target RNA. One possible reason is that rDVRZ has much longer two arms outside its base-pairing region and can easily form a complex secondary or tertiary structure, an obstacle to subsequent base-pairing with target RNA (rBVCF). Its three-dimensional structure simulated by computer (not presented here) supports this conclusion. However, we could conclude that the post-transcribed hammerhead ribozyme should not be too long though we are unsure how long ribozyme and its target RNA are optimal to their interaction.

We found that with the temperature rising, the ribozyme activity increased, possibly because higher temperature helped transit the complex tertiary structure into comparatively extended state and so partially delete the structural obstacle to base-pairing. We also found that the requirement of ribozyme for Mg^{2+} was not strict. It appeared that different Mg^{2+} concentrations from 10 mmol/L to 20 mmol/L could meet the ribozymes' activity. But overhigh Mg^{2+} seemed to cause the non-specific cleavage of target RNA (not presented). The reason for this was unclear. Presently, we are studying the activity of the recombinant ribozyme *in vivo* and its repressive effect on HBV replication.

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A 12-year cohort study on the efficacy of plasma-derived hepatitis B vaccine in rural newborns

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Subject headings hepatitis B vaccine; HBsAg; vaccination; rural newborn; cohort study

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Abstract

AIM To understand the anti-HBs persistence and the long-term preventive efficacy in rural newborns after vaccination with plasma-derived hepatitis B vaccine.

METHODS In the time of expanded program on immunization (EPI), the newborns were vaccinated with 10 µg × 3 doses of hepatitis B vaccine and 762 newborns who were HBsAg negative after primary immunization were selected for cohort observation from 1986 to 1998. Their serum samples were detected qualitatively and quantitatively for hepatitis B infecting markers, including HBsAg, anti-HBs and anti-HBc by SPRIA Kits. The annual HBsAg positive conversion rate was counted by life-table method.

RESULTS ① The anti-HBs positive rate was 94.44% for the babies born to HBsAg negative mothers and 84.21% for those born to HBsAg positive mothers in the 1st year after immunization, and dropped to 51.31% and 52.50% in the 12th year respectively. GMT value was dropped from 31.62 to 3.13 and 23.99 to 3.65 in the 2nd to the 12th year respectively. There was a marked drop in GMT at the 3rd to the 5th year, and in anti-HBs positive rate at the 9th to the 10th year. ② In the period of 12 years observation, the person-year HBsAg positive

conversion rates were 0.12% (5/4150.0) in newborns born to HBsAg negative mothers and 0.20% (1/508.0) in those born to HBsAg positive mothers, and none of the HBsAg positive converted children became HBsAg chronic carriers. Compared with the baseline before immunization, the protective rates were 97.19% and 95.32% respectively.

CONCLUSION The protective efficacy of plasma-derived hepatitis B vaccine persisted at least 12 years, and a booster dose seems not necessary within at least 12 years after the primary three-doses immunization to newborns born to HBsAg negative mothers.

INTRODUCTION

In recent years, many studies on the efficacy of hepatitis B plasma-derived vaccine demonstrated that the anti-HBs antibodies were declined gradually, but the protective rate for children vaccinated with three-doses hepatitis B vaccine was still more than 80%^[1,2]. To understand the anti-HBs persistence and long-term efficacy in newborn, a 1-12 year experimental study on hepatitis B plasma-derived vaccine was carried out among newborns vaccinated with three doses 10 mg/L vaccine in rural area.

MATERIALS AND METHODS

Objects

Newborn infants from one township in Zhengding County, Hebei Province, were vaccinated during 1986-1989. A total of 762 infants who were HBsAg negative after primary vaccination were selected for cohort observation, of them 688 born to HBsAg-negative mothers, and 74 to HBsAg carrier mothers. The annual HBsAg positive conversion rate for newborn infants was 4.72% before immunization.

Vaccine and immunization

Hepatitis B plasma-derived vaccine was produced by Beijing Institute of Biological Product. The newborns were immunized with three doses vaccine at 0mo, 1mo and 6mos of age by well-trained

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nurses between 1986-1989 under the rural area immunization program launched in 1986. The first dose was vaccinated at 2.7 mo-0.3 mo (95% CI). The coverage rate of vaccination with a complete course of 3-dose hepatitis B vaccine was 91.5%.

Blood collection and laboratory study

Blood samples were collected from infants by venipuncture at intervals of one or two years. Serum HBsAg, anti-HBs and anti-HBc were tested by RIAs from Beijing Institute of Biological Product. Those who showed HBsAg (S/N value ≥ 2.1), anti-HBc (CO/S value ≥ 2.0) or both were defined as HBV infection, and anti-HBs (S/N value ≥ 2.1) as immunity.

RESULTS

Persistence of anti-HBs after vaccination

The 688 new born babies were born to HBsAg negative mothers, and 74 were born to HBsAg positive mothers. Anti-HBs positive rate was gradually dropped from 94.44% to 51.31% and 84.21% to 52.50% in the 1st-12th year respectively. GMT value was dropped from 31.62 to

3.13 and 23.99 to 3.65 in the 2nd-12th year respectively. There was a marked drop in GMT value at the 3rd-5th year, and in anti-HBs positive rate at the 9th-10th year. Compared with infants born to HBsAg negative mothers, GMT value was lower in infants born to HBsAg positive mothers (Table 1).

HBsAg annual positive conversion rate for vaccinated children

In the period of 12 years follow-up, 5 children born to HBsAg-negative mothers became HBsAg positively converted. Two of them occurred at the 5th year and the rest occurred at the 9th-10th year. The person-year HBsAg positive conversion rate was 0.12% (5/4150.0). One child born to HBsAg positive mothers became HBsAg positively converted, and it occurred at the 5th year. The person-year HBsAg positive conversion rate was 0.20% (1/508.0). None of 6 HBsAg positive converted children became HBsAg chronic carriers (Table 2). Compared with the baseline before immunization, the protective rates were 97.19% and 95.32% respectively (Table 3).

Table 1 Dynamic change of anti-HBs positive rate and GMT after vaccination

Years vaccinated	Mothers HBsAg (-)			Mothers HBsAg (+)		
	No. tested	Positive rate (%)	GMT (S/N)	No. tested	Positive rate (%)	GMT (S/N)
1	18	94.44	26.30	19	84.21	15.14
2	134	89.55	31.62	48	95.83	23.99
3	232	89.22	26.30	51	92.16	22.91
4	344	85.17	18.62	51	73.33	6.46
6	296	79.39	9.77	54	79.63	8.71
7	608	80.76	9.33	58	86.21	7.78
8	599	78.30	6.17	56	75.00	5.25
9	517	57.79	4.17	48	60.24	3.47
10	412	50.24	3.09	23	34.78	2.51
11	173	51.80	2.86	30	56.00	2.91
12	424	51.30	3.13	20	52.50	3.65
χ^2 m-h		226.12			338.58	
P value		P<0.001			P<0.001	

Table 2 HBsAg person-year positive conversion for immunized children

Follow-up (year)	Mother HBsAg (-)			Mother HBsAg (+)		
	No. person year	No. positively converted	Annual positive conversion rate	No. person year	No. positively converted	Annual positive conversion rate
1	9.0	0	0.00	9.5	0	0.00
2	76.0	0	0.00	33.5	0	0.00
3	183.0	0	0.00	49.5	0	0.00
4	288.0	0	0.00	51.0	0	0.00
5	454.0	2	0.44	55.5	1	1.80
6	428.0	0	0.00	57.0	0	0.00
7	450.0	0	0.00	56.0	0	0.00
8	602.0	0	0.00	57.0	0	0.00
9	482.0	2	0.34	52.0	0	0.00
10	486.0	1	0.20	35.5	0	0.00
11	292.5	0	0.00	26.5	0	0.00
12	298.5	0	0.00	25.0	0	0.00
Total	4150.0	5	0.12	508.0	1	0.20

Table 3 Protective rate to vaccinated neonates

Mother HBsAg	Observed numbers	Person year numbers	HBsAg positive conversion numbers	Annual Positive conversion rate	Effective protective rate
Negative(-)	688	4150.0	5	0.12	97.19
Positive (+)	74	508.0	1	0.20	95.32
Total	762	4658.0	6	0.13	96.96
Baseline	562	1124.0	48	4.27	

DISCUSSION

The ultimate strategy for newborn vaccination with hepatitis B vaccine is controlling and eliminating hepatitis B. Many specialists think that newborns should be vaccinated within 24 hours which is more effective for preventing HBV mother-infant transmission. In recent studies, some reports say that the risk of HBV infection should increase with the decline of anti-HBs positive rate and GMT value after vaccination of hepatitis B vaccine^[2,3]. Thus, a booster of hepatitis B vaccine should be given. In this study, anti-HBs positive rate declined with age, so was GMT value. The anti-HBs positive rate declined markedly from the 9th to the 10th year, and maintained 50% or so at the 10th-12th year. GMT value declined markedly from the 3rd to the 5th year. Therefore, if we only analyze anti-HBs positive rate and GMT value, it is the best schedule to revaccinate at 3-5 years of age after the primary 3-doses regimen.

With the long-term observation of hepatitis B vaccine, more and more scholars think that the protective efficacy for immune responder could persist several years, even if anti-HBs may have declined to undetectable levels. This may be due to the good memory response to hepatitis B vaccination. The higher anti-HBs level after booster immunization dropped quickly with time, and at the end of 3-5 years dropped to the level of children who had not received booster immunization^[4,5]. The evaluation of the immune efficacy of hepatitis B vaccine should induce antibody level, but the dynamic of HBsAg and the chronic carrier rate during ten years of immunization. Cao *et al*^[6] reported that anti-HBs level declined with years after vaccination and HBsAg positive conversion had not been detected among children born to HBsAg negative mothers in Hunan. Our study indicated that none of children born to HBsAg negative mother became HBsAg chronic carrier within 12 years after vaccination with hepatitis B vaccine, although a few children were infected with HBV and positive for HBsAg. The protective rate

to those newborns born to HBsAg negative mother was still more than 95% within 12 years. We believed that long-term efficacy of hepatitis B vaccine was better and the children would have benefited from life-long hepatitis B vaccine. A booster dose seems unnecessary for children born to HBsAg negative mothers within 12 years after primary 3-dose regimen. It was reported by Cao *et al*^[7] that HBsAg positive conversion children born to HBsAg carrier mothers may become HBsAg chronic carriers after vaccination with hepatitis B vaccine, and most of them occurred at 2-3 years of age. In our study, only 1 of 74 children born to HBsAg carrier mother became HBsAg positively converted, but did not become HBsAg chronic carrier within 12 years after vaccination. In order to improve the immune efficacy for those children born to HBsAg positive mothers, the dynamic of HBsAg carrier rate should be observed further in vaccinated children on a large scale.

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Pharmacokinetics of traditional Chinese syndrome and recipe: a hypothesis and its verification (I)

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Subject headings traditional Chinese syndrome/recipe; Chinese medicine; pharmacokinetics; blood stasis; spleen deficiency; hypothesis

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Abstract

AIM To propose a hypothesis defining the absorption, distribution, metabolism and elimination of traditional Chinese recipe (TCR) component in blood of healthy subjects and patients, and estimate its correctness. **METHODS** The pharmacokinetics (PK) of same dose of drug was studied in the animal model of traditional Chinese syndrome (S) and healthy animals. The classification, terminology, concept and significance of the hypothesis were set forth with evidence provided in the present study. The hypotheses consisted of traditional Chinese syndrome PK (S-PK) and traditional Chinese recipe PK (R-PK). Firstly, the observed tetramethylpyrazine (TMP) PK in healthy, chronically reserpinized rats (rat model of spleen deficiency syndrome, RMSDS) and RMSDS treated with Sijunzi decoction (SJZD) for confirmation were used to verify S-PK; secondly, the ferulic acid (FA) PK in healthy and high molecular weight dextran (HMWD)-induced rabbit model with blood

stasis syndrome (RDBSS) was also used to verify S-PK; and lastly, TMP PK parameters in serum of healthy rats afterorally taken - Ligusticum wallichii (LW), LW and Salvia miltiorrhiza (LW&SM) decoctions were compared to verify R-PK.

RESULTS The apparent first-order absorption [K_a , $(13.61 \pm 2.56)h^{-1}$], area under the blood drug concentration-time curve [AUC, $(24.88 \pm 9.76) \mu g \cdot h^{-1} mL^{-1}$], maximum drug concentration [C_{max} , $(4.82 \pm 1.23) \mu g \cdot mL^{-1}$] of serum TMP in RMSDS were increased markedly ($P < 0.05$) compared with those [$K_a = (5.41 \pm 1.91)h^{-1}$, AUC = $(5.20 \pm 2.57) \mu g \cdot h^{-1} \cdot mL^{-1}$, $C_{max} = (2.33 \pm 1.77) \mu g \cdot mL^{-1}$] of healthy rats (HR). The apparent first-order rate constant for α and β distribution phase [$\alpha = (0.38 \pm 0.09)h^{-1}$, $\beta = (0.06 \pm 0.03)h^{-1}$], the apparent first-order intercompartmental transfer rate constants [$K_{10} = (0.24 \pm 0.07)h^{-1}$, $K_{12} = (0.11 \pm 0.02)h^{-1}$, $K_{21} = (0.11 \pm 0.02)h^{-1}$] of serum TMP in RMSDS were decreased significantly ($P < 0.01$) compared with those [$K_{10} = (0.88 \pm 0.20)h^{-1}$, $K_{12} = (1.45 \pm 0.47)h^{-1}$, $K_{21} = (0.72 \pm 0.22)h^{-1}$] of HR. However, no apparent differences occurred between HR and RMSDS treated with SJZD. The serum FA concentration and its AUC [$(5.6690 \pm 2.3541) \mu g \cdot h^{-1} \cdot mL^{-1}$] in RMBSS were also higher than those [AUC = $(2.7566 \pm 0.8232) \mu g \cdot h^{-1} \cdot mL^{-1}$] of healthy rabbits ($P < 0.05$). The K_a $(11.51 \pm 2.82)h^{-1}$, AUC $(0.84 \pm 0.17) \mu g \cdot h^{-1} \cdot mL^{-1}$ of LW & SM-derived TMP in serum were much lower ($P < 0.05$) than those [$K_a = (19.58 \pm 4.14)h^{-1}$, AUC = $(1.27 \pm 0.26) \mu g \cdot h^{-1} \cdot mL^{-1}$] of LW-derived TMP in serum after oral decoctions.

CONCLUSION The SDS and blood stasis syndrome state could affect significantly the pharmacokinetic parameters of drugs and the abnormal SDS pharmacokinetic parameters could be normalized by SJZD. The combination of Chinese medicine in TCR could reciprocally affect the pharmacokinetic parameters of other components absorbed into the systemic circulation. These results support the S and R-PK hypothesis.

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INTRODUCTION

Three progresses in the related subjects arouse us to propose hypotheses defining the traditional Chinese syndrome (S) and recipe PK (S-PK and R-PK)^[1-6]. Firstly, in the field of traditional Chinese medicine (TCM), their characteristic PK has not developed as a consequence of what are the chemical components absorbed into the circulation after administering traditional Chinese recipe (TCR) is not clear yet. Then, the progresses in successful detection of TCR-derived component in serum from 1985 to 1989 supported the concept of above hypothesis^[7,8]. Finally, the theoretical and practical achievements of chronopharmacokinetics are not only an example but also enhanced our intention of presenting the hypothesis^[9-11].

The investigation of TCR-derived compound *in vivo* affords a sound basis for advancing the hypothesis of S and R-PK^[2-8,12,13] and the inspiration from the theory and practice of chronopharmacokinetics greatly encourages us^[9-11]. The knowledge dealing with the rhythmic changes of pharmacokinetic phenomenon in living organisms is called chronopharmacokinetics^[14], in which, PK of administering drug at different time is not constant and is related to its therapeutic and toxic effects^[14]. Similarly, are there possible differences of serum drug concentration and its pharmacokinetic parameters between different TCS and different Chinese medicines of TCR? In other words, does TCS state and the drugs combination in TCR affect significantly the blood drug concentration and their pharmacokinetic parameters after oral administration? We have postulated the above positive differences in the previously published hypotheses^[1-6].

The four applications according to above concepts have been supported by National Natural Science Foundation of China since 1991. Under the support of grants, we choose *Ligusticum wallichii* (LW) prescriptions and its chemical components as the examples to verify the above hypotheses.

MATERIALS AND METHODS

Drugs, chemicals and reagents

Tetramethylpyrazine phosphate (TMPP) intravenous infusion in 2 mL ampule (25 g/L, lot No. 90101) and sodium ferulate (SF) were purchased from Guangdong Limin Pharmaceutical Factory (Shaoguang, China); reserpine injection solution in 1 mL (1mg, lot No. 901008) was purchased from Red-Flag Pharmaceutical Factory of Shanghai Medical University (Shanghai, China).

LW was purchased from Dujiangyan City Pharmaceutical Company (Sichuan, China) which was identified by Professor Hu ZH (the Department of Botany, Northwest University, Xi'an, China). *Salvia miltiorrhiza* (SM), *Panax ginseng* C.A.Mey,

Atractylodes macrocephala Koidz, *Poria cocos* (Schw.) Wolf, *Glycyrrhiza uralensis* Fisch were purchased from Xi'an Pharmaceutical Company (Xi'an, China). All organic reagents were bought from Xi'an Chemical Reagent Factory. All chemicals were of analytical reagent grade unless otherwise stated. Methaqualonum (internal standard) was presented by Institute of Materia Medica, Beijing Medical University (Beijing, China). Coumarin (internal standard) was purchased from Sigma. High molecular weight dextran (HMWD) (M_w500 000) was bought from Tianjin Air Force hospital (Tianjin, China).

Instruments

The following main instruments were used in the experiment: Shimadzu LC-6A HPLC system; SPD-6A ultraviolet detector; shimadzu QP-1000 gas chromatography-mass spectrometer. 7650 infrared spectrometer. RH-90 NMR meter.

Definitions of S and R-PK hypotheses^[1-6]

The S-PK hypothesis indicates that the pharmacokinetic differences between the different TCS have statistical significance and R-PK hypothesis means that one of TCM could influence markedly the pharmacokinetic parameters of other TCM-derived components in blood when administered together in same TCR. The above two pharmacokinetic characteristics are related to the therapeutic, toxic responses and theory of TCM.

The verification of S-PK

The preparation of SJZD (TCR): it consists of *Panax ginseng* C.A.Mey, *Atractylodes macrocephala* Koidz, *Poria cocos* (Schw.) Wolf and *Glycyrrhiza uralensis* Fisch (2:2:2:1). The 7000 g of SJZD drugs were divided into 7 parts. Each part of 1000g was macerated with 7000 mL of distilled water (drug: water=1:7, v/v) at room temperature for 1 h and then boiled for 40 min. The residues were boiled by same volume of water for 40 min once again. Firstly, the two boiled water extracts were mixed, then filtered through several layers of cotton gauze to remove the coarse particles and concentrated by evaporation, repeated preparation of the other parts of SJZD in the same way. The mixture of the seven parts of extraction gives the final concentration of 3 g·mL⁻¹.

Serum TMP and FA PK in healthy and modeled animals were studied to verify the hypotheses. Healthy animals and animal models were divided into five groups and the latter included rat model of spleen deficiency syndrome (RMSDS) treated with SJZD; and rabbit model with blood stasis syndrome (RMBSS). Group 1 (HR-1, *n* = 72): healthy male Wistar rats weighing 240 g ± 20 g afforded from the Experimental Animal Center, the Fourth Military Medical University were injected ip with normal

saline ($0.1 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, 14d). Group 2 (RMSDS, $n = 72$): healthy Wistar rats were injected ip with reserpine ($0.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, 14d). Group 3 (RMSDS treated by SJZD, $n = 72$): healthy Wistar rats were prepared the same as Group 2 and treated with intragastric administration of SJZD ($30 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, 14 d). The above three groups were given *po* $10 \text{ mg}\cdot\text{kg}^{-1}$ of TMPP 24 h after the final injection of normal saline or reserpine. Group 4 (HR-2, $n = 6$): healthy male New Zealand white rabbits afforded from the Animal Center of our university were injected intravenously with normal saline ($15 \text{ mL}\cdot\text{kg}^{-1}$). Group 5 (RMBS, $n = 6$): rabbits were prepared by intravenous injection of 100 g/L HMWD in normal saline ($15 \text{ mL}\cdot\text{kg}^{-1}$). FA was injected intravenously of $5 \text{ mg}\cdot\text{kg}^{-1}$ 30 min after the end of injection of normal saline or HMWD. Rats and rabbits were fasted 12 h before administration.

The 0.5 mL of serum samples of rats were obtained by decapitation after a single oral TMPP at 0.083 h , 0.5 h , 1.0 h , 3 h , 5 h , 8 h , 12 h and 24 h , respectively. At each time point, 6 samples were obtained. The rabbit blood sample of 0.5 mL was directly withdrawn and collected from the ear vein at 2 min , 5 min , 10 min , 20 min , 30 min , 45 min , 60 min , 90 min , 120 min , 150 min and 180 min after intravenous FA. The blood samples were centrifuged ($3000 \text{ r}\cdot\text{min}^{-1}$, 5 min), and 0.2 mL of the resulting serum was used.

Chromatographic condition

Determination of serum TMP concentration^[15-17]: the chromatographic system (Shimadzu LC-6A, Japan) consisted of two pumps (LC-6A), a sample injector (Rheodyne, model 7125), a SPD-6AV detector (Shimadzu) and a C-R3A data processor (Shimadzu). A Shim-Pach CLC-ODS column (particles $5 \mu\text{m}$, $150 \text{ mm} \times 4.6 \text{ mm ID}$) was used for quantitative analysis; AUFs was 0.04 , the flow rate was 1 mL/min , the paper speed was 3 mm/min , column temperature was 38°C , the detection wavelength was 280 nm , the mobile phase consisted of methanol and water ($72:28 \text{ v/v}$). The serum concentration data of HR-1 and RMSDS were analyzed by the 3P87 (Chinese Pharmacological Association) software.

Determination of serum FA concentration^[18]: the detector was set at 320 nm (AUFs: 0.01); the mobile phase was acetonitrile $0.1 \text{ mL}\cdot\text{min}^{-1}$ phosphoric acid ($\text{pH } 2.5$) ($3:7, \text{ v/v}$); the other chromatographic conditions were the same as those in determining serum TMP concentration.

Sample preparation

Serum sample contained TMP: internal standard of Methaqualonum (428 ng) was added to 5 mL of

ground conical centrifuge tube containing $100 \mu\text{L}$ of methanol. The mixture was oscillated on vortex mixer and evaporated to dryness at 48°C on water bath under the stream of nitrogen. Successively added 0.2 mL of blank rat serum, the different amount of TMPP ($0.044 \mu\text{g}\cdot\text{mL}^{-1}$, $0.087 \mu\text{g}\cdot\text{mL}^{-1}$, $0.168 \mu\text{g}\cdot\text{mL}^{-1}$, $0.336 \mu\text{g}\cdot\text{mL}^{-1}$, $0.671 \mu\text{g}\cdot\text{mL}^{-1}$ and $1.342 \mu\text{g}\cdot\text{mL}^{-1}$), $0.2 \text{ mL } 0.05 \text{ mol/L}$ of NaOH solution and 2 mL of trichloromethane to the above centrifugate. After the mixture was vortex mixed again for 15 s , it was centrifuged at $3000 \text{ r}\cdot\text{min}^{-1}$ for 10 min . The organic layer was transferred into another 5 mL ground conical centrifuge tube contained $100 \mu\text{L}$ of 1 mol/L hydrochloric acid-methanol solution (50 mL/L) and evaporated again to dryness on water bath at 48°C under a stream of nitrogen. The residue was dissolved with $100 \mu\text{L}$ of methanol solution and then $20 \mu\text{L}$ was determined by HPLC each time^[15].

Serum sample contained FA: to 0.2 mL of rabbit serum added 0.4 mL of acetonitrile contained $1.5 \mu\text{g}$ of coumarin internal standard. The mixture was vortex mixed and then the deproteinated precipitate was separated by centrifugation ($3000 \text{ r}\cdot\text{min}^{-1}$, 5 min). The supernatant was evaporated at 60°C water bath under a stream of nitrogen. The residue was dissolved in $60 \mu\text{L}$ acetonitrile and $20 \mu\text{L}$ of solution was directly injected into HPLC system for determination. To each 0.2 mL of rabbit blank sera 20 to $800 \mu\text{g}\cdot\text{L}^{-1}$ of FA were added respectively. Their serum samples were analyzed separately by the corresponding method described above^[18].

The verification of R-PK

The preparation of LW decoction, LW&SM (LW: SM=3:1) decoction: the dried roots of them were pounded to about $2 \text{ mm} \times 4 \text{ mm} \times 4 \text{ mm}$ pieces. The other procedure was the same as those for preparing SJZD. The final concentration of LW decoction and LW&SM decoction were $3 \text{ g}\cdot\text{mL}^{-1}$ and $4 \text{ g}\cdot\text{mL}^{-1}$, respectively. TMP content of LW and LW&SM decoctions were determined before administration^[17].

Only healthy rats were used to verify R-PK. Twelve Wistar rats were divided into two groups. LW decoction ($30 \text{ g}\cdot\text{kg}^{-1}$) was given intragastrically to one group ($n = 6$) and LW&SM decoction ($40 \text{ g}\cdot\text{kg}^{-1}$) was given to another group ($n = 6$). The blood samples of 0.2 mL each were obtained by cutting the animal tails at 0.083 h , 0.25 h , 0.50 h , 0.75 h , 1.00 h , 1.50 h , 2.00 h , 3.00 h and 5.00 h after oral TCR.

Analytical method: all quantitative but qualitative detections of TMP were the same as those described above. Chromatographic

conditions: Shim-Pach CLC-ODS column (particles 5 μm , 150 mm \times 4.6 mm ID) was also used for quantitative analysis; the half-preparative column (particles 10 μm , 250 mm \times 10 mm ID) was used for the separation and purification of LW decoction components in serum; Nebulizer and vaporizer temperatures were 250 $^{\circ}\text{C}$. The drift voltage was 70eV.

The identification of LW-derived component in serum^[17]; the related component was firstly separated, purified, enriched and then identified by 3-dimensional HPLC, mass spectrum, and NMR. Calibration curve and statistical analysis were the same as the correspondings described above.

Statistical analysis

The results are expressed as $\bar{x} \pm s$. Comparison of serum drug concentration and pharmacokinetic parameters between controls and studied model of LW and LW&SM decoctions were made by *t* test for paired samples. Differences were considered significant when $P < 0.05$.

RESULTS

The methodological results

One of the chemical components in serum after oral administration of LW extract to rats was identified as TMP by three-dimensional HPLC, UV, IR, MS and NMR (data not shown)^[17].

The chromatographic retention time of TMP, methaqualone (internal standard), FA and coumarin (internal standard) were 3.992 min, 6.223 min, 3.82 min and 7.68 min, respectively (Figure 1, A-B)^[17,18]. They were separated well under their own chromatographic conditions. The ratios between the peak areas of the TMP and methaqualone (internal standard) in serum of rat, and of the FA and coumarin (internal standard) in serum of rabbit were calculated to make the calibration curves and the good linearity over the range (220-6710) $\mu\text{g} \cdot \text{mL}^{-1}$ (TMP, $r = 0.9990$, $n = 6$) and (20-800) $\mu\text{g} \cdot \text{mL}^{-1}$ (FA, $r = 0.9986$, $n = 6$) were obtained. Their equation of the curves were $Y = 0.1889 + 0.0440X$ (TMP) and $Y = 0.5826X + 0.1718$ (FA). If the concentration of FA was above 800 $\mu\text{g} \cdot \text{mL}^{-1}$, the sample was diluted. The detection limit of TMP and FA in sera were 68 $\text{mg} \cdot \text{L}^{-1}$ and 15 $\text{mg} \cdot \text{L}^{-1}$ respectively with a signal-to-noise ratio at 3.

The recoveries of TMP and FA contents from sera of rat and rabbit were determined. Their results are shown in Table 1.

The precision in sera with three levels (0.22 $\mu\text{g} \cdot \text{mL}^{-1}$, 0.8388 $\mu\text{g} \cdot \text{mL}^{-1}$ and 6.7100 $\mu\text{g} \cdot \text{mL}^{-1}$) of TMP and (0.030 $\mu\text{g} \cdot \text{mL}^{-1}$, 0.450 $\mu\text{g} \cdot \text{mL}^{-1}$ and 0.700 $\mu\text{g} \cdot \text{mL}^{-1}$) of FA was detected (Table 2).

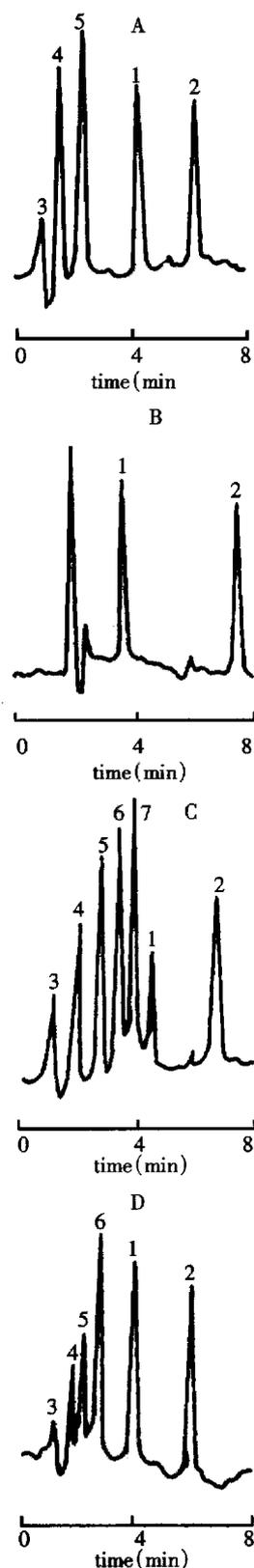


Figure 1 HPLC chromatography of A. TMP (methaqualone, IS) added to rat serum; B. FA (coumarin, IS) in rabbit serum 1. FA (t_R : 3.82 min), 2. coumarin (t_R : 7.68 min); C. serum sample after oral administration of LW&SM extracts in rats; D. sample serum after oral administration of TMPP. 1: TMP (t_R : 3.992 min). 2. coumarin (t_R : 6.233 min). 3. methanol (t_R : 1.017); 4,5: impurities in serum (t_R : 1.725 min and t_R : 2.467 min); 6,7: unknown components (t_R : 3.000 min and t_R : 3.201 min)

Table 1 Recovery of TMP added to rat serum and ferulic acid added to rabbit serum

Drug	Added(mg/L)	Found+SD(mg/L)	Recovery+RSD(%)
TMP	0.2200	0.2126 ± 0.0081	96.64 ± 3.18
TMP	0.8388	0.8294 ± 0.0181	98.99 ± 2.18
TMP	6.7100	0.6657 ± 0.1973	99.34 ± 2.96
FA	0.0300	0.0280 ± 0.0009	95.00 ± 1.60
FA	0.4500	0.43804 ± 0.0043	97.40 ± 1.00
FA	0.7000	0.6883 ± 0.0053	98.30 ± 0.80

Table 2 Precision of tetramethylpyrazine from rat serum and ferulic acid from rabbit serum

Drug	Amount added(mg/L)	Interday cv(%)	Intraday cv(%)
TMP	0.2200	2.45	5.57
TMP	0.8388	1.25	5.67
TMP	6.7100	1.44	4.72
FA	0.030	5.5	6.1
FA	0.450	3.2	4.3
FA	0.700	2.1	3.8

The results of verifying S-PK

RMSDS: Four days after ip injection of reserpine, RMSDS showed decrease in activity, severe muscular hypotonia, palpebral ptosis and diarrhea, which were similar to those reported in our previously paper^[15] and accorded with the published diagnostic standard of spleen deficiency syndrome by Chinese Association of Integrated Traditional and Western Medicine^[19]. In RMSDS, the body weights of rats were reduced by 40% (242 g ± 22 g vs 145 g ± 20 g), and in RMSDS treated with SJZD, they had not shown any significant changes (results not shown).

The above analytical method of TMP was used to study TMP PK following oral TMPP. The pharmacokinetic profiles of oral TMPP in HR-1, RMSDS and SJZD treated RMSDS were the 2-compartment models according to the calculation of serum TMPP concentration-time data (Figure 2), which was in agreement with the results obtained from human and rats^[20,21]. The serum concentrations of TMP in RMSDS were higher than those in HR-1 ($P < 0.01$, Figure 2). The pharmacokinetic parameters of TMPP in RMSDS (Table 3) with the exception of $V/F(C)$ and T peak were significantly different from those in HR-1 ($P < 0.01$); k_a , $T_{1/2\alpha}$, AUC, absorption of TMPP; α , β , $T_{1/2\beta}$, $T_{1/2\alpha}$, CLs, K_{12} , and K_{10} demonstrated that RMSDS decreased the rate of the distribution, transportation and clearance of TMPP ($P < 0.01$). The state of the syndrome in RMSDS affected obviously the absorption, distribution, metabolism and excretion of TMPP.

Table 3 Pharmacokinetic parameters of TMP in serum after oral administration of TMPP (10 mg/kg) to healthy rats and RMSDS with or without SJZD treatment

Parameter	Healthy rat	RMSDS	SJZD
α (h ⁻¹)	2.83 ± 0.70	0.38 ± 0.09 ^b	2.33 ± 0.65
β (h ⁻¹)	0.22 ± 0.02	0.06 ± 0.30 ^b	0.24 ± 0.05
K_a (h ⁻¹)	5.41 ± 1.91	13.61 ± 2.56	5.28 ± 1.39
K_{10} (h ⁻¹)	0.88 ± 0.20	0.24 ± 0.07 ^b	0.82 ± 0.11
K_{12} (h ⁻¹)	1.45 ± 0.47	0.11 ± 0.02 ^b	1.39 ± 0.68
K_{21} (h ⁻¹)	0.72 ± 0.22	0.11 ± 0.02 ^b	0.77 ± 0.18
$t_{1/2\alpha}$ (h)	0.14 ± 0.09	0.05 ± 0.04 ^b	0.16 ± 0.10
$t_{1/2\alpha}$ (h)	0.27 ± 0.11	1.92 ± 0.44 ^b	0.31 ± 0.09
$t_{1/2\beta}$ (h)	3.19 ± 0.39	13.35 ± 5.92 ^b	3.21 ± 0.28
AUC($\mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$)	5.20 ± 2.57	24.88 ± 9.76 ^b	5.11 ± 6.81
T_p (h)	0.30 ± 0.05	0.34 ± 0.03	0.33 ± 0.02
C_{\max} ($\mu\text{g}/\text{mL}$)	2.33 ± 1.17	4.82 ± 1.23 ^b	2.18 ± 1.14
V_{FC} ($\mu\text{g}/\text{mL}$)	2.81 ± 1.30	2.03 ± 0.58	2.19 ± 0.98

^a $P < 0.01$ vs healthy rabbit.

RMBSS: the above method of detecting FA used to study PK in healthy rabbit and FA serum concentration in RMBSS compartment model was fitted and then pharmacokinetic parameters were calculated with a MCPKP program on a COMPAQ 80-386 computer. Compartmental analysis yielded a two-compartment open model. A rapid distribution phase followed by a slower elimination phase was observed. The mean pharmacokinetic parameters are given in Table 4 and serum concentration-time data in Figure 3.

The results of verifying R-PK

The results of detecting TMP concentrations in LW and LW&SM decoctions were 574.50 g·kg⁻¹·L⁻¹ and 463.02 g·kg⁻¹·L⁻¹, respectively.

The chromatograms of TMP and methaqualonum, the latter was added into serum, and then extracted, were the same as shown in Figure 1A, 1B. The serum TMP chromatogram after administration of LW or LW&SM decoctions was different from that of TMP and of IS added to blank serum (Figure 1A) as well as that of post-administrative TMPP injection solution (Figure 1D). There were two more peaks of unknown significance in Figure 1C than that in Figure 1A and one more unknown peak than that in Figure 1D. The serum TCR-derived component parameters of 4 spectra of UV, IR, MS and NMR (data not shown) are similar to that of TMP standard substance and the reference value. Thus it gives an evidence to reveal that this TCR-derived component is undoubtedly TMP. The TMP PK were calculated by 3P87 program (Chinese Pharmacological Association). The fitted figures from two compartment open model and their parameters are indicated in Table 5.

Table 4 Pharmacokinetic parameters of FA in serum after intravenous administration of sodium ferulate (5 mg/kg) in healthy rabbit and rabbit with general blood stasis syndrome ($\bar{x} \pm s, n = 6$)

Parameter	RMBSS	Healthy rabbit
$K_{12}(h^{-1})$	1.01 ± 0.86	1.62 ± 1.18
$K_{21}(h^{-1})$	1.45 ± 0.99	2.47 ± 0.99
$K_{el}(min^{-1})$	4.49 ± 1.10^a	7.37 ± 1.40
$T_{1/2}\alpha(h)$	0.12 ± 0.10	0.07 ± 0.02
$T_{1/2}\beta(h)$	0.62 ± 0.12^b	0.36 ± 0.09
$AUC(\mu g \cdot h \cdot mL^{-1})$	5.67 ± 2.35^b	2.76 ± 0.82
$Cl(L \cdot kg^{-1})$	0.88 ± 0.55	1.81 ± 0.95
$V_{FC}(L/kg)$	0.71 ± 0.03	0.95 ± 0.22

^a $P < 0.05$; ^b $P < 0.01$ vs healthy rabbit. Mean \pm SEM, $n = 6$

Table 5 Pharmacokinetic parameters of TMP in serum after oral administration of LW or LW&SM extracts to rats mean \pm SEM ($n = 6$)

Parameter	LW	LW&SW
$\alpha(h^{-1})$	1.928 ± 0.719	2.328 ± 0.719
$\beta(h^{-1})$	0.479 ± 0.205	0.479 ± 0.289
$K_a(h^{-1})$	19.58 ± 4.139	11.508 ± 2.821^a
$K_{10}(h^{-1})$	1.090 ± 0.502	0.788 ± 0.255
$K_{12}(h^{-1})$	0.470 ± 0.160	0.205 ± 0.092
$T_{1/2}\alpha(h^{-1})$	0.354 ± 0.101	0.298 ± 0.223
$T_{1/2}\beta(h^{-1})$	1.448 ± 0.880	1.447 ± 0.901
$AUC(\mu g \cdot h \cdot mL^{-1})$	1.273 ± 0.255	0.836 ± 0.168^b
$V_{c/r}(L/kg)$	9.665 ± 1.810	7.390 ± 1.089^a

^a $P < 0.05$; ^b $P < 0.01$ vs LW.

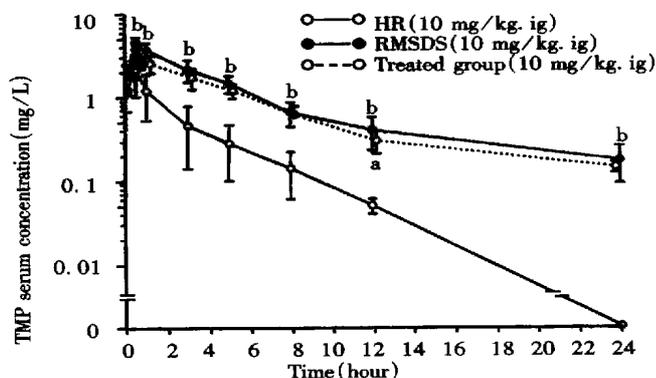


Figure 2 TMP concentration-time curve in serum after oral administration of TMPP in healthy rat ($n = 6$), RMSDS ($n = 6$) and RMSDS treated by SJZD ($n = 6$), ^a $P < 0.05$ vs; ^b $P < 0.01$ vs healthy rat.

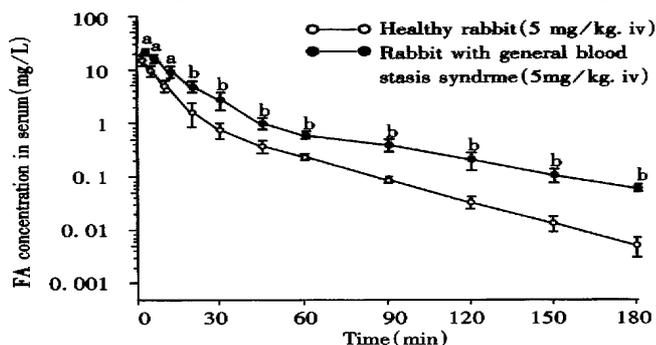


Figure 3 FA concentration-time curve in serum after intravenous administration of sodium ferulate (5 mg/kg). ^a $P < 0.05$; ^b $P < 0.01$ vs healthy rabbit.

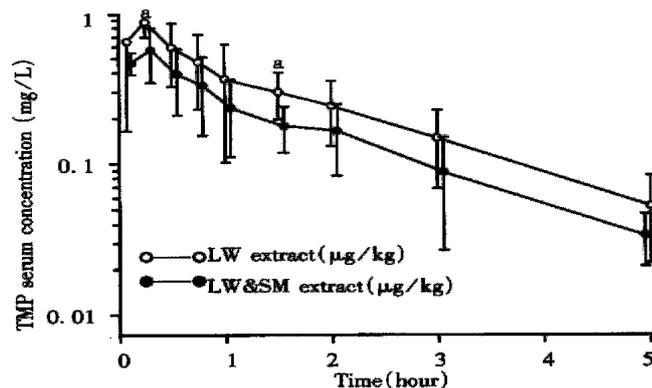


Figure 4 TMP concentration-time curve in serum after oral administration of boiled water extract to rats. ^a $P < 0.05$ vs LW extract.

DISCUSSION

Hypothesis

The concept, terminology, essentials and significance, especially scientific evidences, of S- and R-PK hypothesis were firstly reported and theoretically elaborated in our previous papers^[1-6]. These ideas were exclusively cited^[22-26]. Besides the definition described in above method, we summarized the main elements as follows: ① TCR-derived components *in vivo* are possibly to be detected; ② their number are relatively restricted; ③ they could represent the therapeutic effect of the parent recipe; ④ their concentration and PK could be affected by the combination of TCM in TCR; ⑤ effects of new bioactive components related with those of their parent TCR; and ⑥ the syndrome state could affect their PK significantly. All the ideas of S- and R-PK exhibited the characteristics of combination of TCM theory and PK. The other two published papers only concerned the element 1^[1-6]. So far, over 20 evidences in the available published literatures^[2-8,12,13] supported the element 1.

Methodology

It is necessary to develop scientific analytical method, mainly chromatography, for verifying the hypothesis of S- and R-PK. In general, western drug component (single chemical substance) in blood is only identified by the consistency of retention time of peak in chromatograms as compared with its standard substance derived from the national and international authorized unit^[27-29]. However, this method is not sufficient in analysing the serum TCR-derived component because the coherence of retention time may result from two possibilities, i.e., only a single component peak or an overlap of two or more component peaks according to the theory of chromatography^[17].

We developed the above HPLC method, to determine the serum TMP PK after the administration of TMPP or TCR-LW. When TMPP

solution (Figure 1D) was administered, determination of TMP qualitatively and quantitatively by HPLC is enough corresponding to the method for determining ordinary western drug^[27-29]. As orally administering LW or LW&SM decoctions, the structure of TMP was firstly identified by HPLC in combining with UV, IR, MS and NMR (Figure 1C) and then its concentration and pharmacokinetic value were determined by HPLC. Thus, it is likely to avoid the analytical errors in serum TMP derived from LW or LW & SM in our present experiments^[15-17]. Tables 1 and 2 indicated that these methods for the determinations of serum TMP and FA concentrations were simple, rapid, sensitive, accurate, specific and reduplicable, with high recoveries. All these indexes were sufficient in studying S- and R-PK.

The verification of S-PK

S-PK hypothesis was advanced according to the theories of TCM and PK^[1-6]. To verify this hypothesis, the animal models of spleen deficiency and blood stasis were chosen and their pharmacokinetic characteristics of TMP and FA were studied. One of these models, RMSDS, was induced by injecting reserpine, which had been used as an animal model for "spleen deficiency syndrome" (SDS) in TCM^[30,31] because its physical signs were similar to those seen in the SDS.

The "spleen" digests food, transports, distributes and transforms nutrients and replenishes qi^[32]. We inferred from the above "spleen theory" that the "spleen" should be also responsible for the absorption and disposal of drugs. So, syndrome state of "spleen" is likely to affect the PK. The aim described in the S-PK hypothesis is to demonstrate the pharmacokinetic differences between syndrome and non-syndrome. Our experimental design from S-PK hypothesis is that the poor absorption of drug in SDS state would decrease its blood concentration and bioavailability. On the contrary, Figure 2 and Table 3 show the results of the increases in TMP serum concentration and AUC in RMSDS. Moreover, the parameters of the distribution and elimination of TMP in RMSDS (Table 3) were slowish. The SDS state could markedly affect the absorption, distribution, metabolism and elimination of TMPP in rat, and the observed results from Figure 2 and Table 3 show that SJZD could restore the above abnormal PK of TMPP in SDS model rats. All these TMP pharmacokinetic characteristics in RMSDS and the normalizing effect of SJZD on PK of TMP in RMSDS provided evidences for S-PK.

The mechanism of forming TMP pharmacokinetic characteristics in RMSDS is unknown. It was reported that the gastric and intestinal motility^[33] and the absorptivity of D-xylose^[34] were all decreased in reserpine-induced RMSDS and the patients with SDS. These

observations are paradoxical to the increased serum TMP concentration in RMSDS. This confusion should be settled by further studies. Figure 3 shows that at corresponding time points the FA concentration in RMBSS increased markedly ($P < 0.05$) as compared with normal rabbits. From Table 4 we also found that in RMBSS both the total volume of distribution (V_B) and total elimination rate (CL_B) decreased significantly while FA elimination half-life time ($t_{1/2\beta}$) and AUC increased significantly. This phenomenon may be due to the impairment of microcirculation induced by HMWD infusion. HMWD can lead to abnormal blood rheology (dense, sticky, aggregative, coagulative). So, it is impossible for FA to be metabolized quickly and distributed widely. In conclusion, through this experiment we found a significant difference in the pharmacokinetic parameters between healthy rabbits and RMBSS. This result coincides with S-PK hypothesis^[18].

The verification of R-PK hypothesis

So far, the orthodox academic society has thought that TCR-derived chemical components *in vivo* and their PK are not detectable and enable to study because they or their components are too complicated, trace in quantity or unable to represent curative effect of their parent TCR^[35-38]. Therefore, the article related to above problem is rarely seen in recent years, and resulted in hard to explore the basic pharmacodynamic substance of TCR. The R-PK hypothesis emerged in literature since 1991 holds the idea that TCR-derived chemical components *in vivo* and their PK are possibly to be determined^[1-6]. However, the qualitative and quantitative analysis of LW and LW&SM-derived TMP *in vivo* from Figure 1(C) and its PK from Figure 4 and Table 5 supported the R-PK hypothesis. Similarly, the determinations of over 20 TCR-derived chemical components in blood/urine and their PK^[2-8,12,13,39] in published papers are consistent with the viewpoint of R-PK hypothesis. All these achievements help elucidate the pharmacology of TCM and TCR^[39].

From the results provided in Figure 4 and Table 5 we found that the absorption (K_a), transport (K_{21}) and distribution (V_c/F) of LW&SM-derived TMP in rat serum were decreased significantly compared with those of LW-derived TMP. In Figure 4 and Table 5 it also shows that the concentration and AUC of LW&SM-derived TMP in rat serum were lower than those of LW-derived TMP, which could explain why LW and SM are rarely used alone in a TCR. This viewpoint supported the hypothesis that the combination of drugs in TCR could affect pharmacokinetic parameters of TCR-derived component *in vivo*. It is extremely urgent to explore which component play an important role in the field of interaction of LW or LW&SM derived components *in vivo*.

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Brief Reports

Research on optimal immunization strategies for hepatitis B in different endemic areas in China

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Subject headings hepatitis B vaccine; immunization strategy; cost-benefit analysis; china

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INTRODUCTION

At present hepatitis B vaccine immunization is an unique effective measure for controlling hepatitis B. It is important to determine optimal immunization strategy for controlling HB and to rationally allocate health resources. From the angle of health economics, cost-effective analysis (CBA) is used for the evaluation of economic benefit of the immunization strategies implemented in different endemic areas of HB in China in order to provide the evidences for decision-making and revision of the current HB immunization strategy.

MATERIALS AND METHODS

Basic data

The data for low and medium endemic areas of HB, involving morbidity and mortality of HB and liver cancer, cost of HB vaccine administration, average personal income, GNP, and expenses for medical treatment of patients with acute, chronic hepatitis B and hepatocellular carcinoma, were collected from the medical literature^[1,2]. The corresponding data related to heavy endemic area were obtained from the survey.

Definition of immunization strategies and protective effectiveness

The principal immunization strategies currently

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implemented in China and their effectiveness, which were used as the basis for evaluation, were selected from the related literature^[3,4] for the sake of fair comparison between the different endemic areas. The definition of these strategies is as follows: ① Low-dose immunization strategy is defined as without maternal predelivery HBeAg and HBsAg screening, and infancy vaccination with three or four doses (one dose of booster) of 10 µg plasma-derived hepatitis B vaccine, and yielding a protective effectiveness of 85%. ② Based on maternal predelivery HBeAg and HBsAg screening, high-dose immunization strategy is known as infancy vaccination with one of the following regimens: infant with maternal HBeAg- and HBsAg-negative only receives three doses of 10 µg plasma-derived hepatitis B vaccine; infant with maternal HBeAg and/or HBsAg-positive receives one dose of 30 µg and two doses of 10 µg plasma-derived hepatitis B vaccine; or three doses of 20 µg plasma-derived hepatitis B vaccine and one dose of hepatitis B immune globulin (HBIG); or three doses of 20 µg and one dose of 10 µg (for booster) plasma-derived hepatitis B vaccine. All provided a protective effectiveness of 90%.

Definition of heavy, medium and low endemic areas

The range of HBsAg-positive rate for heavy, medium and low endemic areas of HB is defined as over 11%, from 5% to 10%, and less than 4% respectively. Longan County, Jinan City and Beijing were selected as the representative areas for the heavy, medium and low endemic levels of HB in China, respectively. The HBsAg- positive rate for the whole population and pregnant women was 18.0% and 11.9% in Longan County, 7.59% and 5.40% in Jinan City, and 2.0% and 1.4% in Beijing, respectively. The data collected from these three places were used for CBA.

Study methods

CBA method was used to compare the data of economic benefit between the different places. Comprehensive weighted score analysis (CWSA) was made for determining the optimal immunization strategy. Definition and calculation formula relative to four scales for evaluation of the strategies are as follows: (a) proportion of individuals with HBsAg

carriage prevented among immunized population: HBsAg-positive rate before immunization - HBsAg-positive rate after immunization = HBsAg-positive rate before immunization \times protective effectiveness; (b) net benefit (NB) = total benefit - total cost; (c) benefit cost

ratio (BCR) =

here, the signal B, denotes benefit; C, cost; r , discount rate; t , time; n , lifetime saved. (d) Direct BCR (DBCR) was known as a BCR which only involved the expenses of medical treatment in hospital. The basic principle and procedure of CWSA: First, four kinds of standards, A, B, C, D, used for assessment of every strategy to endemic areas of HB were defined in terms of different weight score (from 0 to 4) of 4 scales. Standard A was designated as comprehensive effectiveness in four scales: 4 scores (very important) to scale I (HBsAg) which denoted the reduced proportion of HBsAg-positive rate between pre- and post-vaccination; 1 score (less important) to scale II (NB); 3 scores (important) to scale III (BCR), and 2 scores (fairly important) to scale IV (DBCR). Standard B: 4 scores to scale HBsAg; 3 scores to scale NB; 2 scores to scale BCR; and 1 score to scale DBCR. Standard C: 4 scores to scale HBsAg; 3 scores to scale NB; 3 scores to scale BCR; and 0 score (not important) to scale DBCR. Standard D: 0 score to scale HBsAg; 4 scores to scale NB; 4 scores to scale BCR; and 3 scores to scale DBCR. Then, the score rank for four scales was calculated according to their corresponding value in each strategy, the maximal score of any scale in all strategies was 10. The calculation of the score rank was that a measured or estimated value of the scale in each strategy was divided by the greatest measured or estimated value of this scale among all strategies being compared, then multiplied by 10. The total comprehensive scores for each strategy was calculated by the following formula: score rank for each strategy \times weighted score for each scale. Finally, based on the same standard, for example, B, the total comprehensive scores for all strategies were compared to screen a strategy with maximal scores. The strategy possessing greatest total comprehensive scores in all standards evaluated was considered as the optimal one.

Data analysis

All data was analyzed with the software of version SAS 6.0 and Excel 5.0.

RESULTS

Benefit from hepatitis B vaccination of different strategies in different endemic areas

Benefit of the low-dose strategy in three places CBA was conducted based on the actual epidemiologic data of Longan County, Shanghai and Jinan City,

and assuming that after the implementation of the infancy low-dose strategy same protective effectiveness of 85% and coverage of 100% could be yielded in these places. The results indicated that the outstanding benefit was obtained for all places in spite of their different economic development level and different endemicity. Longan had a lower value of benefit scales compared with Shanghai and Jinan except the DBCR scale. The greatest value of difference between BCR and DBCR excluding the cost of liver cancer was found in Longan (Table 1).

Table 1 Benefit of HB vaccination of the low-dose strategy for three places with different economic development level and different endemicity

	Place		
	Longan	Shanghai	Jinan
HBsAg-positive rate before vaccination (%)	18.0	10.2	7.6
No. of neonates in 1987	7666	12 000	18 519
Direct benefit*	286	345	562
Indirect benefit*	683	1571	1852
Total benefit*	969	1916	2414
Total cost*	18	22	42
Net benefit*	951	1893	2372
BCR	52.7	85.8	57.9
DBCR	15.5	15.4	13.5
BCR excluding cost of liver cancer	23.6	67.9	34.8
DBCR excluding cost of liver cancer	9.2	14.2	10.1
HBsAg-positive rate after vaccination (%)	3.1	2.2	1.5

*10 000 Yuan RMB.

Comparison of benefit from different strategies in three places Assuming that different immunization strategies had been implemented in each place, the results of CBA for three places showed that the low-dose strategy in Longan and Shanghai would provide the highest values in both scales, BCR and DBCR; the high-dose strategy (30 μ g + 10 μ g \times 2 regimens) in Jinan would yield a slightly higher BCR value compared with the low-dose strategy, 55.35 vs 54.52; and if excluding the influence of difference of economic level between the places (assuming that average personal income for three places was the same i.e. 10 000 in 1998), Longan would have a greater economic benefit, BCR, than Shanghai and Jinan.

Determination of the optimal immunization strategy in different endemic areas of HB

Analysis of sensitivity Based on the assuming parameters involving birth number (10 000), coverage (100%), screening proportion (90%), and sensitivity for screening (90%), the influence of changing endemicity level and strategies on benefit was determined. The results indicated that if the same immunization regimen and strategy was adopted, rank of NB value for every strategy would be seen in order of medium, heavy and low endemic

areas; if BCR of the changing immunization strategies in same endemic area was compared, the low-dose strategy for all endemic areas would yield the greatest BCR and DBCR compared with other strategy, leading to BCR value of 49.91, 54.53 and 37.68 for heavy medium and low endemic area respectively; and DBCR of 14.63, 12.69 and 5.61 for corresponding three endemic areas. No matter which strategy was taken, the greatest difference between DBCR and the BCR excluding liver cancer might be seen certainly in the heavy endemic area; the high-dose strategy would yield the greatest NB compared with other strategy in any endemic area.

Comprehensive weighted score analysis Total comprehensive score for each strategy of different endemic areas were calculated according to four scales of standard, A, B, C, D. The results indicated that when the goal of expectation (representative of standards A, B, C) was to decrease HBsAg-positive rate in general population, the low-dose strategy yielded the highest total comprehensive score. Even through ignoring the decrease of HBsAg-carriage, i.e. only concerning the economic benefit (representative of standard D), the low-dose strategy still yielded the highest total comprehensive score in different endemic areas. But whichever the four standards, the regimen of 10 $\mu\text{g} \times 3$ + HBIG classified into the low-dose strategy always had the least comprehensive score, compared with any regimen of both low and high-dose strategies. The details are shown in Table 2.

Table 2 Comprehensive weighted score analysis of the immunization strategies in different endemic areas

Endemic-area	Immunization strategy	Standard			
		A	B	C	D
Heavy	10 $\mu\text{g} \times 3$	97.3	96.2	96.2	107.9
	10 $\mu\text{g} \times 4$	84.7	88.5	88.5	90.2
	10 $\mu\text{g} \times 3$ + HBIG	83.4	89.9	89.9	86.5
	30 $\mu\text{g} + 10 \mu\text{g} \times 2^*$	93.1	95.9	95.9	100.4
	20 $\mu\text{g} \times 3 + 10 \mu\text{g}^*$	90.6	94.3	94.3	96.8
	20 $\mu\text{g} \times 3 + \text{HBIG}^*$	91.4	94.8	94.8	97.9
Medium	10 $\mu\text{g} \times 3$	97.3	96.2	96.2	107.9
	10 $\mu\text{g} \times 4$	84.7	88.5	88.5	90.2
	10 $\mu\text{g} \times 3$ + HBIG	80.5	88.1	88.1	82.4
	30 $\mu\text{g} + 10 \mu\text{g} \times 2^*$	92.6	95.6	95.6	99.6
	20 $\mu\text{g} \times 3 + 10 \mu\text{g}^*$	90.2	94.1	94.1	96.3
	20 $\mu\text{g} \times 3 + \text{HBIG}^*$	91.2	94.7	94.7	97.7
Low	10 $\mu\text{g} \times 3$	97.3	96.3	96.3	108.0
	10 $\mu\text{g} \times 4$	84.7	88.5	88.5	90.2
	10 $\mu\text{g} \times 3$ + HBIG	78.1	86.6	86.6	79.0
	30 $\mu\text{g} + 10 \mu\text{g} \times 2^*$	90.8	94.5	94.5	97.1
	20 $\mu\text{g} \times 3 + 10 \mu\text{g}^*$	88.3	92.9	92.9	93.6
	20 $\mu\text{g} \times 3 + \text{HBIG}^*$	89.6	93.7	93.7	95.4

*High dose strategy.

DISCUSSION

The results of CBA and sensitivity analysis on the actual data from Longan, Shanghai and Jinan indicated that the low-dose strategy yielded higher NB and BCR in Shanghai and Jinan with higher level of economic development; and DBCR in Longan was slightly higher than that in Shanghai and Jinan after excluding indirect benefit; and the distribution of HBsAg-positive rate in these three places was positively correlated with their DBCR, suggesting that the higher the endemicity level was, the bigger the DBCR obtained; the difference between BCR and BCR excluding expenses of liver cancer in Longan was the greatest compared with that in other two places, and the half of total expenses was used for the treatment of liver cancer in Longan, revealing that a significant economic benefit would be obtained in hyperendemic area of liver cancer assuming that the morbidity of liver cancer could be prevented through the HB vaccination. It is demonstrated from the results mentioned above that the low-dose regimen is an optimal strategy in economic benefit and hepatitis B control for areas with different endemic and economic levels/ However, the strategy, i.e., the maternal HBVMs screening before delivery and high dosage vaccination (30 $\mu\text{g} + 10 \mu\text{g} \times 2$), should be adopted to improve the protective effectiveness of HB vaccine. Also, the results of CWSA used for determining an optimal strategy demonstrated that for any endemicity level the low-dose strategy yielded the maximal comprehensive score compared with other strategies, providing same conclusion with CBA based on the actual data.

It is recommended that in different endemic areas of hepatitis B in China, the high-dose strategy is suitable for the economically developed areas in obtaining better protective effectiveness in decreasing HBsAg-positive rate. However, the low-dose strategy is optimally used for poor rural areas based on its outstanding economic benefit and better protection against HBV.

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Analysis of point mutation in site 1896 of HBV precore and its detection in the tissues and serum of HCC patients

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Subject Headings hepatitis B virus; carcinoma, hepatocellular; precore; polymerase chain reaction; integration; mutation; liver neoplasms

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INTRODUCTION

Hepatitis B is one of the common infectious diseases, which severely impairs the health of the people in our country and has close relationship to the initiation and progression of chronic hepatitis, cirrhosis, and liver cancer. The recent researches indicate that the mutation of HBV precore exists in the patients with these diseases as stated above^[1-5]. According to the recent publications, the mutation of HBV attracts great interests of investigators. The major mutation points in HBV precore are the point in sites 1896 (A1893) and 83 (A83), which are both of G→A point mutations^[6]. Based on the DNA sequence of precore region of HBV, the method of 3'-base specific polymerase chain reaction (3'-BS-PCR) is applied to analyze the 1896 site mutation of HBV^[7] in 126 clinical serum samples and 23 patients' tissues and sera whose tumors have been surgically excised and pathologically diagnosed.

MATERIALS AND METHODS

Reagents and Instruments

Primers were ordered from Life Technology Company, USA; Proteinase K, Taq Polymerase, MgCl₂, PCR reaction buffer ordered from Hua Mei Biotechnology Company; other reagents were of

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analytical purity and made in China. Hema Thermocycler was the product from Zhuhai Hema Medical Instruments Company.

Specimens

The 126 serum samples were collected from outpatients and inpatients of Department of Infectious Disease, the First Affiliated Hospital of Anhui Medical University from November 1996 to August 1997. Seventy-two patients were infected by HBV, and 14 by HAV. Forty were HBsAg negative diagnosed by the method of RPHA. The 23 samples of liver cancer tissues were the surgical specimens from HCC patients in Department of Surgery, the First Affiliated Hospital of Anhui Medical University who were corroborated by pathological diagnosis. The corresponding serum was obtained before operation. These patients are diagnosed according to the diagnostic criteria stipulated by the Sixth National Meeting of Hepatitis, 1990.

DNA extraction from serum

Into 125 μL of the serum to be examined, add 125 μL HBV DNA extraction buffer (200 mmol·L⁻¹ NaCl, 2 mmol·L⁻¹ EDTA, 1% SDS, 100 mmol·L⁻¹ Tris-HCl, pH 8.0) and 6.25 μL Proteinase K (2 g·L⁻¹). Incubate in 37°C water bath for 6 hours. Extract with phenol and chloroform, precipitate the HBV DNA with ethanol, dissolve in 20 μL sterile re-distilled water and store at -20°C.

DNA extraction from the liver cancer tissues

Into the 200 μg HCC tissue, add 500 μL of DNA extract buffer (100 mmol·L⁻¹ NaCl, 1 mmol·L⁻¹ EDTA, 0.5% SDS, 50 mmol·L⁻¹ Tris-HCl, pH 8.0). Homogenize on ice. Add 50 μL of Proteinase K (2 g·L⁻¹). Incubate in 37°C water bath for 6 hours. Extract with phenol and chloroform, precipitate the HBV DNA with ethanol, dissolve in 20 μL sterile re-distilled water and store at -20°C.

PCR

The primers are designed according to the recorded HBV-DNA sequence, the principle of 3'-BS-PCR, and the papers published by Goergen^[6] i.e. 3' primer (GW-1-1d) TCC ACA CTC CAA AAG ACA

(2287-2270), wild type 5' primer (GW-1-1a) GTG CCT TGG GTG GCT TTG (1879-1896) and mutant type 5' primer (GW-1-1b) GTG CCT TGG GTG GCT TTA (1879-1896). Into the 10 μL DNA extracted from serum or tissue diluted into $5\text{ ng} \cdot \mu\text{L}^{-1}$, add PCR reaction buffer [$10\text{ mmol} \cdot \text{L}^{-1}$ Tris, $50\text{ mmol} \cdot \text{L}^{-1}$ KCl, $2\text{ mmol} \cdot \text{L}^{-1}$ MgCl_2 , 0.001% Gelatin, $200\text{ }\mu\text{mol} \cdot \text{L}^{-1}$ dNTPs, $0.5\text{ }\mu\text{mol} \cdot \text{L}^{-1}$ primers (wild type: GW-1-1a+GW-1-1d; mutant type: GW-1-1a+GW-1-1b) and $0.5\text{ }\mu\text{L}$ Taq DNA polymerase ($3\text{ U} \cdot \mu\text{L}^{-1}$)], covered with mineral oil, run PCR: 93°C 1min, 64°C 1min, 72°C 1min, 30 cycles, 72°C 5min. Aspirate $8\text{ }\mu\text{L}$ of PCR products, check the results with 1.5% agarose gel electrophoresis. The positive band is of 408bp.

RESULTS

3'-BS-PCR of HBV DNA

Using the specific oligonucleotides designed according to the DNA sequences of mutant and wild types the results showed that the amplified specific DNA of wild and mutant types of HBV in precode C were both of 408 bp. Figure 1 shows the PCR results of the DNA extracted from 126 patients' serum obtained at different stages, which demonstrates that constant results can be achieved when repeated. The PCR results of the 14 patients infected by HAV were all negative.

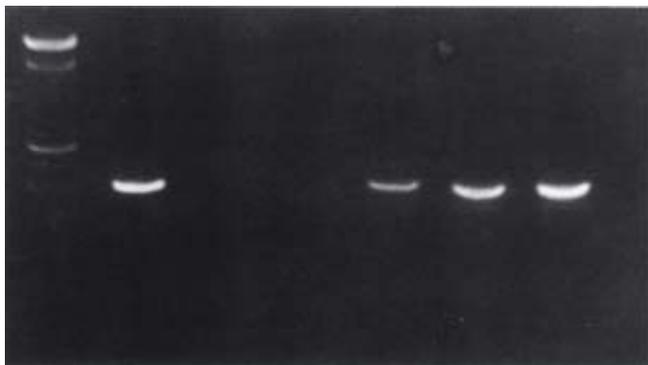


Figure 1 PCR results of HCC patients' point mutation in site 1896 of HBV precore.

1. 100bp DNA Marker; 2. positive control; 3. negative control; 4, 7. sample numbers

Analysis of the 126 patients' point mutation in site 1896 of HBV precore

Of the 72 patients with chronic hepatitis examined with the 3'-BS-PCR of HBV DNA, 58 were positive in wild type, 13 were positive in mutant type only, 21 were positive in both types. The positive rate of mutant type was 47.2% (34/72); the positive rate of mutant type only was 18.1%, the positive rate of both types was 29.1% (21/72). Neither of these types were detected in the 40 patients with negative HBsAg.

Detection of HBV precore gene in the 23 HCC patients (Table 1).

Table 1 The HBV precore and its mutant type detection in 23 HCC Patients n (%)

	Total	HBV precore positive	HBV precore negative	HBV precore positive		
				Mutant type	Wild type	Mixed type
Tissue	23(100%)	12(52.2)	6(26.1)	5(21.7)	12(52.2)	5(21.7)
Serum	23(100%)	7(30.4)	16(69.6)	6(26.1)	4(17.4)	3(13.0)

The positive rate of the 23 HCC patients' surgically excised tissues and pre-operational serum was 52.2% (12/23) and 30.4% (7/23) respectively. In order to exclude the possibility of contamination, negative controls and positive controls were performed each time.

DISCUSSION

The establishment of 3'-BS-PCR method for the analysis of point mutation in site 1896 of HBV precore

The mutation of HBV may have a close relationship to the continuous HBV infection and the deterioration of liver after the infection. The research on the mutation of HBV will greatly promote the clinical analysis of HBV infection and its subsequent diseases. The common methods to detect point mutation are DNA sequencing and SSCP, which are expensive, time-consuming and unsuitable for clinical practice. In order to fulfill the requirements of further researches on HBV mutation and its application to the clinic, we established the 3'-BS-PCR method for the analysis of point mutation in site 1896 of HBV precore.

According to the principle of primer design that only when the last base of primer's 3' end must strictly match its corresponding template could PCR be accomplished, the reports of Georgen^[6] and the phenomenon that the nucleotide of site 1896 of HBV precore mutates from G to A, the corresponding 5' primers of wild and mutant types whose 3' terminal base are G and A respectively were designed to amplify the wild or mutant type DNA of the HBV precore region. Georgen^[6] has successfully applied this kind of method to detect the point mutation of site 1896 in the HBV precore in the patients with positive anti-HBe results, which have been confirmed by DNA sequencing. The positive rates of this site's point mutation were slightly higher than the other reports^[9]. It may be because most patients investigated were inpatients from the Department of Infectious Diseases whose liver damages were more severe than those from outpatients. This explanation is supported by the recorded reports that the positive rates of mutant type are unanimous with the conditions of the liver

damages. The establishment of this method provides a new approach to study the HBV infection and analyze the liver damage.

The study on the integration of HBV precore gene in HCC

Many researches have shown that the infection of HBV has a close relationship with the carcinogenesis of liver cancer and is one of important factors inducing the liver cancer. With the research progresses in molecular oncology, two kinds of genes, oncogenes and anti-oncogenes, that have close relationship to carcinogenesis have been discovered. In the development of HCC, the activation of oncogene and the inactivation of anti-oncogene are frequent events, which cause the changes of the qualities or quantities of the important proteins that they are encoded and eventually lead to the carcinogenesis of normal cells. The integration of HBV DNA in the genome of the cells infected by the HBV is an important factor that brings the instability of chromosomes in liver cells^[8].

Therefore, it is meaningful to investigate the HBV-DNA fragments' integration and its effect on the activation of oncogene and inactivation of anti-oncogene. Our research shows that 12 of 23 primary HCC patients were found to have the HBV precore in the cancer tissues whose positive rate was 52.2%. Among these patients, the HBV precore DNA were detected in 7 patients' sera whose positive rate was 30.4%. The results indicate that the HBV precore gene widely exists in liver cancer tissues and the replication of HBV is accompanied by the development of HCC. HCC patients (21.8%,

5/23) had negative results in serum but positive results in cancer tissues, which indicates that these patients had HBV precore DNA fragments integrated in their liver cells and there was no virus replication. We plan to study whether there is an integration in the genome of liver cells using Southern Blot. Among these HBV precore positive patients, the patients with mutant type detected in the tissues were all wild type. Only half of the patients with mutant type detected in the serum were also wild type. A hypothesis could be made that the HBV precore may mutate during long host in the liver cell and may play an important role in the transformation of normal liver cells.

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Orthotopic liver transplantation for fulminant hepatitis B

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Subject headings hepatitis B; liver transplantation; lamivudine

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INTRODUCTION

When fulminant hepatitis progresses to deep encephalopathy, with stage III or IV coma, it is commonly irreversible with a high mortality rate (80%-100%)^[1]. Liver transplantation may be the sole treatment of choice in such circumstances. However, the early results have been disappointing, largely due to the frequency of posttransplant HBV infection^[2,3]. This recurrent disease often develops in an aggressive manner characterized by high levels of HBV replication and rapidly progressive liver graft damage. In recent years, however, as new antiviral agents, such as lamivudine came into being, the patient's survival has much been improved. The purpose of this study is to assess the effect of OLTx in a series of 7 patients with fulminant hepatitis B, and to evaluate the efficiency of lamivudine on preventing patients from HBV reinfection.

MATERIALS AND METHODS

Between October 1993 and August 1999, 7 adult male patients with hepatitis B were referred for liver transplantation. Their age at the time of operation ranged from 32 years to 49 years (median age 42.9 years). All patients were positive for HBV surface antigen (HBsAg), and 2 had evidence of viral replication. The latter was demonstrated by positive hepatitis B e antigen or/and HBV-DNA. Patient profiles are shown in Table 1.

All patients received orthotopic liver

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transplantation 1 to 10 days after admission. Initial immunosuppression with cyclosporine A (CsA), and methylprednisolone was used in all cases. In the latter 3 cases, tacrolimus (FK506) was given orally when the patients resumed oral intake. Doses were adjusted in the first month to maintain 12 h trough levels of 10 g/L to 15 g/L. Prophylaxis for cytomegalovirus infection was started immediately after surgery with acyclovir or ganciclovir for 2 weeks. Lamivudine was administered orally, 100 mg or 150 mg daily in 5 patients when it became commercially available, and the treatment was not interrupted in any patient.

RESULTS

All patients in this series recovered consciousness 24 h after OLTx. Of them, five recipients were still alive and well with normal liver function during a following up period of 3-7 months. One patient with grade III hepatic encephalopathy died due to HBV reinfection 36 days after liver transplant. Another patient with preoperative esophageal varicosis bleeding died of multi-organ failure 3 days after transplantation. Various medical and surgical complications occurred in two of the five survived patients. One developed recurrent onset of epilepsy necessitated continuous antiepileptic therapy. Another developed stenosis of inferior vena cava, which was successfully treated by balloon dilation and stent replacement.

No acute or chronic graft rejection occurred in any patient. In patient 1, viral replication disappeared within 1 month following OLTx and became positive thereafter. The other 6 patients had a negative HBsAg within 3 to 14 days after OLTx and all had a negative viral replication. No side effects were noted concerning lamivudine treatment.

DISCUSSION

Hepatitis B is a common disease in mainland China. It was estimated that HBsAg carriers accounted for 10% of the total population. However, the natural history of hepatitis B can be quite variable^[4]. Even once signs of portal hypertension develop, many patients can go years before complications develop that impair the quality of their lives. By contrast, others can have stable liver disease for quite some time and then deteriorate quickly, often related with bleeding or infection. Thus, it is difficult to make accurate decisions about the timing of liver transplantation. In general, once the patient's liver

disease begins to impair quality of life, the physician should consider an evaluation for transplantation. Based on this understanding, fulminant or sub-fulminant hepatitis is justifiable for liver transplantation. According to the experience of King's College Hospital in London, the selection criteria for transplantation in hepatitis B are based on the occurrence of three of the followings: a prothrombin time greater than 50 seconds; a jaundice to encephalopathy time of more than 7 days; non-A, non B hepatitis or drug-induced hepatitis; age younger than 10 years or older than 40 years; bilirubin greater than 300 $\mu\text{mol/L}$; or the finding of a prothrombin time of greater than 100 seconds in isolation^[5]. In the present series, all 7 patients met the above mentioned criteria. The overall survival rate of our series was 71.4% after a 3-7 month follow-up period. The mortality rate of those patients with fulminant hepatitis B was 29.6%. Of the two patients who died, one died 5 weeks following OLTx of recurrent fulminant hepatitis B, indicating the high risk of HBV reinfection when the serum HBeAg was positive. The other patient died of multi-system organ failure on postoperative day 3. Nevertheless, the overall outcomes were to our satisfaction and support the continued application of liver transplantation as a therapeutic measure for fulminant hepatitis B.

It has been found that nearly 100% of patients showing evidence of active viral replication, i.e. serum HBV-DNA or/and HBeAg positive, and 70%-80% of those with HBV DNA or HBeAg negative prior to OLTx and received no immunoprophylaxis, developed recurrent HBV infection of the graft following OLTx^[6]. The recurrent disease may manifest as rapidly progressive disease. In our series, patient 1

developed fulminant hepatic failure 5 weeks following OLTx. Interferon (IFN) has been used both prophylactically and therapeutically after OLTx in patients with recurrent HBV, but it has proved to be mostly ineffective and may lead to further complications^[7]. In recent years, perioperative administration of hepatitis B immunoglobulin (HBIg) has been reported to reduce the incidence of recurrence. But, it requires prolonged parenteral treatment and does not suppress viral replication. Additionally, availability of HBIg could be limited and the costs related to prolonged use of HBIg are very high^[8]. These disadvantages of HBIg prevent it from wide clinical use. Because high level viral replication seems to be important in the pathogenesis of HBV recurrence, there has been great interest in the potential role of nucleoside analogue with anti-HBV activity. The most promising are lamivudine and famciclovir. Both agents have been evaluated in both animal model of HBV and clinical trial and have been shown to rapidly suppress viral replication. Lamivudine prophylaxis after liver transplantation resulted in a complete and sustained suppression of viral replication in OLTx recipients^[9]. In our study, 5 patients received lamivudine treatment, no side effect has been identified, and the treatment was not interrupted in any patient. The doses of 100 mg daily suppressed HBV-DNA to undetectable levels in one patient. Serum HBsAg became negative and HBeAb became positive after transplantation in all the patients. No cases of HBV reinfection were noted in lamivudine-treated patients. The results indicated that lamivudine is a beneficial and well-tolerated therapy in OLTx with HBV infection. The long-term effects of lamivudine are being investigated.

Table 1 Details and results of 7 patients underwent OLTx for fulminant hepatitis B

Patients No.	Age/Sex	Medical history	Bilirubin ($\mu\text{mol/L}$)	Encephalopathy	ABO compatibility	Lamivudine regimen	Outcome	Cause of death
1	32/male		478.0	III	Identical	(-)	Died	HBV reinfection
2	44/male	Biliary surgery $\times 2$	756.1	I	Identical	(+)	Alive	
3	49/male	Nasopharyngeal carcinoma	141.3	I	Identical	(-)	Alive	
4	48/male	Epilepsy due to brain trauma, PE $\times 3$	787.1	II	Identical	(+)	Alive	
5	48/male	Hepato renal syndrome duodenal ulcer, PE $\times 4$	500.7	IV	Identical	(+)	Alive	
6	44/male	Esophageal varicosis bleeding, PE $\times 2$	937.5	III	Compatible	(+)	Died	Massive bleeding and multi-organ failure
7	35/male	Tuberculosis, PE $\times 5$	432.6	I	Incompatible	(+)	Alive	

PE: plasma exchange

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Dot immunogold filtration assay for rapid detection of anti-HAV IgM in Chinese

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Subject headings dot immunogold filtration assay; hepatitis A virus; immunoglobulin M/analysis

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INTRODUCTION

The hepatitis A virus specific immunoglobulin M(IgM) antibody is a specific serological marker for early diagnosis of hepatitis A. At present, the methods used at home or abroad for detecting anti-HAV IgM are RIA, ELISA and SPHA. The dot immunogold combination assay that has been developed since 1989 is a new technique with the property of simple and rapid immunological detection, by using the red colloidal gold particles to label the antibodies as indicator, and the millipore filtering membrane coated with antigen as the carrier. Affected by filtration and condensation, the antigen antibody reaction is enabled to go on rapidly. When the reaction is positive, red dots appear on the membrane. It takes about 2 min to 4 min for the whole reaction to be carried out. With the above technique, we have established the dot immunogold filtration assay (DIGFA) for rapid detection of anti-HAV IgM with comparatively satisfactory results.

MATERIALS AND METHODS

Materials

The hepatitis A virus antigen (HAAG) was the cell-cultured antigen, some of which were purchased from the Reagent Factory of Chinese PLA 302 Hospital and the rest was prepared by our institute. The anti-human μ chain monoclonal antibody was purchased from the teaching and research group for immunology of our university. The sheep anti-human IgM antibody was purchased from the immunological room of Chinese PLA 302 Hospital. The chloroauric acid was the product of the Chendu Chemical Plant with the batch number of 93082. Part of the serum samples from hepatitis A patients was supplied by the Department of Epidemiology

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and the rest was collected from the Xi'an Municipal Children Hospital and the Railroad Central Hospital with diagnosis in accordance with the standards revised by the Shanghai Conference held in 1990. The sera of the patients suffering from epidemic hemorrhagic fever were supplied by Professor Sun of the Department of Epidemiology. The remaining serum samples were obtained from the Xijing Hospital. The ELISA kits for anti-HAV IgM detection were purchased from the Nanjing Military Medical Research Institute.

Methods

Principle The serum to be tested was put on the millipore membrane previously coated with HAAG. If there was anti-HAV IgM, the HAAG-anti-HAV IgM colloidal gold complex was formed on the membrane as red dots which were visible to the naked eyes.

Preparation of colloidal gold It was prepared according to the methods by Dar *et al*^[1]. Fifty mL of 0.2 g/L chloroauric acid was heated to the boiling point with 1.2 mL of the 10 g/L sodium citrate added later. The boiling lasted 5 min. The preparation was well done and finished when it became dark red in color.

Anti-human μ chain antibody colloidal gold labelling It was prepared according to reference^[2] with the main procedures as follows: Using 0.1 mol/L K_2CO_3 , 1 mL colloidal gold was regulated to have the pH of 8.0 or 9.0. With the help of magnetic stirring, the F(ab')₂ anti-human μ chain monoclonal antibody or sheep anti-human IgM was added. After 10 min, the bovine serum albumin (BSA) was added to get the concentration of 10 g/L. After that, the mixture was centrifuged at 2 500 \times g for 5 min. The supernatant was further centrifuged at 12 000 \times g for 20 min. The supernatant was discarded and the precipitate was dissolved by 5 g/L BSA-PBS, thus forming the colloidal gold labelling reagent.

Millipore filtering membrane treatment and antigen immobilization The nitrocellulose membrane with millipore diameter of 0.65 μ m produced by the attached factory of the Beijing Chemical School was soaked by triple-distilled water and then dried spontaneously. The disc, 1 cm in diameter was made from the prepared membrane with a punch, was soaked in 0.05 mol/L carbonate buffer and then dried in air. One μ L HAAG solution was dripped onto the center of the disc. After dried at room temperature, the disc was enclosed with 5 g/L BSA, then rinsed with the PBS-T twice for 10 min each time. After being dried, it was put into the self-made immune filtration plate.

Testing methods The immune filtration plate was numbered with the corresponding serum numbers, to the center of the membrane, dripped a drop of 0.01 mol/L PBS-T to activate the surface of the membrane. After the PBS-T was filtered into the membrane, 10 μ L of the serum was dripped slowly to the center of the membrane. Then, the membrane center was flushed by 2-3 drops of washing solution. After that, 30 μ L of the colloidal gold labelling reagent was added. After the latter was filtered into the membrane, the center was flushed with 2-3 drops of washing solution. Red dots in the center denote positive results, while colorless means negative.

Blocking test A: Ten μ L of anti-HAV IgM positive serum was added to 10 μ L of anti-human IgM working solution. B: Ten μ L of the positive serum was added to 20 μ L of the HAAG original solution and mixed evenly. The solution was kept in the water bath at 37°C for 1 h. The sera treated in both ways were put on the membrane. The DIGFA was made following the steps depicted above.

2-ME destruction tests Ten μ L of 0.2 mL/L β -mercaptoethanol (2-ME) was added to 10 μ L of anti-HAV IgM positive serum and mixed evenly. The solution was kept in water bath at 37°C for 1 h. The treated sera were used to do the tests of DIGFA as described above.

ELISA tests The tests were performed according to the operative instructions in a strict way.

RESULTS

Comparison between DIGFA and ELISA Two hundred and seventy nine serum samples were tested in a contrast way with the DIGFA and ELISA. The result was that 148 samples were positive and 125 samples negative with both methods. If the ELISA was used as the reference standard, the specificity of the DIGFA was 98.4% and the sensitivity 97.3%. The coincidental rate of both methods was 97.8%.

Blocking tests and 2-ME destruction tests Ten samples of anti-HAV IgM positive sera tested by the DIGFA and ELISA were chosen at random, all changed to negative after undergoing the blocking affect. Besides, the 10 serum samples which were treated by the 2-ME also changed to negative. This testifies that what was detected by the DIGFA was surely the anti-HAV IgM.

Results of detection on non-hepatitis A sera Forty serum samples from epidemic hemorrhagic fever patients, 10 serum samples with positive anti-HBc IgM and 41 serum samples from the blood donors were all negative when tested by the DIGFA.

Rheumatoid factor interference tests Twenty samples with positive rheumatoid factor (RF) were all shown negative results by DIGFA.

Repetitive tests Ten anti-HAV IgM positive samples and 10 negative samples which were chosen at random were tested repeatedly for five time. They all showed the identical results.

DISCUSSION

The dot immunogold test is a new immunological technique which has been developed in recent years^[1,3,4]. Since the millipore filtering membrane not only absorbs protein, but also affords rapid filtration and acts as capillaries, the antigen or antibody in serum is able to combine rapidly with the counterpart on the membrane. Moreover, as the colloidal gold labelling reagent is red in color, red dots appear after the combination takes place. Therefore, no color developing reagent is needed. This method that has aroused our interest greatly not only keeps with the sensitivity and specificity of the ELISA and RIA, but also with the advantage of affording prompt result.

In China, to detect Anti-HAV IgM, the ELISA and RIA are mainly used^[5,6], but the successful employment of the solid-phase immunoabsorption hemagglutination inhibition test^[7] has been reported. However, the drawbacks of ELISA and RIA lie on their requirement of prolonged operation time, complicated procedures and instruments, and some reagents having carcinogenic or radionuclide effects may be harmful to the handlers or polluted the environment if not properly disposed. Furthermore, the activity of HAAG is unstable and may be likely influenced by temperature, so it is hard to obtain a reagent kits with reliable efficiency. As for the solid-phase immunoabsorption hemagglutination inhibition test, the operation time is also long and no kit is available. The advantages in using DIGFA to test the Anti-HAV IgM are as follows: The operation period is shortened from a few hours to 5 min and the results are reliable and visible to the naked eye. The specificity and sensitivity are approximately equal to those of the ELISA and not influenced by RF. The HAAG from cultured cells is coated on the nitrocellulose millipore filtering membrane in a solid phase, with durable activity; the colloidal gold labelling reagent can be preserved beyond one year; the manipulations are simple and no sophisticated testing instrument required; the operator can be trained in a simple way, and may become acquainted with whole operation technique in a short time.

Therefore DIGFA is an ideal method utilized in the early diagnosis and the epidemiological study of hepatitis A.

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Protective actions of salvianolic acid A on hepatocyte injured by peroxidation *in vitro*

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Subject headings Salvianolic acid A; hepatocyte; carbon tetrachloride; liver injury; lipid peroxidation; water soluble vitamin E; ALT; AST; superoxide dismutase; malondiadehyde; catalase; lactase dehydrogenase; glutathione peroxidase; glutathione

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INTRODUCTION

Salvianolic radix, one of the most commonly used traditional Chinese herbs, was widely studied about its actions against liver injury and fibrosis, and was one of the focuses of recent research^[1,2]. Salvianolic acid-A (SA-A) was an aqueous soluble component of *Salvianolic radix*. In our previous work^[2], SA-A was found to have protective effects against liver injury and fibrosis induced by carbon tetrachloride (CCl₄) in rats. In order to investigate the effect of SA-A on peroxidation in hepatocytes, we induced the injured hepatocyte model by CCl₄ fumigation *in vitro*, treated the cell model with SA-A or aqueous soluble vitamin E (Vitamin E), the latter served as the control drug, and observed the influences of the drugs on the functions of the hepatocytes injured by peroxidation.

MATERIALS AND METHODS

Animals

Wistar rats, male, specific pathogens free (SPF), weighing 140 g-160 g, were provided by the Experimental Center of Animals, Shanghai University of Traditional Chinese Medicine.

Drug

SA-A, molecular formula C₂₆H₂₂O₁₀, molecular structure as Figure 1, weight 494, was extracted and

identified by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Vitamin E was purchased from Hoffman Co. USA.

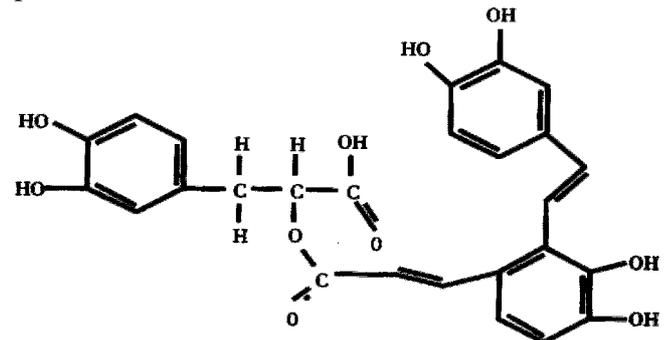


Figure 1 SA-A molecular structure.

Main reagents

Purified type III collagenase (specific activity, 960U/mg), insulin and dexamethasone were purchased from Sigma Co. USA. Soybean trypsin was from Institute of Biochemistry, Chinese Academy of Science; ficoll was from Shanghai Second Chemical Reagent Factory; medium 199 (M199) from Gibco Co., USA; carbon tetrachloride (CCl₄), analytical grade, from Yixin Third Chemical Reagent Factory, Jiangsu Province; and newborn bovine serum (NBS) from Shanghai Sino-American Co.

Isolation and culture of hepatocytes

According to a modified method^[3], hepatocytes were isolated and primarily cultured from rats. In brief, after anesthesia with ether, the rat liver was perfused *in situ* with Ca²⁺ and Mg²⁺- free Hank's solution via the portal vein for 5 min, followed by perfusion with Hank's solution containing 0.5 g/L collagenase for 20 min. The liver was then excised and minced with forceps to remove Glisson's capsule and the liver cells dispersed. The liver cell suspension was filtrated with double layers of gauze and was adjusted to 2 × 10¹⁰/L. Then 5 mL cell suspension was added onto the top of 20 mL 492 g/L Ficoll, and centrifuged at 50 × g, 4°C, 5 min to purify the hepatocytes. The cell recovery was about 1 × 10¹⁰ cells per liver, the purity was more than 95% identified by the cell typical appearance via phase contrast microscope, and the viability was more than 90% assessed by trypan blue exclusion. Hepatocytes were suspended with M199

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containing 5% (v/v) NBS to adjust their density to 5×10^8 cells/L, seeded on plastic dishes (Nunc, Denmark) and primarily cultured at 37°C in a humidified atmosphere of 50 mL/L CO₂ and 950 mL/L air.

Induction of hepatocyte peroxidative injured model

According to David method^[4], the peroxidatic injury of hepatocytes was induced by CCl₄ fumigation. Briefly, after 48 h of isolation and culture, the cells were placed in sealed box, to which 1 mL/L CCl₄ was added, and the cells were fumigated with CCl₄ at 37°C for 24 h.

Drug treatment

Normal hepatocytes in dishes were divided into the following groups: the normal, the control, vitamin E (2×10^{-3} mol/L^[5]) and SA-A treated groups at different concentrations (10^{-4} mol/L- 10^{-8} mol/L). All but the normal group, were incubated with vitamin E or SA-A at different concentrations and fumigated with CCl₄ spontaneously for 24 h, then the culture medium was collected respectively and stored at -70°C until assay.

Biochemical index assay

The ALT and AST in culture medium were assayed with Riman and Frankle method, malondiadehyde (MDA) with Bacon's method^[6]. Superoxide dismutase (SOD), catalase (CAT), lactase dehydrogenase (LDH), glutathione peroxidase (GSH-PX), glutathione (GSH) were measured following the protocols provided by the manufacturer (Jianchueng Biochemical Technological Institute, Nanjing).

Statistics

Chi-square test and *q* test.

RESULTS

The cell morphological changes

After 48 h of isolation and culture, primary hepatocytes gathered, attached and grew very well. At 24 h after fumigation with CCl₄, the hepatocytes partially shrank, their plasma membrane became rough and organelles swollen. When the cell models were incubated with SA-A or Vitamin E, the plasma membrane became smoother and the organelles less smother than those of controlled model cells.

Effects of SA-A or Vitamin E on ALT, AST and LDH activity in hepatocytes injured by peroxidation

The activities of ALT and AST both increased, while the former increase more obviously. LDH activity enhanced approximately 20 folds. SA-A inhibited these pathological increase in dosage

dependent manner, and among all concentrations tested the 10^{-4} mol/L SA-A had the best effect on the cell structure and enzymes. The effect of SA-A was better than that of Vitamin E, but 10^{-7} mol/L- 10^{-8} mol/L SA-A was not effective compared with the control (Table 1).

Effects of SA-A or Vitamin E on MDA contents and the activities of SOD and CAT in hepatocytes injured by peroxidation

MDA content in the control nearly doubled that of the normal, and the activities of SOD and CAT increased remarkably. SA-A decreased these pathological changes, and 10^{-4} mol/L SA-A had a significant inhibitory action. Vitamin E also decreased the MDA content markedly, but had no obvious influence on SOD activity (Table 2).

Effects of SA-A or Vitamin E on GSH content and GSH-PX activity in hepatocytes injured by peroxidation

After the hepatocytes were injured by peroxidation, the GSH-PX activity increased but GSH content decreased remarkably. 10^{-4} mol/L SA-A or Vitamin E inhibited the increase of GSH-PX activity and the decrease of GSH. For the extent of inhibition in GSH lowering, SA-A 10^{-4} mol/L is superior to Vitamin E (Table 2).

Table 1 Effects of SA-A on ALT, AST and LDH activities in hepatocytes injured for 24 h (*n* = 6, $\bar{x} \pm s$)

Group	ALT(U·L ⁻¹)	AST(U·L ⁻¹)	LDH(U·L ⁻¹)
Normal	18 ± 2.8 ^b	56 ± 3.8 ^b	77 ± 38 ^b
Control	103 ± 6.5	176 ± 9.1	1674 ± 128
10^{-4} mol·L ⁻¹ SA-A	49 ± 2.9 ^{bd}	134 ± 5.0 ^{bd}	1050 ± 83 ^{bd}
10^{-5} mol/L SA-A	72 ± 3.9 ^{bd}	177 ± 8.3	1551 ± 88
10^{-6} mol/L SA-A	91 ± 11.1 ^a	177 ± 9.2	1602 ± 88
10^{-7} mol/L SA-A	96 ± 7.9	181 ± 6.7	1657 ± 81
10^{-8} mol/L SA-A	93 ± 11.4	181 ± 10.7	1684 ± 71
Vitamin E	86 ± 7.6 ^b	182 ± 10.7	1509 ± 30 ^a

^a*P*<0.05; ^b*P*<0.01, vs Control; ^d*P*<0.01 vs Vitamin E.

Table 2 Effects of SA-A on the contents of MDA and GSH and the activities of SOD, CAT and GSH-PX in hepatocytes injured by peroxidation (*n* = 6, $\bar{x} \pm s$)

Group	MDA(μmol·L ⁻¹)	SOD(U·L ⁻¹)	CAT(U·L ⁻¹)	GSH(U·L ⁻¹)	GSH-PX(U·L ⁻¹)
Normal	5.11 ± 0.91 ^b	30.4 ± 2.86 ^b	12.8 ± 3.45 ^b	1.27 ± 0.13 ^b	16.7 ± 8.84 ^b
Control	9.17 ± 0.80	59.0 ± 2.23	86.6 ± 13.00	0.36 ± 0.07	90.9 ± 11.00
10^{-3} mol/L SA-A	6.79 ± 0.81 ^a	45.6 ± 3.26 ^{bd}	17.3 ± 3.59 ^{bd}	0.95 ± 0.02 ^{bd}	65.2 ± 1.24 ^a
10^{-4} mol/L SA-A	7.67 ± 1.11	55.2 ± 2.44	38.3 ± 11.82 ^b	0.40 ± 0.04	81.8 ± 17.54
10^{-5} mol/L SA-A	8.27 ± 1.50	57.6 ± 3.27	49.9 ± 6.78 ^b	0.38 ± 0.10	84.1 ± 19.11
Vitamin E	7.52 ± 0.69 ^a	55.8 ± 4.03	27.6 ± 3.22 ^b	0.49 ± 0.07 ^a	63.64 ± 10.57 ^a

^a*P*<0.05, ^b*P*<0.01, vs Control; ^d*P*<0.01 vs Vitamin E.

DISCUSSION

In this study, 24 h after fumigation of hepatocytes with CCl₄, the ALT, AST and LDH all increased remarkably, the rate of elevation was in order of LDH, ALT and AST. It is suggested that the

hepatocytes were acutely injured, cell membrane integrity was broken and the enzymes in cell plasma leaked out. However, after the hepatocytes injured by peroxidation which were incubated with SA-A, the pathological increases of ALT, AST and LDH reduced markedly. It is indicated that SA-A had a potential effect against hepatocyte injury.

The free radicals and its triggered lipid peroxidation were involved in the main mechanisms by which carbon tetrachloride injured hepatocytes. MDA was one of main lipid peroxidatic products, its elevated levels could reflect the degrees of lipid peroxidatic injury in hepatocytes. GSH, a peroxide scavenger with a lower molecular weight, could eliminate superoxide anion and hydrogen peroxide. The content of GSH reflected the ability against peroxidation^[7]. In this study, GSH in hepatocytes of the model group was reduced remarkably, suggesting that the potency of antioxidation in injured cells was decreased. There were many other markers that could reflect lipid peroxidation, e.g. SOD, a scavenger of peroxide anion radicals, which could inhibit the initiation of lipid peroxidation by free radicals; GSH-PX, which could particularly catalyze the reductive action of GSH to H₂O₂ to protect the integrity of plasma membrane and functions; CAT etc. All the above-mentioned enzymes increased in the model cells. This may result from acute compensation after injury, and peroxidatic reaction stimulated by CCl₄ in

hepatocytes. SA-A markedly inhibits the increase of MDA level and the decrease of GSH, also reduced the activities of GSH-PX, CAT, SOD in different extents. Among these results, SA-A had better effect than vitamin E, which is a widely recognized antioxidation drug. It is indicated that SA-A had potential action against lipid peroxidation, this effect perhaps is the main mechanism of protection on liver injury. The results are also in accordance with the other reports^[8] and our previous work^[2].

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Kupffer cell and apoptosis in experimental HCC

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Subject headings liver neoplasms, experimental; diethylnitrosamine; apoptosis; protein P53; Kupffer's cell

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INTRODUCTION

Our previous study has proved that Kupffer cells may have an inhibitory effect on the process of hepatocarcinogenesis^[1], however, their inhibitory mechanism needs exploring deeply. We performed a comparative study on the expression of PCNA, Bax, P53 and apoptosis of liver cancer cells using immunohistochemical technology and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) in the diethylnitrosamine-induced hepatocellular carcinoma (HCC) in rats with or without pretreatment with gadolinium chloride or zymosan which might effectively block or enhance the activity of Kupffer cells in order to know the role of Kupffer cells in apoptosis in the experimental HCC and explore further the inhibitory mechanism of Kupffer cells on the process of hepatocarcinogenesis.

MATERIALS AND METHODS

Establishment of animal models and pathological examination

One hundred and forty male Sprague-Dawley rats were divided into six groups. ① DENA group, 40 rats received diethylnitrosamine (DENA) at a dose of 70 mg/kg in distilled water once/wk till wk15. ② GC+DENA group, gadolinium chloride was injected iv at a dose of 10 mg/kg once 2 wk to suppress Kupffer cells in 40 rats till wk15 and these rats simultaneously received DENA just as DENA group. ③ ZM+DENA group, zymosan was injected iv at a dose of 20 mg/kg once 2 wk to activate Kupffer cells till wk15 and DENA was received just as DENA group. ④ GC group, gadolinium chloride was injected iv at a dose of 10 mg/kg in 0.85% NaCl once 2 wk till wk 15. ⑤ ZM group, zymosan

was injected iv at a dose of 20 mg/kg in 0.85 % NaCl once 2 wk till wk15. ⑥ Control group, these rats were maintained on a standard laboratory diet and tap water. All rats were killed at the wk21 of hepatocarcinogenesis. The liver samples were taken, fixed in 40 mL/L paraformaldehyde and embedded in paraffin. Each specimen was cut into 5 µm serial slices, stained with hematoxylin-eosin and subjected to histopathological examination.

Immunohistochemical staining

The SP method was used, the 1st antibody was mouse-anti-human PCNA monoclonal antibody (Calbiochem Co., dilution 1 : 50), rabbit-anti-human Bax (Santa Cruz, dilution 1 : 50) and mouse-anti-human P53 monoclonal antibody (Novocastra Laboratories, dilution 1 : 50). The SP kit was purchased from Boehringer Mannheim, Germany. DAB staining was used. The dark brown staining of nuclei was taken as PCNA and P53-positive reaction. The dark brown granules in cytoplasm were taken as Bax-positive reaction. We used PBS to replace the 1st antibody as negative control.

Terminal deoxynucleotidyl transferase-mediated biotinylated-dutp nick end labeling method (TUNEL method)

In situ Cell Apoptosis Detection kit was purchased from Boehringer Mannheim, Germany. After deparaffinization and rehydration in ethanol, the sections were incubated with 0.2 mol/L HCl for 20 min at room temperature (RT). They were then deproteinized by incubation with 30mg/L proteinase (in 50mM Tris-HCl, pH 8.0; 5mM EDTA) for 30 min at RT. Terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP in TdT buffer (30mM Trizma base, pH 7.2; 140mM sodium cacodylate; 1mM cobalt chloride) were added to cover the sections, which were then incubated for 60 min in a humid atmosphere at 37°C. The sections then reacted with alkaline phosphatase (ALP) for 30 min at RT. Visualization was carried out with NBT/BCIP. Nuclei with clear blue staining were regarded as positive. TUNEL incubation solution without terminal deoxynucleotidyl transferase was used as negative control.

Proliferating index (PI) and apoptosis index (AI)

PI and AI were determined by Leitz ASM 68K image analyzing system purchased from Germany. Five visual fields (original magnification, 10 × 40)

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from every positive slide would be chosen and the TUNEL and PCNA-positive parenchymal cells as well as the total number of parenchymal cells in the same field would be counted. PI or AI was expressed as the percentage of the number of PCNA or TUNEL-positive parenchymal cells in the total parenchymal cells of the same field.

$$\text{Proliferating index (PI)} = \frac{\text{number of PCNA-positive cells}}{\text{number of total cells}} \times 100\%$$

$$\text{Apoptosis index (AI)} = \frac{\text{number of TUNEL-positive cells}}{\text{number of total cells}} \times 100\%$$

Statistical analysis

Fisher's exact test, Student's test and Spearman rank correlation were employed.

RESULTS

Macroscopic observation and histopathological examination (at wk21 of hepatocarcinogenesis)

Neither the control group, nor the GC group and ZM group showed any changes macro and microscopically; on the contrary, the liver surface in DENA group and GC +DENA group were covered with a lot of white nodules. The diameter of the largest nodules was 0.5 cm in DENA group and 2.0 cm in GC+DENA group. These nodules were diagnosed as HCC histologically. Some white grey focal nodules scattered over the liver surface in ZM+DENA group, were also diagnosed as HCC with many apoptotic cells and apoptotic bodies.

Bax staining

Cytoplasm of cancer cells with clear brown staining was regarded as positive (Figure 1). The positive rates of Bax in ZM+DENA group, DENA group and GC+DENA group were 84.6% (11/13), 28.6% (2/7) and 27.3% (3/11) respectively. It was significantly higher in ZM+DENA group than that in DENA group (Fisher's exact test, $P < 0.05$).

P53 staining

A clear brown staining of the nuclei in cancer cells was regarded as positive (Figure 2). The positive rates of P53 in ZM+DENA group, DENA group and GC+DENA group were 76.9% (10/13), 14.3% (1/7) and 36.4% (4/11) respectively. It was markedly higher in ZM+DENA group than that in DENA group (Fisher's exact test, $P < 0.05$).

PCNA staining

Nuclei with clear brown staining were regarded as positive (Figure 3).

No expressions of Bax, P53 and PCNA in the control group, GC group and ZM group were found.

Apoptosis of cancer cells using TUNEL method
Apoptotic cells were observed in DENA group, GC+DENA group and ZM+DENA group by means of TUNEL method. The cells with clear nuclear labeling were defined as TUNEL-positive cells. Apoptotic cells were scattered in the cancer tissue (Figure 4).

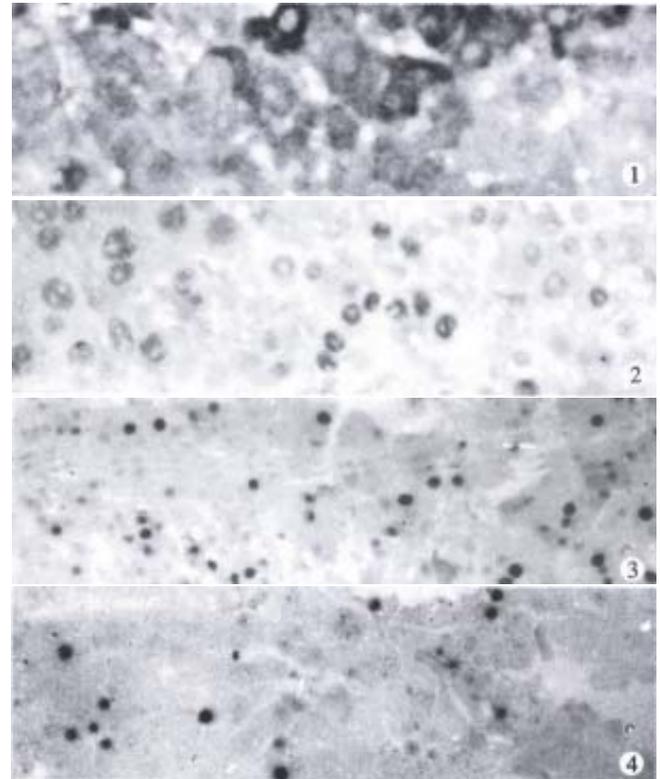


Figure 1 Expression of Bax in rat HCC in ZM+DENA group. SP \times 400

Figure 2 Expression of P53 in rat HCC in DENA group. SP \times 400

Figure 3 Expression of PCNA in rat HCC in DENA group. SP \times 200

Figure 4 Apoptotic cells in rat HCC in ZM+DENA group. TUNEL \times 200

Proliferating index (PI) and apoptosis index (AI) (Table 1)

Table 1 PI and AI in ZM+DENA group, DENA group and GC+DENA group

Groups	Number	PI(% , $x \pm s$)	AI(% , $x \pm s$)	PI/AI
ZM+DENA group	13	15.22 \pm 2.17 ^b	7.53 \pm 1.61 ^b	2.20
DENA group	7	24.97 \pm 2.53	4.36 \pm 1.18	5.73
GC+DENA group	11	38.69 \pm 3.17 ^b	2.52 \pm 0.81 ^b	15.40

^b $P < 0.01$, vs DENA group, Fisher's exact test.

Correlation between apoptosis index, Bax and P53 expression

There was a positive correlation between apoptosis index and Bax or P53 protein reactivity in ZM+DENA group, DENA group and GC+DENA group (Table 2).

Table 2 Correlation between apoptosis index and Bax or P53 expression

Groups	Bax-positive cases/total	AI vs Bax		P53-positive cases/total	AI vs P53	
		r _s	P		r _s	P
ZM+DENA group	11/13	0.63	<0.05	10/13	0.73	<0.05
DENA group	2/7	0.79	<0.05	1/7	0.61	
GC+DENA group	3/11	0.74	<0.01	4/11	0.82	<0.05

Spearman rank correlation.

DISCUSSION

Gadolinium chloride is believed to be a specific suppressor of Kupffer cells^[1,2]. Zymosan is an immunopotentiator and may be used to activate Kupffer cells^[3]. We performed a comparative study on apoptosis in DENA-induced hepatocellular carcinoma in rats with or without pretreatment with gadolinium chloride or zymosan which might effectively block or enhance the activity of Kupffer cells in order to clarify whether the Kupffer cells play a role in apoptosis of the experimental hepatocarcinogenesis or not.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase- δ . It accumulates little in the resting stage cell, but prominently in the nuclei of proliferating cells during G₁-late phase and S-phase and decreases in G₂-phase and M-phase. It is associated with cell proliferation and regarded as a biological marker for cell proliferation^[4]. Bax is a 21 000 protein with extensive amino acid homology with Bcl-2. This protein has been shown to form heterodimers with apoptosis-inhibiting protein, Bcl₂, so it can induce cell apoptosis^[5]. When DNA is injured by toxicant, wild-type p53 drives cell to G₁ and arrests or inhibits cell proliferation until DNA is repaired. When nuclear DNA is badly damaged or cannot be repaired, wild-type p53 induces transcription of apoptotic genes and drives the cell to be in apoptosis^[6]. Gottlieb *et al*^[7] thought that overexpression of P53 was related to cell apoptosis. Zhao *et al*^[8] observed that there was a positive correlation between apoptosis index and P53 protein, and supported the role of P53 in regulating apoptosis in human HCC. The positive correlation between apoptosis index and P53 protein immunoreactivity observed in our study also supports these findings. A new method to detect apoptosis *in situ*, terminal deoxynucleotidyl transferase-mediated (TdT) dUTP-digoxigenin nick end labeling (TUNEL) was recently developed by Gavrieli *et al*^[9], and was applied to detect cell apoptosis of human HCC and other tumors^[10].

Our results showed that the positive rates of Bax and P53 protein were markedly higher in ZM +

DENA group than in DENA group with significant differences. Proliferating index and apoptosis index respectively increased or decreased in ZM+DENA group, DENA group and GC+DENA group successively. These results demonstrated that the blockage of Kupffer cells with gadolinium chloride might suppress cell apoptosis and the activation of Kupffer cells with zymosan might promote cell apoptosis in the experimental hepatocarcinogenesis.

One of the characteristic features of cancer is the continuous growth and the ratio of cell proliferation and cell apoptosis determine the fate of tumor growth^[11-13]. In normal tissues, apoptosis is an efficient way of eliminating transformed cells. However, inability of cells to undergo apoptosis may advance their development, both by allowing the accumulation of dividing cells and by impairing the elimination of genetic mutants that may harbor malignant potential^[11,12]. Our results proved that Kupffer cells could promote apoptosis in the experimental hepatocarcinogenesis and revealed further that they played an inhibitory role in hepatocarcinogenesis through inducing apoptosis of tumor cells.

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Clinical significance of serum intercellular adhesion molecule-1 detection in patients with hepatocellular carcinoma

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Subject headings carcinoma, hepatocellular/diagnosis; intercellular adhesion molecule-1; liver neoplasms/diagnosis; alpha-fetoprotein

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INTRODUCTION

Since the late 1980s, studies on the expression of intercellular adhesion molecule-1 (ICAM-1) in patients with malignancies have demonstrated that ICAM-1 may strongly express in two forms in such diseases: membranous one on the surface of tumor cells (membrane-bound ICAM-1) and soluble one in circulation (soluble ICAM-1, sICAM-1)^[1,2]. Furthermore, increased expression of sICAM-1 in various malignant diseases, such as gastric cancer, colonic and pancreatic cancer was reported by Tsujisaki *et al*^[3], who found that the incidence of positivity of sICAM-1 in the malignant diseases was significantly higher than that in benign diseases. Therefore, the diagnostic value of detecting sICAM-1 for some malignancies has been proposed^[3]. The recent studies on detecting serum sICAM-1 in patients with hepatocellular carcinoma (HCC) have revealed that serum levels of sICAM-1 were well correlated to progression and prognosis of the disease^[4-6], from which it has been considered that sICAM-1 may be useful for monitoring the response to treatment^[4]. However, whether sICAM-1 is a diagnostic marker is still controversial. Our recent study demonstrated that in HCC patients with normal or low levels of serum α -fetoprotein (AFP) measurement of sICAM-1 is of clinical value for detecting early HCC and its recurrence after hepatectomies^[7-9]. To further evaluate the clinical

significance of measuring sICAM-1 in HCC, the serum levels of sICAM-1 in 134 patients with HCC were examined and the results were analyzed.

MATERIALS AND METHODS

Patients

There were 134 patients with HCC, including 115 males and 19 females, aged from 12-80 years, median 49 years. The diagnosis of HCC for all patients was confirmed pathologically either by surgical resection of liver tumors or by liver biopsies. Tumor was less than 5 cm in diameter in 17 patients, less than 10 cm in 37 and more than 10 cm or with extrahepatic metastasis in 80 cases. Hepatitis B surface antigen (HBsAg) was positive in 112 cases and hepatitis C antibody positive in 10 patients. Surgical treatment was carried out in 68 patients, of whom 57 underwent tumor resection and 11 underwent laparotomy or selective catheterization of the hepatic artery. The rest of the patients were treated by transcatheter arterial embolization (TAE) or alcohol injection of tumors. As controls, serum levels of sICAM-1 were measured in 42 patients with chronic hepatitis B (CH), 40 with liver cirrhosis (LC) and 50 healthy blood donors.

Methods

Blood samples were collected early in the morning. The serum was immediately separated by centrifugation and frozen to -80°C . Concentrations of serum sICAM-1 were measured with an enzyme-linked immunosorbent assay kit (Biosource Europe, Fleurus, Belgium). The quantitative determinations of serum AFP in patients with HCC was done by radio-immunoassay (RIA), using an AFP kit (Jiuding Biological, Tianjin, China). The reference ranges of serum AFP concentration were classified as follows: above 200 $\mu\text{g/L}$, positive; 20 $\mu\text{g/L}$ -200 $\mu\text{g/L}$, questionable positive; and below 20 $\mu\text{g/L}$, negative.

Statistical analysis

Data between groups were compared by Wilcoxon's Rank Sum test and Fisher test. $P < 0.05$ means significance statistically.

RESULTS

The median of sICAM-1 in 134 patients with HCC was 1801 $\mu\text{g/L}$, which was significantly higher than

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those in patients with CH (median = 462 $\mu\text{g/L}$), LC (median = 587 $\mu\text{g/L}$) and the healthy subjects (median = 305 $\mu\text{g/L}$) (Table 1). The compared analysis of serum levels of sICAM-1 and AFP in the HCC patients was shown in Table 2. Serum AFP concentration was positive in 85 cases, negative in 22 and questionable positive in 27. The serum levels of sICAM-1, in the corresponding patients were 2018, 1370 and 1453 $\mu\text{g/L}$ respectively. A correlated analysis of serum concentrations of AFP and sICAM-1 demonstrated that there was a close correlation between the two parameters in patients with AFP positive ($r = 0.249$, $P < 0.05$). However, the correlation did not exist in patients with AFP negative or questionable positive. Although the serum concentrations of AFP in these two groups of patients were normal or in a low level, that of sICAM-1 in these patients all exceeded 1000 $\mu\text{g/L}$, the median values of which showed no significant difference compared with the median of the whole group of HCC patients ($P > 0.05$). The ranges of serum levels of sICAM-1 in the patients with HCC are shown in Table 3. One hundred and seven patients (80%) had a sICAM-1 level higher than 1000 $\mu\text{g/L}$ and 126 (94%) higher than 700 $\mu\text{g/L}$. There was no close correlation between the serum value of sICAM-1 and the tumor size among 134 patients with HCC (Table 4). The medians of serum levels of sICAM-1 in patients with different sizes of tumor showed no significant difference ($P > 0.05$).

Table 1 Concentrations of serum sICAM-1 in patients with HCC and control groups

Group	Number of patients	sICAM-1 ($\mu\text{g/L}$) ^a
Normal control	50	305
CH	42	462
LC	40	587
HCC	134	1801 ^b

^a a median $P < 0.01$ vs compared with other groups; ^b $P < 0.01$ vs other groups each.

Table 2 Comparative analysis of serum levels of sICAM-1 and AFP in HCC patients

Group	Number of patients	sICAM-1 ($\mu\text{g/L}$) ^a	AFP ($\mu\text{g/L}$) ^a
AFP < 20 $\mu\text{g/L}$	22	1370	11
AFP 20-200 $\mu\text{g/L}$	27	1453	89
AFP > 200 $\mu\text{g/L}$	85	2018	31610

^a median.

Table 3 Ranges of serum sICAM-1 concentration in 134 patients with HCC

sICAM-1 ($\mu\text{g/L}$)	Number of patients	(%)
330-690	8	6
700-990	19	14
1000-1990	72	54
> 2000	35	26

Table 4 Serum concentrations of sICAM-1 and tumor size in 134 patients with HCC

Tumor size in diameter (cm)	sICAM-1 ($\mu\text{g/L}$) ^a	Number of patients
≤ 5	1518 ^b	17
< 10	1769	37
> 10	1897	80

^a a median; ^b $P > 0.05$ vs others groups each.

DISCUSSION

One obvious phenomenon from the present study was that a much higher level of sICAM-1 was observed in our HCC patients than those reported by the others [4,6,12]. According to the following researches, we may explain the discrepancies: It has been known that ICAM-1 expression can be upregulated by several cytokines [10,11], of which interferon-gamma (INF-gamma) was the main cytokine trigger for ICAM-1 expression in a human hepatoplastoma cell line. In addition, hepatitis B virus-DNA-transfected cells expressed membranous ICAM-1, the triggering mechanisms of which may be gene activation by virus genome or autocrine virus-induced hepatocellular cytokine production. Furthermore, the clinical research of relationship between serum levels of sICAM-1 and HCC showed that the high levels of sICAM-1 were closely related to the progression and prognosis of HCC [4,6,12]. In this study, the rate of hepatitis B infection was 84% (112/134) and 87% of the patients were in the middle or late stage of HCC (117/134). Thus it can be seen from our study that a high infectious rate of hepatitis B and a delayed diagnosis of HCC in this group of patients may be the main reasons for the high expression of serum levels of sICAM-1.

However, with regards to the study on sICAM-1 and HCC, one more important point, which is still controversial and to be resolved is whether detecting sICAM-1 in patients with HCC is of clinical significance for early diagnosing the disease and detecting its recurrence after surgical resection. As the study results by Hyodo *et al.* [4] showed that there was no difference in serum levels of sICAM-1 between their patients with HCC and liver cirrhosis. Based on this they declared that sICAM-1 is only a marker for progression and prognosis of the disease, but not a diagnostic marker for HCC. However, this conclusion was at odds with the observations from other reports [6,8,12], in which significantly higher serum levels of sICAM-1 in patients with HCC than those with LC, HC and healthy controls were demonstrated. The results of present study by the authors further confirmed the observations.

Moreover, the following results from the literatures seem to be more useful to resolve the controversial problem: enhanced expression of ICAM-1 on HCC cell surface exists in most patients with HCC (ranged from 80% to 96.2%) [5,13], which did not exist in peritumor and normal liver tissues. Regarding the association between membranous and soluble ICAM-1 expression, a highly consistent expression rate of the two forms of ICAM-1 was reported by Momosaki *et al.* [5]. They found that in tumor lines, the consistent expression rate of the two forms ICAM-1 was 87.5% (present or absent concomitantly). There were two forms of sICAM-1 in HCC patients: inflammation-associated sICAM-1 and HCC-specific circulating form of sICAM-1. The latter mainly came from HCC cells,

from which the membranous ICAM-1 were shed into the circulation continuously and became the important source of sICAM-1 [14]. In addition, recent studies by the authors [9] found that regardless of positive or negative serum AFP, after a radical resection of liver tumor, the sICAM-1 level would be decreased to the normal within 1-2 months postoperatively. However, in patients underwent non-radical resection due to vascular invasions of the liver or extrahepatic metastasis, the serum levels of sICAM-1 will maintain at a high level. It has been suggested from all of the studies mentioned above that the high level of serum sICAM-1 in HCC may originate mainly from tumor itself. Thus we believe sICAM-1 in HCC patients may be a useful marker for detecting HCC and a monitor of recurrence after hepatectomy.

As the strong correlation between serum levels of sICAM-1 and AFP in patients with positive AFP was disclosed (Table 2), the clinical significance of sICAM-1 detection for diagnosing HCC will be focused on patients with normal or low levels of serum AFP. Serum AFP is still the best diagnostic marker for HCC. But AFP levels may be normal in 20%-40% of patients with HCC, depending on the severity of the disease [15]. In China about 30% of HCC patients can not be diagnosed by this serum marker, which results in delayed diagnosis and, consequently, hampers efforts to improve effective surgical treatment of the disease. Therefore, more sensitive serum markers are required for detecting HCC.

From the study, one of the interesting results was that there were 22 (16.4%) and 27 (20.2%) patients with HCC, whose serum levels of AFP were negative (median = 11 $\mu\text{g/L}$) and questionable positive (median = 89 $\mu\text{g/L}$). However, that of sICAM-1 in those patients were 1370 and 1453 $\mu\text{g/L}$ respectively (Table 2), which showed no significant difference compared with the median level of total group of HCC ($P > 0.05$). Furthermore, analysis of ranges of sICAM-1 levels in patients with HCC demonstrated that 80% of the patients had a high serum level of sICAM-1 exceeding 1000 $\mu\text{g/L}$. According to the study by Shimizu *et al* [6], sICAM-1 level above 1000 $\mu\text{g/L}$ is a determinant for prognosis and progression of HCC, the proportion of patients with high levels of sICAM-1 ($> 1000 \mu\text{g/L}$) in patients with HCC in our study was obviously higher than that of AFP ($> 200 \mu\text{g/L}$). As mentioned in our previous studies the diagnosis of HCC should be strongly suspected when a patient with an uncertain intrahepatic lesion had a serum level of sICAM-1 higher than 1000 $\mu\text{g/L}$ [7]. The results of the present study further confirm our previous conclusion.

Another interesting finding from the study was

that a significant correlation between serum level of sICAM-1 and tumor size of HCC was not observed (Table 4). The median of sICAM-1 concentration in 17 patients with tumor diameter less than 5 cm was 1518 $\mu\text{g/L}$, among them 4 being negative, 8 questionable positive and 5 positive for AFP. In addition, a tumor recurrence was diagnosed in 4 of the 17 patients after 1-3 hepatectomies during the postoperative follow-up. The median of tumor size in the 4 cases was only 2.8 cm when recurrence was confirmed, however, the median of serum level of sICAM-1 in the same patients was 1378 $\mu\text{g/L}$. It was strongly suggested by our observation that measurement of sICAM-1 is of clinical significance in detecting early HCC and monitoring its recurrence postoperatively when tumor is small in diameter, particularly for patients with normal or low serum concentrations of AFP.

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Polymorphism of p16INK4a gene and rare mutation of p15INK4b gene exon2 in primary hepatocarcinoma

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Subject headings p16INK4a gene; p15INK4b gene; polymorphism; mutation; hepatocarcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common cause of death from cancer in China. The mechanisms of hepatocarcinogenesis are not yet known clearly. p16INK4a gene, the multiple tumor suppressor gene 1 (MTS1), encodes P16 protein, which acts as an inhibitor by binding directly to CDK4 and CDK6 and preventing its association with a cyclin. It was supposed to exert negative control on cell proliferation. p15INK4b gene, multiple tumor suppressor gene 2 (MTS2), is a homologue of p16INK4a and has a similar role in control of cell proliferation. Both of them were mapped to chromosome 9p21 region^[1,2]. Although deletion or mutation of p16INK4a occurred in melanoma, biliary tract cancers, gastric carcinoma, hepatocarcinoma, and alterations of p15INK4b were shown in primary lung cancers, acute leukemia, biliary tract cancers and bladder tumors, there has been no report about whether p15INK4b gene altered in primary hepatocellular carcinoma^[3-9]. In the present study, exon 1, exon 2, exon 3 of p16INK4a and p15INK4b exon 2 in 35 HCC, 35 corresponding adjacent noncancerous liver

cirrhosis were analyzed for somatic mutation with PCR-SSCP and one case of aberrant SSCP DNA was cloned and sequenced.

MATERIALS AND METHODS

Specimens and extraction

Tissue specimens used in the study were paraffin embedded and stored in Department of Pathology, the First Affiliated Hospital, West China University of Medical Sciences from 1991-1993. The 35 samples of human primary hepatocarcinoma and 35 corresponding adjacent noncancerous liver cirrhosis were stained with HE and examined under microscope. More than 70% sections used in PCR were hepatocarcinoma sections. And more than 80% cirrhosis sections were the noncancerous liver cirrhosis sections. DNA was extracted from 1-3 sections (10 μ m) of paraffin embedded tissue blocks with xylene, ethanol and phenol method, and dissolved in 50 μ L of distilled water^[10]. Ten samples of normal human blood DNA were extracted with standard method. The concentration of DNA was determined with spectrophotometer.

PCR

The exons of p16INK4a gene and exon 2 of p15INK4b gene were amplified using the following primers (Table 1):

Table 1 Primers for p16INK4a and p15INK4b genes analysis

Gene	Primers	Fragment length (bp)	Ref
P16INK4a exon1	5'GGGAGCAGCATGGAGCCCG 3'(sense) 5'AGTCGCCCCGCCATCCCCT 3'(antisense)	204	[6]
p16INK4a intron1	5'GGAAATGGAACTGGAAGC 3'(sense)	168	[1]
and exon2	5'GCTGCCCATCATCATGACCT 3'(antisense)		
p16INK4a exon2	5'GGCAGGTCATGATGATGGGC 3'(sense)	362	[1]
and exon3	5'TCTGAGCITTGGAAGCTCT 3'(antisense)		
P15INK4b exon2	5'GGCCGGCATCTCCATACCTG 3'(sense) 5'TGTGGCGGCTGGGAACCTG 3'(antisense)	345	[9]

The PCR reaction was performed as follows: 200 ng DNA from paraffin embedded tissue or 100 ng DNA from normal human blood cells, 200 μ mol/L each dATP, dGTP, dCTP and dTTP, 20 pmol primers, 1.5 u of Taq DNA polymerase (Sino-American Biotechnology Company) with a buffer provided by the manufacturer, in a total

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reaction volume of 25 μ L. The thermal cycle profile was 1min at 94°C, 1min at 66°C (p16 exon 1, 204 bp) or 57°C (p16 exon 2, 168 bp) or 55°C (p16 exon 2, exon 3, 362 bp) or 68°C (p15 exon 2), 2 min at 72°C, 40 cycles. The PCR reaction mixture for p16INK4a exon1 (204 bp) exon 2 and exon 3 (362 bp) contained 5% dimethyl sulfoxide.

SSCP

PCR products were directly subjected to silver-staining SSCP analysis according to the method of Peng *et al*^[11]. Ten μ L of PCR products were denatured in 30 μ L of 98% formamide, 10 mmol/L NaOH, 20 mmol/L EDTA, 0.05% (w/v) bromphenol blue and 0.05% (w/v) xylene cyanol, at 98°C for 5 min. The samples were immediately loaded on an 8% polyacrylamide gel and run at 1.25v/cm in 1 \times TBE in 4°C for approximately 14h. Ten μ L of PCR products of p16INK4a exon 2 and exon 3 (362 bp) was digested with *Sma* I. It generated two fragments (114 bp, 248 bp) which were then subjected to SSCP analysis. After electrophoresis, the gels were fixed, and stained with silver.

PCR cloning and sequencing

The gels were stained with silver. The staining was stopped by immersing the gel in 5% acetic acid, 16% methyl alcohol for 30 min. The gel was rinsed for 1 h in distilled water (changed each 5 min). The abnormal migration single strand DNA band of p15INK4b exon 2 was cut from gel and put into the same volume of distilled water. The gels were incubated in 37°C water bath for 4 h, centrifuged at 10 000r/min for 5 min, then the gel was discarded. The supernatant was extracted with phenol/chloroform, and chloroform. The DNA from approximately 90 mg gel was placed in the PCR mixture for amplification under the same conditions as the initial PCR.

Following reamplification by PCR, the PCR products were isolated on 1.8% agarose gel. The 345 bp DNA band was cut from the gel and purified with Glassmilk Isolation Kit (made in our Lab). The DNA was blunted and phosphorylated with *E.coli*- Klenow fragment and T_4 polynucleotide kinase, ligated to *Sma* I site of pUC118. The ligation reaction was transformed into *E.coli*- JM109. The recombinants were screened by minipreparation of plasmid and digesting with restriction endonuclease. The DNA from the clone was screened by PCR-SSCP to identify the clone containing the shift single strand and was sequenced on a ABI 377 DNA sequencer by CyberSyn B.J. Company.

RESULTS

Polymorphism of the p16INK4a gene intron 1 and exon 2

Mutation of p16INK4a gene was analyzed in 31 of

the 35 patients with HCC and 8 healthy blood donors. The PCR amplified 168 bp fragment of p16INK4a intron 1 and exon 2 containing 15 nucleotides within exon 2 and 153 nucleotides of its 5' flanking sequence within intron 1. Three patterns (A, B, B') of p16INK4a intron 1 and exon 2 (168 bp) at SSCP analysis were observed in hepatocellular carcinoma and corresponding adjacent noncancerous cirrhosis (Figure 1). The B pattern (48%, 15/31) outnumbered the B' pattern (26%, 8/31). A pattern was the least (13%, 3/31). Two patterns (B, B') at SSCP analysis were observed in healthy human blood cells. The B' pattern (62.5%, 5/8) outnumbered the B pattern (37.3%, 3/8). The PCR amplified 362 bp fragment covered exon 2, exon 3 and intron 2 sequences. The PCR products were cleaved into two fragments 114 bp and 248 bp by digesting with *Sma* I. Neither band shift nor polymorphism at SSCP analysis was observed. The PCR amplified 204 bp fragment containing exon 1 and 19 nucleotides of 5' flanking upstream sequence and 41 nucleotides of 3' flanking downstream sequence. No band shift was detected in all of the samples.

SSCP analysis of p15INK4b exon 2

A 345 bp fragment containing p15INK4 b gene exon 2 and 60 nucleotides of its 5' flanking sequence within intron 1 was amplified by PCR from all of the hepatocellular carcinomas, adjacent noncancerous cirrhosis and normal blood cells. No evidence of allele deletion was detected (Figure 2). On repeating SSCP analysis, one case of adjacent noncancerous cirrhosis showed an abnormal migration single strand (Figure 3). But no additional shift band was found in either the corresponding tumor tissue from the same patient or the other hepatocarcinoma and cirrhosis tissues. The identical migration single strand was detected in all of the 10 normal human blood cells. No evidence of polymorphism was found.

Cloning and sequencing of the abnormal single strand

The abnormal migration single strand DNA of C₅ (adjacent noncancerous cirrhosis) was purified and cloned in *Sma* I site of pUC118. The recombinant plasmid, pP15E₂, containing the p15INK4a exon 2 insert, was selected after minipreparation. The restriction endonuclease map analysis by *Hind*-III, *Eco*R I, *Bgl*II revealed that pP15E₂ plasmid contained a 345 bp insert (Figure 4) and the insert was placed in antisense orientation. PCR-SSCP analysis of the pP15E₂ showed an identical abnormal migration single strand with that of C₅ (Data not shown). Sequencing analysis of the insert of pP15E₂ indicated that its sequence was identical to that of p15INK4b exon 2 and its upstream 60 nucleotides reported by Kamb (Figure 5).

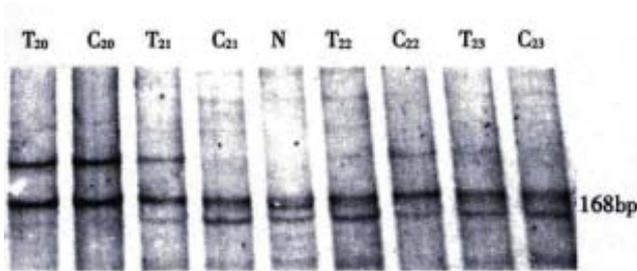


Figure 1 PCR-SSCP analysis of p16 gene intron 1 and exon 2. At the SSCP analysis T₂₀ and C₂₀ showed as A pattern; T₂₃ and C₂₃ showed as B pattern; T₂₁ and C₂₁ showed as B' and B pattern, respectively; T₂₂ and C₂₂ showed as B and B' pattern, respectively. T = Human hepatocarcinoma; C = Adjacent non-cancerous liver cirrhosis; N = Normal human leucocyte

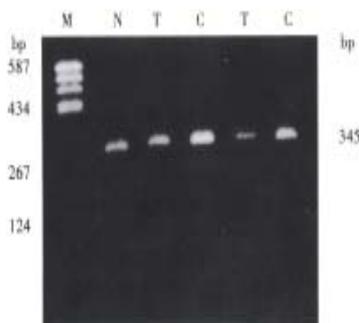


Figure 2 PCR product analysis of p15 gene exon 2 in human hepatocarcinoma on agarose gel. M = pBR322/Hea III; N = Normal human leucocyte; T = Human hepatocarcinoma; C = Adjacent non-cancerous liver cirrhosis

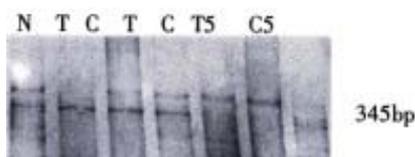


Figure 3 PCR-SSCP analysis of p15 gene exon 2. N = Normal human leucocyte; T = Human hepatocarcinoma; C = Adjacent non-cancerous liver cirrhosis

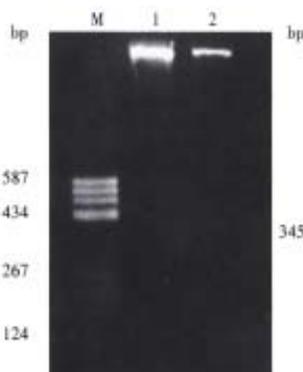


Figure 4 Restriction enzyme analysis of recombinant plasmid which contains aberrant single strand of p15 gene exon 2. M = pBR322/Hea III; 1 = Vector (pUC118) digested with *EcoR* I and *Hind* III; 2 = pP15E₂ digested with *EcoR* I and *Hind* III

Figure 5 The sequence of p15 gene exon 2 in pP15E₂ recombinant plasmid.

DISCUSSION

The p16INK4a gene has been confirmed to be a tumor-suppressor gene by the analysis of p16 gene knockout mice and its abnormalities have been reported in various kinds of primary cancers and cell lines, such as malignant melanomas, gliomas, glioblastomas, and esophageal squamous-cell carcinomas^[1,12-14]. But there have been relatively few studies concerning the alteration of p16INK4a gene in hepatocellular carcinoma. Kita *et al* reported only three (5%) intragenic mutations of p16INK4a in primary HCC^[15]. Chaubert *et al* found four patients carried hemizygous germ-line point mutations of the p16INK4a gene, suggesting the existence of familial HCC involving this gene^[6]. Hui *et al* found higher proportion of HCCs may fail to express p16INK4a at the protein level^[16]. The general conclusion is that alterations of p16INK4a gene are infrequent in HCC. Our study showed polymorphism of p16INK4a intron 1 and exon 2 at SSCP pattern. The A pattern was only presented in HCC patients, not in the healthy blood donors. It is worthy to study whether people with the A pattern has HCC susceptibility. Five patients showed different SSCP patterns in tumor and noncancerous cirrhosis, most of which (4/5) occurred in large HCC (no statistical significance was revealed). It suggested that intragenic mutation may occur during the progression of HCC, and advanced HCC, but not in the early stages of HCC.

There is argument on whether the inactivation of p15INK4b contributes to the carcinogenesis. Okamoto *et al* observed that non-small-cell lung cancer showed homozygous deletions of p15INK4b (23%), somatic mutation (12%) in exon 2, G→A and C→A polymorphism (8%) within the noncoding sequence of 23 nucleotides and 27 nucleotides of 5' of exon 2, respectively. The latter was named 15Int1-27A gene pattern^[7]. But Russin *et al* detected only the 15Int1-27A polymorphism (13%), no mutation in non-small-cell lung cancers^[17]. Sill *et al* reported 15Int1-27A polymorphism (13%), no somatic mutation in 80 acute leukemia^[8]. Orlow *et al* observed deletions of p15INK4b in primary bladder tumors (8%). Yoshida reported no somatic mutation of p15INK4b gene in biliary tract cancers^[4]. The general

consensus is that frequency of mutation of p15INK4b in progression of cancer is very uncommon. In the present study no intragenic mutation of p15INK4b exon 2 was detected. Although one case of adjacent non-cancerous liver cirrhosis showed abnormal migration single strand, the cloning and sequencing of the aberrant SSCP DNA showed the sequence is identical to wild type p15INK4b exon 2 and 60 nucleotides upstream of exon 2^[1]. It suggested that intragenic mutation of p15INK5b exon 2 was an uncommon event in progression of HCC. This result is in agreement to the infrequent mutation of p16INK4a occurring in HCC, and also similar to the studies in AML, bladder cancers and biliary tract cancers^[4,8,9]. Whether the high frequency of p15INK4b somatic mutation in non-small cell lung cancers is related to the different types of tumor deserves further investigation.

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Computed morphometric analysis and expression of alpha fetoprotein in hepatocellular carcinoma and its related lesion

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Subject headings liver neoplasms; carcinoma, hepatocellular; alpha fetoprotein; morphometry

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INTRODUCTION

Hepatocellular carcinoma (HCC) is closely related with hepatitis and cirrhosis. In order to investigate the pathogenesis and early pathologic diagnosis of HCC, HCC and related lesions were analyzed qualitatively and quantitatively by automatic image analyser and immunohistochemical assay.

MATERIALS AND METHODS

Materials

Specimens obtained from surgical resection, autopsy and needle aspiration biopsy of livers during 1966-1997 were fixed in 10% formalin, embedded in paraffin, made into serial sections, and stained with routine HE. They were divided into seven groups: I. normal liver tissues used as controls (10 cases); II. chronic hepatitis (10 cases); III. chronic hepatitis with early cirrhosis (10 cases); IV. micronodular cirrhosis (13 cases); V. micronodular and macronodular mixed cirrhosis (14 cases); VI. paracancerous cirrhosis (27 cases); VII. HCC (39 cases).

All of the specimens were examined and diagnosed by two pathologists. The diagnosis of hepatitis and cirrhosis was referred to the standard of the Beijing Conference in 1995 and WHO's criteria.

Morphometry

Thirteen morphometric parameters were determined by the automatic image analyser (Type Q-900, Cambridge Company). And the sections were

enlarged 1000 times under light microscope on the screen of monitor. The cells and nuclei in the sections were traced by light pen. The automatic image analyser was used to determine the nucleus area (NA), the coefficient of variation of NA (NACV), the nucleus perimeter (NP), the nucleus diameter (ND), the roundness of nucleus (NR), the average volume of nucleus (NAV), the cell area (CA), the cell perimeter (CP), the cell diameter (CD), the cell roundness (CR) the average volume of cell (CAV), the ratio of area of the nucleocytoplasm (A-N/C), the ratio of volume of the nucleocytoplasm (V-N/C). After 50-100 cells in each section were examined at random, of the data were analysed by variance (q-test) and stepwise discriminational analysis. Then the equation of discriminational function was set up, the results were compared based on the histopathological classification.

Immunohistochemical staining

Mouse monoclonal antibody against human AFP and Immunostain S-P Kit were purchased from Fuzhou Maxim Biotechnical Company. Immunostaining of AFP was performed by the S-P method in each case. The procedures of S-P staining were taken according to the manufacturer's recommendations. The color was developed with diaminobenzidine and hematoxylin. Positive and negative controls were simultaneously used to ensure specificity and reliability of the staining.

RESULTS

Morphometry

The values of nucleus parameters (NA, NP, ND, NAV, A-N/C and V-N/C) increased gradually and those of cell parameters (CA, CP, CD and CAV) decreased gradually in the sequence of chronic hepatitis, cirrhosis without tumor, paracancerous cirrhosis and HCC. The difference was statistically significant between the group of HCC and the other groups without tumor ($P < 0.05$). The values of NACV, NR and CR in all groups varied irregularly. The values of most parameters (NA, NP, ND, CA, CP, CD, CAV, A-N/C and V-N/C) of the paracancerous cirrhosis were in between those of the cirrhosis without tumor and HCC. The difference was statistically significant ($P < 0.05$). The difference of the value of most parameters was not significant among chronic hepatitis with early cirrhosis, micronodular cirrhosis, mixed

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micronodular and macronodular cirrhosis ($P < 0.05$). Results of stepwise discriminational analysis: because the difference of most parameters was not statistically significant among chronic hepatitis with early cirrhosis, micronodular cirrhosis, micronodular and macronodular mixed cirrhosis, the three groups were merged into one cirrhosis group of as a whole. Six of 13 parameters processed by stepwise discriminational analysis were chosen in chronic hepatitis (Y_1), cirrhosis without tumor (Y_2), paracancerous cirrhosis (Y_3) and HCC (Y_4). The equation of discriminational function was setup.

$$Y_1 = -526.540 - 32.768(\text{NP}) + 631.477(\text{NR}) + 6.046(\text{CP}) + 1.887(\text{CA}) + 19.264(\text{A-N/C}) - 0.016(\text{CAV})$$

$$Y_2 = -453.402 - 29.633(\text{NP}) + 590.223(\text{NR}) + 5.466(\text{CP}) + 1.636(\text{CA}) + 17.903(\text{A-N/C}) - 0.011(\text{CAV})$$

$$Y_3 = -441.556 - 28.851(\text{NP}) + 603.816(\text{NR}) + 3.875(\text{CP}) + 1.854(\text{CA}) + 17.991(\text{A-N/C}) - 0.014(\text{CAV})$$

$$Y_4 = -623.687 - 36.878(\text{NP}) + 698.493(\text{NR}) + 5.217(\text{CP}) + 2.074(\text{CA}) + 23.555(\text{A-N/C}) - 0.011(\text{CAV})$$

Fifty-three specimens had been tested, only one of the chronic hepatitis with early cirrhosis was falsely classified into chronic hepatitis and the general conformation rate was 98.2% to pathologic diagnosis.

Expression of AFP

Immunostaining of AFP was seen in paracancerous cirrhosis and HCC cytoplasm. The positive rates were 33.3% (9/27) and 43.6% (17/39) respectively. The expression of AFP was negative in hepatitis and cirrhosis without tumor. The positive rate of AFP in the paracancerous cirrhosis (33.3%) was significantly higher than in the cirrhosis without tumor (0%, 0/27) ($P < 0.01$, $\chi^2 = 10.8$).

DISCUSSION

HCC which is related with hepatitis and cirrhosis, is one of the common malignant tumors in the world. Popper *et al* considered the occurrence of HCC was a multistep process: HBV infection → persistent inflammation → necrosis → regeneration and repair → hyperplasia → HCC. The course had been studied by morphometry and the results showed that the values of parameters of nucleus increased gradually and those of cells decreased gradually. These suggested that HCC was associated closely with hepatitis and cirrhosis, especially paracancerous cirrhosis.

Paracancerous cirrhosis differed essentially from cirrhosis without tumor. Ren *et al*^[1] considered the regenerative nodules in the paracancerous cirrhosis had regenerated more actively than those in the cirrhosis without tumor. Watanabe *et al*^[2] reported that the rate of dysplasia in the paracancerous cirrhosis (25.9%) was higher than that in the cirrhosis without tumor (12%). Dai

et al^[3] reported that some liver cells around the HCC triggered the closed gene to resynthesize AFP. Zhang^[4] reported that the re presented different degrees positive expression of AFP in the host hepatocytes around cancer and hepatocytes non-neoplastic animals in late stage experimental hepatocarcinoma in rats. All the results showed that the carcinogenesis in paracancerous cirrhosis was more liable to occur than in cirrhosis without tumor. We discovered that values of most parameters in the paracancerous cirrhosis were in between those of the cirrhosis without tumor and HCC by morphometry ($P < 0.05$). The expression of AFP was positive in the paracancerous cirrhosis and negative in the cirrhosis without tumor ($P < 0.01$). Evidently the paracancerous cirrhosis differed from the cirrhosis without tumor in the respect of function and morphology. It is more likely to be precancerous lesion than cirrhosis without tumor. The values of morphometric parameters in cirrhosis without tumor, regardless of early cirrhosis, micronodular cirrhosis or micronodular and macronodular mixed cirrhosis, were not different significantly. It suggested that the cirrhosis without tumor may be the result of regeneration and repair after HBV infection. Therefore, we inferred that the posthepatitis cirrhosis, the result of persistently affecting HBV, should be evolved into "precancerous cirrhosis" similar to paracancerous cirrhosis, and then some of liver cells were selectively developed into HCC in the circumstance suitable for carcinogenesis. The multisteps in the genesis of HCC may be HBV infection—chronic hepatitis—cirrhosis—"precancerous cirrhosis"—HCC. The paracancerous cirrhosis may be a sequential lesion of precancerous cirrhosis. This inference should be verified by further study. Now, since we can not differentiate the precancerous cirrhosis from the cirrhosis without tumor under light microscope, the precancerous cirrhosis was diagnosed merely by morphometry and immunohistochemical method, which suggested that the patient with precancerous cirrhosis would probably suffer from HCC in the near future or they had already suffered from HCC somewhere in the liver. It was significant to diagnose HCC as early as possible, especially by using needle aspiration biopsy of liver, thus to decrease the possibility of missing small HCC (SHCC, diameter ≤ 3 cm).

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The new proof of neuro-endocrine-immune network—expression of islet amyloid polypeptide in plasma cells in gastric mucosa of peptic ulcer patients

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Subject headings peptic ulcer; plasma cells; gastric mucosa; islet amyloid polypeptide (IAPP); neuro-endocrine-immune network

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INTRODUCTION

Peptic ulcer, as a common disease, seriously affected people's work and life. Its occurrence, development and change have close relationship with the change of people's moods. Animal experiment proved that significant changes occurred in the endocrine system of the gastric ulcer rats^[1]. Recent study also showed that the number of lymphocytes increased markedly in the gastric mucosa of peptic ulcer patients^[2]. All the above indicated that peptic ulcer is closely related neuro-endocrine-immune system. IAPP, a novel islet hormone, not only takes part in the regulation of blood glucose^[3], but also protects gastric mucosa^[4] and regulates gastrointestinal movements^[5]. On the basis of previous studies, we observed the expression change of IAPP and explored the relationship between the endocrine and the immune system in gastric mucosa of peptic ulcer patients, so as to provide morphologic data on the existence of neuro-endocrine-immune network and the changes in peptic ulcer.

MATERIALS AND METHODS

Twenty-one samples, including 6 cases from normal human stomach, 15 cases from gastrectomy of gastric ulcer patients, were collected. The paraffin

sections were prepared as usual. Immunohistochemical PAP method was used to show IAPP-IR cells. Briefly five-micron sections were placed on glass slides deparaffinized in xylene, rinsed in ethanol, and brought to PBS through a series of descending concentration of ethanol; endogenous peroxidase activity was blocked with methanol-H₂O₂ at room temperature for 30 min; rabbit anti-IAPP serum (peninsula, USA) was diluted 1/6000 with PBS, and the sections were incubated overnight at 4°C. Goat anti-rabbit IgG (Huamei, Beijing) (1/50), peroxidase-anti-peroxidase (Capital Medical University) (1/100) and DAB kit (Zhongshan, Beijing) were used for staining. As the negative control, the primary antiserum was replaced by PBS and other steps were the same as stated above. All the sections were counterstained with Mayer hematoxylin.

RESULTS

The IAPP-IR cell was not observed in the gastric mucosa of normal subject (Figure 1A). In comparison, a great number of plasma cells IAPP-IR were found in the gastric mucosa of peptic ulcer patients (Figure 1B,C). Most of IAPP-IR plasma cells were weak and only a few were strong for IAPP staining (Figure 1D). Of the negative control sections, no immunoreactive product to IAPP was found in plasma cells (Figure 1E).

DISCUSSION

The gastric mucosa, in which there are a lot of neurons, endocrine cells and immunocytes that may interact with each other, is an important field for the study of neuro-endocrine-immune network. It will undoubtedly provide valuable data for the study on this network by exploring the change of immune-endocrine of gastric mucosa of peptic ulcer patients. Based on the observation of T and B lymphocytes which increase obviously in the gastric mucosa of peptic ulcer patients^[2] and the action of IAPP, a novel islet hormone, which inhibits gastric acid secretion^[6] and protects gastric mucosa^[4], we further studied the expression change of IAPP in the gastric mucosa. Unexpectedly, it was found that the plasma cells of gastric mucosa increased in number, moreover most of them expressed IAPP to some degree.

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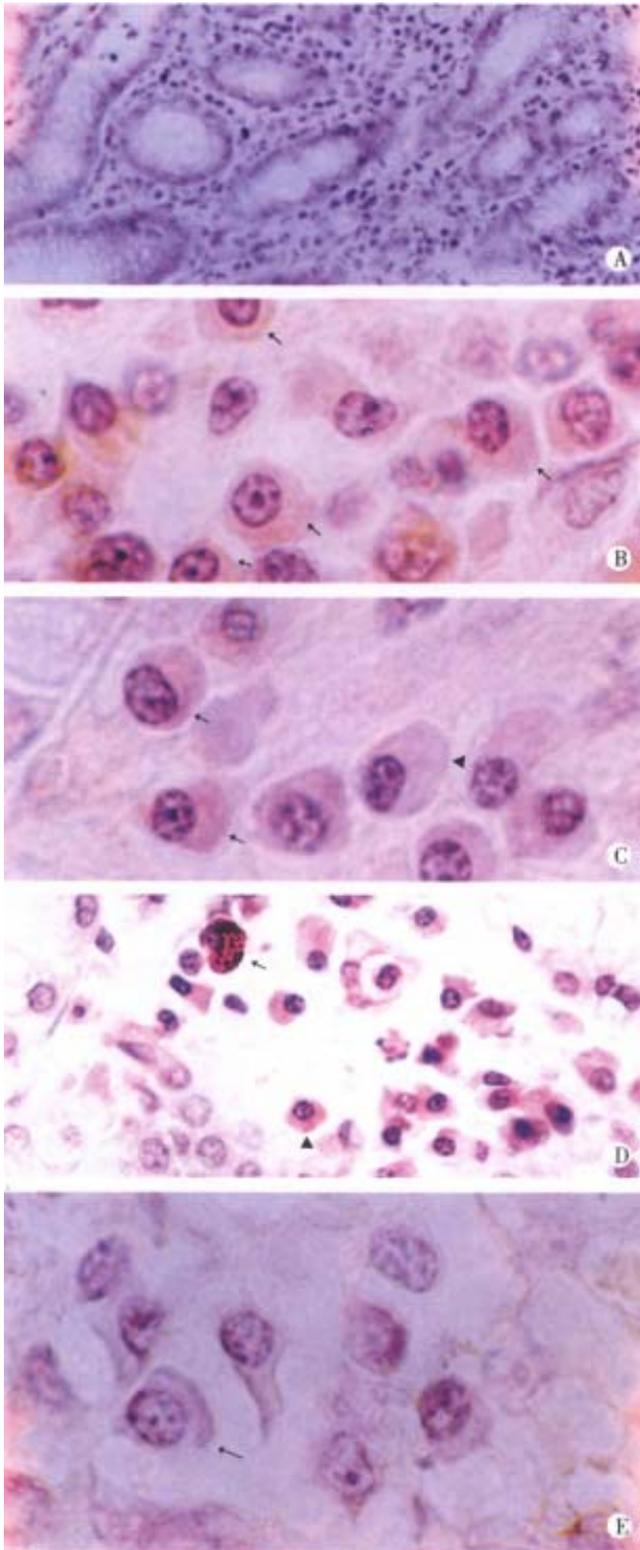


Figure 1 Immunohistochemical PAP method, Mayer Hematoxylin counterstained showing IAPP-IR cells.

A. The gastric mucosa of normal human, no IAPP-IR cell was found. $\times 200$

B-E. The gastric mucosa of peptic ulcer patients.
B. The more IAPP-IR plasma cells (\uparrow). $\times 1000$

C. The weak positive (\uparrow) and negative (\blacktriangle) plasma cells. $\times 1000$

D. The strong (\uparrow) and weak (\blacktriangle) positive IAPP-IR plasma cells. $\times 400$

E. The negative control section in which IAPP antiserum was replaced by PBS. (\uparrow) showing plasma cell. $\times 1000$

Firstly, the specificity of the above findings should be confirmed because there was no IAPP expression in the plasma cells on the negative control sections; and there were also IAPP-IR negative plasma cells around the positive ones. Secondly, the significance of IAPP expression in plasma cells should be studied. IAPP is mainly secreted by islet B cells^[7]. Recent study indicated that besides regulating blood glucose, IAPP could inhibit gastric acid secretion^[4], and protect gastric mucosa^[5]. IAPP-IR cells of islet were markedly increased during the healing process of rat gastric ulcer^[8]. The above-mentioned studies all suggested that IAPP is beneficial to ulcer healing. As it is known, the plasma cells of gastric mucosa come from B lymphocytes and they respond by synthesizing and secreting IgA. It is observed, for the first time, that plasma cells in gastric mucosa of peptic ulcer patients not only increased in the number, but also expressed IAPP. Combined with our previous observation that T and B lymphocytes of gastric mucosa increased in peptic ulcer patients, it is reasonable to infer that some plasma cells in gastric mucosa of peptic ulcer patients may transform to ones expressing IAPP so as to maintain the high level of IAPP in the gastric mucosa and help promote ulcer healing just as a growth factor^[9].

CONCLUSION

It is found, for the first time, that IAPP was expressed in plasma cells of gastric mucosa of peptic ulcer patients, which provides morphologic evidence for the existence of neuro-endocrine-immune network.

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Effect of gastrectomy on G-cell density and functional activity in dogs

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Subject headings gastrectomy; pylorus; G-cell; gastrin; peptic ulcer/surgery

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INTRODUCTION

Billroth gastrectomy has some advantages of inhibiting acid secretion, low ulcer recurrence and low mortality. However, postoperative complications, such as dumping syndrome and reflux gastritis, often occurred as a result of pylorotomy^[1]. To minimize these complications, pylorus-preserving gastrectomy (PPG) had been performed for gastric ulcer with satisfied clinical results. Positive correlation was not found between ulcer recurrence and serum gastrin level^[2]. In this study, we performed distal partial gastrectomy with Billroth II anastomosis (DPG-BII), pylorus-preserving gastrectomy (PPG) and highly selective vagotomy (HSV) on dogs and investigated the relationship between different antrum disposal and gastric acid secretion, serum gastrin level and G-cell density and functional activity.

MATERIALS AND METHODS

Eighteen hybrid adult dogs, with body weight ranging from 10 kg to 20 kg, mean weight 13.9 kg, were randomly divided into 3 groups, and underwent PPG, DPG-BII or HSV respectively. In PPG group, antrum was strictly retained within 1.5 cm-2.0 cm and stomach was resected about 40%. DPG-BII, in which stomach was resected about 75%, and HSV were routinely done. After laparotomy biopsy was taken at antrum 2 cm beyond the pyloric sphincter, the first segmental duodenum and jejunum 4 cm beyond Treitz ligament, 3 mo after operation, biopsies were done again around the original biopsy sites. Gastric acid secretion was analyzed using neutralization method (subcutaneous injection of tetra-gastrin 4 µg/kg). Fasting and

postprandial serum gastrin levels were measured by radioimmunoassay. The G cell density and its functional activity were determined by immunohistochemical assay using an antigastrin antibody (Zymedco) at a dilution of 1:200 in PBS. G cell density was measured according to the method of Creutzfeldt^[3], in which G cell functional activity was divided into 4 grades, as follows: 1+, brown-red cytoplasm, without granule; 2+, minute brown granules, occupied within 1/3 cytoplasm area; 3+, brown granule or clusters occupied, 1/3-2/3 cytoplasm area; 4+, brown-black granules or clusters, above 2/3 cytoplasm area.

RESULTS

Effects of different operative procedures on gastric acid secretion

In DPG-BII, PPG and HSV groups, preoperative basal acid output (BAO) was 1.80 mmol/h, 2.25 mmol/h and 2.19 mmol/h; maximal acid output (MAO) was 5.19 mmol/h, 4.49 mmol/h and 5.30 mmol/h, respectively; 3 mo after operation, BAO decreased to 0.48 mmol/h, 0.98 mmol/h and 0.97 mmol/h; while MAO decreased to 1.04 mmol/h, 1.76 mmol/h and 1.29 mmol/h, respectively. Gastric acid secretion was significantly suppressed by 56%-80%, which showed that all of the three operations can effectively inhibit gastric acid secretion in dogs (Table 1).

Effects of different operative procedure on serum gastrin level

Pre and post-operative fasting and postprandial serum gastrin levels of DPG-BII, PPG and HSV groups are shown in Table 2. In DPG-BII, post-operative fasting and postprandial serum gastrin levels were significantly decreased ($P<0.05$), the inhibiting rate was 49.7% and 48.4% respectively; while in PPG, serum gastrin levels were slightly decreased with an inhibiting rate of 25.9% and 24.4%; in HSV, post-operative serum gastrin levels were increased by 65.2% and 54.1%, respectively.

Effects of different operative procedure on G cell density and functional activity

Postoperatively, G cell density increased in all sites checked. The increasing rate in duodenum was about 75.0% and 50.0% in antrum or residual antrum (Table 3). The increase in jejunum had no statistical significance.

Stained by immunohistochemical method, G cell was stained in brown color and there were brown-black granules in cytoplasm, which were the products of gastrin acted with its antibody and presented as the index of activity of G cell. If 1+

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and 2+ grade cell was taken as normal- or hypofunction, while 3+ and 4+ as hyperfunction, the number of grade 3+ and 4+ G cells as a whole constituted 44% and 60% of the total G cells examined in pre and post-operative specimens respectively, and particularly in duodenum the corresponding postoperative rate was 63%. It reveals that no matter what procedure of gastrectomy was performed, the post-operative G cell functional activity, especially in duodenum was enhanced with statistical significance (Table 4).

Table 1 Effects of different operative procedures on gastric acid secretion

Operation	Group	Preoperation (mmol/h)	Postoperation (mmol/h)	Inhibiting rate (%)
DPG-BII	BAO	1.80 ± 0.25	0.48 ± 0.20 ^b	73.7
	MAO	5.19 ± 0.56	1.04 ± 0.19 ^b	80.0
PPG	BAO	2.25 ± 0.27	0.98 ± 0.26 ^a	56.4
	MAO	4.49 ± 0.34	1.76 ± 0.19 ^b	60.7
HSV	BAO	2.19 ± 0.21	0.97 ± 0.26 ^a	55.9
	MAO	5.30 ± 0.14	1.29 ± 0.47 ^b	75.7

^aP<0.05; ^bP<0.01, vs preoperation.

Table 2 Effects of different operative procedure on serum gastrin level

Operation	Group	Pre-operation (ng/L)	Post-operation (ng/L)	Changing rate(%)
DPG-BII	fasting	179 ± 104	90 ± 117 ^a	↓ 49.7
	postprandial	181 ± 86	94 ± 39 ^a	↓ 48.8
PPG	fasting	190 ± 153	144 ± 63	↓ 25.9
	postprandial	239 ± 115	180 ± 47	↓ 24.4
HSV	fasting	100 ± 10	166 ± 75	↑ 65.2
	postprandial	103 ± 48	186 ± 63	↑ 54.1

^aP<0.05, vs preoperation.

Table 3 Effects of different operative procedure on G cell density

Operation	Site	Preoperation (cell/field)	Postoperation (cell/field)	Increasing rate(%)
DPG-BII	Duodenum	23.1 ± 5.0	41.3 ± 4.9 ^b	78.9
	Jejunum	1.1 ± 1.1	3.2 ± 3.0	190.4
PPG	Antrum	66.2 ± 2.1	103.3 ± 18.8 ^a	56.0
	Duodenum	15.6 ± 5.3	27.1 ± 3.6 ^a	74.3
HSV	Jejunum	1.0 ± 4.2	1.1 ± 1.9	11.0
	Antrum	69.8 ± 23.2	103.3 ± 19.3 ^b	47.6
HSV	Duodenum	33.7 ± 15.1	60.1 ± 21.5	78.5
	Jejunum	5.5 ± 3.3	17.3 ± 9.2	218.3

^aP<0.05; ^bP<0.01, vs preoperation.

Table 4 Effects of different operations on G cell function

Operation	Site	Group	1+	2+	3+	4+
DPG-BII	Duodenum	Preoperation	21	142	106	29
		Postoperation	24	73	157	46 ^b
PPG	Antrum	Preoperation	32	136	71	61
		Postoperation	23	115	64	98 ^a
PPG	Duodenum	Preoperation	50	124	81	45
		Postoperation	24	93	117	68 ^b
HSV	Antrum	Preoperation	55	105	84	56
		Postoperation	38	94	73	95 ^a
HSV	Duodenum	Preoperation	67	107	74	52
		Postoperation	24	99	81	96 ^b

^aP<0.05; ^bP<0.01, vs preoperation.

DISCUSSION

According to the theory "no acid, no ulcer", anti-acid secretion has been the dominant measure in treating peptic ulcer. For suppressing acid secretion, how to treat the antrum has been a much controversial question in general surgery^[4]. Total antrum excision would make the serum gastrin level and gastric acid output lowered, which was accompanied with relatively lower ulcer recurrence; on the other hand, damage of sphincter function resulted in dumping syndrome, reflux gastritis,

dyspepsia and even carcinogenesis of residual stomach^[1]. Under this condition PPG was presented, which not only removed the ulcer lesion and suppressed gastric acid secretion, but also preserved the sphincter function^[5]. Our results showed that all the three procedures can effectively inhibit gastric acid secretion in spite of the different postoperative serum gastrin levels. Clinically, similar results were observed that absolute serum gastrin value of patients were all kept within normal limits, regardless their gastrin level decreased or increased after DPG-BII, PPG or HSV^[2]. This implied that different disposal of antrum did not obviously affect the inhibition of gastric acid secretion.

Gastric acid secretion is a complex physiological process, which was regulated by several factors, such as vagus nerve, G cell, parietal cell and its receptor, some alimentary endocrine substances, gastric mucosal blood supply^[6]. Of them any change may inhibit the gastric acid secretion and keep it at lower output level. In addition to regulating acid secretion, gastrin has important effects on nourishment of gastric mucosa and pancreas^[7]. Our results showed that there were many G cells in duodenum and jejunum besides antrum. After operation, the number of G cells in the nongastric tissue increased and their function enhanced, this was not only associated with the gastric acid depletion, but also was demanded by other physiological effects. Therefore it is evidently impossible and unnecessary to eliminate gastrin from serum by operation of peptic ulcer. To some extent, hypergastrinemia subsequent to treatment of peptic ulcer, such as HSV and antacid drugs, is the main determinant of ulcer healing^[8]. It is the key point that how to keep the whole function of sphincter. Fukushima *et al*^[5] has discovered that the length of preserved antrum was closely related to the residual stomach function. In our study, the length of preserved antrum was strictly limited within 1.5 cm to 2.0 cm, vomiting, decline of food intake and loss of body weight were not found postoperatively in the animals which suggested that the function of sphincter had been fairly maintained.

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Enzymohistochemical study on burn effect on rat intestinal NOS

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INTRODUCTION

The blood irrigate flow obstruction, especially the gastrointestinal (GI) ischemia^[1], is the main factor of the damage to the digestive tract caused by serious burns. The effect of GI ischemia on 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 releases a large quantity of inflammatory media. Neuroendocrine element after burns is closely related to intestinal damage^[2]. As is known at present, abundant nitric oxide synthase (NOS) is distributed in GI tract, whose product NO is a nonadrenergic and noncholinergic (NANC) restraining transmitter in enteric nervous system which participates extensively in various physiological functions in the intestinal tract^[3]. Few reports about the effects on intestinal NOS after serious burns are available. We used scalded rat model with degree III 40% of body surface area (TBSA), and enzymatic histological and biochemical methods to observe dynamically the active changes of empty myenteric plexus NOS and changes of jejunal tissue MDA SOD and GSH-PX and probe into the relationship between NOS and intestinal tissue and function damage as well as their mechanism so as to provide morphological experimental basis for clinical treatment.

MATERIALS AND METHODS

Materials

B-NADPH, TYPE, nitroblue telrazolium (NBT) (Sigma); SOD reagent kit (Wuhan Xiehe Hospital Science and Technology Development Centre); thibarbiluric acid (Shanghai Reagent Factory); self-made phosphate buffer; and healthy SD rats weighing 250 g provided by the Animal Laboratory Centre of Tongji Medical University.

Methods

Eighty rats were randomly divided into five groups: sham-burn control (SBC) and 4 groups of 8, 24, 48

and 72 hours postburn, each group having 16 rats (40 were measured for biochemical indexes, and other 40 for morphological indexes). The rats, before being scalded, had their hair depilated on the neck and back with 80 g/L Na₂S under the anaesthesia with 100 g/L trichloroacetaldehy-demonohydrate after they became conscious, depilated parts were soaked into the 100°C boiling water for 16 sec and scalded to degree III 400g/L TBSA. They were then fed in different cages without treatment. And those in sham-burn control group were soaked into 37°C water.

Rats in different groups, after being scalded, were sacrificed at different time points, a 10 cm empty intestine was taken out and made into thick liquid. SOD activity was determined with modified pyrogallol auto-oxidation^[4]. GSH-PX activity by active DTNB^[5], and the content of MDA with modified thiobarbituric acid fluorescence analysis^[6].

The rats in each group were anaesthetized with 100 g/L trichloroacetaldehy demonohydrate (300 mg/kg), opened up to expose the heart, the blood was quickly rinsed away with 200 mL warm normal saline from left chamber through ascending aorta, and then 450 mL of 40 mL/L cold parafor-maldehyde was instilled for one hour, a 10 cm empty intestine was taken out, the intestinal content was washed off and filled again with the same liquid to make the intestinal track full, both ends were ligated and fixed in the above-mentioned liquid (4°C) for 4 hours. The outer longitudinal tunica muscularis was peeled off carefully from the ring tunica muscularis and tela submucosa, and the specimens were made.

The specimens after being washed with 0.1 mol/L of PBS, was put into reduction type NADPH-d hatching liquid at 37°C for 50 min (consisting of 0.1 mol/L of PB (pH 8.0) which contained 30 g/L tritonx-100, 100 mol/L of nitroblue telbrazolium and 1 g/L of NADPH-d, and washed thoroughly with 0.1 mol/L of PBS. After reaction^[7], it was pasted onto a galative glass piece and conventionally dehydrated, made into transparency, sealed and observed under microscope. The staining result of NOS was achieved using IBAS automatic picture analyzer to determine semi-quantitatively the contents in myenteric plexus neuron NOS and internode bind NOS.

Statistical treatment

The data were expressed as $\bar{x} \pm s$, and *t* test was used to compare the results.

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RESULTS

Changes of the contents of MDA, SOD, GSH-PX in the empty intestinal tissues of burned rats

The content of MDA in the empty intestinal tissues gradually rose with the time of burns; compared with the SBC, the contents in each group increased significantly ($P < 0.01$). But the activity of SOD and GSH-PX decreased markedly the difference being significant as compared with SBC ($P < 0.01$).

Active change of myenteric plexus NOS in the empty intestine of burned rats

Observation under light microscope In the control group, most ganglion cells in myenteric plexus and their scabrosity appeared strongly positive in NOS, while a small number had moderate staining, most being distributed around myenteric plexus and the cell being comparatively large and various in shape, i.e., ovate, triangle or irregular. There were NOS positive products in dark blue in the cytoplasm and the nucleus was negative. Most of intersegment bind fiber contained bulge, and some had division and intersect with varying size (Figure 1). In the group

of 8 hours post-burn, the cell NOS stain appeared obviously lighter but intersegment bind fiber had no obvious change (Figure 2). In the 24 hours post-burn group, ganglion cell NOS staining was intensified obviously with clear cell outline and larger size. Intersegment bind fiber and bulge became markedly dense (Figure 3). In the 48 to 72 hour post-burn groups, ganglion cell and intersegment bind fiber staining gradually became lighter, especially in those of 72 hour group (Figure 4), perikaryon and OD change of myenteric plexus NOS in the empty intestine of burned rats. In burned rats after 8 hours the OD of myenteric plexus and intersegment bind fiber NOS were significantly lower than that in the control group ($P < 0.01$) and in those after 24 hours, the OD of ganglion cells and intersegment bind fiber NOS was significantly higher than that in the control ($P < 0.01$); while in those after 48 to 72 hours, OD of ganglion cells and intersegment bind fiber NOS decreased gradually, being significantly different from the control group and those of 24 hours post-burn ($P < 0.01$).

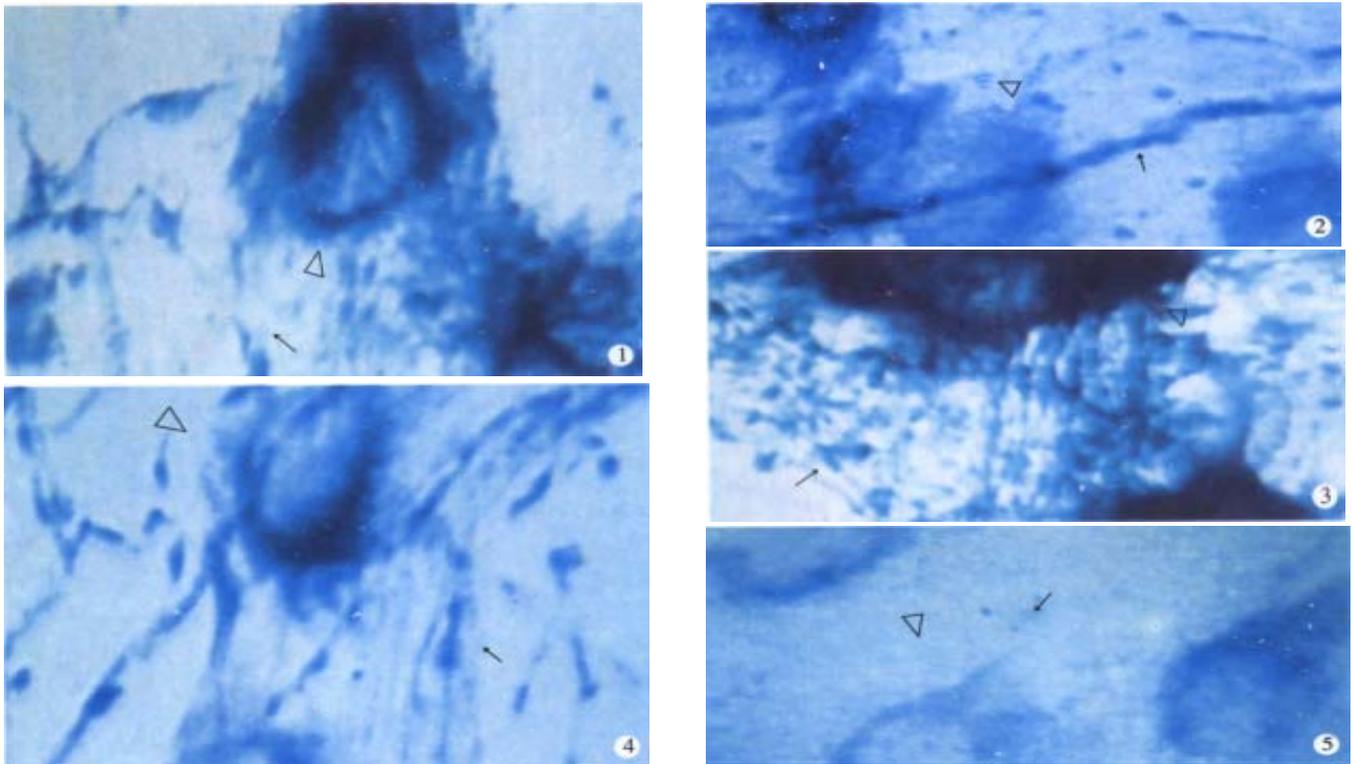


Figure 1 Myenteric plexus neuro bind ganglion cell (Δ) and positive reaction of NOS of intersegmental bundle (\uparrow) in the control group. $\times 400$

Figure 2 Myenteric plexus neuro bind ganglion cell (Δ) and positive reaction of NOS of intersegmental bundle (\uparrow) reduced obviously in the group of 8 hours post-burn. $\times 400$

Figure 3 Myenteric plexus neuro bind ganglion cell (Δ) and positive reaction of NOS of intersegmental bundle (\uparrow) increased obviously in the group of 24 hours post-burn. $\times 400$

Figure 4 Myenteric plexus neuro bind ganglion cell (Δ) and positive reaction of NOS of intersegmental bundle (\uparrow) reduced obviously in the group of 48 hours post-burn compared with the group of 24 hours post-burn. $\times 400$

Figure 5 Myenteric plexus neuro bind ganglion cell (Δ) and positive reaction of NOS of intersegmental bundle (\uparrow) reduced obviously in the group of 72 hours post-burn. $\times 400$

DISCUSSION

NO, a newly discovered active biosubstance in recent years, is a dual-functional messenger and venosity molecule with short half-life and instable nature. The *in vivo* NO, in the form of L-Arg as primer, exists through the catalyza tion of NOS, which indicates that the distribution of NOS is closely related to the physiological functions of NO. It is reasonable to infer bioeffects of NO through the research of NOS, Nathan *et al*^[8] divided NOS into two types, i.e. cNOS and iNOS. cNOS is mainly distributed in neurocyte and endothelium and catalyzes NO which acts chiefly as neurotransmitter and secondary messenger, while iNOS, mainly distributed in macrophage and endothelium cell, catalyzes NO, which has cytotoxic effect. However, the physiological effect of NO to the body depends chiefly on the quality and strength of stimulative elements, the dosage and reactive sites. Thus we can presume that after serious burns bad perfusion of intestinal blood, disorder of motor functions and damaged enteromyocoderm may be related to the abnormal intestinal NOS activity. It was suggested that MDA content in the jejunal tissues of the burned rats increased, the result was consistent with that of Peng *et al*^[9]. At the same time, SOD and GSH-PX antioxidase activities in jejunal tissues decreased after being burned, indicating that lipoperoxidation reactions participate in the pathophysiological course of the mucosal damage of burned rats. Some studies demonstrated that cNOS was distributed extensively in plexuses of GI wall, and its output NO was inhibitory neurotransmitter of NANC nerves in GI tract, which may cause angiectasis of intestinal tract and laxation of smooth muscles^[10]. Eight hours after serious burns myenteric plexus NOS activity decreased obviously in our experiment suggesting burn stress caused adrenergic nerve excitation, and inhibited NANC nerves, leading to the decrease of cNOS activity and the catalyzed NO, causing excessive contraction of smooth muscles. These may be the main causes in bad perfusion of intestinal blood and disorder of motor functions. In this experiment, 24 hours post-burn, myenteric plexus NOS activity increased significantly, possibly because the enterogenous infection after serious burn, activated the endotoxin on macrophage cell of intestine to release a series of such body fluid agents as TNT IL-1, etc, leading to increased iNOS

activity. A large amount of high-concentration NO produced in this way had cytotoxic effects which further damaged the intestinal tissues. Excessive amounts of NO and O₂ free radicals produced more toxic ONOO⁻ free radicals which further react to produce HO[·], NO₂, NO₃⁻, etc. while HO[·] and NO₂ are catalyst of biomembrane lipid peroxidation and can cause a succession of peroxidation of high unsaturation and adipocid occurring on the membrane, thus producing a large amount of metabolin MDA worsening the damage of intestinal tissues. Therefore 48-72 hours post-burn, we found that intestinal myenteric plexus ganglion cell and perikaryon reduced obviously, and changes caused by the lowered NOS activity and other delayed neuronal damage. It can be inferred that NO in the intestine of seriously burned rats and ONOO⁻ reacted by O₂ are important mechanisms that NO damages intestinal structure and functions. It suggests that the damaged structure and functions in seriously burned rats are related to the increase of NOS activity. It is concluded that using the inhibitor of NOS to lower NOS activity level may be beneficial to lessening the degree of post-burn intestinal damage.

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Protective effect of electroacupuncture and moxibustion on gastric mucosal damage and its relation with nitric oxide in rats

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Subjects headings acupuncture and moxibustion; nitric oxide; gastric mucosal damage; gastric mucosal blood flow; transmucosal potential difference; rats

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INTRODUCTION

Gastric mucosal injury is one of the common disorders, there are many reports subjected to its pathogenesis treatment and prevention^[1-4]. We investigated the protective effect of electroacupuncture and moxibustion of Zusanli point on gastric mucosal damage and its relation with nitric oxide (NO) on the animal model with acute gastric mucosal damage induced by ethanol. The detected indexes include the content of NO, gastric mucosal blood flow (GMBF), gastric mucosal lesion index (LI) and transmucosal potential difference (PD).

MATERIAL AND METHODS

Drugs L-arginine (L-arg)—the precursor of NO (purchased from Huamei Biological Engineering Corporation); Natrii Nitroprussidum (SNP)—the donor of NO (provided by Experimental Drug Plant of the Beijing Institute of Pharmaceutical Industry), diluted with distilled water prior to use, Nw-nitro- L-arginine (L-NNA), an inhibitor of NOS (produced by Sigma), and diluted with 0.2 mol·L⁻¹ PBS; 200 g·L⁻¹ pentobarbital sodium was prepared with distilled water immediately before use.

Animal grouping

Wistar rats weighing 180 g-250 g (provided by the Institute of Acupuncture and Moxibustion, Anhui College of Traditional Chinese Medicine) were

fasted for 12 h and allowed free access to water. The animals were divided into 16 groups randomly, including: control group (c), model (m), m+electroacupuncture (EA), m+moxibustion (M), L-arg+m, SNP+m, L-NNA+m, L-NNA+L-arg+m, L-arg+m+EA, L-arg+m+M, SNP+m+EA, SNP+m+M, L-NNA+m+EA, L-NNA+m+M, L-NNA+L-arg+m+EA, L-NNA+L-arg+m+M.

Preparation of acute gastric mucosal damage model^[5]

The rats fasted for 12 h were injected with pentobarbital intraperitoneally at a dose of (30-40) mg·Kg⁻¹. The middle line incision below xiphoid process after anesthesia was performed, then stomach was exposed and injected with 2 mL 70% ethanol, and the abdomen closed, 30 min later the preparation of model was fulfilled.

Experimental procedure

Different procedures were used for different groups. In groups injected drugs or equivalent amount of physiological saline, these were injected respectively into blood flow slowly through the great saphenous vein of the anesthetized rats 15 min before the preparation of the model. In groups which required electroacupuncture or moxibustion, were treated with electroacupuncture or moxibustion on bilateral Zusanli points after the model was successfully prepared. The electroacupuncture was performed with PCE₂ electroacupuncture therapy device on the condition of frequency 20Hz, voltage 5v-8v for 30 min (produced by the Institute of Acupuncture and Moxibustion of Anhui College of Traditional Chinese Medicine). During moxibustion, the lighted pure moxa-cigar was maintained 1 cm from the bilateral Zusanli points. The GMBF and PD values were measured for all rats. Then the blood was obtained through decapitation or from retro-ocular vessel for the NO assay. Subsequently the rat stomach was resected for the analysis of LI, and the mucosa from gastric antrum and the body for assaying NO contents.

Index detection

GMBF was assayed by analyzing hydrogen gas clearance curves^[6]; PD was assayed with direct Ag-

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AgCl. Electrode measurement technique^[7] with few alterations, i.e. the efficient electrode was put on the mucosa while the reference one on the serosa. The electric potential difference between two membranes was recorded. For the detection of LI, the stomach was resected and incised along the greater curvature, washed with physiological saline. Guth index assessment^[8] (slightly modified) was used to compute the damage, on a scale of grades 1-5 as follows: Grade 1, the petechia or ecchymosis, Grade 2, 3, 4 and 5, pathological focus of 1 mm; 1 mm-2 mm, 2 mm-4 mm, and over 4 mm, respectively. The NO content in blood and mucosa of gastric antrum and body were assayed based on the method from Green *et al*^[9]. The Kit for NO assay was provided by the Department of Biochemistry, Institute of Radiology, Academy of Medical Science of PLA. The analysis was based on the protocol, but the mucosa were homogenized before the assay.

Statistical analysis

All data were expressed as $\bar{x} \pm s$, and the Student's *t* test was used for the comparison between groups.

RESULTS

The effect of acupuncture and moxibustion of Zusanli point on GMBF, PD and LI in rats with the gastric mucosal damage (Table 1)

The mean of GMBF, PD in group m showed statistically significant difference compared with the control group ($P < 0.01$), demonstrating marked decrease of GMBF and PD after gastric mucosal damage, the integrity of gastric mucosa was depended on adequate blood flow. GMBF and PD in group m+M was obviously increased ($P < 0.01$) compared with group m, suggesting the electroacupuncture and moxibustion of Zusanli point may relieve and cure the gastric mucosal damage induced by ethanol.

Changes of NO content in blood and gastric mucosa and their relation with LI after electroacupuncture and moxibustion on the Zusanli point of rat models

From Table 1 we can find, NO contents in group m showed significant difference compared with normal control ($P < 0.01$); LI in group m+EA and group m+M was markedly lower than that in group m ($P < 0.01$). The results showed that the gastric mucosal damage aggravated as NO content decreased while NO content increased and the gastric mucosal damage relieved, which suggests that NO content is associated with the integrity of gastric mucosa.

Effect of L-NNA, L-arg and SNP on the response of electroacupuncture and moxibustion (Table 1)

The L-Arg (150 mg·Kg⁻¹), SNP (200 µg·Kg⁻¹) and L-NNA (3 mg·Kg⁻¹) were administered through the great saphenous vein respectively 15 min before administration of 700 mL/L ethanol in different groups. After that the electroacupuncture and moxibustion of Zusanli point were performed for 30 min. Subsequently, the GMBF, PD and LI as well as NO content in blood and gastric mucosa were assayed and detected respectively in each group. It was found that there was little difference in the indexes when group L-arg +m and group SNP+m were compared with group m+EA or group m+M ($P < 0.05$), indicating the function of electroacupuncture and moxibustion on gastric mucosal damage was similar to that of L-arg or SNP. Comparing group L-Arg+m+EA (or M) and group SNP+m+EA (or M) with group m+EA (or M), and group SNP+m+EA (or M) with group m+EA (or M), NO content, GMBF and PD values were markedly increased, and LI value was obviously decreased ($P < 0.01$ or $P < 0.05$), which suggested that electroacupuncture and moxibustion of Zusanli point had the effect of strengthening NO pathway and protecting gastric mucosa.

In comparison of group L-NNA+m with group m, NO content, GMBF and PD value decreased, and LI value increased ($P < 0.01$ or $P < 0.05$), showing that L-NNA aggravated ethanol induced gastric mucosal damage. When we compared group L-NNA+m+EA (or M) with group m+EA (or M), NO content, GMBF and PD value also decreased, but LI value increased ($P < 0.01$ or $P < 0.05$), which suggests L-NNA decreased the protective action of electroacupuncture and moxibustion on gastric damage. While we compared group L-NNA +L-arg +m+EA (or M) with group L-NNA+m+EA (or M), NO contents, GMBF and PD value increased and LI value decreased ($P < 0.01$ or $P < 0.05$), demonstrating L-arg may reverse the inhibitory function of L-NNA. In conclusion, the above mentioned results further suggest that the protective action on gastric mucosa by the electroacupuncture and moxibustion of Zusanli point is NO mediated.

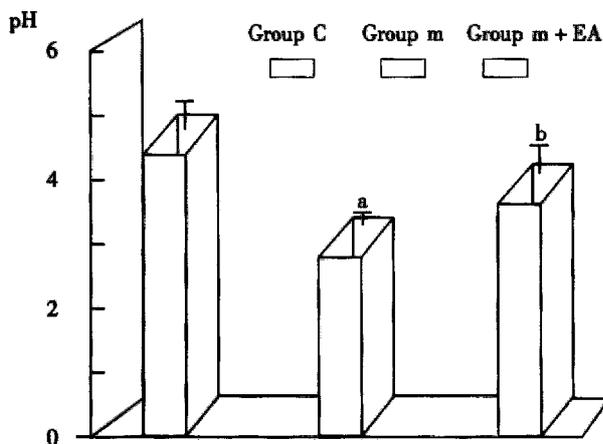
Effect of electroacupuncture of Zusanli point on pH value of gastric fluid (Figure 1)

pH in group c was 4.33 ± 0.40 and that in Group m 2.92 ± 0.37 , decreasing significantly in the latter group ($P < 0.01$). The pH value in Group m+EA, 3.84 ± 0.69 was much higher than that in Group m indicating 700 mL/L ethanol promotes the stomach to secrete acids while electroacupuncture inhibits the secretion.

Table 1 Effect of electroacupuncture and moxibustion of Zusanli point on LI, GMBF, PD and NO after gastric mucosal damage in rats ($\bar{x} \pm s$)

Group	n	LI	GMBF (mL·min ⁻¹ ·100g ⁻¹)	PD (mV)	NO	
					Blood (μmol·L ⁻¹)	Antrum(ng·mg ⁻¹)
C	5	0	138.20 ± 4.37	20.82 ± 0.99	21.12 ± 1.89	53.30 ± 2.65
m	5	45.6 ± 3.2 ^a	60.60 ± 6.09 ^a	11.85 ± 0.82 ^a	12.84 ± 1.54 ^a	35.17 ± 1.57 ^a
m + EA	5	31.4 ± 3.3 ^c	92.55 ± 6.35 ^c	15.84 ± 0.48 ^c	18.65 ± 0.69 ^c	48.51 ± 2.12 ^c
m + M	5	33.8 ± 2.4 ^c	95.91 ± 5.59 ^c	15.96 ± 0.34 ^c	18.53 ± 1.04 ^c	48.49 ± 2.39 ^c
L-arg + m	5	32.4 ± 3.2	106.22 ± 5.30	17.73 ± 0.22	18.93 ± 1.30	47.57 ± 2.86
SNP + m	5	30.0 ± 3.1	104.03 ± 7.57	17.89 ± 0.39	19.07 ± 1.29	47.18 ± 2.17
L-NNL + m	5	55.8 ± 2.8 ^b	56.85 ± 5.96 ^d	6.97 ± 0.32 ^c	8.37 ± 0.27 ^b	27.29 ± 1.71 ^b
L-NNA + L-arg + m	5	46.8 ± 2.6	78.90 ± 5.10	10.93 ± 0.39	12.10 ± 1.23	33.76 ± 2.44
L-arg + m + EA	5	21.0 ± 0.9 ^e	121.50 ± 3.55 ^d	18.14 ± 0.44	24.70 ± 0.75 ^d	50.63 ± 2.34
L-arg + m + M	5	27.1 ± 2.7 ^d	97.46 ± 4.90	16.08 ± 0.45	21.06 ± 1.17	44.26 ± 2.01
SNP + m + EA	5	20.9 ± 0.8 ^e	118.50 ± 4.21 ^d	17.79 ± 0.59	24.80 ± 0.50 ^d	45.17 ± 2.01
SNP + m + M	5	26.8 ± 2.2 ^d	101.11 ± 5.23	14.73 ± 0.61	23.14 ± 1.52 ^d	43.33 ± 1.97
L-NNA + m + EA	6	41.8 ± 2.1 ^e	73.57 ± 5.43 ^d	11.87 ± 0.41 ^d	12.37 ± 1.78 ^e	39.25 ± 1.66 ^d
L-NNA + m + M	6	40.5 ± 3.0 ^d	66.42 ± 5.42 ^e	10.25 ± 0.72 ^e	11.43 ± 1.03 ^e	32.17 ± 2.35 ^e
L-NNA + L-arg + m + EA	5	31.0 ± 1.2 ^g	95.00 ± 3.78 ^f	16.15 ± 0.74 ^g	19.24 ± 0.54 ^g	51.51 ± 2.14 ^f
L-NNA + L-arg + m + M	5	31.5 ± 2.1 ^g	70.76 ± 4.78	11.81 ± 0.53	12.88 ± 0.68	36.37 ± 2.29

^a*P*<0.01 vs Group C; ^b*P*<0.05, ^c*P*<0.01 vs Group m; ^d*P*<0.05, ^e*P*<0.01 vs Group m+EA (orM); ^f*P*<0.05, ^g*P*<0.01 vs Group L-NNA+m+EA (or M).

**Figure 1** Effect of electroacupuncture on pH value with gastric mucosal damage in rats.

^a*P*<0.01 vs Group C; ^b*P*<0.01 vs Group m.

DISCUSSION

Acupuncture and moxibustion may treat and prevent gastrointestinal disorders, the mechanism of which is being further investigated. The acupuncture and moxibustion on Zusanli points show the two-way regulation of gastrointestinal function. In recent years, it is frequently reported that they may relieve symptom and promote the ulcer healing to certain extent^[1-4]. There is relative point specificity, the first choice is the Zusanli point in stomach channel of foot yangming. Our study investigated the therapeutic and protective effect of the acupuncture and moxibustion on acute gastric mucosal damage induced by 700 mL/L ethanol. It is found that

GMBF and PD value as well as NO content, pH value in rat gastric mucosa of model group with acupuncture and moxibustion increased more markedly than those without any therapy (*P*<0.01), while the gastric mucosal damage alleviated (*P*<0.01) which denotes the therapeutic and protective effect of acupuncture and moxibustion on the damage and suggests that the protective action is related with the level of NO content.

NO is a small molecular gas produced from the precursor L-Arg under the NOS catalysis. The process is called as L-Arg-NO pathway, NOS activity plays a key role in NO synthesis. NOS is existed in many kinds of tissues including vascular endothelial cell, thrombocyte, brain cell, renal epithelial cell, macrophage, neutrophil, hepatocyte, etc. Some factors can induce the expression of NOS and increase the NO synthesis. As a special bioinformational molecule, the neurotransmitter or the humoral factor, NO involves in the functional regulation and pathophysiology of many diseases. Our study investigates the protective effect of acupuncture and moxibustion on acute gastric mucosal damage and in the meantime measures NO content in blood and gastric mucosa and finds that NO content is negatively correlated with the severity of gastric mucosal damage, which proves that the protective action of acupuncture and moxibustion of Zusanli point is NO mediated; and in other words NO involves in the regulative effect of acupuncture and

moxibustion on gastrointestinal function.

The good balance between NO and ET keeps the endothelium intact. If the unbalance is developed, the gastric mucosal damage will be formed and the gastric dysfunction presented. Our study showed^[1] that when NO content decreased ET content increased and gastric mucosal damage aggravated, and on the contrary, the damage alleviated. Our results have verified it. When L-arg or SNP was administered 15 min before establishing the model with 700 mL/L ethanol; NO content was higher than that in the model group and concurrently GMBF and PD value increased, and LI value decreased; if NOS inhibitor -L-NNA was given, NO content, GMPF and PD value were lower than those in the model group while LI value increased, which indicates that NO plays an important role in maintaining the intact gastric mucosa. GMBF decrease may prominently respond to the gastric mucosal damage after ethanol induction. It is thought that NO is an EDRF, which is capable of relaxing the vascular smooth muscle obviously. Our research further confirmed that the vascular relaxing action of NO can protect gastric mucosa from damage.

NO initiates the protective effect of acupuncture and moxibustion on gastric mucosa, which has been proved in our work. During experiment, in the model rats, the NO content in their blood and gastric mucosa increased after acupuncture and moxibustion were performed, and GMBF and PD values were also increased while LI value decreased. If L-NNA was preliminarily given, the above effect of acupuncture and moxibustion of Zusanli point disappeared, but concurrently given L-arg, the action of L-NNA could be reversed. In addition, if L-arg or SNP was preliminarily administered and then the model established, NO content in the group with the acupuncture and moxibustion of Zusanli point increased more than that in the corresponding groups without acupuncture and moxibustion, moreover, GMBF and PD values increased and LI decreased correspondingly, showing the synergetic action between them. The above phenomena further confirm that the acupuncture and moxibustion of Zusanli points alleviate gastric mucosal damage, which is NO mediated, and this illustrates that the acupuncture and moxibustion of Zusanli point can activate endogenous NOS.

As far as the possible mechanism why Zusanli point can initiate NO system is concerned, we consider that the main nerves regulating gastric function are vagus nerves, the dorsal vagus nucleus (DMV) being the major motor nucleus. The efficient stimulus transmitted to DMV by the somatic sensory nerves through the spinal cord. DMV also receives afferent information from gastrointestinal tract and integrates other information from central nervous system (CNS), and transfers to the stomach by efferent vagus nerves, resulting in the increase of NO synthesis and release.

The possible mechanism about how NO can protect gastric mucosa from damage is as follows: NO acts as one of endothelial diastolic factors which can cause the dilatation of vascular smooth muscles, resulting in GMBF increase, improvement of blood supply of gastric mucosa, maintaining the integrity of gastric mucosal epithelium, protecting the gastric mucosa from the stimulation of gastric contents and from damage, preventing from H⁺ invasion and keeping normal ionic concentration gradient across gastric mucosa as well as maintaining appropriate PD value, while acupuncture and moxibustion may activate NOS to increase the synthesis and release of NO. Further research remains to be done as how does the acupuncture and moxibustion activate NOS.

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Research of *Helicobacter pylori* infection in precancerous gastric lesions

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INTRODUCTION

Helicobacter pylori (*Hp*) infection has been considered to play significant roles in pathogenesis of peptic ulcer. Additionally *Hp* is associated with the development of gastric epithelial hyperplasia and lymphoid malignancies. The International Agency for Research on Cancer has classified *Hp* as a class I carcinogen and a definite cause of gastric cancer in humans. *Hp* infection first causes chronic active gastritis and may slowly lead to infection of whole stomach. In the late stages of infection, mucosal atrophy and intestinal metaplasia (IM), and even dysplasia (DYS) occur^[1]. Chronic atrophic gastritis (CAG), IM and DYS are considered markers for development of gastric cancer in high-risk individuals. In our study we analyzed *Hp* infection prevalence in 486 patients with precancerous gastric lesions.

MATERIALS AND METHODS

The mucosal biopsy specimens were collected from 486 patients subjected to routine gastroscopy, including 163 cases of CSG, 207 cases of CAG, 71 cases of IM and 45 cases of DYS. Biopsies were taken from five sites in the stomach: one from the angle, four from the lesser and greater curvature of gastric body and gastric antrum. Each biopsy was classified according to the presence or absence of CSG, CAG, IM and DYS, and scanned by Warthin-Starry method.

Data analysis was made with *Chi*-square test. Statistical significance was defined as $P < 0.05$.

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RESULTS

Table 1 Relation between precancerous gastric lesions and age and sex

Variables	n	CSG		CAG		IM		DYS	
		n	%	n	%	n	%	n	%
Sex									
Male	314	96	30.6	142	45.2	43	13.7	33	10.5
Female	172	67	38.9	65	37.8	28	16.3	12	7
Age (yrs)									
≤40	184	64	34.9	98	53.5	13	7.0 ^a	9	4.6 ^b
41-55	171	52	30.3	70	41.2	30	17.3	19	10.8
≥56	131	40	30.5	46	35.4	28	21.1 ^a	17	13.0 ^b

^a $P < 0.05$, ^b $P < 0.05$.

Gastric pathology data was available for 486 cases. As shown in Table 1, there was no significant difference between the two sexes ($P > 0.05$), but the prevalence rates increased with age, being significantly higher in ≥56 age group than ≤40 group for IM and DYS ($P < 0.05$).

Table 2 *Hp* infection rate in precancerous gastric lesions

Variables	n	Hp(+)		Hp(-)	
		n	%	n	%
CSG	163	39	23.9	124	76.1
CAG	207	88	42.5 ^a	119	57.5
IM	71	54	76.1 ^b	17	23.9
DYS	45	40	88.9 ^c	5	11.1

^a $P < 0.05$, CAG vs CSG; ^b $P < 0.01$, IM vs CSG; ^c $P < 0.01$, DYS vs CSG.

The prevalence of *Hp* increased steadily with increasing severity of gastric histopathology (Table 2). The detection rates of *Hp* in IM (76.1%) and DYS (88.9%) were significantly higher than that in CSG (23.9%, $P < 0.01$), and in CAG than in CSG ($P < 0.05$).

Table 3 *Hp* infection distribution in precancerous gastric lesions by biopsy sites

Variables	n	ALC		AGC		A		BLC		BGC	
		n	%	n	%	n	%	n	%	n	%
CSG	39	37	94.9	36	92.3	32	82.1	18	46.2	10	25.6
CAG	88	84	95.4	80	90.9	78	88.6	53	59.9	41	46.2
IM	54	49	90.7	46	85.2	40	74.1	34	62.7 ^a	32	58.8 ^a
DYS	40	36	90.0	33	82.5	30	75.0	28	70.0 ^b	26	63.9 ^b

ALC:antral lesser curvature; AGC:antral greater curvature; A: angulus; BLC:body, lesser curvature; BGC:body, greater curvature

^a $P < 0.05$, IM vs CSG; ^b $P < 0.05$, DYS vs CSG.

As shown in Table 3, the prevalence of *Hp* positivity tended to increase from BLC to BGC, *Hp* positive rates in IM and DYS were significantly higher than that in CSG in both BLC and BGC ($P < 0.05$).

DISCUSSION

The first compelling evidence linking *Hp* infection to gastric carcinoma was generated by seroepidemiologic studies^[2,3], bacterial seropositivity was significantly more common in those with gastric adenocarcinoma, with an odds ratio ranging from 2.8 to 6.0, suggesting a strong association between *Hp* and gastric malignancy.

Hp infection was related to both the intestinal and diffuse types of cancer as well as the precancerous lesion of IM or DYS^[4], and greater than 70% of gastric carcinomas are linked to IM, although DYS may also be seen without neoplastic disease.

Our study demonstrated that the prevalence rates of precancerous lesions varied with age, for IM and DYS, it had an upward trend with aging, while for CSG, it had a downward trend with aging, but were not different between the two sexes. The prevalence

rose steadily with increasing severity of gastric histopathology, the detection rates of *Hp* in CAG (42.5%), IM (76.1%) and DYS (88.9%) were significantly higher than that in CSG (23.9%), suggesting that *Hp* may play a role in late as well as early stages of carcinogenesis.

Our study also showed that *Hp* positive rates in IM and DYS were significantly higher than that in CSG in the lesser and greater curvature of gastric body, suggesting the more serious the gastric histopathology, the higher the *Hp* infection rate, furthermore, the higher level of *Hp* infection site. The study suggests we should take multiple site biopsy for histopathology and *Hp* examination.

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Antisense telomerase RNA induced human gastric cancer cell apoptosis

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INTRODUCTION

Human tissue homeostasis is precisely regulated by cellular division, differentiation and death. Normal human somatic cells progressively lose telomere restriction fragment (TRF) length with each successive cell division, eventually leading to cellular quiescence, chromosomal end-degradation and apoptosis^[1]. On the contrary, stabilization of telomere lengths by expressing telomerase, a an RNA-dependent DNA polymerase, may be involved in cellular immortality and carcinogenesis^[2-4]. Changes of telomerase activity and telomere lengths have been found in almost all human cancers^[4-10], but the evidence of their relationship with immortalization of cancers cell remain to be directly demonstrated. Since shortened telomere was first discovered in Hela cells with antisense RNA techniques in 1995^[11], anticancer agents based on inhibition of telomerase RNA have been reported^[12,13]. However, the relationship between telomerase inhibition and cell apoptosis has not been fully understood. In this study, we investigated the effect of blocking telomerase activity on apoptosis of human gastric cancer cells *in vitro* using an antisense vector for human telomerase RNA component (hTR) into human gastric cancer cells.

MATERIALS AND METHODS

Reagents

EcoRI, *BaI*I, *SaI*I, SP6 or T7 polymerases (Promega), hygromycin (Boehringer Mannheim) and lipofectamine (Gibco BRL) were commercially obtained. Antisense hTR expression construct vector (pBBS212) and hTR recombinant plasmid (pGRN83) were kindly donated by Dr Villeponteall (Geron Corporation, USA).

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Cell line

The human gastric cancer cell line SGC7901 (Fourth Military Medical University, China) was cultured in RPMI1640 (Gibco BRL) containing 100 mL/L fetal bovine serum.

Transfection of antisense hTR expression vector

The TRC₃ piece (hTR) was flanked by 2 *EcoRI* sites and was inserted into *EcoRI* site of pBBS212 to make the plasmids pBBS-hTR, which can express the antisense of hTR under the MPSV promoter^[11,14]. Cells at density of 2×10^5 /well (2 mL) in 6 well plates were transfected with purified plasmids (pBBS-hTR) and a selectable marker, hygromycin, by lipofectin procedure as described^[15]. As a control, cells were transfected either with an hTR-free plasmid (pBBS212) containing hygromycin marker or with hygromycin alone four clonal cells from each group were used for a series of experiments.

hTR expression by blot hybridization

Total RNA was prepared from each group cells by RNA isolation kit (Promega). PGR N83 was lined with *BaI*I or *SaI*I and transcribed *in vitro* using T7 RNA polymerase and SP6 RNA polymerase respectively. The transcription was carried out according to the manufacturer's instructions in the presence of 370GBg [α -³²P]UTP (Beijing Huri Co, China). Then a sense hTR or antisense hTR probe synthesized was used for RNA blot hybridization^[15].

Telomerase assay

Cell extracts were prepared by detergent CHAPS (Pierce), and telomerase activity was measured by telomeric repeat amplification protocol (TRAP) methods as described by Kim *et al*^[5,16]. The TRAP reaction products were separated by 100 g/L polyacrylamide gel electrophoresis and autoradiographed. The basal level of telomerase activity was measured by serial dilution of the protein extracts.

Determination of TRF length

Genomic DNA extracted from the gene transfected cells and the control cells were digested with *Hinf*I and *Rsa*I. DNA samples (8 μ g each) were loaded onto a 8 g/L agarose gel and electrophoresed for bromophenol blue to the bottom of gel at 90V. The gel was dried, denatured, neutralized and hybridized to a 5'-[³²P](TTAGGG)₄ probe (T₄ polynucleotide kinase, Promega) and *autoradi-*

ographed 48 h at -20°C ^[2,17].

Cell cycle analysis

Cells in log phase growth were collected, washed twice with phosphate buffered saline (PBS) and fixed in 70 mL/L ethanol at 4°C overnight. Cells were washed with PBS, digested with 20 mg/L RNase A at room temperature for 1 h, and then resuspended in 50 mg/L propidium iodide solution. Cell cycle was analyzed on the FAC/Scan (ELITEESP, Coulter Co) using a computer program interfaced with the integrator.

Ultrastructural observation

Ultrastructure in the gene transfected cells was observed under a transmissive electron microscope (JEM-2000EX, Japan).

RESULTS

Antisense telomerase RNA inhibited the sense telomerase RNA expression in the gene transfected cells. Antisense hTR expression was high and sense hTR expression was weak in RNA blot hybridization analysis (Figure 1). Sense hTR suppression rate analyzed by thin layer scanning reached 60% (Figure 1). Antisense RNA to hTR mediating telomerase activity was down-regulated in the gene transfected cells but not in the control plasmids transfected cells (Figure 2). Mean TRF length of

the gene transfected cells (11.0 ± 5.6) was also shorter than that of the control plasmids transfected cells (Figure 3). Compared with the control plasmids transfected cells, G1 phase decreased by 25% in the gene transfected cells and its apoptotic peak reached in 46.2% of cell cycle in a computer apoptotic program analysis (Figure 4). Either denaturation and necrosis or chromatin compaction and apoptotic body appeared in the gene transfected cells while the non-gene transfected cells showed no changes of this kind (Figure 5).

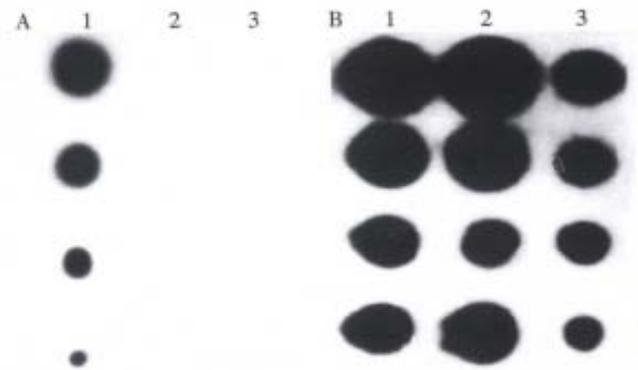


Figure 1 Blotting hybridization of hTR. (A) Antisense hTR expression. (B) Sense hTR expression. (1) SGC7901 cells. (2) SGC7901 cells transduced with control vector (pBBS). (3) SGC7901 cells transduced with vector expressing antisense hTR (pBBS-hTR).

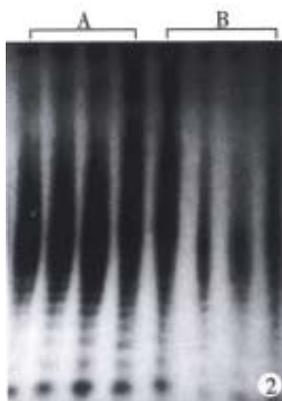


Figure 2 Telomerase activity of SGC7901 cells analyzed by TRAP. (A) SGC7901 cells transduced with control vector (pBBS). (B) SGC7901 cells transduced with vector expressing antisense hTR (pBBS-hTR).

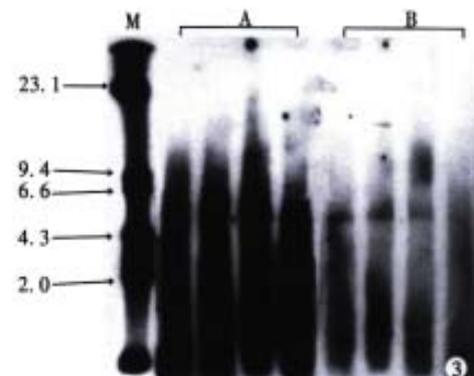


Figure 3 Telomeric lengths of SGC7901 cells analyzed by hybridization of nucleic acids directly in agarose gels. (M) λ -DNA/Hind III Molecular Markers. (A) SGC7901 cells transduced with control vector (pBBS). (B) SGC7901 cells transduced with antisense hTR expressing vector (pBBS-hTR).

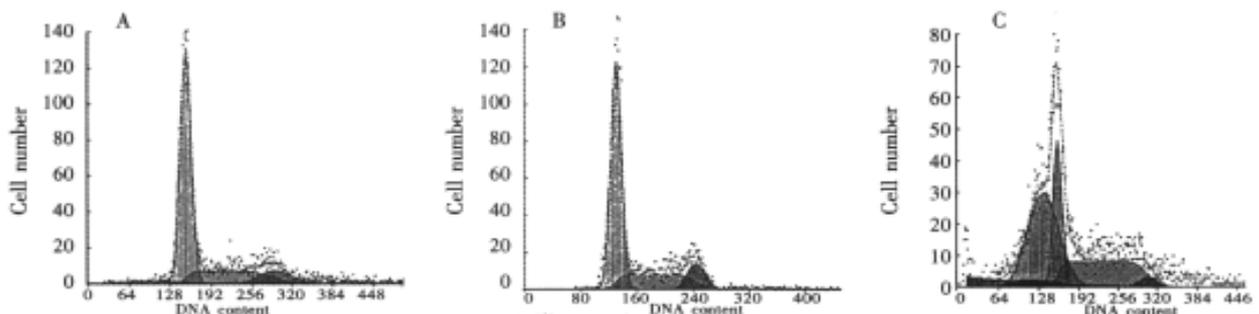


Figure 4 Cell cycle analyzed by flow cytometry. (A) SGC7901 cells. (B) SGC7901 cells transduced with control vector (pBBS). (C) SGC7901 cells transduced with vector expressing antisense hTR (pBBS-hTR).

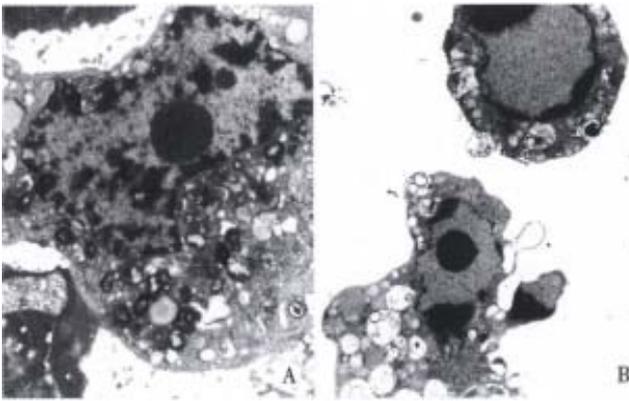


Figure 5 Ultrastructure observed under transmissive electron microscope. (A) SGC7901 cells transduced with control vector (pBBS). (B) SGC7901 cells transduced with antisense hTR expressing vector (pBBS-hTR).

DISCUSSION

Cell apoptosis is closely associated with tumorigenesis, tumor growth and tumor metastasis. Changes of telomere lengths may play an important role in maintaining cell division, proliferation, apoptosis and immortalization^[2,4]. Normal human somatic cells progressively lose their telomeric sequence with replicative senescence till cell "crisis" or apoptosis occurred^[4,18,19]. In contrast, almost all tumor cells and tissues express telomerase and maintain telomere length through an indefinite number of cell divisions^[4-10,19-24]. Antisense hTR, hammerhead ribozyme TeloRZ and antisense oligonucleotide against hTR could suppress tumor cell growth by inhibiting telomerase activity or shortening TRF length^[11-13]. Therefore, it has been proposed that antisense RNA based on telomerase inhibition may potentially reverse uncontrolled proliferation of tumor cells and induce apoptosis of cancer cells^[25,26]. The present study is undertaken to investigate the possible relationship between the antisense hTR expression and apoptosis of human gastric cancer cells.

Our study has demonstrated that the antisense hTR expression level of SGC7901 cells was high. Based on the histogram of flow cytometry, G1 phase was arrested and there was a typical apoptotic peak in the antisense hTR expression vector transfected cells. Meanwhile, compaction of nuclear chromatin and apoptotic body were observed under a transmissive electron microscope. These results suggest that antisense hTR may mediate apoptosis of human gastric cells. Also, we have found that the antisense hTR expression of SGC7901 cell could down-regulate telomerase activity and shorten telomere length. This supports the changes of telomere length and telomerase activity in antisense hTR transfected tumor cells^[11,12], indicating that telomerase can mediate telomeric sequence replication in gastric cancer cells

and that shortening of telomere length by the antisense hTR may be associated with apoptosis of human gastric cancer. Thus, the induction of cell apoptosis in SGC7901 cells expressing antisense hTR demonstrates the potential of telomerase inhibition as a therapeutic target for human cancer.

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Expression of lung resistance protein in patients with gastric carcinoma and its clinical significance

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Subject headings lung resistance protein/expression; pathology ; gastric cancer; drug resistance

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INTRODUCTION

The efficacy of chemotherapy in the treatment of cancer patients is often hampered by the presence or appearance of multidrug resistance (MDR) of tumor cells. One of the most important mechanisms of MDR is overexpression of P-glycoprotein (Pgp) which is encoded by *mdr1* gene^[1]. Recently, another MDR-related protein, lung resistance protein (LRP), has been identified^[2]. Our primary study indicated that LRP overexpressed in gastrointestinal carcinoma^[3]. In this paper, the expression of LRP in human gastric carcinoma and its significance were studied.

MATERIALS AND METHODS

Patients

All the 36 patients (21 men, 15 women; aged 32-78 years, mean age 54.6 years) were in-patients of our hospital admitted between September 1997-August 1998 and surgically resected specimens were diagnosed as adenocarcinoma by pathologists. No patients received chemotherapy before operation.

Methods

The expression of LRP in tumor tissues was detected by SABC (streptavidin-biotin complex) immunohistochemical staining as described before^[4], and in 14 of 36 specimens, the LRP was also analysed by flow cytometry (FCM), the matched mucosas served as normal controls, and peripheral blood lymphocytes as negative controls. The specific LRP monoclonal antibody LRP-56 was kindly supplied by Dr. R.J. Scheper

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(Department of Pathology, Free University Hospital, Amsterdam, the Netherlands), and the SABC immunohisto-chemical kit was purchased from Boster Biotechnology Company (Wuhan).

RESULTS

Expression of LRP in gastric carcinoma tissues

The LRP positive rate was 72.2% (26/36). LRP immunoreactivity was cytoplasmic, and in some specimens, the interstitial cells were also LRP immunostained. The intensity of the reactions was frequently strong and, in general, most of the cancer cells were LRP-positive in LRP-positive tissues.

Expression of LRP and pathologic parameters

The relationship between LRP expression and pathologic parameters is shown in Table 1. The LRP expression in highly and moderately differentiated carcinoma (9/9) was higher than that in mucoid carcinoma (6/11). There was no association between LRP expression and tumor size, lymphodal involvement, serosal invasion, or TNM stages.

Expression of LRP detected by FCM

LRP expression was at low to moderate levels in gastric cancer tissues (0%-20% in 10 patients, 21%-40% in 2 patients, and 41%-60% in 2 patients), and the mean LRP positivity rate was $29.9\% \pm 9.8\%$, significantly higher than that in normal ($16.9\% \pm 7.5\%$, $t = 3.94$, $P < 0.01$) and negative controls ($1.72\% \pm 0.23\%$, $t = 5.63$, $P < 0.01$).

Table 1 The pathologic parameters and expression of LRP

Pathologic parameters	LRP(+) n	LRP(-) n
Tumor size		
<3 cm	7	2
3 cm-5 cm	11	3
>5 cm	8	5
Differentiation		
well	1	0
moderate	8	0
poor	11	5
mucoid	6	5 ^a
Nodal metastasis		
(-)	15	2
(+)	11	8
Serosal invasion		
(-)	7	3
(+)	19	7
TNM stage		
I	6	1
II	8	3
III	9	3
IV	3	3
N	26	10

^a $P < 0.05$, vs well and moderately differentiated carcinoma. n: number of cases

DISCUSSION

LRP is the human major vault transporter protein and is suggested to confer anticancer drug resistance. The mechanism that LRP confers MDR is unknown, but concerning the reduced nuclear accumulation of daunorubicin in the LRP-overexpressing MDR cell line 2R120 and the evidence supporting a role of vaults as transporter unit of the nuclear pore complexes, it is tempting to hypothesize that LRP can mediate drug resistance by regulating both the cytoplasmic redistribution and the nucleocytoplasmic transport of drugs^[5]. LRP was overexpressed in ovarian cancer, leukemia, and several cancer cell lines of MDR phenotype, and LRP is of high predictive value for response to chemotherapy and prognosis^[6,7]. Ikeda *et al*^[8] quantitated the level of LRP mRNA expression in 10 gastric cancer cell lines by RT-PCR, and examined the relationship between its level in these cells and their sensitivities to anticancer drugs. LRP mRNA was expressed in all cell lines, and LRP correlated with the resistance to cisplatin. But up to now, there has been no study about LRP expression in specimens of gastric cancer and the relationship between LRP expression and pathologic parameters. Our study revealed that LRP was frequently overexpressed in untreated gastric cancer, suggesting that gastric carcinoma holds high intrinsic resistance. Analysis of LRP can help evaluate the chemosensitivity of patients to anticancer drugs, and choose more effective drugs.

Meanwhile, our results disclosed that LRP positivity rates in well and moderately differentiated carcinomas were 100%, in poorly differentiated cancer was 11/16, and in mucoid carcinoma was 6/11, showing the descending tendency, and LRP positivity rate in patients with well and moderately differentiated adenocarcinoma was higher than that in mucoid carcinoma, which was consistent with the clinical observation that well-differentiated cancer cells have less satisfactory chemosensitivity than poorly differentiated. LRP expression was independent on tumor size, lymph nodal

involvement, serosal invasion, and TNM stage, indicating that these parameters represent the progression of the tumor only, and have no correlation to chemotherapy drug sensitivity. Our previous studies showed that *mdr1* mRNA and MRP were overexpressed in gastric carcinoma^[9,3], and the present study demonstrated that LRP overexpressed in gastric cancer, suggesting that MDR can be mediated by all of them simultaneously, and combined administration of different MDR reversing agents, which can overcome MDR by increasing the intracellular drug accumulation of cancer cells, could achieve a better effect.

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A study on arsenic trioxide inducing *in vitro* apoptosis of gastric cancer cell lines

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INTRODUCTION

Cell apoptosis, which involves the biologic regulation of the numbers and vital activity of cells, is an important metabolic process in both normal cells and tumor cells. Delayed process of cell apoptosis will probably lead to a disturbance of metabolism, and occurrence and development of neoplasms. Song *et al* have proved the relationship between apoptosis delay and tumor development through inhibition of cell apoptosis induced by tumor promoter^[1]. Thus, induction of cell apoptosis could be a new strategic measure against tumor. In this paper, we studied whether arsenic oxide will induce apoptosis of gastric cancer cell lines (GCCL) to explore the use of such chemical agent against gastric cancer in clinic.

MATERIALS AND METHODS

Target cells

Human gastric cancer cell lines, MKN45 and SGC7901 (provided by Chinese Academy of Sciences) were used as target cells. Human leukemia cell line K562 and human peripheral blood lymphocytes (PBL) were used as controls. All of cells were cultured in RPMI 1640 medium (GIBICO-BRL), supplemented with 10% heat-inactivated fetal calf serum, 100U/mL penicillin and 100mg/mL streptomycin, in a humidified atmosphere of 95% air/5% CO₂ at 37°C. The numbers of four kinds of cells were maintained at 1 × 10⁶/mL by daily adjusting cell concentration.

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Product of arsenic trioxide (As₂O₃)

Ai-Lin No.1 containing As₂O₃ was prepared by pharmacy of our hospital. Stock solution was made at the concentration of 1mmol/L with phosphate-buffered saline (PBS) and diluted with RPMI 1640 to working concentration before use.

Cytocide test

MTT[3-(4,5-dimethylthiazoyl-2-yl)2,5-diphenyltetrazoliumbromide]colorimetric analysis was used to measure the cytocide rate of As₂O₃. Well grown cells were collected and were put into 96 well culture plate at 1 × 10⁴/well. As₂O₃ was added to culture plate at the concentration of 5 μmol/well, while cells stuck on wall, 6 duplicate wells were set up in each sample in both experimental and control groups. Cells were cultured for 24 h, 48 h and 72 h separately, MTT(5 mg/mL) was added at 10 μL/well. Acidulated isopropanol was finally added at 100 μL/well-after 2 h culture; values of OD570 were obtained on autocolorimeter. Cytocide rate was calculated by the following formula:

$$\text{Cytocide rate(\%)} = \frac{\text{Control OD} - \text{Experimental OD}}{\text{Control OD}} \times 100\%$$

Apoptosis detection

Cell treatment MKN45 and SGC7901 cells of 10⁶ were treated with As₂O₃ of the concentration of 5 μmol and 10 μmol, or with 5-Fu (0.5 g/L), or without any treatment as control. Cells were harvested after 24 h, 48 h and 72 h culture for apoptosis detection.

TDT label Consulting Gregory modified method^[2]: PBS containing 1% formalin was added to cells at 4°C. Thirty min later, it was washed twice with PBS, then reacted with 0.5 μg terminal deoxynucleotidyl transferase (TDT) and 0.5 μmol biotinylated dUTP at 37°C for 30 min. Afterwards it was washed again with PBS, finally labelled by affinitin-fluorescein isothiocyanate (FITC) at room temperature for 30 min. DNA strand will be broken off during apoptosis, which can be labelled by dUTP and the positive observable fluorescences were seen under fluorescent microscope.

Flow cytometry(FCM) assays and fluorescent photographing The stimulating wave length of FCM (Becton Dickson FAC Scan) is 488 nm, FITC detection spectrum is between 80 and 630 nm. A total of 2000 cells were counted. Results were recorded and analyzed automatically.

Preparation of specimen for electronic microscopy
MKN45 cells treated with As_2O_3 of concentration of 10 μ mol for 72 h were washed with PBS and fetal calf serum; 25% glutaraldehyde was added to fix the cell specimens for 12 h. Then it was washed twice with 0.1 mol/L phosphoric buffer solution; the specimens were fixed with 1% osmic acid, and were dehydrated step by step with alcohol and acetone; then covered up with Epon 812 epoxy resin. Sections were made by ultramicrotome and dyed with uranium acetate and citric acid, and finally observed under transmission electronic microscope.

RESULTS

Cytocide rate of As_2O_3 on GCCL

Our results showed that As_2O_3 had higher cytotoxicity on GCCL than on K562 ($P < 0.05$). The effect of cytocide was observed at 24 h after reaction, and it increased with time (Table 1).

Table 1 Cytocide effects of As_2O_3 (5 μ mol) on various cell categories (% , $\bar{x} \pm s$)

Group	MKN45	7901	K562	PBL
24 h	69.45 \pm 11.12	68.27 \pm 8.27	51.36 \pm 10.25 ^a	30.31 \pm 5.12 ^b
48 h	71.40 \pm 10.15	69.04 \pm 11.31	57.11 \pm 7.45 ^a	35.67 \pm 4.37 ^b
72 h	80.53 \pm 10.18	82.74 \pm 9.14	65.33 \pm 8.24 ^a	39.74 \pm 8.27 ^b

Comparison of cytocide rate between GCCL and controls by As_2O_3 , ^a $P < 0.05$ ($t > 2.2262$), ^b $P < 0.01$ ($t > 3.250$)

Table 2 Apoptosis rate (%) of MKN45 by different treatment methods ($\bar{x} \pm s$)

Group	Controls	5-Fu	As_2O_3 1	As_2O_3 2
24 h	4.71 \pm 0.36	18.04 \pm 1.50	32.33 \pm 3.75 ^a	60.02 \pm 7.41 ^b
48 h	14.90 \pm 0.94	24.39 \pm 3.45	90.59 \pm 10.35 ^b	97.05 \pm 8.24 ^b
72 h	28.98 \pm 3.12	41.47 \pm 2.24	98.10 \pm 13.24 ^b	98.75 \pm 11.53 ^b

Comparison of apoptosis rate between As_2O_3 and 5-Fu group, ^a $P < 0.05$ ($t > 2.2262$), ^b $P < 0.01$ ($t > 3.250$).

GCCL apoptosis rate induced by As_2O_3

As shown in Table 2, apoptosis rates of 2 kinds of GCCL induced by 5-Fu were also obviously higher than natural cell apoptosis rate, of controls, which suggested that antitumor drug can kill tumor cells by inducing cell apoptosis. Nevertheless, a more significant cytocide effect of As_2O_3 on GCCL was demonstrated in our study compared with that of 5-Fu. We found that apoptosis rate of GCCL induced by As_2O_3 is correlated with the concentration and reaction time of As_2O_3 (Table 3).

Table 3 Apoptosis rate (%) of SGC7901 by different treatment methods ($\bar{x} \pm s$)

Group	Controls	5-Fu	As_2O_3 1	As_2O_3 2
48 h	7.44 \pm 0.60	34.28 \pm 4.15	51.33 \pm 5.16 ^a	78.31 \pm 9.14 ^b
72 h	30.90 \pm 2.54	56.32 \pm 4.56	78.15 \pm 6.78 ^a	89.28 \pm 10.26 ^b

Comparison of apoptosis rate between As_2O_3 and 5-Fu group, ^a $P < 0.05$ ($t > 2.2262$), ^b $P < 0.01$ ($t > 3.250$)

The morphology of GCCL apoptosis under fluorescent microscope:

Under fluorescent microscope, the apoptosis cell can be seen after being terminally labelled (positive), but nonapoptotic cells were not labelled by fluorescein isothiocyanate (negative). The positive staining showed bright green fluorescence. Fluorescent spots appeared in early stage, and these fluorescent bodies gathered like a bunch of grapes in late stage. The cellular volume can be seen shrunken under the microscope.

The morphology of GCCL apoptosis under electronic microscope:

Under the transmission electronic microscope, typical morphologic changes of apoptotic GCCL (mainly cell nucleus) took place after treatment of As_2O_3 . These changes included cell nucleus fixation and shrinkage of GCCL, chromatin condensation, and fragmentation of apoptotic bodies. These changes coexisted.

DISCUSSION

Some researchers have proposed that the uncontrolled growth of neoplasms would be due to the loss of the nature of autoapoptosis rather than over proliferation. Previous studies have proved that there existed autoapoptosis blockage in tumor cells. Lauwers *et al* [3] examined bcl-2 gene in 46 cases of gastric adenocarcinoma by immunochemical method, revealing 75% positivity. Of bcl-2 gene in tumor tissues, which indicated that apoptosis was blocked in gastric cancer. Bcl-2 has been considered as one of survival genes which plays an important role in the specific-inhibition of tumor cell apoptosis [4]. Based on these findings, a new proposal of inducing apoptosis to inhibit tumor growth was introduced [5]. Many factors such as high temperature, cytokine, radiations and all kinds of anti-tumor chemotherapy drugs have a certain effect on inducing tumor cells apoptosis. But some of these are not satisfactory. Our aim is to find a specific-agent which can induce apoptosis of tumor cells. Vollmers *et al* [6] reported the suppressive effects of monoclonal antibody (SC-1) on both *in vitro* proliferation of gastric cancer cell line and growth of a tumor inoculated on nude mice. The inhibition of proliferation of tumor cells was produced through the induction of autoapoptosis, which has been proved by the observation of ultrastructure.

Arsenic is a major composition of traditional Chinese medicine, white arsenic. White arsenic has been considered as a carcinogen. It can inactivate some important enzymes in cells, change the metabolic process and induce chromosome aberration [7]. Zhang *et al* [8] reported a satisfactory result by using As_2O_3 for the treatment of early acute promyelocytic leukemia (APL). Complete remission was 73.3% in patients after the first therapeutic course, and 52.83% in recurrent

patients. The longest remission period of APL patient was over 10 years. No obvious toxic reactions were found when As₂O₃ was given by iv drip, which is appropriate. Similar result has also been obtained by researchers at Shanghai Institute of Hematology of Shanghai Ruijin Hospital^[9,10]. Recently, Zhang *et al*^[11] demonstrated that Arsenic Oxide can inhibit growth of lymphosarcoma cells and induce apoptosis to these cells.

Based on the above studies, we applied As₂O₃ for the treatment of GI solid tumor. The results from *in vitro* study are impressive. Proliferation of MKN45 and SGC7901 was inhibited by As₂O₃ through apoptosis induction. Results also showed that As₂O₃ has a stronger effect of apoptosis induction than 5-Fu. Induction of apoptosis was enhanced with increase of concentration and time of As₂O₃. The question is what the optimal dosage is for clinical use so as to produce maximal effect with no toxicity. Further comprehensive researches are needed to clarify the significance of As₂O₃ for the treatment of GI solid tumors.

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Relationship between collagen IV expression and biological behavior of gastric cancer

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INTRODUCTION

Conceivably the presence of basement membrane (BM) in a neoplasm might be a result of interaction of tumor cells with the extracellular matrix. Collagen IV is one of the major intrinsic components of BM. Recent study^[1] has shown that collagen IV has cell adhesion function and is involved in the process of tumor invasion and metastasis, including colorectal cancer^[2] and breast cancer. But there are few systematic studies on gastric cancer and the results are equivocal. In this study we evaluated the expression of collagen IV immunohistochemically in 148 advanced gastric cancer cases in an attempt to clarify the relationship between the patterns of expression and the biological behavior of gastric cancer.

MATERIALS AND METHODS

Patients

Surgical specimens (148) of gastric carcinomas resected at the Oncology Department of China Medical University from 1988 to 1992 were studied. Routinely formalin-fixed and paraffin-embedded tissue blocks were sectioned at 5 μ m thickness. HE-stained slides were also collected and available.

Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) method by Hsu *et al*^[3] was applied. Briefly, paraffin sections were deparaffinized, dehydrated and pretreated with pepsin (0.1% in 0.1M- HCl for one hour at 37°C) to restore the immunoreactivity to type IV collagen. After blocking of endogenous peroxidase with 0.3% H₂O₂ in methanol and

washing in phosphate-buffered saline (PBS) for 3 \times 5 min, the sections were incubated with anti-collagen IV monoclonal antibody (Dako Putts Co., diluted 1:75) overnight at 4°C in a moist chamber. After washing in PBS the sections were incubated with secondary antibody for 30 min and ABC for 60 min at 37°C, then sections were stained with 0.05% DAB freshly prepared to visualize the immunoreactivity. Tumors were classified as "positive" with regard to the immunoreactivity for collagen IV when there was unequivocal immunostaining of the matrix components at least in one representative area of the tumor.

Statistical analysis

Statistical analysis was performed by χ^2 test.

RESULTS

Collagen IV stained the basement membrane of normal gastric glands and vessels and also the basement membrane of the smooth muscle cells in the muscular layers of the gastric wall.

Relationship between collagen IV expression and histological type, growth pattern of gastric cancer

Seventy patients (47%) has continuous or, more frequently, disrupted linear structures around cancerous gland ducts or solid nests of tumor cells. The positive rates in undifferentiated or signet ring cell cancers were much lower than those in other cancers ($P < 0.01$). Fifty-five percent of carcinomas with the nest-fashioned growth pattern showed collagen IV positive staining (Table 1), which was slightly lower than the percentage of collagen-IV positive carcinomas with mass-fashioned growth pattern, but significantly higher than that of diffuse-gastric carcinomas ($P < 0.01$).

Relationship between the distribution of collagen IV and liver metastasis of gastric cancer

The distribution patterns of collagen IV in cancer tissue in gastric wall are quite different. Collagen IV was seen less often in the deep layer than in the mucosa. Collagen IV positive BM was found in 70 (47%) of 148 patients in the mucosa and only 36 (24%) in the deep layer of stomach. Based on the difference of collagen IV distribution, we divided the cases into three groups as follow: Group A, tumors positive for collagen IV in both mucosa and deep layer; Group B, tumors positive for collagen IV in mucosa but negative in deep layer; Group C,

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tumors negative for collagen IV. From Table 2, we can find that liver metastasis rate in Group A is much higher than that in Group B or C ($P < 0.01$).

Table 1 Relationship between collagen IV expression and histological type, growth pattern of gastric cancer

Type	Number	Collagen IV n(%)	
		(+)	(-)
Growth pattern			
Mass-like	56	43(77)	13(23)
Nest-like	38	21(55)	17(45)
Diffuse	54	7(13)	47(87)
Histological type			
Well differentiated	37	30(81)	7(19)
Moderately differentiated	22	13(59)	9(41)
Poorly differentiated	23	10(43)	13(57)
Undifferentiated	27	3(11)	24(89)
Signet-net cell	25	2(8)	23(92)
Mucoid	14	12(86)	2(14)

Table 2 Relationship between the distribution of collagen IV and liver metastasis of gastric cancer

Liver-metastasis	Group A n = 36(%)	Group B n = 34(%)	Group C n = 78(%)
(+)	10(28)	2(6)	1(1)
(-)	26(72)	32(94)	77(99)

The blood vessels BM in gastric wall are also positively stained

In differentiated carcinoma, a large number of blood vessels were observed in the vicinity of cancer glands and collagen IV was localized around the blood vessels and cancer glands. But in poorly differentiated carcinoma, only a small number of blood vessels were distributed sporadically in stroma. In addition, vasoinvasion of tumor cells were highlighted by collagen IV immunostaining of the blood vessels BM.

DISCUSSION

Normal basement membrane is one of the biological barriers to tumor invasion and metastasis. In carcinomas, a dynamic interaction occurs at the interface between tumor cells and the surrounding extracellular matrix components. Tumor cells not only destroyed the basement membrane by producing collagenase, including the specific type IV collagenase, but also synthesize the components of basement membrane^[4]. And also as a host reaction to the invading tumor, extracellular matrix components may be deposited around the tumor cells. The appearance of these components may symbolize the characteristic of the tumor and reflect its biological behavior. Collagen IV is one of the major components of the basement membrane, Burtin *et al*^[5] showed that expression of collagen IV was related to the differentiation of the colorectal cancer.

Histological growth pattern could be considered as the objective indicator of the biological behavior of gastric cancer. In this study, we found that the presence of collagen IV containing basement membrane was closely related to the growth

pattern. In mass or nest-fashioned growth pattern of gastric carcinomas, continuous or disrupted linear structures stained positively for collagen IV can be seen around cancerous glands or solid nests of tumor cells. But only a few scattered spots or patches were positively stained in diffuse gastric carcinomas, most of which were undifferentiated or signet ring cell carcinomas. Our study demonstrated that the attenuation or absence of collagen IV expression was frequently seen in poorly differentiated and diffusely infiltrating gastric cancers. That is, the loss and irregular distribution of collagen IV expression could be considered as a biological marker of cancer cells which had strong invading ability. This finding may be concerned with these tumor cells which could produce collagenase with higher activity or had poorer potential of collagen IV synthesis.

The prognosis of patients with advanced gastric cancer is poor because the likelihood of recurrence is high. The most common patterns of recurrence are peritoneal implantation and liver metastasis. According to our study, the distribution patterns of collagen IV are related to liver metastasis in gastric cancer. Positive expressions of collagen IV in mucosa and deep layer of gastric cancer are accompanied by much higher incidence of liver metastasis. Vascular spreading of gastric cancer, which is different from direct invasion, is a multi-step process in which cells must migrate from the primary tumor and invade blood vessels. David L *et al*^[6] found that the expression of collagen IV was related to invading potential of tumor cells to blood vessels. All these findings support the hypothesis that collagen IV may play an important role in the metastatic process after migration from the primary tumor. The principal mechanism is an interesting subject for further studies. In conclusion, collagen IV-positive basement membrane in the deep layer of gastric wall in cancer tissue might be a risk factor for liver metastasis. In addition, collagen IV immunostaining facilitates recognition of vasoinvasion by highlighting the basement membrane of vessels.

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The effect of mast cell on the induction of *Helicobacter pylori* infection in Mongolian gerbils

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Subject headings diseases models, animal; Mongolian gerbils; *Helicobacter pylori*; mast cell; *Helicobacter* infections

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INTRODUCTION

Since 1982, *Helicobacter pylori* (Hp) has been successfully isolated and cultured^[1], and the fact many diseases such as gastritis, peptic ulcer, gastric carcinoma and gastric lymphoma were related to Hp, and Hp as an etiological organism, has attracted much attention. In 1991 Yokata *et al*^[2] first induced the Hp infection in Mongolian gerbils. From then on researches on Hp infection and its eradication exploded worldwide. Nakajima *et al*^[3] reported that mast cells were increased in gastric mucosa and submucosa of patients with Hp infection and found degranulated mast cells in these tissues. Now it is known that high affinity IgE and Fc segment receptors exist on the mast cell surface. When IgE bound to these receptors, mast cells degranulated and then many kinds of bioactive mediators were secreted so that the inflammatory process could be effected. Therefore it is extremely important to understand the relationship between degranulation of mast cells in the mucosa infected with Hp and the inflammatory response, and to conjecture of the effect of mast cells on the pathogenesis of Hp infection. This study observed the inflammation of gastric mucosa and the morphology, density, distribution and degranulation of mast cells in the infected gerbils using the histochemical or immunohistochemical methods and electron microscopy to elucidate the effect of mast cells on the pathogenesis of Hp infection.

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MATERIALS AND METHODS

Animals

Thirteen male Mongolian gerbils infected with Hp for 1 mo to 3 mos were allocated to the experimental group, while 5 male Mongolian gerbils with out Hp infection to the control group.

The induction of animal models

Six-week-old specific pathogen-free/sea male Mongolian gerbils and Hp ATCC 43504 were used. The gerbils were subjected to ATCC 43504 and ethanol treatment according to the method described by Hirayama *et al*^[4]. Normal subjects were treated with the same amount of Brucella Broth. The gerbils infected with Hp were injected with BrdU subcutaneously one hour before sacrifice. A portion of the stomach was fixed in MFAA^[5] and embedded in paraffin, and cut in sequential 3 µm-4 µm section for light microscopy; the remainder was fixed in 25 g/L Glutaldehyde, serially dehydrated in ethanol, postfixed in 10 g/L osmium acid, embedded in Epon, and cut in 60 nm ultrathin sections for electron microscopy.

Staining of mast cells

Alcian blue (AB1.0)/ PAS staining The immunohistochemical staining of anti-mast cell monoclonal antibody, MSRM4 (produced by Moredum Scientific Limited Co. Britain, diluted 200 folds) by LsAB method was applied.

Double-staining AB staining was followed by anti-BrdU Ab and anti-CD3 lymphatic Ab complex staining to observe the cellular marking rate of mast cells in S-phase and their relationship with T lymphocytes.

Staining for electron microscopy with uranyl acetate and lead citrate

Bactericidal treatment Positive group was defined at 6 mos after Hp infection and then was cured with triad treatment (Lansoprazole, Ampicillin, Clarithromycin) for 2 wks continuously. At wk 4, wk 8 and wk 12 after withdrawal of medication, the gerbils were sacrificed to observe the changes in distribution, amount and degranulation of mast cells before and after bactericidal treatment.

Counting of mast cells At the magnification employed ($\times 400$), the positive mast cells in the mucosa and the epithelial layer in gastric antrum and body in 5 consecutive areas were counted and

their mean values represented the density of positive cells in this particular area. All the data according to different areas were divided into different groups for further statistic treatment and analysis compared with the normal subjects.

RESULTS

Light microscopy

Mast cells appeared blue stained with AB/PAS and brown when immunohistochemical staining was used. Positive mast cells, filled with granules in cytoplasm, dispersed in epithelium, lamina propria and submucosa and also commonly in muscular layer, having a close relationship with the vessels and nerves in submucosa and muscular layer. All of the above picture rarely appeared in the gastric tissues of normal gerbils. At 2 wk after *Hp* infection, the mast cells began to increase in amount with a tendency of inflammatory-dependence, significantly different from the normal group ($P < 0.001$). On the double staining section, there existed varied amounts of blue heterophilic granules in the cytoplasm and the nucleus was brown. The mast cells with these morphologic characteristics corresponded to the cells in S-phase. The increase of cells in S-phase signified the active hyperplasia in the progression of inflammation. The double staining of CD3 and mast cells indicated that a great deal of mast cells were around the lymphatic follicles in which CD3 existed. The amount of mast cells decreased 1 mo after bactericidal treatment and recovered to the normal level 3 mos after treatment.

Electron microscopy

Under the normal circumstances, the specific high-electron density granules in cells were stable and existed independently, while in inflammatory state the mast cells were activated to degranulate and form vacuoles and tended to be vacuolated after the fusion of vacuoles.

DISCUSSION

It is well known that mast cells exist widely in trachea, digestive tract, skin and many other organs and play an important role in the pathogenesis of allergic reaction characterized by bronchial asthma and urticaria. Recent studies on mast cells found direct or indirect evidences to reveal the close relationship between the mast cells and the injury of gastric mucosa^[6]. The mast cells located in human gastrointestinal tract and induced by pathogen or some antigens, may secrete bioactive substances such as

histamine and prostaglandin, which increase the secretion of gastric acid, and then trigger a series of events: the injury of tissues and the inflammation of mucosa. The rapid development in recent years on the cell culture and molecular biology provides the possibility of explaining that various cytokines can cause the activation and proliferation of mast cells and mast cells can produce and secrete these cytokines. $\text{TNF}\alpha$, GM-CSF, IL-6 and IL-8 can act as the precursor in the local inflammatory reaction which leads to the infiltration of inflammatory cells, and at the same time modulates the development and proliferation of themselves-the autoregulative process. The results of this study showed that the mast cells in gastric mucosa with infection of *Hp* increased significantly, the infiltration of lymphatic cells depended on the appearance of mast cells and the conspicuous increase of degranulation of mast cells in inflammatory areas was related to the degree of gastritis. All of the above supported the fact that mast cells play a role in the occurrence and development of the pathogenicity of *Hp*. It has been reported that some of the mast cells have the function of extending and movement so that the mast cells can penetrate the basal membrane and move toward the interepithelial space. During the moving process, degranulation appears gradually, which results in the phenomena of vacuolation. Therefore, some researchers believe that mast cells of mucosa-type are the active state of mast cells of conjugated-type. At present, the mechanism of the degranulation of mast cells remains unclear. The relationship between mast cells and *Hp* infection and the effect of the mediator secreted by mast cells on the pathophysiology of *Hp* infected gastric diseases need further investigation.

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Effect of *Helicobacter pylori* infection on gastric epithelial cell proliferation

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Subject headings *Helicobacter pylori*; cell proliferation; vacAs1a strain; gastric epithelial cell

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is one of the main pathogens of chronic gastritis and duodenal ulcer (DU), and it may be considered as a risk factor in the incidence of gastric cancer^[1]. *H. pylori* infection may lead to the anomaly of gastric epithelial cell proliferation which is closely related to the development of gastric cancer. Vacuolating cytotoxin (VacA) is an important virulence and vacA subtype determines the toxic activity^[2]. According to its signal sequence, it can be grouped into type s1a, s1b, s1c and s2^[3,4]. Strains harboring vacAs1a are more closely related with digestive diseases^[5] and may be the strains with high toxicity. The effect of *H. pylori* infection on gastric epithelial cell proliferation depends on the vacA subtype^[6]. The report of the effect of strains with vacAs1a on gastric epithelial cell proliferation has not been found in China. We particularly study the effect of this strain in order to reveal whether the patients suffering from *H. pylori* infection have accelerated proliferation of gastric epithelium compared with non-infected patients, and whether the strains harboring vacAs1a have more severe effect on it.

MATERIALS AND METHODS

Patients

Patients suffering from dyspepsia underwent diagnostic endoscopy and biopsy. Those taking H₂ antagonists, proton pump inhibitors, non-steroidal anti-inflammatory drugs, antibiotics or bismuth salts were excluded from the study. Patients with gastric ulcer or cancer were also excluded. Eighty-four patients with chronic gastritis (CSG) and 16 patients with duodenal ulcer (DU) with mean age of 46.45 years (22 years - 76 years) entered the

study. Biopsy specimens were taken from the site approximately 2 cm-5 cm from the pylori.

Histology and diagnosis of *H. pylori* infection

Two antral and one corpus biopsy specimens were routinely processed, and stained with haematoxylin and eosin. Examine *H. pylori* by fast urease test, modified Giemsa stain and culture. At least two positive results of test regarded as *H. pylori* infection.

Immunohistochemistry

An antral biopsy specimen was put immediately into RPMI containing bromodeoxyuridine (BrdU, 5 g/L). It was immersed in a waterbath for 60 min at 37°C and then fixed in Carnoy solution. Sections were stained with anti-BrdU antibody by ABC technique. The nuclei of proliferative cell were stained. Five hundred epithelial cells were counted and the number of positively stained epithelial cell nuclei expressed in percentage as labelling index (LI%). All sections were examined by the same person who was unaware of the subject's *H. pylori* status.

Polymerase chain reaction

H. pylori DNA was extracted routinely, vacAs1a amplified by PCR, 50 µL reaction solution contains the following: 1 × reaction buffer, dNTP mixture (0.2mM each), vacAs1a primers (0.2µM each), 1.25 unit Taq DNA polymerase and 4 µL template. PCR program comprises predenaturation at 94°C for 5 min, followed by 37 cycles of 1 min at 94°C, 90s at 52°C, 45s at 72°C, and a final incubation at 72°C for 7 min. PCR products were inspected by electrophoresis on 2% agarose gels stained by ethidium bromide. It is regarded as vacAs1a positive if a clear band can be seen at 190bp. Primers: 5'-GTCAGCATCACACCGCAAC-3', 5'-CTGCTTG-AATG CGCCAAAC-3'^[3].

Statistics

LI% is transformed to arcsin LI% 1/2, *t* test, Chisquare and multi variate linear-regression analysis were used to deal with the data.

RESULTS

The prevalence of *H. pylori* infection in CSG patients was 50%, and that of DU reached 93.75% ($P < 0.01$), but the disparity of vacAs1a proportion between *H. pylori* positive CSG and DU was not significant (43.59% vs 58.33%, $P > 0.05$).

There is significant difference on LI% between

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CSG and DU ($P < 0.05$), but considering the different prevalence of *H. pylori* infection, we got negative result ($P > 0.05$) from comparing the LI% between positive cases of CSG and DU. The results (Table 1) of analyzing the effect of *H. pylori* infection and its different strains on proliferation, showed that patients with *H. pylori* had higher LI% ($6.14\% \pm 1.21\%$) than *H. pylori* negative ones ($2.43\% \pm 0.61\%$, $P < 0.001$). Patients harboring vacAs1a strains had significantly higher gastric epithelial cell proliferation LI% ($n = 24$, $8.00\% \pm 1.46\%$) than those with non-vacAs1a strains ($n = 27$, $4.51\% \pm 0.86\%$, $P < 0.02$) or noninfected patients ($P < 0.001$).

The sections were graded into mild, moderate and severe according to the extent of inflammation and intestinal metaplasia. Statistics shows a close relationship between inflammation and *H. pylori* status ($P < 0.005$), however it is negative on metaplasia ($P > 0.05$). No significant relationship was found between inflammation and vacAs1a genotype ($P > 0.05$).

The results show that inflammation and neutrophil infiltration were closely related to epithelial cell proliferation ($P < 0.001$), but not to metaplasia ($P > 0.05$). Multivariate linear-regression analysis shows that among the factors, such as age, sex, DU, inflammation, neutrophil infiltration, intestinal metaplasia, vacAs1a strains, non-vacAs1a strains and so on, vacAs1a strain and inflammation are the independent factors influencing the epithelial cell proliferation.

Table 1 BrdU LI% of the patients ($\bar{x} \pm s$)

	LI%	case number
H.pylori-positive	6.14 ± 1.21^a	57 [§]
vacAs1a positive	$8.00 \pm 1.46^{a\#}$	24
vacAs1a negative	4.51 ± 0.86^c	27
H.pylori-negative	2.43 ± 0.61	43

^a $P < 0.001$ vs *H. pylori* negative patients; ^c $P < 0.01$ vs *H. pylori* negative patients; ^e $P < 0.02$ vs non-vacAs1a *H. pylori* patients; [§]vacAs1a were not examined in 6 cases because of loss of specimens.

Table 2 Multivariate linear-regression analysis

LI%	coefficient	SD	t	P> t	95%confidence interval
vacAs1a	4.47	1.37	3.27	0.002	1.76 7.19
inflammatory coefficient	3.89	1.21	3.22	0.002	1.49 6.30
	4.50	1.89	2.38	0.019	0.75 8.26

DISCUSSION

The genesis of gastric cancer is the result of long-term effect of multiple factors of environment and host. Epidemiological investigation and histological evidences showed that *H. pylori* infection was related to gastric cancer independently. *H. pylori* infection induced gastric epithelial cell proliferation, increase of mitosis and mutation^[7]. Because of the instability of the genome of the proliferative cell, hyperproliferation increases the

possibility of DNA damage and aneuploidy. Dysplasia may evolve into carcinoma if damaged DNA cannot be repaired on time or fail in promoting the apoptosis system. Accelerated cellular proliferation rate is the property of malignant tissue and has been confirmed in gastric carcinoma^[8].

The genesis of most gastric adenocarcinomas is believed to follow a series of defined histologic steps from normal gastric mucosa to chronic gastritis, atrophic gastritis, intestinal metaplasia, and neoplasia^[9]. It has been postulated that *H. pylori* plays a causative role at the early phases in this chain of malignant progression^[10]. Therefore, we studied CSG and DU patients (part of them had intestinal metaplasia).

The prevalence of *H. pylori* in DU patients (93.75%) was much higher than that in CSG (50%) which supports the conclusion that *H. pylori* is a closely associated pathogen of DU.

It is reported that gastric epithelial cell proliferation in *H. pylori* associated gastritis patients increased prominently compared with normal control subjects and patients with *H. pylori* negative chronic gastritis, and it reduced after *H. pylori* was eradicated^[11-16]. Our results are in agreement with these reports.

No significant difference was found on BrdU LI% between *H. pylori* positive DU and CSG patients which reveals that the existence of DU does not alter the status of proliferation, some factors other than hyperproliferation such as increased apoptosis may play an important role, in the genesis of DU by keeping the dynamic equilibrium of the epithelium^[17-20]. But some CSG patients cannot keep efficiently this equilibrium, in other words, the proliferation increases without corresponding apoptosis, DNA is prone to be attacked by other carcinogens, resulting in canceration.

About 50% population infected by *H. pylori*, gastric cancer or DU only occurred in a small portion of them. This may be associated with many factors, one of the determinants is the virulence of the strain infected.

Compared with the non-vacAs1a strains infected patients, epithelial cell proliferation of the vacAs1a strains infected patients was much higher. So vacAs1a *H. pylori* strains may be able to promote the epithelial cell proliferation. Multivariate linear-regression shows that vacAs1a strain is an independent influencing factor, which further supports the conclusion that vacAs1a strain is of high virulence. In view of the importance of hyperproliferation during the genesis of gastric cancer, vacAs1a strain may play a critical role in it.

There is much difference on the constitution of the vacA subtype of *H. pylori* according to the reports from different areas. The proportion of vacAs1a strains varied greatly^[3,21]. There have been few reports on the genotype of vacA in China. She *et al* reported the relationship between 60 *H. pylori* strains and the alimentary diseases. The relevance

ratios of vacAs1 in gastric cancer, peptic ulcer and chronic gastritis were 87.5%, 78.9% and 9.1% respectively^[22]. There was obvious geographical discrepancy in the distribution of *H. pylori* vacA subtype.

We found that intestinal metaplasia had nothing to do with *H. pylori* infection, this result corresponds with the report of Cahill *et al.* We also found that intestinal metaplasia was not correlated with proliferation, which differs with some other reports in its clinical importance, the relationship with gastric cancer and effect on cell proliferation. Therefore, we cannot draw conclusion that intestinal metaplasia is not associated with proliferation. Further studies will be conducted.

The mechanism that *H. pylori* and its different strains accelerate proliferation is not clear. Ricci *et al* found VacA can inhibit cell proliferation *in vitro*, while cytotoxin-associated gene (CagA) does not affect proliferation^[23]. *H. pylori* can induce proliferation *in vivo*, so *H. pylori* may act by this suggesting that *H. pylori* based on its ability of inciting inflammatory reaction, influencing the gastrin secretion, but not the direct action of virulences to exact the effect on cell proliferation.

Inflammation and neutrophil filtration are both associated with *H. pylori* infection. That means *H. pylori* infection can arouse acute and chronic inflammation. Accelerated proliferation is related to the extent of inflammation, and the latter is highly related to *H. pylori* infection. This points out that *H. pylori* infection may promote proliferation by inflammation, which was once reported by Lynch *et al*^[11,12]. It is also shown that inflammation acts on proliferation as an independent factor. *H. pylori* infection affects proliferation at least partly by inflammatory action. On the contrary patients harboring vacAs1a strains have similar inflammatory response to those with non-vacAs1a strains, but their ability of inducing proliferation differed. Thereby *H. pylori* may promote proliferation by inflammation, and vacAs1a strains may act by the mechanism other than inflammation, such as the increase of ammonia^[24], gastrin^[25], and the decrease of ascorbic acid concentration^[26,27].

We found that *H. pylori* infection was closely related to gastric epithelial cell proliferation, and the vacAs1a strains had higher activity. The vacAs1a strain and extent of inflammation affect proliferation independently. But the effect of *H. pylori* and its different strains on apoptosis is not clear and needs further studies. Besides, *H. pylori* can be typed into m1 and m2 strains according to vacA middle sequence, and positive or negative cagA. They may have different effects on proliferation and apoptosis, these may play important roles in the pathogenicity of *H. pylori*.

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Effects of radical cholecystectomy on nutritional and immune status in patients with gallbladder carcinoma

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Subject headings gallbladder neoplasms/immunology; cholecystectomy; nutritional status; immune status

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INTRODUCTION

Carcinoma of the gallbladder is the most common neoplasm in biliary tract, and its incidence has been rising in recent years. The rate of correct diagnosis in early gallbladder carcinoma has been raised after the wide use of CT, ultrasound scans and frozen section examination. Now radical cholecystectomy is advocated as the best management for patients with early gallbladder carcinoma. In the present study, the diagnosis of 27 patients with gallbladder carcinoma was confirmed correct, and the patients underwent radical cholecystectomy, and their nutritional and immune assessments were performed pre- and post-operatively.

PATIENTS AND METHODS

Patients

From September 1993 to December 1997, 27 patients with gallbladder carcinoma who had undergone radical cholecystectomy were selected, and they were admitted to the First and Second Affiliated Hospitals of Xi'an Medical University. The patients included, eight males, aged 46-65 years, with a mean of 57 years; nineteen females, aged 50-67 years, with the mean of 59 years. Four patients were diagnosed as having polyposis preoperatively, and diagnosed as carcinoma during the operation by the frozen section technique. In five patients with preoperative diagnosis of carcinoma, metastasis had not been found during the operation. No severe systemic disease was found, e.g. recent myocardial infarction, cerebral vascular accidents, uncontrollable diabetes mellitus or hypertension. Antibiotics were administered

postoperatively to prevent infection, intravenous infusion was routinely administered immediately after surgery and maintained at least for one week. The tumors were graded as stage I, II and III (Nevin stage) by histological examination. All of the resected specimens were sent for histological examination. No chemotherapy or radiotherapy was administered preoperatively.

Methods

The nutritional and immune status of the patients were assessed preoperatively (1 wk before surgery), and on d 3, d 7, d 14 and d 21 postoperatively.

Nutritional assessment Biochemical parameters evaluating the patient's nutritional status consisted of serum levels of albumin, cholesterol, iron, magnesium, zinc, and transferrin determined by an automated calorimetric technique (SMAC), and total iron binding capacity (TIBC) determined by radioimmunoassay.

Immune studies The immune status includes T lymphocyte subpopulation-CD₄, CD₈; immunoglobulins (IgG, IgA and IgM); complements (C₃ and C₄); and serum interleukin-2 and soluble interleukin-2 receptor were evaluated. T lymphocyte subpopulation assessment: blood samples were taken with venopuncture and anticoagulated by heparin. Mononuclear cells were isolated after the sample had been washed twice with Hank's balanced salt solution, and the concentration of the cells was regulated at 2×10^2 /mL with PRMI-1840 (Cow serum). Cell suspensions (0.2 mL) were placed in 24-well plates, and added cow serum 0.1 mL, PHA 0.05 mL, LPS 0.05 mL and 10% RPMI-1640 0.6 mL respectively. The plates were incubated at 37°C with 5% CO₂ for 72 h. Cultured supernatants were harvested and kept at -30°C. The cell suspension was assigned equally into three parts, which was washed once, added with CD₄ and CD₈ monoclonal antibody (monoclonal antibody was provided by Luo Yang Hua Mei Co.), stand for 2 h at 4°C; then each was washed twice again; a sheep-anti-mouse IgG fluorescent antibody 50 µL was added into each tube, stand for 2 h at 4°C, and washed twice again. After harvesting, cell suspension was studied under the fluorescence microscopy and the scintillation cells were quantitated as percentage of 100 or 200 lymphocytes.

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Assay of IgA, IgG, IgM and complements Serum IgG, IgA, IgM, C₃ and C₄ were quantitated by nephelometric immunoassay.

Assay of serum IL-2, sIL-2R Serum IL-2 and sIL-2 levels were measured by a "Sandwich" enzyme-linked immunosorbent assay with two antibodies, the diagnostic reagents were supplied by Norman Bethune Medical University.

Statistical analysis

For each variable, multiple analysis of variance for repeated measurements was used to compare the values measured before operation with those measured at four subsequent time points. The results were presented as mean and standard error ($\bar{x} \pm s$) based on the mixed model of repeated measurement analysis. Statistical analysis was performed with SAS software. *P* values <0.05 were considered statistically significant.

RESULTS

The results of the nutritional and immunological assessment at various time points are demonstrated in Table 1.

Preoperation

As shown in Table 1, in patients with gallbladder carcinoma, the preoperative nutritional and immune status was all within the assigned normal ranges.

Third postoperative day

All the nutritional parameters decreased greatly after surgery, especially the serum iron, transferrin, cholesterol, TIBC, magnesium, and zinc (*P*<0.01, respectively).

The serum levels of immunoglobulins (IgA, IgG, IgM) and C₃, C₄ complements decreased significantly (*P*<0.01), serum IL-2 levels, CD₄ and CD₈ levels also reduced greatly (*P*<0.01), and CD₈ and sIL-2R levels increased significantly (*P*<0.01).

First postoperative week

Compared with the third postoperative day, all the nutritional parameters increased slightly, particularly the serum albumin, magnesium. Serum cholesterol, TIBC, transferrin, iron and zinc were still lower than their preoperative levels (*P*<0.01). CD₄, CD₈, CD₄/CD₈, IL-2, sIL-2 values differed significantly from those of the preoperative stage (*P*<0.01). The serum levels of immunoglobulins (IgG, IgA, IgM) and complements (C₃ and C₄) were significantly higher than those of the third postoperative day, but they were still lower than those of the preoperative day.

Second postoperative week

Compared with the third postoperative day and the first postoperative week, albumin, magnesium and zinc recovered to the preoperative levels (*P*>0.05), however, the levels of TIBC, transferrin and iron were still significantly lower than those of the preoperative ones (*P*<0.01). Compared with the third postoperative day and the first postoperative week, the serum levels of immunoglobulins (IgG, IgA, IgM) and complements (C₃ and C₄) gradually recovered, and IL-2, CD₄, CD₈, sIL-2R levels and CD₄/CD₈ ratio were not statistically different from the preoperative levels.

Third postoperative week

The nutritional evaluation showed continuous improvement in the third postoperative week, most of the nutritional parameters returned to the preoperative levels, except for the serum levels of iron, transferrin and TIBC. The immune parameters IL-2, sIL-2R, CD₄, CD₈, CD₄/CD₈ ratio, C₃, C₄ immunoglobulin levels (IgG, IgA, IgM) also returned to the preoperative levels, with no statistical difference (*P*>0.05).

Table 1 Nutritional and immunity status in patients with gallbladder carcinoma ($\bar{x} \pm s$)

Parameters	Preoperative	Postoperative			
		3rd day	1st week	2nd week	3rd week
Nutrition					
Albumin(g/mL)	3.6 ± 0.07	3.01 ± 0.14 ^b	3.49 ± 0.13 ^a	3.50 ± 0.12	3.58 ± 0.15
Cholesterol(mg/dL)	215 ± 7.81	78.16 ± 7.22 ^b	85.23 ± 7.41 ^b	131.45 ± 6.85 ^b	148.62 ± 8.41 ^b
TIBC(μg/dL)	251.15 ± 12.95	129.18 ± 8.15 ^b	162.67 ± 9.83 ^b	194.15 ± 10.15 ^b	199.34 ± 9.68 ^b
Transferrin(mg/dL)	221.07 ± 12.11	99.77 ± 8.98 ^b	131.29 ± 9.01 ^b	175.25 ± 9.31 ^b	186.21 ± 10.18 ^b
Iron(mg/dL)	65.12 ± 6.15	23.39 ± 4.93 ^b	23.41 ± 5.01 ^b	32.47 ± 5.42 ^b	39.87 ± 5.87 ^b
Magnesium(mg/dL)	2.13 ± 0.06	1.57 ± 0.05 ^b	1.99 ± 0.05 ^b	2.04 ± 0.06	2.06 ± 0.06
Zinc(mg/dL)	106.1 ± 3.56	59.85 ± 5.13 ^b	88.66 ± 4.4 ^{3b}	105.8 ± 3.71	109.9 ± 3.81
Immunity					
C ₃ (mg/dL)	148.1 ± 5.15	70.21 ± 5.6 ^b	96.23 ± 4.68 ^b	118.56 ± 5.11 ^a	123.73 ± 6.22
C ₄ (mg/dL)	25.15 ± 1.15	14.38 ± 1.41 ^b	18.79 ± 1.45 ^a	24.82 ± 1.21	24.98 ± 1.16
T lymphocyte subpopulation					
CD ₄ (%)	44.15 ± 2.01	33.22 ± 1.88 ^b	39.96 ± 2.02 ^b	42.37 ± 2.00 ^a	43.99 ± 1.97
CD ₈ (%)	34.12 ± 1.69	29.47 ± 3.77 ^b	26.21 ± 3.13 ^b	39.69 ± 3.00 ^b	35.81 ± 1.20
CD ₄ /CD ₈ (ratio)	1.3 ± 0.18	1.1 ± 0.08 ^b	1.5 ± 0.19 ^b	1.07 ± 0.14 ^b	1.20 ± 0.16 ^b
IL-2(IU/mL)	9.95 ± 2.95	4.81 ± 1.81 ^b	5.99 ± 2.30 ^b	8.41 ± 2.93 ^a	9.89 ± 3.01
sIL-2R(U/mL)	754.2 ± 141.5	478.3 ± 87.57 ^b	597.4 ± 96.81 ^b	692.1 ± 112.6	748.9 ± 138.8

For each variable, multiple repeated measurements analysis of variance and *t* test were used to compare the values before operation with those measured after operation, ^a*P*<0.05, ^b*P*<0.01, vs before operation.

DISCUSSION

Carcinoma of the gallbladder is one of the most common neoplasms in biliary tract, and 40%-100% cases are complicated with gallstones^[1,2], but correct diagnosis of gallbladder carcinoma in its early stage accounted for only 19.1%, and 53.3% cases are always diagnosed as cholecystitis and gallstone^[3-5]. More and more clinical experiences indicate that radical cholecystectomy for early carcinoma is the most effective treatment^[6-8]. In the present study, though all patients with gallbladder carcinoma were well prepared to receive the radical cholecystectomy, their nutritional and immune status still deteriorated remarkably immediately after the extensive surgical resection. The reasons might be that: ① Large volume of body fluid lost during and after the surgery; ② the radical cholecystectomy is a complex operation needing long time and wide scope of resection. Sumiyoshi^[9] and Wang *et al.*^[10] studied the effect of surgery as an injury factor on nutritional and immune status in patients with carcinoma, it is coincident with our findings in this report. Our investigation showed that all of the nutritional parameters but the serum levels of iron, TIBC and transferrin recovered within 3 wk after operation. Hickey *et al.*^[11] advocated that supplemental vitamins and minerals, e.g. iron should be given postoperatively when deficiencies are suspected. Our conclusion is that adequate iron should be supplemented after the radical cholecystectomy for gallbladder carcinoma in the third postoperative week, and the serum levels of minerals should be monitored routinely after surgery.

The immune study showed remarkable decrease of serum IgA, IgM, IgG and C₃, C₄ complement, IL-2, CD₄, CD₄/CD₈ ratio, and the remarkable increase of serum sIL-2R and CD₈ ($P < 0.01$) on d3 after operation. IL-2 is a T-cell derived soluble lymphokine whose main bioactivity is to stimulate the activated T cell (Th, Ts, Tc) to reproduce continually, proliferate and is the key mediator in cell and humoral immunity and immune regulation. The balance between IL-2 and its receptor regulates the immune status. T cell serves as the center in controlling cellular immune status which can affect directly the occurrence, development and progression of tumor^[12]. T cell's regulating function is mainly performed by CD₄ and CD₈ T cells. CD₄⁺ T cells can help B cell produce antibody and CD₈⁺ T cells can suppress B cell to produce antibody. The stable balance between them keeps normal immune response of the organism. Surgery, as an injurious factor, broke the balance between CD₄ and CD₈, however T cell's immune regulating function is demanded finally by the organism. In gallbladder carcinoma in an early stage, the serum IL-2, CD₄, CD₈, CD₄/CD₈ ratio, sIL-2R recovered remarkably in the first postoperative week. In early

postoperative stage, the serum levels of immunoglobulins and complement reduced remarkably. This evidence suggests the results are possibly influenced by surgical stress and the diluting effect of the postoperative massive fluid therapy. The immune parameters returned to the preoperative levels within 2 wk-3 wk after surgery, suggesting that T cell plays a more important role in the immune regulating system.

The present study suggests that radical cholecystectomy for early gallbladder carcinoma might have a mild and transient adverse effect on the cell-mediated immune response during the early postoperative period. Because of tumor's own direct products, tumor cell's metabolites and immuno-complex in body circulation, which depress the anti-tumor action of the immune cells^[13,14], patients' immune status deteriorated remarkably in the middle and late stage. For gallbladder carcinoma, radical cholecystectomy in its early stage with complete resection of the tumor and removal of lymphnodes should be performed, thus the immune inhibitors in the tumor mass can be excluded. These factors played important roles in the recovery of immune function.

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Intraoperative endoscopic sphincterotomy for common bile duct stones during laparoscopic cholecystectomy

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Subject headings laparoscopic cholecystectomy; common bile duct stones; endoscopic sphincterotomy

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INTRODUCTION

The advent of laparoscopic cholecystectomy (LC) in the late 1980s gained widespread acceptance within a short period of time and has become the preferred treatment for symptomatic gallstones^[1-7], but the management of coexisting gallbladder and common bile duct (CBD) stones has remained controversial because the various strategies proposed have their limitations^[8-12]. In fact, cholelithiasis is found in 10%-15% of patients with cholecystolithiasis, and most authors agree that at least 90% of CBD stones are secondary to those of the gallbladder, and that CBD stones must be extracted in a timely manner in order to avoid consequent complications which would otherwise occur in at least 90% of patients^[1-4]. This paper will introduce and evaluate a new approach of management of coexisting gallbladder and CBD stones—intraoperative endoscopic sphincterotomy (IOES).

MATERIALS AND METHODS

From December 1997 to August 1999, twenty-seven patients with cholelithiasis and CBD stones were treated by LC and IOES. Cholelithiasis was detected in 9 patients by preoperative ultrasonography and 18 routine intraoperative cholangiography (IOC). They were 5 males and 22 females. The youngest patient was 23 years old, the oldest being 74 years, averaging 50.3 years. The preoperative diagnoses are summarized in Table 1. All patients were treated according to a standard

protocol (Figure 1).

Table 1 Preoperative diagnosis of 27 cases

Preoperative diagnosis	No. of patients
Acute cholecystitis with multiple gallstones	3
Chronic cholecystitis with multiple gallstones	15
Acute cholecystitis with multiple gallstones and CBDs	3
Chronic cholecystitis with multiple gallstones and CBDs	6

Figure 1 Protocol of treatment of 27 cases. LF: liver function; US: ultrasonography; LC: laparoscopic cholecystectomy; IOC: intraoperative fluorocholangiography; CBD: common bile duct; IOES: intraoperative endoscopic sphincterotomy.

LC was undertaken by the four trocar technique and IOC was performed according to the technique described by Alfred Cuschieri and Hong^[1,2]. As soon as the CBD stones were confirmed, the endoscopist was expected on call. The patient lay in supine position, after deflating abdominal CO₂, the endoscope (Olympus JF100 or a side-view JF100, JF140) was advanced into the duodenum. Once the position of ampulla of Vater was determined, EST was undertaken routinely. The size of sphincterotomy was about 0.8 cm-1.5 cm. The CBD stones were extracted in different ways according to their size, shape and number. The small stones (less than 8 mm) were expelled into the duodenum with the help of surgeons by injecting normal saline through the cholangiographic catheter, medium sized stones (8 mm-15 mm) were extracted with the basket (Olympus FG-23Q-1) or by a balloon (Wilson-cook EBL-12-200, EBL-8.5-200) monitored under fluoroscopy, large stones (more than 15 mm in diameter) were eliminated by basket or balloon after mechanical lithotripsy. After IOES and removal of CBD stones, the repeated IOC was performed to make sure that there were no retained CBD stones. The endoscope was withdrawn while aspirating the residual air. The LC was then completed.

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RESULTS

The IOES was technically successful in 26 of 27 patients (96.30%) and the CBD stones were removed in all of these cases (100%). In 5 cases, the stones were expelled into the intestinal tract spontaneously, in 17 cases, extracted by basket or balloon and in 4 cases, removed after mechanical lithotripsy. The number of CBD stones ranged from 1 to 15, and the size of the stones varied from 3 mm to 16 mm. In one case, IOES failed because the sphincterome could not cannulate the ampulla of Vater due to stones impacted tightly the distal portion of CBD. Two cases were complicated with mild acute pancreatitis (7.69%) which resolved within 3 d and 7 d being fasted and intravenously supplemented fluid. No other complications occurred. There were no operative mortalities. The average operation time of LC combined with IOES was about 160 min (from 80 min to 210 min). All but 3 cases recovered uneventfully leaving bed and resuming food 8 h-24 h after LC and IOES. One patient developed pneumothorax after LC procedure. In the other two cases recovery was delayed due to the complication of mild acute pancreatitis (mentioned above). The mean duration of hospital stay was 1d to 26 d ($3 \text{ d} \pm 1 \text{ d}$). The patient who had the pneumothorax was discharged on the 26th postoperative day.

DISCUSSION

It is well known that LC has become the primary method of treatment of symptomatic gallstones. Of the 500 000 cholecystectomies performed annually in the United States, 85% of them are estimated now to be performed laparoscopically^[13]. In China LC is also rapidly accepted. Likewise, the introduction of ERCP and EST in the 1970s rapidly revolutionized the management of CBD stones. Numerous series of cases have shown successful endoscopic clearance of the CBD stones in approximately 90% of patients, complications of EST occur in 5%-15%, most of these are minor, and the mortality is less than 1%. New instrumentation is being developed and improved continuously enabling the endoscopist-surgeon to deal more and more successfully and creatively with a variety of intra-abdominal conditions^[4-6]. But the management of coexisting gallbladder and CBD stones is now more controversial than before, because many approaches to resolve this problem have their limitations besides the expense and time (Table 2)^[8-12].

From May 1994 to November 1997, 1794 cases of LC and IOC were performed in our hospital. In about 7%-8% of patients with cholecystolithiasis^[2,3], CBD stones were also found, we removed them by various methods such as converting to open choledochostomy, laparoscopic choledochostomy, laparoscopic choledochoscopy via

cystic duct and postoperative EST. The results revealed that there was no best procedure for the patients, some shortcomings existed as shown in Table 2. Therefore, we employed a new approach of IOES from December 1997 to August 1999 to remove CBD stones identified at the time of LC.

Table 2 Alternatives of management of coexisting gallbladder and CBD stones

Methods	Shortcomings
Convert to open choledochostomy	loss of all advantages of minimally invasive surgery
Laparoscopic transcystic duct choledochoscopy	limited by cystic duct anatomy and risk associated with cystic duct dilation
Laparoscopic choledochostomy	Installation of T-tube and high technical demand
Preoperative ERCP and EST	hard to precisely predict preoperative CBD stones; if failed, subsequent operation should be considered
Postoperative ERCP and EST	If failed, another operation should be performed

The major advantages of combining LC and EST into one procedure are encouraging. The obvious benefit is that the patients have both problems solved at one session. But our 27 cases treated by LC combined with IOES only represent some of the common bile duct explorations done at our hospital during this period due to the following reasons (among 1485 LC cases during this period). First, some surgeons in our department thought that IOES presents significant logistic difficulties in coordinating the necessary personnel and equipment. Second, it was thought that the supine position may increase the technical difficulty of EST^[8-12]. But in our experience it is easier to remove the CBD stones by a combination of LC with IOES than by pre- or postoperative EST. The endoscopist gets benefit from general anesthesia. The surgeons can help the endoscopist to locate the proper position of the ampulla of Vater and irrigate the CBD by injecting normal saline through the cholangiography catheter. Small or fragmented stones can be washed out with normal saline irrigation. Medium or large sized stones in CBD can be removed with the help of a basket or balloon and by lithotripsy. By deflating the peritoneal cavity of CO₂, the endoscopist was able to insufflate the duodenum adequately for performing IOES, after aspirating the gastrointestinal air, LC was more easily accomplished. From our study, IOES was successful in 26 (96.30%) of 27 patients and CBD stones were cleared in all of them (100%), only two cases were complicated with mild acute pancreatitis (7.69%) resolved within 3 d-7 d with intravenous fluid and fasting. No death occurred postoperatively. The case of pneumothorax after LC recovered by conservative management. The total operative time compares favorably with that of

standard LC with CBD exploration in our hospital.

We recommend IOES as a valid alternative approach to the removal of CBD stones during LC. It has been safe and effective, and less traumatic to the patient than other intraoperative procedures or pre- or postoperative EST.

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Pathogenetic effects of platelet activating factor on enterogenic endotoxemia after burn

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Subject headings Platelet activating factor; burn; endotoxemia ; intestinal permeability

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INTRODUCTION

Previous clinical and experimental studies have indicated that an early endotoxemia occurred after a major burn. It is unlikely that burn wound sepsis is the source of circulating endotoxin in less than 12 hour after burn. Increasing evidence demonstrates that the bacteria and endotoxin in the gastrointestinal tract can pass through the gut barrier into blood circulation to form enterogenic endotoxemia following burn^[1-3]. However, its pathogenesis remains poorly understood. Platelet activating factor (PAF), an endogenous phospholipid mediator, has recently been proposed as a critical mediator in shock, sepsis and multiple organ failure^[4,5]. In this study, the relationship between changes of PAF and enterogenic endotoxemia was observed on rat models with 30% TBSA III° burn. The purpose was to investigate the pathogenetic effects of PAF on the occurrence of enterogenic endotoxemia after burn.

MATERIALS AND METHODS

Animals

Wistar rats, male or female, weighing 220 g ± 30 g were used, they were provided by Laboratory of Animal Experiment, Institute of Burn Research, Third Military Medical University.

Experimental design

Animals were randomly divided into three groups. Group 1 (*n* = 10): normal rats served as control. Group 2 (*n* = 40): burned rats that had undergone

30% TBSA III° burn. Group 3 (*n* = 40): treated rats that received PAF antagonist WEB 2170 (5 mg/kg) by intraperitoneal injection after burn. WEB 2170 was provided by Boeringer Ingelheim *Pharmac*-euticals Inc, Germany.

Animals were killed on 6, 12, 24 and 48 hours postburn. Blood and terminal ileum were obtained from all animals for assay of PAF and endotoxin.

Burn model

Rats were anesthetized intraperitoneally with ketamine hydrochloride 80 mL/kg body weight, and their backs were shaved. They were placed in a mould that left approximately 30% area of their body surface exposed. These exposed surface s were immersed in 92°C water for 18s. This type of burn injury is a full-thick ness burn. Animals were resuscitated with 40 mL/kg of lactated Ringer's solution.

Measurement of PAF contents in blood and intestinal tissue

Blood (1mL) was collected into polypropylene tube containing 5 mL of methanol. The methanolic extract was separated by centrifugation. The supernatants were collected and chloroform and water were added to effect phase separation. The lower chloroform-rich phase contained all PAF activity. Chloroform was evaporated under a stream of nitrogen. The samples were stored under -20°C. Segments of ileum tissue (200 mg) were added to 2 mL of 0.25% bovine serum albumin, and after homogenization, the mixture was added to 2 mL of cold acetone, and then centrifuged. Two mL of chloroform as added to the supernatants. After a further centrifugation, the thin layer chloroform containing PAF was collected and evaporated by nitrogen. PAF activity was bioassayed by the aggregation of rabbit washed platelets.

Measurement of intestinal mucosal permeability

After overnight fast, rats were anesthetized, a midline abdominal incision was made. Fifteen cm segments of ileum were isolated, cannulated proximally and distally, and perfused continuously with saline at rate of 1 mL/min-2 mL/min. ^{99m}Tc DTPA (5.55-7.4MBq/kg) was injected via the carotid vein and allowed to equilibrate for 30 min. After that, a 10 min perfusion fluid and 1mL blood were collected for measurement of activity of ^{99m}Tc

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DTPA. The animals were killed and the perfused ileal segment was excised and weighed. DTPA clearance was calculated using the following formula: DTPA clearance = (cpm perfusate × Q)/(cpm plasma × W), and was expressed as mL/min • 100g. Where Q is the rate of perfusion; W, the weight of perfused ileum.

Measurement of plasma endotoxin

Plasma endotoxin was assayed with chromogenic limulus amoebocyte lysate technique.

Statistical analysis

All data were expressed as $\bar{x} \pm s$, and statistical analyses were made using Student's *t* test.

RESULTS

The changes of PAF content

PAF contents of blood and intestinal tissue in burn group were significantly higher than those in control group ($P < 0.01$). The peak level occurred at 12 h postburn. In PAF-antagonist treatment group, the PAF contents of blood and intestinal tissue were significantly decreased compared with burn group, but were higher than those in control group

(Table 1).

The changes of intestinal mucosal permeability

The intestinal mucosal permeability increased significantly at 6h postburn and kept increasing during 48 h postburn compared with control group. The intestinal mucosal permeability in treatment group was lower than those in burn group (Table 2).

The changes of plasma endotoxin

The levels of plasma endotoxin in burn group were significantly higher than those in control group. The levels of plasma endotoxin in treatment group were significantly lower than those in burn group (Table 3).

Correlation analysis

The correlations between intestinal PAF and intestinal mucosal permeability, blood PAF and plasma endotoxin, intestinal mucosal permeability and plasma endotoxin in burn group were analyzed. The results showed positive correlation among the above three pairs with $P < 0.01$, $r = 0.94$, 0.93 and 0.95 respectively.

Table 1 Levels of PAF in blood and intestinal tissue ($\bar{x} \pm s$)

Group	n		After burn (h)			
			6	12	24	48
Control	10					
Blood($\mu\text{g/L}$)		0.56 ± 0.07				
Intestine(ng/g)		0.41 ± 0.06				
Burn	40					
Blood($\mu\text{g/L}$)			1.72 ± 0.21^b	2.76 ± 0.25^b	1.54 ± 0.24^b	1.19 ± 0.13^b
Intestine(ng/g)			1.80 ± 0.21^b	2.34 ± 0.18^b	1.68 ± 0.15^b	1.42 ± 0.16^b
Treatment	40					
Blood($\mu\text{g/L}$)			0.84 ± 0.16^{da}	1.46 ± 0.27^{db}	0.93 ± 0.18^{ca}	0.71 ± 0.15^c
Intestine(ng/g)			0.67 ± 0.07^{da}	1.24 ± 0.13^{dbaa}	0.83 ± 0.12^{da}	0.64 ± 0.08^{da}

^a $P < 0.05$, ^b $P < 0.01$ vs control; ^c $P < 0.05$, ^d $P < 0.01$ vs burn group.

Table 2 Changes of intestinal mucosal permeability ($\text{mL} \cdot \text{min}^{-1} \cdot 100\text{g}^{-1}$, $\bar{x} \pm s$)

Group	n		After burn (h)			
			6	12	24	48
Control	10	0.07 ± 0.02				
Burn	40		0.33 ± 0.14^b	0.58 ± 0.18^b	0.21 ± 0.07^b	0.13 ± 0.04^b
Treatment	40		0.19 ± 0.05^{db}	0.27 ± 0.06^{db}	0.10 ± 0.04^{da}	0.08 ± 0.03^c

^a $P < 0.05$, ^b $P < 0.01$ vs control; ^c $P < 0.05$, ^d $P < 0.01$ vs burn group.

Table 3 Levels of plasma endotoxin (ng/L , $\bar{x} \pm s$)

Group	n		After burn (h)			
			6	12	24	48
Control	10	34 ± 8				
Burn	40		93 ± 28^b	129 ± 32^b	90 ± 22^b	59 ± 16^b
Treatment	40		57 ± 15^{ca}	66 ± 13^{db}	50 ± 10^{da}	43 ± 8^c

^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$, ^d $P < 0.01$ vs burn group.

DISCUSSION

The pathogenesis of enterogenic endotoxemia remains poorly understood. It is considered that the main cause is injury of intestinal mucosal barrier. Under physiological condition, the intestinal mucosa functions as a major local defense barrier preventing intestinal bacteria and endotoxin from invading distant organs and tissues. However, under the circumstances of trauma, shock and sepsis, the impairment of intestinal barrier may result from ischemic damage of the intestinal mucosa, the bacteria and endotoxin in gastrointestinal tract can pass through the intestinal barrier to mesenteric lymph nodes and systemic organs, resulting in enterogenic sepsis (endotoxemia)^[6,7]. There is increasing evidence that enterogenic sepsis may play an important role in the development of systemic infection as well as multiple organ failure^[8,9]. The present study results show that increased intestinal permeability after burn is an important cause of enterogenic endotoxemia.

PAF is a phospholipid mediator released from stimulated leukocytes, platelets, endothelial and mast cells^[10,11]. It has been regarded as an important endogenous mediator of shock, sepsis and multiple organ failure. Our study demonstrated that PAF contents in blood and intestinal tissue after burn were all significantly increased and were positively correlated with the increase of intestinal permeability and plasma endotoxin. Treatment with PAF antagonist can significantly decrease intestinal permeability and plasma endotoxin. These suggest that PAF is involved in the process of increasing the intestinal permeability after burn and is also an important factor leading to enterogenic endotoxemia.

Previous studies showed that endotoxin can stimulate directly or indirectly macrophages and endothelial cells to release PAF, which also

mediates some endotoxin-induced pathologic processes of multiple organ injury^[12,13]. It is suggested that a PAF-endotoxin positive feedback relationship existed in the body. Therefore, administration of PAF antagonists has an important effect on preventing and treating enterogenic endotoxemia.

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Ultrastructural localization of glutathione S-transferase-pi in human colorectal cancer cells

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Subject headings colorectal neoplasms; GST-pi; immunoelectron microscope; colloid gold

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INTRODUCTION

Placental form glutathione S-transferase (GST-pi) is a group of isoenzymes with protein combining ability. Since the reports showing that GST-pi was significantly increased in proliferative hepatic nodule induced by chemical carcinogen and in well differentiated carcinoma^[1], more studies have shown^[2-4] that GST-pi expressed highly in neoplasm, and could be regarded as a tumor marker. GST-pi has the antimutagenesis and antitumor ability, and is related closely with the resistance of tumor to anticancer drugs. As the description about the immunoelectron microscopical localization of GST-pi in human colorectal cancer cells was not available, we have observed the ultrastructural localization of GST-pi in colorectal cancer cells, and explored the distribution of GST-pi in these cells and its relationship with colorectal carcinogenesis.

MATERIALS AND METHODS

Fresh colorectal cancer tissues ($n = 6$) were obtained from surgical specimens, and the colorectal mucosal tissues ($n = 3$) more than 10 cm away from the cancer served as controls.

Reagents Monoclonal mouse anti-GST-pi antibody (Dako Company); 10 nm colloid gold labeled goat anti-mouse IgG (Institute of Basic Medicine, Academy of Military Medical Science).

Tissue processing All fresh blocks of tissue were fixed with 2.5 mL/L glutaraldehyde and 40 mL/L

paraformaldehyde, routinely dehydrated and embedded in Epon 812. Ultrathin sections were placed on nickel grids, treated with 30 mL/L H₂O₂ (15 min), washed thoroughly with 0.05M, pH 7.4 TBS, blocked with 1% bovine serum (30 min), dropped mouse anti-GST-pi monoclonal antibody (1:20), and placed in refrigerator (4°C) overnight. Washed with 0.05M, pH 7.4 TBS, then with 0.02M, pH 8.2 TBS, dropped 10 nm colloid gold labeled goat anti-mouse IgG (1:10), stand in room temperature for 1 h, washed with TBS, and restained with uranyl acetate followed by lead citrate. The first antibody was replaced by TBS as control. These specimens were observed under electron microscope.

RESULTS

The most significant ultrastructural change of colorectal cancer cell occurred in the nucleus. It was large and irregular in shape, with peripheral aggregation of heterochromatin and plenty of ribosomes and large nucleoli. There were colloid gold labeled GST-pi positive particles in all of the six cancer specimens. The positive particles were round in shape, high and clear electron density, distributed as clusters or spots in cancer cells. The majority of positive particles were in mitochondria, lysosomes and some plasma, and some also presented in nuclei (Figure 1) or in the periphery of nuclei (Figure 2). The positive particles accumulated in lumps. Visible positive particles were never found in normal control cells.

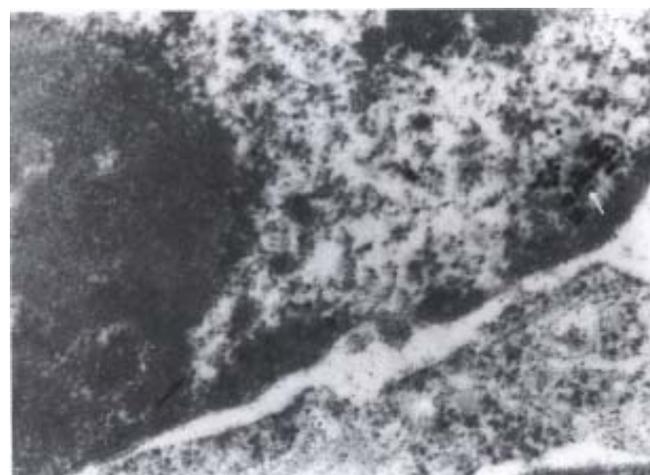


Figure 1 GST-pi positive particles present in nuclei. EM × 40 000

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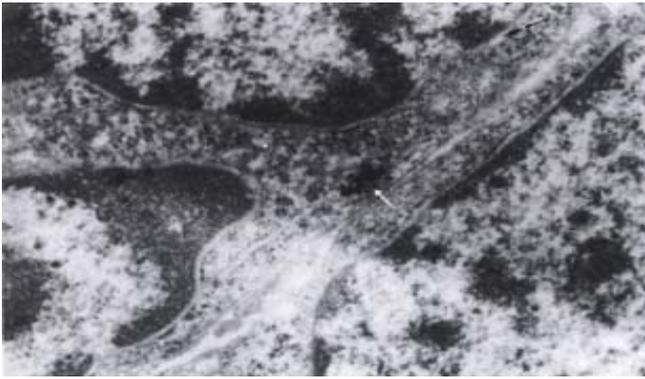


Figure 2 GST-pi positive particles present in the periphery of nuclei. EM \times 25 000

DISCUSSION

Biological characters of GST-pi

Placental form of glutathione included rat and human forms named GST-p and GST- π respectively. Because they presented generally in placenta according to the new category, they were nominated as GST-placental isoenzyme (GST-pi) as a whole. GST-pi could detoxify chemical mutagenesis, cancer promoter, lipid and DNA hyperoxidase, and protect normal cells from the influence of cancerigenic materials, so that GST-pi played an important role in the anti-mutation and anti-cancer process. As an important enzyme of detoxification, GST-pi was controlled by the gene. The high expression of GST-pi gene occurred while the carcinogen acted on the cells to induce their mutation^[5]. The high expression of GST-pi in many neoplasms was related to the resistance of anticancer drugs. Katagiri *et al* reported that the expression of GST-pi negatively correlated to the sensitivity of anticancer drug in testicular tumor^[6]. Dong J suggested that the expression of GST-pi closely related to resistance of anticancer drug in ovarigenic neoplasm^[7]. GST-pi could be regarded as a marker in evaluating the effect of tumorectomy, or in predicting the drug resistance of tumor cells. High expression of GST-pi in precancer and cancer of liver, and the protein synthesis ability could be blocked by cyclohexamide, which showed that GST-pi was controlled in transcription level. This may be the molecular base of the cancer cells in

getting cytotoxic drug resistance. We found that GST-pi expressed only in cancer cells, but not in normal cells, suggesting that this might be related to the action of carcinogen. GST-pi specific expression in colorectal cancer cells may be used as a marker for diagnosis of colorectal carcinoma.

Ultrastructural localization of GST-pi

We found that GST-pi was located in cytoplasm, mitochondria, lysosomes and nucleus adjacent to nuclear membrane of colorectal cancer cells. These agreed with immunohistochemical studies^[8]. But normal mucosa occasionally showed slight positivity in immunohistochemical studies. This is different from our findings. It might be related to the weak expression in normal mucosa. There were few studies about the GST-pi ultrastructural localization. In our study, GST-pi positive particles were found not only in cytoplasm, but also in nucleus. Chen M reported the same result in bladder carcinoma^[9]. Why was GST-pi positive particles present in nucleus? We think this can be an atavism, and if so it could not be used as a marker indicating colorectal cancer. Whether it is a right concept or not needs further studies.

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Case Reports

Life threatening vitamin B₁₂ deficiency: will timely screening make a difference?

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Subject headings vitamin B₁₂ deficiency/therapy; anemia, sickle cell; gastric mucosa; biopsy; anemia, pernicious; toxicity

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INTRODUCTION

While Vit.B12 deficiency is common, with a prevalence of about 15% in the elderly^[1-3], and recommendations for treatment available, detection of deficiency at the pre-clinical stage by appropriate screening does not always take place. Our report is an example of life threatening Vit.B12 deficiency diagnosed at age 56, with the onset of Vit.B12 depletion likely to have begun in the previous decade. Further, the patient had sickle cell trait along with Vit.B12 deficiency, a combination only sporadically reported.

CASE REPORT

A 56 year old caucasian male with no significant past medical illness presented to our University hospital with a history of lethargy, weakness, anorexia, pallor and icteric sclera for a few months. He denied use of medications inclusive of acid lowering agents (H₂ blockers or proton pump inhibitors) and vitamins of any kind. Dietary habits were omnivorous. Physical examination confirmed marked pallor and jaundice. Vital signs were stable; rest of the examination including nervous system was unremarkable.

Hemoglobin: 24g • L⁻¹, Hematocrit: 6.9%, MCV: 75fl, Platelet: 64 × 10⁹ • L⁻¹, Corrected reticulocyte count: 0.004, Blood smear: anisocytosis, poikilocytosis and macroovalocytosis with hypersegmented neutrophils, Folate: 12.1 µg • L⁻¹, Iron: 1.57 µg • L⁻¹, TIBC: 59, Ferritin: 764 µg L⁻¹, LDH: 15500U/L, and Haptoglobin: 31 mg • L⁻¹. Hemoglobin electrophoresis: HbA 0.67, HbS 0.30. Direct

Coomb's test: negative. Vitamin B12: 57ng L⁻¹ (Normal 200-900ng L⁻¹), Intrinsic factor antibodies: positive. Bone marrow aspirate and biopsy: erythroid hyperplasia with megaloblastic features. Upper endoscopy and gastric biopsy revealed chronic gastritis with mucosal atrophy. Colonoscopy was normal except for hemorrhoids.

Therapy included a total of 6 units of packed red blood cells. Vitamin B12 therapy was initiated as daily intramuscular injections, 1 000 µg daily for 3 days. Clinical improvement was dramatic and the patient was discharged shortly.

DISCUSSION

Our patient presented with pancytopenia and a life-threatening drop in Hb to 24 g • L⁻¹. Though the hematologic manifestations were severe, interestingly, there was no evidence of peripheral neuropathy, myelopathy, dementia or any neuropsychiatric manifestations. In view of omnivorous dietary habits, a dietary deficiency from inadequate consumption of Vit. B12 was unlikely. Neither was the patient on any antacids or other acid lowering agents. Proton pump inhibitors and H₂ blockers, widely used as prescription and over the counter agents for upper gastrointestinal symptoms have been associated with Vit. B12 deficiency. Acid peptic activity is important in the initial step where Vit. B12 is separated from food protein; lack of acid may cause food-cobalamin malabsorption, a common cause of Vit.B12 deficiency^[4,5].

An average omnivorous American diet provides 5 µg-15µg of Vit.B12 per day. Animal products such as meat, poultry, fish, eggs and dairy products are rich sources of Vit.B12. Plant sources contain Vit.B12 only if they are contaminated with microorganisms^[6,7]. As a result, vegans are at a higher risk of becoming Vit. B12 deficient over a period of time. When consumed, Vit.B12 attaches to an 'R' binder (haptocorrin) present in saliva, after it is separated from food protein by acid in the stomach. The pancreas secretes proteases, which at an alkaline pH beyond the stomach digests the 'R' binder facilitating the attachment of intrinsic factor (IF) to B12. IF-B12 complex is absorbed via receptors in the distal ileum. An efficient enterohepatic circulation of Vit.B12, wherein most of the Vit.B12 secreted in the bile is reabsorbed, is the reason why it takes anywhere upto 20 years to become Vit.B12 deficient if one stops consuming

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Vit.B12^[6,8].

PA was the most likely cause of Vit.B12 deficiency in our patient. The age of onset with advanced disease by mid fifties and absence of any other basis like gastric or small intestinal surgery, chronic pancreatitis, *Crohn's* disease and other causes of malabsorption makes PA the probable diagnosis. The presence of IF antibodies, which is more specific for PA than parietal cell antibodies and seen in about 40% of patients with PA, lends credence to our assumption^[9]. The additional finding of Vit.B12 deficiency with sickle cell trait in this patient is not common. Patients with severe sickle cell disease may have unrecognized Vit. B12 deficiency^[10]. Furthermore, routine folate supplementation in sickle cell anemia prior to determining Vit.B12 status has been considered risky, as it can mask the findings of Vit.B12 deficiency^[11].

The spectrum of Vit.B12 deficiency has been elaborately described in 4 stages^[8,12]. Stages 1 and 2 represent Vit.B12 depletion and stages 3 & 4 represent Vit.B12 deficiency with lab abnormalities and clinical manifestations. Our patient presented with full-blown stage 4 disease, suggesting that he would have been in the pre-clinical stage for many years prior to presentation. Screening for Vit.B12 deficiency would avert the morbidity associated with deficiency states. While in the past there have been no precise guidelines for screening, more recently several approaches have been described. Screening is aimed at reaching a diagnosis at the onset of depletion, i.e. at the pre-clinical stage. The literature suggests several options—from doing nothing until one is symptomatic, to screening all individuals, or an individualized approach^[4,13,14].

Our approach to screening and treatment of Vit. B12 deficiency has been described previously^[13]. Here initial screening is recommended for a select group of individuals at first contact. Included are patients with unexplained anemia, gastritis, acid lowering states from use of certain drugs, autoimmune diseases, HIV disease, *Crohn's* disease, multiple sclerosis, thyroid disease, malabsorption syndromes and vegans. In all other patients the initial screening is recommended at age 50, and thereafter every 5 years until age 65. Annual screening is suggested after age 65. Although normal Vit.B12 levels range from 200 to 900ng • L⁻¹, values between 200 and 400ng • L⁻¹ may need further evaluation including serum (or urine) homocysteine and methyl malonic acid to assess for presence of true deficiency^[13,14].

Treatment for Vit.B12 deficiency is generally initiated with intramuscular injections of Vit.B12, the usual dose being 1 000 µg daily for 3-5 days. Doses vary from 100 to 1 000 µg • d⁻¹ larger doses are accompanied by greater losses in the urine^[6]. Maintenance therapy may be by any of 3 routes intramuscular (IM), oral or intranasal. IM

injections are given every 1 to 3 months. Oral administration necessitates larger doses; 500 to 1 000 µg • d⁻¹ are needed to ensure absorption in PA where 1% maybe absorbed even in the absence of IF^[15]. However compliance with oral administration will always remain in question. Intranasal administration of Vit.B12 has been approved in 1998; this form of Vit.B12 is administered weekly (500 µg • wk⁻¹) and attains levels comparable to maintenance with IM route^[13,15].

CONCLUSION

The case illustrates the importance and need for timely screening for Vit.B12 deficiency. Delay in diagnosis and treatment resulted in a near fatal presentation of a common disease. The primary care physician should be aware that there is a window of opportunity for diagnosis and treatment; several complications of Vit.B12 deficiency are irreversible if early treatment is not provided. The treatment modalities are several and inexpensive, with no side effects. Selection of screening tests and choice of maintenance therapy may be individualized based on patient and physician preferences. Timely screening and treatment of Vit. B12 deficiency will make a difference.

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Liver inflammatory pseudotumor or parasitic granuloma ?

Xiao Long Ji, Ming Shi Shen and Tong Yin

Subject headings inflammatory pseudotumor; parasitic granuloma; ascariasis larva; liver disease

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INTRODUCTION

Liver pseudotumor is a very rare benign lesion. Since the first case reported by Pack and Baker in 1953^[1], only 40 cases had been reported up to 1996. The diagnostic challenge of hepatic inflammatory pseudotumor is emphasized by the fact that most of the reported cases were diagnosed by surgical procedures.

Pathogenesis and etiology of hepatic inflammatory pseudotumor are uncertain. We report a case of hepatic pseudotumor that was suspected to be a well-differentiated hepatocellular carcinoma based on abdominal ultrasound, CT and MRI, but the final diagnosis is parasitic granuloma of ascariasis larva after hepatic lobectomy.

CASE REPORT

A solitary multiple focal hepatic lesion was discovered in a 52-year-old man under the examination of ultrasonography when he was undergoing the regular physical examination in 1998-12. The ultrasonography showed that the nodule appeared as well defined, hypoechoic and hypovascular irregular solid mass without posterior acoustic enhancement on ultrasound (Figure 1). Subsequently, CT scan and MRI also demonstrated the existence of the lesion in the frontal segment of the right hepatic lobe (Figures 2, 3). In January 1999, a fine needle aspiration biopsy was performed on the lesion and the cytological examination showed the possibility of the well differentiated hepatocellular carcinoma. Therefore, the patient underwent the hepatectomy on January 12, 1999.

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Pathological findings

Gross findings: The specimen of partial hepatic lobectomy measured 10 cm × 8 cm × 6 cm in size. The cut surface of the lesion contained several pale nodules, which were well-defined and slightly hardened. The center of the nodule was soft and pale-brownish in color, but the contour appearance of the surrounded liver was normal.

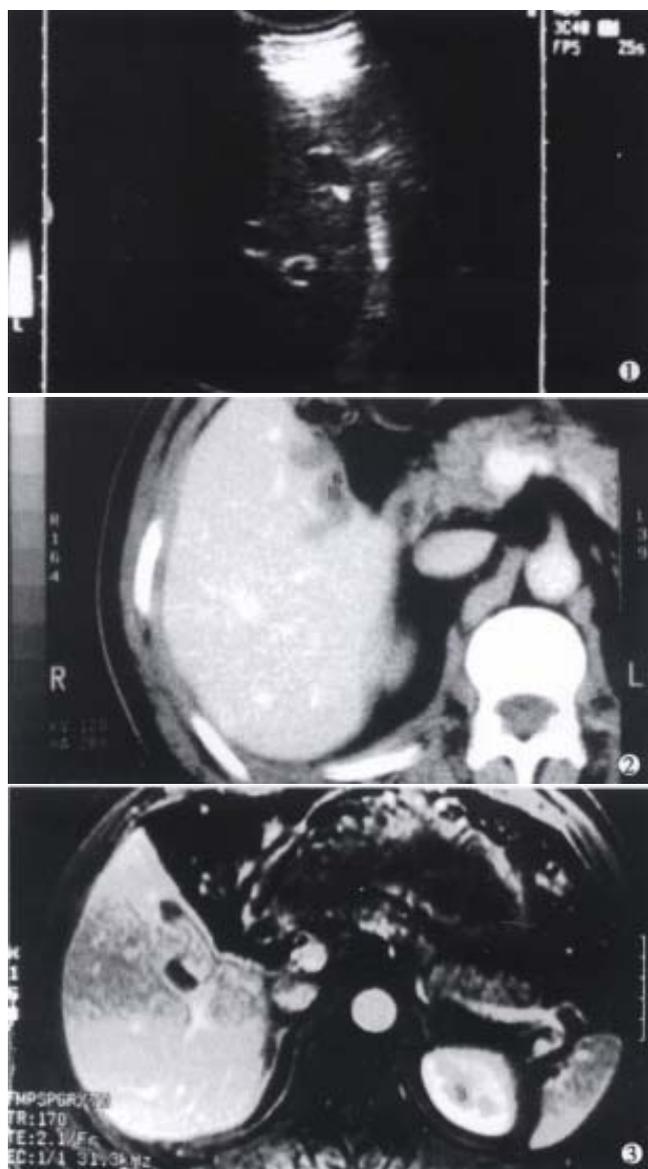


Figure 1 A nodule appeared as well-defined, hypoechoic and hypovascular irregular solid mass by ultrasonography.

Figure 2 Two nodules of low density without early enhancement detected on CT scans.

Figure 3 Two nodules of low signal intensity on T1WI and isosignal intensity on T2WI by MRI.

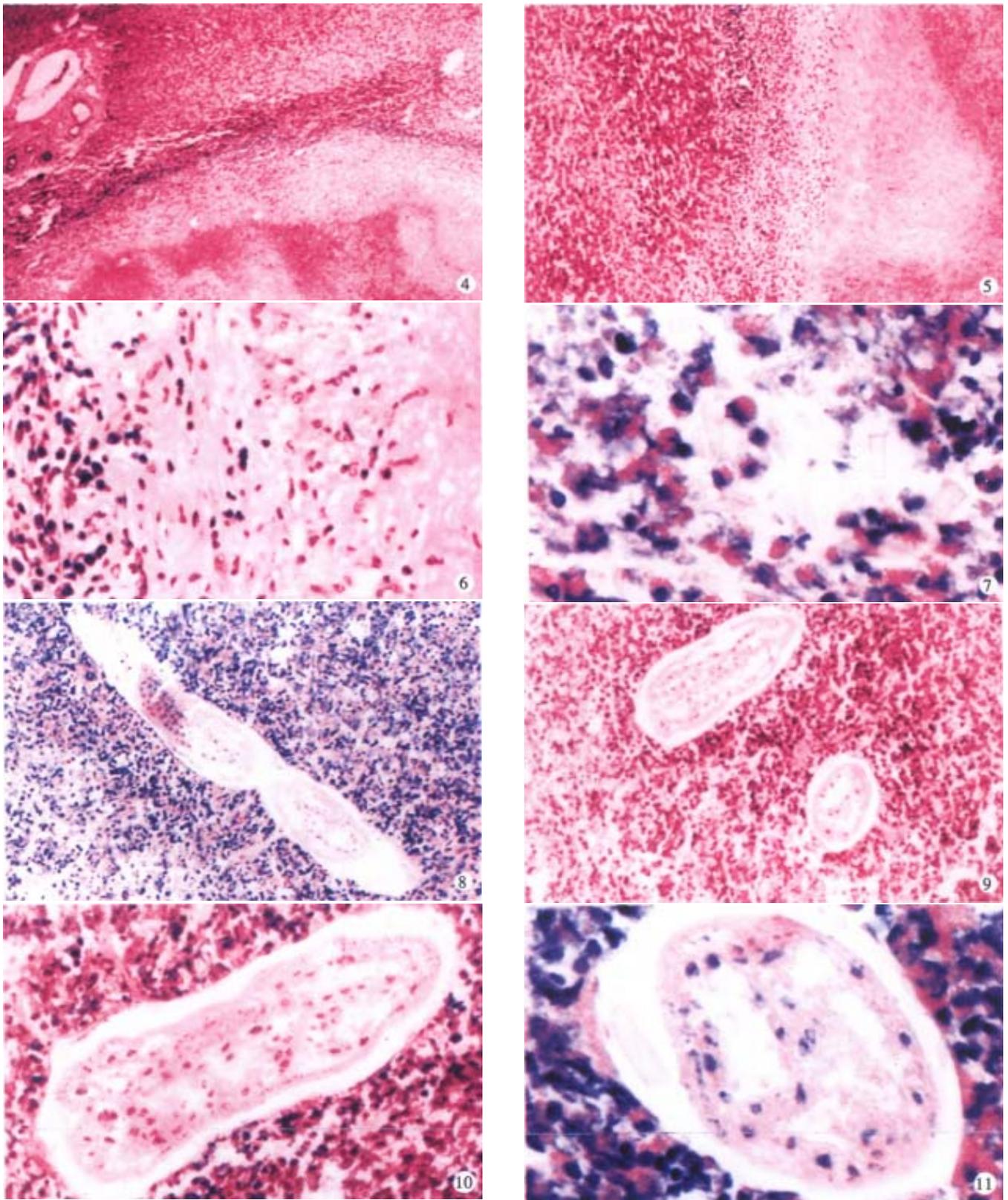


Figure 4 Solitary necrotic nodules of the liver. HE \times 50

Figure 5 Central necrotic core enclosed by a hyalinised fibrotic capsule. HE \times 100

Figure 6 Hyalinised fibrotic capsule infiltrated with eosinophils and lymphocytes. HE \times 200

Figure 7 Charcot-Leyden's crystals and eosinophils in the necrotic center. HE \times 400

Figure 8 One degenerated larva in the center of necrotic nodule. HE \times 100

Figure 9 Two pieces of the degenerated larva in the necrotic center of the nodule. HE \times 200

Figure 10 Necrotic cells around the larva. HE \times 200

Figure 11 Degenerated larva with Charcot-Leyden's crystals. HE \times 400

Microscopic findings

Histopathologic examination. The lesion contained a large area of necrosis with irregular border (Figure 4), around which there are fibrosis and infiltration of lymphocytes, plasma cells and eosinophils (Figures 5,6). In the center of the necrotic lesion, there are many Charcot-Leyden's crystals (Figure 7). The parasite infection was suspected and 26 pieces of tissues from the specimen were sectioned and embedded to find out the pathogen. Finally several parasites with certain morphologic architecture could be found in the sphaelus of the same section (Figures 8,9) only in one of the slides. Although the cells of the parasites were degenerated or necrosed, the viscus and somatic texture could still be recognized (Figures 10,11).

DISCUSSION

Inflammatory pseudotumors are rare benign lesions which may occur in anywhere of the body. They are usually associated with fever, pain and mass formation, and are frequently misdiagnosed as malignant neoplasm. Clinical manifestations and laboratory findings are similar in hepatic pseudotumor and hepatocellular carcinoma. The differential diagnosis between these two is difficult merely based on the images from ultrasonic and radiological examinations. The diagnostic challenge of hepatic inflammatory pseudotumor is emphasized by the fact that most of the reported cases were diagnosed after surgical procedures.

Liver pseudotumors are especially rare and the etiology and pathogenesis are uncertain. Infection was considered as a possible etiology^[2]. Only two cases with organisms were reported: *Escherichia coli* was detected in one case^[3], and Gram-positive cocci in the other^[4]. Other suggested mechanisms include an immune reaction, liver hemorrhage and necrosis,

occlusive phlebitis of hepatic veins, and local reaction to biliary tract^[5]. This is the first case in the literature which demonstrated that the ascariasis larva was found in the necrotic focus of the liver and it was primarily diagnosed as inflammatory pseudotumor. It was suggested that more sections should be taken when hepatic inflammatory pseudotumor was suspected.

The life cycle of ascariasis is complex but well understood. Adult male and female worms live in the small intestine, usually the jejunum, where each gravid female worm produces 200 000 to 250 000 eggs daily. Fertilized eggs pass in the feces and develop into infective eggs in 3 to 4 weeks. The eggs hatch in the small intestine, and the emerging larvae penetrate the intestinal wall, enter the portal vein or intestinal lymphatic vessels, migrate through the liver to the heart, and are pumped through the pulmonary arteries to the lungs. In the lungs, the larvae break out of capillaries into the air spaces. These larvae migrate up from the bronchi to the trachea and down to the esophagus. In the intestine, the larvae develop into sexually mature adults^[6]. From this life cycle, we postulate that the larvae are arrested in the liver during their migration through the liver and become a necrotic nodule.

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Renal amyloidosis as a late complication of Crohn's disease: a case report and review of the literature from Japan

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Subject headings Crohn's disease/complication; renal amyloidosis; amyloid deposits

Saitoh O, Kojima K, Teranishi T, Nakagawa K, Kayazawa M, Nanri M, Egashira Y, Hirata I, Katsu KI. Renal amyloidosis as a late complication of Crohn's disease: a case report and review of the literature from Japan. *World J Gastroentero*, 2000;6(3):461-464

INTRODUCTION

Secondary amyloidosis is a rare but serious complication of Crohn's disease (CD). The incidence of the association of secondary amyloidosis in patients with CD has been reported to be 0.5%-8% in Western countries [1-6]. However, in Japan, the number of patients with CD complicated by amyloidosis is limited. The characteristics of their clinical manifestations and the incidence of association are uncertain. Therefore, we report herein a patient with Crohn's disease who developed amyloidosis 13 years after the onset of CD. The diagnosis of renal amyloidosis was confirmed by renal biopsy. We also reviewed the literature concerning amyloidosis associated with CD in Japan.

CASE REPORT

The patient was a 29-year-old Japanese female. In May 1985, at the age of 14, she developed diarrhea. In March 1986, she developed fever and arthralgia. A diagnosis of CD was made by endoscopic and radiographic examinations. Thereafter, she received corticosteroids and sulfasalazine. However, hospitalization was required several times. In May 1993, severe stenosis of the ascending colon was found as shown in Figure 1. Therefore, subtotal colectomy, ileocecal resection, and ileosigmoid anastomosis were performed. As shown

in Figure 2, surgical specimen of the terminal ileum, cecum, and ascending colon (May 26, 1993) showed thickening of the bowel wall, cobblestone appearance, and longitudinal ulceration. Histological findings showed transmural inflammation and noncaseating epithelioid cell granuloma. In February, 1997, the patient was admitted because of fever, anemia, and hypoproteinemia. As shown in Figure 3, gastrografin enema examination performed on May 8, 1997 showed a stricture around the ileosigmoid anastomosis. The anastomosis and part of the remaining ileum were resected because of the stenosis. Cholecystectomy was also performed because of gallstones (the biggest stone measured 11 mm × 5 mm, five stones in total, pigmented). Immediately after surgery, right ureteral stricture due to retroperitoneal involvement was found and indwelling double J-catheter was placed. In August 1998, she was admitted because of anemia and hypoproteinemia. Her family history was unremarkable. Her height was 158 cm and body weight was 39.5 kg. Physical examination revealed marked pretibial edema. Laboratory findings included the followings: WBC $7.9 \times 10^9/L$, RBC $2.25 \times 10^{12}/L$, Hb-69 g/L, Ht 0.216, platelet $360 \times 10^9/L$, total protein 53 g/L, albumin 11 g/L, CRP 8.9 mg/dL (normal: less than 0.25 mg/dL), serum amyloid A protein (SAA) 235 $\mu g/dL$ (normal: less than 8 $\mu g/dL$), BUN 7.5 mmol/L, creatinine 145 $\mu mol/L$, creatinine clearance 0.38 mL/s, and urinary protein 9.3 g/day. Renal biopsy was performed in November 1998. As shown in Figure 4, amyloid deposition was found in the mesangial areas and blood vessels. The deposits were Congo red positive. The positive staining disappeared after pretreatment with potassium permanganate. The deposits immunoreacted with the antibody directed against amyloid A (AA)-amyloid. Colonoscopy revealed small discrete ulcerations around J-pouch. The amyloid deposition was not observed in the digestive tract. The electrocardiogram was normal. Ultrasonography did not show any abnormal findings in the heart. Thyroid was not swollen. Thyroid function tests were normal. Administration of prednisolone (40 mg/day) and 5-aminosalicylate (5-ASA) (2.25g/day) normalized serum levels of

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acute phase protein such as CRP and SAA. However, massive proteinuria and hypoalbuminemia persisted. Then, when the dosage of prednisolone was reduced to 10 mg/day, serum CRP and amyloid A protein became positive. Thereafter, azathiopurine (50 mg/d ay) was administered in addition to 5-ASA (2.25 g/day) and prednisolone (10 mg/day).

The onset of CD in this patient was May 1985. Diagnosis was made in March 1986. Proteinuria developed in July 1997. Massive proteinuria developed in April 1998. Creatinine clearance was decreased in July 1997. Amyloid deposits were supposed to have already been present in mid 1997.

DISCUSSION

There is wide geographic variation in the incidence of secondary amyloidosis, occurring in 6%-8% of patients with CD in Northern Europe^[2,4,5], 2% in England^[3], but only 0.5%-0.9% in the United States^[1,6]. However, in Japan, the incidence of secondary amyloidosis in patients with CD remains uncertain. Only 18 Japanese patients with CD complicated by secondary amyloidosis have been reported in the literature from Japan (Table 1)^[7-22].

Secondary amyloidosis is caused by extracellular deposition of the N-terminal AA fragment of the circulating acute phase plasma protein SAA. Secondary amyloidosis can complicate any inflammatory condition in which there is a sustained acute-phase response including CD, rheumatoid arthritis, and chronic sepsis. It has been reported that the activity of the underlying inflammation is an important factor in the development and progression of secondary amyloidosis. In the 18 Japanese patients with CD complicated by

amyloidosis, there were no patients who maintained prolonged remission. Therefore, in CD as well, the disease activity is considered an important factor in the development of secondary amyloidosis. In the present patient, surgery had already been performed two times as the disease activity could not be controlled by medical treatment. Nevertheless, serum CRP remained positive. Moreover, in the present patient, the involvement of the retroperitoneum caused right ureteral stricture.

Inflammation is thought to precede the development of secondary amyloidosis. However, the time-course and progression of secondary amyloidosis are not understood well. In animal experiments, amyloid deposits were found 18h or a few weeks after inflammatory stimuli^[23-25]. Various factors have been reported to influence the susceptibility, onset, and progression of murine amyloidosis^[26,27]. It remains controversial whether amyloidosis is a late complication of CD. In the present patient, nephrotic syndrome developed and the diagnosis of secondary amyloidosis was made 13 years after the onset of CD. When proteinuria was initially detected, it had already been 12 years since the onset of CD. In the literature, the time lapse between the onset of CD and the diagnosis of amyloidosis has been reported to range from 3 to 15 years or from 1 to 21 years^[28,29]. In 11 of the 18 Japanese patients, more than 5 years had passed after the onset of CD. The longest period was 18 years. Conversely, in 2 patients, the diagnosis of amyloidosis preceded that of CD. When CD was diagnosed in these 2 patients, the lesion of CD had already become typical. These findings suggest that secondary amyloidosis usually occurs as a late complication of CD. However, as the onset of CD is usually gradual, CD and secondary amyloidosis may be diagnosed almost simultaneously.

Table 1 Japanese Crohn's disease patients complicated by secondary amyloidosis

Case	Age	Sex	Sites of CD	Duration of CD prior to amyloidosis diagnosis	Clinical course of CD before amyloidosis diagnosis	Major clinical manifestation of amyloidosis	type of amyloid	Proteinuria	Author (year)
1	23	M	I,C	6 year	Not well controlled	Intestinal	AA	Present	Oshima T <i>et al.</i> (1988)
2	28	M	I,C	9 year	Not well controlled	Renal	AA	Present	Tsutsui R <i>et al.</i> (1988)
3	37	M	I,C,R	11 year	Not well controlled	Renal	AA	Present	Araki T <i>et al.</i> (1989)
4	20	F	C	4 year	Not well controlled	Renal	AA	Present	Kikuchi H <i>et al.</i> (1989)
5	25	M	I	0 [#]		Renal	AA	Present	Momiyama Y <i>et al.</i> (1989)
6	28	M	J,C	0		Renal	AA	Present	Takashima H <i>et al.</i> (1990)
7	24	M	I	0		Intestinal	AA	None	Itoh T <i>et al.</i> (1991)
8	44	M	I	8 year	Not well controlled	Intestinal	AA	None	Sakai Y <i>et al.</i> (1992)
9	26	M	I,C	13 year	Not well controlled	Renal	AA	Present	Ohwan T <i>et al.</i> (1994)
10	34	M	I,C	15 year	Unknown	Intestinal	AA	None	Yamamoto J <i>et al.</i> (1994)
11	28	M	I	12 year	Not well controlled	Renal	AA	Present	Itoh F <i>et al.</i> (1996)
12	28	M	I	7 year	Not well controlled	Renal	AA	Present	Itoh F <i>et al.</i> (1996)
13	26	M	C	4.5 year	Not well controlled	Renal	AA	Present	Yoshinaga Y <i>et al.</i> (1996)
14	43	M	C,R	15 year	Not well controlled	Renal	AA	Present	Yoshinaga Y <i>et al.</i> (1996)
15	21	M	I,C	3.5 year	Not well controlled	Renal	AA	Present	Horie Y <i>et al.</i> (1997)
16	43	M	C,R	18 year	Not well controlled	Renal	AA	Present	Muro K <i>et al.</i> (1998)
17	35	F	C	0 [#]		Renal	AA	Present	Taki F <i>et al.</i> (1998)
18	26	M	C	11 year	Unknown	Thyroid	AA	Present	Habu S <i>et al.</i> (1999)
19	29	F	I,C,R	13 year	Not well controlled	Renal	AA	Present	Present case (1999)

#:The diagnosis of amyloidosis preceded that of Crohn's disease. CD: Crohn's disease, J: jejunum, I: ileum, C: colon, R: rectum, AA: amyloid A

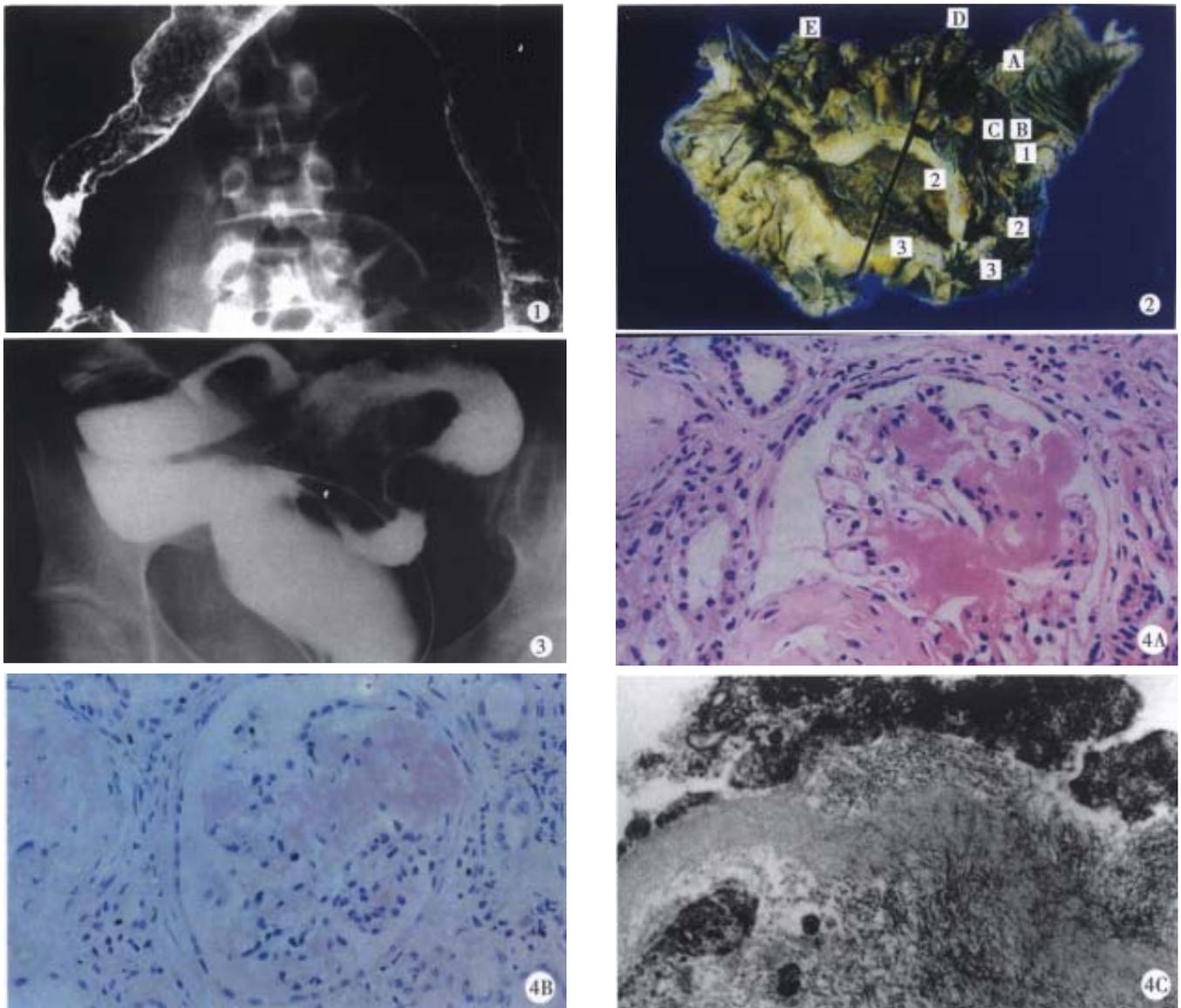


Figure 1 Barium enema examination performed on March 18, 1993. Stricture associated with cobblestone appearance was seen in the ascending colon. Small inflammatory polyps were observed in the transverse colon and descending colon.

Figure 2 Surgical specimen of the terminal ileum, cecum, and ascending colon (May 26, 1993). Thickening of the bowel wall, cobblestone appearance, and longitudinal ulceration were found. Histologically, transmural inflammation and noncaseating epithelioid cell granuloma were found.

Figure 3 Gastrografin enema examination performed on May 8, 1997. A stricture was seen around the ileosigmoid anastomosis.

Figure 4 Findings of the renal biopsy.

A. Histological findings (hematoxylin and eosin). Amorphous, eosin-stained deposits were seen in the mesangial areas.

B. Histological findings (Congo red stain). The deposits were Congo red positive. Congo red stain showed reddish pink deposits that demonstrated apple-green birefringence when examined under polarized light.

C. Electron microscopic findings. Fine fibrils (8 to 10-nm in diameter) arranged randomly or in bundles were found in the mesangium.

The kidney is involved in the majority of patients with secondary amyloidosis. The resulting renal insufficiency caused a deterioration in the prognosis of the patient. Azathiopurine^[30], colchicine^[31,32], dimethylsulfoxide^[33], and elemental diet^[19] have been proposed as the treatment for secondary amyloidosis. However, the effectiveness of this regimen has not been established. Therefore, prevention or early diagnosis and treatment are important. In 15 of the

18 Japanese patients, the kidney was involved by amyloidosis. And in 13 of the 15 patients, renal involvement was the main clinical manifestation of amyloidosis. In only one of the 15 patients^[19], the amount of urinary protein decreased to less than 0.3 g/day since the initiation of elemental diet therapy. In the remaining 14 patients, however, neither proteinuria nor impaired renal function improved after various therapeutic attempts.

Therefore, regular urine test for proteinuria

would be useful for early diagnosis of amyloidosis regardless of the interval since onset of CD. To prevent the development and progression of secondary amyloidosis, it is probably important to maintain CD in the remission.

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Molecular biology of liver disorders: the hepatitis C virus and molecular targets for drug development

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INTRODUCTION

Molecular biology has made a tremendous impact on the diagnosis and treatment of liver diseases^[1,2]. In particular, advances in molecular biology made possible the discovery of the virus that causes hepatitis C. In this review, we use hepatitis C as an example of the impact that molecular biology has made in the area of liver disorders. We emphasize how our growing understanding of the hepatitis C virus (HCV) has led to the identification of targets for development of new treatments.

THE HEPATITS C VIRUS (HCV)

Basic molecular virology of HCV

Investigators at Chiron Corporation were the first to discover HCV and reported this in a landmark paper published in 1989^[3]. The virus was identified by antibody screening of cDNA expression libraries made from DNA and RNA from the plasma of chimpanzees. These chimpanzees were inoculated with serum from humans with what was then called post-transfusion "non-A, non-B" hepatitis. The DNA expression library was screened with antibodies from sera of other patients with "non-A, non-B" hepatitis. This led to the isolation of clones that were derived from portions of the viral genome and encoded fragments of viral polypeptides. Treatment with RNase and DNase showed that HCV was a positive-stranded RNA virus^[3]. In an accompanying paper, the investigators who discovered HCV and their collaborators showed that the vast majority of individuals with chronic "non-A, non-B hepatitis" had antibodies against the newly identified viral

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polypeptides^[4].

After the discovery of HCV, its entire genome was cloned and sequenced in several laboratories^[5-8]. HCV is a member of the Flaviviridae family. Once HCV infects cells, the positive, single stranded RNA genome is translated into a polyprotein of 3010 to 3033 amino acids, depending upon the strain (Figure 1). The viral RNA is not capped and translation occurs via an internal ribosome entry site (IRES) at the 5' end of the viral RNA^[9,10]. The mechanism of translation of uncapped viral RNA therefore differs from that used by virtually all cellular mRNAs which are capped at their 5' ends.

Both host cell and viral proteases cleave the HCV polyprotein into several smaller polypeptides (Figure 1). The major structural proteins are core protein and two envelope proteins called E1 and E2. Core protein forms the nucleocapsid of the mature virion and E1 and E2 are present in the viral envelope. A small polypeptide called P7 is also generated as a result of cleavage at the E2-NS2 junction but its function is not clear. Four major non-structural proteins called NS2, NS3, NS4, and NS5 are generated, two of which, NS4 and NS5, are further processed into smaller polypeptides called NS4A, NS4B, NS5A, and NS5B. The non-structural proteins have enzymatic functions that are critical for viral replication in cells, such as RNA helicase (NS3), protease (NS2, NS3-NS4A complex) and RNA polymerase (NS5B) activities. NS5A has been implicated in determining sensitivity to interferon alpha.

HCV replication and interactions with host cells

Little is known about the fundamental aspects of HCV replication, primarily because a robust cell culture has not been established. Although viral proteins and RNA components involved in critical steps in HCV replication are known, very little is understood about the mechanistic details or the role of accessory host cell factors. Some of the basic steps in HCV replication that occur in infected cells are outlined here.

After infection of cells, HCV RNA must be translated into protein. HCV RNA translation is initiated by internal ribosome binding, not by 5'-end dependent mechanisms^[9,10]. Internal initiation is specified by an IRES element. Such elements were first discovered in the genomes of picornaviruses^[11]. The IRES is believed to require the set of canonical translation initiation factors in order to function. In addition, IRES function is also thought to be dependent on other cell proteins. However,

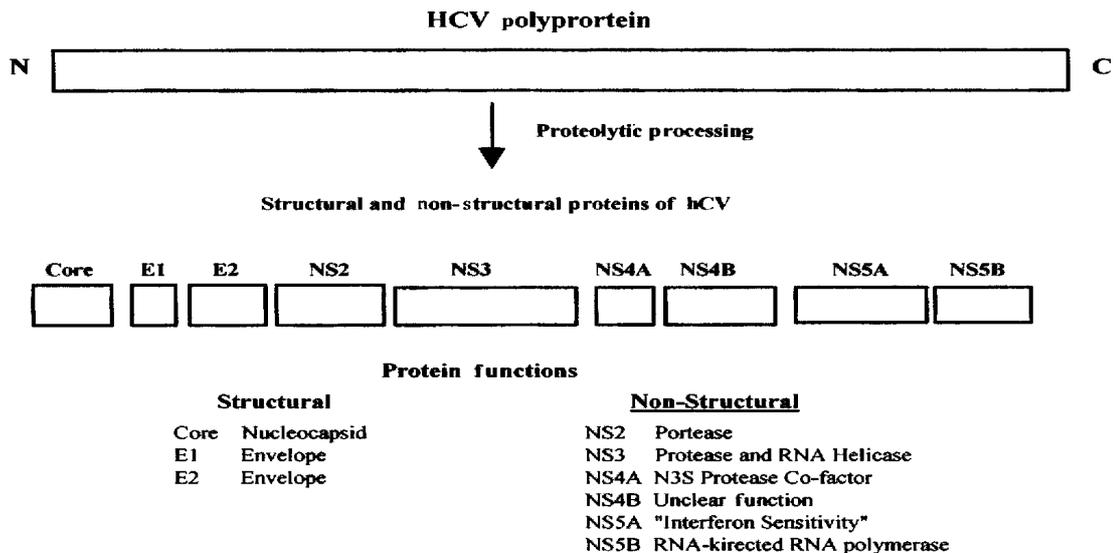


Figure 1 HCV proteins and their functions. The positive-stranded RNA of about 10,000 nucleotides is translated into a polyprotein of approximately 3000 amino acids. The polyprotein is proteolytically cleaved into several smaller proteins. Core, E1, and E2 are structural polypeptides. Core protein is the virus nucleocapsid and E1 and E2 are viral envelope proteins. A small polypeptide known as P7 (not shown) is also produced by additional cleavage between E2 and NS2. The major non-structural proteins are NS2, NS3, NS4, and NS5. NS4 is further processed into NS4A and NS4B and NS5 into NS5A and NS5B. NS2 and part of NS3 are proteases that process the viral polyprotein. NS3 also has RNA-helicase activity. NS4A is a cofactor for the NS3 protease and NS5B is an RNA-dependent, RNA polymerase. The functions of NS4B and NS5A are less well understood but NS5A is thought to play a role in determining sensitivity to interferon.

no single cell protein has been shown to be dispensable for the function of all IRESes.

HCV RNA must be unwound for efficient protein synthesis to occur. This process is catalyzed by a RNA helicase that is part of the viral NS3 protein. The three-dimensional structure of the HCV NS3 helicase domain has been determined and details about its function are emerging^[12-14]. At the present time, it is not known if host cell co-factors are necessary for optimal functioning of the NS3 helicase. Cellular RNA helicases have also been shown to bind to the HCV core protein^[15-17], however, it is not known if they also play a role in unwinding viral RNA.

After its synthesis, the HCV polyprotein is processed into the structural and non-structural proteins. Proteolytic cleavages between structural polypeptides are catalyzed by signal peptidase in the endoplasmic reticulum. Two virally encoded proteases, NS2 and NS3, catalyze the other cleavages of the HCV polyprotein. The NS3 protease contains a trypsin-like fold and a zinc-binding site and is complexed with the viral protein NS4A^[18,19].

HCV RNA must be replicated to produce more virions. The viral protein NS5B is an RNA-dependent RNA polymerase. NS5B bears some similarity and motif organization to poliovirus polymerase and human immunodeficiency virus 1 (HIV-1) reverse transcriptase but adopts a unique shape due to extensive interactions between the fingers and thumb polymerase subdomains that encircle its active site^[20]. The precise mechanism of action of the

HCV NS5B polymerase is not known. Cellular or viral protein or RNA binding partners that function as subunits or initiation factors may be necessary for optimal activity.

The replication rate of HCV in human hosts is estimated to be extremely high. It appears that the estimated half-life of a viral particle is 2.7 h with production and clearance of about one trillion viral particles a day^[21]. This rate of virion production is approximately 1,000 times greater than that estimated for HIV-1. Factors responsible for the high rate of HCV replication are not entirely understood. This rapid rate of replication can explain the development of mutant strains or quasispecies that occur after HCV infection. It may also make development of an effective vaccine difficult.

DRUG TARGETS FOR THE TREATMENT OF HCV INFECTION

"Non-specific" anti-viral agents for HCV infection

The currently available drugs for the treatment of hepatitis C are anti-viral agents not specifically directed against HCV. The United States Food and Drug Administration (FDA) has approved several preparations of recombinant interferon alpha for the treatment of chronic hepatitis C. Interferon alpha is a suboptimal treatment in that only about 20% or less of patients who complete a one year course of treatment respond successfully as determined by the inability to detect HCV in serum 6 mo after the drug is stopped^[22]. Numerous adverse events are also associated with interferon alpha, most notably flu-like symptoms,

neutropenia, thrombocytopenia, and depression. Interferon alpha must be administered by injection 3 times a week. Newer preparations of interferon alpha-2b complexed with polyethylene glycol have been developed^[23]. These so-called “PEG-ylated” interferon alphas are released more slowly and evenly into the bloodstream and need only be administered by injection once a week. “PEG-ylated” interferon alphas will likely be approved for the treatment of chronic hepatitis C in the United States in the year 2000 or 2001.

The combination of interferon alpha-2b plus oral ribavirin is approved in many countries for the treatment of chronic hepatitis C. Combination treatment for 6 mo leads to no detectable virus in serum 6 mo after stopping therapy in approximately 40% of subjects^[24-26]. The major adverse event associated with ribavirin is hemolytic anemia, which in rare cases can be life threatening. VX-497 is a compound in development that inhibits inosine monophosphate dehydrogenase and may have antiviral effects similar to those of ribavirin^[27]. VX-497 is being studied in combination with interferon alpha to establish if it is as effective as ribavirin with a similar or preferable adverse events profile.

Other cytokines have also been tested in the treatment of HCV infection. A recent report of a pilot study suggests that interleukin-10 may slow the development of liver fibrosis in subjects with chronic hepatitis C^[28]. Interleukin-10, however, was not shown to have anti-viral activity against HCV.

Agents directed against HCV non-structural proteins

The next generation of drugs for the treatment of hepatitis C will likely be directed against non-structural HCV proteins with known enzymatic activities. Three major targets are the NS3 protease, NS3 helicase, and NS5B RNA-directed RNA polymerase. The fact that these proteins have enzymatic activities that can be measured *in vitro* make them amenable to high-throughput screening techniques favored by pharmaceutical chemists. This obviates the need to grow HCV in cell cultures or in small animals, tasks that have eluded investigators.

The three-dimensional structure of the HCV NS3 protease domain has been determined by X-ray crystallography^[18,19]. In addition, the structure of the NS3 protease domain complexed with an inhibitor has recently been established^[29]. Armed with this knowledge, chemists can use rationale drug design to synthesize compounds to inhibit protease activity. Rational drug design can be combined with combinatorial chemistry in which a library of thousands or more structurally similar molecules is tested against the target. By combining rational drug design and combinatorial chemistry with high throughput screening techniques that measure enzymatic activity, NS3 protease inhibitors can be identified, further developed and ultimately tested in infected chimpanzees and humans.

Similar methods can be used to identify inhibitors of the NS3 helicase domain and NS5B RNA

dependent RNA polymerase. The three-dimensional structures of these proteins are also known^[12-14]. Although human cells have RNA helicases, their mechanism of action is probably different than RNA helicases of viruses^[30]. Animal cells do not have RNA-dependent RNA polymerases, making NS5B an attractive target for an anti-viral agent.

Agents directed against HCV RNA

HCV RNA differs from cellular mRNA. First, it has a unique ribonucleotide sequence. Second, as outlined above, HCV RNA is uncapped and translation is initiated via an IRES. Third, the viral RNA must be efficiently packaged into the mature virions. These features make the HCV RNA a potential target for anti-viral drugs.

Ribozymes are catalytic RNA molecules that can be designed to cleave specific RNA sequences. Ribozymes therefore have potential utility as drugs against RNA viruses, including HCV. Investigators at Ribozyme Pharmaceuticals have developed ribozymes against conserved genomic sequences in HCV^[31]. These ribozymes cut the viral RNA at specific sequences and are able to inhibit HCV RNA-directed protein synthesis and HCV RNA replication in *in vitro* systems. It is anticipated that a ribozyme against HCV will be tested in human clinical trials in the next couple of years.

HCV RNA is translated by internal ribosome binding mediated by an IRES^[9,10]. The IRES adopts a tertiary structure that is necessary for function^[32,33]. Interference with IRES structure or function is a logical approach to attacking HCV replication. Antisense oligonucleotides targeted to a stem-loop structure within the IRES have been shown effective at inhibiting HCV gene expression^[34,35]. Other small molecule inhibitors can potentially be designed to inhibit HCV IRES function, which can be measured using *in vitro* assays adaptable to high throughput screening methods.

HCV RNA genomes must be packaged into newly synthesized virions. This is likely mediated by specific interactions between sequences in RNA and core protein. Synthetic oligonucleotides corresponding to sequences in the 5' region of the HCV genome have been shown to bind to core protein^[36]. Agents that block HCV RNA binding to core protein could be useful as inhibitors of virion production.

Agents directed against other targets of HCV

HCV presumably gains access to hepatocytes, and possibly other cells, by binding to a plasma membrane protein receptor or receptors. HCV envelope proteins E1 and E2 have been shown to interact with plasma membranes of hepatocytes and other cells^[37]. E1 and E2 may form a heteromeric complex^[38], however, it is not clear if their association is necessary for binding to cell membranes.

The receptors for HCV entry into liver cells are also not presently known. However, an interaction between HCV E2 and a plasma membrane protein CD81 has been described and characterized in some detail^[39,40]. It is difficult to establish if this interaction mediates HCV entry

into cells, primarily because a robust cell culture system for HCV is not currently available. Even if CD81 or other proteins that bind to HCV E1 and E2 are not receptors that mediate viral entry, knowledge of these interactions could lead to the development of drugs that inhibit the binding of HCV to cells. Additional experimental work that may lead to the definitive identification of HCV receptors could also lead to the development of viral entry inhibitors. Structural analysis of the interactions of viral envelope proteins with cellular receptors should be of tremendous value in the development of drugs as it will be in the case of HIV-1^[41,42].

The core protein of HCV is another potential target for the development of anti-viral drugs. In infected cells, HCV core protein is synthesized on the endoplasmic reticulum membrane with a large domain facing the cytoplasm^[43]. HCV core protein has been shown to form multimers^[44] and the self-interaction of core protein is likely important in the assembly of the virion nucleocapsid. HCV core protein expression may also influence critical processes that have implications for cellular pathophysiology. Core protein may play a role in transformation and oncogenesis^[45] or be involved in regulating apoptosis as it has been shown to bind to the cytoplasmic domain of lymphotoxin- β receptor, a member of the tumor necrosis receptor protein family^[46]. HCV core protein also binds to a cellular RNA helicase and this interaction may adversely affect host cell protein synthesis and provide the viral RNA with enhanced access to the cell's protein synthesis machinery^[15-17]. Inhibitors of core protein self-assembly or its interactions with other cellular proteins could therefore be useful in the treatment of hepatitis C.

CONCLUSIONS

The innovative application of standard technique in molecular biology led to the discovery of HCV. The development of treatments for HCV infection has lagged our understanding of the molecular biology of the virus because neither a small animal model of the disease nor a robust cell culture system for the virus currently exist. Recent advancements such as the development of "sub-genomic replicons"^[47] and an infectious viral RNA clone that can infect chimpanzees^[48] may partially overcome these limitations. Nonetheless, based on our present understanding of the molecular biology of HCV, several steps in the viral life cycle can currently be targeted for the development of anti-viral drugs.

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New insights into the pathogenesis of intestinal dysfunction: secretory diarrhea and cystic fibrosis

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INTRODUCTION

A major function of the intestinal epithelium is to control the amount of fluid entering into and being absorbed from the lumen^[1]. In healthy conditions, net fluid movement follows an absorptive vector, although significant secretion also takes place to subserve digestive function. Thus, the secretion of fluid, driven by the active secretion of electrolytes, is important for maintaining the fluidity of intestinal contents during various stages of digestion and thereby allowing for diffusion of enzymes and nutrients. In the setting of disease, dysregulation of intestinal transport mechanisms may alter the balance between absorptive and secretory processes such that secretion predominates, leading to the clinical consequence of diarrhea. However, under conditions of both health and disease, fluid secretion is driven largely by the active secretion of chloride ions. Thus, there are both basic and clinical reasons for wishing to gain a full understanding of the basis and regulation of this transport process. The goal of my article, therefore, will be to review our understanding of intestinal chloride secretion and the ways in which it is regulated. Recent insights in this are enhancing our ability to intervene in diseases where chloride secretion is over-expressed, such as infectious and inflammatory diarrheal illnesses will also be discussed. This article will also cover the implications of intestinal secretory mechanisms for a genetic disease where chloride secretion is under-expressed, namely cystic fibrosis, where significant intestinal dysfunction, including

obstruction and malabsorption, may also ensue.

MECHANISMS OF INTESTINAL CHLORIDE SECRETION

The secretory mechanism

The details of the ion transport pathways making up the intestinal chloride secretory mechanism have been quite well worked out at this point^[1,2]. The mechanism is predominantly expressed, at least as assessed functionally, in epithelial cells lining the crypts of both the small intestine and colon, although there may be a small degree of secretion derived from villus or surface epithelial cells in addition^[2]. Chloride is taken up from the bloodstream across the basolateral membrane of epithelial cells via a sodium/potassium/2 chloride cotransporter that has been cloned and designated as NKCC1^[3]. This cotransporter is driven secondarily by the low intracellular sodium concentration established by the active sodium/potassium ATPase, also localized to the basolateral membrane. This allows chloride to accumulate in the cell cytosol above its electrochemical equilibrium. When apical chloride channels are opened, this chloride is then free to flow out of the cell down this electrochemical gradient, resulting overall in net transepithelial transfer of the anion. Chloride exit occurs predominantly through a channel referred to as CFTR, which is the product of the gene that is defective in the setting of cystic fibrosis^[4]. More recently, in addition, it has been recognized that the apical membrane of intestinal epithelial cells may also contain calcium-activated chloride channels of the CLCA family, and perhaps other channels for these and/or other anions^[5,6]. The transport mechanism is also critically dependent on the presence of basolateral potassium channels which serve to recycle co-transported potassium back across the basolateral membrane, and thus prevent cell depolarization^[7,8].

Positive regulation of chloride secretion

Chloride secretion is stimulated in the intestine by a broad array of substances derived from local and more distant endocrine cells, enteric nerves, neighbouring cell types such as immune cells and subepithelial myofibroblasts, and exogenous factors such as bacterial enterotoxins^[2]. These regulatory pathways provide for minute-to-minute physiological increases in the extent of secretion (such as in response to ingestion of a meal) but also contribute to the marked upregulation of secretion that can occur in the setting of a disease^[1]. Despite many hormones, neurotransmitters and other mediators that have been identified and characterized to date as stimuli of chloride

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secretion, however, appear to exert their effects through two main signaling pathways^[1]. The first of these is mediated by changes in intracellular cyclic nucleotides, and results in large magnitude, sustained secretory responses. At an intracellular level, the primary locus for regulation appears to be the cyclic-nucleotide mediated phosphorylation and opening of the CFTR chloride channel, particularly via cAMP and the cAMP-dependent protein kinase, PKA^[9,10]. It is increasingly recognized that at least one type of PKA is "scaffolded" in the proximity of CFTR via various interacting proteins, which likely contributes to the efficiency of signal transduction in response to cAMP-mobilizing agonists^[11,12]. Chloride secretion can also be evoked by agonists that increase intracellular levels of cGMP, the best physiological example of this pathway being the stimulation by the peptide agonist guanylin, which binds to an apical receptor also shared by the heat-stable toxin of *E.coli*^[13]. cGMP-dependent secretion involves a cGMP-dependent protein kinase and/or cross-activation of PKA, and is very similar to that induced by increases in cAMP in terms of its magnitude, kinetics and dependence on CFTR activation. Cyclic nucleotide-dependent secretion, in general, may also involve regulatory events at additional levels within the transport machinery. For example, there is evidence to suggest that sustained secretion requires the insertion of additional NKCC1 molecules into the basolateral membrane (or at least an alteration in their rate of endocytosis), and cAMP-activated potassium channels have also been postulated on functional grounds^[3,8,14].

Chloride secretion can also be evoked by agonists that are capable of causing an increase in cytosolic calcium concentrations. In contrast to the cyclic-nucleotide dependent chloride secretory responses discussed above, calcium-dependent responses are distinctive in that they are smaller and also considerably more transient^[2]. However, calcium-dependent secretion may nevertheless play a physiological role, particularly in the setting where only a brief, self-limited secretory response is called for. Moreover, synergism occurs between the secretory effects of cAMP- and calcium-dependent agents, allowing the host, to call on markedly upregulated rates of secretory function at times of threat (such as invasion by pathogenic microorganisms). Finally, calcium-dependent secretory responses may assume far greater significance in the setting of cystic fibrosis, where the cAMP-dependent chloride channel, CFTR, is lost or dysfunctional^[15]. At an intracellular level, at least part of the chloride secretory effect occurring in response to an increase in cytosolic calcium is secondary to the opening of basolateral, calcium-sensitive potassium channels. This, in turn, increases the driving force for chloride to exit across the apical membrane through the small proportion of CFTR channels that are hypothetically open even in resting cells^[16]. However, evidence is also accumulating to suggest that elevations in calcium, acting in concert with the calmodulin-dependent protein kinase CaMKII, open

additional apical chloride channels of the CLCA family^[17-20]. This view lends additional credence by the observation that calcium-dependent agonists can cause chloride secretion in at least some epithelial cells even in the absence of any measurable CFTR function^[17,21].

Negative regulation of chloride secretion

Work from our laboratory has also indicated that certain intracellular signaling pathways may lead to the inhibition of ongoing chloride secretion, and/or may result in its termination^[2]. Such negative signals appear to be particularly pertinent in the case of calcium-stimulated chloride secretion, with the transience of this secretory response implying that endogenous factors may serve to limit its extent. We have identified two main pathways whereby calcium-dependent chloride secretion is inhibited. The first of these, interestingly, is activated by substances that also serve to initiate calcium-dependent chloride secretion. An example is the muscarinic agonist carbachol, which evokes a transient chloride secretory response that renders intestinal epithelial cells refractory to re-stimulation by another calcium-dependent secretagogue^[22]. Both the stimulatory and inhibitory effects of carbachol are dependent on cytosolic calcium^[23]. The inhibitory pathway involves the generation of a novel inositol phosphate mediator, inositol 3,4,5,6 tetrakisphosphate [Ins (3,4,5,6) P₄], which reduces the open probability of calcium-activated chloride channels^[22,24]. The upstream pathways leading to generation of this messenger involve tyrosine-kinase dependent events, and the transactivation of the receptor for epidermal growth factor (EGF) and recruitment of the MAP kinase signaling cascade^[25]. Inhibitors of both the EGF receptor and MAP kinase pathway potentiate and prolong secretory responses to carbachol^[25].

A second inhibitory mechanism also centers around the EGF receptor, but the messengers and targets involved in the inhibition of chloride secretion are different^[26-28]. Thus, when the EGF receptor is activated by its cognate ligands, it heterodimerizes with another member of the ErbB receptor family, ErbB2, and thereby recruits alternative signaling events than those resulting from EGF receptor transactivation in response to carbachol^[29]. This signaling diversification, in turn, appears to activate phosphatidylinositol 3-kinase, a novel isoform of protein kinase C (PKC-), and ultimately leads to the inhibition of a basolateral potassium channel, thereby inhibiting the overall process of chloride secretion by preventing potassium recycling^[27,28,30]. It is of interest to note that EGF, and related growth factors, inhibit chloride secretion without themselves serving as agonists in the process. Since such growth factors and their receptors are known to be upregulated in the setting of mucosal injury, their ability to inhibit chloride secretory responses may represent an adaptive response that would limit diarrhea under these conditions.

ALTERATIONS IN CHLORIDE SECRETION IN THE SETTING OF DISEASE

Secretory diarrhea

An excess of chloride secretion into the intestine, above that which can be compensated for by the reserve capacity of absorptive mechanisms, manifests clinically as diarrhea^[1]. The classical example of this response is seen in cholera, where up to 20 liters of stool fluid can be lost per day^[1]. Cholera, in common with a number of other intestinal pathogens, elaborates enterotoxin that interact with epithelial cell signal transduction machinery to elicit profound and sustained chloride secretion due to an irreversible increase in intracellular cyclic nucleotide concentrations. Cholera toxin is internalized and induces a massive increase in intracellular cAMP, whereas the heat stable enterotoxin of *E. coli* bind to an apical receptor that contains an integral guanylyl cyclase activity, and thereby stimulates chloride secretion secondary to an increase in intracellular cGMP. The direct effects of enterotoxins on secretory epithelial cells are also amplified by a number of additional actions. For example, cholera toxin also activates enteric nerve endings, enterochromaffin cells and mast cells, all of which are capable of releasing neurotransmitters and other agonists that themselves can stimulate chloride secretion either directly or indirectly^[2].

Secretory diarrheal illness is also seen in the setting of infections with pathogens that are not known to elaborate enterotoxins activities. For example, *Salmonella* Dublin, an invasive bacterium, appears to activate a program of gene expression within the epithelial cell that predisposes to excessive fluid secretion. Thus, there is upregulation of cyclooxygenase-2 and nitric oxide synthase expression, and in cell culture models, both of these enzymes appear to contribute to an enhanced capacity of the epithelial cell to secrete chloride in response to a range of stimuli^[31]. Similarly, over-expression of cyclooxygenase-2 leads to an increased production of prostaglandins, particularly those of the E series that are known to be potent chloride secretagogues^[31]. *Salmonella* invasion also evokes the synthesis of a wide range of chemokines and other cytokines by intestinal epithelial cells, and the resulting inflammatory influx that is targeted to infected cells almost certainly further amplifies secretory responses in the intact tissue setting^[32]. Furthermore, some workers have shown that *Salmonella* infection is associated with the de novo expression of receptors for the neuropeptide galanin, a known secretory agonist^[33,34]. Antibodies that interrupt galanin signaling can significantly dampen intestinal fluid secretion in a murine model of salmonellosis^[33]. Thus, the host defense response of diarrhea can be generated even by pathogenic microorganisms that do not elaborate any known enterotoxins. Indeed, upregulation of the secretory capacity of intestinal epithelial cells can even occur in the setting of infection with a non-invasive parasite also associated with diarrheal illness, *Giardia lamblia*. These parasites adhere to, but do not penetrate, the intestinal epithelium. Co-culture of intestinal epithelial cells with *G. lamblia*

trophozoites results in enhanced chloride secretory responses, and a apparent upregulation of expression of membrane transport proteins involved in the chloride secretory mechanism such as CFTR and NKCC1 (Resta-Lenert *et al*, submitted). However, paradoxically, *Giardia* co-incubation appears to significantly reduce the ability of certain agonists to mobilize intracellular calcium. These data, in addition to being potentially significant for our understanding of infectious diarrhea, are also of interest because they underscore the concept that calcium-dependent secretagogues may exert negative as well as positive effects on secretion.

Diarrhea is also a common sequella of non-infectious, inflammatory conditions of the intestine, such as ulcerative colitis and Crohn's disease^[1]. A substantial body of evidence now suggests that cells of the immune system that presumably are activated in the course of such conditions can release a wide range of products capable of evoking chloride secretion. Conversely, inflammation and tissue injury is also often accompanied by upregulation of the expression of growth factors, such as EGF, and their receptors, which may serve ultimately to counteract excessive secretion associated with inflammatory conditions of the gut^[35]. Such mechanisms may underlie the efficacy of growth factors such as EGF and insulin-like growth factor that has been demonstrated in diarrheal conditions in various models of inflammation^[35].

Cystic fibrosis

The disease states discussed above are characterized by excessive chloride secretion. However, inadequate chloride secretion, as occurs in the setting of cystic fibrosis, can be equally or perhaps even more disadvantageous for the patient. Cystic fibrosis is characterized functionally by the absence of a cAMP-regulated chloride secretory pathway^[15]. This is due to a series of more than 800 known mutations although about 70% of cases are related to a single mutation, of the CFTR gene, $\Delta F508$, that results in a channel protein that fails to traffic normally to the apical plasma membrane of secretory epithelial cells. Much of the morbidity and mortality in cystic fibrosis relates to pulmonary manifestations^[36]. However, with advances in diagnosis and treatment, the median survival age for patients with cystic fibrosis has increased dramatically over the last several years. This means that disease manifestations in organs other than the lungs are becoming more recognized, and may carry a substantial burden in terms of morbidity.

There are a number of gastrointestinal manifestations of cystic fibrosis, as well as complications related to dysfunction of the biliary and pancreatic systems. A large proportion of newborns with the disease are born with a form of intestinal obstruction known as meconium ileus, assumed to result from inspissated intestinal secretions that are poorly hydrated and thus difficult to clear from the lumen. In some cases, prompt surgical intervention is necessary, and evidence suggests that children presenting

with this complication will go on to have poorer nutritional status and growth, even if treated successfully, than cystic fibrosis patients without this manifestation^[37,38]. Similarly, intestinal obstruction associated with cystic fibrosis may also be seen in older children and also in the increasing number of adults living with this disease^[39]. The role of CFTR in promoting biliary and pancreatic ductular secretion also means that the majority of patients suffer from pancreatic insufficiency, up to half experience biliary disorders, and approximately 5% may display Frank liver disease^[39]. In addition, it has recently been postulated that unsuspected cystic fibrosis (of genotypes other than those associated with pancreatic insufficiency, such as $\Delta F508$) may be the cause of at least some cases of idiopathic pancreatitis^[40]. In each of these cases of gastrointestinal complications of cystic fibrosis, the pathology suggests that any alternate chloride secretory mechanism that are present in intestinal, pancreatic or biliary epithelial cells are insufficient to compensate for the loss of cAMP-regulated chloride secretion. In this respect, the knowledge that intestinal epithelial cells, at least, express intrinsic negative signaling pathways that limit the extent of calcium-dependent chloride secretion may offer both an explanation for this situation, as well as a possible therapeutic target if such negative signaling events can be countermanded.

The prevalence of cystic fibrosis in, especially, Caucasian populations, where estimates suggest that more than 3% of the population are heterozygous carriers of the disease, raises questions about the evolutionary pressures that have driven the retention of mutant CFTR alleles in the gene pool. Hypothetically, such individuals might have thereby gained a heterozygote advantage in resistance to enterotoxin-mediated diarrheal illnesses, such as cholera, although this may be inconsistent with the relative prevalence of cystic fibrosis in Caucasian compared with other ethnic groups. Moreover, studies in mouse models of cystic fibrosis also fail to suggest that heterozygotes show any diminished intestinal secretory responses to cholera toxin^[41,42]. However, more recent studies suggest that tissues obtained from mice with one mutant CFTR allele do display an increased resistance to invasion by bacteria such as *Salmonella*, although the mechanism of this effect is unknown^[43]. This does provide an attractive hypothesis for cystic fibrosis prevalence, especially given that typhoid fever was common in Western Europe until relatively recent times.

CONCLUSION

Advances in our understanding of the molecular basis of intestinal chloride secretion, as well as its regulation, may offer significant insights into disease states where this transport mechanism is either over or under-expressed in the intestine, such as secretory diarrhea and cystic fibrosis. Ultimately, it is hoped that such insights will spawn new therapies for these conditions. Secretory diarrheal illness, in particular, still constitutes a major burden of morbidity

and mortality, particularly among children in developing countries. Moreover, in creased international travel, and the emergence of more highly pathogenic strains of certain bacteria, such as *E.coli*, will also increase the prevalence of diarrhea. Thus, improved therapies are urgently needed, in concert with the public health measures that will reduce exposure to the infectious agents that cause such disease.

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Alterations in gastric mucin synthesis by *Helicobacter pylori*

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INTRODUCTION

Helicobacter pylori is recognized as a cause of chronic active gastritis, gastric and duodenal ulcers, and gastric cancer, though the mechanisms of pathogenesis for *H. pylori*-associated diseases are not yet well understood^[1-4]. The ecological niche to which *H. pylori* is well-adapted is the mucous layer of the human gastric antrum, which has mucin glycoproteins as major constituents. Mucins, high-molecular weight carbohydrate-rich glycoproteins that coat the surface of the stomach and are secreted into the lumen, function to protect the stomach and could be important in *H. pylori* colonization. For further understanding the pathogenesis of *H. pylori* related diseases, it is important to consider whether *H. pylori* colonization of the surface epithelium is associated, as cause or effect, with changes in the gastric mucin synthesized by surface mucous cells.

MUCINS PRODUCED IN NORMAL STOMACH

The entire gastrointestinal tract is coated with a protective mucous layer. The main components of the viscoelastic mucous are mucin glycoproteins. Mucins are thought to protect the surface of the gastrointestinal tract from mechanical damage, from dessication, and from chemical irritants. Gastric mucins are the major components of an unstirred mucous-bicarbonate layer that protects the gastric epithelium from the high concentrations of acid in the stomach lumen and from autodigestion by pepsin. The protective functions of the gastric mucous layer imposes rigid requirements on the structure of gastric mucins (Figure 1). They must be very high in molecular weight

and highly hydrated to provide the viscoelasticity necessary for protection from mechanical damage, and must also be acid-stable and have little non-glycosylated polypeptide exposed as a target for pepsin.

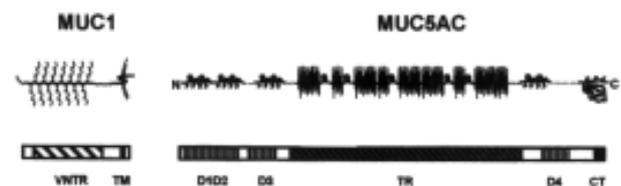


Figure 1 Models of gastric mucin structure. Lower bars represent cDNA sequences of MUC1 cell-surface mucin and MUC5AC secreted mucin, with different domains labeled. Resulting structures of the proteins with attached carbohydrate are schematically represented above.

Like mucins from a number of sources, human gastric mucins are very high in molecular weight and are heavily substituted with O-linked oligosaccharides. Human mucins are encoded by at least nine distinct mucin genes, of which three, MUC1, MUC5AC, and MUC6, are expressed at high levels in the normal stomach (Table 1).

MUC1 mucin is well characterized^[5]. The protein encoded by the MUC1 gene has a large central domain (VNTR, Variable Number of Tandem Repeats) composed of a variable number (25 to 125) of tandem repeats of a 20-amino acid sequence with 25% threonine and serine and 7% proline. Unlike most other mucins, the MUC1-encoded protein has a transmembrane segment and a cytoplasmic tail that can interact with the cytoskeleton. The O-linked carbohydrates on MUC1 mucin are heterogeneous, differ between tumors and normal epithelial cells^[6], and can influence the recognition of the mucin protein by different monoclonal antibodies. MUC1 mucin is produced to some extent by most epithelial cells, but has been studied most extensively in mammary, pancreatic, and colon cancer cells.

MUC5AC is expressed in the stomach and in tracheobronchial cells. Immunohistochemical studies indicate that MUC5AC apomucin is present in surface mucous cells of the gastric epithelium^[7,8]. MUC5AC mucin has a small tandem repeat sequence of 8 amino acids, interspersed with cysteine-containing regions (Figure 1). The glycoprotein is very large with the bulk of the

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molecule made up of heavily glycosylated tandem repeats of an 8-amino acid peptide sequence rich in threonine (to which O-linked oligosaccharides are attached) and proline. Interspersed irregularly within the tandem repeat region are cysteine-containing motifs. Like MUC2 intestinal mucin, both the N-terminal and the C-terminal have cysteine-rich globular domains^[9] with sequence similarity to the D domains of pre-provon Willebrand factor and an inferred cysteine-knot motif, topologically similar to epidermal growth factor (EGF). The cysteine-rich globular domains of secreted mucins may be involved in the oligomerization of mucin or in binding to collagen of basement membranes^[10]. MUC5AC is expressed in the normal stomach, but not in normal colon. Aberrant expression of MUC5AC has been reported, however, in colorectal cancers and adenomas^[8,11,12] and may be related to the progression of colon cancers.

MUC6 is also expressed in the normal stomach, but in mucous glands rather than surface mucous cells^[7,8,13,14]. This mucin has a very large tandem repeat sequence of 169 amino acids, very high in amounts of Thr, Ser, and Pro^[13]. The high content of Thr and Ser likely accounts for the large amount of carbohydrate present on this molecule. Thus, there are (at least) two completely different secreted mucins produced by the normal stomach. How these mucins differ in carbohydrate (and whether they differ in function) is speculative. Genes for both MUC5AC and MUC6 have been assigned to a region of chromosome 11 that also codes for two other secretory mucins^[15], with a gene order of HRAS-MUC6-MUC2-MUC5AC-MUC5B-IGF2.

Like mucins from other sources, the carbohydrate portion of gastric mucin is heterogeneous. Several neutral oligosaccharide structures have been published^[16,17], but the overall complement of oligosaccharides is not known. The results of histochemical studies suggest that the carbohydrate portion of the mucin in surface mucous cells is different from that in mucous glands. The surface mucous cells are stained by PAS, while mucous glands (cardiac gland, mucous neck cells, and pyloric gland cells) are stained by alcian blue. The "neutral" mucins in surface mucous cells are also stained by galactose oxidase/Schiff, suggesting the presence of terminal Gal or GalNAc^[18]. The mucous gland cells uniquely show periodate-enhanced binding of concanavalin A, called paradoxical ConA staining. The structural basis for this is unclear, but it may detect terminal alpha-GlcNAc^[18]. The mucous gel layer covering the surface epithelium has been shown to have clearly demarcated layers of two distinct mucin types^[19]. On the basis of staining properties, these likely correspond to the surface-type neutral mucin (MUC5AC) reactive with galactose oxidase and to the gland-type acid mucin (MUC6), possibly sulfomucin, revealed by periodate-enhanced (paradoxical) ConA staining.

EFFECT OF *H.pylori* ON GASTRIC MUCIN IN VIVO

Though both the thickness and the hydrophobicity of the mucous gel layer is decreased in the gastric mucosa of ulcer patients^[20], it has not been established whether this is associated with increased mucin degradation, decreased mucin synthesis, or a change in mucin type. It has been postulated that one important pathogenic property of *H.pylori* is its ability to weaken the mucous gel that protects the gastric epithelium^[21,22], but the presence or absence of mucinase activities in *H.pylori* is controversial^[23-25]. Direct analysis of mucins from *H.pylori* infected and uninfected patients show no decrease in viscosity, arguing against *H.pylori* dependent mucin degradation^[26]. In spite of histochemical observations of mucous depletion accompanying *H.pylori* infection, qualitative alterations in the type of mucin produced have only recently been studied.

In order to determine the effect of *H.pylori* infection on mucin gene expression in the gastric epithelium^[7], biopsies from *H.pylori*-positive and *H.pylori* negative patients were examined by immunohistochemistry (Table 2). MUC6 was limited to mucous glands of *H.pylori*-negative patients, but 72% of *H.pylori* positive patients also expressed MUC6 on surface mucous cells. In contrast, MUC5AC mucin was seen in significantly fewer surface mucous cells of *H.pylori*-positive specimens. Overall, the percent of the surface epithelium stained by anti-MUC5 was significantly lower in *H.pylori*-positive specimens than in *H.pylori*-negative specimens ($P < 0.01$). Carbohydrates recognized by LeX and paradoxical ConA staining were aberrantly expressed in the surface mucous cells of 16/27 and 17/23 of *H.pylori*-positive tissues, respectively. There was a suggestive but non-significant decrease in staining for MUC1 mucin. Retrospective examination of clinical histories and histological findings showed that the mucin alterations occur in *H.pylori* infected individuals with and without ulcers, but not in patients with non-*H.pylori*-associated gastritis or gastric ulcers (Figure 2). This indicates that the mucin alterations are not simply a secondary effect of inflammation.

For more direct examination of mucin gene expression, the presence of MUC5AC and MUC6 message in antral biopsies were examined by in situ hybridization^[7]. In antral biopsy specimens from *H.pylori*-negative patients, MUC5AC mRNA was homogeneously expressed in surface epithelial cells. MUC5AC expression in the surface epithelial cells of *H.pylori*-positive patients was patchy, however, and often absent from large areas of the surface epithelium. Concordance between the pattern of MUC5AC expression as determined by in situ hybridization and immuno-histochemistry was 100%. MUC6 expression was limited to cells of the deep glands in *H.pylori*-negative patients, seen by both in situ hybridization and immunohistochemistry. In contrast, 6 of 8 *H.pylori*-positive specimens

Table 1 Human mucin genes

Gene	Locus	mRNA size	Tandem Repeats	Other structural features	Expression in normal tissues
MUC1	1q21-24	4-7 kb	20 AA	Transmembrane	Most epithelia
MUC2	11p15.5	14-16 kb	23 AA, -16 AA	D-domains, cystine knot	Colon>small intestine>respiratory tract
MUC3	7q22	16-17.5 kb	17 AA, 59 AA	Cystine knot	Small intestine>colon, gall bladder
MUC4	3q29	16.5-24 kb	16 AA		Respiratory tract, cervix>GI tract
MUC5AC	11p15.5	17-18 kb	8 AA	D-domains, cystine knot	Stomach (surface)>respiratory tract
MUC5B	11p15.5	17.5 kb	29 AA, interrupted	D-domains, cystine knot	Respiratory tract, salivary gland, cervix>GI tract
MUC6	11p15.5	16.5-18 kb	169 AA	Cystine knot	Stomach (glands), gall bladder
MUC7	4	2.4 kb	23 AA	No homology to large mucins	Salivary glands
MUC8	12q24.3	-9 kb	18 AA, 41 AA	Not Thr/Ser rich	Trachea

Table 2 Histochemical staining of mucins in gastric biopsy specimens

Antigen		<i>H.pylori</i> negative	<i>H.pylori</i> positive
MUC5AC	% Stained ^a , intensity score (surface)	(69.8 ± 3.5)%, 2.8 ± 0.1	(51.2 ± 5.7)%, 2.6 ± 0.1
MUC6	% Stained ^a , intensity score (surface) ^a	4%, 0.1 ± 0.1	72%, 1.8 ± 0.2
Le ^a +b	% Stained ^a , intensity score (surface) ^a	63%, 0.9 ± 0.2	96%, 1.9 ± 0.2
Paradoxical ConA	% Stained ^a , intensity score (surface) ^a	18%, 0.4 ± 0.2	79%, 2.1 ± 0.3
LeX	% Stained ^a , intensity score (surface) ^a	4%, 0.4 ± 0.4	59%, 1.0 ± 0.2
MUC1	Intensity score (surface), intensity score (glands)	2.6 ± 0.2, 1.7 ± 0.1	2.7 ± 0.1, 1.4 ± 0.2
Sialyl Tn	Intensity score (surface), intensity score (glands) ^a	1.8 ± 0.2, 1.9 ± 0.1	1.6 ± 0.1, 1.4 ± 0.1

^a% of surface epithelium stained; ^b% of patients with surface staining; ^c*P*<0.05, *H.pylori* positive vs *H.pylori* negative

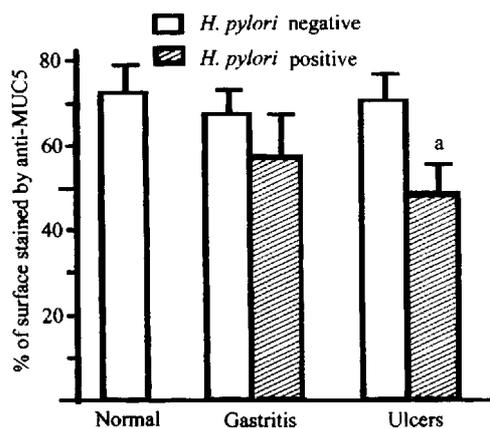


Figure 2 Expression of MUC5AC in surface epithelium of normal human stomach and gastritis and gastric ulcer specimens. ^a*P*<0.05 vs corresponding *H. pylori* negative group.

(0/7 *H.pylori* negative specimens) had focal MUC6 mRNA expression in surface epithelial cells. MUC5AC and MUC6 gene expression were examined in antral biopsies obtained from patients with *H.pylori*-associated antral gastritis (biopsy-proven) before and after documented eradication of the bacterium. In 7 of 10 cases MUC5AC expression increased (*P* = 0.004) after *H.pylori* eradication (Figure 3). Eradication of *H.pylori* also resulted in reversal of MUC6 antigen expression toward normal patterns.

The effect of *H.pylori* on gastric mucin expression was further examined by purification and immunochemical analysis of mucins from gastric juice of *H.pylori*-positive and *H.pylori*-negative patients. For *H.pylori*-infected patients and uninfected patients that had been examined for immunohistochemical staining of biopsy tissues, gastric

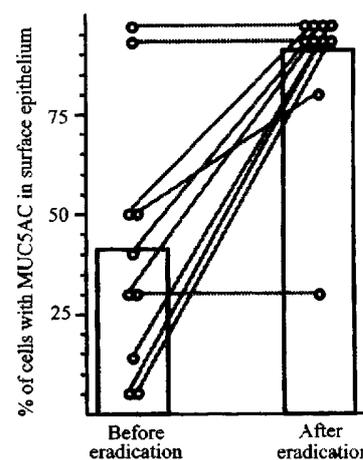


Figure 3 MUC5 expression in patients before and after eradication of *H.pylori* infection. MUC5 gene expression was determined by in situ hybridization. Bars show mean percent of epithelial cells expressing MUC5. Lines show changes in MUC5AC expression in individual patients.

aspirates were used as a source for mucin purification by gel filtration and CsCl density gradient centrifugation. There was no significant difference in yield of mucin or carbohydrate content between *H.pylori*-positive and *H.pylori*-negative specimens (Figure 4). The purified mucins were examined by ELISA for MUC6 and Le^b antigenic activity. MUC6 activity was higher (*P* = 0.026) in mucins from the *H.pylori*-infected patients than in mucins from uninfected patients. Mucins from the *H.pylori*-infected patients also bound monoclonal antibody to Le^b antigen to a significantly greater extent (*P* = 0.014) than mucins from the uninfected patients (Figure 4). Subsequently, these purified mucins have been examined

by SDS-PAGE and Western analysis. Infection with *H. pylori* was associated with an increase in MUC6 (detected with anti-M6P and also with anti-Le-b and *Ulex europaea agglutinin*) and a decrease in MUC5AC (detected with anti-M5P and 45M1 antibodies and also with peanut agglutinin and *Vicia villosa agglutinin*) in these secreted mucins.

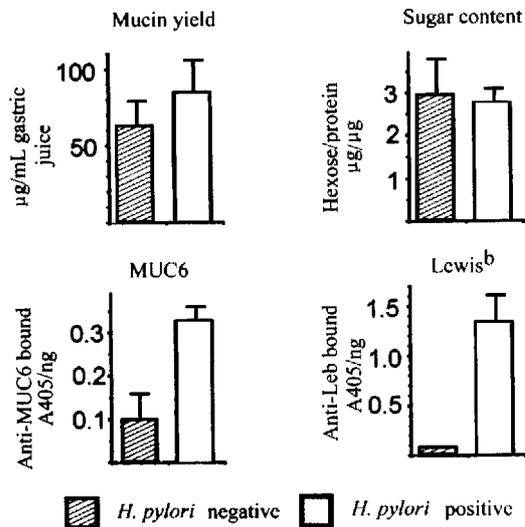


Figure 4 Purification and analysis of mucins from gastric juice. Upper left, yield of mucins purified from gastric aspirates of 5 *H. pylori*-negative and 5 *H. pylori*-positive patients. Upper right, carbohydrate content of purified mucins. Lower left, binding of antibody to MUC6 peptide in ELISA. Lower right, binding of antibody to Lewis-b antigen.

These results establish that there is aberrant surface expression of gland-type gastric mucin in surface mucous cells of *H. pylori* infected patients, accompanied by focally decreased MUC5AC mucin. This decrease in MUC5AC mucin and aberrant expression of MUC6 might be expected to disrupt the protective surface mucin layer. How or whether alterations in gastric mucins would influence processes that lead to disease is an important question which requires that the specificity and mechanisms of mucin depletion be better understood.

EFFECT OF *H. pylori* ON MUCIN SYNTHESIS IN VITRO

Analyses of tissue specimens and purified mucin glycoproteins indicate that gastric surface-type mucin expression is reversibly decreased in *H. pylori*-infected patients^[7] but do not allow direct examination of mucin synthesis. Gastric cells in culture were examined to determine the effect of *H. pylori* on mucin synthesis^[27]. KATO III gastric epithelial cells were incubated in the presence or absence of *H. pylori*, and the mucin produced was quantitated by labeling with [³H]glucosamine and size exclusion HPLC on Superose 6 columns. The ³H-labeled high-molecular weight glycoprotein was confirmed to be

mucin by CsCl density gradient centrifugation, chemical and enzymatic degradation treatments. *H. pylori* (type strain NCTC11637), under conditions that had little effect on viability, inhibited the synthesis of mucin by 82% (Figure 5). There was no inhibition of mucin synthesis by the non-gastric pathogen *Campylobacter jejuni*, and little inhibition by a strain (Tx30a) of *H. pylori* that is CagA-negative and non-toxicogenic. Similar results were seen in five other gastric cell lines tested (Figure 5). Inhibition of mucin synthesis was detected as early as 4h after addition of bacteria, and was partially reversible, though with a slower time-course than the onset of inhibition. Inhibition of mucin labeling was concentration dependent (Figure 6) and did not require the presence of intact bacteria. There was no inhibition by a soluble extract of *H. pylori*, but the *H. pylori* pellet fraction gave inhibition equivalent to intact bacteria.

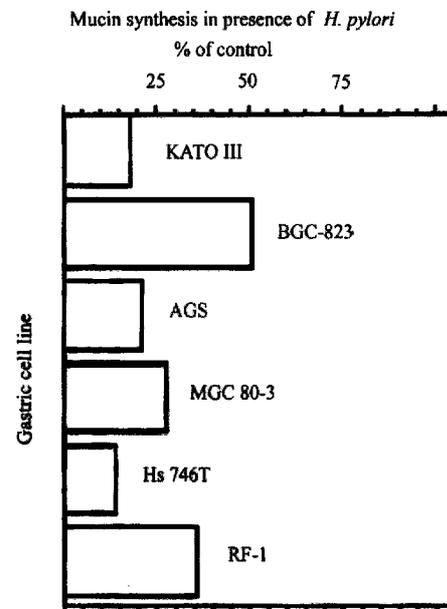


Figure 5 Effect of *H. pylori* on mucin synthesis in six gastric cell lines. Cells were labeled 22 h with [³H]glucosamine in the presence or absence of 1 OD₆₀₀ *H. pylori*, and labeled glycoproteins were analyzed by size-exclusion HPLC.

In a pulse-chase analysis, *H. pylori* had no effect on mucin secretion. Furthermore, there was little or no degradation of mature mucin in the presence or absence of *H. pylori*. Further experiments, to examine the effects of *H. pylori* on mucin glycosylation, used benzyl-GalNAc, which specifically inhibits synthesis of peripheral carbohydrate on mucin-type glycoproteins^[28]. Incubation of KATO III with *H. pylori* decreased labeling of mucin to a similar extent in the presence or absence of benzyl-GalNAc (Figure 7), indicating that the effect of *H. pylori* is not due to inhibition of peripheral glycosylation per se, but results from inhibition of synthesis of mucin core structures.

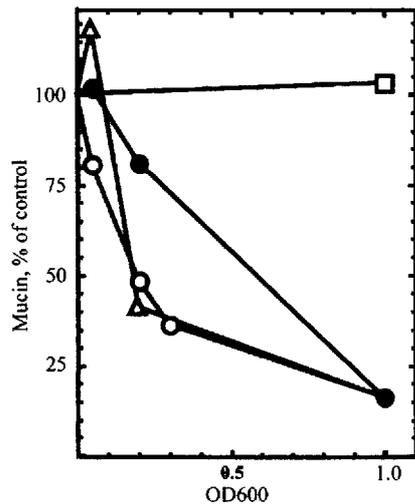


Figure 6 Inhibition of mucin synthesis by subfractions of *H. pylori*. KATO III cells were labeled with [³H]glucosamine in the presence of different concentrations of intact *H. pylori* (open circles), *H. pylori* lysate (filled circles), the 100,000 × g pellet (open triangles), or the 100,000 × g supernatant (open squares).

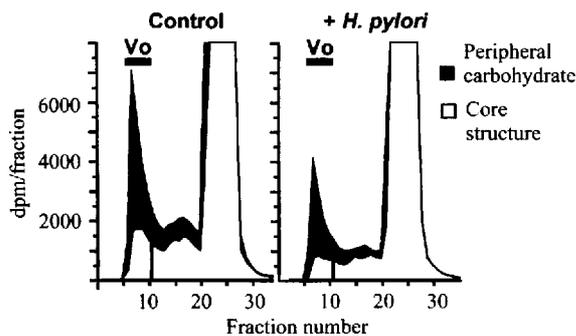


Figure 7 Effect of *H. pylori* on synthesis of peripheral and core carbohydrate structures of mucin. KATO III cells were incubated with or without 2 mmol/L benzyl-GalNAc, and labeled for 4 h in the presence or absence of 1 OD600 *H. pylori*. Bars show void volume (Vo) containing labeled mucin. Solid areas show labeling of the peripheral carbohydrate (inhibitable by benzyl-GalNAc). Open areas show residual labeling of core structures in the presence of benzyl-GalNAc.

KATO III produces MUC5AC and MUC1 mucins, and the amount of both mucin proteins is decreased by co-incubation with *H. pylori* (Figure 8). Expression of another high molecular weight glycoprotein, CEA, and another control protein, galectin-3, was unaffected by *H. pylori*. *H. pylori* also decreased the amount of MUC5AC protein in BGC-823 gastric cells and the amount of MUC1 protein in the BGC-823, AGS, and MGC 80-3 cell lines. The inhibition of synthesis of both MUC5AC and MUC1 protein was concentration dependent and associated with the insoluble fraction of *H. pylori* lysates. Kinetically, the onset of inhibition of MUC1 expression was more rapid than inhibition of MUC5AC expression. MUC1 inhibition was seen within 4 h while MUC5AC inhibition was slower. MUC1 recovery was also more rapid than recovery of

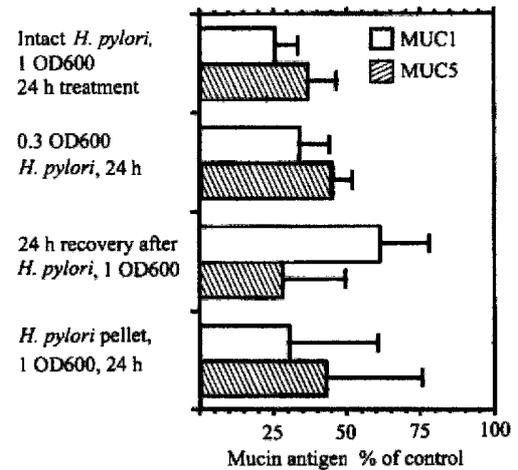


Figure 8 Effect of *H. pylori* on expression of MUC1 and MUC5AC in KATO III gastric epithelial cells in vitro. KATO III cells were incubated with or without *H. pylori*, and cell lysates were subjected to Western analysis. Content of MUC1 antigen, detected with monoclonal antibody HMFG2, and of MUC5AC antigen, detected with monoclonal antibody CLH2, is expressed as percentage of untreated cells.

MUC5AC.

These experiments^[27] demonstrate that *H. pylori* decreases the amount of total mucin, and MUC5AC and MUC1 proteins in gastric epithelial cells. Indirect evidence indicates that this is due to a decrease in the synthesis of mucin protein rather than changes in glycosylation, secretion, or degradation of mucins. These results may help to explain the mucin depletion associated with *H. pylori* infection *in vivo*^[7,29].

INFLUENCE OF MUCINS ON *H. pylori* ADHESION

Most of the *H. pylori* in the stomach are present in the mucus gel layer, and appear to cause little harm to the host; adhesion of *H. pylori* to the gastric epithelial cell surface may be required for causing disease. For example, induction of the proinflammatory chemokine interleukin-8, requires that bacteria be in contact with the epithelial cell surface^[30,31]. Mucin glycoproteins produced by the gastric epithelial surface could influence the process of *H. pylori* adhesion in two ways: First, secreted glycoproteins could bind to bacterial adhesins and help to keep the bacteria in the mucous gel layer, preventing their approach to the epithelium. Although *H. pylori* has several different adhesins which could be involved in binding to mucins^[32-35] and human gastric mucin has been shown to inhibit bacterial binding to other cell types, e.g., erythrocytes and HEp-2 cells^[36,37], it is not known whether secreted gastric mucin can inhibit the adhesion of *H. pylori* to gastric epithelial cells. Second, cell-surface mucin glycoproteins could shield the epithelial cell surface from exposure to contact-dependent virulence factors, preventing adhesion-dependent synthesis of pro-

inflammatory chemokines. In MUC1-expressing cells, the highly glycosylated tandem repeat domain extending out from the cell surface can interfere with cell-cell interactions, for example, integrin-mediated aggregation^[38]. Since adhesion of *H.pylori* to gastric epithelial cells requires their close proximity to the cell surface where they can interact with integrins or other cell-surface receptors, cell-surface mucins might be expected to block adhesion of *H.pylori* to gastric epithelial cells.

Since previous results indicated that gastric surface-type mucins are decreased by *H.pylori* both in vivo^[7] and in vitro^[27], we sought to determine the influence of mucin on adhesion of *H.pylori* to cultured gastric epithelial cells. For measurement of the adhesion of *H.pylori* to gastric epithelial cells, an assay was established using biotinylated *H.pylori*, with bacteria attached to the BGC-823 gastric epithelial cells quantitated with avidin-biotin-peroxidase complex and ABTS as chromogen. The binding of bacteria was characterized with regard to time dependence, temperature dependence, and bacterial strain dependence. Optimal conditions for adhesion were found to be 30 min incubation at 37°C. Under conditions where the CagA/cytotoxin positive type strain of *H.pylori*, NCTC 16137, bound well to BGC-823 cells, there was little binding of the CagA-negative, cytotoxin-negative strain of *H.pylori*, Tx30a or of the non-human pathogen *Helicobacter mustelae*. As further validation, the standard binding assay was compared to colony counts for detection of viable *H.pylori* bound to BGC-823 cells (Figure 9). Binding of biotinylated bacteria was equivalent to binding of viable bacteria.

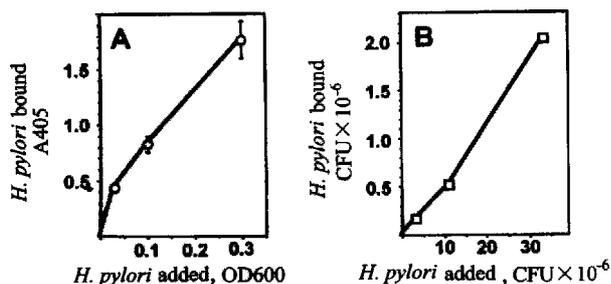


Figure 9 Assay of adhesion of *H.pylori* to gastric epithelial cells. Biotin-labeled *H.pylori* were incubated with BGC-823 cells for 30 min at 37°C, and attached bacteria were quantitated using the avidin-biotin-complex assay (A, left panel) or by colony counts (B, right panel).

BGC-823 cells are well differentiated, attach well to tissue culture plastic, and are susceptible to *H.pylori*-dependent inhibition of mucin synthesis^[27,39]. In comparison to other gastric epithelial cell lines, there was more binding of *H.pylori* to BGC-823 cells (which produces MUC5AC mucin and has relatively low levels of MUC1 cell-surface mucin) than to two cell lines (AGS and MGC-803) which do not produce MUC5AC mucin but

produce high levels of MUC1 mucin^[27,40]. This inverse correlation between MUC1 expression and *H.pylori* adhesion in gastric epithelial cell lines suggests that MUC1 mucin could interfere with *H.pylori* adhesion.

In order to test the role of mucin in *H.pylori* adhesion, we sought to inhibit mucin synthesis in BGC-823 gastric epithelial cells and examine the effect on binding of bacteria. It was initially confirmed that treatment of BGC-823 cells with the mucin-specific glycosylation inhibitor benzyl-GalNAc inhibits total mucin synthesis (measured by labeling with [³H] glucosamine and size-exclusion chromatography) by approximately 80% (Figure 10). Treatment of 823 cells with benzyl-GalNAc significantly increased the adhesion of *H.pylori* (treated/control = 1.43 ± 0.14, n = 6). These results indicate that inhibition of mucin glycosylation is associated with an increase (rather than a decrease) in *H.pylori* adhesion, suggesting that mucins protect against (rather than facilitate) binding of the bacterium to the gastric epithelial surface.

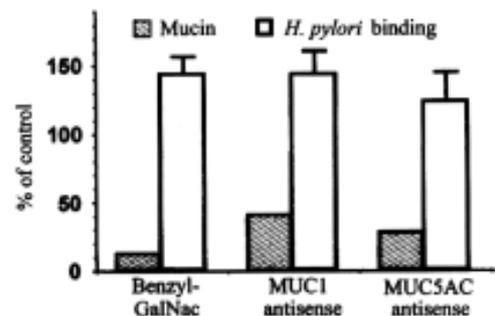


Figure 10 Effect of inhibitors of mucin synthesis on *H.pylori* adhesion. BGC-823 cells were treated with 2 mmol/L benzyl-GalNAc, with 5 μmol/L MUC1 antisense (5'-GGG-CTG-GGG-GGG-CGG-TGG-3'), or with 5 μmol/L MUC5AC antisense (5'-AGA-GGT-TGT-GCT-GGT-TGT-3'). Solid bars show, as percent of control, amounts of total mucin (left), MUC1 mucin (middle), and MUC5AC mucin (right). Open bars show binding of biotinylated *H.pylori*, expressed as percent of control.

In order to specifically decrease the synthesis of MUC1 and MUC5AC phosphorothiolate antisense oligodeoxynucleotides were designed and targeted against the VNTR regions (MITR) of MUC1 and MUC5AC genes. By Western analysis (Figure 10), MUC1 antisense treatment decreased MUC1 protein (treated/control = 0.39 ± 0.15, n = 6), and MUC5AC antisense treatment decreased MUC5AC protein (treated/control = 0.27 ± 0.13, n = 3). MUC1 antisense oligodeoxynucleotide treatment of BGC-823 cells significantly increased the adhesion of biotinylated *H.pylori*, (treated/control = 1.43 ± 0.18, n = 4). MUC5AC oligodeoxynucleotide treatment had no significant effect on adhesion (treated/control = 1.23 ± 0.22, n = 5), but it should be noted that the culture medium, which would contain most of the secreted (MUC5AC) mucins, was removed before the

adhesion assay was performed.

These results indicate that cell-surface mucin glycoprotein decreases adhesion of *H. pylori* to gastric epithelial cells. Since inhibition of mucin synthesis *in vitro* is associated with an increase in *H. pylori* adhesion, *H. pylori*-dependent mucin depletion *in vivo* would be expected to facilitate further binding of the bacterium to the gastric epithelial surface.

CONCLUSION AND WORKING HYPOTHESIS

Our working hypothesis (Figure 11) is that *H. pylori* alters the synthesis of gastric mucin in surface mucous cells and that the resultant alteration in the surface mucous gel layer facilitates adhesion of *H. pylori* to the epithelial cell surface, which could lead to increased inflammation. Based on *in vitro* results, *H. pylori* adhesion decreases mucin synthesis, and decreased mucin synthesis increases *H. pylori* adhesion. If a similar cycle applies *in vivo*, the pathogenetic effects of *H. pylori* infection could be mechanistically tied to the mucin depletion observed histologically.

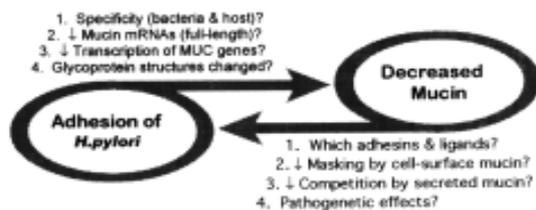


Figure 11 Working hypothesis: interaction of *H. pylori* and gastric mucins.

The diseases caused by *H. pylori*, gastroduodenal ulcers and gastric cancer, take years to develop, but only short-term effects can be studied *in vitro*. *H. pylori* infection causes little overt damage to the gastric epithelium in most of the host population. *H. pylori* may, therefore, cause ulcers through a subtle disturbance in the interaction between the bacteria and the host, rather than acute damage to one critical component of the host cell. Non-physiological conditions are necessary for observable effects in cultured cell systems. Caution must therefore be exercised in extrapolating these data to the *in vivo* interactions between *H. pylori* adhesion and mucin expression. Nevertheless, if, as we hypothesize, *H. pylori* adhesion to gastric epithelial cells decreases mucin synthesis, and decreased mucin synthesis further increases *H. pylori* adhesion, even small effects on both processes could eventually lead to disease.

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Current medical therapy of inflammatory bowel disease

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INTRODUCTION

The 1990's have brought a significant promise and the hope for a better and brighter future in the new millennium for patients with inflammatory bowel disease (IBD). A better understanding of the pathophysiology of IBD symptoms has led to newer treatment modalities and streamlining of therapy for specific subsets of patients.

ULCERATIVE COLITIS

The treatment for ulcerative colitis (UC) is aimed at modulating the inflammatory response. The drugs which are found to be effective are sulfasalazine (Azulfidine, Salazopyrin) and its 5ASA derivatives, glucocorticosteroids, immunomodulators/immunosuppressants, and other new potential drugs (Table 1).

Table 1 Medical therapy in IBD

Sulfasalazine and 5-amino salicylates	Azulfidine Olsalazine, Asacol, Pentasa, Balsalazide
Corticosteroids	Hydrocortisone, ACTH, Prednisone, Budesonide
Immunosuppressive/Immunomodulators	Immunan/6MP, Cyclosporin A, Methotrexate, Anti-TNF α Antibody, (Remicade, CD P571) FK506, IL-10, IL-11
Antibiotics	IL-1 Receptor Antagonist, Anti-CD4 Antibody Metronidazole, Ciprofloxacin, Clarithromycin, Trimethoprim-sulfamethoxazole
New potential drugs	Nicotine Heparin ISIS 2302 (ICAM-1 inhibitor) Hydroxychloroquine, Leukotrienes inhibitors, Short chain fatty acids Antioxidants & free radical scavengers Probiotics

Sulfasalazine and 5-ASA compounds

Initially developed in the 1940s for the treatment of rheumatoid arthritis, salicyl-azo-sulfapyridine, or

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sulfasalazine was quickly recognized as being effective in the treatment of colitis. Consisting of a molecule of 5-aminosalicylic acid (5-ASA) joined by an azo bond to a molecule of sulfapyridine (Figure 1), sulfasalazine has been a mainstay in the treatment of UC for more than 50 years^[1].

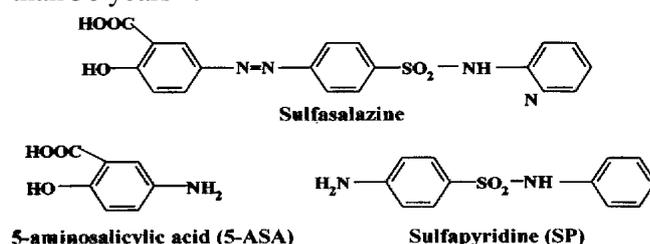


Figure 1 Structure of salicyl-azo-sulfapyridine or sulfasalazine (azulfidine, salazopyrin).

The active moiety in sulfasalazine is 5-ASA, with the sulfapyridine acting as a carrier to prevent absorption of 5-ASA in the small bowel. In the distal ileum and colon, bacteria that possess azo reductase split the molecule, releasing free 5-ASA and sulfapyridine (Figures 1,2). Almost all colonic bacteria have azoreductase enzyme. The sulfapyridine is readily absorbed from the colon, acetylated in the liver, and conjugated with glucuronic acid, and excreted in the urine^[1]. The 5-ASA is only minimally absorbed, with the majority being excreted in the feces unchanged (Figure 2). 5-ASA's mechanism of action is by direct contact with colonic mucosa to suppress various pro-inflammatory pathways including both cyclooxygenase and lipoxygenase derived products such as prostaglandins and leukotrienes from arachidonic acid and from suppression of superoxide dismutase and possibly by other mechanisms.

Sulfasalazine has been well studied in UC and has proven efficacy in inducing remission in patients with mild-to-moderate disease, as well as in maintaining remission. Its use has not been studied in a controlled manner for severe UC, but it is commonly used as an adjunct to approximately 80% of patients with mild-to-moderate disease, compared with 30% to 35% of those receiving placebo. Studies examining maintenance of remission have shown that sulfasalazine's effect is dose-dependent, with relapse rates of 33% with 1 g/day, 14% with 2 g/day, and 9% with 4 g/day^[2].

The toxic effects of sulfapyridine are the limiting factor in using sulfasalazine. Common adverse reactions include headache, nausea, anorexia, and dyspepsia. These

symptoms relate to plasma levels of sulfapyridine and usually occur at doses greater than 3 g/day [3]. Because of sulfasalazine's substantial toxicity and the limitation of dosing due to side effects, efforts were made to develop 5-ASA products with other delivery systems to prevent proximal small-bowel absorption. Three such oral preparations are now available in the United States: two products containing mesalamine (Asacol, Pentasa), olsalazine sodium (Dipentum) and balsalazide. Each uses a different mechanism to deliver the 5-ASA moiety to the sites of inflammation such as distal small bowel and colon, bypassing the absorption by jejunum.

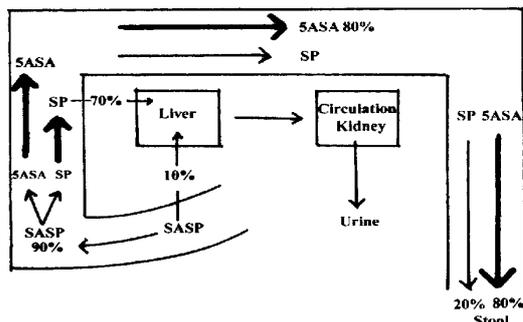


Figure 2 Metabolism and pharmacokinetics of salicylazosulfapyridine. Adopted from the Ph.D. thesis entitled, "Salicylazosulfapyridine Metabolism in Clinical Practice" submitted by Das KM to the University of Edinburgh, U.K., January 1973.

Asacol (mesalamine) contains 5-ASA coated with an acrylic-based resin. This releases 5-ASA in a pH-dependent manner at pH 6 or above. This causes release of 5-ASA in the distal small bowel and colon, making this drug ideal for the treatment of UC. Another mesalamine product, Pentasa, is 5-ASA encapsulated in ethylcellulose microgranules. This time-release formulation allows for release of 5-ASA throughout the small and large intestines. Thus, this drug appears to be superior for small bowel Crohn's disease (CD) in addition to its use in UC. While these products are effective and safe, there have been rare reports of granulomatous hepatitis, interstitial nephritis and recurrent thrombocytopenia on rechallenge with mesalamine/mesalazine^[4,5,6].

Olsalazine sodium consists of two molecules of 5-ASA linked by a diazo bond. Balsalazide consists of 5-ASA linked with 4-amino-benzoyl- β -alanine (an inert compound) by a diazo bond. The presence of the azo bond in both of these compounds, similar to sulfasalazine's, prevents small-bowel absorption and allows for delivery of the drug mainly in the colon, where bacterial azo reductase liberates the 5-ASA. These drugs would therefore be useful in the same circumstances as sulfasalazine. However, the 5-ASA products may cause diarrhea due to decreased water absorption in the small bowel, and this side effect occurs most frequently with olsalazine. Since the diseases these

drugs are used to treat are diarrheal illnesses, their use may be limited by this untoward effect. In a recent study^[7] balsalazide was found to be more effective and better tolerated than mesalamine as treatment for UC.

In the treatment of distal colitis, enema preparation of 5-ASA (Rowasa enema) is efficacious. In the initial study to identify the active moiety in sulfasalazine, patients with distal UC were treated with sulfasalazine, 5-ASA, or sulfapyridine enemas^[8]. Three quarters of the patients in the sulfasalazine and 5-ASA groups showed improvement, while only about one third of patients in the sulfapyridine group improved. These data supported the hypothesis that 5-ASA was the active therapeutic moiety, and subsequent studies confirmed the efficacy of 5-ASA enemas in distal colitis^[9]. 5-ASA is also available in suppository form (Rowasa) that is beneficial for the treatment of proctitis.

Distal ulcerative colitis

About two thirds of the patients with proctitis and proctosigmoiditis respond to hydrocortisone enema (Cortenema) or 5-ASA enemas (Rowasa). However, in the patients with proctitis and/or proctosigmoiditis that do not respond to this conventional treatment of hydrocortisone enema or 5-ASA enemas, several maneuvers are helpful. A clinical trial that compared the efficacy of nightly 4 g 5-ASA retention enemas with continued administration of 100-mg hydrocortisone enemas in distal UC after failure of a 3-week trial of the latter, with or without oral sulfasalazine, demonstrated that a significantly greater number of refractory patients responded to 5-ASA enemas than to continuation of standard therapy^[10].

Several studies show that mesalamine enemas 4 g/day along with oral mesalamine 2.4-4 g/day are useful in inducing and maintaining remission. The data also suggest that a higher dosage (such as 4 g/day orally) of all but one 5-ASA agent offers an important therapeutic advantage in some of the patients who are apparently "refractory" to conventional dosage. The exception to this is olsalazine, which, because of dimer-induced intestinal secretion, delivers more fluid to the colon and may cause diarrhea. Other data suggest that daily or every-other-day administration of 5-ASA enemas is sufficient to maintain remission, but that this effect is lost if administration is reduced to every third day.

A recent 6-week multi-center, randomized, double-blind comparative study of oral mesalamine versus rectal mesalamine versus combination therapy in the treatment of mild-to-moderate distal UC evaluated the differences among these regimens. A total of 60 patients were enrolled in the study; patient demographics, including UC history, did not differ significantly among treatment groups^[11]. This study suggests that a combination of oral and rectal mesalamine in patients with mild-to-moderate UC produces earlier relief of rectal bleeding than either therapy alone and more complete relief of rectal bleeding than oral therapy alone. 5-ASA by the oral route and intermittently by topical route was found to be more effective than oral

therapy alone in maintaining remission^[12].

Glucocorticoids

Like sulfasalazine, glucocorticoids and adrenocorticotrophic hormone (ACTH) have been used in the treatment of UC for more than 40 years. The first controlled trial was done in 1955 by Truelove and Witts^[13], and this, and other subsequent studies, clearly established the efficacy of glucocorticoids. Several studies have established that for mild-to-moderate disease, a daily dose of 40 mg of prednisolone is optimal, while for severe disease the optimal dosage is 60 mg of prednisolone or equivalent in divided doses. Even in severe disease, intravenous glucocorticoids have been shown to induce remission in up to 80% of patients. While ACTH is effective, studies have shown that it has no significant benefit over glucocorticoids, and it is now rarely used due to its expense and the fact that it must be used intravenously. For distal colitis, local therapy with glucocorticoid enemas is effective. However, unlike sulfasalazine and 5-ASA compounds, glucocorticoids have not been found to have any benefit as long-term maintenance therapy in UC.

Because of the numerous systemic side effects of glucocorticoid therapy, attempts have been made to develop poorly absorbed, topically active steroids and glucocorticoids with high topical activity and high rate of metabolism in the liver. The most promising of these agents appears to be budesonide. It is readily absorbed from the gut and rapidly degraded to metabolites with low glucocorticoid systemic activity during the first passage through the liver. It is not yet available in the United States, but several recent studies with oral administration suggest its efficacy in treating UC^[14], and ileal Crohn's disease^[15,16], as well as distal UC in the form of enemas^[17,18].

In a randomized trial of Budesonide 8 mg/day vs prednisone 40 mg/day for 8 wk in patients with CD, there was equivalent remission by CDAI < 150 in both groups. However, twice as many responders in the Budesonide groups responded to treatment with no side effects as compared to the prednisone group^[19].

Immunosuppressive/immunomodulatory agents

The first immunosuppressive agents used in the treatment of IBD were 6-mercaptopurine (6-MP) and its *S*-imidazole precursor, azathioprine. Azathioprine was developed with the intent of allowing delayed release of 6-MP, which is the active metabolite. In clinical practice, azathioprine and 6-MP have similar efficacy and toxicity, and their use is usually based on personal preference and experience.

Several controlled and uncontrolled trials have been conducted to evaluate the efficacy of 6-MP (1-2 mg/kg body weight per day) and azathioprine which is metabolized in the liver and releases 6-MP. The overall results show a response rate of about 70%, with a significant steroid-sparing effect in most patients. In patients who stop taking these agents after remission is

induced, the relapse rate is about two thirds^[20,21].

The main impediment to use these agents by many gastroenterologists is the concern of toxicity. While many patients receiving either agent may develop a decreased white blood cell (WBC) count, few develop marked leukopenia. Because of the potential for bone marrow suppression, blood counts must be checked frequently (e.g. initially bi-weekly and then once a month), but at the dosages used problems are rare. In one of the largest series reviewing the toxicity of 6-MP in inflammatory bowel disease, the authors found marked bone marrow suppression in 2% of patients (WBC < 2500/mm³), infections in 7.4%, and severe infections in 1.8%. Off note, however, many patients were receiving concomitant corticosteroids, and it is unclear what role this may have played in the infections. Pancreatitis can be seen in 3.3% of patients^[22]. This is believed to be an allergic reaction and usually occurs within 1 month of onset of therapy and abates on withdrawal of the agent. Pancreatitis precludes further use of these drugs, while leukopenia may be supported with low-dose corticosteroids or by using a smaller dosage of 6-MP. Rarely, patients may develop a cholestatic hepatitis-like picture even after long term use. Therefore, periodic check-ups (about every 6 months) for liver function tests are necessary. Abnormal results are reversible after cessation of therapy.

A theoretical risk with the use of purine analogues is teratogenicity and the development of malignancy, particularly lymphoma. Although there have been occasional reports of malignancy in patients receiving 6-MP or azathioprine, little data support a causative effect in patients with IBD^[23]. In reviews looking at teratogenicity, no difference in premature births or congenital anomalies was found in patients taking 6-MP compared with the general population^[24].

Cyclosporine is an immunomodulatory agent that has shown some promise in refractory IBD. It acts by inhibiting T-lymphocyte function, does not have the myelosuppressive effects of the purine analogues, and produces an effect quickly, usually within 1 wk. The first report of cyclosporine use in IBD was in 1984, and multiple open trials for use in UC have been conducted since then.

Several recent trials have investigated the efficacy of cyclosporine. These studies, although with small patient numbers, indicate that cyclosporine, at least temporarily, and induced remission in up to 80% of patients with severe UC unresponsive to corticosteroid therapy^[25]. However, the majority of these patients do not sustain clinical remission with oral cyclosporine and eventually required colectomy before one year^[26]. Recently, a study showed lower relapse rate with azathioprine maintenance therapy after remission was induced with intravenous cyclosporine^[27].

Antibiotics

Antibiotics are not generally useful as a primary therapy for

UC. Although antibiotics do have a clear role in treating complications of UC, such as abscesses, they have never been shown to be efficacious as a direct treatment for the underlying disease^[28].

A recent double blind, placebo controlled trial found that ciprofloxacin treatment was slightly better than placebo at 3 months but approached placebo without significant difference in response at 6 months in UC patients^[29].

Nicotine

While UC is more common among the previous smokers, the efficacy of nicotine as a therapeutic agent in UC remains controversial^[30,31]. Given the addictive property of nicotine and significant adverse effects on cardiovascular system and the lack of clear benefit in UC, nicotine cannot be recommended at this time^[32].

Nutrition

Since UC is a condition of bowel inflammation, several investigators have studied bowel rest as therapy. Total parenteral nutrition (TPN) allows for bowel rest, eliminates dietary macromolecules and, thus, reduces mucosal immune response, and helps to correct the malnutrition associated with UC. Several randomized controlled trials have looked at the benefit of TPN in active UC. They found that TPN offered no benefit over the control group with regard to UC^[33]. Patients with distal colitis/proctitis should be on a high roughage diet^[34] and those with anemia refractory to iron and vitamin supplements may benefit from treatment with oral iron and recombinant erythropoietin^[35].

Other agents

Heparin was found to paradoxically induce remission in 9 of 10 patients with UC refractory to standard therapy^[36]. It may act as an anti-thrombotic agent or may be directly anti-inflammatory. Further multicenter studies are currently in progress to examine the efficacy of heparin in patients with active UC.

Deficiency of short-chain fatty acid (SCFA) is associated with diversion colitis, and studies with SCFA enemas in patients with distal UC showed good response in an initial study^[37] although a subsequent study could not reproduce these data^[38]. Further studies are warranted to explore this simplified treatment.

CROHN'S DISEASE

The management of CD is similar in many respects to the management of UC since the two diseases share many common features. However, there are differences.

Sulfasalazine and 5-ASA products

Being a transmural disease, the medical management of the inflammatory process in CD is often more difficult than it is in UC. Pharmacologically active compounds that exert their effect from the luminal side of the intestine as a local anti-inflammatory agent have limited response because of the

lack of transmural availability of the drug. Oral sulfasalazine or 5-ASA derivatives are commonly employed initially for the colonic manifestations of the disease. A multicenter study that evaluated the role of Pentasa in active CD demonstrated that a dose of 4 g/day was more effective in inducing remission than placebo^[39].

Glucocorticoids

Corticosteroids are often used on a short-term basis to manage acute exacerbations of the disease. However, intra-abdominal and perineal sepsis, common complicating factors, must be ruled out before corticosteroids are administered. Oral budesonide (9 mg daily) treatment has been found to be useful to induce remission with less steroid-related side effects^[40]. The efficacy was lower than prednisone^[41]. Recently, budesonide (9 mg daily) was also found to be superior in inducing remission than mesalamine (2 g twice daily) over 16 wk of therapy in active ileocolonic CD^[42]. However, on a long term basis, (over 12 months), the use of budesonide (6 mg/day) did not sustain clinical remission^[43]. Further studies are currently in progress to ascertain the dosage, duration and clinical response.

Immunosuppressive/immunomodulatory agents

In a double-blind placebo controlled study, 6-mercaptopurine (6-MP) was found to induce remission or significant clinical improvement in about two-thirds of patients with symptomatic CD^[44]. If symptoms cannot be controlled with corticosteroids or if it is not possible to taper steroid dosage while maintaining symptom control, then immunomodulatory agents such as azathioprine or 6-MP may be used in conjunction with reduced doses of steroids to induce and maintain remission. 6-MP may be used in conjunction with reduced doses of steroids to induce and maintain remission. 6-MP has also been demonstrated to be effective in closing fistulas and reducing steroid requirements in patients with CD. Unfortunately, fistulas frequently recur upon cessation of immunosuppressive treatment^[44]. IV loading of 6-MP has not been shown to decrease the time to respond in patients requiring ongoing steroid therapy for Crohn's disease. However, Casson has a report of a rare response of IV 6-MP in patients with fulminant colitis who failed steroid management and refused to have surgery^[45,46]. Cyclosporine and methotrexate have been found to be beneficial in subgroup of patients with chronically active CD^[47,48]. Tacrolimus (FK506) has a mechanism similar to cyclosporine and preliminary results have shown some benefits to proximal small bowel or fistulizing CD^[49]. In a recent small study, 7 of 11 patients with steroid refractory Crohn's disease and UC, tacrolimus with azathioprine and mesalamine achieved rapid remission and allowed for tapering of steroids^[50]. However, the data related to the use of these drugs is very limited, and at this time, these drugs may be considered in a subgroup of patients who are refractory to more conventional treatment.

Nutrition

Low residue diets have been found to be beneficial in patients with CD^[51]. In patients with proximal CD there was a significantly increased incidence of lactose malabsorption^[52]. A defined formula diet can induce remission, probably through a decrease in immunostimulation by luminal contents^[53]. This treatment is controversial because remission lasts only as long as the diet is continued. Because fishoil has anti-inflammatory actions, studies have shown a reduction in relapse rates in CD, but its use is limited by the unpleasant taste and smell^[54].

Antibiotics

Antibiotics (metronidazole, ciprofloxacin, and clarithromycin and trimethoprim-sulfamethoxazole) can be useful in treatment of CD^[55,56]. Antibiotics are also used to treat complications such as intra-abdominal sepsis and perineal fistulas. Although long-term use of antibiotics for treatment of CD has been advocated by some physicians, there is no convincing support for this strategy.

Maintenance of remission in CD

One of the most vexing issues in the treatment of CD is maintaining remission. The recent data come from trials that compare maintenance regimens following surgical resection. Both mesalamine and metronidazole have been useful in prolonging the time from surgery to symptomatic relapse^[57,58,59].

A meta-analysis of sulfasalazine and 5-ASA for maintenance therapy of CD demonstrated that 5-ASA was more likely to maintain remission than sulfasalazine and that it demonstrated greater efficacy for patients with ileal disease than for those with combined ileocolonic disease; ileocolonic disease responded better than colonic disease^[60].

Although oral mesalamines have demonstrated some benefit in maintaining remission, there are many unresolved issues, e.g. which mesalamine preparation is preferable, in which clinical group such as phlegmonous type, stricture type or perforating type of CD, what dose should be used, and whether drugs are equally effective in maintaining remissions induced initially by medical and/or surgical interventions. A recent study suggested that 6-MP is also effective in preventing post-operative recurrence of CD and may be more effective than 5-ASA preparations^[61].

Newer agents

Tumor necrosis factor (TNF- α) is a proinflammatory cytokine present in excess in the mucosa of patients with active CD. A chimeric mouse/human anti-TNF- α monoclonal antibody (cA2, infliximab, Remicade; Centocor, Malvern, PA) was developed and its efficacy was assessed in patients with severe CD^[62]. There was no apparent dose relation between 5 mg per kg to 20 mg per kg body weight given as intravenous infusion as a single

dose over a 2 h period. After a single dose of infusion 5 mg/kg clinical response was achieved in 65% of the patients. About half of these patients in the treatment group (compared with 4% in the placebo group) went into remission by wk 4. However, by wk 12, the number of patients maintaining their clinical response after the single dose infusion had decreased to 41% (compared to 12% in the placebo group). Repeated doses in initial responders appear to maintain remission at least in the short term study reported so far. The patients with no response to the first infusion of cA2 were less likely to have a response to a second infusion. Thus, this group of patients may clinically differ from the responder group. Preliminary results from another anti-TNF antibody (CD P571) also showed promising results^[63]. The adverse effects of cA2 therapy were mostly transient and not serious and they included headache, nausea, upper respiratory tract infection, fatigue, myalgia, rhinitis, pain, pruritus and dyspnea^[62]. Of the 29 patients who received two cA2 infusions, two had a reaction with chest pain, dyspnea and nausea necessitating discontinuation of the infusion. At least 6% of the patients developed anti-cA2 antibody by 12 wk. This percentage may be higher because cA2 was still detectable in serum samples in two-thirds of the patients and the presence of cA2 may have interfered with the anti-cA2 assay. As use of infliximab has increased, reports of hypersensitivity have been published. In our experience, this can be avoided by premedication with benadryl and glucocorticoids^[64]. It is unknown at this time whether the presence of anti-cA2 will influence the efficacy of the therapy, particularly after subsequent infusions. Remicade has recently been approved by the FDA for use in moderate and severe Crohn's disease, with or without fistula since closure of fistula has also been reported with this therapy. In another review, infliximab was effective in achieving fistula closure 68% vs 26% placebo^[64].

Interleukin-10 (IL-10) is a cytokine with both anti-inflammatory as well as immunosuppressive properties. In a recent study in patients with steroid-refractory active CD, daily administration of IL-10 for one week resulted in 50% of patients achieving complete remission within 3 wk (compared to 23% in the placebo group)^[65].

IL-11 has been known to have a mucosal protective effect. Besides its anti-inflammatory effects, it also has a trophic effect on intestinal villi. Sands *et al* are currently investigating dosing strategies for possible use of this cytokine in the treatment of active Crohn's disease^[66,67].

Intercellular adhesion molecule-1 (ICAM-1) is an inducible transmembrane glycoprotein involved in the activation of leukocytes and is upregulated in inflamed mucosa in CD. ISIS 2302 is a 20-base phosphorothioate oligodeoxynucleotide which selectively inhibits cytokine-induced ICAM-1 expression. In patients with steroid-resistant active CD, it was found to achieve remission in 7 of 15 patients immediately after treatment and 5 of 7 patients were still in remission at the end of six months^[68]. This modality of treatment opens up yet another novel

approach of administering antisense therapy for IBD.

It is recognized that NSAIDs may be harmful in patients with inflammatory bowel disease. Since the arrival of COX₂ inhibitors on the market, only one study has attempted to evaluate their safety in IBD. The results do not point to any advantage or safety of COX₂ drugs over NSAIDs^[69].

Probiotics

There has been recent investigation into the bacterial makeup and possible association of intestinal bacteria with inflammation. Investigators have focused on use of nonpathogenic *E.coli* and other probiotic preparations in maintaining remission of UC. In one study by Rembacken *et al* at 12 months there was a statistically significant difference between two groups, one taking mesalamine, the other taking nonpathogenic *E.coli*^[70,71].

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Pathogenesis and management of pain in chronic pancreatitis

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INTRODUCTION

Of the three cardinal manifestations of chronic pancreatitis—pain, diabetes mellitus and steatorrhea, it is pain that brings the patient to the physician and is the most difficult to manage. The intractable pain that is quite debilitating disrupts lifestyle and leads to functional incapacity, drug and alcohol dependency, and a drug-seeking behavior that occasionally might push the desperate patient to suicidal tendency. Painless CP is an exception that has been observed in nearly 5% to 10% of patients with all forms of chronic pancreatitis. Lack of pain is also a feature of the late onset idiopathic CP.

CHARACTERISTICS OF PANCREATIC PAIN

The pain of pancreatitis is steady and agonizing, is felt in the epigastrium, sometimes in the left upper quadrant with radiation to the back between T12 and L2 or to the left shoulder. Typically pain is postprandial in nature suggesting a gastric lesion. Colicky pain is unusual in CP. Associated nausea and persistent vomiting that does not relieve the pain are in contrast to the pain of gastritis or pyloric obstruction. The pain may be accompanied by flushing and shortness of breath. The severity of pain varies greatly in different patients and indeed in the same individual in different episodes, for no obvious reason. The onset of the persistent abdominal pain 12 to 48 h after a drinking bout or “on the afternoon after the night before” is said to be characteristic of alcoholic pancreatitis, but exacerbation of pain may occur even during abstinence from alcohol with no identifiable cause. At times of pain patients bend forward to the so-called “pancreatic position” or lie in the knee-chest position on their right or left side.

The duration of pain-free intervals is unpredictable and may last from weeks to many months, making it difficult to assess the value of different modalities of pain therapy. As the disease advances, the postprandial relationship is lost and pain becomes steady and unrelenting^[1].

Recently Ammann *et al* reported characteristics of pancreatic pain in 207 patients with alcoholic chronic pancreatitis^[2]. They identified two patterns of pain Type A and B based on natural history. In Type A pain in nearly 44% of patients, who never, who never needed surgery for pain relief the duration of pain was short usually less than 10 days. In contrast Type B pain occurred in 56% of patients who had episodes of constant pain defined as prolonged periods of daily persistent pain, occurring 2 or more days per week for at least 2 months. All these patients underwent surgery. The two types of pain noted by Ammann in Switzerland has not been observed in other series^[3]. What Ammann has noted in his Zurich patients based on the natural history of pain in chronic pancreatitis as “burning out of the pancreas” and pain relief after 10 or more years is also not seen in many other countries^[4-6]. Although it is not clear why geographical differences occur in the natural history of chronic pancreatitis, one observation is that the different studies quoted include patients with a alcoholic and non-alcoholic pancreatitis while Ammann’s group is exclusively of alcoholic pancreatitis.

PATHOGENESIS OF PAIN IN CP

Pain in CP is multifactorial in pathogenesis^[1]. The current thoughts include (a) increased intraductal pressure, (b) increased pancreatic tissue pressure (interstitial-hypertension), (c) pancreatitis-associated neuritis, (d) pancreatic ischemia, and (e) ongoing pancreatic injury. Complications of CP, such as pseudocyst, common bile duct obstruction, and associated gastroduodenal diseases (e.g., peptic ulcer), may contribute to the pain. Pancreatic cancer may mimic CP and may be a complication in some patients with CP.

Intraductal hypertension

Increased intraductal pressure is the most important cause of pain. Intraoperative ductal pressure measurements, direct measurements through endoscopic approach, and studies on surgical and autopsy specimens of pancreas have substantiated the concept.

The normal intraductal pressure, depending on the

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methodology used in the unstimulated pancreas, is about 7 mmHg, and it is markedly increased in CP. DuVal in 1958 measured the pancreatic duct pressures in nine patients with CP using a small-caliber Foley catheter inserted into the distal pancreatic duct at pancreaticojejunostomy and in four patients with cutaneous pancreatic fistulae who served as controls^[1]. In those with CP the intraductal pressure was invariably higher. Histopathological studies reveal that strictures of the ducts and ductules, obstruction by calculi in major ducts or its branches are the essential features of CP and could all cause pre-stenotic intraductal hypertension.

Interstitial hypertension

The pathogenesis of interstitial or tissue hypertension is the same as intraductal hypertension^[7]. Even minimal obstruction to the ductules can lead to tissue fluid hypertension. Indeed, interstitial hypertension can theoretically occur much before intraductal hypertension and ERCP changes of ductal morphology are detectable. In the so-called minimal change chronic pancreatitis, the severe pain cannot be explained with the above^[8].

Pancreatic pain maybe ischemic in nature analogous to the pain in compartment syndromes. High interstitial pressure could greatly increase vascular resistance and reduce pancreatic blood flow. Karanjia *et al* have shown in their cat model of CP that the blood flow to the pancreas was 40% lower than in normal pancreas, whereas secretory stimulation of the pancreas further decreased the blood flow^[9]. Decompression of the obstructed pancreatic duct reversed all these changes. Increase in ductal or interstitial pressure has been questioned in a few studies and no correlation has been found between ductal morphology and pain^[10]. In those with normal ERCP findings, one cannot rule out the possibility of interstitial hypertension.

Ongoing pancreatic injury

Kloppel hypothesizes that the dynamics of the disease rather than the end result are possible factors for pain^[11]. During progressive scarring the pancreatic nerves and ducts become irregularly entrapped in fibrotic tissue. Recurrent tissue necrosis causes pain in early stages, whereas the persistent pain in an advanced CP is a result of incomplete obstruction of the ducts.

The role of pancreatic stellate cells in this regard is notable. Pancreatic stellate cells are perivascular and derived from Vitamin A-containing cells. These cells because of their contractile potential and perivascular location could cause microvascular ischemia and pain^[12].

Inflammatory mass

Nearly 30% of patients with painful chronic pancreatitis have enlargement of the pancreatic head caused by inflammation^[13]. The inflammatory mass caused pain by involving pancreatic nerves and producing obstruction of

bile duct, pancreatic duct or duodenum^[14,15]. Operations have been devised to remove inflammatory masses^[16,17]. The studies of Ammann *et al* did not find these inflammatory masses in the head of the pancreas^[2].

Neuronal changes

Keith *et al* (1985) studied pancreatic tissue obtained from 50 patients who underwent pancreatic resection or decompression^[18]. They found perineural accumulation of inflammatory cells, predominantly eosinophils correlating with severity of pain and alcoholism-scores, but not with ductal morphology, indicating a role of these inflammatory cells in the causation of pain.

It is well known that eosinophils are toxic to nerve tissue. The mean diameter of nerves in the pancreas of CP patients is significantly greater than in controls^[19]. The perineural sheath is altered such that it no longer provides a barrier between the surrounding connective tissue and the internal neural components. The absence of the normal barrier provided by the perineurium exposes the nerves to activated enzymes, plasma components, and bioactive materials released from inflammatory cells. Increased mean diameters of nerves argue against an old thought that pain is caused by strangulation of nerves by fibrosis. The weakness of the neuronal theory of pain lies in its failure to explain the relief of pain with the cessation of pancreatic function when it shows the same histologic changes in nerves, as in painful pancreatitis. It also does not explain the relief of pain with surgical procedures that reduce intraductal pressure but do not alter the neuronal changes.

NATURAL HISTORY OF PAIN IN CP

Pain in CP as the disease progresses to pancreatic exocrine and endocrine insufficiency is unpredictable in an individual case. Levra *et al* (1970) reporting on a longitudinal study of 113 patients followed for 4 years, observed that the pain decreased in 42%, was stable in 32%, and worsened in 26%^[20]. The often quoted study of Ammann *et al* (1984) from Switzerland concluded that 85% of patients obtained lasting relief from pain at a median of 4.5 years from the onset of disease accompanied by a marked increase in pancreatic dysfunction and calcifications suggesting a relation between pain and the onset of pancreatic insufficiency.

Although there seems to be no consensus with regard to the percentage of patients who get spontaneous relief, some observers have confirmed a final pain-free stage in the natural history of CP in 30% to 50% of patients. However, in an individual case no one can predict how long it will take to reach a pain-free stage of CP or whether that individual will ever get a "burned out pancreas". Surgical management cannot be delayed hoping that spontaneous relief would ever occur.

The study of Ammann *et al* quoted earlier is relevant here^[2]. Nearly 44% of patients had only intermittent pain

and had a favorable course without invasive therapy. In evaluating various treatment modalities the group of patients who underwent the therapy has to be clarified. An endoscopic therapy of clearing stones from the pancreatic duct by a group claimed that the best predictor of pain-free interval after therapy was infrequent attacks before therapy^[22]. One might criticize studies with similar results as expected response even without the therapy.

MANAGEMENT OF PAIN IN CP

Management of pain in CP is to be approached by a team of physicians that includes a gastroenterologist, surgeon, radiologist, and a psychiatrist. It is difficult to assess the severity of pain in patients with CP since many of them are addicted to alcohol and/or narcotics. Equally important is to recognize that in patients with alcoholic pancreatitis it is not uncommon to see malingering or a drug-seeking behavior in the form of exaggerated complaints of pain. The available modes of therapy in the management of pain are tabulated. Before the initiation of therapy, treatable complications such as pseudocysts, bile duct obstructions, and peptic ulcer disease should be ruled out.

Table 1 Management of pain in chronic pancreatitis

Medical (conservative)	Abstinence from alcohol Analgesics (nonopioid and opioid NSAIDs) Tricyclics Pancreatic enzyme supplements Parenteral nutrition Octreotide
Endoscopic therapy	Sphincterotomy, stone extraction, extracorporeal shock wave Lithotripsy (ESWL) of pancreatic calculi Septotomy+ Stent placement
Neurolytic therapy	Drainage procedures Surgical therapy Resections Denervation procedures
Experimental	Dissolution of calculi with oral medications Direct chemical dissolution of calculi Duct injection to cause complete obstruction (prolam, acrylate, latex)

Abstinence from alcohol

The help of a psychiatrist or an alcoholics anonymous group is often necessary. The rate of pain relief is usually higher in abstinent patients, and deterioration of pancreatic function is slower. The importance of alcohol abstinence thus cannot be overemphasized. Considerable time should be spent in discussing with the patient and making him or her understand the inevitable progression of disease if alcohol use is continued.

It is difficult to interpret the results of studies showing no correlation between abstinence from alcohol and abdominal pain. It is likely that the clinical stage of the disease alters the effect of alcohol or pain. In the early stages, with well-preserved pancreatic function, secretagogues such as alcohol may exaggerate pain, whereas in advanced stages they may have very little

stimulatory effect on the fibrotic pancreas^[21].

Diet

The goal is to provide rest to the pancreas or avoid excessive stimulation while maintaining adequate nutrition. The conventional diet is one that is low in fat and small in quantity. In some patients hospitalization, total cessation of oral intake of food, and short-term use of partial or total parenteral nutrition may be needed for urgent management of severe pain. The diet is also determined by the fact whether there is associated diabetes and/or steatorrhea. Supplementation with oral antioxidants is reported to reduce the intensity and frequency of pain.

Antioxidant supplementation

The basis of antioxidant therapy was the hypothesis that CP is a disease caused by unopposed free radical (FR) injury^[23,24]. Banks and colleagues in their attempt to prevent generations of FR used allopurinol 300 mg/d. There was no significant reduction in pain. Despite a definite proof for FR injury in relation to CP or clinical trials confirming the use of antioxidants, there is widespread enthusiasm for the therapy. Antioxidant therapy is inexpensive and harmless. For a disease with no better substitute for treatment the simplicity of antioxidant therapy is attractive.

Cigarette smoking is noted to be an associated factor in the pathogenesis of CP, it is prudent to advise against smoking^[25,26]. Most alcoholics who develop CP are heavy cigarette smokers.

Analgesics

The first step is to try nonopioid analgesics, such as acetaminophen, salicylates and nonsteroidal anti-inflammatory drugs (NSAIDs) with or without antidepressants. However, most patients need opioid analgesics for symptomatic relief, and the initial doses should be low and administered less frequently. Small doses of codeine derivatives with acetaminophen are the usual drugs of choice. The use of an H₂ receptor antagonist or a proton pump inhibitor to suppress gastric acid production, reduce destruction of orally administered pancreatic enzymes and theoretically reduce pancreatic stimulation is advocated. As an adjunct to the therapy, a small dose of an antidepressant may be helpful. If a potent narcotic is needed to offer pain relief, surgery should be considered as early as possible before the development of narcotic addiction.

Pancreatic enzyme therapy

The basis for oral enzyme therapy for pancreatic pain is as follows. Secretory status of exocrine tissues and obstruction to outflow determine intraductal pressure. Deficiency of proteases (trypsin, chymotrypsin) within the duodenal lumen causes release of cholecystokinin from the mucosa,

leading to enhanced stimulation of the exocrine tissues [27-29]. Experimental evidence indicates that the presence of intraluminal proteases in the proximal small intestine inhibits the release of cholecystokinin and thereby stimulation of the exocrine parenchyma.

To effect feedback inhibition of pancreatic secretion, it is important to administer large doses of commercially available pancreatic enzymes. One should choose the type of preparation that increases the intraduodenal proteases [28]. The ideal patient for enzyme therapy is a female with idiopathic pancreatitis with normal fecal fat excretion. Individuals with large dilated ducts tend not to respond to enzyme therapy. Enzyme therapy is simple, easy to initiate, but somewhat expensive. However the cost of enzyme therapy cannot be considered high when the alternative is major pancreatic surgery. The selection of an enzyme preparation is to be based on the protease concentration within the preparation, the stability of the enzyme in withstanding gastric acidity, and the timely release of the enzyme from capsules in the proximal intestine. The inconvenience of taking large doses of enzyme preparations is justified if the preparations relieve pain or reduce the need for analgesics. Hence, it is worth trying enzyme therapy in all patients with painful CP for at least 6 to 8 weeks.

If there is no pain relief with a nonenteric coated preparation, addition of an H₂ receptor antagonist or sodium bicarbonate (650 mg) before and after each dose of pancreatic enzymes to protect them from gastric acid is a consideration.

The side effects of enzyme therapy are few. Pancreatic extracts from insoluble complexes with folic acid, as a result folic acid deficiency can develop. Hyperuricemia and hyperuricosuria are described in cystic fibrosis patients treated with large doses of pancreatic extracts. Allergic reactions to the porcine proteins in extracts may occur. There is a valid criticism that studies supporting response to enzyme therapy are few and have used only a small number of patients.

The vast majority of patients with CP are those with longstanding history of alcoholism, more often men, with large duct disease. Even the protagonists of enzyme therapy do not consider the above group as ideal patients to obtain relief with oral pancreatic enzymes. It appears that at this time the majority of gastroenterologists are not quite enthusiastic about enzyme therapy for pancreatic pain [30]. The role of enzyme therapy is limited despite its wide popularity which is solely based on its simplicity and the fact that it appears to be a non-invasive form of therapy compared to endoscopic therapy or surgery.

Somatostatin and octreotide

Somatostatin is a naturally occurring hormone that inhibits pancreatic secretion. Octreotide is a synthetic long-acting analogue of somatostatin that inhibits cholecystokinin

release and both basal and oral stimulated pancreatic secretion. The duration of action of octreotide is longer than that of somatostatin, but it increases the contractability of the Sphincter of Oddi while somatostatin decreases it. One multi-center randomized study of 91 patients with CP showed promising results [31]. At a dose of 200 mg TID octreotide offered good pain relief especially in those with constant pain. Another recent study by Malfertheiner *et al* however did not show any benefit [32].

Endoscopic therapy in CP

Endoscopic therapies are based on the premise that the most important mechanism of pain in CP is impairment of outflow of pancreatic secretion by strictures or calculi in the main pancreatic duct [33-36]. The attractive feature of endoscopic drainage procedures is that it offers an alternative to surgical forms of drainage. The optimistic belief that these methods may achieve the same results as surgical drainage but without its operative morbidity (20% to 40%) or mortality (2% to 5%) rates is attractive.

The forms of endoscopic procedures currently available include sphincterotomy, internal drainage of pancreatic cysts, extraction of stones from the pancreatic duct, guidewire-catheter dilation of strictures, and placement of pancreatic stents. Endoscopic intervention can be considered in the following situations: (1) biliary strictures, (2) pain or recurrent pancreatitis associated with a dominant stricture at the proximal end (head), (3) recurrent pancreatitis with pancreas divisum, (4) pancreatic cysts, (5) pancreatic stones, and (6) Sphincter of Oddi dysfunction.

Lithotripsy

Although it is controversial whether stones or calculi directly cause pain it is logical to assume that removal of stones would relieve intraductal hypertension. Although endoscopically it is possible to remove one or two stones in the head end of the pancreas, it is only after fragmentation by shock wave or laser lithotripsy that the more proximal innumerable stones can be drained. Some studies have noted excellent pain relief [35-38]. In a series of 123 patients with pancreatic calculi ESWL was helpful in 122. After ESWL the pancreatic duct could be completely cleared with endoscopic papillotomy in 595 of patients [37]. Adamack and group recently (1999) studied 43 patients who were successfully treated with ESWL [38]. The only feature associated with treatment success was the presence of a single stone rather than multiple stones. Successfully treated patients achieved some degree of reduction in pain. The factors favoring stone removal and clinical response include three or less stones, absence of multiple strictures, stones confined to the head of the pancreas, stone diameter of <10 mm and absence of impacted stones [35]. Lithotripsy with stone removal is not yet a well accepted form of therapy for pain relief in chronic pancreatitis. Lack

of expertise, a skepticism generated by lack of adequate well controlled trials and the theoretical opinion that removal of stones is only cosmetic to the total histology of the pancreas have dampened the enthusiasm for this form of therapy in the U.S. among academicians.

Sphincterotomy

The best indication for endoscopic sphincterotomy is when the patient has a solitary ductal stone in the head of the pancreas without any evidence of proximal stricture. Relief of pain can be predicted if a postsphincterotomy pancreatogram demonstrates immediate reduction in size of the main pancreatic duct.

Stenting

Another procedure used in the treatment of pain is endoscopic stenting of the main pancreatic duct after sphincterotomy. The stents have side holes at approximately 1-cm intervals that permit better drainage of pancreatic juice from side branches. Plastic stents have been noted to be inferior to self-expandable metallic stents (Wallstent), which provide better dilation of the stricture and do not clog as often. Cremer's group noted that in addition to pain relief marked improvement in nutritional status of patients occurred as a result of better delivery of bile and pancreatic secretions in the duodenum helping digestion^[37]. Excellent improvement in pain has been reported. Prolonged symptomatic improvement even after the stent has been removed is observed. This unexpected result may be explained by the fact that before or during stenting, many small calculi are removed, eliminating small ductular obstructions.

The risk of pancreatic stenting is beginning to be appreciated^[33,39,40,41]. A number of complications are reported after stent placement: (1) clogging of the endoprosthesis is frequent, and it must be changed every 4 to 6 months; (2) migration of the prosthesis may be a serious problem (3) the stent can be passed spontaneously without any adverse effect; (4) migration, clogging, and occlusion of the stent may lead to pancreatic abscess formation or infect a pseudocyst that is in communication with the main pancreatic duct; (5) duodenal erosion may be caused by improper positioning of the stent; (6) pancreatitis may be induced by stent placement but is usually mild and self-limiting; and (7) ductal changes mimicking CP may develop even after stent placement.

Celiac ganglion blocking

It is reasonable to expect that pharmacologic denervation of the celiac plexus will result in pain relief. Celiac plexus blocking is technically difficult; the pain relief is transient and sometimes lasts only a few days. It is also associated with complications such as hypotension, epidural or intraperitoneal hematomas, and sexual dysfunction. Neurolytic block is thus considered a last resort therapy.^[41]

Recent reports on celiac ganglion blocking using endoscopic ultrasound guidance offer much hope^[42,43].

A variant of celiac block is intrapleural analgesia achieved by instilling local anesthetic into the pleural space^[44]. Dramatic pain relief lasting for 4 months has been reported. Diffusion within the interpleural space seems to block several intercostal nerves. The scientific explanation is that pancreatic pain is transmitted along afferent fibers from the pancreas to the splanchnic nerves and also by the lower intercostal nerves, which innervate the peritoneum.

Acupuncture and transcutaneous nerve stimulation (TENS)

A pilot study showed that 3 patients with chronic pain responded well to both electroacupuncture and TENS^[45]. In 23 patients with chronic pancreatitis with daily pain for 3 months the efficacy of TENS was studied. In two prospective studies with cross over design active acupuncture was compared with sham acupuncture and TENS of the segmental points of the pancreas with sham treatment. Neither acupuncture nor TENS brought about pain relief that could substitute for or supplement medical treatment (Ballegaard, et al. 1985). This small study conducted in an institution not specializing in acupuncture should not discourage experts in the field from trying it. In the absence of an effective therapy for pain in CP, there is a great need to look at alternate medicine for possible answers. The expertise of the Chinese is greatly needed here.

Surgery for pain in CP

Surgery is a consideration when pain is severe enough to interfere with day-to-day life and when it cannot be managed safely with medical treatment alone. Recent review articles well describe the surgical aspects of CP^[46,47]. Evaluation for surgery must be individualized and the following should take into consideration: frequent hospitalizations, disruption of employment and social life, nutritional status, depression of other psychiatric manifestations, and drug dependence. Preoperative assessment should include abdominal computed tomography, ERCP, and if appropriate, psychiatric evaluation.

The operative candidates can be divided into two broad groups: those with dilated pancreatic ducts (who more likely benefit from ductal drainage) and those with normal size ducts (who may need pancreatic resection or a denervation procedure).

Drainage procedures Many authors recommend longitudinal pancreaticojejunostomy or lateral pancreaticojejunostomy (modified Puestow procedure) when the pancreatic ducts are large enough for anastomosis^[46]. Long-term pain relief is achieved in more

than two thirds of patients with CP and a dilated (more than 7 mm diameter) pancreatic duct. Pain relief is immediate in 80% to 90% of patients, although there is an unpredictable recurrence rate in some of them. Some 60% to 75% of patients are pain free for 5 to 6 years. Recurrence of pain after an initially successful longitudinal pancreaticojejunostomy suggests stricture formation and may indicate the need for reoperation.

The operative mortality rate averages about 4%, and diabetes, does not result from the procedure. However, many patients may go on to become insulin-requiring diabetics because of continual destruction of the pancreas as a result of CP.

Pancreatic resection Resection is the procedure of choice in a pancreas whose ducts are not dilated, when a previous drainage procedure has failed, or if pathologic changes predominantly involve a particular area of the gland. Resection offers good pain relief that tends to be more permanent than that after pancreaticojejunostomy. Resections generally involve one of the following procedures: (1) pancreaticoduodenectomy, (2) local resection of the head of the pancreas, and (3) subtotal or distal pancreatectomy. However, exocrine and endocrine insufficiency is a major drawback with resections.

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Therapeutic methods for diarrhea in children

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DEFINITION

Acute diarrhoea is defined as passage of loose or watery stools at least three times in a 24 h period. When loose stools contain blood, it is called bloody diarrhoea (dysentery). It is the consistency of the stools which is most important rather than the frequency. Breast-fed babies often pass "pasty" stools frequently which is not diarrhoea. The mother can often tell accurately whether child has diarrhoea or not.

MAGNITUDE OF THE PROBLEM

Acute diarrhoea is an important cause of mortality and morbidity particularly in young children in the developing countries. Of the 11.6 million deaths among children less than five years old in all developing countries (1995) due to infectious diseases, 19% deaths are attributed to diarrhoea^[1]. In 1993, an estimated 3.2 million children below five years of age died from diarrhoea alone; 80% of these deaths occurred in the first two years of life^[2].

AETIOLOGY

Acute diarrhoea is usually caused by different infectious agents. The microbial agents causing diarrhoea may be classified as bacterial (*Vibrio cholerae* O1 and O139, *Vibrio parahaemolyticus*, enterotoxigenic *E.coli*, *Shigella*, *Salmonella*, *Campylobacter jejuni*, *Aeromonas* etc.), viral (Rotavirus, Norwalk virus etc.), parasites (*E. histolytica*, *Giardia lamblia*, *Cryptosporidium* etc.). The various agents produce diarrhoea either by production of toxin(s) or by invasion of the gut mucosa. Those organisms which produce diarrhoea by production of toxin(s) produce watery diarrhoea. *Vibrio cholerae* and enterotoxigenic *E.coli* are the prototype organisms producing watery (also called

secretory) diarrhoea. Those organisms which invade the gut mucosa usually produce bloody diarrhoea (dysentery). *Shigella* and *E.histolytica* are the prototype organisms producing bloody diarrhoea (also called invasive diarrhoea).

In watery diarrhoea usually there is loss of lots of fluid and electrolytes from the body which results in dehydration which is a conspicuous clinical feature in watery diarrhoea. In contrast, in invasive diarrhoea not much fluid and electrolytes are lost in the stool. Therefore, dehydration is not a major feature. It is most practical to base treatment of acute diarrhoea on the clinical type of illness (watery or bloody). Laboratory studies are usually not needed.

DEHYDRATION

Management of acute watery diarrhoea includes replacement of fluid and electrolytes losses, proper feeding and use of appropriate antibiotic in selected cases. It has been mentioned that dehydration occurs due to loss of fluid and electrolytes from the body. Dehydration is now clinically assessed as diarrhoea with "no signs of dehydration", diarrhoea with "some dehydration" and diarrhoea with "severe dehydration". Table 1 describes how to determine the degree of dehydration clinically. The signs typical of children with no signs of dehydration are shown in column A, signs of some dehydration in column B and those with severe dehydration in column C. The sign in bold print with asterisks (*) are the most valuable signs for assessing dehydration and are called "key signs". If two or more of the signs in column C are present including at least one key sign the child has severe dehydration. If this is not the case, but two or more signs from column B (and C) are present, including at least one key sign, the child has some dehydration. If this also is not the case, the child is classified as having no signs of dehydration.

PREVENTION OF DEHYDRATION (PLAN A)

Diarrhoea with no signs of dehydration may be managed safely and effectively at home with the administration of extra fluid, proper feeding and watching for danger signs. The mother may be educated to give her child extra fluid in the form of coconut water, salt and sugar solution, rice water with salt, mild tea (these fluids are called "home available fluid"-HAF) or oral dehydration salt solution (ORS). At any stage if the child becomes either very thirsty, passes many watery stools, vomits repeatedly, or has fever, or blood in stool, the mother should be alerted to take the child to a doctor for further management.

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Table 1 Assessment of diarrhoea patients for dehydration

1. Look at:	Condition ^a	Well, alert	Restless, irritable	'Lethargic or unconscious; floppy'
	Eyes ^b	Normal	Sunken	Very sunken and dry
	Tears	Present	Absent	Absent
	Mouth and tongue ^c	Moist	Dry	Very dry
	Thirst	Drinks normally, not thirsty	*Thirsty, drinks eagerly'	'Drinks poorly, or not able to drink'
2. Feel:	Skin pinch ^d	Goes back quickly	'Goes back slowly	'Goes back very slowly'
3. Decide:		The patient has no signs of dehydration	If the patient has two or more signs, including at least one 'sign', there is some dehydration	If the patient has two or more signs, including at least one 'sign', there is severe dehydration
4. Treat:		Use Treatment Plan A	Weigh the patient, if possible, and use Treatment Plan B	Weigh the patient and use Treatment Plan C urgently

^a Being lethargic and sleepy are not the same. A lethargic child is not simply asleep: the child's mental state is dull and the child cannot be fully awakened; the child may appear to be drifting into unconsciousness.

^b In some infants and children the eyes normally appear somewhat sunken. It is helpful to ask the mother if the child's eyes are normal or more sunken than usual.

^c It is necessary to look inside the child's mouth. The mouth may be dry in a child who habitually breathes through the mouth. The mouth may be wet in a dehydrated child owing to recent vomiting or drinking.

^d The skin pinch is less useful in infants or children with marasmus or kwashiorkor, or obese children.

TREATMENT OF DEHYDRATION (PLAN B & C)

Studies have shown that 90% of cases of watery diarrhoea with some dehydration can be safely and effectively managed with ORS solution alone^[3]. WHO/UNICEF recommended ORS contains sodium chloride 3.5 g, 85 mm potassium chloride 1.5 g, sodium bicarbonate 2.5 g, or trisodium citrate, dihydrate 2.9 g and glucose 20 g dissolved in 1 L of water. For the treatment of some dehydration ORS should be administered (50-100 mL per kg). Table 2 gives the guidelines for treating children and adults with some dehydration. The mother should be taught to prepare and give ORS solution. The solution should be given to infants and young children using a clean spoon or cup. Use of a feeding bottle is strongly discouraged. If vomiting occurs (usually during the first hour of treatment) the mother should wait for 5-10 minutes and then start giving ORS solution again but more slowly. The disadvantages of WHO/UNICEF ORS are that it does not reduce the stool volume or duration of diarrhoea and thus are sometimes not acceptable to the mothers. Several clinical trials have shown that an ORS solution containing cooked rice powder in place of glucose substantially reduces the rate of stool loss due to acute diarrhoea. Rice based ORS solution significantly reduces the rate of stool output during the first 24 hours of treatment by 36% in adults with cholera and by 32% in children with cholera. In contrast, the rate of stool loss in infants and children with acute non-cholera diarrhoea treated with rice ORS solution was only reduced by 18%^[4]. A small but significant proportion of dehydrated patients might benefit from using a low osmolarity solution in which glucose concentration has been slightly reduced^[5]. However, the real benefit of using such a solution as well as their exact composition remains to be determined.

The preferred treatment for children with severe dehydration is rapid intravenous rehydration. Such treatment should preferably be carried out by admitting the patient to the hospital. Guidelines for intravenous

rehydration are given in Table 3. The preferred solution is Ringer's Lactate.

FEEDING DURING DIARRHOEA

During diarrhoea the child should be fed properly. Previously it was thought that during diarrhoea, nutrients are not absorbed adequately and hence the bowel should be given a rest. Recent studies indicate that during and after diarrhoea most of the nutrients are sufficiently absorbed. In fact, proper feeding during diarrhoea has been shown to be beneficial and prevents malnutrition. Breast feeding should be continued throughout the duration of diarrhoea. Easily digestible, energy-rich, high potassium containing, non-fibrous food should be given to the child. During convalescence at least one extra feed daily for several weeks is recommended. Locally available and culturally acceptable foods are preferred.

ROLE OF DRUGS

All cases of bloody diarrhoea where dehydration is present should be managed with rehydration therapy as detailed for the acute watery diarrhoea. In addition, in all the cases of bloody diarrhoea in children below 5 years of age should be treated with an appropriate antibiotic assuming that the child is suffering from shigellosis. Studies have shown that antibiotic therapy definitely hastens recovery^[6]. The drug of choice for shigellosis is nalidixic acid^[7]. Other drugs (ampicillin^[8] or cotrimoxazole^[9]) may be used depending upon the drug resistance pattern of the circulating shigella strains in the area. It has been shown that norfloxacin^[10] or ciprofloxacin^[11] are also highly effective in the treatment of shigellosis. These drugs are contraindicated for young children because of the potential cartilage toxicity reported in experimental animals^[12]. However, more and more information is coming that these drugs may turn out to be safe even in children for such short term use^[13]. Amebiasis rarely occurs in children below 5 years of age and therefore random use of antiamebic drugs for childhood diarrhoea is

Table 2 Guidelines for treating children and adults with some dehydration

	Approximate amount of ORS solution to give in the first 4 hours					
Age ^a	Less than 4 months	4-11 months	12-23 months	2-4 years	5-14 years	15 years or older
Weight:	Less than 5 kg	5-7.9 kg	8-10.9 kg	11-15.9 kg	16-29.9 kg	30 kg or more
In mL	200-400	400-600	600-800	800-1200	1200-2200	2200-4000
In local measure						

^a Use the patient's age only when you do not know the weight. The approximate amount of ORS required (in mL) can also be calculated by multiplying the patient's weight in kg by 75.

- If the patient wants more ORS than shown, give more.
 - Encourage the mother to continue breastfeeding her child.
 - For infants under 6 months who are not breast fed, also give 100-200 mL clean water during this period.
- NOTE: During the initial stages of therapy, while still dehydrated, adults can consume up to 750 mL per hour, if necessary, and children up to 20 mL per kg body weight per hour.

Table 3 Guidelines for intravenous treatment of children and adults with severe dehydration

• Start IV fluids immediately. If the patient can drink, give ORS by mouth until the drip is set up. Give 100 mL/kg Ringer's Lactate Solution ^a divided as follows:			
	Age	Fist give 30 mL/kg in:	Then give 70 mL/kg in:
	Infants (under 12 months)	1 hour ^b	5 hours
	Older	30 minutes ^b	2.5 hours

- Reassess the patient every hour. If hydration is not improving, give the IV drip more rapidly.
- After six hours (infants) or three hours (older patients), evaluate the patient using the assessment chart. Then choose the appropriate Treatment Plan (A, B or C) to continue treatment.

^a If Ringer's Lactate Solution is not available, normal saline may be used.

^b Repeat once if radial pulse is still very weak or not detectable.

not recommended. However, if antibiotic therapy fails and *E. histolytica* trophozoites are seen by microscopic examination of stool, metronidazole or tinidazole may be used in the recommended doses.

Routine use of antibiotic(s) in acute watery diarrhoea is not recommended and is actually harmful. The only indication for use of antibiotics is for the treatment of suspected severe cholera cases as an adjunct to rehydration therapy. Cholera should be suspected when a child of more than two years of age suffers from acute watery diarrhoea with severe dehydration in an endemic area. The drugs of choice for the treatment of cholera are tetracycline and doxycycline. Norfloxacin^[14] and ciprofloxacin^[15] have been shown to be also highly effective. Antibiotic therapy shortens the volume and duration of diarrhoea thereby reducing the fluid requirement, and duration of hospitalisation and excretion of *Vibrio cholerae* in stool. Other drugs, as for example, antiemetics, anticholinergics, antidiarrhoeals like opium, charcoal, kaoline and pectin, steroid and cariotonics are not required for the treatment of diarrhoea. In fact some of them are not only useless but may also be harmful. Antiemetics produce sedation and therefore, interfere with oral rehydration therapy and may produce or aggravate hypotension thereby interfering with the renal circulation. Anti-cholinergics may produce paralytic ileals.

Recently, supplementation with micronutrients especially zinc as an adjunct to rehydration therapy for the treatment of acute diarrhoea has been suggested^[16].

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A study on relationship of nitric oxide, oxidation, peroxidation, lipoperoxidation with chronic cholecystitis

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Subject headings nitric oxide; oxidation; peroxidation; lipoperoxidation; chronic cholecystitis

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Abstract

AIM To study relationship of injury induced by nitric oxide, oxidation, peroxidation, lipoperoxidation with chronic cholecystitis. **METHODS** The values of plasma nitric oxide (P-NO), plasma vitamin C (P-VC), plasma vitamin E (P-VE), plasma β -carotene (P- β -CAR), plasma lipoperoxides (P-LPO), erythrocyte superoxide dismutase (E-SOD), erythrocyte catalase (E-CAT), erythrocyte glutathione peroxidase (E-GSH-Px) activities and erythrocyte lipoperoxides (E-LPO) level in 77 patients with chronic cholecystitis and 80 healthy control subjects were determined, differences of the above average values between the patient group and the control group and differences of the average values between preoperative and postoperative patients were analyzed and compared, linear regression and correlation of the disease course with the above determination values as well as the stepwise regression and correlation of the course with the values were analyzed.

RESULTS Compared with the control group, the average values of P-NO, P-LPO, E-LPO were significantly increased ($P < 0.01$), and of P-VC,

P-VE, P- β -CAR, E-SOD, E-CAT and E-GSH-Px decreased ($P < 0.01$) in the patient group. The analysis of the linear regression and correlation showed that with prolonging of the course, the values of P-NO, P-LPO and E-LPO in the patients were gradually ascended and the values of P-VC, P-VE, P- β -CAR, E-SOD, E-CAT and E-GSH-Px descended ($P < 0.01$). The analysis of the stepwise regression and correlation indicated that the correlation of the course with P-NO, P-VE and P- β -CAR values was the closest. Compared with the preoperative patients, the average values of P-NO, P-LPO and E-LPO were significantly decreased ($P < 0.01$) and the average values of P-VC, E-SOD, E-CAT and E-GSH-Px in postoperative patients increased ($P < 0.01$) in postoperative patients. But there was no significant difference in the average values of P-VE, P- β -CAR preoperative and postoperative patients. **CONCLUSION** Chronic cholecystitis could induce the increase of nitric oxide, oxidation, peroxidation and lipoperoxidation.

INTRODUCTION

Chronic cholecystitis is a frequently encountered disease of the digestive system. Some studies point out that in blood of patients with acute cholecystitis the levels of inducible nitric oxide (iNO) and lipoperoxides are markedly increased, while the level of vitamin C, the activities of superoxide dismutase and glutathione enzyme are significantly decreased^[1-5]. However, up to now, there has been no reports on the above in patients with chronic cholecystitis. In order to observe the metabolic state of nitric oxide and other free radicals in patients with chronic cholecystitis, and the degree of injury induced by oxidation, peroxidation, lipoperoxidation due to the chronicity of cholecystitis, we determined nitric oxide (P-NO), vitamin C (P-VC), vitamin E (P-VE), β -carotene (P- β -CAR) and lipoperoxides (P-LPO) levels in the plasma as well as superoxide dismutase (E-SOD), catalase (E-CAT), glutathione peroxidase (E-GSH-Px) activity and lipoperoxides (E-LPO) level in the erythrocytes in 77 patients with chronic cholecystitis and 80 healthy controls.

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We also analyzed and compared differences of the above average determination values between the patient and the control group, and between preoperative and postoperative patients. Additionally, we analyzed the relationship between the course of the disease and the above values in the patients by the linear regression and correlation as well as the stepwise regression and correlation.

SUBJECTS AND METHODS

Subjects

Patients Seventy-seven patients suffering from chronic cholecystitis with gallstones who were confirmed diagnostically through abdominoscopy and biopsy in the People's Hospital of Jinhua City were randomly sampled. Their ages ranged from 31 to 69 years (52.4 ± 10.3 a), and their courses of disease were from 2 to 20 years (5.2 ± 5.6 a). Of them, 32 were male and 45 were female. No patients had abnormality in the routine examination of blood, urine, feces, ECG and X-rays, and medical history about heart, brain, lung, liver, kidney, diabetes, autoimmune disease, peripheral vascular disease, cataract, tumor, and so on. The gallbladders of all the patients with chronic cholecystitis were removed by abdominoscopy.

Control Eighty healthy adults confirmed through the comprehensive health examination by the 2nd Affiliated Hospital of Zhejiang University were randomly sampled, their ages were from 31 to 70 years (52.7 ± 9.6 a), and 40 were male and 40 were female. The healthy adults were all normal in the routine examination of blood, urine, feces, ECG and X-rays, with no medical history regarding heart, brain, lung, liver, kidney, cholecystic disease, diabetes, autoimmune disease, peripheral vascular disease, cataract, tumor, etc.

All the patients and the healthy adults had neither exposure to kind of radiation, nor contacted any kind of pesticide and poison. Within a month prior to the study they had not taken any antioxidants such as vitamin C, vitamin E, ginkgo leaf agents, tea-polyphenol etc. There was neither any significant difference ($P > 0.05$) between the average age of the patient and the control group as determined by *t* test, and nor any significant difference ($P > 0.05$) between the gender proportion of the patient and the control group as determined by χ^2 test.

Methods

Blood samples Fasting venous blood samples were collected in the morning for all the subjects with heparin sodium as an anticoagulant. The separated plasma and erythrocytes were stored immediately at 4°C ^[6].

Plasma NO (P-NO) level Colloidal aluminium hydroxide without nitrite was used to absorb yellow pigments and to cause protein sedimentation in the plasma. The nitrite in the supernatant, which contained sodium acetate (0.20 mol/L) and disulphanilic acid (3.30 mmol/L), reacted with β -Naphthylamine and formed a

colored product, which was detected spectrophotometrically, using sodium nitrite (2.50 $\mu\text{mol/L}$) as the standard and at a wavelength of 520 nm. The P-NO concentration was expressed in nmol/L^[6,7].

Plasma LPO (P-LPO) level Trichloroacetic acid (TCA) solution (20.0 g%, w/v) was used to cause protein sedimentation in the plasma. The protein sediment reacted with thiobarbituric acid (TBA) solution (0.67 g%, w/v) and produced red colored compounds following incubation in a water bath at 100°C . This was detected spectrophotometrically at 532 nm, using tetraethoxypropane (TEP, 5.0 $\mu\text{mol/L}$) as the standard. The P-LPO concentration was expressed as $\mu\text{mol/L}$ ^[6,8].

Plasma VC (P-VC) level TCA (5.0 g%, w/v) was used to cause protein sedimentation in the plasma, and ferric trichloride was added to the supernatant. Vitamin C in the supernatant reduced Fe^{3+} in ferric trichloride to Fe^{2+} . Fe^{2+} , on reacting with ferrocene, produced a colored product which was detected spectrophotometrically at 563 nm, using vitamin C as the standard. The P-VC concentration was expressed as $\mu\text{mol/L}$ ^[6,9].

Plasma VE (P-VE) level Absolute ethyl alcohol was used to cause protein sedimentation in the plasma and to extract vitamin E. Vitamin E in the supernatant reduced Fe^{3+} in ferric trichloride to Fe^{2+} . Fe^{2+} reacted with ferrocene to form a colored product that was detected spectrophotometrically at 563 nm, using vitamin E as the standard. The P-VE concentration was expressed as $\mu\text{mol/L}$ ^[6,10].

Plasma β -CAR(P- β -CAR)level A mixture of absolute ethyl alcohol and petroleum ether was used to cause protein sedimentation in the plasma and to extract β -carotene. The petroleum ether extract containing β -carotene was analyzed colorimetrically, using β -carotene as the standard at a wavelength setting of 440 nm. The P- β -CAR concentration was expressed as $\mu\text{mol/L}$ ^[6,11].

Erythrocyte LPO (E-LPO) level A mixture of absolute ethyl alcohol and trichloromethane (5 : 3) was used to precipitate hemoglobin (Hb) from a hemolytic solution (HS) of RBC without WBC and platelets. Hb level was determined in the HS. LPO in the extracted solution reacted with TBA-glacial acetic acid solution (1.0 g%, w/v) in a water bath at 100°C and produced red colored compounds. These were detected using TEP (5.0 $\mu\text{mol/L}$) as the standard at 532 nm. The E-LPO concentration was expressed as nmol/g Hb^[6,12].

Erythrocyte SOD (E-SOD) activity A mixture of absolute ethyl alcohol and trichloromethane (5 : 3) was used to precipitate Hb from the HS of RBC without WBC and platelets. Hb level was determined in the HS. Pyrogallol (6.0 mmol/L) auto-oxidized in Tris-HCl buffer (50

mmol/L, pH 8.20), SOD was added to the buffer to inhibit its auto-oxidation and SOD activity was calculated according to the auto-oxidation rate of pyrogallol and the rate of SOD-inhibited pyrogallol auto-oxidation. The WL of 420 nm was used and the E-SOD activity was indicated as U/g Hb^[6,13].

Erythrocyte CAT (E-CAT) activity H₂O₂ (0.20 mol/L) was added to phosphate buffer (10 mmol/L, pH 7.0) containing HS of RBC without WBC and platelets. The Hb level was determined in the HS. After a reaction time of 60 s, a solution of potassium dichromate (0.169 mol/L) and glacial acetic acid (1 : 3) was added to the reacting mixture to stop the reaction, and the reacting mixture was heated for 10 min at 100°C. Colorimetry was done at 570 nm. The E-CAT activity was indicated as K/g Hb^[6,14].

Erythrocyte GSH-Px (E-GSH-Px) activity A mixture of absolute ethyl alcohol and trichloromethane (5 : 3) was used to precipitate Hb from the HS of RBC without WBC and platelets. Hb level was determined in the HS. GSH-Px in the extract catalyzed the reaction of glutathione and 5, 5'-Dithiobis-*p*-nitrobenzoic acid (DTNB) and produced yellow colored compounds which were detected at 422 nm, using glutathione (1.0 mmol/L) as the standard. The E-GSH-Px activity was expressed as U/g Hb^[6,15].

Major analytical reagents such as Vitamin C, Vitamin E, β -Carotene, Superoxide dismutase, Catalase, β -Naphthylamine, 1,2,3-Trihydroxybenzene (pyrogallol), 1, 1,3,3-Tetraethoxypropane, 2-Thiobarbituric acid were all purchased from SIGMA CHEMICAL COMPANY, USA; and the other analytical-grade reagents were all procured from China. The main analytical instruments were 721-spectrophotometer and UV-754-spectrophotometer.

Statistic analysis

All data were analyzed with SPSS/8.0 and Statistica/6.0

statistic software using Compaq Pentium III/600 computer. Statistical testing methods included unpaired and paired *t* test and chi square test (χ^2 test), linear regression and correlation analysis, stepwise regression and correlation analysis, and confidence interval (CI) of 95%. The level of significance of hypothesis testing was $P < 0.05$ and the power of test (power) > 0.75 .

Results

Comparison between the above mentioned determinations in the patient and the control group

The average values determined for P-NO, P-LPO and E-LPO in the patient group were significantly increased ($P < 0.01$) with respect to the control, whereas the average values of P-VC, P-VE, P- β -CAR, E-SOD, E-CAT and E-GSH-Px in the patient group were significantly decreased ($P < 0.01$) (Table 1).

Comparison between the above mentioned determinations in the preoperative and the postoperative patients

The average values of P-NO, P-LPO and E-LPO in the postoperative patients were significantly decreased ($P < 0.01$), whereas the average determination values of P-VC, E-SOD, E-CAT and E-GSH-Px in the postoperative patients were significantly increased ($P < 0.01$), but there was no significant difference between the average values of P-VE, P- β -CAR in the pre- and postoperative patients (Table 2).

Linear regression and correlation analysis between the course of disease and the above mentioned values determined in the patients

In pace with gradual prolonging of the course of disease in the patients, the values of P-NO, P-LPO, E-LPO in the patients were gradually increased ($P < 0.01$), the values of P-VC, P-VE, P- β -CAR, E-SOD, E-CAT, E-GSH-Px were gradually decreased ($P < 0.01$) (Table 3).

Table 1 Comparison of various determinations between patient group and control group (CI 95%, $\bar{x} \pm s$)

Group	n	P-NO nmol/L	P-VC μ mol/L	P-VE μ mol/L	P- β -CAR μ mol/L	E-SOD U/g Hb	E-CAT K/g Hb	E-GSH-Px U/g Hb	P-LPO μ mol/L	E-LPO nmol/g Hb
Patient	77	514 \pm 142 482-546	44.3 \pm 10.9 41.8-46.8	19.1 \pm 4.6 18.0-20.1	1.35 \pm 0.38 1.26-1.44	1813 \pm 249 1757-1869	233 \pm 57 220-246	22.7 \pm 4.8 21.6-23.8	13.6 \pm 1.9 13.2-14.0	38.2 \pm 7.2 36.6-39.8
Control	80	365 \pm 157 330-400	55.2 \pm 12.8 52.4-58.0	25.4 \pm 5.3 24.2-26.6	1.72 \pm 0.45 1.62-1.82	2057 \pm 212 2010-2104	309 \pm 61 295-323	27.2 \pm 5.5 26.0-28.4	11.3 \pm 1.7 10.9-11.7	29.4 \pm 6.7 27.9-30.9
<i>t</i>		6.2290	5.7344	7.9415	5.5559	6.6197	8.0588	5.4535	8.0001	7.9316
<i>P</i>		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 2 Comparison of various determinations between preoperative and postoperative patients (CI 95% $\bar{x} \pm s$)

Group	n	P-NO nmol/L	P-VC μ mol/L	P-VE μ mol/L	P- β -CAR μ mol/L	E-SOD U/g Hb	E-CAT K/g Hb	E-GSH-Px U/g Hb	P-LPO μ mol/L	E-LPO nmol/g Hb
Postoperative	77	514 \pm 142 482-546	44.3 \pm 10.9 41.8-46.8	19.1 \pm 4.6 18.0-20.1	1.35 \pm 0.38 1.26-1.44	1813 \pm 249 1757-1869	233 \pm 57 220-246	22.7 \pm 4.8 21.6-23.8	13.6 \pm 1.9 13.2-14.0	38.2 \pm 7.2 36.6-39.8
Postoperative	77	436 \pm 139 404-468	48.5 \pm 11.3 45.9-51.1	19.3 \pm 5.1 18.1-20.4	1.34 \pm 0.36 1.26-1.42	1915 \pm 242 1860-1970	274 \pm 59 261-287	25.1 \pm 5.3 23.9-26.3	12.4 \pm 1.8 12.0-12.8	34.9 \pm 6.9 33.3-36.5
<i>t*</i>		8.9773	7.5376	0.8739	0.5732	7.9814	10.8058	9.5453	9.2384	10.3493
<i>P</i>		<0.01	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*Paired *t* test

Table 3 Linear regression and correlation analysis between the course of disease and the values determined in the patients

Correlative item	n	Regression equation	r	t _r	P
Course with P-NO	77	Y = 368.6527+18.8688X	0.7162	8.8874	<0.01
Course with P-LPO	77	Y = 11.8836+0.2212X	0.6958	8.3888	<0.01
Course with E-LPO	77	Y = 31.5301+0.8807X	0.6369	7.1553	<0.01
Course with P-VC	77	Y = 53.7124-1.1505X	0.6495	7.3982	<0.01
Course with P-VE	77	Y = 23.3849-0.4532X	0.5572	5.8114	<0.01
Course with P-β-CAR	77	Y = 1.7887-0.0486X	0.7428	9.6093	<0.01
Course with E-SOD	77	Y = 2075.87-29.3446X	0.6239	6.9146	<0.01
Course with E-CAT	77	Y = 282.0238-6.7284X	0.7227	9.0545	<0.01
Course with E-GSH-Px	77	Y = 28.6710-0.7006X	0.7233	9.0702	<0.01

Stepwise regression and correlation analysis for the course of disease and the above-mentioned values determined in the patients Supposing the course of disease in the patients to be y, the determination values of P-NO, P-VC, P-VE, P-β-CAR, E-SOD, E-CAT, E-GSH-Px, P-LPO and E-LPO in the patients to be X₁, X₂, X₃, X₄, X₅, X₆, X₇, X₈ and X₉ respectively, after stepwise regression and correlation, the stepwise regression equation was $y = -0.2706 + 0.0203x_1 + 0.6844x_3 - 11.3731x_4$, $r = 0.7902$, $F = 40.4440$, $P < 0.01$. The equation suggested that the correlation of the course of disease was the closest with the values determined for P-NO, P-VE and P-β-CAR.

DISCUSSION

The metabolic status of nitric oxide and functional status between oxidation and antioxidation systems in human body are in close relationship with health^[6-10,12-73]. If the metabolism of nitric oxide is abnormal and the dynamic balance between oxidation and antioxidation is disturbed, free radicals (FRs) concentration will unusually increase and a series of FRs chain reactions will pathologically aggravate in human body. This status can speed senility of human cells, and induce many diseases^[6-10,12-73]. Vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) are the most important antioxidants in human body, and they play an important role in scavenging superoxide anions (O[•]₂), hydroxyl radical (•OH), hydroperoxyl radical (HO[•]₂), lipid FRs, lipoxyl FRs, alkyl FRs, alkoxy FRs, singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂) and others, thereby protecting biological membranes against oxidation, peroxidation and lipoperoxidation^[6,9,10,44-54, 56-69] injury. And they can promote synthesis and stabilization of immunoglobulin in human body and obstruct formation of carcinogens such as nitrosamine^[6,9,10,44-54,56-69]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are the most important specific antioxidases in human body, SOD is able to clean O[•]₂, obstruct and prevent the pathological aggravation of a series of FRs chain reactions induced by O[•]₂, CAT enables toxic active mass H₂O₂ to degrade into non-toxic O₂ and H₂O, GSH-Px may decompose toxic active mass LPO^[6,13-15,29-39,41,42,46-54,56-69]. LPO and its metabolic products such as malondialdehyde (MDA), conjugated

diene (CD) and others are important poisonous residual products that enable biological membranes to be injured by lipoperoxidation. Marked increase in LPO level in human body can strongly attack DNA, proteins, enzymes, biological membranes and so on, which leads to the lipoperoxidation injury of the biological membranes, etc^[5-8,39-43,48-53,62-71]. Nitric oxide (NO) is a neurotransmitter and endothelium-derived factor that reduces tone of vascular smooth muscle, and disorder of NO metabolism can induce many diseases^[6,7,16-28,46-49,55,62-66].

In this study the results that the average values of P-NO, P-LPO and E-LPO in the patient group were significantly higher than those in the control group ($P < 0.01$), and that the average values of P-VC, P-VE, P-β-CAR, E-SOD, E-CAT and E-GSH-Px in the patient group were significantly lower than those of the control group ($P < 0.01$) showed that there was a severe disorder of the NO metabolism and imbalance between oxidation and antioxidation, and there was the pathological aggravation of the oxidation, peroxidation, lipoperoxidation reactions in the bodies of the patients with chronic cholecystitis and gallstones. The causes probably were as follows. The cytokines, particularly interleukin -1 (IL-1), which were released out by inflammatory cells such as phagocytes namely lymphocytes, neutrophilic granulocytes, macrophages in the cholecystic inflammatory reaction, can activate inducible nitric oxide synthase (iNOS). The iNOS enables NO to be produced excessively in the body of patients, thereby resulting in a significant increase in the P-NO value in the patients^[6,7,16-28,46-49, 55,62-66]. Excessive NO was diffused into near by tissues and cells, thus further leading to injury of the tissues and cells^[6,7,16-28,46-49,55,62-66]. The excess NO can combine with iron ions in heme group, with activated guanylate cyclase and lipoperoxidation reaction. The excess NO also inactivated antioxidases such as SOD, CAT, GSH-Px by means of the reaction of NO and hydrosulfide group (-SH) in the enzymes, which further resulted in marked decrease in SOD, CAT, GSH-Px activities and further injured cells and biological membranes. Excessive NO in the body was able to be speedily oxidated into nitrogen dioxide (NO₂). Both NO and NO₂ themselves are extremely active FRs, NO₂ was still able to react with the organic molecules in cystic bile, and activate the neutrocytes and phagocytes in the cholecystic focus, thereby releasing out a vast amount of O[•]₂, •OH, HO[•]₂, and H₂O₂ etc. Meanwhile, the phagocytes such as polymorphonuclear leukocyte were speedily activated, and a large number of O[•]₂, •OH, HO[•]₂ etc were released out, and continuously got into the blood stream in the patients, thereby inducing the pathological aggravation of a series of FRs chain reactions^[6,7,16-28,46-49,55,62-66].

It must be stressed that excessive NO was capable of reacting speedily with O[•]₂, thereby forming another kind of free radical, i.e. superoxide nitroso free radical (ONOO⁻) which possessed still more strong oxidative

properties. ONOO⁻ can further attack and injure the various cells in the body, and deactivate the antioxidants such as SOD, CAT and GSH-Px. Excess NO and NO₂ in human body injured DNA by way of the deamination of the base and the chain scission^[6,7,46-49,55,62-66]. So, on one hand the P-NO level in the body of patients was significantly increased, and on the other hand the body had no choice but to put to good use a great quantity of antioxidants and antioxidants in the body so as to catch and clear these excess O[•]₂, •OH, HO₂• and others^[6-10,12-73], which resulted in significant decrease of the levels of P-VC, P-VE, P-β-CAR and the activities of E-SOD, E-CAT, E-GSH-Px in the patients. Besides the gallbladder calculi such as bilirubin, cholesterol and other organic substances themselves also produce a large number of FRs^[2-5,29,34,35,70-78].

NO₂ is a very active catalyst, and NO₂ can aggravate lipoperoxidation of the polyunsaturated fatty acids (PUFAs) through hydrogen-extractive process. The excess NO, NO₂, O[•]₂, •OH, HO[•]₂ also can attack upon directly PUFAs, aggravate significantly the lipoperoxidation, thereby resulting in a large number of PUFAs which get lipoperoxidated, and subsequently form LPO. With the addition of the significant reduction in the synthesis or regeneration of GSH-Px decomposing LPO, and the marked loss of the GSH-Px activity, it goes without saying that finally this status resulted in significant increase of P-LPO and E-LPO levels^[6,7,46-49,51,52,55,62-66].

In general most anti-oxidative vitamins such as VC, VE, β-CAR, etc, must be acquired from dietary sources because they cannot be synthesized in the body. It is generally recognized that the chronic cholecystitis patients have poor appetite because their diets are controlled, and digestion of VC, specially digestion of fat-soluble vitamins such as VE and β-CAR, markedly reduced. And the anti-oxidative vitamin-poor diets cannot provide sufficient free radical scavengers to keep the balance between oxidation and antioxidation. For this reason, the values of P-VC, P-VE, P-β-CAR in the bodies of the patients were further significantly decreased^[6,9,10,47-54,56-68].

In this study the average values of P-NO, P-LPO and E-LPO in the postoperative patients were significantly decreased, the average values of P-VC, E-SOD, E-CAT, E-GSH-Px were significantly increased, but there was no significant difference in the average values of P-VE and P-β-CAR between the preoperative and postoperative patients. The findings showed that the series of FRs chain reactions in the body of patients were marked lysis, and the dynamic balance between oxidation and antioxidation, obtained resumption to a very marked degree because of the elimination of inflammatory focus after operation. However, before the compensation of common bile was established the absorption of fats and lipids was still limited, thus the absorption of fat-soluble VE and β-CAR obviously reduced^[6,9,10,47-54,56-68]. Therefore, the normal levels of P-VE, P-β-CAR in the patients were

difficult to be resumed shortly after operation.

In this study there was the linear correlation between the course of disease and the above determined values, specially the stepwise correlation with the course with P-NO, P-VE, P-β-CAR values was the closest. This status suggested that in chronic cholecystitis, a large amount of NO produced by iNOS induced and activated by the long-time infection and stimulation of the calculi in gallbladder provoked the pathological aggravation of a series of FRs chain reactions^[2-5,16-29,34,35,46-54,56-78]. As a result, the patients with chronic cholecystitis over a long time were in the state of serious imbalance between oxidation and antioxidation as well as injuries induced by oxidation, peroxidation and lipoperoxidation^[2-5,29,34,35,70-78]. The findings also showed that the metabolic status of nitric oxide and the changes in vitamin E and β-carotene levels in the body played an important part in chronic cholecystitis. Therefore, the above values, particularly the dynamic determination of P-NO, P-VE and P-β-CAR values, to a great degree, contribute to wards monitoring the condition and course in patients with chronic cholecystitis.

We think that in treating preoperative and postoperative patients with chronic cholecystitis with suitable dosage of antioxidants such as vitamin C, vitamin E, β-carotene, ginkgo leaf agents, tea-polyphenol daily to the patients may alleviate the injuries induced by oxidation, peroxidation and lipoperoxidation.

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A comparison between intravenous and peritoneal route on liver targeted uptake and expression of plasmid delivered by Glyco-poly-L-lysine

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Abstract

AIM To compare the effects of intravenous route and peritoneal route on liver targeted uptake and expression of plasmid delivered by galactose-terminal glyco-poly-L-lysine (G-PLL). **METHODS** The plasmid pTM/MMP-1 which could be expressed in eukaryotic cells was bound to G-PLL, and was then transferred into Wistar rats by intravenous and intraperitoneal injection. The expression and distribution of the plasmid were observed at different time periods by *in situ* hybridization and immunohistochemistry. **RESULTS** The plasmid could be expressed significantly within 24 h after being transferred *in vivo* by both intravenous and intraperitoneal routes. One week later the expression began to decrease, and could still be observed three weeks later. Although both the intravenous and intraperitoneal route could target-specifically deliver the plasmid to the liver, the effect of the former was better as compared to that of the latter.

CONCLUSION Intravenous route is better for liver targeted uptake and expression of G-PLL-bound plasmids than the peritoneal route.

INTRODUCTION

The efficient transference and the high expression of exogenous genes in specific cells or tissues are critical steps

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for both *in vitro* and *in vivo* gene therapy^[1-12]. Gene transference mediated by receptors is carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, and then the foreign gene can be delivered into the cells by phagocytosis^[13,14]. There also exist some specific receptors on the surface of hepatocytes such as asialoglycoprotein receptors (ASGP-R)^[15-19], which facilitate the researchers to deliver exogenous genes into hepatocytes specifically using the ligand-receptor interaction. Galactose-terminal glyco-poly-L-lysine (G-PLL) contains the saccharide group of galactosan that can be specifically ligated to the asialoglycoprotein receptor (ASGP-R) on the surface of hepatocytes. At the same time, the cationic poly-L-lysine can bind to nucleotides with high affinity, so it can serve as a good carrier to deliver exogenous DNA to liver specifically and steadily^[20-24].

Both peripheral veins and abdominal cavity can be used as the delivery route to target drug or nucleotide to liver^[25-28], but the comparison of their effects on the targeted liver uptake has seldom been reported.

Using rats as the experimental animals, we compared *in vivo* the difference in distribution and expression of the plasmid given through intravenous or intraperitoneal route.

MATERIALS AND METHODS

Preparation of the carriers

The original plasmid of rat interstitial collagenase was kindly provided by Prof. John J Jeffrey^[29], and we reconstructed it with the plasmid of pTarget (TM) (Promega Co., Madison, MI, USA), which could be expressed in eukaryotic cells. We also inserted a segment of nucleotides (GAC TAC AAG GAC GAC GAT GAT AAG) before the terminator codon (TAA) of the rat interstitial collagenase. The 'Flag Domain' peptide (DYKDDDDK) encoded by the segment of nucleotide above, which was usually called 'Tag', could be fused in the rat interstitial collagenase^[30] and could be specifically recognized by an M2 monoclonal antibody (Kodak, New Haven, CT, USA). This recombinant plasmid was named pTM/MMP-1. The plasmid of pTM/MMP-1 was extracted and purified using QIAGEN-Tip 500 kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. The plasmid was mixed with different amounts of galactose-terminal glyco-poly-L-lysine (kindly provided by Dr. Shou-Ming Wen of Air-Force General

Hospital of PLA, China. The mean molecular weight of this GPLL was 8500 and the ratio of galactose to poly-L-lysine was 15:28. The optimal proportion of the plasmid pTM/MMP-1 binding to galactose-terminal glyco-poly-L-lysine was determined by electrophoresis in 1% agarose gel.

Animal experiments

Eighteen male Wistar rats, with body weight of 130-150 g, were randomly divided into three groups of six rats each. Poly-L-lysine intravenous (PI) group: 50 µg plasmid pTM/MMP-1 bound to galactose-terminal glyco-poly-L-lysine was administered through cauda vein; poly-L-lysine intra-peritoneal (PP) group: each rat was given the same amount of pTM/MMP-1 bound to galactose-terminal glyco-poly-L-lysine intraperitoneally; normal group: control animals. Twenty-four hours, 48 h, 72 h, 1 wk, 2 wk, 3 wk after the administration of the plasmid, one rat from each group was randomly selected and anaesthetized with 2% pentobarbital sodium intraperitoneally. Then 1 mL blood was obtained by cardiac puncture for the assay of alanine transaminase (ALT), aspartic transaminase (AST), and creatinine (Cr) to observe the functions of important organs. After perfusion of the whole body with 20 mL phosphate-buffered saline and 40 mL 4% paraformaldehyde through ventricular injection, the tissues of liver, spleen, lungs, and kidneys were collected and fixed in 4% paraformaldehyde, encapsulated in paraffin and cut into sections 4-µm thick. This procedure above was approved by the Laboratory Animal Committee of Shanghai Medical University.

Immunohistochemistry

Immunohistochemistry was performed according to the literature^[31,32]. The first antibody used was M2 monoclonal antibody which was specific for flag-domain tag (Kodak, New Haven, CT, USA) and the second antibody used was Horse anti-mouse IgG, labeled with biotin (Vecter, Burlingame, CA, USA). After the treatment with avidin and biotin (ABC kit, Vecter, Burlingame, CA, USA), color development was followed using dimethylaminoazobenzene (DAB) and counterstained with hematoxylin. Five fields were observed under high power from every immunostained section and the positive signals were counted.

In situ hybridization

The procedure of *in situ* hybridization was also described previously^[2,5,26]. To state briefly, the oligonucleotide probe (5'-TGG TGT GAC TAC AAG GAC GAC GAT GAT AAG-3') was synthesized in Cell Biology Institute of Chinese Academy of Sciences (Shanghai), which could hybridize with the (mRNA) of the flag-domain tag in the plasmid pTM/MMP-1, and the 5' end of the probe was labeled with biotin. After the hybridization of the target mRNA with the probe, the rest of the procedure was similar to that followed in immunohistochemistry excluding

the step of hematoxylin counterstaining.

Other biochemical assays

ALT, AST, and Cr were assayed using the 7170A Automatic Analyzer (HITACHI, Japan) to observe the changes in the important organs' function.

Data analysis

All of the data were analyzed by the software SPSS 7.0 for windows (one-way ANOVA).

RESULTS

Ratio of G-PLL to plasmid

According to the electrophoresis results, we found that 0.3 µg of galactose-terminal glyco-poly-L-lysine could thoroughly bind to 1 µg of the plasmid, which meant that about 72 molecules of G-PLL could carry one molecule of the plasmid pTM/MMP-1 (Figure 1).

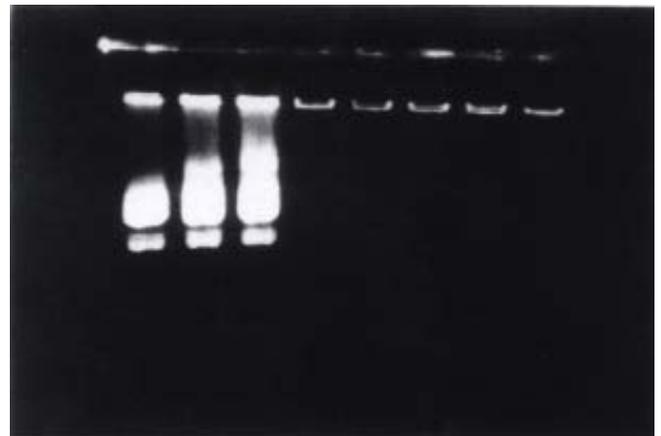


Figure 1 Determination of optimal proportion of G-PLL bound to plasmid by 1% agarose electrophoresis. Lane 1-8 are respectively 0.05 µg, 0.1 µg, 0.2 µg, 0.3 µg, 0.4 µg, 0.5 µg, 1.0 µg, and 1.5 µg G-PLL mixed with 1 µg pTM/MMP-1 plasmid. pTM/MMP-1 1 µg plasmid could only be bound completely by more than 0.4 µg G-PLL.

The changes in the functioning of important organs

Compared with the normal group, there was no obvious elevation of the ALT, AST, and Cr levels in the PI and PP groups.

The distribution and expression of the plasmid in liver, spleen, lung and kidney

The results of the immunohistochemistry and *in situ* hybridization showed that the plasmid binding to G-PLL could be expressed *in vivo*, regardless of the introducing route and the results of immunohistochemistry were more sensitive and stable. In addition, the protein product of the plasmid could be secreted extracellularly (Figures 2 and 3), similar to the expression of interstitial collagenase in the physiological state^[30,33]. We found that both intravenous route and intraperitoneal route could make liver as the major distribution organ of the plasmid bound to G-PLL.

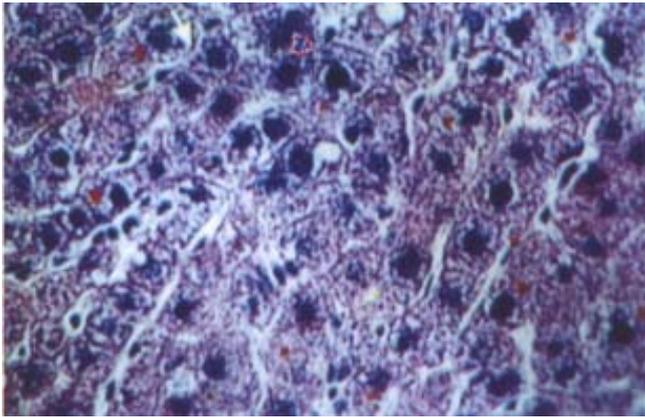


Figure 2 Immunostaining of flag-domain tag in the liver all hours after administration of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) via cauda vein. × 200

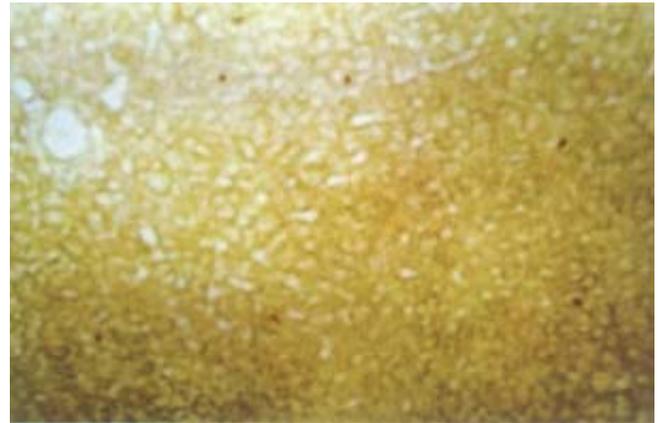


Figure 3 *In situ* hybridization with biotin labeled oligonucleotide probe in the liver 3 wk after the administration of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) via abdominal cavity. × 100

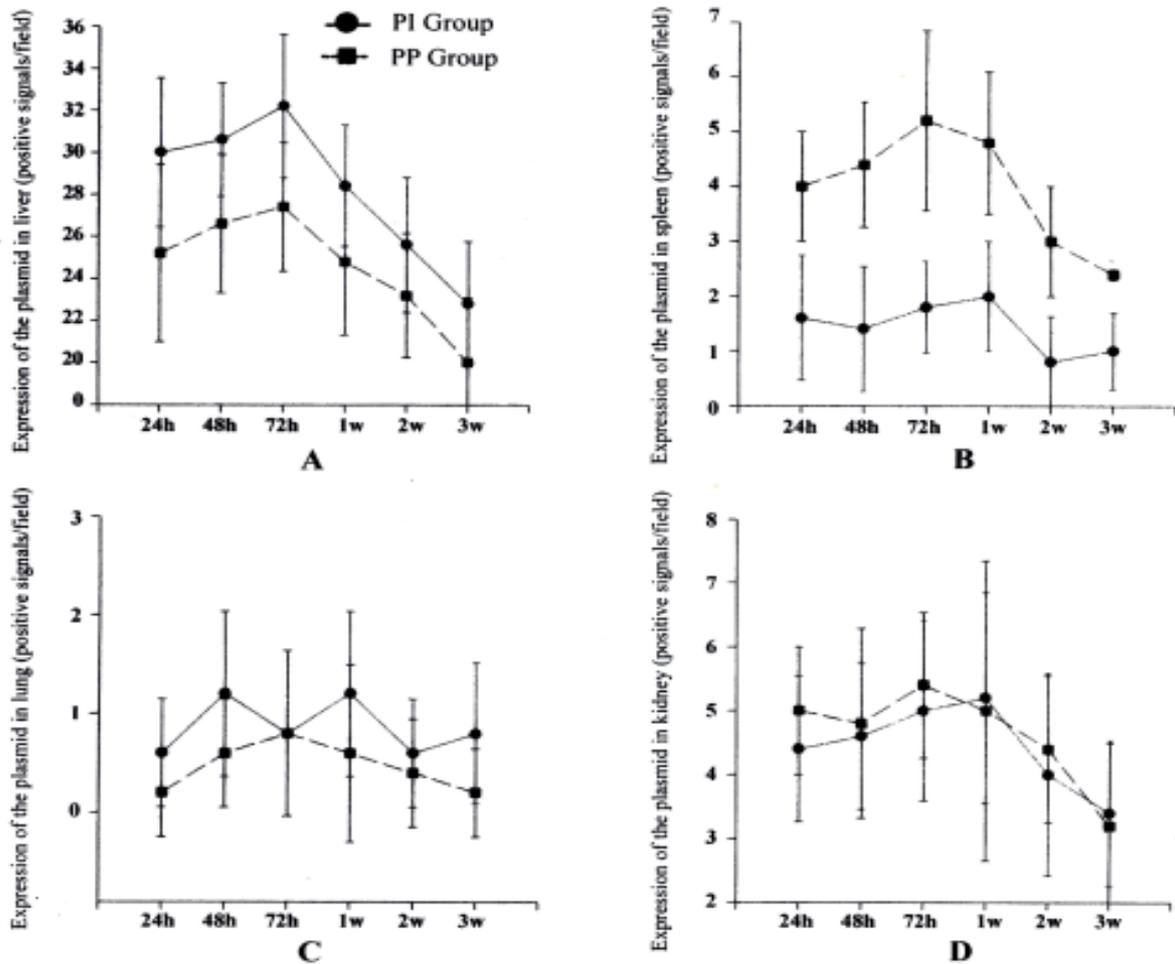


Figure 4 The distribution and expression of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) in different tissues and at different time period and administered intraperitoneally or intravenously. A: liver, B: spleen, C: lung, D: kidney. PI: plasmid bound to G-PLL introduced intravenously, PP: plasmid bound to G-PLL introduced intraperitoneally.

The obvious expression of the plasmid could be observed 24 h after the administration and began to decrease one

week later, although it could still be observed weakly even two or three weeks later. Among the two groups, we

observed that the expression and distribution of the plasmid in the liver of the PI group was significantly higher than that of the PP group. Besides the liver, the plasmid in PI group could also be expressed in lung at a lower level, and almost could not be expressed in spleen and kidney. As for the PP group, most of the distribution and expression was located in the liver and a relatively higher level could be seen in the spleen and kidney, whereas low expression could be observed in the lungs (Figure 4).

DISCUSSION

Regarding the gene therapy of hepatic diseases, efficient delivery of the exogenous genes to the liver and its high expression there could increase its local accumulation and minimize the side-effects on other tissues and organs as well^[34-36].

For the gene therapy of hepatic diseases in the animal experiments, exogenous genes were usually delivered to the liver through the portal vein, bile duct injection, or even by direct liver injection^[37-41]. From the viewpoint of clinical application these methods have limitations concerning its invasive trauma and possible risk. If the gene transference to liver could be accomplished by a peripheral vein or abdominal cavity, the limitations could then be avoided or decreased and it would also be easily accepted by the patients. So we observed the efficacy of liver targeted gene delivery using intravenous and intraperitoneal routes.

Receptor mediated gene transfer was carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, such that the foreign gene could be delivered into the cells by phagocytosis^[24,42]. It has been reported that the ratio of the targeted uptake by the liver delivered by G-PLL could reach up to 70%-90% *in vivo*^[43,44]. In our experiments we found that in addition to its main location in the liver, the plasmid binding to G-PLL could be also expressed in kidneys, spleen, and lungs. It might be due to the existence of AS GP-R in the other extrahepatic tissues^[45,46].

Recently Zhang *et al*^[42] found that intravenous injection was an useful method for delivery to liver by a target carrier. We found that the peripheral vein route was better than abdominal cavity in the targeted delivery of the plasmid bound to G-PLL. We conjectured that although the majority of the DNA/cARRIER complex would reach the liver through portal vein after the absorption by the abundant capillary bed of peritoneum, quite a lot of the complex would possibly be absorbed or degraded by other organs in the abdominal cavity, thus leading to the decreased distribution and expression of the plasmid in the liver. In the same way, it might be one of the reasons that its distribution in the spleen reached to a relatively high level. In this experiment, we also observed whether G-PLL binding to the plasmid would induce some toxicity to the body. We did not find any detrimental effect on the functioning of important organs such as liver, heart, and

kidney, and this further indicated that G-PLL could be used safely *in vivo* as the delivery carriers for drug or nucleotides.

In conclusion, we found that the plasmid bound to GPLL could be delivered to the liver efficiently and safely, and the intravenous transference was better than peritoneal transference for the targeted uptake by the liver when G-PLL was used as a carrier. But whether G-PLL can be used to deliver drugs or nucleotides for the treatment of liver diseases in human beings by intravenous route deserves further investigations.

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Overexpression of p27^{KIP1} induced cell cycle arrest in G₁ phase and subsequent apoptosis in HCC-9204 cell line

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Subject headings p27^{KIP1}; apoptosis; cell cycle; inducible expression system; carcinoma, hepatocellular; liver neoplasms

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Abstract

AIM We have previously reported that inducible over-expression of Bak may prolong cell cycle in G₁ phase and lead to apoptosis in HCC-9204 cells. This study is to investigate whether p27^{KIP1} plays an important role in this process.

METHODS In order to elucidate the exact function of p27^{KIP1} in this process, a zinc inducible p27^{KIP1} stable transfectant and transient p27^{KIP1}-GFP fusion transfectant were constructed. The effects of inducible p27^{KIP1} on cell growth, cell cycle arrest and apoptosis were examined in the mock, control pMD vector, and pMD-KIP1 transfected HCC-9204 cells.

RESULTS This p27^{KIP1}-GFP transfectant may transiently express the fusion gene. The cell growth was reduced by 35% at 48 h of p27^{KIP1} induction with zinc treatment as determined by trypan blue exclusion assay. These differences remained the same after 72 h of p27^{KIP1} expression. p27^{KIP1} caused cell cycle arrest after 24 h of induction, with 40% increase in G₁ population. Prolonged p27^{KIP1} expression in this cell line induced apoptotic cell death reflected by TUNEL assay. Forty-eighth and 72 h of p27^{KIP1} expression showed a characteristic DNA ladder on agarose gelelecrophoresis.

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CONCLUSION Bak may induce cell cycle arrest in G₁ phase through upregulating expression of p27^{KIP1} and subsequently lead to apoptosis in HCC-9204 cells. The p27^{KIP1}-GFP fusion protein can be transiently expressed in HCC-9204 cells. The inducible p27^{KIP1} expressing cell line provides a model to assess p27^{KIP1} function.

INTRODUCTION

Cell cycle regulatory proteins play a critical role in both normal cell growth and tumorigenesis. The cyclin dependent kinases (CDKs) are the major regulators of cell cycle progression, and thus are important candidates for therapeutic tumor suppression^[1]. The CDKs, which are responsible for the phosphorylation of the retinoblastoma protein (pRb) and pRb related proteins, are in turn regulated by changes in cyclin levels, phosphorylation and the presence of cyclin kinase inhibitors (CKIs)^[2-3]. Two classes of CKIs are present within mammalian cells. One class, the INK4 (inhibitors of CDK4) family, is a group of ankyrin repeat protein whose members p16^{INK4A}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} are specific inhibitors of cyclin D1/CDK4 or CDK6 complexes^[4-6]. The second class of CKIs, the p21 family, consists of p21^{CIP/WAF1}, p27^{KIP1} and p57^{KIP2} which are general inhibitors of the G₁/S CDKs^[7-9]. Homology between the family members is limited to a conserved amino-terminal 60-residue domain responsible for kinase binding and inhibition^[10].

p27^{KIP1} regulates cell cycle progression by interacting with, and thereby inhibiting, various cyclin-CDK complexes. Physiologically, p27^{KIP1} is believed to act primarily to regulate progression of cells from late G₁ into S through its interaction with cyclin E-CDK2 complexes^[11]. p27^{KIP1} has been implicated as a mediator of growth arrest due to TGF-β, cAMP, and other extracellular factors^[12]. Moreover, elevated expression of p27^{KIP1} leads to G₁ arrest in many cell types and promotes neuronal differentiation in mouse neuroblastoma cells, while inhibition of p27^{KIP1} expression through use of antisense technology prevents G₁ arrest and/or suppresses entry of fibroblasts into a state of quiescence in response to mitogen depletion^[13-14]. The phenotype of mice null for the p27^{KIP1} gene includes increased body size, female sterility and a high incidence

of spontaneous pituitary tumors^[15].

We have previously shown that overexpression of Bak might prolong cell cycle in G₁ phase though this new property of Bak had never been reported before. We provided a hypothesis that Bak's translocated expression from the cytoplasm to the nuclei of HCC-9204 cells may trigger the expression of certain cell cycle regulators such as cyclins, CDKs, or CDK inhibitors through DNA-protein or protein-protein interaction. Since p27^{KIP1} is one of major cyclin-CDK regulators in G₁ phase, we currently executed an investigation of whether overexpression of Bak in HCC-9204 cells upregulates the expression of p27^{KIP1} and whether p27^{KIP1} itself may induce cell cycle arrest in G₁ phase and even apoptosis in HCC-9204 cells.

MATERIALS AND METHODS

Cell line and cell culture

HCC-9204 cell line derived from a human hepatocellular carcinoma and established in our laboratory was cultured in Eagle's medium containing phenol red, with 50 mL/L fetal bovine serum (FBS) (HyClone Laboratories, USA) in a humidified atmosphere of 95% air, 5% CO₂ at 37°C^[16]. All media were supplemented with 2 mmol/L L-glutamine, 100 mg/L penicillin and 100 IU/L streptomycin. Culture medium and supplements were obtained from GIBCO BRL. Of the HCC-9204 cell line, p53 was mutated. Medium was changed every 3 days. Cells were removed from culture flasks for passage by washing once with Hank's balanced salt solution, followed by a 5 min incubation with 0.5 mmol/L EDTA and 0.5 g/L trypsin at pH 7.4, RT.

RT-PCR analysis

Total RNA was extracted from HCC-9204 cells using an RNA extraction reagent, TRIzol (Life Technologies, USA), according to standard acid-guanidinium-phenol-chloroform method^[17]. About five µg of total RNA were reverse transcribed at 42°C for 60 min in a total 20 µL reaction volume using a first-strand cDNA synthesis kit (Boehringer Mannheim, Germany). cDNA was incubated at 95°C for 5 min to inactivate the reverse transcriptase, and was served as template DNA for 32 rounds of amplification using the GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, CA, USA). PCR was performed in a standard 50 µL reaction mixture consisting of 10 mmol/L Tris-HCl, 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride (pH 8.3), 0.2 mmol/L dNTPs, 50 pmol of each sense and antisense primer and 2.5 U of Taq DNA polymerase (MBI, Canada). Amplification was performed for 30 s at 94°C, 45 s at 58°C and 45 s at 72°C after heat-start for 5 min. Finally, an additional extension step was carried out for 7 min. As negative control, the DNA template was omitted in the reaction. The amplification products were separated on 12 g/L agarose gels and visualized by ethidium bromide staining. PCR primers for p27^{KIP1} were as follows: forward primer, 5'-ggggtaccatgtcaaactgctgag-3'; reverse

primer, 5'-gcgtcgacacgtttgacgtcttc-3': according to the p27^{KIP1} gene structure in Genebank. PCR product of 597 bp was obtained. Kpn-I and Sal-I sites were designed in the 5' and 3' end of cDNA encoding p27^{KIP1} and the terminal code for p27^{KIP1} was mutated so as to construct a fusion protein.

DNA sequencing

The PCR product was purified through Wizard Plus Minipreps DNA Purification System (Promega, USA). The pGEM-T Easy Vector Systems (Promega, USA) was applied for the cloning of the PCR product. Then the recombinant plasmid DNA was transformed into *E. coli* and was sequenced by dideoxy-mediated chain-termination method, using ABI PRISM-TM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA). This kit was developed specifically for the preparation of samples for sequence analysis on the ABI PRISM 377 DNA Sequencer. Cycle sequencing was performed on the GeneAmp PCR systems 2400 (Perkin Elmer, USA) according to the instructions.

Plasmid construction

Plasmids were constructed using standard molecular biology techniques^[18]. In order to construct a hybrid DNA encoding a chimerical p27^{KIP1}-GFP fusion protein, pGEM-T Easy KIP1 was digested with Kpn-I and Sal-I to yield a 0.6 kb fragment. The resulting fragment then ligated to Kpn-I and Sal-I digested pGREEN LANTERN-1 (Clontech, USA) containing the humanized T_{hr}⁶⁵ GFP cDNA under the control of CMV enhancer/promoter and the SV40 polyadenylation signal. Correct reading framework of the fusion gene was confirmed by DNA sequencing. Human p27^{KIP1} (pBluescript SK-p27) was a gift from Dr. Polyak^[19-20]. PCR primers for p27^{KIP1} were as follows: forward primer, 5'-ggggtaccatgtcaaactgctgag-3'; reverse primer, T7 primer. PCR product of 1.0 kb was obtained and subsequently ligated into pGEM-T Easy Vector System. To construct pMD-KIP1, pGEM-T Easy KIP1 was digested with EcoR-I and Xho-I to yield a 1.0 kb fragment. The resulting fragment then ligated to EcoR-I and Xho-I digested pMD-neo. Plasmids for transfection were purified using a kit (Quiagen Inc., USA).

Transient and stable transfection

pKIP1-GFP and pMD-KIP1 were used for transient and stable transfections respectively. Clonfectin (Clontech, USA) was used to transfect plasmids into HCC-9204 cells. According to the instructions, Clonfectin transfection reagent was an effective liposome transfection reagent for many mammalian cell types with high transfection efficiencies and required only 1-4 hour incubation for optimal results, especially effective transfections in serum-containing media. Green fluorescence was detected 4, 24 and 48 h after transfection. For stable transfection, the transfected HCC-9204 cells were grown for 2 weeks in a

medium containing 0.5 g/L G418 (Life Technologies, Inc., USA), after which the G418 concentration was reduced to 0.2 g/L. Stable cell line of HCC-9204 transfected with pMD-KIP1 vector was treated by continuous exposure to 100 μ mol/L of ZnSO₄.

Cell viability

Cells were seeded at 10⁵ per dish. HCC-9204 cells viability was determined by Nikon Eclipse TE200 inverted microscopic examination of cells stained by 1 g/L trypan blue (trypan blue exclusion), counting cells on a hemocytometer at the time point indicated^[21].

Apoptosis analyzed by TUNEL assay

Cells were harvested for TUNEL staining. The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein isothiocyanate (FITC)-12-dUTP into DNA by using terminal deoxynucleotidyltransferase (TdT)^[22]. Briefly, a kit from Boehringer Mannheim (In Situ Cell Death Detection Kit, FITC) was used. After a 30 min (RT) incubation with 30 g/L BSA, 200 mL/L normal bovine serum in PBS, pH 7.4, slides were covered with the TUNEL mix (calf thymus TdT, FITC-12-dUTP and cobalt chloride in 1 \times reaction buffer) for 1.2 h at 37 $^{\circ}$ C. The morphologic features were visualized by fluorescence microscopy. Routine HE staining was also conducted. Negative control was performed by omitting TdT. We used paraffin-embedded sections of HCC as a positive control^[23].

DNA laddering

Laddering of DNA in extracts from untransfected and transfected cells was carried out. DNA was extracted from 5 \times 10⁶ cells 24 h post-induction. DNA fragmentation was assayed as described previously^[24]. Briefly, 24 h following addition of 100 μ mol/L ZnSO₄, 5 \times 10⁶ cells were lysed in buffer containing 5 mmol/L Tris (pH 8.0), 20 mmol/L EDTA and 5 g/L Triton X-100 on ice for 30 min. High molecular weight DNA was removed by centrifugation at 14 000 r/min for 15 min at 4 $^{\circ}$ C, and the supernatant was sequentially extracted with phenol:chloroform, and chloroform. The low molecular weight DNA was recovered by ethanol precipitation, resuspended in 30 μ L of TE buffer, and treated with RNase A for 3 h at 37 $^{\circ}$ C prior to electrophoresis on 12 g/L agarose gels.

Western blot analysis of transgene expression

Monolayers were rinsed with PBS and lysed with SDS-PAGE loading buffer (50 mmol/L Tris HCl pH 6.8, 100 mmol/L dithiothreitol, 20 g/L SDS). Mock and pMD-neo transfected cells served as a control. Samples were analyzed by SDS-PAGE and transferred into Hybond-C super membranes (Amersham, UK). The membranes were blocked with 50 mL/L skim milk, 1 g/L Tween-20 and then probed with primary antibody overnight according to manufacturer's instructions, washed in PBS, 2 g/L Tween-20 and then incubated with the appropriate horseradish-

peroxidase (HRP) conjugated secondary antibody. After washing, the membranes were developed by DAB detection reagents according to manufacturer's guide (Dako Co. USA). p27^{KIP1}, rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) was detected followed by HRP-conjugated anti-rabbit IgG (Fc) was used. The level of β -actin was used as a control for equal loading of protein.

Immunohistochemistry analysis

HCC-9204 cells were harvested at different times of induction with ZnSO₄. Cell preparations were fixed with 700 mL/L ethanol for two hours, washed in PBS, and incubated with p27^{KIP1} antibody diluted 1 \times 100 in PBS containing 10 mL/L bovine serum albumin (BSA, Sigma, USA). Immunostaining was performed using LSAB1 kit (Dako, Peroxidase, USA), according to the instructions of the manufacturer.

Fluorescence microscopy and laser confocal microscope

Nikon Eclipse TE200 inverted fluorescence microscope was used to observe and photograph. Images were collected on a laser confocal microscope, model MRC-1024 (BioRad, UK) with a Plan-Neofluar 40 \times 1.3 NA Apochromat objective (Zeiss, Germany). The 488 nm line of a krypton/argon laser was used for fluorescence excitation of FITC. Images were processed using Adobe Photoshop 5.0 software (Adobe Systems Inc., USA)

RESULTS

RT-PCR for p27^{KIP1} in Bak transfected HCC 9204 cells

Bak transfected HCC-9204 cells were collected 0, 24 and 48 h post-induction as the previous study mentioned and were used to extract total RNA by TRIZOL reagent. The findings as shown in Figure 1 suggested that overexpression of Bak constantly induced expression of CDK inhibitor, p27^{KIP1}, at mRNA level in HCC-9204 cell line.

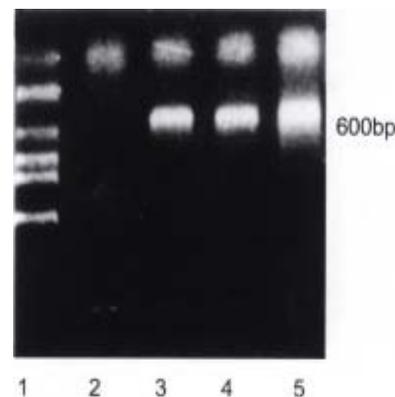


Figure 1 RT-PCR results of human p27^{KIP1} RNA in Bak transfected HCC-9204 cell line. Predicted PCR product size is about 600 bp. lane 1: Molecular weight Marker (Takara, DL 2000); lane 2: Bak (0 h); lane 3: Bak (24 h); lane 4: Bak (48 h)

Sequencing of p27^{KIP1} and p27^{KIP1}-GFP fusion genes

The purified p27^{KIP1}-PCR product was cloned into pGEM T-Easy vector and sequenced. Comparison of the sequences with published data in Genbank indicated only one base pair of disparity while the amino acid residue encoded was the same as what the Genbank indicated (Figure 2). pGEM-T Easy KIP1 was digested with Kpn-I and Sal-I to yield a 0.6 kb fragment. The resulting fragment then ligated to Kpn-I and Sal-I digested pGREEN LANTERN-under the control of CMV enhancer/promoter and the SV40 polyadenylation signal. Correct reading framework of the fusion gene was confirmed by restriction enzyme analysis or DNA sequencing.

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gtcaaacgtgcgagtgctaacgggagccctagcctggagcggatggacgccaggcag 60
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tttcgatttcagaatcacaaacccctagagggcaagtacgagtggaagaggtaggag 240
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zccggcgcaggagagccaggatgtcagcgggagcccccggggcgcctttaattggg 360
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zaccgggttagcggagcaatgcaggaataaaggaagcagacctgcaaccgacgattct 440
tactcaaacaaaagagccaacagaaacagaagaaaatgttttagacgggttccccaat 540
:ggttctgtggagcagacgcccagaagcctggcctcagaagacgtcaaacgt 595

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Figure 2 The nucleotide sequences of p27^{KIP1}. Only one nucleotide is changed from c→t at site No 524, while the sequences of amino acids are the same

Transient expression of p27^{KIP1}-GFP fusion proteins in HCC-9204 cell line

Green fluorescence was visible 8 h after transient transfection with pKIP1-GFP fusion constructs. The p27^{KIP1}-GFP fusion proteins localized primarily to cell nuclei, with some GFP presented in the cytoplasm (Figure 3). However, 24 h post transfection, the green fluorescence disappeared. Some cells exhibited typical active cell death including condensed and rounded cells, cell detachment under Nikon Eclipse TE200 inverted microscope as shown in Figure 4.



Figure 3 Cell slides under Nikon Eclipse TE200 fluorescence microscope using excitation light at either 488 nm to detect GFP (emission filter 522 nm). Distribution of GFP p27^{KIP1} fusion protein expressed in living HCC-9204 cells 8 after 100 μmol/L ZnSO₄ treatment, indicated by an arrow. × 200

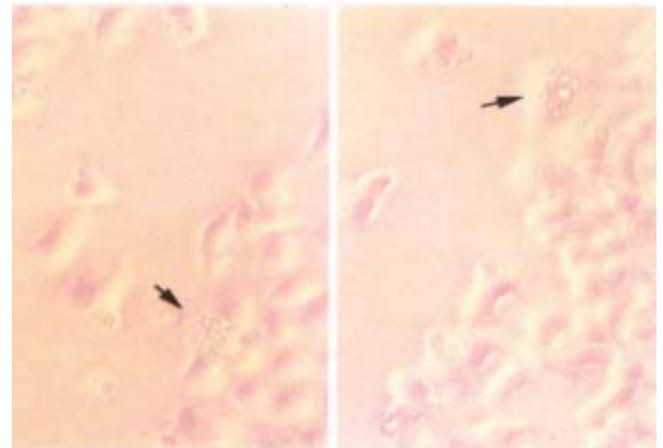


Figure 4 Transient transfected HCC-9204 cells exhibiting typical active cell death such as cell blebbing, condensed and rounded cells as seen under, Nikon Eclipse TE200 phase contrast microscope indicated by an arrow. × 200

pMD vectors direct the inducible expression of p27^{KIP1}

Inducible pMD-KIP1 vectors were constructed to express human cyclin kinase inhibitors, p27^{KIP1}, as shown in Materials and Methods. Transgene expression was under the control of the metallothionein-II (MT-II) promoter and the BGH polyadenylation signal. Previously it was found that MT-II promoter gave higher expression levels in HCC-9204 cells as compared with those obtained with the HCMV promoter. To study the effects of pMD-vector driven expression of p27^{KIP1}, HCC-9204 cells were left untreated (mock), or were transfected with control pMD-vector or pMD-KIP1, at 4, 24 and 48 h, respectively after addition of 100 μmol/L ZnSO₄. Western blot analysis showed a strong signal of Mr 27 000 protein appeared in HCC-9204 24 h post induction. However no band was detected in normal HCC-9204 cells as shown in Figure 5. Immunohistochemistry of p27^{KIP1} as shown in Figure 6 was consistent with Western blot analysis. Nuclear staining signals were observed in p27^{KIP1} stable transfectant 24 h post addition of ZnSO₄.

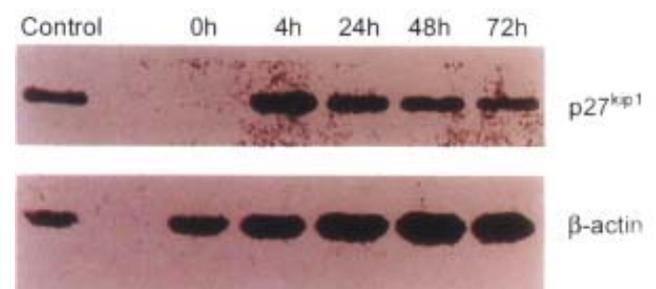


Figure 5 The results of Western blots of lysates from transfected HCC-9204 cells, probed with antibodies against human p27^{KIP1}. Excellent p27^{KIP1} expression was observed in HCC-9204 cells even 4 h after induction with zinc.



Figure 6 p27^{KIP1} detected by DAKO LABC kit in transfected HCC-9204 cells after 24 h of addition of ZnSO₄. p27^{KIP1} dark-brown granules are present in the nuclei of p27^{KIP1} transfected HCC-9204 cells, as indicated by an arrow. × 200

Inhibition of tumor cell growth by exogenous p27^{KIP1}

p27^{KIP1} gave a very pronounced inhibition of growth. HCC-9204 cells viability was determined by microscopic examination of cells stained by 1 g/L trypan blue (trypan blue exclusion) (Figure 7). Time course effect of p27^{KIP1} on HCC-9204 cells growth was observed under Nikon Eclipse TE200 inverted microscopic (Figure 8). Within one day post-induction, a block in cell growth was observed in HCC-9204 cells with a few cells staining positive with Trypan blue, indicating that the exogenous p27^{KIP1} was having a cytostatic effect on growth. More dramatic effects were observed 24 h after addition of zinc. Mock and control pMD vector transfected cells grew until confluent (day 3) and then entered a growth arrest following which significant numbers of trypan blue-staining cells were observed, indicative of cell death, accounting for the decline in the cell number, which was especially apparent in confluent HCC-9204 cells. By 3 days post-induction, cells staining positive with trypan blue-staining cells were observed in the cultures transfected with p27^{KIP1} even though the monolayers were not confluent, and this cell death accounted for the overall reduction in cell number that was observed during the remainder of the experiment. Cell death was greatest in HCC9204 cells transfected with pMD-KIP1.

From Figure 7, we could obviously figure out that the cell growth was reduced by 35% after 48 h of p27^{KIP1} induction with zinc treatment as determined by trypan blue exclusion assay. These differences remained the same at 72 h, and even at 96 h post addition of zinc.

pMD vector expression of p27^{KIP1} alters cell cycle distribution profiles

To see if the p27^{KIP1} had an ability to induce G₁ arrest, cell cycle analysis was performed on transfected HCC-9204 cells. At 24 h post-induction, HCC-9204 cells expressing exogenous p27^{KIP1} showed an accumulation of cells in G₁

compared to mock and control pMD transfected cells, consistent with p27^{KIP1} inhibiting tumor cell growth. (Figure 9)

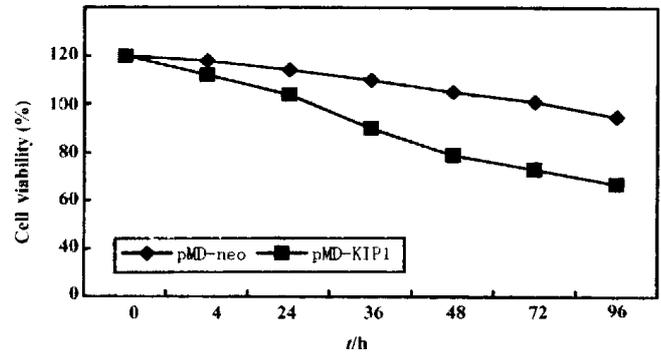


Figure 7 Time course of growth of cells transfected with pMD-KIP1 expressing p27^{KIP1}. HCC-9204 cells were induced by 100 μmol/L ZnSO₄. HCC-9204 cells viability was determined by microscopic examination of cells stained by 1 g/L trypan blue, counting cells on a hemocytometer at the time point indicated. Each treatment was triplicate.

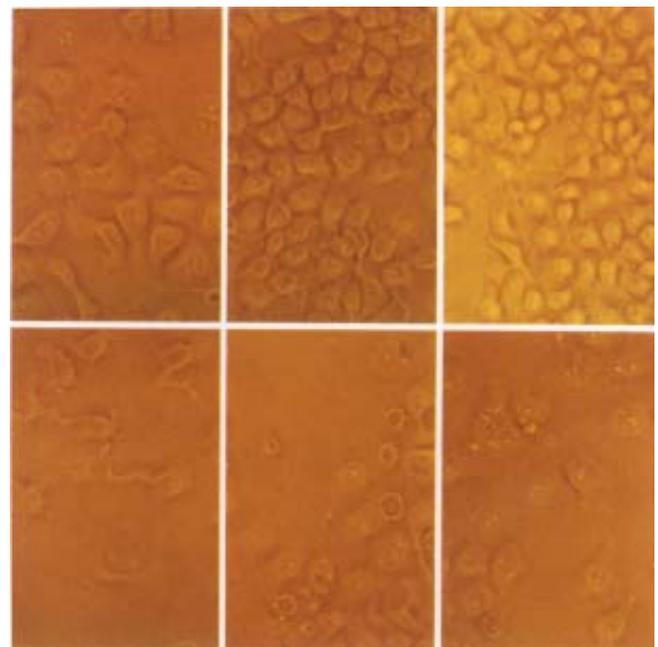


Figure 8 Time course effect of p27^{KIP1} on HCC-9204 cells growth. Cells were seeded at 10⁴+5 per dish. HCC-9204 cells were induced by 100 μmol/L ZnSO₄ at the time indicated. Cells morphology was observed under Nikon Eclipse TE 200 phase-contrast microscopic at 0, 24 and 48 h post addition of zinc. “Upper-row” represents pMD-neo transfected cells; “Lower-row” represents pMD-KIP1 transfected cells. × 200

FACS analysis showed that overexpression of p27^{KIP1} resulted in an increase of G₁ population in HCC-9204 cells from 35.21% to 76.31%. Together, these data indicated that overexpression of p27^{KIP1} alone is sufficient to suppress the proliferation of human tumor cells. We are currently investigating the role of p27^{KIP1} in mediating cellular responses to a variety of external signals and the mechanism of inhibition.

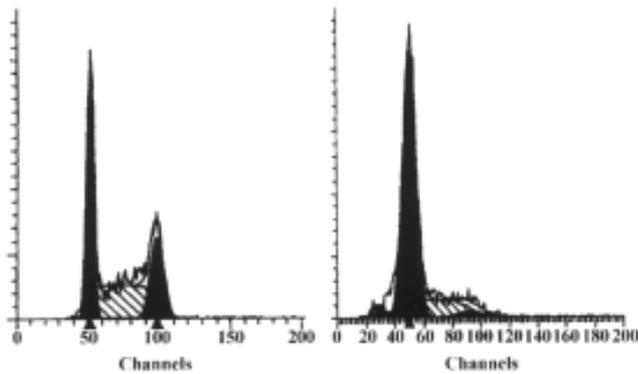


Figure 9 Result of flow cytometric analysis. Cells were stably transfected with p27^{KIP1} and induced by 100 $\mu\text{mol/L}$ ZnSO₄. The percentage of each phase for each group (before or 24 h after addition of zinc) is indicated in each panel.

Induction of apoptosis in cells transfected with vectors expressing p27^{KIP1}

Since by day 3, a significant proportion of HCC-9204 cells transfected with the vectors expressing p27^{KIP1} stained positive with trypan blue, indicative of cell death, we attempted to ascertain whether p27^{KIP1} was inducing apoptosis. To confirm that over-expression of p27^{KIP1} induced cell death was apoptotic, a variety of apoptosis associated detection assays were applied. In stable transfectants after addition of 100 $\mu\text{mol/L}$ ZnSO₄, cell growth was significantly inhibited according to FACS analysis as shown in Figure 9. TUNEL analysis (Figure 10) and DNA laddering (Figure 11) confirmed that part of cells underwent apoptosis 48 h or 72 h after addition of 100 $\mu\text{mol/L}$ ZnSO₄.

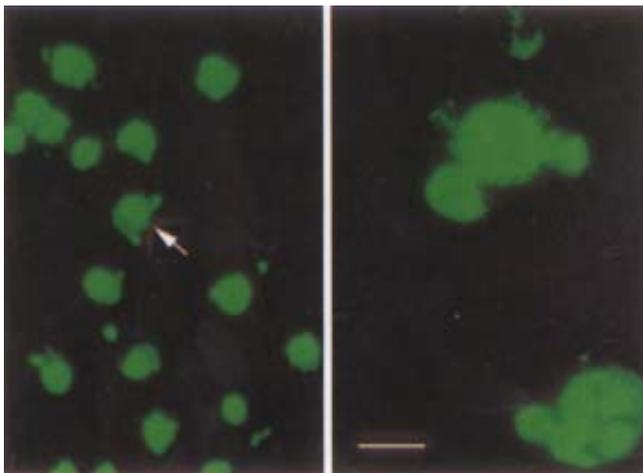


Figure 10 TUNEL assay demonstrating obvious apoptotic changes in HCC-9204/p27^{KIP1} 24 h after addition of zinc under laser confocal microscope as indicated by an arrow. (Bar represents 10 μm)

Taken together, p27^{KIP1} overexpression induced rapid morphological changes in HCC-9204 cells. p27^{KIP1} overexpression inhibited proliferation of HCC-9204 cells and subsequently induced apoptosis.

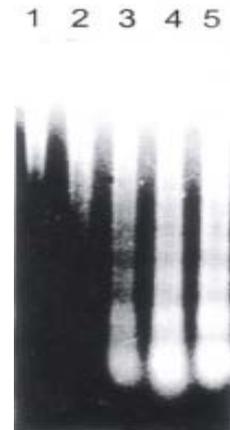


Figure 11 Analysis of DNA ladder formation at 0 (lane 1), 4 h (lane 2), 24 h (lane 3), 48 h (lane 4), 72 h (lane 5) post addition of zinc in HCC-9204 cell line. Each lane was loaded with 10 μg DNA.

DISCUSSION

Since its introduction into cell biological research, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has become a versatile tool for the analysis of protein function and dynamics at the cellular level^[25]. GFP, which consists of 238 amino acids, has been used as a tag for localization of a broad range of proteins in a wide variety of eukaryotic cells^[26]. A mutant of GFP, S65T with an excitation peak of 489 nm and an emission peak of 511 nm, emits four to six times more fluorescence energy compared with wild-type GFP. GFP may be fused generically to a target protein, and the fluorophore of GFP forms spontaneously in the presence of oxygen, thus rendering it an ideal probe for *in vivo* applications. The *in vivo* expression of GFP obviates the fixation and permeabilization of cells for immunofluorescence or the microinjection of labeled proteins^[27].

GFP fusion proteins constitute a major advance in the study of the dynamics of intracellular processes in living cells^[28-32]. A major concern in the application of GFP as a fluorescent tag relates to whether the distribution of GFP fluorescence is identical to that of the protein to which it is fused. We have constructed GFP p27^{KIP1} fusion gene and transfected into HCC-9204 cells. The results indicated that the fusion protein was still expressed in the nuclei of HCC-9204 cells.

Previous studies indicated that cell survival following transfection with pGREEN LANTERN-1 was similar to transfection with pCMV • SPORT- β gal. No trypan blue-stained cells were seen to fluoresce green, and the fluorescence intensity of green fluorescent cells treated with trypan blue remained the same when judged by microscopic observation indicating that fluorescent cells were viable cells^[33]. A comparative study has been performed to determine whether apoptotic cells still can express GFP. A

GFP positive clone was chosen and counterstained with PI. The cells which had a property of apoptosis were stained with PI and no longer expressed GFP. We used PI counter-staining to show that the cells appearing as red fluorescence were not viable and only viable cells had a potential to express GFP. The fluorescence green vanished as soon as the cells become unviable (Figure 12). Therefore, transient expression of KIP1 - GFP fusion protein was disrupted when these cells underwent apoptosis.

The cell cycle progression is controlled by a family of cyclins and their specific catalytic partners, cyclin-dependent kinases (CDKs). D-type cyclins govern the G₁/S transition in association with their proper physiological partners, CDK4 or CDK6, and CDK2, respectively^[34-35]. For cells to complete the G₁/S transition, the activities of both D-type cyclins and cyclin E are essential. CDK inhibitors stop the cell cycle progression by negatively and stoichiometrically regulating cyclin/CDK activities and are the targets of various extracellular growth-modifiers. These inhibitors have similar N-terminal regions which are responsible for the blockade of CDK kinases. They can inhibit any G₁ cyclin/CDK activity. The CDK inhibitors also served as the common mediators of various biological processes including DNA-damages, cell differentiation, cell to cell contact inhibition and cell senescence^[36]. Recent studies indicate that oncogene signals converge to cyclin/CDK and CDK inhibitors^[37]. We have reported that overexpression of Bak may prolong cell cycle in G₁ phase in HCC-9204 cells. We postulated that translocated expression of Bak in this process transferred signals to the cell cycle regulators in the nuclei, and thus induced cell cycle arrest in G₁ phase.

p27^{KIP1}, as a member of CDKIs, is constitutively expressed in many cells, and the level of this protein is elevated in G₀ phase and declines as cells in culture enter the cell cycle^[38-40]. p27^{KIP1} may play an important role in governing the growth factor restriction point by ensuring that CDK activity is suppressed during G₀ and early G₁ phase^[41]. Interestingly, in many types of tumors including breast, colon, prostate and ovarian carcinomas, the expression of p27^{KIP1} gene was down-regulated^[42-43]. From the sequencing analysis, we could figure out that in some types of tumor, p27^{KIP1} gene was null or mutated, while in other types it was still intact. HCC-9204 cells, as a target cell line, has intact p27^{KIP1} which was confirmed by fluorescence *in situ* hybridization (FISH) as shown in Figure 13 and DNA sequencing. Researchers are attempting to discover the mechanism of controlling the expression of p27^{KIP1} and cyclin-CDK activation in these malignant cells.

The correct timing of cyclin-CDK activation is regulated at several levels, including the control of cyclin-encoding gene expression, cyclin stability and cellular localization, as well as CDK subunit phosphorylation and dephosphorylation^[44-46]. Another level of regulation is provided by the CDKIs which consist of two families. The

potential role of p27^{KIP1} in control of the hepatocyte cell cycle is suggested by the implication of mitogens and anti-mitogens known to control p27^{KIP1} levels in the regulation of liver cell proliferation^[47]. The discovery of p27^{KIP1} has facilitated the elucidation of these mechanisms. In addition, in all organs including the liver, p27^{KIP1}, but not p21/mice show an increased cell density without any obvious alteration in liver cells differentiation or structure^[48].

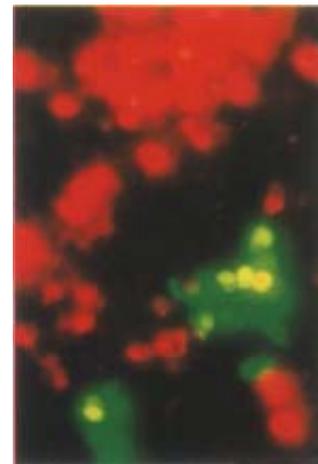


Figure 12 Comparative study of whether apoptotic cells can still express GFP. A GFP positive clone was chosen and counterstained with PI. Apoptotic cells were stained with PI and no longer expressed GFP. HCC-9204 cells were observed under Nikon Eclipse TE200 fluorescent phase-contrast microscopic. $\times 200$



Figure 13 In situ fluorescence hybridization with FITC labelled p27^{KIP1} probe. Two signals were observed in the chromosome under laser confocal microscopy. (Bar represents 5 μm)

Most studies examining the function of p27^{KIP1} had been performed in tissue culture systems using transformed cell lines or embryonic cells. Previous studies suggested that p27^{KIP1} overexpression causes cell cycle arrest in G₁ phase and/or apoptotic death of mammalian cells and supported the potential utility of gene therapeutic approaches aimed at elevating p27^{KIP1} expression for

treatment of human cancers and inhibition of tumorigenicity^[49]. Recent evidences demonstrated that high levels of p27^{KIP1} protein, induced by adenovirus vector, led to growth arrest as well as enhancement of apoptosis in several cell lines from different species and tissues of various origins^[50]. However, as far as we know, no study has been conducted previously to examine the expression of p27^{KIP1} in human hepatocellular carcinoma tissue in relation to apoptosis. We obtained p27^{KIP1} gene fragment in Bak transfected HCC-9204 cells and constructed a transient or stable transfectant. The purpose of the study was to examine the potential role of p27^{KIP1} during Bak overexpression in HCC-9204 cells. The results suggest that overexpression of Bak may induce expression of p27^{KIP1} at least in mRNA level. p27^{KIP1} may exert its effect on G₁ phase elongation induced by Bak and p27^{KIP1} itself may play an important role in the regulation of cell cycle during this process. To our knowledge, this is the first time that a significant correlation between p27^{KIP1} expression and cell cycle arrest and apoptosis has been demonstrated in HCC-9204 cell line.

However, the mechanism of tumor growth suppression appears to include not only p27^{KIP1} but also various other proteins, such as p53 etc. As we know, p53 is mutated in HCC-9204 cell line and is often null in other cell lines^[51-53]. Therefore, how these cell types undergo cell death is of concern since the p53-dependent apoptosis pathway has been abolished. p53 serves as a major G₁ checkpoint regulator of the induction of p21^{CIP1} but not of p27^{KIP1} and p57^{KIP2}, while it induces apoptosis^[54]. The p53-mediated CDK inhibitor p21^{CIP1} does not lead to significant apoptosis, though both p21^{CIP1} and p27^{KIP1} induce G₁ arrest through their potential abilities to inhibit CDK activity^[55-56]. These findings suggest that the inhibition of CDK activity may not be sufficient to induce apoptotic cell death. A possible explanation of these differences between p27^{KIP1} and p53-mediated p21^{CIP1aa} functions is that p27^{KIP1} may have other functions in addition to its notable function as a CDK inhibitor. It can also be speculated that p27^{KIP1}, which has the potential to induce growth arrest and apoptosis, may play a more important role in tumor growth suppression than p53-mediated p21^{CIP1}.

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Bile acid formation in primary human hepatocytes

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Subject headings bile acid formation; cell culture; cholesterol metabolism; cyclosporin; human hepatocytes

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Abstract

AIM To evaluate a culture system for bile acid formation in primary human hepatocytes in comparison with HepG2 cells. **METHODS** Hepatocytes were isolated from normal human liver tissue and were cultured in serum-free William's E medium. The medium was collected and renewed every 24 h. Bile acids and their precursors in media were finally analysed by gas chromatography-mass spectrometry.

RESULTS Cholic acid (CA) and chenodeoxycholic acid (CDCA) conjugated with glycine or taurine accounted for 70% and 25% of total steroids. A third of CDCA was also conjugated with sulphuric acid. Dexamethasone and thyroid hormone alone or in combination did not significantly effect bile acid formation. The addition of cyclosporin A (10 µmol/L) inhibited the synthesis of CA and CDCA by about 13% and 30%, respectively.

CONCLUSION Isolated human hepatocytes in primary culture behave as in the intact liver by converting cholesterol to conjugated CA and CDCA. This is in contrast to cultured HepG2 cells, which release large amounts of bile acid precursors and unconjugated bile acids into the medium.

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INTRODUCTION

The degradation of cholesterol to bile acids is a major way for elimination of cholesterol from the body. Disturbances of bile acid formation may lead to clinical disorders in humans like atherosclerosis, cholesterol gallstone formation and liver diseases. Primary bile acids-cholic acid (CA) and chenodeoxycholic acid (CDCA) are formed from cholesterol in human liver and excreted via the bile as conjugates to the intestine. In the intestine, the bile acids are partly deconjugated and 7 α -dehydroxylated by microbial enzymes to the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA). The bile acids, with the exception of lithocholic acid, are efficiently absorbed from the intestine and return to the liver via the portal vein. In the liver (70-90)% of the bile acids are reextracted and resecreted to the bile. Thus, the main bile acids in human bile are CA, CDCA, and DCA. Small amounts of LCA and ursodeoxycholic acid (UDCA), a metabolite of CDCA are also found.

Two major pathways of bile acid synthesis from cholesterol have been described^[1-5]. The main pathway, also called the neutral pathway, is initiated by a 7 α -hydroxylation, catalyzed by the rate-limiting enzyme cholesterol 7 α -hydroxylase. This hydroxylation is followed by further transformations of the steroid nucleus and oxidative cleavage of the side chain. An alternative pathway, also called the acid pathway, is initiated by a 27-hydroxylation of cholesterol. The quantitative importance of the latter pathway is not yet clear. It may be of importance for the formation of particularly CDCA and as a "back-up" pathway in conditions in which the neutral pathway is repressed for some reasons.

Bile acid formation in humans has been studied by different *in vivo* and *in vitro* techniques. Also cultures of human hepatoblastoma cells (HepG2) have been employed. However, these cells have been found to be defective in CA formation and conjugation by some authors^[6-9]. The cells have also been found to produce large amounts of bile acid precursors.

More recently, techniques have been developed for preparation of primary cultures of normal human hepatocytes. These offer now possibilities to study bile acid synthesis in normal hepatocytes under various experimental conditions.

MATERIALS AND METHODS

Preparation and culture of human hepatocytes

Primary hepatocytes were prepared from normal donor liver tissue. The project was approved by the Ethics Committee at Huddinge University Hospital and by Human Ethics

Committees of the Western Sidney Area Health Service, Australia. Liver sections weighing 100-200 g and with only one cut-surface were used. Hepatocytes were isolated using a 2-step perfusion technique utilizing EGTA and collagenase type IX-S as recently described^[10]. They were seeded on 60-mm dishes at a density of 3.5×10^6 cells per dish. Dishes were precoated with 0.2 mL of matrigel, a laminine-rich extracellular matrix prepared from Engelbreth-Holm-Swarm mouse sarcomas^[11]. Hepatocyte viability was >85% as determined by trypan blue exclusion. The cells were then incubated in serum-free William's E medium (3 mL/dish), supplemented with glutamine (292 mg/L), Na_2SeO_3 (173 $\mu\text{g/L}$), insulin (2 mIU/mL), penicillin G sodium, streptomycin sulphate and gentamycin. In some experiments, triiodothyronine (T3) (0.1 $\mu\text{mol/L}$) and/or dexamethasone (0.1 $\mu\text{mol/L}$) were added. In some other experiments, on d 4, cyclosporin A (CsA), an inhibitor of the sterol 27-hydroxylase^[12,13], was added to the medium (0-10 $\mu\text{mol/L}$). The medium was collected and renewed every 24 h and frozen at -20°C for later analysis of bile acids. At the end of the incubation, the hepatocytes were harvested and stored at 20°C until extraction.

Analytical procedures

For determination of the primary bile acids CA and CDCA formed, deuterium-labeled bile acids were added to the collected culture medium. After alkaline hydrolysis and extraction, the bile acids were analysed by gas chromatography-mass spectrometry as described recently^[10]. For more detailed analysis of neutral and acidic steroids-unconjugated and conjugated with glycine or taurine and/or sulphuric acid-group separation was performed by anion exchange chromatography after addition of isotopically labeled steroids. Deconjugation was performed by an enzymatic method. Sulphate groups were cleaved by solvolysis in acidified tetrahydrofuran. The identification of steroids was performed by gas chromatography-mass spectrometry. Further details are given in a recent paper^[14].

RESULTS AND DISCUSSION

Formation of bile acids

CA and CDCA were the two dominating steroids formed, accounting for as much as 70% and 25% of total, respectively (Figure 1). CA and CDCA are the two bile acids normally synthesized in human liver but the ratio between the production rates of the two bile acids in the hepatocytes was higher (about 3:1) than that of normal subjects (about 2:1). However, the ratio is similar to that obtained in patients with complete biliary drainage^[15]. Small amounts of two other bile acids, 3β -hydroxy-5-cholenoic acid and 3β , 7β -dihydroxy-5 β -cholanoic acid were also detected (about 1% of total of each).

Bile acid formation was lowest on d 1 and 2 and then increased several-fold on d 4 to 6, whereafter it declined slightly (Figure 2). The reason for this is not clear.

However, it has previously been observed that the activity and mRNA levels of cytochrome P450 enzymes involved in drug metabolism show a similar profile in human hepatocytes e.g., a sharp recovery followed by a steady decline (Liddle C *et al*, unpublished observations).

About 3% of the total amount of the isolated steroids consisted of bile acid precursors. Among those, 7α -hydroxy-cholesterol and 7α -hydroxy-4-cholesten-3-one are established intermediates in the neutral pathway and 27-hydroxycholesterol is the first intermediate in the acidic pathway. Since 7α -hydroxycholesterol can also be formed by autooxidation of cholesterol, 7α -hydroxy-4-cholesten-3-one is a better marker for the neutral pathway. The daily release of 7α -hydroxy-4-cholesten-3-one increases in parallel to those of CA and CDCA, whereas the release of 27-hydroxycholesterol decreases. Since the hepatocytes are incubated in a serum-free medium devoid of lipoprotein cholesterol, the decrease in amounts of 27-hydroxycholesterol is consistent with the hypothesis that cholesterol utilized for the acidic pathway is mainly derived from plasma lipoproteins^[2,7].

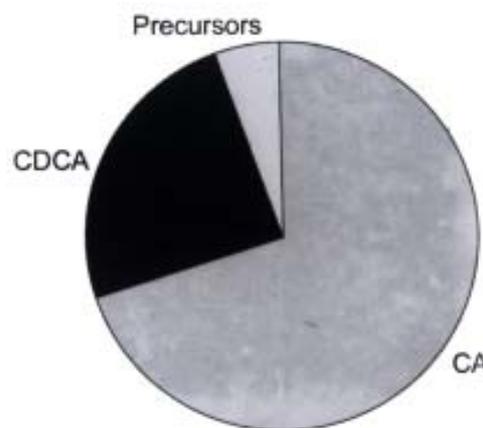


Figure 1 Distribution of cholic acid, chenodeoxycholic acid and precursors formed in primary cultures of human hepatocytes.

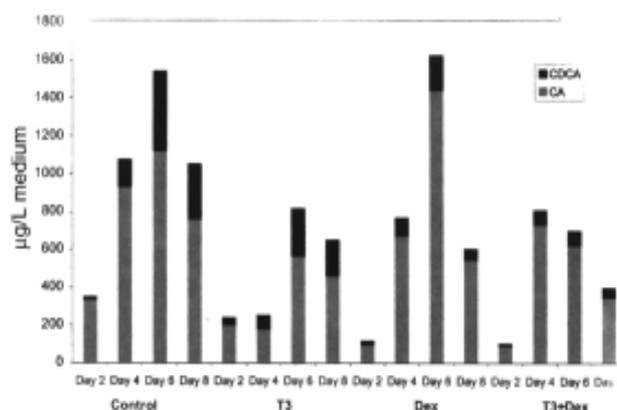


Figure 2 Formation of cholic acid and chenodeoxycholic acid in primary cultures of human hepatocytes during d 2, 4, 6 and 8. Effects of adding T3 and dexamethasone (DEX) alone and in combination.

As can be seen in Figure 3, HepG2 cells release large amounts of bile acid precursors into the incubation medium. It cannot be excluded that the bile acid synthetic pathways also may be abnormal in cultured HepG2 cells.

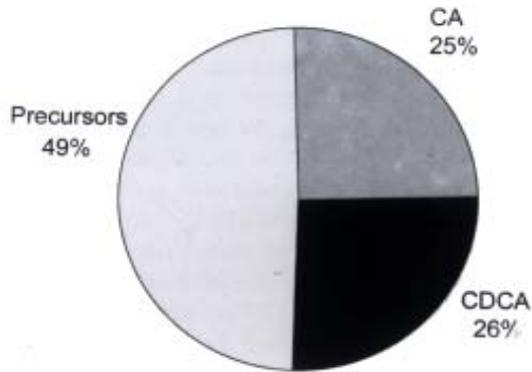


Figure 3 Distribution of cholic acid, chenodeoxycholic acid and precursors formed in cultures of HepG2 cells (data from ref. 7).

Conjugation

CA and CDCA were conjugated with glycine or taurine to more than 99.5% (Figure 4). About one-third of CDCA was also conjugated with sulphuric acid (Figure 5). 3β-Hydroxy-5-cholenoic acid and 3β, 7α-dihydroxy-5β-cholanoic acid were also sulphated. 27-Hydroxycholesterol and other oxysterols were partly sulphated.

In contrast to primary human hepatocytes, HepG2 cells release large amounts of unconjugated bile acids into the medium (Figure 6).

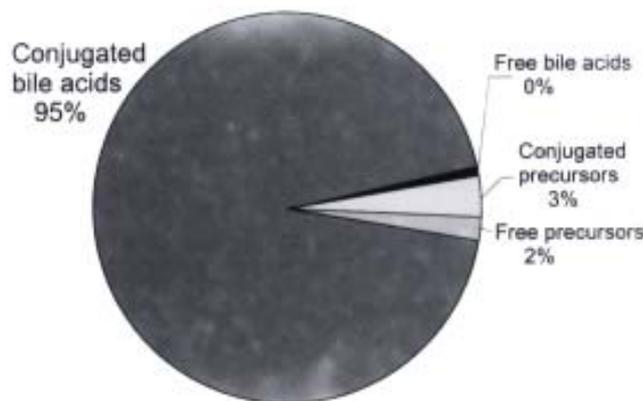


Figure 4 Conjugation of bile acids and precursors formed in primary cultures of human hepatocytes.

Effect of cyclosporin A

Cyclosporin A (CsA) is a potent inhibitor of the sterol 27-hydroxylase activity, especially towards nonpolar substrates like cholesterol^[12,13]. When added to the culture medium, 1 μmol/L and 5 μmol/L CsA had little or no effect on the formation of bile acids, but 10 μmol/L CsA decreased the formation of CA by about 13% and CDCA by about 30% (Figure 7). No accumulation of bile acids occurred in the hepatocytes. However, a significant accumulation of 7α-

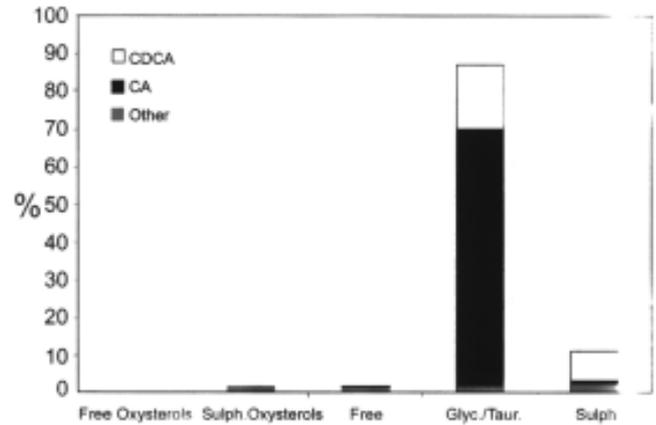


Figure 5 Distribution of free and conjugated bile acids and potential intermediates isolated from medium on the fifth day of incubating primary human hepatocytes. CDCA = chenodeoxycholic acid; CA = cholic acid.

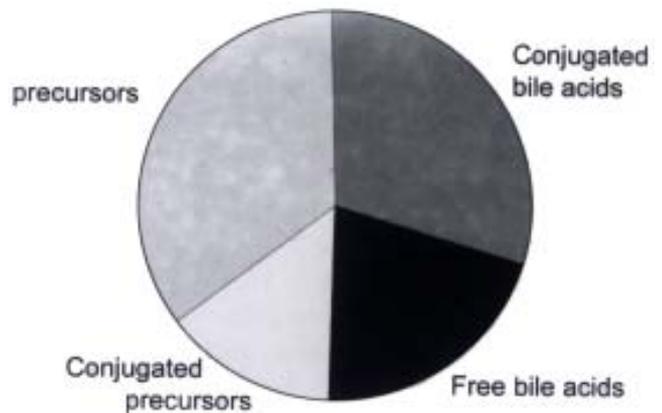


Figure 6 Conjugation of bile acids and precursors formed in cultures of HepG2 cells (data from ref. 7).

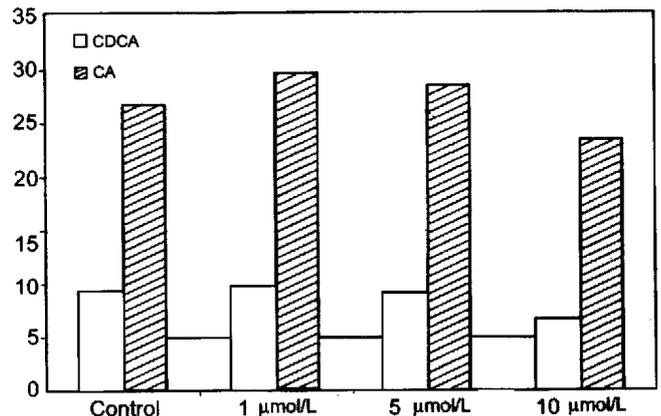


Figure 7 Effect of cyclosporin A on bile acid formation in primary human hepatocytes.

hydroxy-4-cholestene-3-one and 7α, 12α-dihydroxy-4-cholestene-3-one, an intermediate in CA synthesis, was found indicating that not only the acidic pathway but also the neutral pathway of bile acid formation was affected by CyA. In agreement with these results, it has previously been reported that patients with cerebrotendinous xanthomatosis, who lack the sterol 27-hydroxylase^[16,17],

accumulate 7α , 12α -dihydroxy-4-cholestene-3-one more than other intermediates in the liver^[18]. However, the decreased formation of total bile acids (CA and CDCA) during CsA treatment may mainly be due to an inhibitory effect of CsA on the hepatic synthesis of cholesterol^[12].

Influence of hormones

Previous studies have shown that the combined addition of T3 and dexamethasone stimulates bile acid formation, cholesterol 7α -hydroxylase activity and mRNA levels in rat hepatocytes, whereas the addition of T3 or dexamethasone alone has little or no stimulatory effect^[10,19]. The addition of T3 and dexamethasone alone or in combination to human hepatocytes did not increase bile acid synthesis (Figure 2). If anything, bile acid formation tended to decrease. The different effects of T3 and dexamethasone in rat and human hepatocytes agree well with recent studies of the hormonal regulation of the cholesterol 7α -hydroxylase gene promoter^[20,21]. These studies showed that the rat cholesterol 7α -hydroxylase gene promoter is stimulated by dexamethasone whereas thyroid hormone has no significant effect. The promoter of the human cholesterol 7α -hydroxylase gene, on the other hand, is suppressed by both dexamethasone and thyroid hormone.

In conclusion, isolated human hepatocytes in culture behave as in the intact liver by almost quantitatively converting cholesterol to conjugated CA and CDCA. In contrast, cultured HepG2 cells release large amounts of bile acid precursors and unconjugated bile acids into the medium. Human hepatocytes in culture represent a useful model for further studies on the synthesis and regulation of bile acids.

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Glyco-poly-L-lysine is better than liposomal delivery of exogenous genes to rat of liver

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Subject headings liposomes; glyco-poly-L-lysine; targeted liver uptake; exogenous gene

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Abstract

AIM To compare the effects of liposomes and glyco-poly-L-lysine on liver targeted uptake and expression of plasmid in rat liver.

METHODS After binding with lipofectamine or galactose-terminal glyco-poly-L-lysine, the plasmid could be expressed in eukaryotic cells when injected into Wistar rats by intravenous route. At different time intervals after the injection, the distribution and expression of the plasmid in liver of rats were observed and compared using *in situ* hybridization and immunohistochemistry.

RESULTS The expression of the plasmid binding to liposomes or G-PLL could be markedly observed 24 h later, and began to decrease one week later, but it still could be observed up to three weeks. Both liposomes and G-PLL could deliver the plasmid to the liver effectively, but the effect of the latter was better than the former concerning the distribution and expression of the plasmid targeted uptake in the liver.

CONCLUSION G-PLL is better than liposome as the targeted carrier for delivering exogenous genes to the liver.

INTRODUCTION

Liver is one of the important metabolizing organs, and is closely related to many kinds of diseases, such as hereditary disease, infectious disease, metabolic disease, tumors and so on. Since the development of chemical

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drugs for the treatment of these diseases is comparatively slow, gene therapy has opened up a prospective way for them^[1-5].

The efficient delivery and the high expression of exogenous genes in specific cells or tissues are critical steps for gene therapy both *in vitro* and *in vivo*^[6-15]. As for the gene therapy of hepatic diseases, efficient delivery of the exogenous genes to the liver and its high expression could increase its local accumulation while minimize the side-effects on other tissues and organs as well.

Both liposome and glyco-poly-L-lysine (G-PLL) are often used as carriers to deliver exogenous genes to the liver in gene therapy experiment^[16-22]. Most of the liposomes are cationic when used as the carriers of gene transference. Both *in vitro* and *in vivo* studies have showed that liposomes could be applied to transfer exogenous genes to hepatocytes^[23,24], but its specificity varies in different studies^[24-26]. Poly-L-lysine is a kind of polycation which can be bound to DNA by interacting with the opposite electric charge on DNA. After binding with other specific ligands through covalent linkage, the resulting ligand-poly-L-lysine-DNA complex can be formed^[27] and can thus be used to deliver foreign DNA to specific cells or tissues. Similarly, after being saccharified by galactose, glyco-poly-L-lysine (G-PLL) formed is then capable of delivering exogenous genes to the liver specifically^[28-30].

Using rats as the experimental animal, we compared the *in vivo* potency of liposomes and glyco-poly-L-lysine on delivering the plasmid, which could be expressed in eucaryotic cells.

MATERIALS AND METHODS

Preparation of the carriers

The original plasmid of rat interstitial collagenase was kindly provided by Prof. John J Jeffrey^[31], and we reconstructed it with the plasmid of pTargeT (TM) (Promega Co., Madison, MI, USA), which could be expressed in eucaryotic cells. We also inserted a segment of nucleotides (GAC TAC AAG GAC GAC GAT GAT AAG) before the terminator codon (TAA) of the rat interstitial collagenase. The 'Flag Domain' peptide (DYKDDDDK) encoded by the segment of nucleotide above, which was usually called 'Tag', could be fused in the rat interstitial collagenase^[32-33] and could be specifically recognized by a M2 monoclonal antibody (Kodak, New Haven, CT, USA). This recombinant plasmid was named

pTM/MMP-1. The plasmid of pTM/MMP-1 was extracted and purified using QIAGEN-Tip 500 kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturers of the instructions. The plasmid was mixed with different amounts of lipofectamine (GIBCO, Grand Island, NK, USA) or galactose-terminal glyco-poly-L-lysine (kindly provided by Dr. Shou-Ming Wen of Air-force General Hospital of PLA, China. The mean molecular weight of this kind of G-PLL was 8500 and the ratio of galactose to poly-L-lysine was 15:28). The optimal proportion of the plasmid pTM/MMP-1 to lipofectamine or galactose-terminal glyco-poly-L-lysine was determined by electrophoresis in 1% agarose gel.

Animal experiments

Eighteen male Wistar rats, with body weight 130-150 g, were randomly divided into three groups. Lipofectamine intravenous (LI) group: 50 µg of plasmid pTM/MMP-1 encapsulated by lipofectamine was given through cauda vein; Poly-L-lysine intravenous (PI) group: 50 µg plasmid pTM/MMP-1 binding to galactose-terminal glyco-poly-L-lysine was administered through cauda vein; Normal group: control animal. Twenty-four hours, 48 h, 72 h, 1 wk, 2 wk, 3 wk after the administration of the plasmid, one rat from each group was randomly selected and anaesthetized with 2% pentobarbital sodium intraperitoneally. Then 1 mL blood from each rat was obtained by cardiac puncture for the assay of alanine transaminase (ALT), aspartic transaminase (AST), and creatinine (Cr) to follow the functions of important organs. After perfusion of the whole body with 20 mL phosphate-buffered saline and 40 mL precold 4% paraformaldehyde through ventricular injection, the tissues of liver, spleen, lungs and kidneys were collected and fixed in 4% paraformaldehyde, encapsulated in paraffin and cut into sections of 4 µm thick. The animal experiments above were approved by the Laboratory Animal Committee of Shanghai Medical University.

Immunohistochemistry

Immunohistochemistry was performed according to the literature^[34,35]. The first antibody used was M2 monoclonal antibody which was specific for flag-domain tag (Kodak, New Haven, CT, USA) and the second antibody used was Horse anti-mouse IgG, labeled with biotin (Vecter, Burlingame, CA, USA). After the treatment with avidin and biotin (ABC kit, Vecter, Burlingame, CA, USA), color development was followed using dimethylaminoazobenzene (DAB) and counterstained with hematoxylin. Five fields were observed under high power from every immunostained section and the positive signals were counted.

In situ hybridization

The procedure of *in situ* hybridization was also described

previously^[34,35]. To state briefly, the oligonucleotide probe (5'-TGG TGT GAC TAC AAG GAC GAC GAT GAT AAG-3') was synthesized in Cell Biology Institute of Chinese Academy of Sciences (Shanghai), which could hybridize with the (mRNA) of the flag-domain tag in the plasmid pTM/MMP-1, and the 5' of the probe was labeled with biotin. After the hybridization of the target mRNA with the probe, the rest of the procedure was similar to that followed in immunohistochemistry excluding the step of hematoxylin counterstaining.

Other biochemical assays

ALT, AST, and Cr were assayed using the 7170A Automatic Analyzer (HITACHI, Japan) to observe the changes in the important organs' function.

Data analysis

The data was analysed using the software SPSS 7.0 for windows (One-way ANOVA).

RESULTS

Ratio of liposomes and G-PLL to plasmid

According to the electrophoresis results, we found that 5 µL of lipofectamine could encapsulate 1 µg of plasmid pTM/MMP-1 completely; While 0.3 µg of galactose-terminal glyco-poly-L-lysine thoroughly could bind to 1 µg of the plasmid, which meant that about 72 molecules of G-PLL could carry one molecule of the plasmid pTM/MMP-1 (Figures 1 and 2).

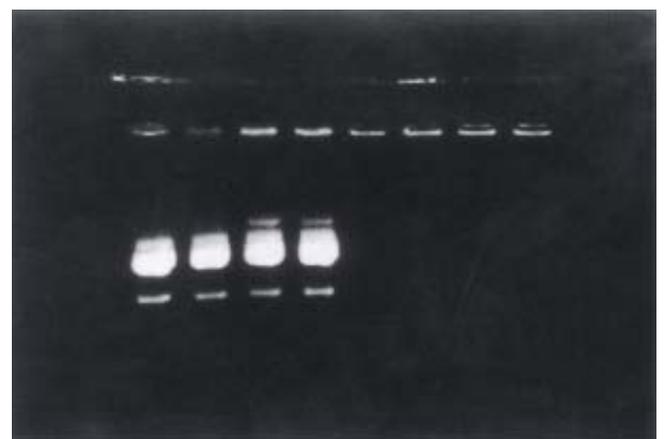


Figure 1 Determination of the optimal proportion of liposome bound to plasmid by 1% agarose electrophoresis. Lane 1-8 are respectively 1-8 µL lipofectamine mixed with 1 µg pTM/MMP-1 plasmid. Plasmid 1 µg could only be encapsulated completely by more than 5 µL lipofectamine.

The changes in the functioning of important organs

Compared with the normal group, there was no obvious elevation of the ALT, AS T, and Cr levels in the LI, PI groups.

The distribution and expression of the plasmid in liver, spleen, lung and kidney

The results of the immunohistochemistry and *in situ* hybridization showed that the plasmid binding to liposomes or G-PLL could be expressed *in vivo*, and the results of immunohistochemistry were more sensitive and stable. In addition, the protein product of the plasmid could be secreted extracellularly (Figures 3 and 4), similar to the expression of interstitial collagenase in the physiological state^[36]. We found that both liposomes and G-PLL could deliver the plasmid to the liver very efficiently, making liver as its major distribution organ. The obvious expression of the plasmid could be observed 24 h after the administration and began to decrease one week later, although it could still be observed weakly even two or three weeks later (Figure 5). Among the three groups, we also observed that the expression and distribution of the plasmid in the liver was most in the PI group, followed by the LI group. Besides the liver, the exogenous gene could also be expressed highly in lungs, and expressed in kidney in a relatively lower level in the LI group; while for the PI group, a relatively lower level of the expression could be seen in the kidney, the spleen, and the lung.

DISCUSSION

Both liposomes and G-PLL can be used as the targeted carriers to deliver drugs or nucleotides to liver^[17-21,24-29], but the comparison of their effects on the liver targeted uptake have not been reported extensively.

Gene transfer mediated by receptors is carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, and then the foreign gene can be delivered into the cells by phagocytosis^[37-40]. There also exist some specific receptors on the surface of hepatocytes such as asialoglycoprotein receptor (ASGP-R)^[41,42], which facilitate the delivery of exogenous genes into hepatocytes specifically using the ligand-receptor interaction. Galactose-terminal glyco-poly-L-lysine contains the saccharide group of galactosan that can be specifically ligated to the ASGP-R on the surface of hepatocytes. At the same time, the cationic poly-L-lysine can bind to nucleotides with high affinity, thus forming a good carrier to deliver exogenous DNA to liver specifically and steadily^[28-30]. It has been reported in the literature, that the ratio of the liver targeted delivery could reach up to 70%-90% *in vivo*^[27,43]. In our experiments we found that in addition to its main location in the liver, the plasmid binding to G-PLL could be expressed in kidney, spleen, and lung also. It may be due to the existence of ASGP-R in other extrahepatic tissues^[44,45].

Liposomes are a kind of annular closed vesicles made from double layers of lipid molecules^[46]. Having no toxicity and no immunogenicity^[47]. Gene transfer mediated by liposomes is achieved by the fusion between liposomes



Figure 2 Determination of the optimal proportion of G-PLL bound to plasmid by 1% agarose electrophoresis. Lane 1-8 are respectively 0.05 µg, 0.1 µg, 0.2 µg, 0.3 µg, 0.4 µg, 0.5 µg, 1.0 µg, and 1.5 µg G-PLL mixed with 1 µg pTM/MMP-1 plasmid. Plasmid 1 µg could only be bound completely by more than 0.4 µg G-PLL.

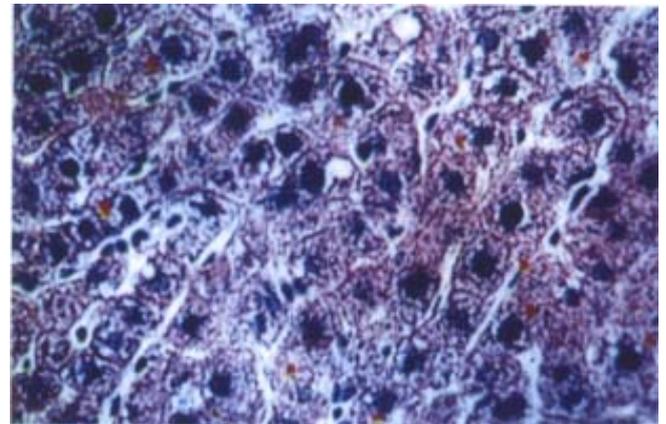


Figure 3 Immunostaining of flag-domain tag in the liver of rat 24 h after the administration of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) via cauda vein. × 200

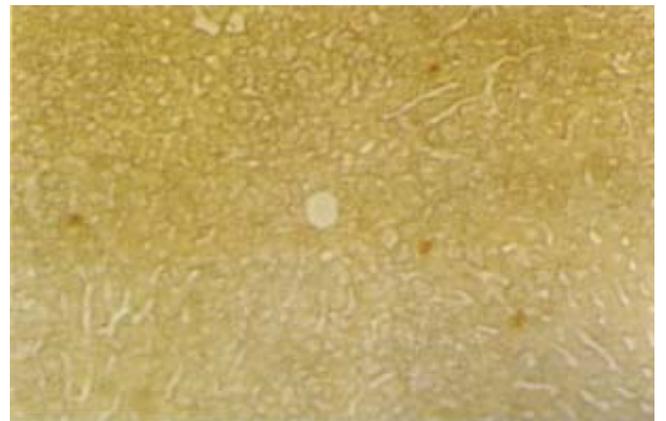


Figure 4 *In situ* hybridization with biotin labeled oligonucleotide probe in the liver 3 wk after the administration of the plasmid encapsulated by liposome (lipofectamine) via cauda vein. × 100

and the membrane of the cells. Liposomes encapsulating the foreign DNA can be integrated with the membrane of

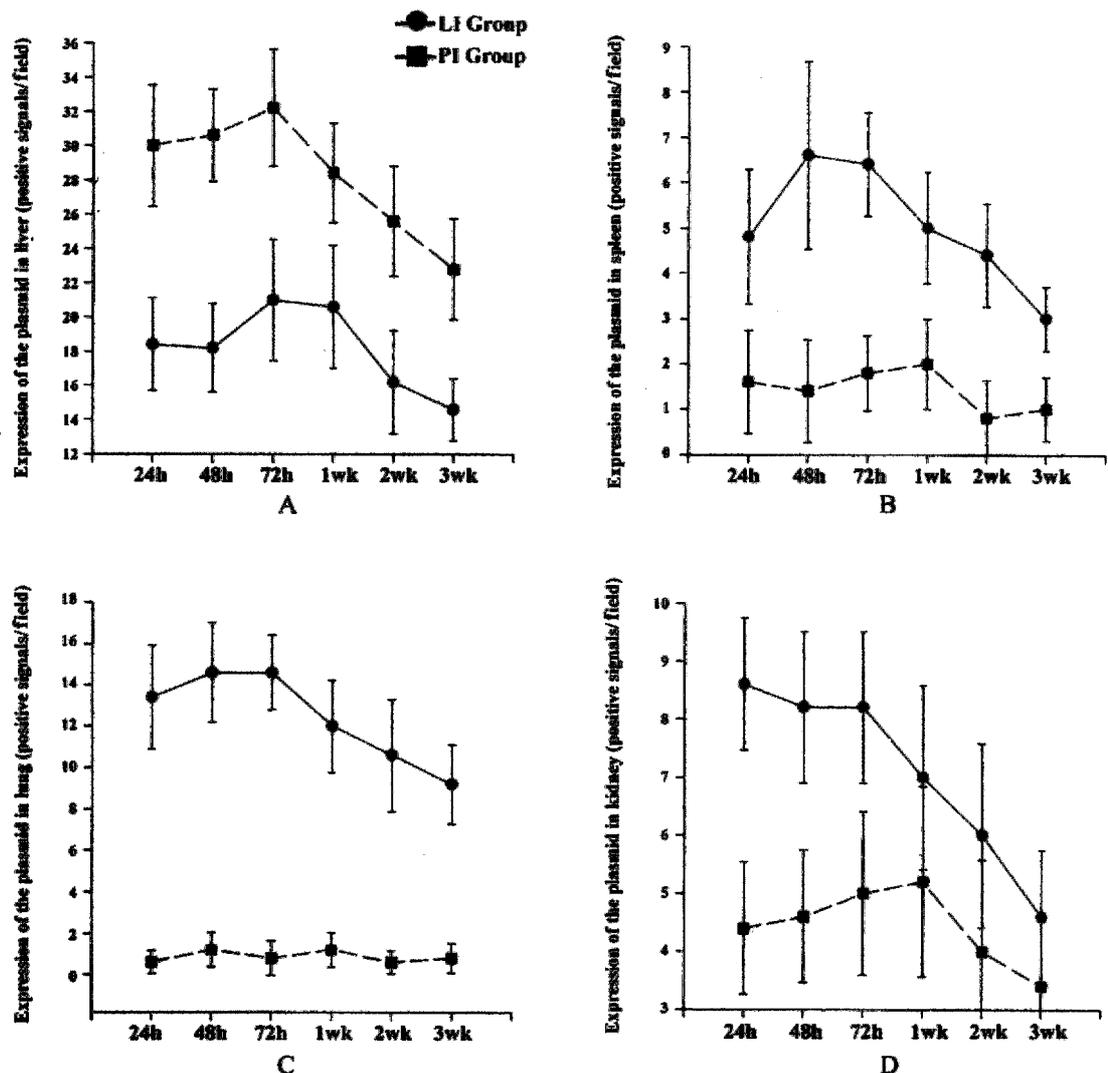


Figure 5 The distribution and expression of the plasmid bound to liposomes or G-PLL (galactose-terminal glyco-poly-L-lysine) in different tissues and at different time points. A: liver, B: spleen, C: lung, D: kidney. LI: plasmid encapsulated by liposomes given through cauda vein, PI: plasmid bound to G-PLL introduced intravenously.

the cells, and thus the foreign gene can be delivered inside the cells by phagocytosis^[24,48-50]. Because it contains lecithin and lactosylceramide that can bind to ASGP-R on the surface of hepatocytes specifically, liposome could also be used as targeted delivery carrier to the liver^[51,52]. Our experimental results demonstrated that the plasmid encapsulated by liposomes could also be expressed in lung and spleen to a certain extent despite its major expression in the liver, maybe due to the macrophage system which existed in the lung, spleen, and other tissues. This phenomenon demonstrates further that the efficacy of liver targeted delivery by liposomes is inferior to that of G-PLL.

In this study, we also observed whether liposomes and G-PLL, both binding to the plasmid, would induce some toxicity to the body or not. We did not find any detrimental effects on the functioning of important organs like liver, heart, and kidney and this indicated further that liposomes and G-PLL could be used *in vivo* safely as delivery carriers for drug or nucleotides.

In conclusion, we found that the plasmid binding to liposomes or G-PLL could be delivered to the liver efficiently and G-PLL was better than liposomes regarding the distribution and expression of the plasmid in the liver. Both liposomes and G-PLL can be used as carriers to deliver drugs or nucleotides to rat liver, but whether they can be used in human beings in the future deserves further investigation.

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Induction of apoptosis by arsenic trioxide and hydroxy camptothecin in gastric cancer cells in vitro

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Subject headings gastric cancer; apoptosis; arsenic trioxide; hydroxycamptothecin

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Abstract

AIM To study the effects of arsenic trioxide and HCPT on different degrees of differentiated gastric cancer cells (SGC-7901, MKN-45, MKN-28) with respect to both cytotoxicity and induction of apoptosis *in vitro*.

METHODS The cytotoxicity of As₂O₃ and HCPT on gastric cancer cells was determined by MTT assay. Morphologic changes of apoptosis of gastric cancer cells were observed by light microscopy and transmission electron microscopy. Apoptosis and cell cycle changes of gastric cancer cells induced by HCPT and As₂O₃ were investigated by TUNEL method and flow cytometry.

RESULTS As₂O₃ and HCPT had remarkable cytotoxic effects on different degrees of differentiated gastric cancer cells. The IC₅₀ of As₂O₃ on well differentiated gastric cancer cell MKN-28, moderately differentiated gastric cancer cell SGC-7901, and poorly differentiated gastric cancer cell MKN-28 were 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respectively. The IC₅₀ of HCPT on MKN-28, SGC-7901, and MKN-45 were 9.35 mg/L, 10.21 mg/L, and 12.63 mg/L respectively after 48 h treatment. After 12 h of exposure to both drugs, gastric cancer cells exhibited morphologic features of apoptosis, including cell shrinkage, nuclear condensation,

and formation of apoptotic bodies. A typical subdiploid peak before G₀/G₁ phase was observed by flow cytometry. The apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively after 48 h exposure to 10 μmol/L As₂O₃. The apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 21.88%, 12.35%, and 30.26%, respectively after 48 h exposure to 10 mg/L HCPT. The apoptotic indices were 7%-15% as assessed by TUNEL method. The effect of As₂O₃ on SGC-7901 showed remarkable cell cycle specificity, which induced cell death in G₁ phase, and blocked G₂/M phase. HCPT also showed a remarkable cell cycle specificity, by inducing cell death and apoptosis in G₁ phase and arrest of proliferation at S phase.

CONCLUSION As₂O₃ and HCPT exhibit significant cytotoxicity on gastric cancer cells by induction of apoptosis. As₂O₃ and HCPT might have a promising prospect in the treatment of gastric cancer, which needs to be further studied.

INTRODUCTION

Gastric cancer is one of the most common malignant tumors in China. Evidences have demonstrated that stomach cancer is a disease caused not only by excessive cellular proliferation and poor differentiation, but also by decrease in apoptosis of the gastric cells^[1]. Though the disease in its early stage can be treated by surgical resection, in advanced stage its response to conventional chemotherapy or radiotherapy is usually not satisfactory. Therefore, we think, that induction of apoptosis of gastric cancer cells might be a new means in the treatment of gastric cancer. Arsenic trioxide (As₂O₃) and hydroxycamptothecin (HCPT) have long been used in China. The former was first reported by investigators in Harbin and Shanghai to be an effective drug in the treatment of patients with a cute promyelocytic leukemia (APL)^[2,3]. It induces apoptosis of the leukemic cells at a concentration achieved in the plasma of treated patients (0.5 × 10⁻⁶ mol/L-1 × 10⁻⁶ mol/L), as demonstrated by studies on all -trans-retinoic acid (ATRA)-susceptible or resistant APL cell lines, on primary APL cell culture, and on patients' blood samples

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obtained during treatment with arsenic^[4]. It is not known whether As₂O₃ is effective in the treatment of solid tumors such as gastric cancer or not.

HCPT is a unique antitumor drug that has been extracted and synthesized by Chinese scientists from *Camptotheca accuminata* which is a native plant in China^[5]. HCPT can act directly on topoisomerase I inhibiting its activity. HCPT possesses stronger cytotoxicity to tumor cells with less side effects, as compared to camptothecin^[6]. More recent studies have shown that camptothecin have strong apoptosis induction effects in human leukemia cell lines^[7]. However, there is yet no report about HCPT-induced apoptosis in stomach cells. Therefore, we studied the effects of HCPT and As₂O₃ on induction of apoptosis in gastric cancer cells.

MATERIALS AND METHODS

Cell culture and chemicals

Human moderately differentiated gastric adenocarcinoma cell line SGC-7901 was obtained from Shanghai Sixth People's Hospital, human poorly differentiated stomach adenocarcinoma cells line MKN-45 and well differentiated stomach adenocarcinoma cell line MKN-28 were kindly provided by Japanese Cancer Research Resources Bank Corp (Tokyo, Japan). SGC-7901, MKN-45 and MKN-28 were maintained in a humidified, 5% CO₂ atmosphere and cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 2 μmol/L L-glutamine, 100 units/mL penicillin, and streptomycin. HCPT 1 g/L and 0.1% As₂O₃ preparation for iv administration were kindly provided by Hubei Huangshi Second Pharmaceutical Company, and Shanghai Institute of Hematology, respectively.

MTT method and cytotoxicity

One hundred μL cancer cells in exponential growth at 1 × 10⁴/mL were added into flat-bottomed 96-well plates (NUNC) 24 h prior to drug treatment. Cells were treated with 1 mg/L-100 mg/L HCPT, (0.5-10) μmol/L As₂O₃ and with no drugs (control) in triplicate for 24 h and 48 h, respectively. After washing the medium was replaced by 100 μL RPMI 1640 (GIBCO) medium containing 1 g/L 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium (MTT, MERCK). After 4 h, plates were centrifuged at 800 × g for 5 min, the MTT medium was removed, and the blue dye was dissolved in 200 μL of warm dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm. The cytotoxicity rates were measured by the formula:

$$\text{The cytotoxicity rate} = \left(1 - \frac{\text{OD}_{570 \text{ test}}}{\text{OD}_{570 \text{ control}}}\right) \times 100\%$$

The IC₅₀ was evaluated from the cytotoxicity curve.

Apoptosis cell morphology

One hundred μL cancer cells at concentration of 5 × 10⁶/

mL were incubated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation with drugs for 12 h, 24 h, 48 h, and 72 h, glass slides with cancer cell growth were fixed with 4% polyformalin, and stained with hematoxylin-eosin. Cell morphology was examined under light microscopy.

Transmission electron microscopy

Culture cells were fixed in 2% glutaraldehyde in 0.1 mol/L, pH 7.4 PBS at 4 °C and postfixed with 1% osmium tetroxide for 2 h, then the cells were embedded with Epon 812 and ultrathin sections were cut. Cells were observed under transmission electron microscope (H-500, Japan).

TUNEL assay

Apoptosis was assessed by dUTP labeling of DNA nicks with terminal deoxynucleotidyl transferase (TUNEL). One hundred μL cancer cells at concentration of 5 × 10⁶/mL were inoculated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation with drugs for 24 h and 48 h, glass slides with cancer cells growth were fixed with 4% polyformalin. The TUNEL assay was performed according to the instructions in the *In Situ* Cell Death Detection Kit (Boehringer-Mannheim, Germany). Briefly, after washing twice with pH 7.4 PBS, 50 μL TUNEL reaction solution was added to the well, then incubated at 37 °C for 2 h. After substrate reaction, stained cells were examined under light microscopy. Apoptotic cells were scored and expressed as the number of positively stained cells per 500 cells (*n* = 4-5).

Flow cytometry

Apoptotic cells were also detected by flow cytometry which was performed as described previously. About 1 × 10⁶ cells were treated with drugs for 24 h and 48 h. After trypsin digestion, the cells were collected by centrifugation, then fixed in 70% ethanol /phosphate buffered saline for at least 12 h at 4 °C. After 100 μL (1 g/L) RNase treatment, cells were stained with 50 mg/L propidium iodide. Cells were examined by flow cytometry using a FACScan (Becton-Dickinson, USA). The results were analyzed with Lysis II software (Becton-Dickinson).

RESULTS

As₂O₃ and HCPT cytotoxicity

HCPT and As₂O₃ had strong cytotoxic effect on gastric cancer cells (Figure 1). The IC₅₀ of As₂O₃ on MKN-28, SGC-7901, and MKN-45 was 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respectively. The IC₅₀ of HCPT on MKN-28, SGC-7901, MKN-45 was 9.35 mg/L, 10.21 mg/L, and 12.63 mg/L, respectively.

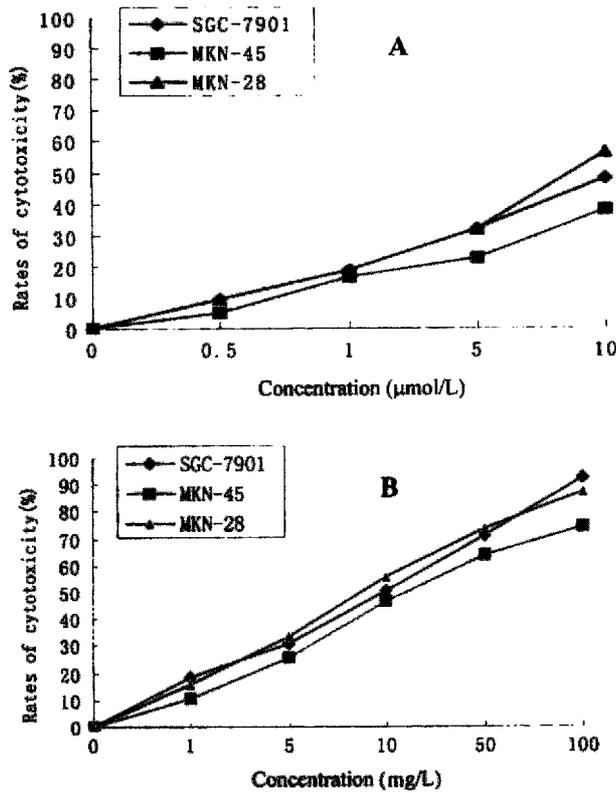


Figure 1 Cytotoxicity of As₂O₃ (A), and HCPT (B), on gastric cancer cells.

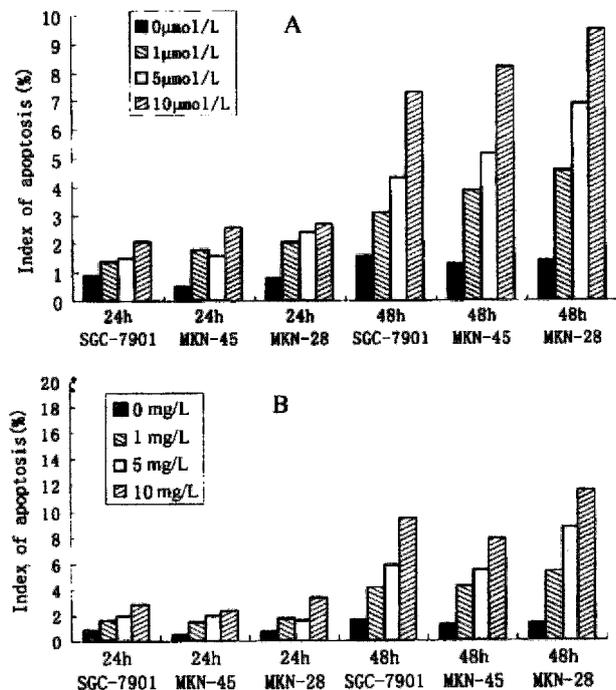


Figure 3 The index of apoptosis of gastric cancer cells induced by As₂O₃ (A) and by HCPT (B).

Induction of apoptosis by As₂O₃ and HCPT

After 12 h of exposure to As₂O₃ and HCPT, gastric cancer cells MKN-28 and SGC-7901, began to show morphologic features of apoptosis. The apoptotic cells increased on

prolongation of exposure time to drugs. The ultrastructural features of apoptosis was observed in MKN-45 and SGC-7901 by transmission electron microscopy, including cell shrinkage, cytoplasmic blebs, condensation of chromatin, nuclear condensation, fragmentation of nucleus, and formation of apoptotic bodies, and so on. The results are presented in Figure 2.

As₂O₃ and HCPT induced time- and dose-dependent apoptosis in three strains of gastric cancer cells. The indices of apoptosis were measured by TUNEL. The results are presented in Figure 3A and 3B.

To further study the inducing effect of As₂O₃ and HCPT on gastric cancer cells, we analysed the DNA fragment reflecting the endonuclease activity during apoptosis. (Figure 4). Examination of histogram related nuclear DNA contents on FACS showed a distinct region below G₁ phase, which is the typical profile of apoptotic cells in which DNA stainability is reduced due to degradation and subsequent leakage of DNA from cells. After 48 h exposure to 10 mg/L HCPT, the apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 21.88%, 12.35%, and 30.26%, respectively. After 48 h of exposure to 10 µmol/L As₂O₃, the apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively (Figure 4).

Effect of As₂O₃ and HCPT on cell cycle of SGC-7901 cells

The effect of As₂O₃ and HCPT on SGC-7901 cells show remarkable cell cycle specificity. There was no significant change in cell cycle after 10 µmol/L As₂O₃ treatment for 24 h. The fraction of G₀/G₁ was decreased from 54.2% to 17.7%, while the fraction of G₂/M was significantly increased from 20.2% to 63.4% with 10 µmol/L As₂O₃ treatment in SGC-7901 cells for 48 h. The results showed that As₂O₃ induced gastric cancer cell death was in G₁ phase, blocked at G₂/M phase. The fraction of G₀/G₁ phase was decreased from 54.2% to 37.6%, while the fraction of S phase was increased from 25.6% to 38.6% with 10 mg/L HCPT treatment in SGC-7901 cells for 48 h. The results showed that HCPT-induced apoptosis was at G₁ phase, and arrested at S phase.

DISCUSSION

Since the first observation of the relationship between arsenic and skin cancer in 1820s, arsenic compounds have been generally recognized as potent environmental carcinogens, more likely as a co-mutagen and co-carcinogen for human skin and lungs^[8], although no animal model had been established^[9]. Biochemically, it is documented that arsenic can inactivate some important enzymes by binding their sulfhydryl groups. Arsenic can also interfere with the phosphorylation-dephosphorylation process by replacing the phosphorylation reaction. It has

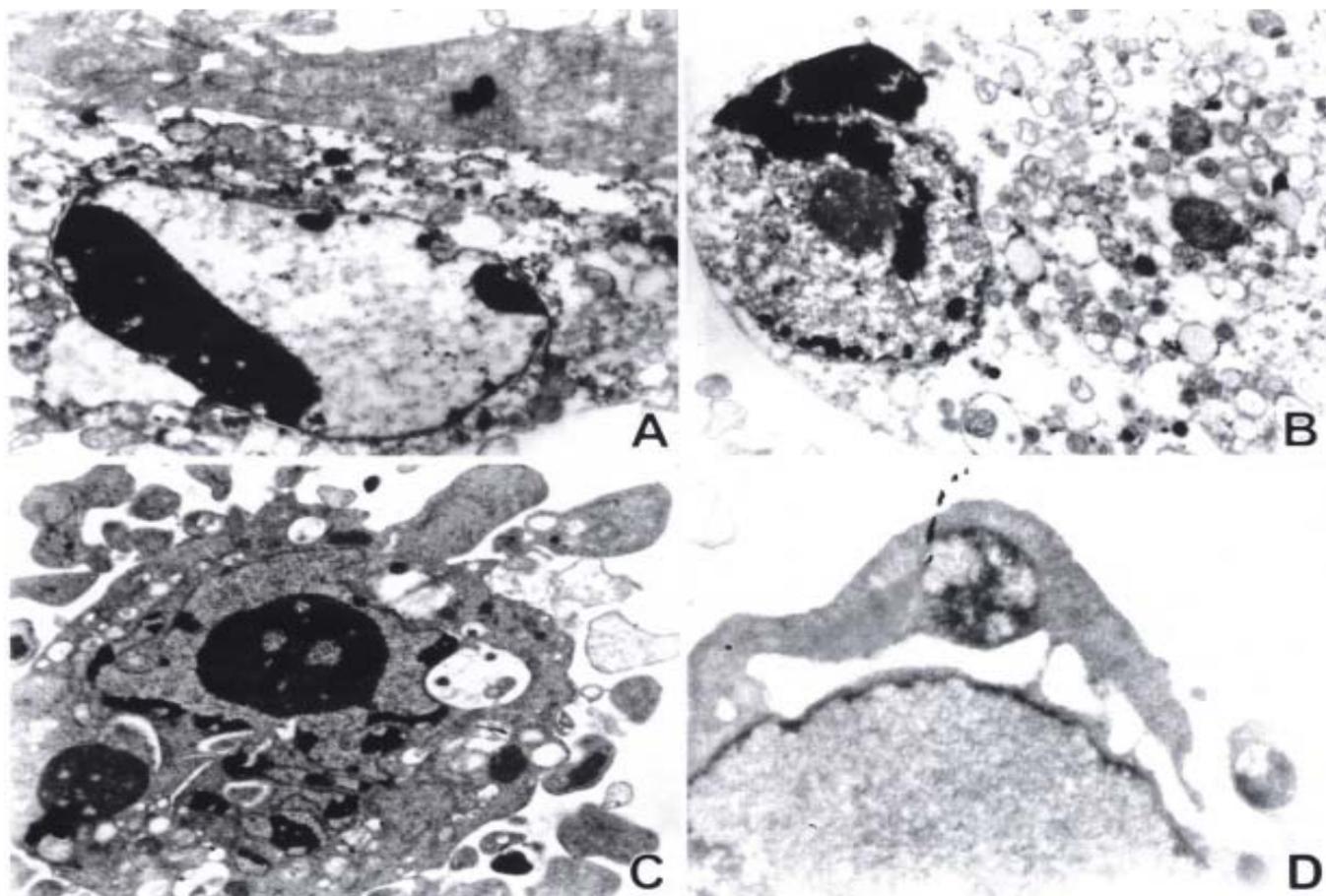


Figure 2 Electron microscopic observation of ultrastructural changes in gastric cancer cells treated with 10 $\mu\text{mol/L}$ As_2O_3 for 48 h. The early change of apoptosis, nuclear chromatin condensation, looks like a new moon subjacent to the nuclear membranes (A). The intermediate stage of apoptosis presents cytoplasmic blebs, nuclear condensation, overflow of nuclear chromatin, and like-sprout (B). The late stage of apoptosis is characterized by splitting of nuclear membrane, nuclear chromatin condensation, overflow of some parts of nuclear chromatin, and fragmentation of nucleus (C), and formation of apoptotic bodies (C) (D).

also been shown that arsenic can induce chromosome aberrations, sister-chromatin exchanges, DNA-protein cross links and protein-associated DNA-strand breaks in mammalian cells^[10,11]. However, low concentration of some arsenic compounds also had some benefits to human physiologically, such as stimulation of human hematopoiesis. The use of arsenic compounds as drugs has a long history in Chinese traditional medicine. For example, it was recorded that arsenic had therapeutic effects on some human diseases such as psoriasis, syphilis, and rheumatism. Recently, it has been shown that two arsenic compounds As_2O_3 ^[2] and arsenic disulfide^[12], which were used in some traditional Chinese prescriptions, are very effective in APL treatment. For instance, a report from the northeastern region of China showed that As_2O_3 (10 g/L via intravenous infusion for 28 to 60 days) induced clinical complete remission (CR) in 65.6% of APL patients. More interestingly, 28.2% (9/32) of patients survived more than 10 years. A more recent clinical trial with As_2O_3 treatment also demonstrated that

CR was achieved in 15 of 16 APL patients who relapsed after ATRA-induced and chemotherapy-maintained CR^[4].

It has been suggested that As_2O_3 might induce apoptosis selectively in APL cells^[13]. Furthermore, at pharmacological concentrations, it has no effect on the growth and survival of the leukemia myeloid cells U937 and HL60^[3]. However, preliminary reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells but can be observed in various lines of either myeloid or lymphoid origin^[14-18] and in blast cells from patients with non-M3 acute myeloid leukemia^[19]. Another arsenic-containing compound, the melaminyl-phenyl-arsenoxide melarsoprol, which is used in the treatment of human African trypanosomiasis, has a broad efficacy against leukemia cells of both lymphoid and myeloid lineage^[20]. Both As_2O_3 and melarsoprol also markedly induce apoptosis in plasma cell lines and in plasma cells from multiple myeloma^[21]. Recent reports have demonstrated that As_2O_3 can induce apoptosis in various cell lines of solid tumor. As_2O_3 inhibited the growth and survival of solid tumor cell

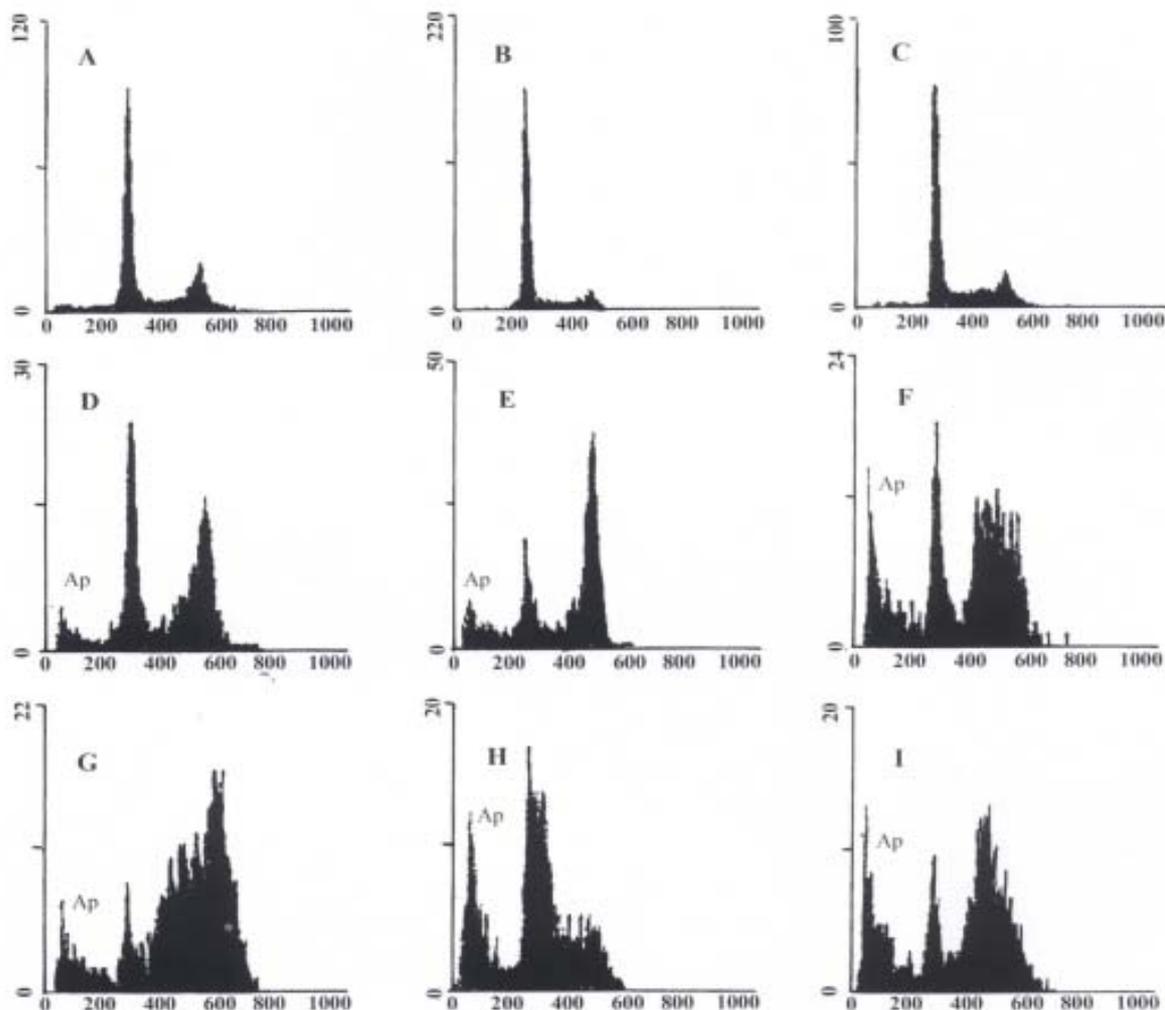


Figure 4 Nuclear DNA contents measured by flow cytometry in As_2O_3 and HCPT-induced apoptosis in gastric cancer cells at 48 h. Ap represents apoptotic cells. A: Untreated MKN-45 cells; B: Untreated SGC-7901 cells; C: Untreated MKN-28 cells; D: MKN-45 cells treated with $10 \mu\text{mol/L } As_2O_3$; E: SGC-7901 cells treated with $10 \mu\text{mol/L } As_2O_3$; F: MKN-28 cells treated with $10 \mu\text{mol/L } As_2O_3$; G: MKN-45 cells treated with 10 mg/L HCPT ; H: SGC-7901 cells treated with 10 mg/L HCPT ; I: MKN-28 cells treated with 10 mg/L HCPT .

lines in gastric^[22], esophageal^[23,24], lung^[24], cervix^[25], liver^[26] tumor and neuroblastoma^[27] by triggering programmed cell death. These results suggest that As_2O_3 may be an effective drug in treatment of solid tumors. As_2O_3 has been intensively investigated for the treatment of cancer.

There is growing evidence indicating that apoptosis plays a crucial role in both carcinogenesis and development of stomach tumors^[1,28-30]. Induction of apoptosis in gastric cancer cells might be a new means in the treatment of gastric cancer. It was reported that both radiation and chemotherapy (5-fluorouracil, cisplatin, mitomycin and trichostatin) can induce apoptosis in gastric cancer^[31-33]. Our previous study showed that As_2O_3 had an effect on inhibiting proliferation and inducing apoptosis in gastric cancer cell SGC-7901^[22]. In this study, we found that As_2O_3 exhibited a dose- and time-dependent cytotoxicity on gastric cancer cells at different

degrees of differentiation. The IC_{50} of As_2O_3 on MKN-28, SGC-7901, and MKN-45 were $8.9 \mu\text{mol/L}$, $10.5 \mu\text{mol/L}$, and $11.6 \mu\text{mol/L}$, respectively. Toxicity of As_2O_3 was not significantly different among gastric cancer cells at different degrees of differentiation. As_2O_3 -treated gastric cancer cells presented characteristic morphological changes of apoptotic cells. Results showed DNA degradation into oligonucleosomal fragments and these changes occurred in a time- and dose-dependent manner. The apoptotic index was 7%-15% as assessed by TUNEL. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. After 48 h of exposure to As_2O_3 , the apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.8%, 22.5%, and 9.68%, respectively. We concluded that one of the main effects of As_2O_3 on gastric cancer cells at the concentrations used is to induce cell death by apoptosis.

It was recently shown^[45] that the cell cycle time was prolonged in As_2O_3 treated malignant lymphocytes

Namalwa and Raji cells, and that no substantial increase in cell cycle time was found in Jurkat cells treated with As_2O_3 , as compared with untreated cells. This result suggests that As_2O_3 can inhibit proliferation of some malignant lymphocyte cell lines by prolonging the cell cycle instead of arresting cells in a specific phase. In the present study, we demonstrated that the effect of As_2O_3 on SGC-7901 showed remarkable cell cycle specificity in inducing cell death at G_1 phase, and blocking proliferation at G_2/M phase. This result is consistent with the report of Deng *et al.*^[46], in which As_2O_3 induced apoptosis in HeLa cells by arresting G_2/M phase of the cell cycle.

Camptothecin and its analogues are agents with a unique spectrum of antitumor activity mediated by a selective inhibition of eukaryotic DNA topoisomerase I (Topo I). The cytotoxicity of these compounds is predominantly exerted during S phase, and is associated with an inhibition of DNA replication. This inhibition is generally thought to be mediated by stabilization of the CPT-Topo I-DNA cleavable complex^[34]. Recent studies have shown that camptothecin and its analogues can strongly induce apoptosis in human leukemic cells^[35-36], prostate^[36-37], colon^[38] and breast^[39-40] cancer cells as well as glioma cells^[41].

HCPT has been used in the treatment of gastrointestinal tumor^[42,43], but its mechanism of anticancer action is still not completely understood. Recently, there were reports that HCPT can induce apoptosis of cancer cells of colon^[47], pancreas^[48], and bladder^[49]. Our previous study showed HCPT can induce apoptosis in gastric cancer cell SGC-701^[44]. In this study, we found that HCPT had a strong dose- and time-dependent cytotoxicity to gastric cancer cells at different degrees of differentiation. The IC_{50} of HCPT on MKN-28, SGC-7901, and MKN-45 was 9.35 mg/L, 10.21 mg/L, and 12.62 mg/L, respectively. There was no significant difference in toxicity of HCPT on gastric cancer cells at different degrees of differentiation. Gastric cancer cells treated with HCPT presented characteristic morphological changes of apoptosis. The effects of inducing apoptosis in gastric cancer cells could be correlated with time and dosage of HCPT treatment. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. After 48 h of exposure to 10 mg/L HCPT, the apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.8%, 22.5%, and 9.68%, respectively. In this study, the fraction of G_0/G_1 phase was decreased from 54.2% to 37.6%, while the fraction of S phase was increased from 25.6% to 38.6% with 10 mg/L HCPT treatment of SGC-7901 cells for 48 h. The result suggested that HCPT also showed a remarkable cell cycle specificity, induced cell death and apoptosis at G_1 phase, and arrested proliferation at S phase.

The molecular mechanism of As_2O_3 on APL cells

showed that the inhibition of cell proliferation was due to a direct induction of apoptosis through downregulation of bcl-2 expression and modulation of PML/RAR α /PML protein *in vitro* studies^[3]. Furthermore, the activation of caspases was also involved in As_2O_3 -induced apoptosis in APL cells^[50]. Akao *et al.* also reported that As_2O_3 induced apoptosis through the down-regulation of Bcl-2 protein and activation of caspases in B-cell leukemia cell lines^[51]. It was also reported that As_2O_3 induced apoptosis in neuroblastoma cell lines through the activation of caspase-3^[27]. Litvak *et al.* reported that inhibition of gastric cancer by camptothecin involves apoptosis and multiple cellular pathways, and induction of apoptosis of gastric cancer cell was mediated by up-regulation of p53, p21Waf1/Cip1, and p27Kip1 and the down-regulation of Bcl-2 and Bcl-XL^[52]. Camptothecin induced apoptosis through activation of caspase in U-937 cells^[53]. Our results showed that HCPT and As_2O_3 exerted significant cytotoxicity on gastric cancer cells and induction of apoptosis *in vitro*. The molecular mechanism of apoptotic effect of HCPT and As_2O_3 on gastric cancer cells remains to be further investigated, and the effect of apoptosis must be confirmed by *in vivo* studies. However, the results of the present study suggest that HCPT and As_2O_3 might be candidate drugs to be used in the treatment of gastric cancer, thus needing further studies.

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Effects of AT1 receptor antagonist, losartan, on rat hepatic fibrosis induced by CCl₄

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Subject headings liver cirrhosis/drug therapy; renin-angiotensin system; angiotensin II type 1 receptor antagonist; losartan

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Abstract

AIM To investigate effect of losartan, an AT1 receptor antagonist, on hepatic fibrosis induced by CCl₄; and to determine whether or not AT1 receptors are expressed on hepatic stellate cells. **METHODS AND RESULTS** Fifty male Sprague-Dawley rats, weighing (180 ± 20) g, were randomized into five groups (control group, model group, and three losartan treated groups), in which all rats were given the subcutaneous injection of 40% CCl₄ (every 3 days for 6 weeks) except for rats of control group. Rats of losartan-treated groups were treated with losartan (20mg/kg, 10mg/kg, 5mg/kg, daily gavage). After 6 weeks liver tissue and serum samples of all rats were examined. Serum hyaluronic acid (HA), procollagen type III (PC III) were detected by radioimmunoassays. van Gieson collagen staining was used to evaluate the extracellular matrix of rats with liver fibrosis. The expression of AT1 receptors, transforming growth factor-beta (TGF-β), and alpha-smooth muscle actin (α-SMA) in liver tissue were determined by immunohistochemical techniques. Compared with model group, serum ALT and AST of losartan-treated groups were significantly reduced ($t = 4.20$, $P < 0.01$ and $t = 4.57$, $P < 0.01$). Serum HA and PC III also had significant differences ($t = 3.53$, $P < 0.01$ and $t = 2.20$, $P < 0.05$). The degree of fibrosis was improved by losartan and

correlated with the expressions of AT1 receptors, TGF-β, and α-SMA in liver tissue. **CONCLUSION** AT1 receptor antagonist, losartan, could limit the progression of the hepatic fibrosis induced by CCl₄. The mechanism may be related to the decrease in the expression of AT1 receptors and TGF-β, a meliorating the injury of hepatocytes; activation of local renin-angiotensin system might relate to hepatic fibrosis; and during progression of fibrosis, activated hepatic stellate cells might express AT1 receptors.

INTRODUCTION

Hepatic fibrosis, which may ultimately lead to cirrhosis, is associated with most chronic liver diseases, and is characterized by the net accumulation of extracellular matrix (ECM), including collagen, glycoproteins, and proteoglycans^[1,2]. Many reports have suggested that hepatic stellate cells (HSCs) are the major producers of ECM in liver injury, and play a prominent role in liver fibrosis^[3-7]. Tissue repair after acute liver damage involves "activation" of "quiescent" HSCs to myofibroblast-like cells^[8-12]. Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that has been assigned a key role in epithelial repair, and HSCs were shown to its main source^[13-16]. In cultured HSCs, TGF-β-mediated up-regulation of collagen and other ECM components mRNA was time and dose-dependent^[17,18]. In the past years, significant progress has been made in our understanding of this pathologic mechanism, however, few effective drugs can slow the progression of the fibrosis^[19,20].

Over the past decade, preventing the formation of angiotensin II by angiotensin-converting enzyme (ACE) inhibitors has revolutionized the therapy of hypertension and especially of congestive heart failure^[21]. Recently, a number of studies demonstrated that ACE inhibitors also effectively limited the progression of cardiac, renal and pulmonary interstitial fibrosis^[22-26]. Recent work has shown that angiotensin II type 1 (AT1) receptor antagonist, losartan, can also ameliorate the renal and cardiac fibrosis^[27,28]. The prevailing hypothesis for the main mechanism was suppressing the expression of TGF-β in kidney and heart, rather than its dynamic effects^[29-32].

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Based on this and other information, we hypothesized that the AT1 receptor antagonist, losartan, could also limit the progression of hepatic fibrosis. To explore our speculation, the present study was designed to investigate the effect of losartan on rat's hepatic fibrosis induced by CCl₄, and determine whether or not there was expression of AT1 receptor on hepatic stellate cells.

MATERIALS AND METHODS

Animals and reagents

Fifty male Sprague-Dawley rats, weighing 180 ± 20 g, were purchased from Animal Center of Shanghai Medical University (Shanghai, China). Losartan was obtained from MSD Co. (England). Polyclonal rabbit antibody to rat TGF- β was purchased from Boster Biotech Co. (Wuhan, China). Monoclonal antibody of α -smooth muscle actin (α -SMA) was purchased from Maixin Biotech Co. (Fuzhou, China). Hyaluronic acid (HA) and procollagen type III (PC III) radioimmunoassays kits were purchased from Navy Shanghai Medical Institute (Shanghai, China).

Serum function tests

Fifty rats were randomized into five groups (control group, model group and three losartan-treated groups) in which all rats were given subcutaneous injection of 40% CCl₄ (0.3 mL/100 g, every 3 days for 6 weeks) except for rats of control group (only given injection of same dose of olive oil). In an initial experiment, rats of losartan-treated groups were treated with losartan (20 mg/kg, 10 mg/kg, 5 mg/kg by daily gavage). After six weeks, all rats were sacrificed. Serum was collected and stored at -20°C for analysis of aspartate transaminase (AST) and alanine transaminase (ALT) activity by standard enzymatic methods. The serum levels of PC III and HA was determined by radioimmunoassays.

Immunohistochemical detections and histological data

The liver sections were fixed in a 10% solution of formaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4), and embedded in paraffin. Five-micrometer slides were prepared. van Gieson collagen staining was used to evaluate the ECM of rats. According to van Gieson collagen staining, the degree of fibrosis was divided into five grades (0-5). Specimens were scored blindly by the histologist and were also ranked blindly for severity of fibrosis. The expression of AT1 receptor (anti-rat rabbit polyclonal antibody was the product of Santa Cruz Biotech Co), TGF- β and α -SMA were detected by immunohistochemical techniques.

Statistics

Data are presented as $\bar{x} \pm s_x$. Comparison between two groups was made using Student's *t* test. Difference of

fibrosis between model and losartan-treated groups was compared using Ridit analysis. The test was considered significant at $P < 0.05$.

RESULTS

Serum function tests

Compared with control group, ALT and AST increased significantly in fibrotic rats in the model group, but only marginally in losartan treated rats. ALT and AST activities were significantly lower in 20 mg losartan-treated group than in model group of CCl₄ rats. The effects were associated with doses of losartan (Table 1).

Table 1 Serum function tests

	<i>n</i>	LT (U/L)	AST (U/L)
Control	10	99.50 \pm 18.78	244.50 \pm 46.52
Model	7	1863.29 \pm 893.68 ^a	2680.00 \pm 1039.12 ^a
Losartan (20 mg/kg)	7	432.14 \pm 112.26 ^d	824.57 \pm 265.41 ^d
Losartan (10 mg/kg)	7	535.25 \pm 200.78 ^d	77.50 \pm 270.32 ^d
Losartan (5 mg/kg)	6	771.71 \pm 237.18 ^c	1643.00 \pm 810.36

^a $P < 0.05$ vs control; ^c $P < 0.05$ vs model; ^d $P < 0.01$ vs model

Serum components of ECM

As expected, serum levels of HA and PCIII increased in rats of model group. Serum HA levels were approximately three times higher in rats of model group than rats of control group. There was a tendency towards a decrease in HA and PC III levels in losartan treated group (Table 2).

Table 2 Serum component of ECM

	<i>n</i>	HA (ng/L)	PC III ($\mu\text{g/L}$)
Control	10	331.42 \pm 42.31	19.06 \pm 4.43
Model	7	911.66 \pm 345.49 ^a	31.82 \pm 6.90 ^a
Losartan (20 mg/kg)	7	425.05 \pm 115.80 ^d	22.78 \pm 8.38 ^c
Losartan (10 mg/kg)	7	556.11 \pm 195.22 ^c	24.49 \pm 2.73 ^c
Losartan (5 mg/kg)	6	734.03 \pm 318.93 ^c	24.19 \pm 6.76

^a $P < 0.05$ vs control; ^c $P < 0.05$ vs model; ^d $P < 0.01$ vs model

Histological data

Piecemeal and lobular necrosis was obvious in the CCl₄ model compared to rats in control group. The lobular necrosis was significantly decreased by losartan in three treated groups. There was an increase in the area of fibrosis in model rats compared with rats in control group. There was a significant decrease in the losartan treated rats (Table 3, Figure 1).

Table 3 Degree of fibrosis

	<i>n</i>	0	I	II	III	IV	<i>U</i> -value
Control	10	10	0	0	0	0	
Model	7	0	0	1	2	4	
Losartan (20 mg/kg)	7	0	2	3	1	1	2.05 ^a
Losartan (10 mg/kg)	8	0	2	4	1	1	2.31 ^a
Losartan (5 mg/kg)	7	0	1	3	2	1	1.49

^a $P < 0.05$ vs model

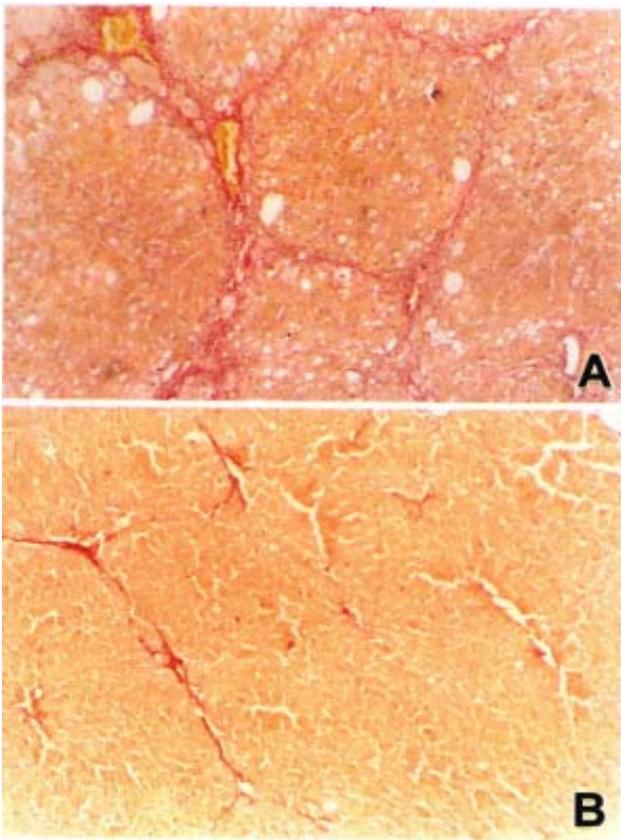


Figure 1 Collagen expression in liver section of model rat (van Gieson staining, $\times 200$) (A), and of losartan treated group (van Gieson staining, $\times 200$). Significantly less fibrosis can be noted compared to that in the model group (B).

Expression of AT1 receptors, TGF- β , and α -SMA

Compared with normal liver tissue, in which AT1 receptors mainly locate in vasculature, in the fibrotic liver tissue, the expression of AT1 receptors significantly enhanced, and scattered in fibrotic areas. The expression of AT1 receptors was markedly reduced by losartan (Figure 2). The expression of α -SMA was a marker of HSC activation. Immunohistochemical detection demonstrated that vascular smooth muscle cells and pericytes were positive for α -SMA in control livers, whereas HSCs strongly positive of α -SMA were observed in rats of model group, and they were scattered along the sinusoidal walls. Many α -SMA-positive HSCs were detected in the area of centrilobular and periportal fibrotic bands in rats of model group. Compared with model group, liver of rats treated with losartan, showed markedly reduced numbers of α -SMA-positive HSC. At same time, its serum levels of PC III and LN were also significantly decreased ($P < 0.05$).

Similar to the α -SMA, TGF- β was also strongly expressed in areas of periportal fibrotic bands in rats of model group. In contrast, livers of rats treated with losartan showed significantly reduced numbers of TGF- β -positive cells (Figure 3).

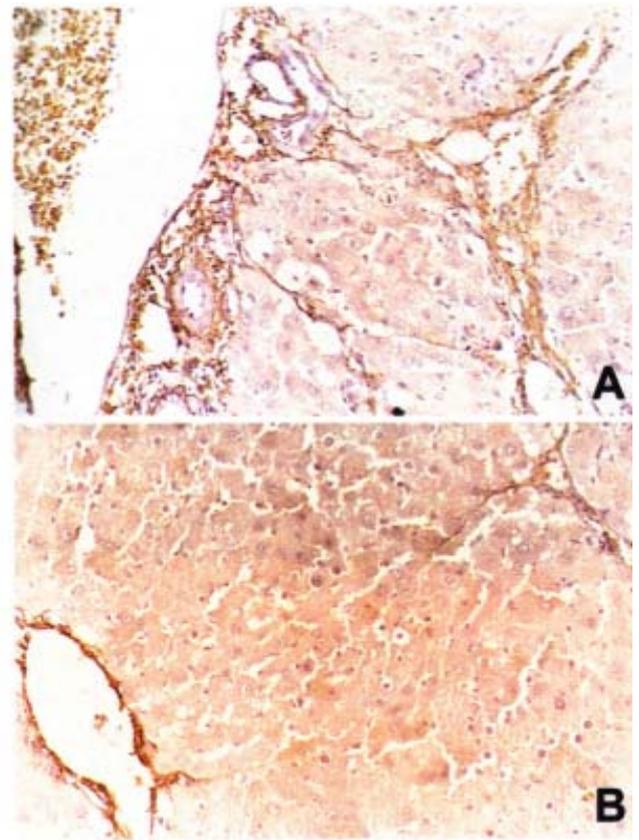


Figure 2 AT1 receptor expression in liver section of model rat (DAB staining, $\times 200$). AT1 receptor is seen mainly scattered in fibrotic areas and vascular wall (A), and of losartan treated group (DAB staining, $\times 200$). Note significantly less AT1 expression than that seen in the model group (B).

DISCUSSION

Our study firstly demonstrated that AT1 receptor antagonist, losartan, could slow the progression of hepatic fibrosis induced by CCl_4 . Activated HSC might express AT1 receptors in fibrotic liver tissue.

Over the last decade, many lines of evidence have demonstrated that local RAS activation was the major mechanism of cardiac and renal interstitial fibrosis^[33-37]. *In vivo* studies have shown that ACE inhibitors and AT1 receptors antagonists can limit the progression of cardiac, renal, and pulmonary fibrosis^[23,38-41], and the mechanism is independent of their dynamic effects. This is based on several *in vitro* and *in vivo* findings. First, all cardiac and renal fibroblasts express AT1 receptors; secondly, Ang II induces mitogenic response, increases protein synthesis, production of collagen, and TGF- β in fibroblasts in a dose-dependent manner^[29,42,43]. These results support the notion that Ang II can both directly act on fibroblasts and/or enhance the expression of TGF- β .

In the present study, the three different doses (20 mg/kg, 10 mg/kg, 5 mg/kg) of losartan were given to the rats. As the results shown, losartan could limit the progression of the hepatic fibrosis in a dose-dependent

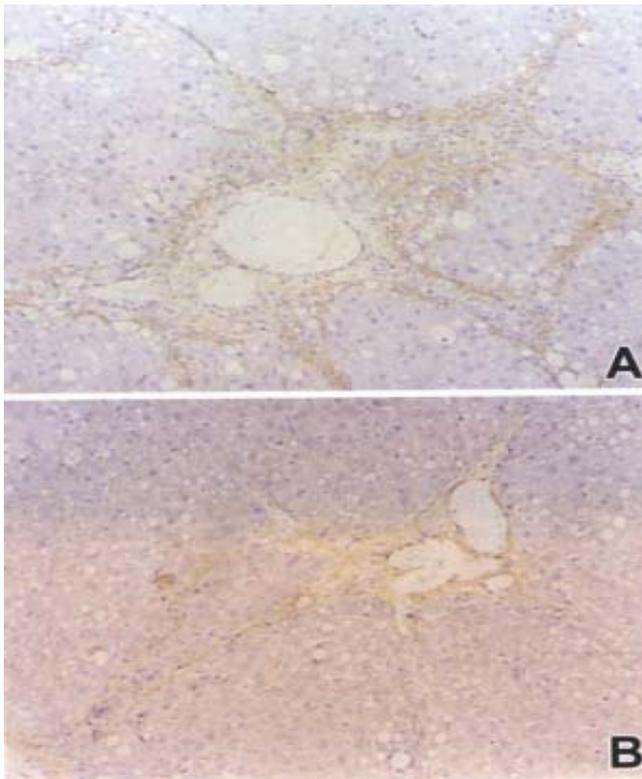


Figure 3 TGF- β expression in liver section of model rat (DAB staining, $\times 200$). TGF- β expression is seen scattered in the fibrotic areas and vascular wall (A), and of losartan treated group (DAB staining, $\times 200$). Note significantly less TGF- β expression than that seen in the model group (B).

manner. At the dose not influencing systolic blood pressure (10 mg/kg) in the normotensive rats^[44], losartan could attenuate the fibrosis, but even at a very low dose (5 mg/kg), had a weak but significant effect. In agreement with this study, Boffa *et al*^[45] also reported that losartan completely prevented collagen I gene activation and attenuated the degree of fibrosis without influencing the systolic pressure in the kidneys of transgenic mice. This suggested that Ang II might act as an important regulator in the progression of hepatic fibrosis induced by CCl₄.

Using the immunohistochemical methods, we observed the expression of AT1 receptors in liver tissue. As the results shown, this is the first report that confirms that the liver tissue expresses AT1 receptors. Compared with normal rats, where AT1 receptors are mainly located in the vasculature, the expression of AT1 receptors were significantly enhanced in rats of model group, which were mainly located in fibrotic area, and correlated with the degree of fibrosis. The results suggested that the expression of AT1 receptors might relate to hepatic fibrogenesis. Furthermore, immunostaining indicated that the distribution of AT1 receptor correlated with the expression of TGF- β and α -SMA. As has been recently reported, in a model of chronic cyclosporine (CsA), TGF- β played a role in CsA-

induced tubulointerstitial fibrosis and arteriopathy by stimulating ECM protein synthesis and inhibiting ECM degradation. Losartan resulted in decreasing expression of TGF- β and synthesis of ECM^[46]. Zhang *et al*^[28] have also reported that losartan is effective in reducing the increasing expression of AT1 receptors and ECM protein in infarcted heart tissue. Based on the above data, we postulated that expression of AT1 receptor might be related to the activation and ECM synthesis of HSCs.

Another interesting finding in our study was that losartan could ameliorate the hepatocyte injury, as reflected by the release of liver enzymes. The explanation for this observation is not known, but it might relate to protection of the hepatocyte from free radical-mediated damage. Recently, Anthuber *et al*^[47] demonstrated that non-thiol-containing ACE inhibitor, enalapril, could attenuate the hepatocyte injury induced by ischemia/reperfusion. The prevailing mechanism of action was considered to relate with modulation of the angiotensin, bradykinin, and prostacyclin metabolism. Whether losartan has some as-yet-unknown, specific, protective property, remains to be determined in future studies.

That transforming growth factor β (TGF- β) is a key molecule responsible for tissue fibrosis, provides a basis for targeting TGF- β as an antifibrotic agent^[48-50]. Recently, Sun *et al*^[31] found that the early induction of TGF- β 1 via the angiotensin II type 1 receptor played a major role in the development of cardiac fibrosis in infarcted heart. Shihab *et al*^[46] described that losartan reduced TGF- β overproduction in a dose-dependent manner, slowing the rate of renal interstitial fibrosis^[22].

Overproduction of TGF- β and activation of HSC are key processes in the progression of hepatic fibrosis. Our results demonstrated that losartan could reduce the expression of TGF- β and α -SMA in liver tissue and suppress fibrosis progression. It is suggested that RAS also participates in the progression of hepatic fibrosis induced by CCl₄ in rats.

In conclusion, our results demonstrated that (1) AT1 receptor antagonist, losartan, could limit the progression of the hepatic fibrosis induced by CCl₄. The mechanism may be related to the decrease in the expression of AT1 receptors and TGF- β , ameliorating the injury of hepatocyte; (2) activation of local renin-angiotensin system might relate to hepatic fibrogenesis; (3) in the progression of fibrosis, activated hepatic stellate cells might express AT1 receptor.

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Participation of CD45, NKR-P1A and ANK61 antigen in rat hepatic NK cell (pit cell) mediated target cell cytotoxicity

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Subject headings hepatic NK cells; pit cells; cytolysis; apoptosis; perforin/granzyme pathway

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Abstract

AIM Several triggering receptors have been described to be involved in natural killer (NK) cell-mediated target cytotoxicity. In these studies, NK cells derived from blood or spleen were used. Pit cells are liver-specific NK cells that possess a higher level of natural cytotoxicity and a different morphology when compared to blood NK cells. The aim of this study was to characterize the role of the NK-triggering molecules NKR-P1A, ANK61 antigen, and CD45 in pit cell-mediated killing of target cells.

METHODS ⁵¹Cr-release and DNA fragmentation were used to quantify target cell lysis and apoptosis, respectively.

RESULTS Flow cytometric analysis showed that pit cells expressed CD45, NKR-P1A, and ANK61 antigen. Treatment of pit cells with monoclonal antibody (mAb) to CD45 (ANK74) not only inhibited CC531s or YAC-1 target lysis but also apoptosis induced by pit cells. The mAbs to NKR-P1A (3.2.3) and ANK61 antigen (ANK61) had no

effect on pit cell-mediated CC531s or YAC-1 target cytolysis or apoptosis, while they did increase the Fcγ receptor positive (FcγR⁺) P815 cytolysis and apoptosis. This enhanced cytotoxicity could be inhibited by 3,4-dichloroisocoumarin, an inhibitor of granzymes.

CONCLUSION These results indicate that CD45 participates in pit cell-mediated CC531s and YAC-1 target cytolysis and apoptosis. NKR-P1A and ANK61 antigen on pit cells function as activation structures against FcγR⁺ P815 cells, which was mediated by the perforin/granzyme pathway.

INTRODUCTION

Natural killer (NK) cells can kill certain tumor cells or virus-infected cells without prior sensitization and thus play an important role in host anti-tumor defense^[1]. Cytotoxic activity of NK cells is achieved by two distinct mechanisms, i.e. necrosis (cytolysis) and apoptosis, and is believed to be regulated by triggering and inhibiting receptors on NK cells^[1-3]. Several candidate receptors involved in triggering a cytotoxic reaction, such as NKR-P1A (CD161A) and an NK-activation structure recognized by monoclonal antibody (mAb) ANK61 (ANK61 antigen), have been described on rat NK cells^[2,4,5].

NKR-P1A is a lectin-like surface molecule expressed on all rat NK cells^[4]. The ligands for NKR-P1A are oligosaccharides. Interactions of these oligosaccharides on target cells with NKR-P1A on NK cells are crucial for target cell recognition and for NK cytotoxicity^[6]. Monoclonal antibody to NKR-P1A (3.2.3) has been shown to stimulate NK cell phosphoinositide turnover, calcium mobilization, degranulation and NK cytotoxicity against certain Fcγ receptor positive (FcγR⁺) tumor target cells^[4,7]. These findings demonstrate that NKR-P1A is an activating molecule on rat NK cells that recognizes target cells and induces cytotoxicity.

Recently, a rat NK-activation structure (ANK61 antigen) recognized by mAb ANK61 has been described^[5]. Cross-linking of the ANK61 antigen and FcγR⁺ tumor targets with mAb ANK61 resulted in enhanced killing of the targets by NK cells^[5].

CD45, which is expressed by most leukocytes, including NK cells, has been shown to be involved in NK

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cell-mediated target cell cytotoxicity^[8-10]. It is reported that mAbs against CD45 inhibit NK cell-mediated target cell killing^[8,9] and CD45-negative NK cells fail to lyse tumor targets^[10]. The cytoplasmic domain of CD45 has tyrosine phosphatase activity, which is believed to play an important role in the regulation of NK activity^[8,11].

Pit cells are liver-specific NK cells^[12]. Compared to blood NK cells, rat pit cells possess a higher level of cytolytic activity against a variety of tumor target cells, including NK resistant P815 cells, which is comparable to the cytotoxicity level of lymphokine-activated killer (LAK) cells^[13]. These data indicate that pit cells have different characteristics from blood NK cells. Although much is known about the NK receptors that participate in NK cell activation and target cell killing, the surface receptors involved in pit cell-mediated target cell killing have not yet been clarified. Moreover, involvement of the above mentioned antigens in NK cell-mediated target cell apoptosis has remained elusive, since ⁵¹Cr-release assay employed in the previous studies^[4,5,10] indicates only cytolysis, rather than apoptosis. In the present work, we used mAbs against CD45, NKR-P1A, and ANK61 antigen to evaluate their roles in pit cell-mediated target cell lysis and apoptosis.

MATERIALS AND METHODS

Isolation and purification of pit cells

Male Wistar rats (Proefdierencentrum, Leuven, Belgium) weighing (250–280) g were used at an age between 8 to 12 wk. Hepatic NK cells (pit cells) were isolated according to protocols described before^[14]. The purity of the recovered pit cells was at least 90%, as evaluated by morphology on May-Giemsa-stained cytospin preparations and by flow cytometric analysis using mAb 3.2.3. The viability of the recovered cells was more than 95%, as determined by Trypan blue exclusion. The procedures used in this study were approved by the local ethical committee (license No LA1230212).

Tumor cell line

CC531s, a dimethylhydrazine-induced colon carcinoma of Wag/Rij (inbred strain of Wistar) rats^[15], P815, a murine mastocytoma, and YAC-1, a mouse T cell lymphoma, were maintained in culture medium RPMI-1640 (Gibco, Life Technologies, Gent, Belgium), supplemented with 10% fetal calf serum (Eurobiochem, Bierges, Belgium), benzylpenicillin (100 kU/L), streptomycin (100 mg/L), and glutamine (0.2 mmol/L) (Gibco, Life Technologies, Gent, Belgium).

Reagents and antibodies

The following mouse anti-rat mAbs were used: 3.2.3 (anti-NKR-P1A, IgG_{2b} or IgG₁)^[4], ANK74 (anti-CD45, IgG₁)^[8], and ANK61 (anti-rat NK activation structure, IgG₁)^[5] were developed in Department of Surgery and Pathology, Leiden University Medical Center, the

Netherlands. 3,4-dichloroisocoumarin (DCI), a granzyme inhibitor^[16] was purchased from Sigma (Bornem, Belgium).

Flow cytometry

The expression of surface antigens on the cells was measured by one or two-color flow cytometric analyses as described previously^[17]. Briefly, a quantity of 0.5×10^6 cells per sample was incubated (30 min, 4 °C) with the primary antibodies, 3.2.3, ANK74, and ANK61. Cells were then washed thrice with cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.02% sodium azide. Subsequently, cells were incubated with fluorescein-conjugated anti-mouse IgG₁ and biotin-conjugated anti-mouse IgG_{2b} (Gilbertsville, PA). After incubation and washing, cells were incubated with streptavidin-phycoerythrin (Gilbertsville, PA). Then, cells were washed and fixed with 2% paraformaldehyde in PBS and analyzed (FACstar, Becton Dickinson, Mountain View, CA). Isotype-matched irrelevant antibodies were used as a control.

⁵¹Cr-release assay

Cytolysis was measured after 4 h incubation of P815 and YAC-1 cells, and 18 h for CC531s cells. ⁵¹Cr-release assay was performed using round-bottomed 96-well microplates as described previously^[18]. Briefly, 1×10^6 target cells/0.5 mL were labeled with 9.25 MBq Na ⁵¹CrO₄ (Amersham Belgium, Gent, Belgium) for 80 min at 37 °C. Before the incubation, freshly isolated pit cells were preincubated with either saturating amounts of the mAbs (final concentration for each mAb was 10 mg/L), 3.2.3, ANK61, ANK74, or irrelevant mouse IgG₁ for 15 minutes at room temperature. The mAbs were also present during the assay and the treatments with these mAbs did not affect the viability of the cells. The target cells were seeded at a concentration of 1×10^4 cells/well. Suspensions of effector cells were then added to the wells at effector to target (E:T) ratios of 10:1 in a final volume of 200 μL. All assays were done in triplicate. For the experiments using DCI, pit cells were preincubated with 50 μmol/L DCI for 30 min at 37 °C. After washing twice with medium, the cells were incubated with the target cells as mentioned above. After a incubation at 37 °C for 4 or 18 h, 100 μL of supernatant per well was aspirated and counted in a gamma counter to determine experimental release. Results were expressed as percentage of specific lysis according to the formula:

$$\% \text{ specific cytolysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100\%$$

Experimental release represents cpm (counts per minute) release from target cells in the presence of effector cell and mAbs. Spontaneous release was obtained from wells containing labeled target cells and medium only. Maximal release of ⁵¹Cr-labeled target cells was determined

after adding 20 μL 10% sodium dodecyl sulfate (SDS) detergent to the labeled target cells.

Quantitative DNA fragmentation assay

DNA fragmentation in the target cells was determined as described previously^[19]. Briefly, to label the DNA of the target cells, 5×10^5 cells in 250 μL medium were incubated with 0.37 MBq [methyl-³H] thymidine (³H]-TdR) (Amersham, Buckinghamshire, U.K) for 3 h at 37°C. Before the coincubation, freshly isolated pit cells were preincubated with the mAbs as mentioned above. The pit cells (10^5 cells in 100 μL) and the target cells (10^4 cells in 100 μL) (E:T = 10:1) were placed in triplicate in 1.5 mL microcentrifuge tubes. The total volume per microcentrifuge tube was 200 μL . For the experiments using DCI, pit cells were preincubated with 50 $\mu\text{mol/L}$ DCI for 30 min at 37°C. After washing twice with medium, the cells were coincubated with the target cells as mentioned above. After 3 h coincubation at 37°C and centrifugation at $300 \times g$ for 10 min, the incubation medium was removed from the tubes. Subsequently, the pelleted cells were lysed with 0.5 mL of cold lysis buffer (4°C) (5 mmol/L Tris, 2 mmol/L EDTA, 1% Triton X-100, pH 7.4) for 30 min at 4°C. Then, the lysates were ultracentrifuged ($10,000 \times g$ for 15 min at 4°C) to separate fragmented DNA from intact DNA. Radioactivity (cpm) in the incubation medium, in the $10,000 \times g$ supernatant and in the $10,000 \times g$ pellet was determined in a beta counter (Beckman, Fullerton, CA, USA). The percentage fragmented DNA was calculated using the following formula:

$$\% \text{ specific DNA fragmentation} = \frac{\text{cpm}_{\text{fr,exp}} - \text{cpm}_{\text{fr,spont}}}{\text{cpm}_{\text{total}} - \text{cpm}_{\text{fr,spont}}} \times 100\%$$

in which: cpm_{fr} = the radioactivity in the incubation medium plus the cpm in the $10,000 \times g$ supernatant; $\text{cpm}_{\text{total}}$ = cpm_{fr} + radioactivity in the $10,000 \times g$ pellet; exp = experimental (CC531s cells with pit cells); spont =

spontaneous (CC531s cells and medium only).

Hoechst 33342 (HO 342)/propidium iodide (PI) staining

The method of HO 342 and PI staining has been described previously^[19]. In short, target cells, at a concentration of 1×10^4 cells per well in a 96m multiwell plate, were coincubated with pit cells (E:T = 10:1). After 3 h coincubation, the cells were stained with HO 342, which stains DNA blue, and PI, which only penetrates necrotic cells through the damaged cell membrane and stains DNA red. Cells were viewed under a Leica DM IRB/E inverted fluorescence microscope (Leica, Heidelberg, Germany) with ultraviolet excitation at 340 to 380 nm. The apoptotic target cells were determined by their characteristic apoptotic morphological changes of the nucleus, i.e. condensation of chromatin and nuclear fragmentation. Target cells alone acted as spontaneous apoptosis control.

Statistical analysis

Results were given as the mean with the corresponding standard deviation. Statistical analysis was performed by Student's *t* test or one-way ANOVA with post-hoc multiple comparison analysis made by Duncan test, using SPSS statistical package (SPSS Inc., Chicago, IL, USA). Statistical significance between two groups was considered at the level of $P < 0.05$.

RESULTS

Expression of surface antigens on pit cells

Flow cytometric analysis was used to determine the expression of surface antigens on pit cells and target cells. In two-color flow cytometric analysis, mAb 3.2.3 was used to stain pit cells^[20]. Pit cells were shown to express CD45, which was recognized by mAb ANK74, and an NK activation structure, which was recognized by mAb ANK61 (Figure 1). CC531s, P815, and YAC-1 cells did not express these NK-associated antigens (data not shown).

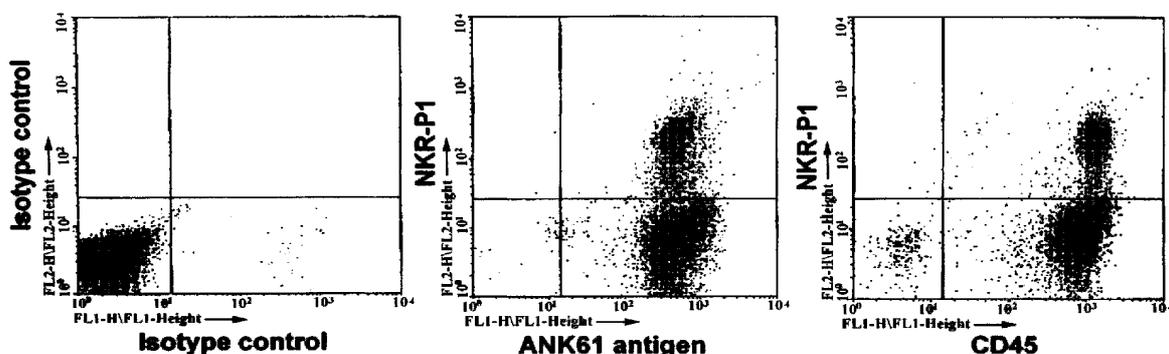


Figure 1 Expression of CD45 and ANK61 antigen on hepatic NK cells (pit cells). The cells were washed out of the liver by sinusoidal lavage. After Ficoll-Paque gradient centrifugation and nylon-wool adherence to remove erythrocytes, granulocytes, monocytes and B lymphocytes, the cells were stained with anti-NKR-P1A mAb (3.2.3) (phycoerythrin) and anti-ANK61 antigen (ANK61), anti-CD45 mAb (ANK 74) (fluorescein) and analyzed by two-color flow cytometry. CD45 and ANK61 antigen were expressed on the x-axis (fluorescein), NKR-P1A on the y-axis (phycoerythrin).

Effect of CD45, NKR-P1A, and ANK61 antigen on pit cell-mediated target cell lysis

To investigate the function of these surface antigens in pit cell-mediated cytolysis of targets, the mAbs against these antigens were used in ^{51}Cr -release assays. In the presence of anti-CD45 mAb ANK74, the lysis of CC531s and YAC-1 cells by pit cells was significantly inhibited ($P < 0.01$, $P < 0.05$) (Figure 2A, 2B). The presence of mAbs 3.2.3 and ANK61 had no effect on pit cell-mediated lysis against-CC531s and YAC-1 targets (Figure 2A, 2B). These data indicate that CD45 is involved in pit cell-mediated cytolysis against CC531s and YAC-1 targets.

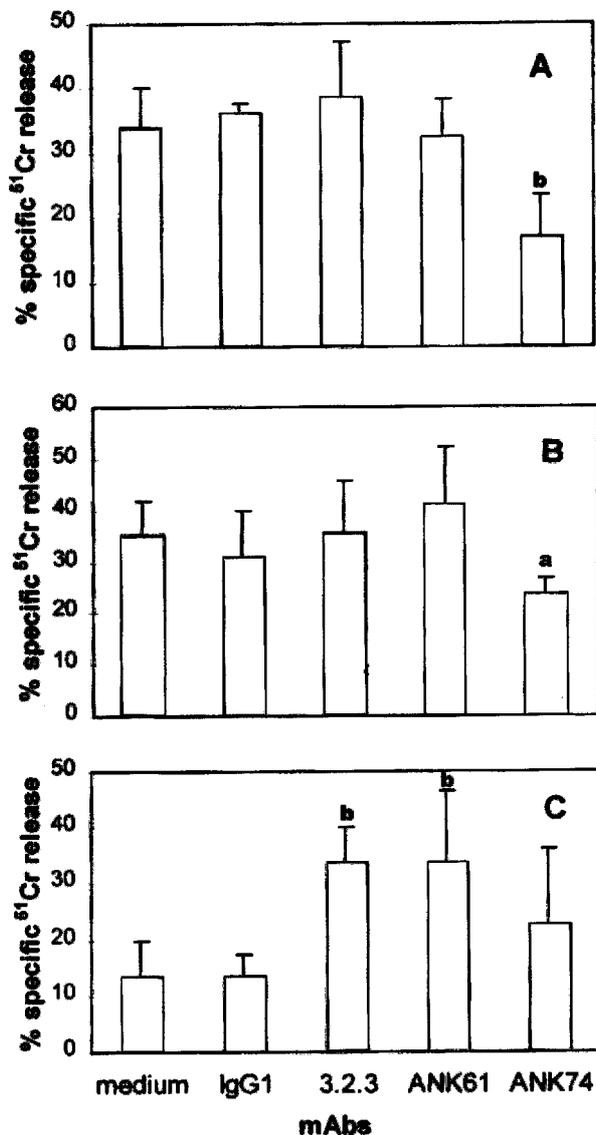


Figure 2 Effect of the mAbs on pit cell-mediated CC531s (A), YAC-1 (B) and P815 (C) target cell cytolysis. ^{51}Cr -labeled target cells were incubated at an E:T ratio of 10:1 with freshly isolated pit cells in the absence or presence of the mAbs. (A), Cytolysis of CC531s cells was measured in an 18h ^{51}Cr -release assay. (B), Cytolysis of YAC-1 cells was measured in a 4-h ^{51}Cr -release assay. (C), Cytolysis of P815 cells was measured in a 4-h ^{51}Cr -release assay. Values are mean \pm SD from four different experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs the control.

It is reported that mAbs 3.2.3 or ANK61 can induce cytolysis in $\text{Fc}\gamma\text{R}^+$ target cells by NK cells by redirected antibody-dependent cellular cytotoxicity (rADCC)^[4,5]. In order to investigate whether these mAbs play a similar role in pit cells, cytolytic activity of pit cells was measured by a 4-h ^{51}Cr -release assay using the $\text{Fc}\gamma\text{R}^+$ P815 cell line^[21], a murine mast ocytoma, as target cells. As shown in Figure 2C, the mAbs 3.2.3 and ANK61 significantly increased the cytolysis of P815 cells by pit cells ($P < 0.01$). Anti-CD45 mAb ANK74 did not significantly increase the cytolysis (Figure 2C).

Effect of CD45, NKR-P1A, and ANK61 antigen on pit cell-mediated target apoptosis

When CC531s^[19], P815 (Vermijlen D, unpublished data) or YAC-1 targets (Figure 3) were coincubated with pit cells, these target cells showed typical morphological characteristics of apoptosis like nuclear fragmentation. To address the effect of the surface antigens in pit cell-mediated apoptosis of these targets, a quantitative DNA fragmentation assay was used. The results showed that the anti-CD45 mAb ANK74 significantly inhibited pit cell-mediated DNA fragmentation of CC531s and YAC-1 cells ($P < 0.05$, $P < 0.01$) (Figure 4A, 4B). The presence of mAbs 3.2.3, ANK61 had no effect on DNA fragmentation of CC531s and YAC-1 cells by pit cells (Figure 4A, 4B), but increased the DNA fragmentation of P815 cells induced by pit cells ($P < 0.01$) (Figure 4C). The anti-CD45 mAb ANK74 did not significantly affect the DNA fragmentation of P815 cells by pit cells (Figure 4C).

Inhibition of anti-NKR-P1A and ANK61 antigen mAb-enhanced P815 cell cytotoxicity by DCI

To address whether mAbs 3.2.3 and ANK61 enhanced $\text{Fc}\gamma\text{R}^+$ P815 cell killing by pit cells via the perforin/granzyme pathway, we used DCI, an inhibitor of granzymes in intact cells, to investigate the role of granzymes in the process. When pit cells were preincubated with 50 $\mu\text{mol/L}$ DCI, the mAb-enhanced P815 cytolysis and apoptosis by pit cells were completely inhibited ($P < 0.05$, $P < 0.01$) (Figure 5A, 5B). These results suggest that mAbs 3.2.3 or ANK61 induce cytotoxicity in $\text{Fc}\gamma\text{R}^+$ P815 cells by pit cells via the perforin/granzyme pathway.

DISCUSSION

Pit cells are liver-specific NK cells and, together with Kupffer cells, constitute the natural cellular defense against invading cancer cells in the liver^[12]. It has been shown that pit cells are four to eight times more cytotoxic against YAC-1 and CC531s cells than blood NK cells and are able to kill NK-resistant, LAK-sensitive P815 cells^[13]. However, the surface receptors involved in pit cell-mediated cytotoxicity against tumor targets have remained elusive. Present data show that the mAbs against the NK-triggering molecules NKR-P1A and ANK61 antigen increased not only cytolysis but also apoptosis of $\text{Fc}\gamma\text{R}^+$ P815 cells, but had no effect on $\text{Fc}\gamma\text{R}$ YAC-1 and

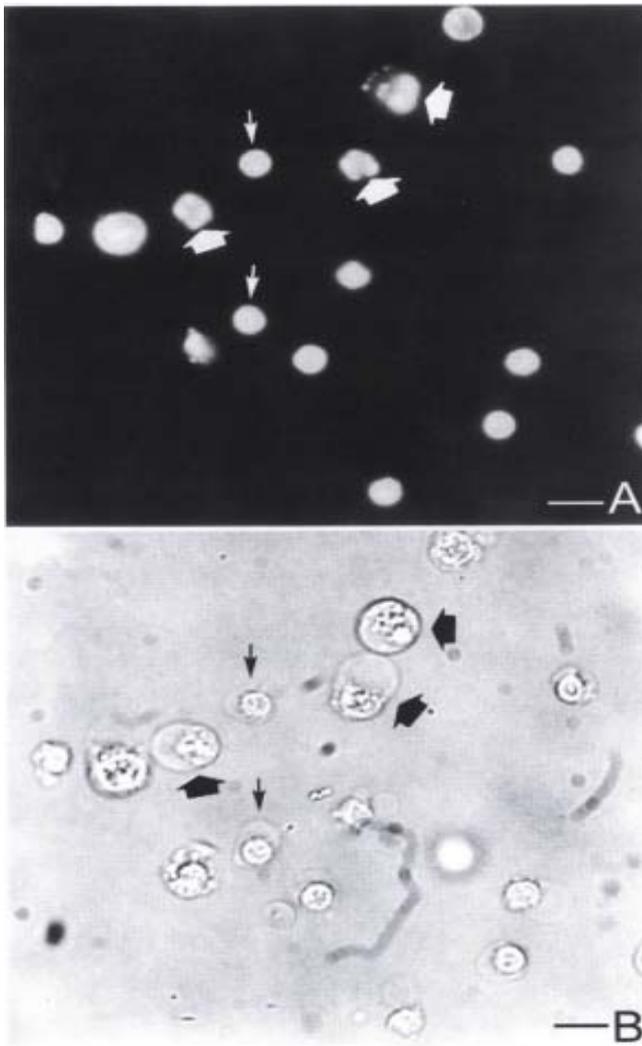


Figure 3 Fluorescence and light micrographs of YAC-1 cells coincubated with pit cells at an E:T ratio of 10:1 for 3 h. (A) Fluorescence micrograph showing the apoptotic YAC-1 cells with fragmented nuclei (thick arrows) and pit cells (thin arrows). Light micrograph shows the same field as (A). Bar = 5 μ m.

CC531s cytotoxicity induced by pit cells. These results indicate that NKR-P1A and ANK61 antigen on pit cells function as triggering molecules against $Fc\gamma R^+$ target cells, but not against $Fc\gamma R^-$ targets. It has been demonstrated that mAbs 3.2.3 and ANK61 enhance cytolytic activity of NK and LAK cells only against $Fc\gamma R^+$ tumor target cells by rADCC^[4,5,7]. Other evidence to support this observation was that, in our experiments, cross-linking of NKR-P1A on pit cells with mAb 3.2.3 did not induce granule exocytosis from pit cells, as observed in May-Giemsa-stained cytospin preparations (data not shown). This is in contradiction with a report describing that mAb 3.2.3 can induce degranulation and release of granzymes from mNK and IL-2 activated NK cells^[22]. Furthermore, after incubation of pit cells with mAb 3.2.3, the medium and the pit cells were separated by centrifugation. The medium

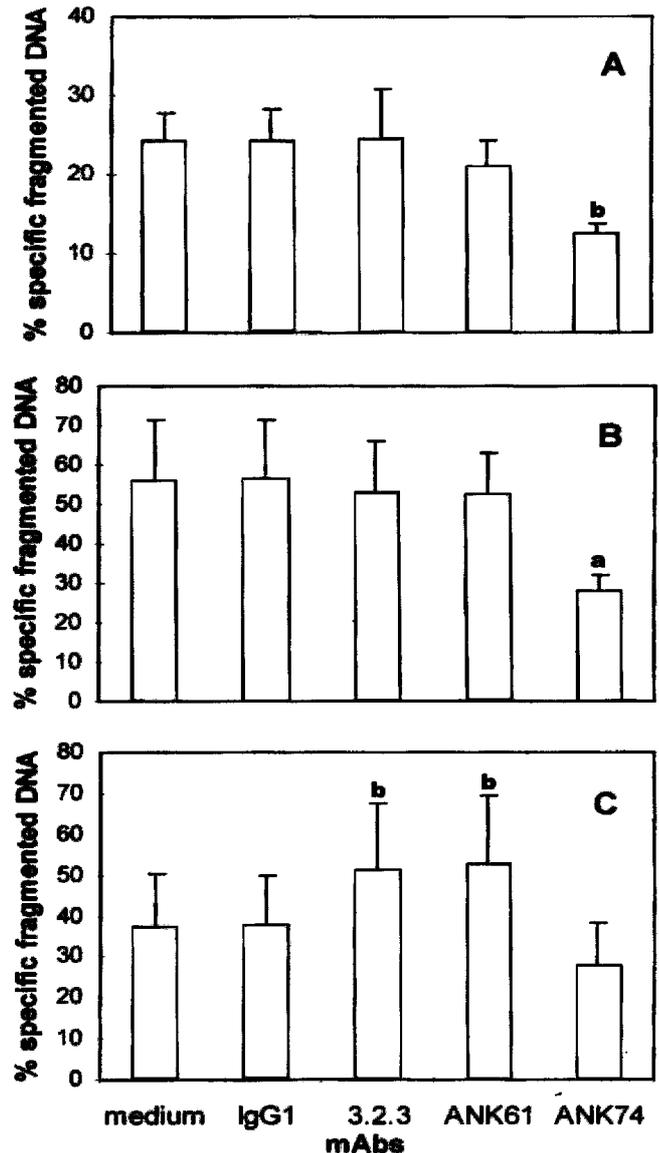


Figure 4 Effect of the mAbs on pit cell-induced CC531s (A), YAC-1 (B) and P8 15 (C) target cell apoptosis. [3 H]-TdR labeled target cells were incubated at an E:T ratio of 10:1 with freshly isolated pit cells for 3 h in the absence or presence of the mAbs. Values are mean \pm SD from four different experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs the control.

did not kill CC531s and YAC-1 cells, whereas the pit cells' cytotoxicity against these targets was comparable to freshly isolated pit cells (data not shown). Moreover, it has also been shown that NKR-P1A negative NK clones are able to lyse the same type of target cells as NKR-P1A positive NK clones^[23] and expression of NKR-P1A is not an absolute requirement for NK activity^[24]. These data suggest that NKR-P1A is not the sole activation molecule on NK cells. Actually, a diversity of activation molecules on NK cells has been described^[2,3,5,25]. NK cells might use these different activation molecules to lyse different target cells.

It is believed that mAbs 3.2.3 or ANK61-enhanced cytotoxicity in $Fc\gamma R^+$ target cells by NK cells is a rADCC reaction^[4,5]. The present data show further that these

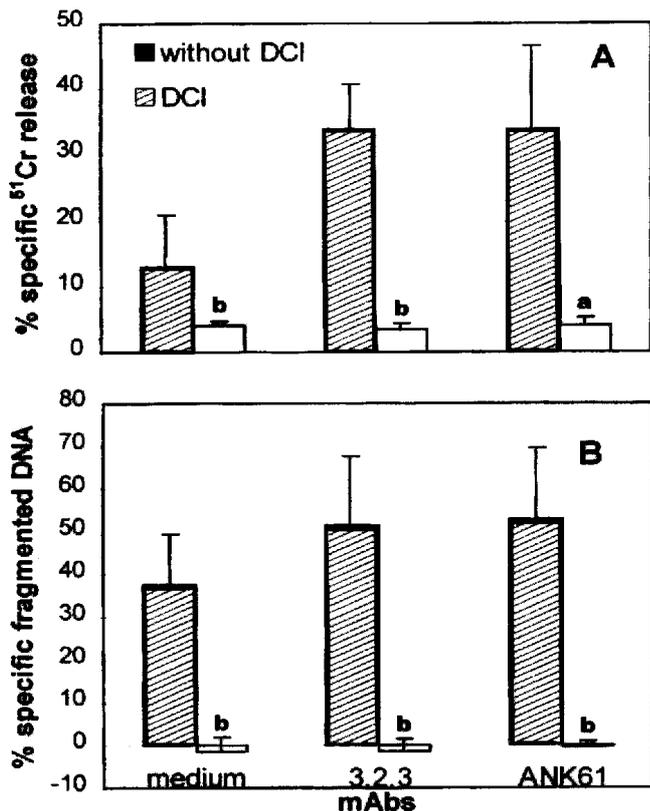


Figure 5 Effect of DCI on mAbs 3.2.3 and ANK61-enhanced Fc γ R⁺ P815 cell cytotoxicity (A) and apoptosis (B) by pit cells. Pit cells were preincubated with 50 μ mol/L DCI for 30 min at 37°C. After washing twice, the cells were coincubated with P815 cells as mentioned in materials and methods. Values are mean \pm SD from three different experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs the corresponding control.

mAb-enhanced cytotoxicity and apoptosis of P815 cells by pit cells are mediated by the perforin/granzyme pathway because this enhanced cytotoxicity can be completely inhibited by the granzyme inhibitor DCI.

Although the function of CD45 is well known in NK cells^[8-11], its role in pit cells has not yet been clarified. We showed here that CD45 was involved in the pit cell induced cytotoxicity and apoptosis of CC531s and YAC-1 cells but not of P815 cells. The reason that CD45 on pit cells had different effects on pit cell-mediated cytotoxicity against different targets is not clear. It has been reported that CD45 is not essential for Fc γ R-mediated function in NK cells, since NK cells in CD45^{-/-} mice have normal cytotoxic activities, comparable to normal mice (CD45^{+/+})^[26]. Another possible explanation is that CD45 plays a regulatory role in signal transduction through surface receptors on NK cells, such as NKR-P1A, LFA-1, and killer cell inhibitory receptors (KIR) in NK cell-mediated target killing^[8,10,11]. A diversity of receptors is found to be involved in NK cell activation^[2,3]. It could be that CD45 plays a different regulating role in different NK activation receptors. On the other hand, the regulatory role of CD45 in the inhibitory signal pathway via KIR is dependent on the expression level of MHC class I on target

cells^[8]. The anti-CD45 mAb inhibits only the lysis of target cells expressing low level of MHC class I, such as CC531s^[8] and YAC-1^[27], whereas the lysis of target cells expressing high level of MHC class I, such as P815^[27], is not inhibited^[8].

In summary, CD45 on pit cells participated in pit cell-mediated CC531s and YAC-1 but not P815 cytotoxicity and apoptosis. NKR-P1A and ANK61 antigen on pit cells were involved in pit cell-mediated Fc γ R⁺ P815 cell cytotoxicity and apoptosis, but not in Fc γ R⁻ CC531s and YAC-1 cell cytotoxicities. The mAbs 3.2.3 and ANK61-induced cytotoxicities of Fc γ R⁺ P815 cells by pit cells were mediated by the perforin/granzyme pathway. These findings provide evidence that the same molecules on pit cells and blood or spleen-derived NK cells regulate target cell lysis and apoptosis. The difference in cytotoxic capacity between these cell types therefore may be on a quantitative rather than a qualitative level.

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Study on the risk factors of lymphatic metastasis and the indications of less in vasive operations in early gastric cancer

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INTRODUCTION

The principle of surgical treatment for gastric cancer is the radical resectioning although the suitable resecting range for different cases of gastric cancer is still being argued upon^[1-9]. However, the diagnostic accuracy of early gastric cancer (EGC) without lymphatic metastasis has obviously improved with an improvement in the diagnostic technique and due to the accumulation of knowledge on the biological profiles of EGC^[10-17]. The D₂ lymph node excision was used as a regular operation to treat the EGC previously. But the concept for the EGC without lymphatic metastasis has gradually changed and the less invasive resections has been applied in some cases^[18-20]. This study aimed at investigating the risk factors of lymphatic metastasis in EGC in order to find out the proofs for the suitable indications for less invasive operations such as endoscopic mucosal resectioning (EMR), laparoscopic and laparotomic resectioning.

MATERIALS AND METHODS

From 1978 to 1999, 520 cases of EGC with operative and pathological analyses were studied. Among them, 287 were male and 233 female, with an average age of 62 years (32-81). The samples from the primary tumor and the

metastatic lymph nodes were collected and examined as previous reports distinguishing the tumor was limited in mucosal layer (m Ca) or submucosal layer(sm Ca) of stomach^[10] were scanty. According to the gross differences of the following groups were constituted of II_c+III, II_c, II_b, II_a and I.

Sixty-six cases diagnosed by the ultrasound endoscopic examination received the less invasive operative technique. This resection retained the partial omentum and resected the 1st station and No 7 of lymph nodes. In this operation, the dissection was made and the omentum was cut off in (3-4) cm places away from or along the blood vessels in greater and less curvature of the stomach. And the No 7 lymph nodes were resected and blocked or picked out. Hepatic branch of vagus had better to be protected possibly. Mouth margin and anal margin for gastr ectomy was over 2 cm away from the tumor site. All of 259 cases received radical resectioning of N₂ lymph nodes or a more radical resection.

The rate of lymphatic metastasis and the morbidity of intestinal obstruction after operation and the expression of long-term survival were statistically analyzed by Check test or by log-rank test respectively. *P* value less than 0.05 was considered statistically significant.

RESULTS

In this study, the risk factors regarding the occurrence of lymphatic metastasis were investigated. The long-term survival analysis and the complications relating to the less invasive operation were studied at the same time.

Gross type

Lymphatic metastasis happened the most easily in II_c+III subgroups of both m Ca and sm Ca, and secondly in II_c subgroups. In II_c+III subgroups, the lymphatic metastasis could be identified in 2 cases of m Ca and 20 cases of sm Ca, and one n₂ lymphatic metastasis was observed in m Ca and 10 in sm Ca (Table 1). No n₂ lymphatic metastasis could be found in other subgroups of m Ca. However, this n₂ lymphatic metastasis could be observed in sm Ca in all groups except II_b.

Invading depth

Forty-four cases of n₁ lymphatic metastasis and 14 cases of n₂ lymphatic metastasis (58 cases of lymphatic metastasis in

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total, 11.2%) could be demonstrated in all cases of EGC. Four (1.5%) of 275 cases suffering from the m Ca had lymphatic metastasis, of which one case shared n_2 lymphatic metastasis (Table 2). Lymphatic metastasis was identified in 54 (22.0%) out of 245 cases in sm Ca group, of which 13 cases were of n_2 lymphatic metastasis. This result showed that lymphatic metastasis was more frequent in sm Ca than in m Ca ($P < 0.05$).

Table 1 Lymphatic metastasis relating to gross types (%)

Gross types	m Ca (n = 275)	sm Ca (n = 245)
II _c +III	2.5 (2/85) ^{a,b}	25.3 (20/79)
II _c	1.7 (2/120) ^b	20.2 (17/84)
II _b	0 (0/3)	0 (0/4)
II _a +II _c	0 (0/29)	23.8 (10/42)
II _a	0 (0/34)	25.0 (2/8)
I	0 (0/4)	17.9 (5/28)

*Number of positive lymph nodes/total numbers of cases.
^b $P < 0.01$, vs each subgroup of II_b, II_a+II_c, II_a or I types.

Table 2 Lymphatic metastasis relate(LMR) of m and sm Ca (%)

Invading depth	n_1 LMR	n_2 LMR	Total LMR
m Ca (n = 275)	1.1 (3) [*]	0.4 (1)	1.5 (4) ^b
sm Ca (n = 245)	16.7 (41)	5.3 (13)	22.0 (54)
Total cases (n = 520)	8.5 (44)	2.7 (14)	11.2 (58)

*Number of cases. ^b $P < 0.01$, vs sm Ca.

Tumor size with or without ulcer

With tumor size less than 20 mm in m Ca, there was no lymphatic metastasis in 122 of 275 cases regardless whether accompanied with ulcer or not. In 153 cases with tumor larger than 20 mm, there were 4 cases of lymphatic metastasis in m Ca ($P < 0.01$, Table 3). Among 164 cases with m Ca and without ulcer, the lymphatic metastasis could be observed only in one with 25 mm of tumor. On the other hand, 3 of 111 cases of lymphatic metastasis could be found in ulcer group, in which all tumors were larger than 30 mm. These findings indicated that lymphatic metastasis was more significantly in cases with tumor size larger than 20 mm subgroups, especially those with ulcer ($P < 0.05$).

Table 3 Lymphatic metastasis related to tumor size with or without ulcer (%)

Ulcer diameter (cm)	m Ca (n = 275)		sm Ca (n = 245)	
	With ulcer (n = 111)	Without ulcer (n = 164)	With ulcer (n = 110)	Without ulcer (n = 135)
≤ 2	0 (0/43)	0 (0/79)	16.7 (3/18)	3.1 (1/32)
~ 3	0 (0/21)	2.4 (1/42)	20.8 (5/24)	21.1 (8/38)
~ 4	5.3 (1/19)	0 (0/15)	20.0 (4/20)	24.0 (6/25)
~ 5	6.7 (1/15)	0 (0/14)	18.8 (3/16)	18.2 (4/22)
≥ 6	1 (1/13)	0 (0/14)	43.8 (14/32)	33.3 (6/18)
Total No	2.7 (3/111) ^a	0.6 (1/164)	26.4 (29/110) ^a	18.5 (25/135)

^a $P < 0.05$, vs ulcer subgroup in each group.

In sm Ca, 4 out of 50 (8.0%) cases of lymphatic metastasis could be identified in cases with tumor size less

than 20 mm with or without ulcer, which showed that the radical resection with standard lymph node excision could be carried out even in small tumor sized sm Ca.

In 135 cases of sm Ca without ulcer, 25 (8.5%) cases were demonstrated to have lymphatic metastasis, out of which 12 (11.3%) cases belonged to n_2 lymphatic metastasis. But 29 out of 110 (26.4%) cases of sm Ca with lymphatic metastasis were proven to occur in the ulcer subgroup, in which 7 (6.4%, $P < 0.05$) cases had n_2 lymphatic metastasis. Therefore, the n_2 lymphatic metastasis happened more significantly in sm Ca than in m Ca.

Pathological classification

One hundred and forty-three out of 275 cases (52.0%) with m Ca had well-differentiated adenocarcinoma in which there was no lymphatic metastasis. Two out of 58 cases (3.4%) and 2 out of 57 cases (3.5%) sharing lymphatic metastasis were respectively identified in the poorly-differentiated group and the non-differentiated group of EGC. Fifty-four out of 245 (22.0%) cases with lymphatic metastasis were observed in sm Ca, out of which there were 21 (8.6%), 11 (4.5%), 15 (6.1%), and 7 (2.9%) cases respectively in each group of well-differentiated, poorly-differentiated, non-differentiated and ringed cells cancer. Difference in pathological classification could not significantly result in the increase of lymphatic metastasis, but n_2 lymphatic metastasis was more easily recognized in worst differentiated groups.

Comparison of D₂ and D₁₊₇

resections In a study regarding the less invasive D₁₊₇ resection, this resection and D₂ or more radical lymph node excision were separately carried out in 66 cases and 259 cases during 1988-1999. All cases resulted in the curable resection and the longest follow-up was of 10 years. In D₁₊₇ group, only one case was histopathologically demonstrated for the occurrence of lymphatic metastasis. Twelve cases diagnosed as m Ca preoperatively were sm Ca and others were m Ca. In investigating the postoperative complications, 1 out of 66 cases (1.5%) with D₁₊₇ resection and 39 out of 259 cases (15.1%) with D₂ resection suffered from the intestinal obstruction ($P < 0.01$). But no mortality occurred in D₁₊₇ resection group and 2 cases (0.8%) died within one month after D₂ operation.

The investigation of long-term survival showed that the survival rates of 1 and 5 years in D₁₊₇ and D₂ operation groups were respectively 98.3% vs 97.6% and 90.2% vs 92.1%, in which no significant differences could be observed.

DISCUSSION

As is well known, the degree and the range of lymphatic metastasis is dependant on the biological features of the primary tumor^[10,12-14]. This study showed that one of the main risk factors for the lymphatic metastasis was the tumor invading depth infiltrating through the mucosal layer and

the tumor size being larger than 20 mm. In sm Ca with tumor size larger than 20 mm, the morbidity of n_2 lymphatic metastasis happened with an increase in the tumor size. Therefore, correct determination of the invading depth and the tumor size are key points for the less invasive surgeries in EGC. The indications for endoscopical mucosal resection (EMR)^[20-21], laparoscopic resection^[22-31] and less invasive resection by laparotomy^[20,32-33] should be limited in the cases of EGC without lymphatic metastasis. Even for less invasive resection by laparotomy, it should be indicated only for n_1 lymphatic metastasis without ulcer, I, II_a and II_b gross types as II_c types with tumor size less than 20 mm although such indications have not been accepted ubiquitously^[10,20,32-33]. The less invasive surgery by laparotomy has become one of the main choices in surgical treatment of EGC currently. The range of less invasive operation includes the partial gastrectomy and regional lymph node excision as well as the retains of the omentum, vagus, pylorus and so on^[10,14,27]. The less invasive operations for m Ca or sm Ca have been proven to share the good results of long-term survival^[14,33], and at the same time, less invasive surgery is required.

Development of less invasive operations by laparotomy have made both MER and laparoscopic resection applicable in the treatment of EGC^[18-32]. The rate of complete resection for primary tumors and metastatic lymph nodes by EMR or laparoscopic resection was between 45%-85%. This resection rate was lower because most tumors were larger than 10 mm and some were located in the cardia, lesser curvature of gastric body as well as latter wall of stomach^[9,11,14]. Mucosal resectioning by laparoscopic surgery including lesion lefting resection^[31] and gastric mucosal resection^[20,23] comes in between EMR and less invasive laparotomy. Advantages of this laparoscopic surgery are to get more satisfactory resectioning of the entire layer of the gastric wall at the lesion site and the regional lymph nodes of n_1 around the tumor in comparison to EMR^[20,29-31]. If the tumor infiltrated to the sm Ca or the vessels in the gastric wall, the radical resectioning at the 2nd station of lymph nodes should be carried out as the first choice^[1,9,32,34].

Judgements for the invading depth of EGC before operation are mainly based on the gastric fiber examination with the ultrasound. On the application of the ultra sound endoscopy, especially at 20 MHz, correct diagnostic has been observed in over 80% cases and identification of m Ca or sm Ca was easier^[15-17,34]. At the same time, n_2 lymphatic metastasis happened more easily in sm Ca. Therefore, D₂ lymph node excision should be applied in sm Ca if the diagnosis is doubtful before or during operation. The investigations on the metastasis and the regional invasion of gastric cancer has the support of not only clinicopathological studies but the genetic and molecular biological studies also^[35-42]. At present, a very careful choice should be made for the less invasive operations,

especially for EMR and laparoscopic surgery, as making a correct judgement for the invading depth of tumor in stomach before operation^[16-17,34] is very difficult.

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Sutureless end-to-end bowel anastomosis in rabbit using low-power CO₂ laser

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Subject headings laser surgery; anastomosis, surgical; intestine, small; animals, laboratory; microscopy, electron; rabbits

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INTRODUCTION

The use of laser energy to weld biological tissues and produce sutureless anastomosis has its advantages over conventional silk-sutured anastomosis since it was reported in small vessels^[1] and fallopian tubes^[2], in the late 1970s. Since then, more investigators have welded a larger variety of tissues^[3-13] and have expanded its application to welding trials of enterotomies of rabbit and rat small intestine^[14-17]. Sauer *et al*^[18] reported results from Nd: YAG laser in reconstruction of end-to-end welding in rabbit small intestine. Recently, controlled temperature during YAG and argon laser-assisted welding of enterotomies of rabbit and rat was implemented to eliminate exponential increases in the rate of denaturation associated with rapidly increasing temperature^[19,20]. Yet there was no report of sutureless end-to-end bowel anastomosis using low-power CO₂ laser. This is a report of a circumferential end-to-end laser welding bowel anastomosis in rabbit by using 3 different CO₂ laser powers to explore the feasibility of CO₂ laser welding of a circumferential intestinal tissue and to determine the optimal laser-welding parameter. Then the appropriate CO₂ laser power was chosen to weld bowels in rabbit and its long-term healing effect was evaluated.

MATERIALS AND METHODS

Animals and equipment

Twenty-eight Japanese white rabbits weighing between 1.8 kg and 2.5 kg (6 for a cut and 22 for chronic phase of experiment) regardless of sex were supplied by Experimental Animal Center of Wenzhou Medical College.

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A hand-held Model JZ-5, power 500 mW-5 W adjustable CO₂ laser made in Shanghai Optical Machinery Institute, which was modified to provide a 100 mW-1000 mW potentiometer, was used. The laser spot diameter was 0.4 mm. The laser power output was calibrated by a Model SD2490 CO₂ laser power meter produced by Subei Electronic Equipment. The pressure was measured by a PT-6B pressure transducer and PTM-6B physiological pressure meter, jointly produced by Shanghai Fudan University and Zhejiang Ouhai Electronic Equipment Factory, with normal saline intraluminal infusion at an average rate. The flow was monitored by a computed infusion pump (DYB-1) made in Jia ngsu Haimeng Electronic Equipment Factory.

Acute phase

A rabbit was fasted for 24 h prior to surgery and sodium pentobarbital (30 mg/kg, im) was used for anesthesia. Using a 5-cm upper abdominal incision, the ileum was identified. At 10 cm proximal to the ileocecal junction, intestinal circumference was measured. Ileum was cut apart and then reconstructed. Each subsequent test was moved approximately 10 cm and a bowel anastomosis was welded by using 3 different CO₂ laser powers of 250, 500 and 1000 mW respectively. At two circumferential cut-edges of ileum, 3 silk stay sutures were placed to hold tissue together and allow for accurate approximation and inversion. Before and after each welding, the laser power output was calibrated and modified. Tissue blanching and constriction, and fusing of both edges of the anastomosis were marks of the completion of a tissue weld. The circumferential welding was completed in sequence.

One min after each laser welding, a 10-cm segment of bowel including the anastomosis was isolated between occlusive bowel ligature, and the bursting pressure of anastomosis was tested by the intraluminal infusion of normal saline at a rate of 16 mL/min. The pressure was recorded until the anastomosis burst which was visible both as leakage of infusate from the anastomosis and as a sharp drop of pressure. Each rabbit was welded 3 to 5 times and all subjects were sacrificed after the experiment.

Chronic phase

A rabbit was fasted for 24 h prior to surgery and sodium pentobarbital (30 mg/kg, im) was used for anesthesia. Using upper midline abdominal incision, ileum was circumferentially cut apart for a laser-welded anastomosis and a conventional one-layer silk sutured anastomosis. It was randomly determined which anastomosis was 10 cm or

35 cm proximal to ileocecal junction. Three silk stay sutures were placed to hold the cut intestinal edges in welded anastomosis, and 16-20 stitches of interrupted one-layer 0 silk suture were used in sutured anastomosis.

The laser power output 500 mW was used in the laser welding. Tissue blanching and constriction, and fusing of both edges of the anastomosis by naked-eye were marks of the completion of a tissue weld. The time used for laser welded anastomosis was about 5-7 min while sutured anastomosis needed 10-15 min.

Gentamycin (4 mg, im) was administered 12 h pre and post-operatively. All rabbits were sacrificed at d 3, wk 1, 3 and 5 and pathological study was done macroscopically and microscopically.

RESULTS

Acute phase

The intestinal circumference was 26-30 (28.2 ± 1.3) mm. The laser was delivered within 1 mm of both intestinal cut-edges. Based on this calculation, the laser delivery area of each anastomosis was $(26-30) \times 2$ mm. The delivery time for 250 mW group, 500 mW group and 1000 mW group was 92 s-153 s (average 123 s), 59 s-84 s (average 70 s) and 25 s-45 s (average 40 s) respectively. According to the above parameters, the laser power density and energy density of each anastomosis could be calculated. The number of bowel anastomosis using 3 different CO₂ laser powers and the bursting pressure are shown in Table 1.

Table 1 The bursting pressure of the welded and sutured anastomoses

Groups	Number of anastomosis	Bursting pressure ($x \pm s$) kPa (mmHg)
Silk-sutured	5	6.1 ± 1.9 (46 ± 14)
250 mW-welded	7	1.1 ± 0.4 (8 ± 3)
500 mw-welded	6	2.7 ± 0.7 (20 ± 5) ^a
1000 mW-welded	6	1.7 ± 0.8 (13 ± 6)

^a $P < 0.05$ vs other three groups

Chronic phase

In chronic phase experiment, except that one animal died at 1 day and another at 3 days after operation from unknown causes without anastomotic disruption by autopsy, the remaining 20 rabbits, which were dissected at a different postoperative times, were divided into four groups, each consisting of 5 rabbits.

At d 3 after operation, both modes of anastomoses healed well without leaks. Loose fibrous adhesion, stiffness, and edema were found in the adjacent bowel including the anastomosis and mesentery. Suture was seen in the sutured anastomosis while the laser welded anastomosis was smooth. Microscopically, both anastomoses showed edema, acute inflammatory cell infiltration, consisting of mainly neutrophils, lymphocytes, and macrophages, but comparatively less in the laser-welded anastomosis. At 1 wk after operation, edema was markedly reduced but the cut-edge in the sutured

anastomosis was still swollen. Adhesion was observed surrounding both anastomoses, less fibrous adhesion was seen in all laser-welded anastomoses except in one that had a small walled-off leak surrounded by fibrous formation without pus or purulent fluid in the abdominal cavity. Some inflammatory cells infiltration, mainly lymphocytes and plasmacytes, was found in both groups. Broad gap of mucous due to excessive inversion was seen in the sutured anastomosis while the gap was much narrower in the laser-welded anastomosis.

At 3 wk after operation, anastomotic tissues were morphologically normal and soft. Linear scar along the cut-edge of sutured anastomosis was seen while laser anastomosis showed less scar formation. Fibrous adhesion was remarkably decreased in the sutured anastomosis than before. No adhesion in 2 cases and very little adhesion in 3 cases was found in the laser-welded group. Microscopically, inflammatory cell infiltration and fibrous proliferation still existed in both anastomoses. In the sutured anastomosis, there was more and thicker fibrous tissue than in the laser-welded anastomosis, especially at the sutured site. At 5 wk after operation, white linear seams were seen around the sutured anastomoses. Slightly sunken scar was found on one anastomosis and some fibrous adhesion in another. At both cut-edges of anastomosis dotted line of scars resulted from the silk suture were vaguely observed, but no residual black suture remained. Two laser-welded anastomoses were hardly distinguishable and the calibre of the other three anastomoses was identical to that of the normal bowel, though thread-like scarring was found at the cut-edges.

DISCUSSION

Laser which is used to weld living tissue differs from high energy laser for other medical purposes in that the former requires specific low power energy. Which type of laser is to be chosen depends on the structure of the target tissue and welding requirement as well. Sauer *et al*^[16] used CO₂ laser to weld a 0.5 cm longitudinal incision in rabbit bowel and acquired the bursting power of 5.4 kPa. In this experiment low-power CO₂ laser was used to weld circumferential bowel anastomosis, which attempted to make laser-welding more applicable to clinical practice. The bursting pressure of 500 mW group was 2.7 kPa. The bursting pressure was lower probably because the welded anastomoses in this group were circumferential instead of a small longitudinal incision and therefore greater tension was expected. Besides, the distance between each stay suture to be welded was as long as 1.0 cm, much longer than Sauer's 0.5 cm^[16]. This study shows that the 500 mW group produced remarkably higher bursting pressure than the other two groups. Group 250 mW cannot produce a desired welding effect due to much too low power and prolonged delivery while charring tissue occurs in the 1000 mW group so that the tensile strength of the welded anastomosis is affected. Furthermore, Cilesiz *et al*^[19,21] believed that temperature feedback control improved the

quality and stability of laser-assisted enterotomy closures in surviving animals, temperature (90-95) °C was considered optimal.

Though no information is available about the maximum bursting pressure of bowel of the conscious rabbit, Abbott *et al* reported that the basic pressure of the distal bowel was 0.83-1.03 kPa, and Fink reported that the pressure of normal ileum was no more than 0.92 kPa with the peak pressure less than 2.03 kPa in the human body. Therefore we infer that the bursting pressure produced in the anastomoses of the 500 mW group is adequate to avoid the anastomotic disruption caused by peak tensile strength.

The healing process of the welded bowel reveals that fibrous tissue or intestinal adhesion to various degrees forms around both kinds of anastomoses early after operation. Later, the laser-welded anastomosis is hardly distinguishable from the normal intestine, while suture remnant and white linear scar are found in the sutured anastomoses. Microscopically, compared with the sutured anastomosis, the laser-welded anastomosis has less inflammatory cells early after operation and less fibrous proliferation, less scar formation, and no suture granulation later postoperatively. We therefore conclude that the laser-welding in intestinal anastomosis, as a new type of sutureless surgical technique, has its potential clinical application value with the advantages including sutureless anastomosis, no foreign material, avoidance of needle trauma and suture remnant, minimal inflammatory response with reduction in stricture and infection. It is especially suitable for mini-bowel anastomosis in congenital intestinal atresia.

It is universally accepted that laser thermal effect is involved in the mechanism of laser-welding of biological tissue. It is also shown that adequate tissue seals occur in laser-welded wounds only when the tissue edges are directly opposed, whereas any blood in the interface selectively absorbs the laser energy and forms a fibrin seal which is too weak to tolerate increased intraluminal pressure^[14,22]. According to Jain^[1] in his study of YAG laser welding of the microvessels, it is the physical coherence of fibrous collagen that welded successfully the vascular walls, meanwhile laser does not result in degeneration, coagulation, and necrosis. In contrast, it is held by most scholars that, based on the observation that coagulation and necrosis exists at the welded site, strong weld is not the result of physical coherence of collagen, but rather the result of chemical bonding caused by laser thermal effect which can lead to collagenic degeneration and tissue necrosis^[23-26]. In our welding experiments, ideal bonds formed when the opposed cut edges of the bowel were directly and strongly held together and tissue blanching was seen during laser welding. Microscopically, slight tissue degeneration and necrosis could also be seen near the laser-welded anastomosis. However, recent studies show that solid protein used as solder can improve vascular welding effect^[27,28]. Meanwhile, it is reported that collagen

synthesis is stimulated during the healing process after laser welding^[29,30], therefore, further study on mechanism of laser-welding is needed.

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Can the rat donor liver tolerate prolonged warm ischemia ?

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Subject headings liver transplantation; cadaver; tissue donors; organ procurement; tissue survival; non-heart-beating donors; warm ischemia

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INTRODUCTION

The last two decades of the twentieth century have witnessed increasingly successful rates of liver transplantation. The number of liver transplantations has increased steadily while the number of organ donors has remained relatively constant. Thus a great disparity has developed between the demand and supply of donor organs and remains a major limiting factor for further expansion of liver transplantation. Although many procedures, such as split liver^[1], living-related transplantation^[2], and xenotransplantation^[3], have been attempted clinically to overcome the shortage, it is hoped that livers harvested from non-heart-beating donors (NHBDs) would alleviate the problem of organ shortage, which again becomes the focus of attention^[4-9]. However, sensitivity of the liver to warm ischemia remains a major worry for use of the NHBDs. The aim of this animal study was to assess if murine liver could tolerate prolonged period of warm ischemia and to determine the optimum timing of intervention in the cadaver donor in order to preserve liver viability.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 180 to 220 g, were obtained from Shanghai BK Lab Animals Co. Ltd. Donor and recipient rats were matched for size. Rats were housed in the standard animal room without fast before the surgery and with free activity and standard diet after surgery.

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Experimental design

Orthotopic liver transplantation was carried out in 5 groups with non-heart-beating time in the donor ranging from zero to 15, 30, 45 and 60 min respectively (Group 1-5). The period of non-heart-beating was counted from the donors cardiac arrest to the beginning of the cold perfusion of the liver. Each group consisted of 13 rats; 5 rats were sacrificed one hour after surgery for sample collection and graft biopsies; the other 8 rats were used to observe one-week survival rate; and rats that survived for more than 7 d after transplantation were considered as survivors. Except for 1 or 2 survivors that were randomly selected to observe the possibility of long-term survival, the others were also sacrificed 7 d after surgery.

Animal surgery

Both donor and recipient surgery was performed at room temperature of 21°C to 22°C under ether anesthesia. No immunosuppressive agents were given to either donor or recipient. Five min after the intravenous administration of 500 IU of heparin, donor cardiac arrest was induced by opening the chest and cross clamping the base of the heart with hemostats. Donor surgery began 15 min prior to the end of the designated warm ischemic time in each group. All livers were perfused with 10 mL of 4°C Ringer's lactate solution through the abdominal aorta, and stored in the same cold solution for an average cold ischemia of 60 min. The donor liver was implanted to the recipient according to the cuff technique of Kamada and Calne^[10] with an average anhepatic phase of 16 min. No heparin was administered to the recipient rat.

Sample collection and analysis

The blood samples were obtained one hour and seven days after hepatic implantation for measurement of serum ALT and AST, meanwhile liver tissues were also taken for histologic examination. Furthermore, one hour after reperfusion serum TNF level was detected by the bioassay using L-929 cell lines as described elsewhere. The graft tissues were fixed in 10% formaldehyde, and sections were stained with hematoxylin and eosin.

Statistics

Values are expressed as the $\bar{x} \pm s$. Statistical significance was compared with group 1 and tested by unpaired Student's *t* test. The survival rates in each group were compared with group 1 using Fisher's exact test. *P* values less than 0.05 were considered significant.

RESULTS

Survival

No recipient death was directly related to operative technical failure in this study. The one-week survival rate and the long-term survival period of rats that underwent liver transplantation are listed in Table 1. With a stepwise increase of the non-heart-beating period from zero to 15, 30, 45, and 60 min in donors, the survival rates in recipients were 100, 75, 62.5, 25, and 0 percent respectively. Moreover, rats that received grafts having suffered 15, 30, or 45 min warm ischemia before implantation showed the possibility to survive more than 60 d. The longest time for using NHBDs in our study was 45 min. A total of 19 recipient rats died within 7 d, among which 15 deaths were caused by primary graft nonfunction, with massive ascites and patchy liver necrosis at autopsy. Two rats died of biliary complications and 2 died of pulmonary infection. The survivor in Group 3 also died of pulmonary infection 18 d after surgery.

Table 1 Survival status

Group	Warm ischemia/min	One-week survival rate	Long-term survival/day
1	0	100% (8/8)	>60, >60
2	15	75% (6/8)	>60, >60
3	30	62.5% (5/8)	18, >60
4	45	25% (2/8) ^a	>60
5	60	0% (0/8) ^a	

^a*P*<0.01 vs Group 1

Hepatic function

The mean serum ALT and AST concentration one hour after reperfusion was significantly higher in Groups 2, 3, 4, 5 compared with Group 1, and the concentration of enzymes dropped markedly after 7 d. The postoperative increase in enzyme concentration appeared to be proportional to warm ischemia time.

Table 2 ALT and AST values after transplantation

Group	One hour after reperfusion		7 d after operation	
	ALT(u/L)	AST(u/L)	ALT(u/L)	AST(u/L)
1	362 ± 49	623 ± 75	38 ± 9	115 ± 19
2	546 ± 68 ^a	873 ± 95 ^a	64 ± 11 ^{a,b}	345 ± 66 ^{a,b}
3	1096 ± 195 ^a	1435 ± 238 ^a	124 ± 35 ^{a,b}	454 ± 120 ^{a,b}
4	1505 ± 326 ^a	2001 ± 376 ^a	184	732
5	2357 ± 534 ^a	2886 ± 590 ^a		

^a*P*<0.01 vs Group 1; ^b*P*<0.01 vs 1 h after RPF

Serum TNF

The mean serum TNF levels one hour after reperfusion in Group 1 to 5 were (1.84 ± 0.21) U/mL, (2.62 ± 0.29) U/mL, (4.0 ± 0.4) U/mL, (4.5 ± 0.4) U/mL and (7.4 ± 0.6) U/mL respectively. The degree of serum TNF elevation was associated with the severity of warm ischemia injury to donor liver.

Histological examination

One hour after reperfusion, the livers whether or not subjected to warm ischemia all showed basically well-preserved hepatic architecture without lymphocyte infiltration in the portal area. In Group 5, mild diffused hepatocyte vacuolization was identified, accompanied by dilatation of the sinusoids. One week after transplantation, the livers of the different groups manifested lymphocyte infiltration in the portal area and congestion in the sinusoids. Neutrophil infiltration in the portal area was also identified in Groups 3 and 4. Furthermore, mild lobular necrosis surrounding hepatic vein was found in Group 3; moderate lobular necrosis and ballooning degeneration were found in Group 4.

DISCUSSION

Ever since the guidelines for defining brain death have been established, brain dead donors with beating hearts have been the commonest source of transplant organs^[8]. However, the concept of utilizing organ allograft from non-heart-beating donors for transplantation is not new. During the early stage of transplantation, NHBDs were the only source of unpaired allograft, i.e. hearts and livers. With refinements of surgical techniques, improvements in immunosuppression and development of effective organ preservation, the increasing success of and widening indication for liver transplantation has exacerbated the shortage of donor organs, and resulted in an increasing number of potential allograft recipients dying on waiting list. To increase the number of livers available for liver transplantation, grafts procurement from NHBDs has again become the focus of attention. Furthermore, recent estimates indicate that an increase of 20% to 25% in organ donors could be realized if NHBDs were used routinely^[12].

During the past two decades, the belief in the extremely high sensitivity of hepatic parenchyma cells damage from warm ischemic injury has been challenged. In both clinical and experimental studies, it has been suggested that the liver can tolerate warm ischemia up to 60 min^[13-18]. Warm ischemia of graft is considered a risk factor for postoperative graft dysfunction after liver transplantation. However, in liver transplantation from NHBDs, the effect of warm ischemic damage is subsequently compounded by preservation (cold ischemia) and reperfusion injuries. The tolerance of the liver to warm ischemia in such situations is still controversial.

Generally liver allografts can be preserved at low temperatures safely for up to 24 h in University of Wisconsin solution. In transplantation setting, however, there are a few studies about the limit of the period of warm ischemia before transplantation. In rodents, Soejima *et al*^[19] reported that none of their control rats exposed to 30 min warm ischemia before transplantation survived more than 2 d, and also the similar result was seen in Sumimoto's study^[20]. However, Ikeda^[21] reported that all

the rats suffering 30 min warm ischemia before transplantation survived more than 3 wk. Moreover, in 1995 Xu and Jones reported 50 per cent of one-week survival rate after 100 min of non-heart-beating time^[22], and also Takada reported his successful pig liver transplantation from NHBDs exposed to 60 min warm ischemia^[23]. Our data were compatible with Ikeda's^[21] and Richter's^[24]. The present study unexpectedly found that rat livers harvested from NHBDs after 30 min of cardiac arrest could be successfully grafted to the recipients with a 62.5% one-week survival rate and have the possibility to survive more than 60 d. No statistical significance of one-week survival rates was found between Group 1 and 3. In Group 4, even the donor liver suffered 45 min of warm ischemia, two of eight (25%) rats survived more than a week, also with a chance of long-term survival. The results confirmed that the liver is less sensitive to warm ischemia than formerly believed.

Hepatic ischemia-reperfusion injury is characterized by sinusoidal perfusion failure, accumulation and adherence of leukocyte in sinusoids, loss of the endothelial integrity with concomitant extravasation of blood cells, and, finally, breakdown of the liver microcirculation^[25]. Under inflammatory conditions, there are several factors that would favor neutrophil trapping. First swelling of Kupffer cells and endothelial cells occurs during exposure to inflammatory mediators. Second, an imbalance of vasoconstrictor (e.g. endothelin-1) and vasodilator (e.g. nitric oxide) formation can further narrow the sinusoidal diameter^[26,27]. Moreover, inflammatory mediators such as activated complement factors reduce the deformability of neutrophil. The combination of these factors appears to be responsible for the microcirculation failure.

It is widely accepted that warm ischemia and cold ischemia represent two different and distinct types of injury to the liver. While cold ischemia mainly causes injury to the cells of the sinusoidal lining, hepatocytes are the most vulnerable to warm ischemia^[21,28,29]. The postoperative elevations in the serum liver enzyme concentration observed in the present study may reflect the extent of hepatocyte disruption, which was more severe in proportion to the duration of warm ischemia. TNF produced by Kupffer cells plays an important role in the pathogenesis of early graft failure^[30-33]. In this study, we demonstrated an elevation of serum TNF levels in association with increasing prolonged warm ischemia time. The degree of histological damage and serum ALT and AST value changes correlated positively with the TNF levels.

In conclusion, rat liver can tolerate a prolonged warm ischemia time up to 45 min for transplantation with a chance of recipient survival. Based on our experience, warm ischemia is not the only factor limiting the success of liver transplantation from NHBDs^[34]. More attention should be paid to other factors such as the mechanical injury during the harvest of cadaver donor liver and to the

cold preserve time and anhepatic phase. Actually, clinical experience with the use of liver allograft from NHBDs has been reported^[4,5,35]. The patients, who had irreversible neurologic damage but did not meet standard brain death criteria, were transported to the operating room where ventilation was discontinued, and rapid cold perfusion was performed after declaration of death. In such controlled circumstances, the warm ischemia time was less than 30 min in most cases. Some of the procured grafts were actually transplanted, exhibiting satisfactory initial function. However, in the patients who suffered a sudden cardiac arrest, in whom cardiopulmonary resuscitation was necessary before organ procurement (referred to as uncontrolled NHBDs), there was a high incidence of primary graft nonfunction. In this regard, the present study is a simulation of well-controlled NHBDs, and further intensive experimental studies are required to approach this goal, because human NHBDs have much more complicated clinical conditions than healthy rat donor. In clinical liver transplantation, unstable hemodynamic conditions before cardiac arrest may produce a warm ischemia injury. New strategies to attenuate ischemic liver injuries will be required to establish the safety and efficacy of liver transplantation from human NHBDs.

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Serum vascular endothelial growth factor is a potential biomarker of metastatic recurrence after curative resection of hepatocellular carcinoma

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Subject headings endothelium, vascular; endothelial growth factor; carcinoma, hepatocellular; enzyme-linked immunosorbent assay; liver neoplasms; liver cirrhosis; immunohistochemistry

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China. To date, surgery is still the best solution to it. However, metastatic recurrences after curative hepatic resections are very common. Tang *et al* have reported that recurrence rate within 5 years of curative hepatic resection is 61.5%^[1]. As curative hepatic resection has a high tendency for metastatic recurrence, therapeutic interventions such as transarterial embolization and antiangiogenesis have been tried to further improve prognosis of HCC patients. Therefore, establishing a dependable, sensitive, easy, and economical method to predict metastatic recurrence following curative hepatic resection is of clinical urgency.

Neovascularization has been shown to be essential for the growth and metastasis of solid tumors. Vascular endothelial growth factor (VEGF), a dimeric heparin-binding glycoprotein with a molecular weight of about Mr45000, is one of the most important angiogenic factors. In addition to increasing permeability of blood vessels, VEGF has potent mitogenic effect on vascular endothelial cells^[2-9]. Serum VEGF levels have previously been shown to be raised in patients with various tumors, including brain, renal, melanoma, breast, gastrointestinal,

and liver malignancies particularly in metastatic diseases^[10-15]. Because VEGF plays an essential role in tumor angiogenesis and hence the metastasis and recurrence of HCC, its elevation in serum may be a candidate biomarker of metastatic recurrence. Consequently, we set out to study whether preoperative serum VEGF could be used as a biomarker of metastatic recurrence following curative hepatic resection in HCC. Since 84.6% of HCC patients have accompanied cirrhosis to some extent we also examined serum concentrations of VEGF in cirrhotic patients and normal healthy controls^[16]. In addition, we studied the relationship between serum VEGF concentrations and immunohistological expressions of two known metastatic recurrence parameters-p53 and PCNA in tumor tissues.

MATERIALS AND METHODS

Subjects

The current study registered 12 normal healthy controls, 12 patients with cirrhosis, 8 patients with benign liver tumors including hemangioma and focal nodular hyperplasia, and 85 HCC patients who received curative resection. The healthy controls were selected randomly from people coming to our hospital for a medical checkup and found to be healthy. Cirrhotic patients were diagnosed clinically. HCC and benign liver tumor were diagnosed histologically. All HCC patients had underlying cirrhosis to some degree as confirmed by operations. Thrombi, intra- and extra-hepatic dissemination, were confirmed by operation, and/or ultrasonography, and computed tomography. According to generally recognized standards, we set HCC patients with thrombi, intra- and extra-hepatic dissemination, and tumor size larger than 5 cm as high-tendency metastatic recurrence (HTMR) group, and less than 5 cm as low-tendency metastatic recurrence (LTMR) group. Hepatic resection with no signs of tumor lesion within the liver, and no metastatic lesion outside the liver after operation as well as no tumor thrombi in major branches of portal, hepatic vein, and intrahepatic biliary ducts before operation was considered as curative hepatic resection.

Blood samples were taken from all subjects. The serum was separated after 20-30 min of coagulation at room temperature and was stored at -80°C until the assay. Repeated thawing and freezing of samples was avoided.

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VEGF assay

The VEGF determinations were performed in duplicate following the manufacturer's instructions using the R&D Systems Quantikine enzyme-linked immunosorbent assay (ELISA) kit. The VEGF concentration in a sample was determined by computer software-generated interpolation (Microsoft Origin software) from the standard curve. The internal VEGF standards ranged from 0 to 2000 ng/L, and the intensity of chromogen was measured at a wavelength of 450 nm with a reference wavelength of 595 nm using the dual wavelength mode on the BIO-RAD 450 Microplate Reader. Standard curve was generated and plotted using a log-log linear regression.

Immunohistochemistry

For the immunohistochemical demonstration of p53 and PCNA protein, formalin fixed, paraffin embedded sections were deparaffinized in xylene and alcohol and placed for 15 min in alcohol-H₂O₂ for blocking endogenous peroxidase. The samples were processed in a microwave oven, placed in a thermoresistant plastic box with 10 mmol/L pH 6.0 citrate buffer. Tissue sections were treated in the oven twice for 5 min while the buffer was boiling. Tissue sections were left at room temperature in the buffer solution for 20 min without drying. Sections were treated with bovine serum albumin to prevent background staining and incubated for 1 h with a primary nondiluted ready-to-use murine anti-p53 antibody (Dako, Carpinteria, CA) or murine anti-PCNA antibody (Dako, Carpinteria, CA) diluted at 1:500 at room temperature in a humidified chamber. Slides were rinsed with phosphate buffered saline for 3 min and incubated first with the biotinylated linked goat anti-mouse antibody for 30 min and then with the labeling reagent, peroxidase conjugated streptavidine, for 30 min. After the slides were rinsed, the peroxidase label was demonstrated using 3-amino-9-ethylcarbazol (AEC) for 15 min, and counterstained with Mayer hematoxylin. AEC produced a red product which was soluble in alcohol and was used with an aqueous mounting media. Positive and negative controls were included in each experiment. Specifically, for the latter the primary antibody was substituted with nonspecific mouse IgG. p53 or PCNA immunopositivity was recorded when more than 15 carcinoma cell nuclei were stained in one or more fields^[10].

Statistics

Analyses were performed using SAS (Version 6.12; SAS Institute, Inc., Cary, NC). Student's *t* test and Oneway ANOVA were used to determine the differences between the means of different groups. Results were expressed as mean \pm SD. The level of significance was $P < 0.05$.

RESULTS

The VEGF concentrations in the normal controls and groups

of cirrhotic, benign liver tumor, and HCC patients were 158.46 ± 41.84 ng/L, 90.00 ± 22.42 ng/L, 156.34 ± 41.32 ng/L, 164.42 ± 76.07 ng/L, respectively (Table 1). Cirrhotic patients had the lowest levels of VEGF in the four groups. Compared with the cirrhotic group, HCC group had a significantly higher level of VEGF in the serum ($P < 0.01$). Yet, no significant differences could be found between serum levels of VEGF in HCC and benign liver tumor or normal healthy control group ($P > 0.05$) (Figure 1). Since large HCC has a high tendency to recur after hepatic resection, we next divided HCC patients into small HCC and large HCC group. The VEGF concentrations of large HCC group were a little higher than those of small HCC patients (173.52 ± 52.34 ng/L vs 154.46 ± 37.23 ng/L, $P > 0.05$). However, this difference was not significant. In patients with thrombi, VEGF levels were significantly higher than those in patients without (182.46 ± 35.61 ng/L vs 157.62 ± 53.42 ng/L, $P < 0.05$). On dividing the HCC patients into HTMR and LTMR groups, HTMR patients were observed to have significantly higher VEGF concentrations in the serum than LTMR patients (185.33 ± 92.88 ng/L vs 144.75 ± 51.37 ng/L, $P < 0.05$) (Figure 2). A notable case observed was that of a female patient having a tumor growth of just 1.8 cm diameter with no thrombi but with the highest levels of VEGF (819.37 ng/L), she was the first to metastasize (within three months). As p53 is reported to play a role in regulating the production of VEGF, we further divided HCC patients into p53 positive and p53 negative groups. We found that serum VEGF levels in p53 positive patients were significantly higher than those in p53 negative patients (176.56 ± 53.29 ng/L vs 149.26 ± 41.29 ng/L, $P < 0.05$). Despite PCNA being a commonly used clinical indicator of metastatic recurrence after curative hepatic resection in HCC, we did not find any significant difference in VEGF levels between PCNA positive and PCNA negative groups (176.56 ± 53.29 ng/L vs 165.26 ± 54.29 ng/L, $P > 0.05$).

Table 1 Serum VEGF levels in different HCC groups which received curative hepatic resection, benign liver tumor group, and normal control group ($\bar{x} \pm s$)

Group	Case (n)	VEGF concentrations (ng/L)
Normal control	12	158.46 ± 41.84
Liver cirrhosis	12	90.00 ± 22.42
Benign	8	156.34 ± 41.34
HCC	85	$164.42 \pm 76.07^{a,b}$
Small HCC	34	154.46 ± 37.23
Large HCC	51	173.52 ± 52.34^c
Without thrombi	71	157.62 ± 53.42
Thrombi	14	182.46 ± 35.61^d
p53 negative	34	149.26 ± 41.29
p53 positive	38	176.56 ± 53.29^e
PCNA negative	36	165.26 ± 54.29
PCNA positive	31	176.56 ± 53.29^f

^a $P < 0.01$, vs liver cirrhosis; ^b $P > 0.05$, vs benign; ^c $P < 0.05$, vs small HCC; ^d $P < 0.05$, without thrombi; ^e $P < 0.05$, vs p53 negative; ^f $P > 0.05$, vs PCNA negative.

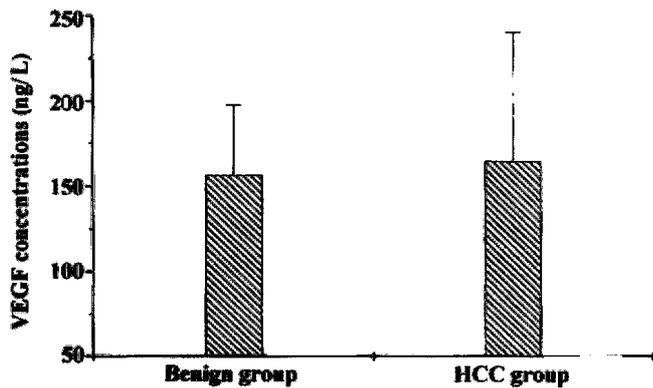


Figure 1 Serum VEGF levels in benign liver tumor and HCC (hepatocellular carcinoma) groups.

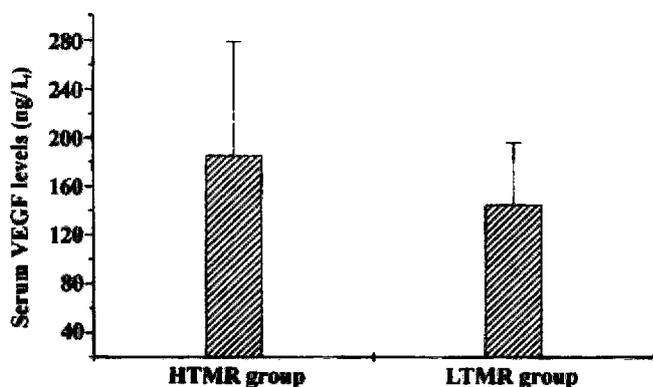


Figure 2 Serum VEGF levels in HTMR and LTMR groups. HTMR: high-tendency metastatic recurrence group; LTMR: low tendency metastatic recurrence group.

DISCUSSION

VEGF is produced by a wide variety of tumor cells, helping the growth and dissemination of the solid tumor by making it more vascular. In HCC, it acts in a paracrine fashion and plays an essential role in tumor angiogenesis^[2-5,17].

The prognostic value of VEGF has been shown in breast and gastric cancer based on VEGF expression in tumor tissue detected by immunohistochemistry, with VEGF concentrations being high in highly vascular rich breast tumors. The VEGF positivity in gastric cancer correlates with vessel involvement, lymph node metastasis as well as liver metastasis and is associated with an overall poor prognosis^[18-23]. Because local tumor invasion and metastatic spread are angiogenesis-dependent, it is hypothesized that metastatic recurrence after curative hepatic resection in HCC may be associated with up-regulation of angiogenic factors. Our present study showed that HTMR patients had significantly higher levels of VEGF than LTMR patients. This indicates that VEGF is a potential biomarker of metastatic recurrence in HCC patients after curative hepatic resection. The sensitive elevation of VEGF in one female patient further strengthens the hypothesis that raised VEGF levels may predict metastatic recurrence in HCC. The range of serum VEGF levels among healthy controls was from undetectable to

481.02 ng/L. The relevance of normal levels of VEGF is not clear at present and further studies are required to clarify it. As about 84.6% of Chinese HCC patients have some degrees of cirrhosis, it would be proper to compare VEGF levels between cirrhosis group and HCC group, rather than normal healthy controls and HCC patients^[16]. Compared with cirrhotic patients, HCC patients had significantly higher levels of VEGF in their serum. It indicates that VEGF could play an important role in transforming liver cirrhosis into HCC. Unexpectedly, the mean serum levels of VEGF in HCC and benign liver tumor patients were observed to be very close. This suggests that VEGF can not be used as a marker to distinguish benign liver tumor from malignant one (HCC). The main reason for this phenomenon may be that all of the benign liver patients in our study have noncirrhotic liver. Since tumor thrombi is a putative indicator of early metastatic recurrence following curative hepatic resection and poor prognosis^[24,25], we detected VEGF levels in HCC patients with thrombi and found that consistent high levels of VEGF reflected a high tendency towards metastatic recurrence in the thrombi group. Meanwhile, we found that there was no significant difference between small HCC group and large HCC group regarding VEGF concentrations although mean serum VEGF levels in big HCC patients were higher than those in small HCC patients. Despite the fact that tumor size is a commonly used prognostic indicator of HCC, our result does not sufficiently reflect its role as an important parameter of metastatic recurrence in HCC^[26,27]. Here the point to contemplate is that the ability of metastatic recurrence of HCC cannot be predicted by a single parameter alone, such as presence of tumor thrombi, intrahepatic dissemination or tumor size, rather all of them combined together can give a more precise indication.

p53 and PCNA have been reported to be indicators of HCC metastatic recurrence^[28-31]. Meanwhile, *p53* also plays an important role in regulating the production of VEGF. Wild-type *p53* down-regulates whereas mutated *p53* up-regulates VEGF expression according to some studies^[32-35]. In the present study, *p53* positive patients had significantly higher levels of VEGF than *p53* negative counterparts, confirming previous reports^[25]. However, the difference in VEGF levels between PCNA positive and PCNA negative groups was not significant. The role of PCNA in the regulation of VEGF is currently being investigated in our lab.

In conclusion, this study demonstrates that serum VEGF is a potential biomarker of metastatic recurrence in HCC patients following curative hepatic resection. However, it can not distinguish HCC from benign liver tumor. *p53* positive patients have a significantly higher VEGF level in the serum than their counterparts. Further follow-up studies are needed to delineate the ability of VEGF in predicting metastatic recurrence after curative hepatic resection in HCC.

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Comparative ultrastructural study of endoplasmic reticulum in colorectal carcinoma cell lines with different degrees of differentiation

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Subject headings colorectal neoplasms; endoplasmic reticulum; ultrastructure; microscopy, electron; microscopy, electron, scanning; cell differentiation

Feng S, Song JD. Comparative ultrastructural study of endoplasmic reticulum in colorectal carcinoma cell lines with different degrees of differentiation. *World J Gastroentero*, 2000;6(4):569-571

INTRODUCTION

The endoplasmic reticulum (ER) consists of a complex system of tubules, lamellae, and flattened vesicles, and has a variety of morphologies in different cells. It is believed to play a central role in the biosynthesis of cholesterol, phospholipids, steroids, prostaglandins, membrane and secretory proteins^[1]. Cancer cells have different functions and ultrastructure from their original cells^[2-4]. The studies on ER membrane system of cancer cells are of great significance in understanding their malignant behavior. In the present work, the ultrastructural characteristics of ER in human colorectal carcinoma cell lines with different differentiation degrees were investigated.

MATERIALS AND METHODS

Materials

Well differentiated human colorectal carcinoma cell line CCL229 and poorly differentiated cell line CCL227 were generous gifts from Dana-Farber Cancer Institute of Harvard Medical School, USA.

Methods

Cell culture The human colorectal carcinoma cell lines

CCL229 and CCL227 were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, BRL) supplemented with 10% calf serum and 2 kU/L gentamycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

Transmission electron microscopy Exponential cells cultured in flasks were collected, washed thrice in phosphate buffer saline (PBS), and then fixed in 2.5% glutaraldehyde in a buffer containing 0.1 mol/L sucrose and 0.1 mol/L sodium cacodylate (cacodylate buffer, pH 7.4) for 2 h. After being washed in cacodylate buffer, the cells were post-fixed in 1% osmium tetroxide for 30 min, dehydrated through graded alcohol and acetone, and embedded in Epon 812. Ultrathin sections were cut with glass knives on LKB 2088 ultratome, stained with uranyl acetate and lead citrate and examined under a Hitachi H600 transmission electron microscope.

Scanning electron microscopy Cells grown on coverslips were rinsed thrice in a buffer containing 60 mmol/L sodium citric acid, 25 mmol/L KCl, and 35 mmol/L MgCl₂ (sodium citric acid buffer, pH 7.4), then fixed in 125 mmol/L potassium permanganate in sodium citric acid buffer for 7 min. Cells were rinsed in sodium citric acid buffer, dehydrated through graded alcohol, replaced with iso-amyl acetate, dried at CO₂ critical point in a Hitachi HCB-2 critical point drier, gilded on a BIKO TB-3 ion film-plating machine and then examined under a Hitachi S-450 scanning electron microscope.

RESULTS

Well differentiated colorectal carcinoma CCL229 cells appeared round or polygonal with many microvilli and pseudopodia and a large elliptic nucleus containing 1-2 dense nucleolus. The cytoplasm contained some mitochondria, Golgi apparatus, lysosomes, polyribosomes as well as abundant ER. The ER consisted of a complex system of tubules, lamellae, and flattened vesicles distributed throughout the cytosol. There was a great amount of vesicle-like and flattened cisternal ER in pseudopodia (Figures 1, 2).

More apparent heteromorphism was observed in poorly differentiated colorectal carcinoma CCL227 cell. There were less microvilli and pseudopodia on the cell surface. The ratio of nuclei to plasma was higher than that found in CCL229 cell. An abundance of free polyribosomes and less

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of mitochondria, Golgi apparatus and lysosomes were found in the cytoplasm, and its ER, which mainly consisted of vesicles and short tubules was much less than that in well differentiated CCL229 cell (Figures 3, 4).

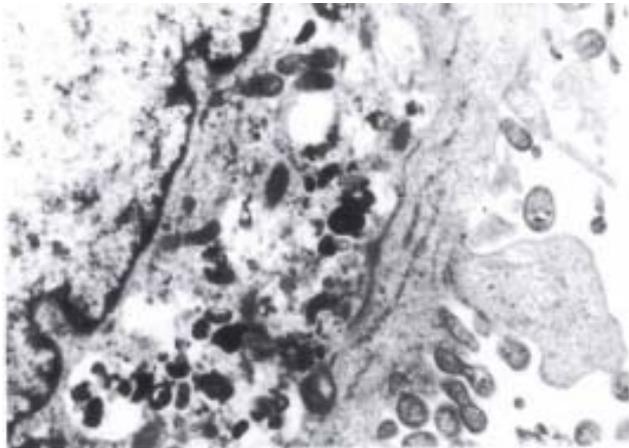


Figure 1 Ultrastructure of well differentiated colorectal carcinoma cell line CCL229 under TEM. $\times 800$

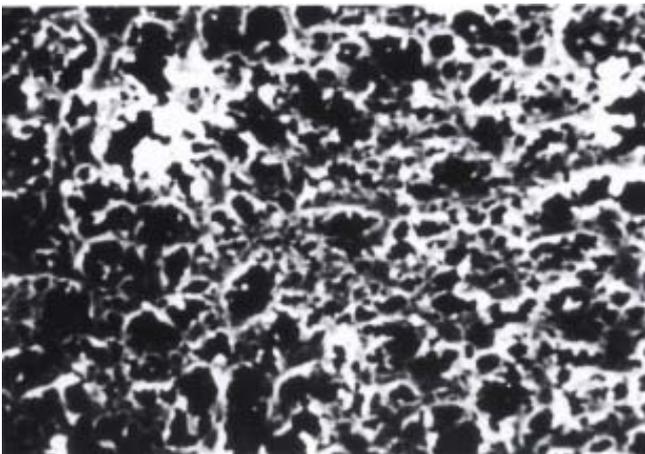


Figure 2 Ultrastructure of well differentiated colorectal carcinoma cell line CCL229 under SEM. $\times 7500$

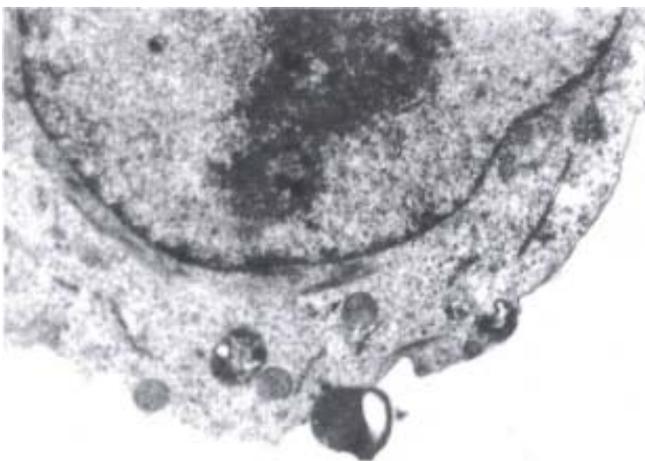


Figure 3 Ultrastructure of poorly differentiated colorectal carcinoma cell line CCL227 under TEM. $\times 500$

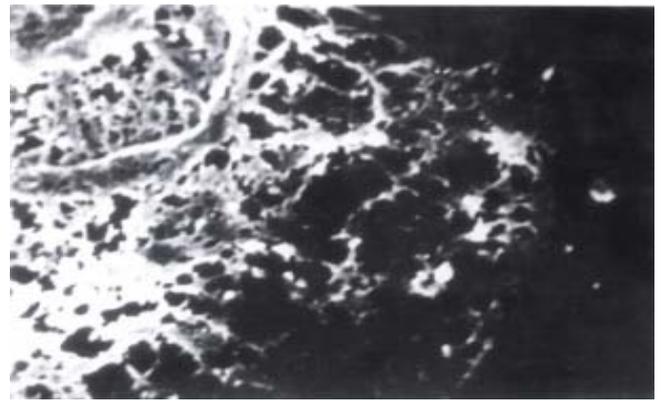


Figure 4 Ultrastructure of poorly differentiated colorectal carcinoma cell line CCL227 under SEM. $\times 5500$

DISCUSSION

Overlap with other compartments and limits of regular light and electron microscopy make it difficult to study the ultrastructure and cellular distribution of ER membrane system. Terasaki *et al*^[5] reported a rapid and simple technique for localizing the structure of whole-mount ER both in living and glutaraldehyde-fixed cells by fluorescent microscopy which can also be detected by phase-contrast microscopy and scanning electron microscopy in potassium permanganate-fixed cells. With this technique, the ultrastructure and distribution of ER in many normal and tumor cells have been studied^[6-11].

The ER is a highly specialized structure which performs many distinct functions. Hence a well developed ER may be looked upon as an expression of cell differentiation and functional activity. It is abundantly clear that immature or undifferentiated cells such as stem cells, embryonic cells, and cells in culture have, as a rule, a poor complement of ER as compared with their normal maturing counterparts, and this concept also applies to tumor cells. In general, there is a meaningful correlation between ultrastructural signs of anaplasia and malignancy. In this study, using regular transmission microscopy and whole-mount ER scanning electron microscopy of potassium permanganate fixation, the ultrastructure and distribution of ER in well and poorly differentiated colorectal carcinoma cell lines CCL229 and CCL227 was investigated comparatively. The results showed that well differentiated cell line CCL229 had abundant endoplasmic reticulum, which consisted of a complex system of tubules, lamellae, and flattened vesicles distributed throughout the cytoplasm. A great amount of vesicle-like and flattened cisternal endoplasmic reticulum were found in pseudopodia. Poorly differentiated cell line CCL227 had relatively less ER. This result is coincident with the above-mentioned concept.

In poorly differentiated CCL227 cell, the ER is not abundant, but free polyribosomes are very rich in the cytoplasm. This presumably reflects the active synthesis of endogenous proteins needed for cell growth and division.

Well differentiated cell CCL229 is a highly invasive colorectal carcinoma cell line, its high invasiveness correlates to its cell's ultrastructure^[12,13]. Pseudopodia of tumor cells play an important role in the invasion process^[14], tumor cells are supposed to secrete a great amount of proteolytic enzymes to cleave the basement membrane^[15-18]. In this work, cell CCL229 was found to have many microvilli and pseudopodia with abundant ER which presumably reflect its active synthesis of secretory proteins. This may be the ultrastructural basis of its high invasiveness.

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Study on environmental etiology of high incidence areas of liver cancer in China

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Subject headings liver neoplasms; etiology; epidemiology; water pollution; environmental carcinogens; nitrosamines; china; geology

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INTRODUCTION

China is a country with a high incidence of liver cancer in some areas^[1]. Liver cancer has a wide distribution and threatens human health seriously. A rough estimation shows that out of a population of 1.2×10^8 in liver cancer areas patients are more than 1.0×10^5 . The environment of the liver cancer area is very complicated and has different characteristics in different regions. But the epidemic regularity of liver cancer is obvious, and the environment in the cancer areas has also distinct characteristics that contribute to the study on the environmental etiology of liver cancer^[2-5].

THE EPIDEMIOLOGICAL CHARACTERISTICS OF LIVER CANCER

The liver cancer discussed here has epidemiological characteristics like endemic, comparison and trend. The general investigation of liver cancer all over the country of China or local area shows that the high rate of liver cancer has been found in some provinces, counties, towns, and islands with the background of the low death rate ($10/10^5$)^[1,6]. The main areas of high rate of liver cancer chiefly concentrate in the coastal areas such as Jiangsu, Guangxi, Fujian, Zhejiang, Guangdong Provinces, and Shanghai City^[7,8] (see Figure 1). Qidong in Jiangsu Province and Fusui in Guangxi Province are two counties of the highest death rate of liver cancer in China and the death rates are $47.76/10^5$ and $46.87/10^5$ ^[1,9]. As far as macroscopic environment is concerned, the risk area often

has focused distribution and has a strict and steady dividing line between light and non-liver cancer areas. The risk area has peculiar characteristics regarding landmark, geology, hydrogeology, and geochemistry^[9,10]. It is an important way to study the cause of high incidence of liver cancer from the angle of environmental etiology.

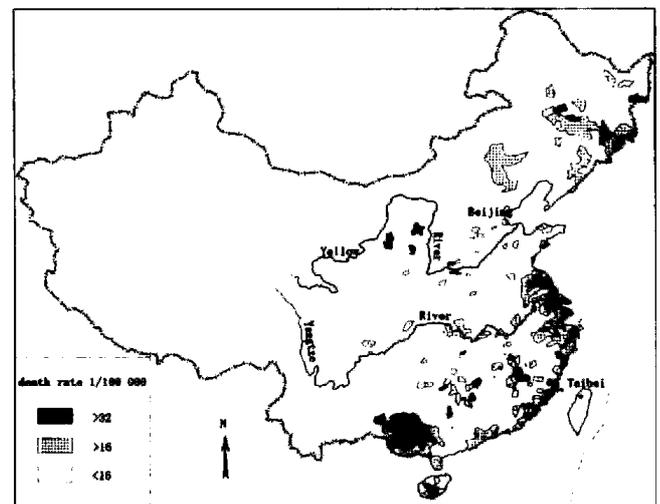


Figure 1 Sketch map of liver cancer in China.

The area of high incidence of liver cancer is adjacent to that of the low incidence area and the death rate has great disparity. For example, the death rate in Fusui is $46.87/10^5$ while that in Niming County is $7.47/10^5$, and the death rate in Qidong is $47.76/10^5$ while that of Rudong County adjacent to Qidong is $20.6/10^5$. The death rate in Tongxiang Town in Qidong County is $50.67/10^5$ while that in adjacent Xining Town is $17.28/10^5$. All these show that the carcinogens are distinctly different in the two adjacent environments^[9].

The three epidemic trends of liver cancer may be summarized as follows: the change in epidemic curve from low to high, parallel development or from high to low. The trend reflects the change in the intensity of carcinogenic factor with time, and the epidemic curve of many high incidence areas of liver cancer belongs to the first type. The curve rose in the 1970s, while it gently went down from the end of the 1980s to the beginning of the 1990s. We think that organic pollution of the drinking water may be the reason for the rising of the curve, while the downward trend of the curve may be the result of changed water.

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THE ECO-ENVIRONMENTAL GEOLOGICAL TYPES IN LIVER CANCER AREAS

The cancer of digestive system in China has wide distribution and threatens human health seriously and liver cancer is the most serious one of all^[1,11]. Distinct environmental characteristics were discovered in the high incidence areas of liver cancer according to a survey and analysis on a large scale. It reflects comprehensively the climate, hydrology, landmark, geology, geochemistry, drinking water, the degree of pollution, economic condition, the food custom and so on. However, the organic and reduction environment and the drinking water polluted by organisms are the most obvious and common characteristics. The high incidence areas are classified into five geo-environmental types^[10,12-14].

(1) The denuded mountain environmental type: it is mainly distributed at the junction of three provinces of Hebei, Henan, and Shanxi in south Taihang Mountain. Esophagus, liver and stomach cancer are the common cancers. The climate is dry and the rainfall is about 500 mm-600 mm. The slopes are bare and are made up of carbonate and clastic rock. The ground has sparse plantation and the surface water and groundwater are very scarce. The drinking water condition is poor, and only a few residents drink stream and spring water, while most have to drink pool and pond water polluted with organic waste.

(2) The Karst mountain environmental type: it is mainly distributed in Fusui and Longan County in Guangxi Province, the main cancers are liver and stomach cancer. The rainfall is sufficient about 1100 mm-1300 mm. The carbonate rock layer distributes extensively in the cancer areas belonging to Karst geographical areas. Underground rivers are developed while surface water is very scarce. People here mainly drink the water from dirty ponds or wells besides the ponds.

(3) The river network plain environmental type: it is mainly distributed in Jianhu and Huai'an in the north of Jiangsu Province, Yangzhong and Taixing in the middle and lower reaches of Yangzi River. Main cancers are of the esophagus and liver. The area belongs to the alluvial and lake plains of the lower reaches of Yangzi River, and most of the cancer areas are in the lake-marsh depression which is rich in humus sludge, peat, and biological deposition and CH₄ is easily found escaping from the earth's surface. The surface water and groundwater is rich but the runoff is stagnated.

(4) The delta type: it is distributed in Qidong, Haimen, Rudong, Chongming Island in Yangzi River Delta and Nanhui County belonging to Shanghai City, Shunde, Jiangmen, and Foshan in the Zhujiang delta. Liver and esophagus cancer are the main cancers. The climate is humid with plenty rainfall. Most of the areas are sea and land mutual depositions that are mainly made up of sand, humus, peat, and biological deposition. The surface

water and groundwater flow is sluggish and is severely polluted by organisms.

(5) Coastal plain and island type: stomach, liver, and esophagus cancer are the main diseases. The cancer areas of coastal plain type are mainly distributed in Changle and Putian in Fujian Province. Most of them are sea and land mutual depositions of coastal plains that are made up of clay, soil, and biological deposition. The groundwater and surface water are rich, but severely polluted by organisms. Dongshan Island in Fujian and Nan'ao Island in Guangdong Province belong to the island type which is a rocky hill and is covered by thin Quaternary deposition, and fresh water is scarce.

The classification of eco-environmental geology has contributed towards a deep study and contrast between the macroscopic and microscopic characteristics in disease areas, and searching for questionable carcinogenic factors of environment, and giving comprehensive protective measures.

DRINKING WATER TYPES AND LIVER CANCER

Comparing the high incidence areas of liver cancer in China, we discovered that there exists a common faction in cancer incidence in these areas although the natural environment is different. It is that the groundwater or surface water is rich in humus, the water flow is stagnant and is in an organic and reduction environment consisting of pools, ponds, cellars, trenches, channels, and the groundwater in the swampland. The people in non-cancerous areas mainly drink groundwater or surface water which is not polluted by organisms. The water flow is smooth and is in an oxidation environment such as springs, rivers, and shallow or deep well water^[9]. Taking Fusui and Qidong as examples, we have constructed a figure that reflects the relation between the type of drinking water source and the death rate due to liver cancer (Figure 2). The figure illustrates that the death rate due to drinking the pond and pool water is the highest and due to deep well water is lowest.

RELATIONSHIP BETWEEN DRINKING WATER AND LIVER CANCER

After affirming a close relationship between drinking water type and liver cancer, we tested the inorganic and organic compositions of all kinds of drinking water. We did not find any peculiar contents regarding common and trace elements, but some cancer areas were low in minerals and Se in water^[13-16]. However the organisms in water were different and the death rate due to liver cancer was found to have a very close relationship with drinking water type and the degree of water pollution^[17-18] (Table 1).

From Table 1, it can be observed that water pollution indicator such as nitrate, nitrite, COD, and humic acid have an obvious relation with the drinking water type and death rate from liver cancer increases with a rise in the

above indicators. In order to study the relationship between death rate and water quality, 29 water samples that corresponded to death rate due to liver cancer were chosen. The death rate and COD, DO, BOD, NO_2^- , and humic acid were used to make a correlation analysis to study the relationship between the death rate and the five factors. The regression equation is $y = a + bx$, y stands for the death rate due to liver cancer, x stands for the five factors. The results of the analysis are listed in Table 2.

Table 1 The organic pollution indicators of drinking water and death rate from cancer

The drinking water type	Qidong in Jiangsu					Fusui in Guangxi		
	Pond	Channel	River	Shallow well	Deep well	Pond	River	Deep well
Death rate	61.53	58.17	41.99	18.80	0.0	115.05	37.49	0.0
Number of samples	20	18	22	21	16	7	18	25
COD	4.66	3.85	3.55	3.20	1.28	48.27	0.75	0.197
Humic acid	0.43	0.36	0.28	0.18	0.08	0.58	0.18	0.042
NO_3^-	14.52	18.20	21.46	67.91	1.53	0.74	0.75	0.480
NO_2^-	0.54	0.48	0.38	0.28	0.04	0.061	0.008	0.0096

The death rate is $1/10^5$ and the unit of NO_3^- , NO_2^- is ppm.

Table 2 The analysis between various drinking water factors and death rate due to liver cancer

Content PPM	Regression a	Coefficient b	Interrelation coefficient r
Humic acid	29.4985	78.6384	0.5981 ^b
NO_2^-	53.1249	75.9767	0.4232 ^a
COD	43.8098	0.4280	0.4881 ^b
BOD	45.0576	0.6231	0.4872 ^b
DO	191.8340	-19.0087	-0.7285 ^b

^a $P < 0.05$, ^b $P < 0.01$, vs control.

The results shows that humic, COD and BOD have a very remarkable positive relation with the death rate ($P < 0.01$), NO_2^- has a significant positive correlation. While DO has a very marked negative correlation ($P < 0.01$). That is to say, the lower the DO is in the water, the higher is the death rate. Pond water is severely polluted by organisms and is in a strong reduction environment lacking of DO. We postulate that the five factors given above can be used as important signs to judge the water quality and the death rate due to liver cancer.

THE RELATION BETWEEN NITROSAMINE AND LIVER CANCER

It has been affirmed that nitrosamine is carcinogenic. Nitrosamine contents were observed in water from high incidence areas of liver cancer in the 1970s and 1980s in China, but the test method used was not very sensitive for semi-quantitative^[18].

We collected 24 water samples from cancer-prone villages and non-cancer-prone villages in Fushui County to quantify the contents of nitrosamine compound in water. Nitrosamine was not found in sixteen water samples taken

from river, stream, and deep well water except in 8 samples of pond water. Dimethyl nitrosamine was the main compound in the pond water samples, only three pond water samples contained diethyl nitrosamine. The death rate seems to have positive relation with the contents of nitrosamine (Table 3).

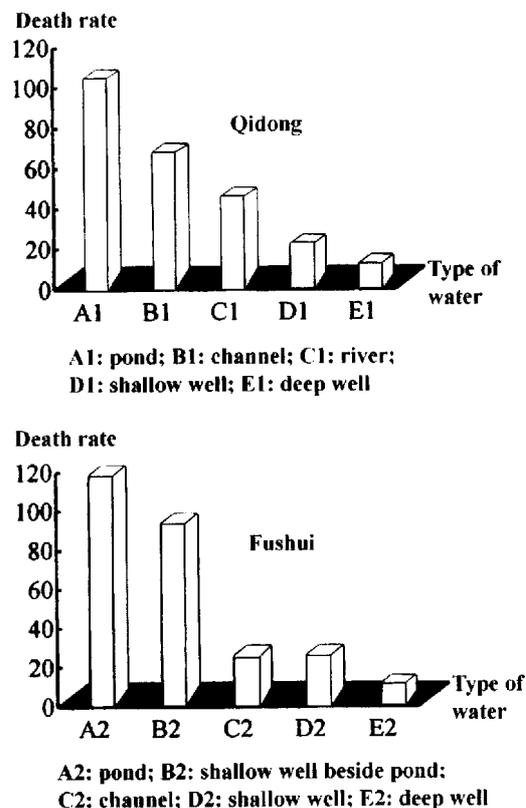


Figure 2 Relation between the types of drinking water source and the death rates from liver cancer.

Table 3 The content of nitrosamine in pond water and the death rate due to liver cancer in Fusui

Location of samples	Death rate of liver cancer 1/10 ⁵	Dimethyl nitrosamine ppb	Diethyl nitrosamine ppb
Sihe	74.96	3.8	
Jiuchen	35.42	3.2	
Yiliu	31.68	3.6	
Mumin	80.80	2.4	2.2
Qutun	112.59	5.6	
Zhongyuan	148.49	4.8	2.4
Lailu	95.36	4.2	
Jiuta	18.38	0.8	0.4

MUTATION TEST OF DRINKING WATER

Professor De Long Su^[11] holds that the high incidence of liver cancer has relation with water polluted by micro organisms. He carried out an Ames test on the drinking water in Qidong disease area and got a positive result. Li Sheng Zhang^[9] carried out a micronucleus test using Zilu Grass from America on pond water in Fusui disease area and extracted liquid from the pond mud. His results show

that high pollution degree corresponds to high micronucleus rate of plant cell and high death rate due to liver cancer. He also found that the micronuclei in clean deep well water in Karst area and corresponding water are very low.

Following some studies^[19-24], we decided to carry out an HHPL mutation test on pond water, shallow well water, and deep well water. Distilled water was used as a blank contrast.

The first test was the cell micronuclei test and the results showed that HHPL micronuclei rate found in the water that has been poisoned had a dose-effect relationship with the density of the samples and the higher the density of samples content was, the higher the micronuclei rate was. The micronuclei rate had a marked difference (pond water > shallow well water > deep well water \geq blank). On evaluation by *t* test, the micronuclei rates of poisoned pond water and shallow well water were significantly different ($P < 0.001$).

The second test used was the mutation test of cell chromosome malformation test. The results showed that the mutation rate of chromosome aberration of HHPL has dose-effect with the density of each polluted water sample. Rates of chromosome aberration caused by polluted water are statistically significant and similar to micronuclei test as evaluated by *t* test, $P < 0.001$ (Table 4). This can be an important test for studying environmental etiology of liver cancer.

Table 4 Result of cell mutation test and polluted water samples

Water source type	Cell micronuclei rate %	Cell chromosomal aberration rate %
Pond water 1	63.34	6.42
Pond water 2	59.66	4.76
Shallow well water 1	48.32	3.76
Shallow well water 2	42.66	3.21
Deep well water	3.30	0.75
Blank contrast	2.43	0.17

STUDY ON THE WATER QUALITY BEFORE AND AFTER THE CHANGE IN WATER SOURCE

It has been accepted that liver cancer has close relations with the polluted drinking water. The changed water project has been made in many cancer prone areas since 1980's, especially in Fusui County, the changed water project has been implemented on a large scale, and the present water source is from the limestone layer. The depth of well is about 100 m-200 m. Most cancer prone villages have used this supply of water with beneficial results. We chose nine villages supplied with changed water and observed the change in the water quality and human health before and after. Before the water was changed, the pond water was severely polluted, and the color of water was brown and grey green, the water smelled terrible, and residents had many unhealthy symptoms such as pale cheeks, fatigue, distension of abdomen, menoxenia and liver cancer. After the water was

changed, the deep well water was clear and sweet, the symptoms noted above gradually disappeared, residents felt sound and the liver cancer death rate lowered. The results of water quality before and after the water was changed and mutation test are listed in Table 5.

Table 5 Contrast in water quality before and after the change in water

Test item (ppm)	NO ₂	Humic acid	COD	DO	NH ₄
1	0.0224	0.53	44.24	6.10	0.34
2	0.0106	0.05	0.34	8.21	0.07
Test item (ppb)	Se	Fe	Mn	Zn	nitrosamine
1	0.56	1110.88	198.33	9.29	4.20
2	0.39	164.60	10.14	50.47	0
Test item	HHPL				
	Plant cell micronuclei rate/%	micronuclei rate/%	chromosome aberration rate/%		
1	10.34	59.61	4.76		
2	5.96	3.30	0.75		

Note: 1. pond water, 2. deep well water.

From Table 5 it can be observed that the water quality of deep well is better than that of pond water. Thus using a change in water source and additional treatments, an improvement in human health and prevention in the occurrence of liver cancer^[25-32] may be observed.

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Influence of splanchnic vascular infusion on the content of endotoxins in plasma and the translocation of intestinal bacteria in rats with acute hemorrhage necrosis pancreatitis

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Subject headings acute hemorrhage necrosis pancreatitis; microcirculation/splanchnic organ; endo toxins/plasma; intestinal bacterial trans-location

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INTRODUCTION

The main reason for the death of the patient with acute hemorrhage necrosis pancreatitis (AHNP) is pancreatic infection and multi-organ failure caused by endotoxemia and intestinal bacterial translocation^[1-7]. However, the pathogenesis of endotoxemia and intestinal bacterial translocation remains a question^[8-10]; moreover, no effective method of prevention and cure for it has been found till now^[11-15]. In the present study, we infused low dose dopamine and low molecular weight dextran through the catheters to abdominal aorta and portal vein, and observed its influence on the endotoxin concentration in plasma and the rate of translocation of intestinal bacteria in AHNP rats.

MATERIALS AND METHODS

Animals

A total of 48 Sprague-Dawley rats weighing 295-320 g were divided into 4 groups (with 12 rats in each group): Group A (healthy rats), Group B (AHNP rats), Group C (femoral artery and femoral vein infused rats), and Group D (abdominal aorta and portal vein infused rats).

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Experimental methods

Bacterial labeling Following the way previously described by Wells, we directly labeled O₅₅B₅ Escherichia coli (*E. coli*) with fluorescein isothiocyanate to prepare the solution of 5×10^6 cfu/L tracer.

Induction of AHNP model and blood vessels catheter insertion and infusion

Firstly, all rats were deprived of food 24 h before laparotomy, and were given a gavage of fluorescein-labeled *E. Coli* (0.7 mL/100 g) 12 h later, then they were kept for 12 h before being anesthetized ip with 2% pentobarbital sodium (0.15 mL/100 g). Secondly, after the abdominal hair was removed and the abdominal cavity was opened through a midline laparotomy, the pancreas was exposed and 5% taurodexychoic acid sodium solution (0.15 mL/100 g; Sigma) was slowly injected into the pancreatic duct with retrograde pressure. Five minutes later, hemorrhage, necrosis, swelling and exudation appeared in the pancreas. Thirdly, 2 h after the AHNP models were completed, the animals of group B were infused continuously with saline through the catheters which were connected to the femoral artery and femoral vein and portal vein (the catheter was inserted from the ileocolic vein to the main trunk of portal vein), the animals of group C were infused continuously and alternately with low dose dopamine ($5 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and low molecular weight dextran (1.5 mL through catheters inserted into femoral artery and femoral vein, while being infused continuously with saline through the portal vein. The animals of Group D were infused continuously and alternately with low dose dopamine and low molecular weight dextran through the portal vein and abdominal aorta (catheters were inserted from the femoral artery and above the junction between the abdominal aorta and abdominal cavity artery), while being infused continuously with saline through the femoral vein. The total amount of infusion (6 mL/100 g) was the same in each group. Lastly, after 4 h of sustained infusion, the abdominal cavity was found to be hemorrhagic on being re-opened, and it was more evident in both Group B and Group C than in Group D. The pancreas showed pathological changes such as hemorrhage, necrosis, swelling and exudation, while Group A (control group) had no pathological changes.

Indicators and methods

Plasma endotoxin One mL vein blood was put into the heparin-containing test tube under aseptic and non-pyrogenic conditions, and after being centrifuged 500 rpm at for 10 min, the plasma was absorbed and preserved in a refrigerator at -20°C . The content of plasma endotoxin was investigated by quantitative azostromatic coloration limulus test microassay (kit from Institute of Medicine in Shanghai).

Investigation of mesenteric lymph node (MLN) labeled-bacteria MLN of the ileocecum were excised, weighed, triturated and diluted into 10% tissue plasma and was observed under fluorescein microscope for the existence of labeled-bacteria. The bacterial translocation rate was also calculated.

The microcirculation of pancreas and mesentery

The diameter of pancreatic and mesenteric small vein at the ileocecum end was directly measured under the microscope and recorded on the video-camera.

Pathological changes in the intestinal mucosa:

Observed under light and transmission electron microscopy.

RESULTS

Changes in content of plasma endotoxin and rate of translocation of labeled-bacteria in MLN (Table 1)

Table 1 Changes in plasma endotoxin concentration ($\bar{x} \pm S_x$, EU/mL) and the rate of translocation of labeled-bacteria in MLN

Group	Plasma endotoxin (2 h)	Plasma endotoxin (6 h)	Rate of translocation of labeled-bacteria (%)
A	0.023 ± 0.004	0.033 ± 0.006	0
B	0.028 ± 0.002	0.340 ± 0.038	91
C	0.025 ± 0.007	0.270 ± 0.048	83
D	0.027 ± 0.001	0.103 ± 0.018	33

There was a significant statistical difference between group D and either group B or C ($P < 0.05$), regarding the content of plasma endotoxin and translocation rate of MLN labeled-bacteria. However, there is no statistical difference between group B and group C ($P > 0.05$), while a significant difference exists when group A is compared with either group B, C, or D ($P < 0.05$).

Microcirculatory changes in pancreas and mesentery (Table 2)

Table 2 Change in the mesenteric small vein diameter after 2 h and 4 h AHNP

Group	2 h MVD	6 h MVD
A	0.66 ± 0.04	0.63 ± 0.04
B	0.69 ± 0.05	1.03 ± 0.05
C	0.72 ± 0.03	1.09 ± 0.05
D	0.77 ± 0.07	0.68 ± 0.05

Between 2 h and 4 h after AHNP, in Group B and Group C, the diameter of pancreatic and mesenteric small vein increased significantly ($P < 0.05$), and the velocity of blood was observed to be retarded or even blocked, while there was no significant increase in the diameters of pancreatic and mesenteric small veins in group D ($P > 0.05$) and no statistical difference regarding the velocity of blood stream between group D and group A ($P > 0.05$).

Pathological changes in intestinal mucosa

Optical microscopic observation It was seen that a large-number of mucosal chorionic epithelium were exfoliated, the upper parts of villus intestina were in significant edema, the central chylectasia was expanded, the blood vessels congested, the proprietary membrane was in moderate edema and the inflammatory cells infiltrated in group B and group C. While the damage of mucosa in group D was alleviated as compared to group B or group C, it was seen that only the villus became shorter, the proprietary membrane was in edema and the inflammatory cells infiltrated.

Electronmicroscopic observation Rarefaction and exfoliation of the epithelium microvilli of intestinal mucosa, exudation of matrix vacuolar degeneration of mitochondria, swelling of endoplasmic reticulum, and break-down of epithelium bridges were observed in groups B and C; and in group D only slight derangement of intestinal mucosa epithelium and slight swelling of mitochondria and endoplasmic reticulum were seen.

DISCUSSION

An extensive amount of experimental and clinical work reveals that the disorder of pancreatic microcirculation, the production of many inflammatory mediators and cytokines and the translocation of intestinal bacteria are all thought to play a critical role in the pathogenesis of acute hemorrhage necrosis pancreatitis^[15-26]; furthermore, the disorder of splanchnic organic microcirculation, especially the disorder of pancreas microcirculation in AHNP is closely connected with the production of many inflammatory mediators and cytokines^[26-37]. Dopamine has been seen to possess complicate pharmacological functions^[38-40], in above $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, alpha adrenergic receptors are additionally activated, causing splanchnic vascular contraction. At a dose range of $1-4 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, the effect is predominantly on dopaminergic receptors, leading to splanchnic dilatation. At the $4-10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, beta adrenergic receptors are increasingly stimulated, which stops the increase in microvascular permeability caused by histamine and bradykinin. It also activates dopaminergic receptors leading to an increase of blood flow in splanchnic organs. Low molecular weight dextran can lower blood viscosity and hemagglutination leading to a halt in microvascular thrombogenesis in portal vein system^[41-42]. Therefore, low dose dopamine and low molecular weight dextran can be used to improve splanchnic

microcirculation. With the aid of catheters inserted into the aorta and portal vein, we infused low dose dopamine and low molecular weight dextran continuously and alternately, which enhances the drug concentration in pancreas, liver and intestinal tract. The results have revealed that by this method the microcirculation was improved, and the content of endotoxin and the rate of intestinal bacterial translocation were decreased with increasing blood supply to the pancreas and intestinal tract of AHNP rats. This effect can be related to the following factors: Firstly, low dose dopamine and low molecular weight dextran can directly improve the ischemic status of intestinal tract and inhibit the damage of the barrier function of intestinal mucosa. It can also inhibit the pancreatic hemorrhage, necrosis and decrease the production of inflammatory mediators by improving microcirculation of pancreas, liver, and intestinal tract. On the other hand they can lighten the damage of inflammatory mediators and endotoxin on intestinal tract by enhancing the ability of the liver in clearing from inflammatory mediators and endotoxin. Hence, the method can alleviate the injury of intestinal mucosa and protect its barrier function, and inhibit endotoxemia and translocation of the intestinal bacteria, and so indicate that the disorder of microcirculation of pancreas and intestinal tract and liver are critically important to endotoxemia and the bacterial translocation from the intestine. The study also shows that the infusion through catheter to femoral vein and artery has no obvious influence on the content of endotoxin in plasma and the bacterial translocation in AHNP rats, the reason of which may be related to the low concentration of drugs in pancreas, liver and intestinal tract.

To achieve clearance of inflammatory mediators in patients with AHNP, in addition to drainage and removing the necrotic tissues by operation, we can also infuse low dose dopamine and low molecular weight dextran in to the abdominal cavity after operation through catheter inserted either from the right gastroduodenal vein to the portal vein or from the femoral artery, which can improve the microcirculation disorder of pancreas, liver and intestinal tract. Moreover, we can infuse enzyme inhibitors and other anti-inflammatory mediators through the catheter to the portal vein, so as to eliminate inflammatory mediators before they reach the liver.

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Regulatory effects of electro-acupuncture at Zusanli on ir-SP content in rat pituitary gland and peripheral blood and their immunity

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Subject headings electro-acupuncture; pituitary; point ST36; substance P (SP); erythrocytes; flow cytometry; rats; immunity; immunosuppression

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INTRODUCTION

It has been reported in many studies that electro-acupuncture (EA) can positively regulate erythrocytic immunity and T-lymphocytic subgroups^[1-8]. Nevertheless, its mechanism remains to be explored. In the present study, a multi-group, multi-stepped and multi-indexed observation was conducted on the effects of EA on erythrocytic immunity and T-lymphocytic subgroups. A simultaneous assay of the changes in immunoreactive substance-P (ir-SP) content in the pituitary gland and peripheral blood was also carried out. The objective of the study was to investigate the regulatory effects of the immune system and their possible mechanism in the treatment of relevant diseases with EA.

MATERIALS AND METHODS

Animals and groups

Forty healthy Shanghai SD white rats weighing 150 g-180 g, aged 12 wk-14 wk, provided by the Center for Laboratory Animals, 4th Military Medical University, Xi'an, were divided into 5 groups with 8 rats in each group: the normal control group, the Zusanli group, the immunosuppressive model group, the non-acupoint group, and the Zusanli + immunosuppressive group.

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Instruments and agents

G6805-A EA Instrument was purchased from Shanghai Medical Instrument Factory, Shanghai, China. Radioimmunoassay kit was provided by Beijing Haikerui Biotech Co. Ltd., Beijing, China. Epics-profile II Flow Cytometer was product of Coulter, USA. Anti-rat CD4 FITC/CD8PE was provided by SEROTEC, Britain.

Methods

Rat model of immuno-suppression Rat model of immuno-suppression was established by injecting 100 mg/kg of newly-prepared cytoxan (CY) solution into the rats' abdominal cavity on the 1st and the 4th day. Same amounts of normal saline was injected into the abdominal cavity of the rats of the other 4 groups.

Electro-acupuncture All the rats were raised in a quiet place. The acupoint chosen for EA was Zusanli (He-Sea Point, St 36)^[9]; the non-acupoint for EA was 0.5 cm off Zusanli. EA parameters were intermittent waves at 2 Hz and 3 V. The visual parameter was the slight shivering of the hind limbs of the rats. After they were fixed on a wooden board, the rats of the Zusanli-group were electro-acupunctured at Zusanli continually for 30 min at a fixed time for 7 d on a daily basis. The immunosuppressive group and the Zusanli + immunosuppressive group were also established according to the above method. EA treatment for the Zusanli + immunosuppressive group was the same as that for the Zusanli group. Rats of the control and the immunosuppressive group were tied by the same method for 30 min at a fixed time on a daily basis but did not undertake EA stimulation.

Sampling and assay Thirty mg/kg of 1% pentobarbital sodium was injected into the abdominal cavity of all the rats on the 7th day of EA. Blood samples were taken by decapitation after anesthesia with 15 U/ml of heparin as an anticoagulant. Ir-SP content in the pituitary gland and peripheral blood was assayed by radioimmunoassay (RIA) according to the manufacturer's instructions. By micro-whole-blood direct immunofluorescence staining and flow cytometry, T-lymphocytic subgroups were assayed to reflect the cellular immunity. The rats' RBC-C₃bRR and RBC-ICR were assayed by immune adherence rosette^[10].

Statistical analysis SPSS 8.0 (a statistical analysis system) was applied for the data analysis. The methods included variance analysis and linear correlation.

RESULTS

In the Zusanli group, the values of CD4+, RBC-C₃bRR and RBC-ICR and the content of SP in pituitary gland and peripheral blood were all markedly higher than that of the control ($P < 0.01$). The values of CD8+ did not change significantly ($P > 0.05$), and the rate of CD4+ positively correlated with RBC-C₃bRR ($r = 0.719$, $P < 0.05$), and so did the content of SP. The values of CD4+ and RBC-C₃bRR and the content of SP in rats' pituitary gland and peripheral blood of the immunosuppressive group were significantly lower than that of the control ($P < 0.01$, $P < 0.05$). The values of CD8+ did not change significantly ($P < 0.05$). In contrast, after EA at Zusanli, there was significant improvement in the indexes of the immuno-suppressive group ($P < 0.01$) whereas there was no statistical difference between the non-point and the control group (Tables 1 and 2).

Table 1 Changes in the values of T-lymphocytic subgroups and erythrocytic immunity ($n = 8$, $\bar{x} \pm s$)

Group	CD4+	CD8+	RBC-C-3bRR	RBC-ICR
Control	43.1 ± 3.1	23.6 ± 2.6	8.6 ± 2.1	6.8 ± 2.3
Non-point	44.7 ± 4.6	21.0 ± 4.3	11.1 ± 1.2	8.9 ± 2.9
Immuno-suppressed	34.5 ± 2.5 ^a	26.2 ± 8.4	5.2 ± 1.2 ^b	9.8 ± 4.4
Zusanli	65.6 ± 8.4 ^a	29.5 ± 8.2	15.9 ± 3.0 ^a	12.1 ± 1.2 ^a
Zusanli+Immuno suppressive	48.8 ± 6.0 ^c	22.6 ± 7.0	16.4 ± 4.1 ^c	15.0 ± 5.2 ^c

^a $P < 0.01$, ^b $P < 0.05$ vs control group; ^c $P < 0.01$ vs immuno-suppressed group. EA: electro-acupuncture

Table 2 The contents of substance P in pituitary gland and peripheral blood in rats ($n = 8$, $\bar{x} \pm s$)

Group	Pituitary gland	Peripheral blood
Control	569 ± 8	20 ± 7
Non-point	547 ± 22 ^b	20 ± 8 ^b
Immuno-suppressed	273 ± 106 ^a	12 ± 4
Zusanli	592 ± 142 ^{ab}	92 ± 22 ^a
Zusanli+immuno-suppressive	554 ± 32 ^b	30 ± 9 ^b

^a $P < 0.01$ vs control group; ^b $P < 0.01$ vs immuno-suppressed group. EA: electro-acupuncture

DISCUSSION

Regulatory effects of EA at Zusanli on the neuroendocrine system

Zhao *et al*^[11] discovered the cubic structure of the microangium of Zusanli and Lin *et al*^[12] reported that there might be a nerve network in the colloid of the spine and brain stem that corresponds with the stomach channel of Foot Yangming, St, runs through the spine, and reaches the nucleus of spinal tract of trigeminal nerve. These studies have proved the regulatory effect of EA at

Zusanli on neuroendocrines. With the development of studies on the mechanism of acupuncture, the relation between acupuncture and endocrine hormone has become a focus of interest for many researchers. Many studies have indicated that acupuncture can regulate thyroid hormones, sex hormones, adrenocortical hormones, insulin, brain-gut peptide, etc^[13]. Substance P, an important cerebral and brain-gut peptide is widespread in the central nervous system and gastrointestinal tract. It exists not only in the endocrine and paracrine cells, playing the roles of hormones and the local transmitter, but also in the endogenous and exogenous neurons, playing the role of a neural transmitter. The present study has proved that EA at Zusanli can significantly increase the synthesis of substance P in the pituitary gland, and its content in the peripheral blood, thus bringing into play its unique physiological and pathophysiological regulatory functions.

The general regulatory effects of Zusanli EA and the theory of neuroendocrine-immune network

It has been reported that many hormones can modify the body's immunity by their intervention at macrophages, T-lymphocytes and B-lymphocytes^[14-20] receptor sites whereas immune cells can also secrete various neuropeptides and hormones as immune transmitters. What is more, some neuroendocrine cells can secrete cellular factors. These cells can influence both the neuroendocrine system and the immune system. The three systems of nerve, endocrine and immunity depend mainly on the peptidergic factors they generate and the receptors of the latter to communicate with each other. Therefore, immune cells can also be regarded as a kind of receptors in the body that receive stimulation from antigens, secrete immune transmitter, transmit information to the central nervous system before it regulates, as a feedback, the body's immunity by means of transmitting nerves and hormones. This is the so-called "theory of neuroendocrine-immune regulatory network". The regulatory effect of EA at Zusanli on the nervous, endocrine and immune systems cannot be separated from its internal association with the neuroendocrine-immune regulatory network. In addition to its direct regulation of the above systems, acupuncture can also indirectly act upon the systems through their internal association with each other. This is probably the theoretical basis of Traditional Chinese Medicine's general regulatory principle. It is also a point of collision between Traditional Chinese Medicine and modern medicine, namely the holistic medical mode^[21].

Role of SP in the mechanism of acupuncture's effects on the immunoregulation

SP is an important biologically active substance of information channel transmission^[22]. Modern immunological research has proved that cells of the immune system can release SP and contain SP conjugative sites of

high affinity^[23]. SP can affect the immune system in the following ways. It can 1) stimulate and proliferate lymphocytes *in vivo* and *in vitro*; 2) improve the proliferative reaction of the spleen, mesenteric lymph nodes and intestinal aggregated lymphatic follicles^[24]; 3) at milli-Molar level, it can induce interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factors (TNF) from monocytes^[25]; 4) increase lipopolysaccharide (LPS)-induced secretion of IL-10 from monocytes in human umbilical cord blood and reverse the inhibitory effect of INF- γ on LPS-induced secretion of IL-10^[26]; 5) stimulate the synthesis of PGE₂, which in turn stimulates the generation of cellular factors^[27]. The present study indicates that after electro-acupuncture at Zusanli, ir-SP in the rats' pituitary gland and peripheral blood increased significantly in comparison with that of the control and were positively correlated with the change in CD4⁺. This attests that electro-acupuncture can regulate cellular immunity by exciting organism synthesis and releasing SP and affect the neuroendocrine-immunoregulatory network through the indirect effect of SP on the change of various cellular factors. However, the change in SP after electro-acupuncture at Zusanli had nothing to do with the immune indexes of RBC, which indicates that electro-acupuncture probably does not depend on SP to influence RBC immune indexes.

The effects of EA at Zusanli on the mucosal immunity of gastrointestinal tract

Modern immunological studies have confirmed the significant regulatory effects of gastrointestinal hormone on the intestinal mucosal immunocytes^[28-30]. The intestinal mucosal immuno-tissues are controlled by peptidergic nerve fibres, which were located adjacent to lymphatic systems in the epithelial layer, lamina propria and immunocytes such as macrophages, mastocytes, etc. can directly regulate the intestinal immune system. In the intestine-associated lymphatic tissues, the existence of T-lymphocytes and SP receptors specific to B cells in dense SP teloneurons and aggregated lymphatic follicles indicates that SP might be acting as a nutritious factor for the intestinal immunocytes. It is by stimulating its organic synthesis and release that EA at Zusanli regulates the intestinal immunity, thus adjusting gastrointestinal diseases and functions. Furthermore, EA at Zusanli can also positively regulate the general immune system by the exchange between GALT and the general lymphatic tissues. According to Traditional Chinese Medicine, pathogenesis boils down to three factors, i.e., spiritual, exopathic and constitutional factors. The three factors constitute the gist of the theory of neuroendocrine-immunoregulation network.

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The serological prevalence and risk factor analysis of hepatitis G virus infection in Hubei Province of China

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Subject headings hepatitis G virus; enzyme linked immunoabsorbent assay; risk factors; polymerase chain reaction; prevalence; transcription, genetic

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INTRODUCTION

Hepatitis G virus (HGV), also known as GB virus C, is a recently cloned virus which may be associated with human non A-E hepatitis^[1,2]. It is parenterally transmitted and usually coinfects or superinfects with hepatitis B or hepatitis C virus^[3-5]. Some investigations have been reported on the seroprevalence and molecular prevalence of HGV infection in different areas and different population^[6-15]. Current infection of HGV is diagnosed by detection of HGV RNA, and past infection with HGV is detectable by testing anti-HGV envelope protein (E2)^[16-17]. To investigate the prevalence of HGV in Hubei Province, a central area of the People's Republic of China, ELISA and RT-PCR were employed to detect serum anti-HGV and HGV RNA in 1516 patients who were divided into 16 groups.

MATERIALS AND METHODS

Samples

One thousand five hundred and sixteen serum samples were obtained from 16 groups in Hubei Province, including 299 drug users (19.72%), 98 prostitutes (6.46%), 504 blood donors (33.25%), 61 hemodialysis patients (4.02%), 351 cases with viral hepatitis (23.15%), 41 with primary hepatocellular carcinoma (PHC, 2.70%), 51 with hemopathy (3.36%), 53 renal transplant recipients

(3.49%) and 3 liver transplant recipients (0.20%). 55 freshmen, nurses and doctors (3.63%) were employed as healthy controls.

Reagents and detecting methods

Serum HBV markers, HAV IgM, HEV IgM, and anti-HCV were detected by enzyme linked immunoabsorbent assay (ELISA). Anti-HGV was also detected by ELISA and the test kits were procured from Wantai Biological Preparation Co Ltd, Beijing. HGV RNA was assayed by reverse transcript polymerase chain reaction (RT-PCR) with the primers of 5'-UTR, and the kits were purchased from the Center of Hepatitis Reagents, Beijing. Both anti-HGV and HGV RNA were simultaneously measured in the drug users, prostitutes, and healthy subjects. For other groups, HGV RNA was detected only when their anti-HGV was positive due to insufficient outlay.

Data statistics

All data were analyzed by means of the Chi-square test.

RESULTS

HGV infections in drug users and prostitutes

The positive rates of anti-HGV and HGV-RNA in drug users were 9.06% (34/375) and 27.20% (102/375); those in prostitutes were 38.77% (38/98) and 20.41% (20/98). The analysis of risk factors of HGV infections in drug users and prostitutes is listed in Tables 1 and 2.

Table 1 Positive rates of anti-HGV and HGV RNA in drug users and prostitutes

	n	Anti-HGV	HGV RNA
Drug users [*]			
po	211	6.63 (14)	27.96 (49)
iv	164	12.20 (20)	32.92 (54)
Prostitutes			
Non-addict	22	9.09 (2)	13.36 (3)
Addict	76	14.43 (36)	22.36 (17)
Healthy group	55	1.82 (1)	0 (0)

*Seventy-six prostitutes who were addicted to drugs were added to this group.

The positive rate of HGV RNA in intravenous drug users (IVDU) was higher than in oral users ($\chi^2 = 4.36$, $P < 0.05$). The positive rates of anti-HGV in the prostitutes who were addicted to drugs were higher than those who were not ($\chi^2 = 12.19$, $P < 0.01$).

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Table 2 Risk factors of HGV infections in drug users

	Sex		Addiction duration		HBV infection	
	Male	Female	< 2 years	≥2 years	HBsAg (+)	HBsAg (-)
Anti-HGV	8.65	9.23	6.48	12.58	13.33	9.37
HGV RNA	3.46	32.84	9.72	52.56	16.67	22.78

The statistical difference of anti-HGV positive rate was tested between users addicted for more than 2 years and users for less than 2 years ($\chi^2 = 4.29$, $P < 0.05$). The positive rate of HGV-RNA in female users was higher than that in male users ($\chi^2 = 11.55$, $P < 0.01$), that in the patients who were addicted for more than 2 years was also higher than those addicted for less than 2 years ($\chi^2 = 79.06$, $P < 0.01$).

HGV infections in blood donors

Seven percent (29/417) of blood donors were found to be anti-HGV positive and 27.5% anti-HGV positive donors were detected to be HGV RNA positive.

HGV infections in the cases of PHC, hemodialysis, liver or kidney transplantation (Table 3)

Table 3 HGV infections in the cases of PHC, hemodialysis, liver or kidney transplantation

	n	Anti-HGV	HGV RNA in anti-HGV (+) cases
Hemodialysis	61	13.11 (8/61)	37.50 (3/8)
PHC	41	4.80 (2/41)	100.00 (2/2)
Liver transplantation	3	33.30 (1/3)	100.00 (1/1)
Kidney transplantation	53	1.82 (1/53)	0
Total	158	7.5 (12/158)	50.00 (6/12)

HGV infections in the patients with hemopathy

Anti-HGV was detected in 6 (11.7%) out of 51 cases with hemopathy, among them, 4 cases with acute or chronic leukemia, one with aplasia anaemia and one with leukopenia. No HGV RNA was assayed in six anti-HGV positive cases.

HGV infections in the patients with viral hepatitis (Table 4)

Table 4 Positive rates of anti-HGV and HGV RNA in patients with viral hepatitis

	n	Anti-HGV	HGV RNA in anti-HGV (+) cases
Hepatitis A	35	14.29 (5/35)	20.00 (1/5)
Hepatitis B	214	17.29 (37/214)	35.14 (13/37)
Hepatitis C	62	14.52 (9/62)	55.56 (5/9)
Hepatitis E	25	4.00 (1/25)	0
Hepatitis NA-E	15	26.67 (4/15)	50.00 (2/4)
Total	351	15.05 (56/351)	37.50 (21/56)

The positive rates of anti-HGV and HGV RNA did not correlate with the sex, age, duration, and severity of diseases. The mean levels of ALT and T-Bil in patients

with hepatitis B whose anti-HGV was positive were higher than those whose anti-HGV was negative.

DISCUSSION

HGV is a new pathogenic agent which was discovered in 1995, its genome structure resembles other flaviviruses containing a positive, single plus-strand RNA^[1,2]. It has been demonstrated that the distribution of HGV is global^[1,2,6-14]. The transmission route of HGV is similar to that of HBV and HCV^[1,3,4,11,12]. The investigation on HGV infection rates in Hubei Province of China showed that the infection rates of HGV in cases with viral hepatitis, hemodialysis, hemopathy, transplantation, intravenous drug users (IVDU) and prostitutes were 15.05%, 13.11%, 16.7%, 9.1% and 14.43%, higher than that in healthy controls (1.82%). The result indicates that HGV infection is common in China.

The infection rate of HGV in drug users varies widely on different documents with a range from 23.8% to 77.4%^[7,18-20]. However, there is no controversy regarding the fact that drug addiction is a high risk factor for HGV infection. We found that the positive rate of HGV RNA in IVDU was higher than that in oral drug users (12.82% vs 2.97%, $P < 0.05$). This suggests that the unsterilized injections contribute to HGV transmission among IVDU.

It is known that sexual contact is a common route for horizontal transmission of HBV, HCV and HDV. Recently, sexual transmission of HGV has been demonstrated^[18,21-25]. Our survey found that most of the prostitutes were also addicted to drugs, this may be one of reasons that they had a high HGV infection rate. HGV infection was found in 15.05% of patients with viral hepatitis. This is much higher among the blood donors in the same geographic area. However, there may be bias in this statistical inference, since blood donors who are found to be positive for HBsAg or for anti-HCV through screening programs are told not to donate blood. Thus, the low prevalence of HGV infection may represent a selection bias. This viewpoint was demonstrated by Handajani *et al*^[14].

No agreement has been reached on the pathogenicity of HGV. Most of the recent investigations show that HGV infection in patients with hepatitis B and hepatitis C is not associated with any changes in indices of liver diseases, including serum ALT level, Knodell score or histology activity index (HAI)^[25-28], and the consequences of hepatitis^[27,29]. However, some reports including this paper found that HGV infection was associated with liver damage, even fulminant hepatitis^[31,32]. So further prospective studies are needed to demonstrate its relative significance in causing hepatitis and other diseases.

High infection rates of HGV were also discovered in patients with hemodialysis, PHC, and transplantation. This result may be associated with the more transfusions received by these cases than healthy persons.

A conclusion which can thus be drawn from this study

is that HGV infection is common in this area, and the drug users, prostitutes, transplant recipients, blood donors, patients with hemopathy, hemodialysis, and liver diseases are high risk groups for HGV infection.

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High-level expression of human calmodulin in *E.coli* and its effects on cell proliferation

Xiao Jun Li, Jian Guo Wu, Jun Ling Si, Da Wen Guo and Jian Ping Xu

Subject headings calmodulin; gene expression; biological activity; *Escherichia coli*; cell proliferation; trifluoperazine; polymerase chain reaction; monoclonal antibodies

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INTRODUCTION

Calmodulin (CaM), widely distributed in almost all eukaryotic cells, is a major intracellular calcium receptor responsible for mediating the Ca^{2+} signal to a multitude of different enzyme systems and is thought to play a vital role in the regulation of cell proliferative cycle^[1,2]. Recently, many studies showed that CaM is also present in extracellular fluid such as cell culture media and normal body fluid and has been reported to stimulate proliferation in a range of normal and neoplastic cells, apparently acting as an autocrine growth factor^[3-11]. In 1988, Crocker *et al* reported for the first time that addition of extracellular pure pig brain CaM could promote DNA synthesis and cell proliferation in K₅₆₂ human leukaemic lymphocytes^[7]. After that, more and more research was done on extracellular CaM and evidences demonstrated that extracellular CaM could also stimulate cell proliferation in normal human umbilical vein endothelial cells^[5], keratinocytes^[4], suspension-cultured cells of *Angelica Dahurica*, etc^[6]. CaM is a monomeric protein of 148 amino acids that contains four homologous Ca^{2+} binding domains. CaM has been highly conserved throughout the evolution. Only 1 out of 148 amino acids of human CaM is different from that of fish CaM. Complementary DNAs encoding rat, eel, chicken, human, and trypanosome CaM have been cloned.

In this paper, we describe the expression and purification of recombinant human CaM (rhCaM) and the

role of extracellular rhCaM in SP2/0 mouse myeloma cell proliferation.

MATERIALS AND METHODS

Construction of expression plasmid

PCR amplification was used for the insertion of the coding sequence of human CaM III (hCaM III) between BamHI and EcoRI sites of the expression vector pBV220 (constructed by Dr. Zhang^[12,13]). The primers for PCR based on the reported sequence of hCaM III were synthesized by DNA synthesizer (Sangon). Upper primer: 5'-CGGAATTCATATGGCTGACCAGCTGAC-3', containing EcoRI restriction site. Down primers: 5'-CGGGATCCTTACTTTGCAGTCATCA TC-3', containing BamHI restriction site. The vector pUC/hCaM III (a generous gift from Dr. Strehler EE^[14]) was used as template. PCR amplifications were performed on a thermocycler (PE 9600). The PCR conditions used were 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, for 30 cycles. The product was a single band of about 400 bases detected by 1.2% agarose gel electrophoresis. After purifying the PCR product by High Pure PCR Product Purification Kit (Boehringer Mannheim) and isolating the vector pBV220 by the Wizard Plus Minipreps DNA Purification Kit (Promega), the amplified gene fragments of hCaM III digested with EcoRI and BamHI were ligated into the pBV220 vector that had been previously digested with the same enzymes. The recombinant construct (pBV220/hCaM III) was then used to transform *E.coli* DH5 α (a generous gift from Beijing Institute of Basic Medical Sciences) competent cells by the $CaCl_2$ method.

Expression and purification of rhCaM

A single colony from *E.coli* DH5 α cell harboring the pBV220/hCaM III construct was used to inoculate a 5-mL LB culture. After overnight growth at 30°C, the 5 mL culture was used to inoculate a 1-L culture in LB medium and grown at 30°C for 4-6 h until the absorbance reached 0.5 at 600 nm. The bacterial culture was then induced at 42°C for additional 5 h. Stable rhCaM-expressing recombinant *E.coli* was harvested by centrifugation at 5000 r/min for 5 min. The pellets were washed twice with PBS, resuspended in 0.05 mol/L Tris-HCl buffer (pH 7.5, containing 0.25 mmol/L PMSF, 2 mmol/L EGTA and 2 mmol/L β -mercaptoethanol) and disrupted by sonication (Cyclone/Tempest IQ₂) at 100 W for 30 min with 10 s of pulse and 10 s of pulse off time. Supernatant fractions were obtained by centrifugation at 12 000 r/min for 30 min. $CaCl_2$ solution 0.1 mol/L was added to the supernatant to

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adjust to 5 mmol/L CaCl₂ final concentration. The supernatant was applied to a Phenyl-sepharose CL-4B affinity column (Pharmacia) equilibrated with buffer I (0.05 mol/L Tris-HCl buffer, pH 7.5, 0.1 mmol/L CaCl₂), then the column was washed with buffer I followed by another wash with buffer I containing 0.5 mol/L NaCl. CaM then was eluted from the column with buffer I containing 1 mmol/L EGTA. The solution containing purified rhCaM was dialysed against ddH₂O for 48 h. The protein concentration was determined by Bradford assay. RhCaM was filtered through micropore filter membrane (0.22 μm) to remove bacteria and stored at -20°C.

SDS-PAGE and immunoblot assay

Expression and purified protein were analyzed by 15% SDS-PAGE. Gels were stained with Coomassie brilliant blue G. For immunoblot analysis, protein were separated by 15% SDS-PAGE and electroblotted on to nitrocellulose (NC) membrane (BioRad). After blotting, nonspecific protein binding sites were blocked with 3 g/L BSA in 50 mmol/L Tris-HCl, pH 8, and 150 mmol/L NaCl, 0.5% Tween 20. The NC membrane were incubated with a 1:200 dilution of anti-CaM McAb (Sigma). After washing, the membrane were incubated for 1 h with HRP conjugated goat anti-mouse immunoglobulin antibody (1:200, Boehringer Mannheim). Color development was obtained by adding DAB substrate.

Amino acid composition

The amino acid composition of purified expression product was analyzed using an automatic amino acid analyzer, Hitachi 835-5Q.

DNA sequence analysis

The DNA sequence was determined with ABI PRISM™ 377 DNA sequencer.

NAD kinase assay

The activity of CaM-dependent NAD kinase (NADK) was detected as described by Harmon^[15].

Cell culture

SP2/0 cells were cultured in RPMI 1640 medium (GIBCO), supplemented with 20% neonatal calf serum (NCS) under 37°C, 5% CO₂ conditions.

Measurement of cell proliferative rate

Cell proliferative rate was determined by MTT colorimetric assay^[16,17]. SP2/0 cells were collected at the logarithmic growth phase by centrifugation. The cells were washed twice and seeded in 96-well plate and cultured for 48 h with rhCaM and CaM-antagonist trifluoperazine (TFP). MTT 10 μL (Sigma, 0.5 mg MTT in 1 mL PBS) was added to each well of 96-well plate and cultured for another 4 h. After the formazan was dissolved with 100 μL DMSO, absorbance value of each well at 490 nm (A_{490nm})

was read on a BioRad Model 550 microplate reader.

Proliferative rate = A_{490nm} value of experimental group / A_{490nm} value of control group × 100%. Improved proliferative rate = Proliferative rate - 100%.

RESULTS

Expression of rhCaM in *E. coli*

The results of both restriction enzyme digestion of pBV220/hCaM III and PCR identification in which pBV220/hCaM III was used as template showed a specific single band with the same molecular weight as reported hCaM gene on agarose gel (Figure 1). DNA sequence analysis also indicated that the recombinant vector had been constructed successively. After heat induction, the total extract of the *E. coli* DH5α cell harboring pBV220/hCaM III was analyzed by 15% SDS-PAGE under reducing conditions. As shown in Figure 2, a unique protein band with an apparent molecular weight (17 000) was similar to that of standard human brain CaM. This protein accounted for over 20% of the total cellular protein. The study on solubility of expression protein indicated that CaM protein was expressed predominantly in the soluble form. Western blot analysis showed that anti-CaM McAb specifically bound to the 17 000 band of expression product. The expression product purified by phenyl-sepharose CL-4B affinity chromatography was shown as a single band on agarose gel by SDS-PAGE. The protein concentration was determined by Bradford method and approximately 3-4 mg of the purified protein were obtained from 1 L of bacterial culture.

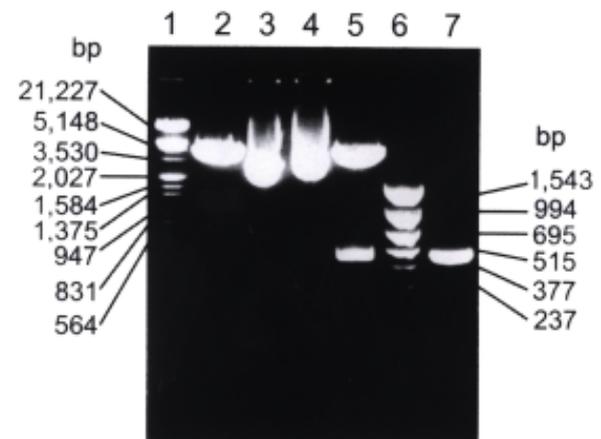


Figure 1 Restriction enzyme digestion and PCR analysis of recombinant plasmid pBV220/hCaM III.

1. λDNA/Hind III+EcoR I marker
2. pBV220 digested with EcoR I and BamH I
3. pBV220
4. pBV220/hCaM III
5. pBV220/hCaM III digested with EcoR I and BamH I
6. pBR322/Hinf I marker
7. PCR product of hCaM III

Amino acid composition analysis

The amino acid compositions of purified rhCaM were identical to those of the previously reported bovine brain

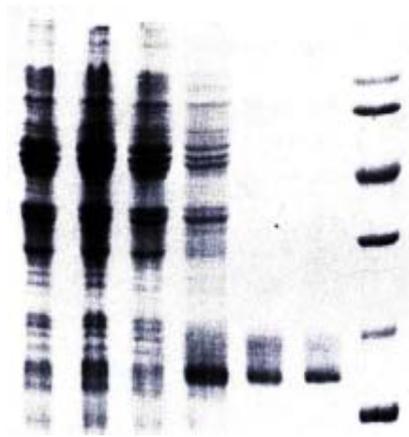


Figure 2 SDS-PAGE analysis of hCaM expression and purification.

1. Uninduced DH5 α /pBV220
2. Induced DH5 α /pBV220
3. Uninduced DH5 α /pBV220-hCaM III
4. Induced DH5 α /pBV220-hCaM III
5. Purified rhCaM by Phenyl-sepharose CL-4B column
6. Standard Human brain CaM (Sigma)
7. Protein molecular weight marker

CaM. The acidic amino acid (such as Asp and Glu) composition was about 30% or more.

NAD kinase assay

The results of NAD kinase (NADK) assay showed that purified rhCaM was able to activate CaM-dependent NADK activity to the same extent as the standard human brain CaM (Sigma, Figure 3).

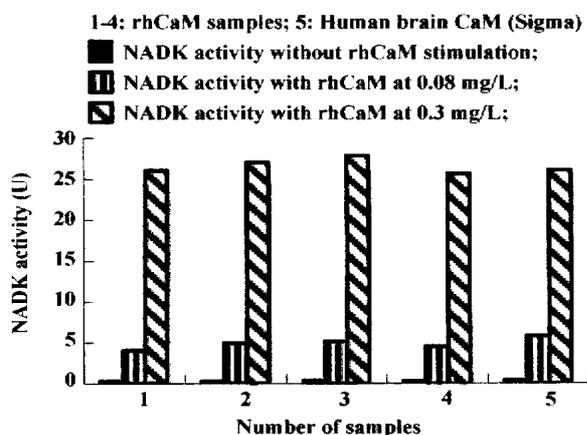


Figure 3 Activation of NADK by rhCaM.

Effect of extracellular rhCaM on cell proliferation

Cells were diluted with RPMI 1640 (containing 0.5% NCS) to $1-5 \times 10^5$ /mL and seeded in 96-well plates. rhCaM was added to the final concentration of 0.1-10 μ g/mL and each concentration was triplicated. Cells were incubated with different concentrations of rhCaM under 37°C, 5% CO₂ conditions for 48 h prior to MTT assay. The effect of extracellular rhCaM on cell proliferation was investigated (Figure 4). Evidence indicated that rhCaM within a certain concentration (0.1-7.5 mg/L) could stimulate cell proliferation. The stimulatory effect declined

when the rhCaM concentration was higher than 10 mg/L. Effect of a addition of pure rhCaM was also dependent in some degree upon the cell density of the initial culture. For a certain CaM concentration, the lower the cell density of the initial culture, the higher the promoting effect. Moreover, the effect of exogenous rhCaM was also influenced by the amount of NCS added to the culture medium. When NCS in medium accounted for 0.5%-1%, a significant stimulatory effect could be observed. However, when NCS in medium was increased to 2%-10%, no stimulatory effect was observed for rhCaM (data not supplied).

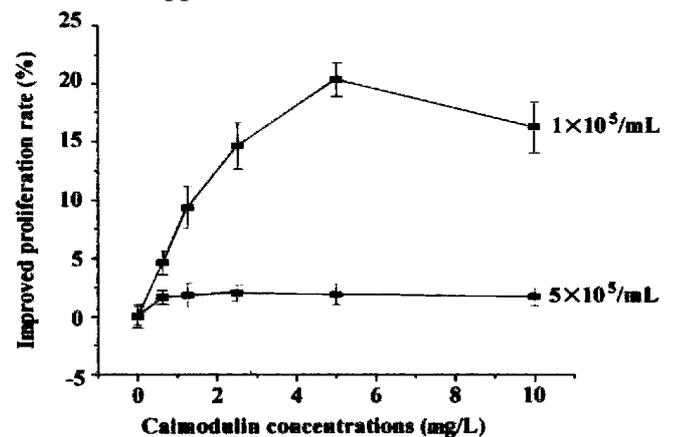


Figure 4 The effect of extracellular rhCaM on cultured SP2/0 cells.

Inhibitory effect of TFP on cell proliferation

Cells were seeded in 96-well plates as above. CaM-antagonist TFP (Sigma) was added to the final concentration of 0.025-250 μ mol/L. Each concentration was done in triplicate. Cells were incubated with TFP under 37°C, 5% CO₂ conditions for 48 h and determined by MTT assay. Results indicated that addition of various concentrations of TFP to cell culture medium could significantly inhibit cell proliferation rate and the inhibitory effect strengthened with increase in the concentrations of TFP (Figure 5).

Effect of extracellular rhCaM on TFP-inhibited cells

TFP was added to the SP2/0 cell (1×10^5 /mL) culture medium until the final concentrations were 25 μ mol/L. Cells were cultured in 5% CO₂ incubated under 37°C for 6h, washed twice with RPMI 1640, then seeded into 96-well plate at the density of 1×10^5 /mL. Various concentrations of rhCaM were added and cells were cultured for 48 h. Results demonstrated that addition of rhCaM could alleviate the inhibitory effect of TFP. rhCaM 5 mg/L could offset the inhibition almost to normal (Figure 6).

DISCUSSION

CaM, a heat-stable, acidic and multifunctional calcium-binding protein, exists in almost all eukaryotic cells. On the gene level, a multigene family of three maximally

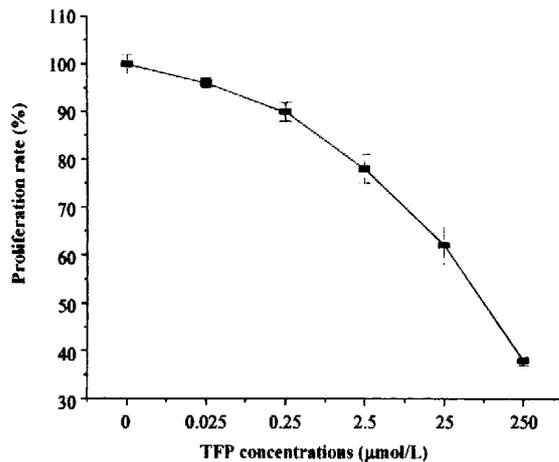


Figure 5 The inhibitory effect of CaM-antagonist TFP on cell proliferation.

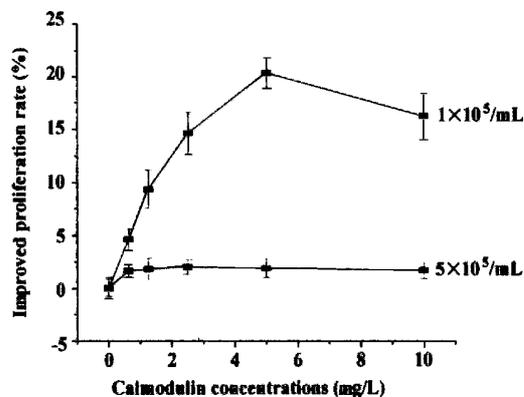


Figure 6 The effect of extracellular rhCaM on TFP inhibited cells.

divergent members is responsible—at least in mammals—for the generation of the single CaM protein with the same amino acid sequence. Three human CaM cDNAs called hCaM I, hCaM II, and hCaM III have been cloned^[14,18-23]. In this paper we described the construction of expression plasmid for human CaM III gene and expression of hCaM protein in *E. coli*. About 3-4 mg of purified rhCaM was obtained from 1 L of *E. coli* culture. This level of production is higher than that achieved by the classic purification method. If the genetic engineering techniques are used for a large scale expression, the product can be much higher.

It is well known that intracellular CaM plays an important role in the regulation of cell proliferation. The levels of intracellular CaM and CaM mRNA vary during cell cycle and accelerate cell proliferation. Moreover, CaM antagonists TFP, W₇, W₁₃ and anti-CaM antibodies can block the effect of CaM and arrest cell cycle at G₁/S boundary^[24-27]. In the last few years, extracellular CaM was detected and evidence accumulated that extracellular CaM could also affect cell proliferation.

In our study, effects of purified rhCaM and CaM antagonist TFP on SP2/0 proliferation rate were examined. The results revealed that certain concentrations of extracellular rhCaM (0.1-7.5 mg/L) could stimulate cell

proliferation in a dose-dependent manner and the stimulatory effect was dependent upon the cell density of the initial culture. The lower the cell density, the higher the stimulatory effect. The results are consistent with previous literature^[4-7]. TFP inhibited cell proliferation and the inhibition could be alleviated by addition of extracellular rhCaM, which further supports that extracellular CaM could accelerate cell proliferation. CaM was also detected in many extracellular body fluids such as breast milk, saliva and serum. Serum, which is necessary for the successful culture of many cell types, contains high levels of CaM which could amount to 0.9-8 mg/L^[28]. This might explain why the effect of CaM was influenced by NCS added to culture media. While CaM could facilitate cell proliferation, compared with NCS, the effect of CaM was less important.

So far, how extracellular calmodulin achieves its effects is yet unclear. Evidence indicates that the mechanisms may be as follow: (1) CaM can interact with some cell proliferation-related factors such as epidermal growth factor (EGF), transforming growth factor (TGF) and platelet derived growth factor (PDGF). It is possible that extracellular CaM exerts its effects by strengthening these factors' binding to their membrane receptors. It was reported that EGF receptor contained a CaM-binding domain^[29] and the binding of EGF to cells could be inhibited by CaM antagonist^[30]. Mac Neil *et al* showed that there was a significant positive correlation between CaM level and EGF concentrations in normal body fluids, indicating that CaM together with EGF, TGF, PDGF etc may be members of a functionally co-ordinated group of mitogens^[28]. (2) CaM may directly interact with CaM-binding proteins (CaMBPs) on cell membrane. CaMBPs were found to exist on cell wall of wheat coleoptiles^[31]. Extracellular CaMBPs were also detected in the suspension-cultured cells of *Angelica Dahurica* and carrot^[32]. CaMBP is a kind of glycoprotein which can bind with CaM and may function as a bridge between CaM and intracellular metabolic processes.

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Lactosamination of liposomes and hepatotropic targeting research

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Subject headings liposomes; asialoglyco-protein; liver; interferon-alpha; antigens, viral; drug carriers; drug therapy; rats

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INTRODUCTION

Site-specific delivery of therapeutic drugs to their target cells is a major scientific challenge for the pharmaceutical sciences. It offers a number of advantages over conventional drug administration. With drug targeting, high local concentrations of the drug can be achieved, thus circumventing many unwanted side effects. Various carriers have been suggested for the delivery of drugs, including liposomes^[1-5] and (neo) glycoproteins^[6-8]. The asialoglycoprotein receptor (ASGP-R) has frequently been utilized for targeting drugs to the parenchymal liver cell^[6-12]. Liposomes have several advantageous characteristics as drug carrier, and particularly, ligand-tacked liposomes achieve a highly effective targeting^[13]. Hara *et al* reported that asialofetuin (AF)-tacked liposomes distributed to rat hepatocytes selectively *in vivo*^[14], and ASGP-R mediated the uptake of AF-liposomes encapsulating IFN- γ by isolated rat hepatocytes *in vitro*^[15]. Lactosaminated human serum albumin (L-HSA) is a neoglycoprotein taking number of galactose residue as terminal sugar^[6].

In this paper, we studied the preparation and rat hepatocyte uptake of the conjugate of L-HSA and liposomes, and the inhibitory effect of L-HSA-liposomes containing IFN- α on replication of hepatitis B virus (HBV) on 2.2.15 cells line.

MATERIALS AND METHODS

Materials

Sodium cyanoborohydride was purchased from Aldrich, Chemical Co., Milwaukee, WI, USA, α -lactose,

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phosphatidylcholine (PC), cholesterol (Chol), and N-succinimidyl-S-acetylthioacetate (SATA) were obtained from Sigma, St. Louis, MO, U SA. Maleimido-4-(p-phenylbutyryl) phosphatidylethanolamine (MPB-PE) was purchased from Avanti Polar lipids, Birmingham, AL, USA. Dulbecco's modified eagle medium, penicillin and streptomycin were obtained from GIBCO, Grand Island, NY, USA. MTT was from Boehringer Mannheim, Germany. Fetal calf serum was a product of Hyclone, USA. Other reagents were of analytical grade.

Methods

Lactosamination of human serum albumin

Lactosamination was performed according to Schwartz & Gray^[16]; human serum albumin (HSA) 500 mg was allowed to react at 37°C with 1.0 g α -lactose and 1.0 g NaBH₃CN in 20 ml 0.05 mol/L potassium phosphate buffer solution (PBS), pH 8.0. The reaction was continued for 144 h. And the mixture was dialyzed against double distilled water. The lactose content of lactosaminated human serum albumin (L-HSA) was determined by the phenol-sulphuric acid method of Dubois *et al*^[17].

Liposomes preparation PC, Chol, and MPB-PE were mixed in a molar ratio of 23:16:1 and dissolved in ether, dried by a rotary evaporator, dissolved in HN-buffer (10 mmol/L HEPES, 135 mmol/L NaCl, pH 6.7) and lyophilized. Phospholipid phosphorus of liposome preparation was measured by phosphate assay after perchloric acid destruction. Conventional liposomes were prepared with PC and Chol in a molar ratio of 23:16.

Coupling of L-HSA to MPB-PE containing liposomes (MPB-liposomes)

L-HSA was coupled to MPB-liposomes by a sulfhydryl-maleimide coupling technique according to Derksen *et al*^[18]. Using SATA as a heterobifunctional reagent, free sulfhydryl groups were introduced in L-HSA^[19]. After separation of SATA from the protein by Sephadex G-25 gel permeation chromatography, the acetylthioacetate-L-HSA was deacetylated by a freshly prepared solution of 0.5 mol/L hydroxylamine-HCl, 0.5 mol/L HEPES, 25 mmol/L EDTA, pH 7.0. After deacetylation, the thioacetyl-L-HSA was allowed to react with the MPB-PE containing liposomes for 4 hours at room temperature, in a ratio of 3 mg of protein per mg liposomes. N-ethylmaleimide was added to cap un-reacted sulfhydryl groups. Liposomes were separated from unconjugated protein by Sepharose 4B

infiltration. The L-HSA-liposomes conjugates were characterized by determining protein and phospholipid phosphorus content, lyophilized, and stored at 4°C.

Tissue distribution of L-HSA-liposomes L-HSA-liposomes were labeled with ^{131}I by Iodogen's method. Male Sprague-Dawley rats weighing 200-250 g were anaesthetized by intraperitoneal injection of 20-25 mg sodium pentobarbital. Radiolabeled liposomes were injected via the penile vein and the abdomen was opened. At the end of the experiment, liver lobules and other tissues were removed and weighed. Radioactivity was determined by γ -counter. And the results were registered as cpm per gram tissue.

Inhibitory effect of L-HSA-liposomes encapsulating interferon- α on HBV replication To assess the effects of L-HSA-liposomes encapsulating interferon- α on HBV replication, 2.2.15 cells were plated at a density of 2×10^5 per 17 mm culture dish and pre-incubated in Dulbecco's modified eagle medium containing 10% fetal calf serum for 24 h. After being washed with phosphate-buffered saline, they were cultured at 37°C for 12 d, with fresh medium changed every 3 d supplemented with L-HSA-liposomes encapsulating interferon- α (LL-IFN), conventional liposomes encapsulating interferon- α (CL-IFN), and free interferon- α (IFN) at an appropriate concentration. The culture medium was collected every 3 days and the cells were used in subsequent experiments. The viability of cells was examined spectrophotometrically by the MTT methods, and the cytotoxicity of the compounds was also monitored by the MTT as say.

RESULTS

Synthesis and characterization of L-HSA-liposomes Having reacted with lactose, the HSA and lactose mixture was dialysed against water for 3 days. There was no lactose in the final dialysis solution, suggesting the L-HSA had been purified completely. Each HSA molecule was modified with about 17 molecules of lactose. When L-HSA was coupled to MPB-liposomes, the amount of L-HSA that could be coupled to 1 μmol MPB-liposomes was 538.7 μg . A number of about 107 000 L-HSA molecules per liposome particle were calculated, assuming that the molecular weight of L-HSA was 7 600 and the average diameter of MPB-liposomes was 400 nm. The liposomes conjugate was stored at 4°C for at least 8 weeks and filtered by Sepharose 4B. The infiltration figure showed as a single absorbing peak, suggesting that the liposomal conjugate was stable at 4°C.

Tissue distribution of L-HSA-liposomes

Forty minutes after i.v. injection of ^{131}I labeled L-HSA-liposomes, radioactivity in liver was higher than the other organs ($P \leq 7.84 \times 10^{-6}$) (Table 1). Spleen uptake of L-HSA-liposomes was less than half of that of liver, and the uptake by kidney, heart, and lung was 1/4 to 1/9.

When L-HSA was pre-injected, the liver uptake decreased significantly, showing no statistic difference with spleen ($P = 0.38$) (Table 2). However, ^{131}I labeled L-HSA-liposomes uptake was also observed in the other organs.

Table 1 Tissue distribution of ^{131}I -labeled L-HSA-liposomes in rat

Rat No.	Tissue distribution <i>in vivo</i> (cpm per gram tissue)				
	Liver	Spleen ^a	Kidney ^b	Heart ^c	Lung ^d
1	31777.2	15394.2	7912.1	4427.5	9568.5
2	30476.1	15464.1	5424.5	3180.8	6044.1
3	30013.1	14041.4	7932.3	3777.5	5886.1
4	36099.3	10817.5	6372.3	3722.7	8717.3
5	36479.8	17882.6	7228.6	3885.5	7695.8
Average	32969.1	14719.9	6974.1	3798.8	7582.4

^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min after injection as described in *Materials and Methods*.

Compared with liver: ^a $P = 7.84 \times 10^{-6}$; ^b $P = 1.06 \times 10^{-7}$; ^c $P = 2.98 \times 10^{-8}$; ^d $P = 2.1 \times 10^{-7}$

Table 2 Tissue distribution of ^{131}I labeled L-HSA-liposomes in rat, after L-HSA pre-injection

Rat No.	Tissue distribution <i>in vivo</i> (cpm per gram tissue)				
	Liver	Spleen ^a	Kidney ^b	Heart ^c	Lung ^d
1	34682.0	35187.6	5535.7	2474.2	6368.0
2	20587.4	19791.1	4541.8	2277.2	8507.7
3	24288.8	27297.1	4564.4	2089.5	7280.7
4	33733.1	17637.5	5560.3	2698.3	12561.1
5	25600.9	18994.5	6999.2	3227.0	11087.1
Average	27778.4	23781.6	7699.1	2553.2	8679.4

Fifteen min after L-HSA pre-injection, ^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min after injection as described in *Materials and Methods*.

Compared with liver: ^a $P = 0.38$; ^b $P = 4.35 \times 10^{-5}$; ^c $P = 1.66 \times 10^{-5}$; ^d $P = 2.52 \times 10^{-4}$

Table 3 Inhibitory effect on HBeAg expression in 2.2.15 cells of different types of interferons

Treatment	Inhibitory effect on HBeAg expression (%)			
	3 days	6 days	9 days	12 days
Liposomes	0	1.8	0	0
L-HSA-Liposomes	0	0	0	0
IFN 0.08 MU ^a	0	4.7	12.7	2.3
CL-IFN 0.08 MU ^b	0	0	22.8	5.8
LL-IFN 0.08 MU	44.8	60.7	43.5	45.5
LL-IFN 0.04 MU	6.8	47.4	28.6	12.1
LL-IFN 0.02 MU	0	24.6	23.8	4.6
LL-IFN 0.01 MU	0	4.8	15.4	5.2

IFN: interferon; CL-IFN: interferon entrapped in conventional liposomes; LL-IFN: interferon entrapped in L-HSA-liposomes.

Compared with LL-IFN 0.08 MU: ^a $P = 0.003$, ^b $P = 0.015$

Anti-HBV activity of L-HSA-liposomes encapsulating interferon- α

Table 3 shows the antiviral activity of L-HSA-liposomes encapsulating interferon- α tested against HBV *in vitro*. The liposomes and L-HSA-liposomes did not show any anti-

HBV effect. However, when entrapped in L-HSA-liposomes, interferon was 3 to 5 times more effective against HBV replication in 2.2.15 cells than CL-IFN and IFN (*P* value was 0.015 and 0.003, respectively). To achieve similar antiviral effects, the dose of LL-IFN was only 1/4, 1/8 of CL-IFN and IFN, respectively. The treatments did not appear to be cytotoxic to 2.2.15 cells at the concentrations used in the anti-HBV assay.

DISCUSSION

Worldwide, HBV infection is the main cause of chronic liver disease. HBV carriers are at risk for chronic hepatitis, cirrhosis, and hepatocyte carcinoma. Previous studies have indicated the correlation of HBV X gene to hepatocyte carcinoma^[20]. An effective treatment for HBV infection is therefore urgently needed. Interferon- α , nucleotide analogs, Chinese medicinal herbs, and other immunological approaches have shown promising results in a subset of patients treated for prolonged periods^[21-24]. But overall response rates have been unsatisfactory. Selective drug delivery to the parenchymal liver cell by drug conjugation to a carrier would improve curative effect, reduce therapeutic dosage and side effects of drugs. In this report we describe the preparation of a liposomal drug carrier system, which could target the therapeutic drug to liver via the asialoglycoprotein receptor. Lactose was coupled to human serum albumin by reductive lactosamination in the presence of sodium cyanoborohydride. Then L-HSA was covalently coupled with liposomes using the heterobifunctional reagent N-Succinimidyl-S-acetylthioacetate. This is a well established method for coupling proteins to liposomes, based on the reaction of thiolated proteins and liposomal maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine^[10]. Thiolation of L-HSA, in which about 17 of the free ϵ -amino groups of lysine were derivatized with lactose, still allowed the introduction of several sulfhydryl molecules in this protein. This indicates that SATA is suitable for coupling derivatized protein to liposomes.

Mammalian liver contains a unique asialoglycoprotein receptor responsible for the rapid serum clearance and lysosomal catabolism of desialylated glycoproteins bearing terminal, non-reducing galactose residues. Previous studies demonstrated that, for bovine serum albumin, at least 13 lactosyl groups were needed for high affinity recognition by the ASGP-R^[25]. In our study, about 107 000 molecules L-HSA per liposome were coupled, and each HSA molecule was modified with 17 lactose molecules, hence the liposomal conjugate had a high affinity to ASGP-R. The *in vivo* study indicated that the liver uptake of liposomal conjugate was higher than other organs, which was about 2 times higher than that of spleen, and 4 to 9 times higher than kidney, heart, and lung. L-HSA pre-injection almost prevented the selective liver delivery of liposomal conjugate, suggesting that the galactose-specific nature of hepatic uptake. Modified with 17 lactose molecules, L-HSA was capable of inhibiting the hepatic uptake of

liposomal conjugate, owing to the high affinity for the hepatic galactose-recognizing receptor. Another study also showed that liposomes modified with several galactose residues had a similar liver targeting^[26].

2.2.15 cell is a stable expression system of transfected HBV DNA, which could produce HBV particle, and is often taken as a cell model for screening anti-hepatitis B virus drugs^[27,28]. Interferon had the inhibitory effect on HBV replication in this system^[29]. Wu *et al.*^[30] proved that this system contained ASGP-R. *In vitro*, interferon- α entrapped in liposomal conjugate had a higher activity against HBV free than interferon and than interferon entrapped in conventional liposomes. To achieve similar antiviral effects, the doses of LL-IFN capable of inhibiting virus growth were 4 to 8 times less than that of CL-IFN and IFN.

In conclusion, the results obtained in this study indicated that the conjugate of lactosaminated human serum albumin and liposomes achieved good liver targeting, and allowed the development of a potent liver-targeting drug carrier system.

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Intestinal acariasis in Anhui Province

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INTRODUCTION

The mites found in stored food and house comprise a large group of subclass Acari, belonging to the suborder Acaridida of the order Acariformes. They can be found in dust and vacuum samples from floors, furniture, mattresses, Chinese herbal medicine, dry fruit, grain, flour, sugar, and bedding. These mites are nidicolous and feed on organic debris, including sloughed human skin, fungi, spilled food, pollen, etc. These mites are particularly prevalent in Chinese herbal medicine, dry fruit, grain, flour, sugar, beds, though carpeted floors near beds or couches may also have large numbers. The most common species are *Acarus siro*, *Tyrophagus putrescentiae*, *Dermatophagoides farinae*, *D. pteronyssinus*, *Glycyphagus domesticus*, *G. Ornatus*, *Carpoglyphus lactis* and *Tarsonemus granarius*, etc. The viability of mites in storage is quite strong and they can invade and parasitize the intestines of humans^[1-15]. They can cause pulmonary acariasis^[16-25], urinary acariasis^[26-33] and so on. The dejecta of mites is a quite strong allergen and can cause different allergic diseases^[34-44]. Intestinal acariasis can be caused by some mites related to the way of diet intake and invading against intestinal mucosa, intestinal muscle^[45-58]. The first report of intestinal acariasis caused by these mites was made by Hinman *et al* (1934)^[45]. From then on, all kinds of studies on the disease have been reported gradually. In order to make an epidemiological survey of intestinal acariasis the investigation of the disease was taken in some areas of Anhui Province from 1989 to 1996.

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MATERIALS AND METHODS

Materials

The site of investigation was in the Chinese traditional medicine storehouses, factories and rice storehouses, rice mills, primary schools and so on, the subjects of investigation were sellers of the traditional Chinese medicine, workers of granary and processing, primary school pupils in grades 1-6 and 152 patients with diarrhea who sought medical advice and workers in certain processing factories.

Methods

Case history Included the history of present illnesses, the history of past illnesses, the patients' occupation.

Feces examination First, the stool of the subject was collected in disposable feces boxes. Secondly, mites were separated by saturated saline flotation methods and identified with the help of microscope.

Blood examination Differential leukocyte counting was made in patients who had positive results of feces mite examination.

RESULTS

The rate of detection of mites in stool and clinical symptoms

The total number of samples of feces examination were 3416 (male 1879, female 1537), the positive number of mites in stool were 225 (male 128, female 97) and the detectable rate of mite was 6.59% (male 6.81%, female 6.31%). Among them, the samples of feces examined in the groups of age 5-14, 15-24, and >24 were 1829 (5.74%), 508 (3.54%), 927 (6.36%) respectively. There were 43 positive samples in 152 patients with diarrhea who were suspected to suffer from intestinal acariasis, the detectable rate was 28.29%. Of the 225 mite positive cases observed after stool examination, the detectable number of mite cases together with other intestinal parasites was 131, the number of cases with only mite symptoms was 94.

Results of analysis of digestive canal symptoms in 30 cases positive of mites all one in stool showed that the number of patients with diarrhea, abdominal pain, abdomen discomfort, mucous stool, pus and blood stool, burning sensation of anus were 26 (86.67%), 20 (66.67%), 19 (63.33%), 15 (50.00%), 13 (43.33%), and 14 (46.67%) respectively. Besides, some of the patients were accompanied with the symptoms of hypodynamia, magersucht, lassitude, asthma, vomiting, anorexia, low fever, dysphoria, general

malaise, etc.

Mites separated from stool

The detectable rates of adult mites, larval mites, both adult and larval mites, both adult mites and eggs, adult and larval mites and eggs, both larval mites and eggs, and both hypopus and eggs were 44.00% (99), 10.22% (23), 34.67% (78), 2.67% (6), 4.89% (11), 2.22% (5), and 1.33% (3) respectively. The population identification analysis showed that there were *Acarus siro*, *Tyrophagus putrescentiae*, *Dermatophagoides farinae*, *D. pteronyssinus*, *Glycyphagus domesticus*, *G. Ornatus*, *Carpoglyphus lactis* and *Tarsonemus granarius* in stool. Furthermore, the hypopus of mites could be found. The statistics of this investigation showed that the concentration of mites with a count of 1-2/cm³, 2-4/cm³, >5/cm³ was 11, 55, 159 respectively.

Blood examination

Among the 30 patients, differential leukocyte count in most cases was $(5.55-10.4) \times 10^9/L$ with the exception of 4 cases $[(11.0-12.9) \times 10^9/L]$. The eosinophilic granulocyte count was high $[(0.32-0.78) \times 10^9/L]$. The average value of constituent ratio of eosinophilic granulocyte was 0.09 (0.04-0.11) and was higher than that of normal control ($P < 0.01$).

The rate of detection in different occupations

The field investigation showed that different rate of invasion existed in different occupations. The detectable rates of mites in stool were 6.71% (20/298), 6.59% (89/1350), 6.14% (98/1596), 4.17% (5/120) and 25.00% (13/52) in herb stuff processors and sellers, food storage and processor workers, primary school pupils, machinery workers, and some others.

Anamnesis

Among the 30 patients with obvious symptoms, the total misdiagnosis rate was 63.33%. The patients who had been misdiagnosed for chronic colitis, allergic enteritis, intestinal neurosis, amebiasis, hookworm disease, ascariasis, chronic appendicitis, pelvic inflammation, metroendometritis, intestinal adhesion, and chronic diarrhea were 2, 5, 3, 1, 1, 1, 2, 1, 1, 1, and 1 respectively.

Mites separated from work environment

Different breeding mites were separated from the samples of work environment and food. Collecting the samples of mill floor dust (30 shares), stores of traditional Chinese medicine, and traditional Chinese herbs (146 species) of wolfberry fruit, ophiopogon root, liquorice, boat-fruited sterculia seed, safflower, and so on. The results showed that the number of breeding mites per gram were 91-1862, 21-186, 0-483, 10-348, 51-712, and 311-1192, in mill floor dust, traditional Chinese medicine stores, traditional Chinese herbs such as candied fruit, dry

fruit, brown sugar, and expired cake. Twenty-six species of mites could be identified out of them (most of them were unidentifiable), and belonged to nine families of Acaridae, *Carpoglyphidae*, *Glycyphagidae*, *Pyroglyphidae*, *Histiostomidae*, *Chortoglyphidae*, *Lardoglyphidae*, *Tarsonemidae* and *Cheyletidae*. The results of this study showed that the mites separated from work environment were identical to those from stored food.

DISCUSSION

The main pathogenic mites have been verified as *Acarus* and *Tarsonemidae* by Zuo Zuoxue (1951). However, most of the mites causing intestinal acariasis that have been confirmed by Li Yousong (1980), Zhou Hongfu (1986), Li Chaopin (1987)^[56] *et al*, belong to 6 families Astigmatid order like, *Acaridae*, *Carpoglyphidae*, *Glycyphagidae*, *Pyroglyphidae*, and *Tarsonemidae*. The results of our investigation are in basic agreement with the views that had been previously reported. With regard to the path of infection of intestinal acariasis, the general idea was that the infection of the disease was caused by the mites coming from stored and swallowed food^[50-58]. Our study has verified that mites separated from stool were identical to those from storage, and belonged to common species of mites in our living environment and stored food. With the help of dust sampler, eight sampling sites were set up in a traditional Chinese medicine plant. Thirteen mites (adult 9, larval 4) were separated from the 640 L volume of air in the work environment of the plant. Thus another path of infection may be through respiration and swallowing of the mites in the air. The results of our study also showed that intestinal acariasis was associated with the hobby of drinking of tea, including ophiopogon root, liquorice, boat-fruited sterculia seed; and eating dry fruit, including da teplum persimmon, preserved fruit, candied fruit, dried persimmon, and even dried flesh of fish. Previous reports^[45-58] and the conclusions of this study verify that the disease is associated with breeding of mites in the diet, work environment, and stored food; but unassociated with age or sex.

The vitality of some mites causing intestinal acariasis is quite strong. The study report of Kodama *et al*^[48] had confirmed that the mites could live in 10% formalin for 12 days and then oviposit after two weeks, they could live in stool for 83 days and then oviposit and hatch to larva. In our study, twenty mites were soaked in distilled water at the temperature of 20-25°C, and another twenty in running water at room temperature for 7 weeks. The results showed that the mites collected from the bottom of the test tubes were all alive. After the stool with *Acarus siro* had been stored in a sealed plastic bag for 7 weeks, active mites could still be found. Kampmeier's report verified that not all mites could be killed by gastric acid or digestive juices^[49]. Mites could be found alive by differential leukocyte count.

After mites came into the intestinal tract, mechanical stimulation and injuries of tissues were caused by the

gnathosoma, chelicera, feet, and so on^[59]. The dejecta, products of metabolism, and cleaved body parts of mites could cause different allergic reactions of the digestive system, such as abdominal pain, diarrhea, burning sensation of anus, and so on. The reports in past have shown that the dejecta, metabolites, cleaved body parts were all quite strong allergens and could result in high levels of eosinophilic granulocytes, total IgE, special IgE for mites in serum. The clinical manifestation of this was allergic urticaria, allergic rhinitis, allergic asthma et al^[34-44]. The results of our studies showed that the levels of eosinophilic granulocytes in patients with intestinal acariasis were high too. Its possible mechanism could be similar to that of the allergic reaction caused by other mites. The intestinal acariasis may not only cause injury of the intestinal mucosa and invade the intestinal muscle but also stimulate the immune system by the dejecta, metabolites, and cleaved body of the mite itself. The disease may be easily misdiagnosed for allergic enteritis, chronic colitis, intestinal neurosis, amebiasis, pelvic inflammation, or schistosomiasis japonica^[60]. As a result, proper treatment might be thus delayed.

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Study of T-lymphocyte subsets, nitric oxide, hexosamine and *Helicobacter pylori* infection in patients with chronic gastric diseases

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Subject headings stomach disease; T-lymphocyte; nitric oxide; *Helicobacter pylori*; hexosamine; Helicobacter infections; gastric mucosa

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INTRODUCTION

Chronic gastritis (CG) and peptic ulcer (PU) are frequently-occurring diseases. It is now well recognized that *Helicobacter pylori* (Hp) is a major factor that leads to CG and PU^[1-8]. In order to study the relationship among T lymphocyte subsets, NO, Hexosamine and Hp infection in patients with chronic gastric diseases, the levels of blood T lymphocyte subsets, plasma NO and hexosamine in gastric mucosa were measured respectively in 30 patients with CG and 32 patients of PU+CG.

MATERIALS AND METHODS

Clinical materials

Thirty-two patients with PU and CG (23 males and 9 females, aged 20 to 68 years, mean age 43.2) and 30 patients with CG (18 males and 12 females, aged 24 to 66 years, mean age 44.3) were enrolled in the study. Twenty healthy people with comparable ages acted as control. All subjects were excluded of hepatic and other diseases.

Methods

Vein blood 2 mL from each fasted patient was obtained for blood T lymphocyte subsets and NO determinations. Four gastric biopsies were taken from gastric antrum 3 cm from pylorus (2 from curvatura ventriculi minor and the other 2 from ante-wall) for Hp, pathology and hexosamine determinations. T-lymphocyte subsets were determined by FCM methods (FACS 420, by Becton-Dickinson

Company, U.S.A.). Laser light source was 2 Wargon ionic laser, wavelength 488 nm. The emerging green fluorescence by FITC was used for fluorimetry by 520 nm long filter disc. The data were processed by an HP-300 computer programme. Hp infection was diagnosed if any two of the following methods were positive: (1) rapid urase test (test kit procured from San Qiang Company); (2) gastric mucosa smear with gram's stain. The Hp density gradients were according to Monshoy staging grades: 0, free; I, a few; II, obvious (in all fields of microscopy); III, plenty or piled; (3) Warthin-Starry stain of gastric mucosa.

Pathology

HE stain was used for observing mucosal inflammation. The severity of gastritis was graded as: mild: infiltrated inflammatory cells only at gastric pit or intestinal villas; moderate: infiltrated cells in gland lamina propria; and heavy: infiltrated inflammatory cells in muscular layer of mucosa. Determination of hexosamine: two gastric biopsies were ground into 1 mL liquid, centrifuged it, and supernatant was kept at -20°C for measuring. Protein content in supernatant was measured by taking 20 µL sample and 500 µL Coomassie brilliant blue G250 reagent at the wavelength of 600 nm by automatic analysis apparatus. 0.5 mL of the supernatant and 0.5 mL enriched hydrochloric acid were mixed together at high pressure (147 KPa) for 30 min, put into 0.5 mL NaOH (8.3 mol/L) and acetyl acetone solution, boiled for 15 min and cooled, and then analyzed. The results were expressed as mg/mL. Hexosamine was calculated as following: hexosamine = supernatant hexosamine (mg/mL)/supernatant protein (g/L). NO measurement: (1) pre-measurement: Took 200 µL plasma and the same amount of a cetonitrile, shook them, and centrifuged for 10 min (5000 r/min), repeating once again as above, taking 10 µL of supernatant overflow for measurement. Baseline 810 high effect liquid phase apparatus, made by Waters Company, U.S.A. and 486 outer purple detector were used. Conditions of chromatostrip included: mobile phase 2.5 mmol/L LiOH water solution +50 g/L acetonitrile, velocity of flow 1.2 mL/min, column temperature 40°C, detection wavelength 214 nm, analysis column IC-Pak Anion, 4.6 mm × 5 cm (Waters Company, U.S.A.), detection time 10 min.

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RESULTS

T-lymphocyte subsets in CG patients

Total lymphocyte (CD_3^+), helper lymphocyte (CD_4^+), suppressor lymphocyte (CD_8^+), CD_4^+/CD_8^+ in CG patients were significantly lower than those of normal control ($P < 0.05-0.01$, Table 1). Eighteen out of 30 CG patients had *Hp* infection (60%). CD_3^+ , CD_4^+ in Hp^+ group were significantly decreased ($P < 0.01$, < 0.05), but CD_4^+/CD_8^+ was not significantly changed (Table 2). In addition, the study of the relationship between different pathological changes and T lymphocyte subsets indicated that: CD_3^+ , CD_4^+ , CD_4^+/CD_8^+ in severe CG were significantly decreased than those in mild to moderate CG ($P < 0.01$, < 0.05 , < 0.05 respectively, Table 3).

Table 1 T-lymphocyte subsets in CG patients ($\bar{x} \pm s$, %)

Lymphocyte	CG (n = 30)	Normal control (n = 20)
CD_3^+	60.7 \pm 2.4 ^b	68.3 \pm 3.9
CD_4^+	35.8 \pm 2.5 ^b	43.0 \pm 3.8
CD_8^+	25.2 \pm 2.4 ^a	26.4 \pm 1.7
CD_4^+/CD_8^+	1.43 \pm 0.15 ^a	1.64 \pm 0.18

^a $P < 0.05$, ^b $P < 0.01$, vs normal group

Table 2 T-lymphocyte subsets in CG patients with *Hp* infection ($\bar{x} \pm s$, %)

lymphocyte	<i>Hp</i> (+) (n = 18)	<i>Hp</i> (-) (n = 12)
CD_3^+	59.7 \pm 2.5 ^b	62.2 \pm 0.9
CD_4^+	34.8 \pm 2.6 ^a	36.9 \pm 2.0
CD_8^+	25.3 \pm 2.5	25.0 \pm 2.3
CD_4^+/CD_8^+	1.42 \pm 0.17	1.45 \pm 0.12

^a $P < 0.05$, ^b $P < 0.01$, vs *Hp* (-)

Table 3 Pathological stages and T-lymphocyte subsets in CG ($\bar{x} \pm s$, %)

Lymphocyte	Mild-moderate (n = 13)	Severe (n = 17)
CD_3^+	62.3 \pm 1.7	59.5 \pm 2.1 ^b
CD_4^+	37.4 \pm 1.8	35.4 \pm 2.7 ^a
CD_8^+	25.5 \pm 2.1	24.9 \pm 2.6
CD_4^+/CD_8^+	1.50 \pm 0.13	1.40 \pm 0.13 ^a

^a $P < 0.05$, ^b $P < 0.01$, vs mild-moderate

Hexosamine levels

The hexosamine levels in patients with severe lesions (38.0 mg/g \pm 3.8 mg/g) were significantly lower than those with mild and moderate CG (47.0 mg/g \pm 7.6 mg/g, $P < 0.01$). The hexosamine levels in Hp^+ group were significantly lower than those in Hp^- group ($P < 0.05$, Table 4). In addition, 24 out of 32 PU+CG patients (75.0%) had *Hp* infection. The hexosamine levels in Hp^+ group were significantly lower than those in Hp^- group ($P < 0.01$, Table 4).

Table 4 Changes in hexosamine levels in CG, PU+CG patients with *Hp* infection ($\bar{x} \pm s$, mg/g)

	CG		PU + CG	
	<i>Hp</i> (+) n = 18	<i>Hp</i> (-) n = 12	<i>Hp</i> (+) n = 24	<i>Hp</i> (-) n = 8
Hexosamine	40 \pm 6	45 \pm 7	39 \pm 8	51 \pm 7

^a $P < 0.05$, ^b $P < 0.01$, vs *Hp* (-)

Plasma NO levels

In CG, PU+CG patients, the levels of plasma NO (2514 μ g/L \pm 364 μ g/L, 2824 μ g/L \pm 673 μ g/L) were significantly higher than those of normal control (2228 μ g/L \pm 214 μ g/L, $P < 0.05$, $P < 0.01$), and the level of plasma NO in PU+CG patients (2824 μ g/L \pm 673 μ g/L) was higher than those of CG patients (2514 μ g/L \pm 364 μ g/L, $P < 0.05$). The levels of NO in CG, PU+CG *Helicobacter pylori* positive group were significantly higher than in Hp^- group ($P < 0.01$, $P < 0.05$, Table 5). The study of the relationship between different pathological changes and NO levels indicates that the levels of NO in both groups with severe lesion were significantly higher than those in mild to moderate lesions ($P < 0.05$, Table 5).

Table 5 Changes in plasma NO, pathological stages and *Hp* infection in CG and PU+CG ($\bar{x} \pm s$, μ g/L)

	CG	PU+CG
<i>Hp</i> (+)	2671 \pm 258	3071 \pm 398
<i>Hp</i> (-)	2282 \pm 387	2579 \pm 668
Mild-moderate	2328 \pm 413	2403 \pm 284
Severe	2656 \pm 251	2880 \pm 802

^a $P < 0.05$, ^b $P < 0.01$, vs *Hp* (-); ^c $P < 0.05$, vs severe.

DISCUSSION

As we all know, CG is closely related to *Hp* infection [9-12], but so far any report regarding the relationship between CG and T lymphocyte has not been observed. Our results showed that CD_3^+ , CD_4^+ , CD_8^+ , CD_4^+/CD_8^+ T cells in CG patients were observed to be significantly lower than in normal control. This indicated that the functioning of cellular immunity was impaired in CG patients. In order to explore the relationship between T lymphocyte subsets and *Hp* infection, this experiment divided CG patients into Hp^+ group and Hp^- group. The results showed that CD_3^+ , CD_4^+ T cells in Hp^+ group were significantly decreased showing that *Hp* infection is related to the decrease in the function of cellular immunity. In addition, the majority of the severe cases of CG (71%) had *Hp* infection, and the levels of CD_3^+ , CD_4^+ , CD_8^+ , CD_4^+/CD_8^+ T cells were significantly decreased than in mild to moderate CG patients. This implied that the impaired function of cellular immunity in CG patients especially in severe cases, may possibly be related to *Hp* infection.

Gastric mucosin-bicarbonate barrier is one of main constituents of gastric mucosal barrier^[13,14]. Gastric mucosin contains polymer glycoproteins, electrolytes, peptides, lipides, etc. The present study showed that: the orientation of *Hp* is related to pathogenic factors, such as lipopolysaccharide, urea enzyme^[15-18], vacuole toxin^[19-21] and adhesiveness. Hexosamine^[22] reflects the glycoprotein. So, the purpose of this study was to evaluate the lesion of gastric mucosal barrier by measuring the levels of hexosamine in gastric mucosa. The results showed that in CG, and PU+CG patients, the levels of hexosamine in *Hp*⁺ group were significantly decreased than in *Hp*⁻ group. The more severely the gastric mucosa was impaired, the higher the *Hp* infection rate was (moderate 60.7%, severe 74.2%). *Hp* infection leads to the decrease in hexosamine levels in gastric mucosa especially in severe cases. It is thus indicated that *Hp* is one of main pathogenic factors for endogastritis or severe gastritis.

NO is a multifunctional regulatory substance of the body^[23-30]. It has many biological activities. It participates in many physiological functions and pathogenesis in digestive tract. Endogenous NO is an important transmitter maintaining the blood flow of gastric mucosa, and has a role in inhibiting platelet aggregation in gastric mucosa microcirculation. The results in this study showed that the levels of plasma NO in CG and, PU+CG patients were increased to varying extents as compared to normal control, and the levels of plasma NO in PU+CG patients were significantly increased than those in CG patients. In addition, the plasma NO levels in *Hp*⁺ group in CG and PU+CG patients were significantly increased as compared to *Hp*⁻ group. According to the pathological stages, the more the gastric mucosa was impaired by *Hp* infection, the higher the levels of NO were. It is suggested by our results that *Hp* infection in gastric mucosa may induce an increase in NO production by inflammatory cells^[31]. It might be possibly an immune response of our body to resist *Hp* infection, but the exact pathogenesis needs to be further confirmed.

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Helicobacter pylori vacA genotypes and *cagA* status and their relationship to associated diseases

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Subject headings *Helicobacter pylori*; gastritis; peptic ulcer; stomach neoplasms; genotype; polymerase chain reaction

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a major causative bacterium of chronic gastritis, peptic ulcer and mucosa-associated lymphoid tissue lymphoma in humans, and associated with an increased risk of gastric cancer^[1-8]. An important virulent factor of *H. pylori* is the vacuolating cytotoxin (VacA) encoded by *vacA* that induces cytoplasmic vacuolation in target cells both *in vitro* and *in vivo*^[9-11]. VacA is produced as a 140 kDa precursor which contains an N-terminal signal peptide and an approximately 33 kDa C-terminal outer membrane exporter. The precursor is cleaved at both N-terminal and C-terminal and secreted into the extracellular milieu as a 95 kDa mature protein. The mature protein further undergoes specific cleavage to yield 37 kDa and 58 kDa subunits^[12-14]. Although *vacA* is present in all *H. pylori* strains, only about 50% to 60% of strains can induce vacuolation of epithelial cells as assessed by the HeLa cell assay. *vacA* shows considerable genetic variation in *H. pylori* isolated from all over the world and contains at least two variable regions. The s region exists as s1 or s2 allelic types. Among type s1 strains, subtypes s1a and s1b have been identified. The m region occurs as m1 or m2 allelic types. Specific *vacA* genotype of *H. pylori* strains are associated with the production of the cytotoxin *in vitro*, epithelial damage *in vivo*, and clinical consequences^[15-27]. The other virulent factor is the cytotoxin-associated protein (CagA) encoded by the cytotoxin-associated gene (*cagA*). The *cagA* gene is present in about 60% to 70% of strains and all of these

strains express the *cagA*. The presence of *cagA* is also associated with the production of the cytotoxin *in vitro*, and clinical outcome^[24-30]. The aim of this study was (i) to identify *vacA* genotypes and *cagA* status of *H. pylori* isolated from Chinese patients; (ii) to evaluate the relationship between *vacA* genotypes, *cagA* status and related gastroenterological disorders.

MATERIALS AND METHODS

Patients

Seventy-four clinical isolates of *H. pylori* were obtained from patients which underwent gastroduodenoscopy in Changhai Hospital, Shanghai, China. *H. pylori* strain CCUG17874 and G50 obtained from IRIS, Italy were also used in this study. No patient had received nonsteroidal anti-inflammatory drugs or antacids. Of these, 31 patients (mean age 47 years) had peptic ulcer including 21 duodenal ulcers, 5 gastric ulcers and 5 complete ulcers; 39 patients (mean age 41 years) had gastritis including 18 suppur gastritis, 6 atrophic gastritis and 15 erosive gastritis; and 4 patients (mean age 56 years) had gastric adenocarcinoma.

Isolation and culture of *H. pylori*

Two gastric biopsies were obtained respectively from gastric antrum and corpus of each patient by using endoscopy. Each specimen was placed in a transport medium and sent to the laboratory within 3 h. The specimens were then incubated onto Campylobacter selective agar (MERCK) containing 10% sheep blood for 3 d at 37°C under microaerobic conditions (10% CO₂, 5% O₂, 85% N₂). All *H. pylori* strains were positive for urease, oxidase, catalase and were identified by Gram and Giemsa staining under light microscopy. Colonies directly harvested from the plates were used for RNA extraction.

Preparation of total RNA and RT-PCR amplification

The extraction of total RNA was performed using Promega's SV total RNA isolation system according to manufacturer's instructions. The DNA sequences of the primer oligonucleotides used for RT-PCR and the size of the corresponding PCR products are listed in Table 1. RT-PCR reactions were performed as follows: denaturation at 95°C for 2 min, followed by 30 cycles consisting of 95°C for 40 s, 55°C for 1 min, and 67°C for 2 min, and final extension at 67°C for 7 min. The RT-PCR products were electrophoretically separated on a 1.5% agarose gel and stained with ethidium bromide.

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Statistical methods

Analysis of data was performed using the χ^2 test. Probability levels (P) of <0.05 were considered statistically significant.

Table 1 Oligonucleotide primers used in this work

Region amplified	Primer sequence	Size of PCR product
cagA	5'ATAATGCTAAATAGACAACCTTGAGCG 3' 5'TTAGAATAATCAACAAACATCAGGCCA 3'	297 bp
vacA		
s1a	5'ATGGAAATACAACAAACACAC 3' 5'GTCAGCATCACACCGCAAC 3'	190 bp
s1b	5'ATGGAAATACAACAAACACAC 3' 5'AGCGCCATACCGCAAGAG 3'	187 bp
s2	5'ATGGAAATACAACAAACACAC 3' 5'CTGCTTGAATGCGCCAAAC 3'	286 bp
m1	5'GGTCAAAATGCGGTTCATGG 3' 5'CCATTGGTACCTGTAGAAAC 3'	290 bp
m2	5'GGAGCCCCAGGAAACATTG 3' 5'CATAACTAGCGCCTTGCAC 3'	352 bp

RESULTS

vacA genotypes of *H.pylori* strains

Typing of the *vacA* gene of 74 clinical isolates and strain CCUG17874 and G50 was performed based on signal and middle sequences by RT-PCR as described in materials and methods. All of the 74 clinical isolates and strain CCUG17874 were s1a type whose fragment was 259 bp products as predicted by RT-PCR-amplification. Strain G50 was s1b type whose fragment was 286 bp products predicted by RT-PCR-amplification. No clinical isolates was of type s1b. Neither strain CCUG17874 and G50 nor clinical isolates was of type s2. Seven isolates (9.5%) and strain CCUG17874 were of m1 type whose fragment was 290 bp products. Sixty-seven isolates (90.5%) and strain G50 were of m2 type whose fragment was 352 bp products. Overall, 7 strains (9.5%) were type s1a/m1, 67 isolates (90.5%) were type s1a/m2. An s1a/m2 type strain was predominantly found in clinical isolates from Chinese patients living in Shanghai.

cagA positivity of *H.pylori* strains

The *cagA* gene was determined in 74 isolates by RT-PCR. Sixty-nine Chinese *H.pylori* strains (93.2%) were *cagA* positive. When the *vacA* genotype was compared with *cagA* status, all (100%) of 7 s1a/m1 type strains and 62 (92.5%) of 67 s1a/m2 type strains were *cagA* positive.

Relationship between *vacA* genotype, *cagA* status and related gastroenterological disorders

Infection with type s1a/m1 strain was found in 4 (12.9%) of 31 patients with peptic ulcers compared with 3 (7.7%) of 39 patients with gastritis. Infection with s1a/m2 strain was found in 27 (87.1%) of 31 patients with peptic ulcer compared with 36 (92.3%) of 39 patients with gastritis. There was no statistical significance between *vacA* genotype and the clinical outcome. ($P > 0.05$). *cagA* positivity was found in 29 (93.5%) of 31 patients with peptic ulcer compared with 37 (94.9%) of 39 patients

with gastritis. There was also no relationship between *cagA* status and related gastroenterological diseases ($P > 0.05$).

DISCUSSION

Based upon the methodology for typing *vacA* gene developed by Atherton *et al*^[19], we analysed and typed *vacA* genotype of clinical isolates from Chinese patients living in Shanghai by using RT-PCR. The product amplified by RT-PCR was obtained in all clinical isolates, CCUG17874 and G50 strains. The results suggested that *vacA* transcription occurred in each of *H.pylori* strains tested and was in agreement with previous reports^[19]. According to typing of *vacA* alleles, these Chinese patients infected prevalently with *H.pylori* contained the type s1a/m2 of *vacA* alleles. The finding reported here was different from that in United States, Germany, and Japan^[15-26]. The reason why the s1a/m2 strains have accumulated in Shanghai, China is unclear. It is known that genomic rearrangement may be associated with the uptake of DNA by natural transformation. Chinese have the same habits and customs, and little intermarriages with other nations. The high incidence of the s1a/m2 allele in Chinese population may indicate that it was first acquired in this population and the chance for genomic diversity may be little. Alternatively, the s1a/m2 allele may have a selective advantage in the Chinese population due to human polymorphism and ethnic background.

Recently, van Doorn *et al* found a novel subtype of *vacA* signal region from s1a subtype, designated as s1c. Type s1c allele was obtained exclusively in isolates from East Asia^[32]. We have done *vacA* gene sequencing from 5 clinical isolates (in press). The s region was considered as type s1a. Compared with s1c sequence, two of the five strains were of type s1c. These results indicated that s1c subtype could be identified as subtype s1a. Whether s1c strains are phenotypically different from s1a strains remains to be determined.

The findings here showed that all strains but five possessed the *cagA* gene and suggested that *cagA* positive strains were predominantly found in Shanghai also. The relationship between type s1a *vacA* allele and *cagA* positive has been described^[31]. All (100%) of Chinese (Shanghai) *H.pylori* strains were of type s1a *vacA* allele and 93.2% of strains were *cagA* positive and were highly prevalent in China.

Subtype analysis of *vacA* alleles and *cagA* positivity demonstrated that the *vacA* subtype and *cagA* status was not independently associated with the clinical outcome of *H.pylori* infection. The factors affecting the clinical consequences need further studies.

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Case Reports

Hepatic angiomyolipoma-misdiagnosis as hepatocellular carcinoma: A report of 14 cases

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Subject headings angiomyolipoma; liver neoplasms/diagnosis; immunohistochemistry; microscopy, electron; carcinoma, hepatocellular/pathology; case-control studies

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INTRODUCTION

Angiomyolipoma (AML) is a rare benign mesenchymal tumor of the liver, composed of a varying heterogeneous mixture of three tissue components: blood vessels, smooth muscle, and adipose cells. It has recently been proposed that the perivascular epithelial cell (PEC) is the common progenitor^[1,2]. Since its first description by Ishak in 1976^[3], there have been more than 100 cases reported in the English literature^[4-6]. With the advance of radiological techniques, many more tumors are being diagnosed by the means. But radiological findings of AML may only be suggestive of the lesion; its definitive diagnosis requires histological confirmation^[9-19]. Some authors regard renal and hepatic AMLs, pulmonary and soft tissue lymphangiomyomatosis^[2], pulmonary and pancreatic clear cell "sugar" tumor, and cardiac rhabdomyoma as closely related groups of tumors, based on their morphologic overlap and common immunoreactivity for HMB-45^[11]. They show different microscopic appearances, however, according to their organ of origin. The goals of this study were to highlight more subtle morphology and to gain possible insights into the differential diagnosis that could provide important information about this disease.

MATERIALS AND METHODS

Fourteen cases of AML were identified in the pathology files at the Department of Pathology, Chinese Military General Hospital, four of which were consulted cases. All the cases were independently reviewed by two pathologists, and the most important diagnostic criterion was the

presence of HMB-45-positive cells. The clinical data and follow-up information were obtained in each case (Table 1). All the tumor tissues had been fixed in neutral buffered formalin and were routinely embedded in paraffin. Hematoxylin and eosin-stained sections were examined. According to the morphological aspect, one block was selected for each of these cases. Immunohistochemical study was performed on representative blocks by using an avidin-biotin peroxidase complex technique. Selective cases were also examined with antibodies to CD68, CD31, CD34, factor VIII-related antigen.

RESULTS

Clinical findings

The clinical findings in all 14 patients are summarized in Table 1. There was an marked female predominance (women: men = 9:5) and the average age of patients at diagnosis was 40.07 years (range 30-63 years). Only four cases (case 4,7,10,13) had symptoms of space-occupying lesions while others were incidentally discovered on imaging studies (Figure 1). Two cases had two masses each (the other mass was diagnosed as a hemangioma by a pathologist) and one of them had calcified nodes (confirmed by a pathologist). None of 14 cases had tuberous sclerosis syndrome, angiomyolipoma of kidney, lymphangiomyoma or clear cell tumor of lung or other parts. For patients with complete follow-up data, all were well with no tumor recurrence and metastasis after excision.

Gross findings

Thirteen cases were solid and one was cystic (the content of cyst was a brown liquid). All tumors were well circumscribed, but not encapsulated (Figure 2). The average size of all thirteen cases was 9.4 cm (range 2.5 cm-26 cm). The surfaces of tumor sections were yellowish but not uniform and some parts of tumors were fish-like.

Histological features

The most common pattern was that of solid sheets of myoid cells intermixed with areas of adipose cells and vessels, most of which were thin-walled (Figure 3) and few of which were thick-walled but had to be carefully observed. In 12 out of 14 cases there were clusters of hematopoietic

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Table 1 The clinical findings in all 14 patients

NO.	Sex	Age(years)	Tumor size (CM)	Location (LOBE)	Primary diagnosis	Follow-up
1	F	38	4.5	R	Angiosarcoma	Well 44 mo after excision
2	F	33	2.5	R	Hepatic carcinoma	Well 35 mo after excision
3	F	33	6.0	R	Hepatic carcinoma	Well 33 mo after excision
4	F	49	26.0	R,L	Hemangioma	Well 153 mo after excision
5	F	48	3.5	R	Hepatic carcinoma	Well 31 mo after excision
6	F	38	12.5	L	Hepatic carcinoma	Well 12 mo after excision
7	F	35	14.5	L	Hepatic carcinoma	Well 9 mo after excision
8	M	63	17.0	R	Cystic adenoma or cystic adenocarcinoma	Well 9 mo after excision
9	M	37	3.5	R	Hepatic carcinoma	Well 16 mo after excision
10	M	34	10.0	R	Hepatic carcinoma	Well 11 mo after excision
11	M	30	7.5	L	Hepatic carcinoma	Well 16 mo after excision
12	F	43	6	L	Hepatic carcinoma	Well 3 mo after excision
13	M	37	12	L	Hepatic carcinoma	Well 2 mo after excision
14	F	43	6	L	Angiosarcoma	Well 1 mo after excision

Table 2 Immunohistochemical findings in 12 cases

Serial number	HMB45	Actin	S-100	EMA	AFP	Ki-67%
1	++	++	local+	-	-	<1
2	++	++	Local+	-	-	<1
3	++	++	Local+	-	-	<1
4	++	+	Local+	-	-	<1
5	++	+	Local+	-	-	<1
6	++	+	Local+	-	-	<1
7	++	+	Local+	-	-	<1
8	+	+	Local+	-	-	<1
11	++	+	Local+	-	-	<1
12	++	++	Local+	-	-	<1
13	++	++	Local+	-	-	<1
14	++	++	+	-	-	<1

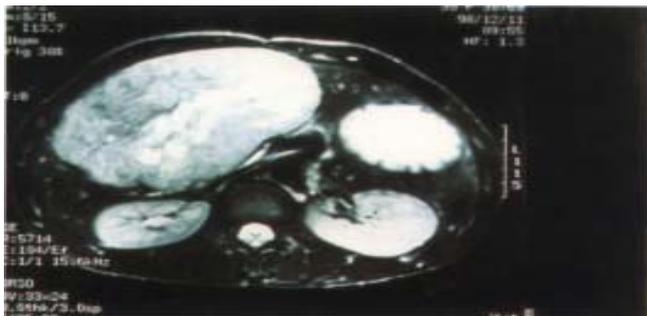


Figure 1 MRI image of case 7.



Figure 2 Macroscopic appearance of AML with yellowish fatty areas (case 7).



Figure 3 Thin-walled vessels and trabecular tumor cells of AML. IH: CD31 × 100

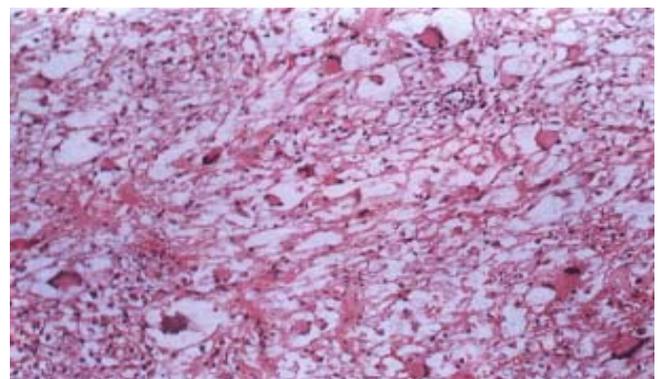


Figure 4 Multinucleus cells in a large number in AML of case 9. HE × 100

cells and in 1 of 14 cases there were lots of multinuclear cells (Figure 4). The myoid cells usually predominated and their morphology varied from epithelioid to intermediate (ovoid or short spindle) spindle. The epithelioid cells in our 12 cases were main cell pattern, cytoplasm of which varied from clear to vacuolated and eosinophilic granular, nuclei of which were partly normal chromatic with moderate pleomorphism, with delicate chromatin, and a single distinct eosinophilic nucleolus. Furthermore, there were some large bizarre cells in 10 cases out of 14 cases but without nucleus mitoses

(Figure 5). Long spindle cells with elongated eosinophilic cytoplasm could be seen but very rarely lipoblast-like cells were observed in sheets in local areas. Only two cases had multiple local necrotic areas but their total size was smaller than 5 percent of the tumor (Figure 6).

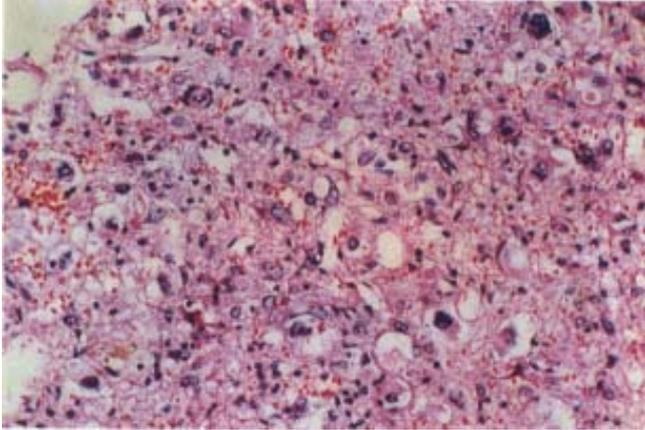


Figure 5 Pleomorphic and large bizarre cells. HE \times 200

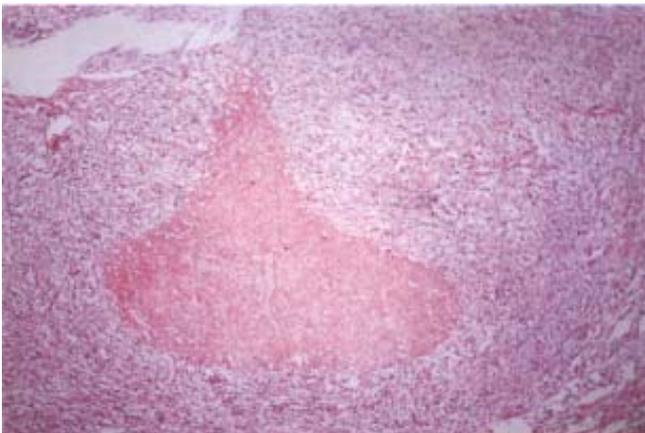


Figure 6 Local necrotic area of case 9. HE \times 50

Immunohistochemical findings (Summarized in Table 2) The tumor cells were positive for HMB-45, but negative for EMA and AFP in all cases. Local tumor cells were positive for Actin and S-100. HMB-45 staining was intense, granular, and concentrated in the perinuclear pink cytoplasm. Although the epithelioid cells were most consistently stained, the spindle cells were also weakly positive. Actin staining in spindle tumor cells was more intense than in other cells. The lipoblast-like cells were positive for both HMB-45 and S-100 protein. Lots of multinucleus cells presenting in 1 of 12 cases were positive for HMB45, Actin, S-100 and CD68. Furthermore, positive rate for Ki-67 of tumor cells in all 12 cases was no more than 1 percent.

Ultrastructural finding Six cases were examined under H-7000 electron microscopy. Neoplastic cells generally were polygonal and closely arranged with minimum intercellular material. In the cytoplasm, glycogen and round-to-oval mitochondria were common features,

together with a characteristic finding of lots of electron-dense, membrane-bound granules (these granules were 60-100 nm in diameter with some filament-like structures in them which could be seen under high magnification. These were deemed to be either premelanosomes or atypical lysosomal bodies) (Figure 7).

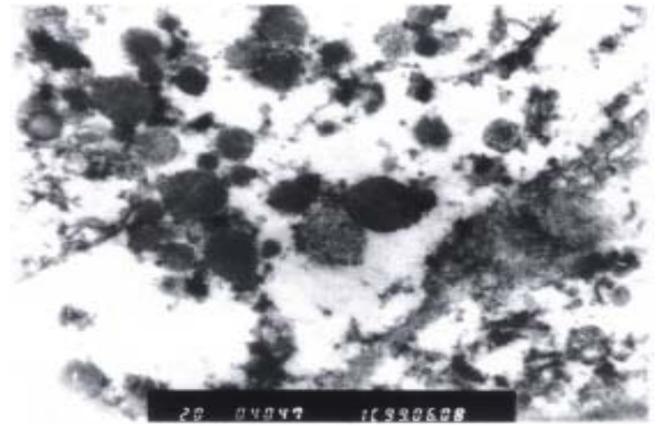


Figure 7 Ultrastructure of neoplastic tissue: glycogen, electron-dense granules. EM \times 20 000

DISCUSSION

Angiomyolipoma, which occurs frequently in kidney but rarely in other sites^[7,8], previously considered as a hamartomatous growth rather than a true neoplasm, is a rare benign mixed mesenchymal clonal neoplasm of the liver. Since the first case described by Ishak^[3], AML has been diagnosed with increasing frequency with advances in MRI, CT, CD-SO and angiography. Despite the claim that the imaging features are highly characteristic^[9-19], the preoperative diagnoses are erroneous in more than half of the cases in the present series. None of our 14 cases were correctly diagnosed before operating, furthermore, 5 cases were misdiagnosed as hepatic cell carcinoma or sarcoma by pathologist even after operating.

The diagnosis of AML is usually made on recognition of three or four components of the tumor, namely blood vessels, smooth muscle, mature fat, and hematopoietic tissue^[4-6,20]. These elements, however, are variable in proportion and distribution. The adipose cells have no characteristic features in themselves to distinguish between various lipomatous tumors. Extramedullary hematopoiesis is not an integral part of AML and is seen in many primary benign and malignant hepatic tumors. Its occurrence is probably more closely related to the hepatic sinusoidal endothelium which plays an important part in hematopoiesis in the fetal liver. This also explains that hematopoietic cells are found only in hepatic but not renal AMLs. Blood vessels are present in all kinds of tumors which easily escape attention. The more distinctive features in AML are their tortuosity, thick walls (but rare in AML of liver) and perivascular muscle proliferation. It thus appears that the myoid component is the only specific and diagnostic

component in AML and it can exist in epithelioid, spindle, and intermediate forms. The essential component in this tumor appears to be PECs^[1-6]. It has been speculated that the distinctive epithelioid cells are primitive mesenchymal cells that have an ability to differentiate towards both myoid and fat cells. Spindle myoid cells and lipocytes probably represent the mature derivatives of the epithelioid cells. Immunohistochemistry is particularly useful for diagnosing, as many authors have found. HMB-45 has been shown to be a promising marker for renal and hepatic AMLs and can even be applied to minute samples such as fine-needle aspirates^[21,22].

Because of the rarity and the pleomorphism of histological features of hepatic AMLs, histological diagnosis may be difficult, especially with needle biopsy (one of our cases was diagnosed by this means). Many features in AML can mislead the unwary pathologist to a diagnosis of HCC: polygonal cells in trabecular arrangement, peliosis, nuclear pleomorphism, prominent eosinophilic nucleoli, deficient reticulin framework, presence of glycogen, eosinophilic globules, and tumor necrosis. Four of our cases were misdiagnosed by pathologists because of causes mentioned above. In AML with spindle cells and pleomorphic features, sarcoma is the most common incorrect diagnosis. For this reason, one case in our series was misdiagnosed as angiosarcoma. Lipomatous AML has to be differentiated from true lipoma and focal fatty change. Evidently, a careful histologic survey of the entire specimen usually reveals the presence of the classic mixed pattern of the tumor. Immunohistochemical result (positive for HMB45 and Actin but negative for AFP or EMA), ultrastructural structure and the low proliferation of tumor cells can further confirm the diagnosis^[25-35]. The histogenesis of AML is unclear, but immunohistochemical and ultrastructural studies provide important insights into its cell differentiation^[4-6,20,23,24]. The concept of "perivascular epithelioid cells" as the unified feature has been proposed^[1]. Presumably, the PECs are an aberrant type of mesenchymal cells proliferating to form tumorous lesions, possibly due to chromosomal aberrations in the tuberous sclerosis (TS) complex gene. Because they are capable of dual differentiation, the argument for a hamartomatous proliferation is unsound. Recent molecular studies have shown tumor clonality and 5q deletions with a common region of deletion spanning 5q33 to 5q34, indicating a clonal neoplastic process^[36-40].

Preoperative identification of AML is desirable because of differences in clinical course and treatment between this disease and other hepatic neoplasms^[41]. Although some imaging features may suggest hepatic AML, in such cases, histological analysis must be performed. No matter whether or not the liver biopsy contains fat component, the diagnosis can be established based on myoid component positive for HMB45.

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progress of gastric cancer etiology: N-nitrosamides 1999s

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INTRODUCTION

Stomach carcinoma is still the leading cause of cancer death in China and the second one in the world. Its possible causes include: A) chemical factors such as intragastric formation of *N*-nitroso compounds (NOC) and high salt intake; B) biological factors such as infection of *Helicobacter pylori* and biotoxins intake; and C) nutritional factors such as deficiency of vitamin C, selenium, and other antioxidants. Nitrogenous precursors of NOC, e.g. alkylamines, alkylureas, alkylguanidines, and alkylamides, occur widely in nature and potential nitrosating agents, e.g., nitrite (NO₂⁻) and NO_x (the gaseous oxides of nitrogen) are similarly widespread. Relationship between exposure to NOC and causes of human cancer was investigated extensively ten years ago. Results indicated that the exposures of NOC might contribute to the occurrences of malignancy in the upper digestive tracts including stomachs. It was also observed that both high salt intake and deficiency of some micronutrients enhanced NOC-induced carcinogenicity. Recent studies show that infection of *H. pylori* can lead to atrophic gastritis and achlorhydria, and promote endogenous formation of NOC indirectly^[1]. Much attention has been paid to stomach cancer and NOC regarding the characterization of natural *N*-nitrosamides in human environment in the 1990s.

N-nitrosamides, one kind of direct-acting NOC, can

be synthesized endogenously in stomach lumens and damaged DNA of gastric mucosal epithelium *in situ*. Most of epidemiological investigations showed that the occurrence of stomach cancers was correlated positively with exposure levels of nitrosating agents and nitrogenous precursors of NOC^[2]. Laboratory synthesized *N*-nitrosamides are strong animal stomach carcinogens. Intragastric *N*-nitrosamide formation may play an important role in the etiology of gastric carcinomas^[3]. However, most *N*-nitrosamides are chemically reactive, thermal, photo, and alkali-labile compounds. It is very difficult to detect *N*-nitrosamides in human environments chemically. Little was known about the existence of natural *N*-nitrosamides before the 1980s because of lack of a convenient sensitive method to detect them precisely^[4]. Several progresses have been made on the study of *N*-nitrosamides in the past ten years, including setup of detection methods for trace *N*-nitrosamides in the early 1990s^[5,6] resulting in recent discovery of natural *N*-nitrosoureas in human environments^[7-9].

This article will present and discuss results of studies on stomach cancer and *N*-nitrosamides in the past decade.

DEVELOPMENT OF SENSITIVE AND SELECTIVE METHODS TO DETECT *N*-NITROSAMIDES CHEMICALLY

Many *N*-nitrosamines can be analyzed sensitively (detection limit, less than 1 ng/injection) by a standard commercial Thermal Energy Analyzer Detector (TEA), relying on their thermal cleavage of the *N-N* bond to produce a nitrogen oxide (NO) radical. However, *N*-nitrosamides and related compounds, unlike *N*-nitrosamines, typically rearrange on pyrolysis to yield molecular nitrogen (N₂) instead of nitrogen oxide. Because of the possible etiological role of *N*-nitrosamides in human gastric carcinogenesis, it is necessary to setup a sensitive and selective method to detect *N*-nitrosamides in human environments.

A liquid chromatography (HPLC) with postcolumn photolysis device was assembled first at Shuker and Tannenbaum's laboratory, Massachusetts Institute of Technology in 1983 (Figure 1A)^[10]. In the device *N*-nitrosamides were cleaved photolytically by ultraviolet (UV) irradiation to produce nitrite ion in aqueous solution, which was determined colorimetrically with Griess reagent in a postcolumn reactor. However this method is not *N*-

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nitrosamide-specific. It is of low sensitivity (detection limit, ng/injection): 20 for *N*-me thyl-*N*-nitrosoarea (NMU) and 8 for *N*-methyl-*N*'-nitro-*N*- nitrosoguanidine (MNNG). The polarity of *N*-nitrosamides ranges widely from strongly polar to non-polar. Non-polar *N*-nitrosamides do not undergo photohydrolysis because of their nondissolubility in aqueous solution. Though the device could not be used to detect trace amounts of *N*-nitrosamides in nature, it has been often used to further develop sensitive methods (Figure 1B and 1C)^[5,11]. Fine *et al*^[12] at New England Institute for Life Sciences have modified the pyrolysis chamber in a standard TEA such that *N*-nitrosamides release nitric oxide on pyrolysis (sensitivities for standards, less than 1 ng/injection) in 1987. However, details of the instrument were not provided and further development was needed.

An HPLC-photolytic interface-TEA method was reported to precisely detect *N*-nitrosamides and other non-volatile NOC at Hotchkiss's laboratory, Institute of Food Science, Cornell University in 1988 (Figure 1B)^[5]. A chromatographic effluent containing separated NOC is introduced into a glass coil with a purge stream of He and irradiated with UV in a photolysis device. Nitric oxide, cleaved by photolysis, is separated rapidly from the solvent through a series of cold traps and carried by the He into the reaction chamber in a standard TEA. The maximum sensitivity of the approach was approximately 8 ng for NMU and 16 ng for MNNG, and less than 1 ng for *N*-nitrosoamino acids and *N*-nitrosamines. The selectivity for *N*-nitrosamides was not mentioned.

A selective and sensitive HPLC-Photolysis/Pyrolysis-TEA method (Figure 1C) was set up for *N*-nitrosamides at Beijing Institute for Cancer Research, Beijing Medical University School of Oncology (Chen *et al*; Li and Deng)^[6,11]. In the photolysis device, *N*-nitrosamides are first cleaved photolytically by UV irradiation to produce nitrite ion in aqueous chromatographic effluent. Then nitrite ion in the effluent is introduced into a pyrolysis tube (made of Al_2O_3) at 550°C with carrying gas stream of He or N_2 , and releases nitric oxide on pyrolysis. Nitric oxide is separated rapidly from the solvent-spray through a series of cold traps, and is lead into the reaction chamber in TEA by the carrying gas. Detection limits (ng/injection) are: 5.2 for NMU, 8.5 for MNNG, about 1 for *N*-nitrosamines, and 16 for *N*-nitrosoproline. In addition, when UV lamp of the photolysis device is turned off, the responses decreased up to 90%-100% for *N*-nitrosamides, but only 0%-45% for other kinds of NOC. It indicates that the method is a selective one, which can be used to differentiate *N*-nitrosamides preliminarily from other kinds of NOC by comparing the difference in response UV when the lamp is on or off. This special feature can be useful for chemists to select the right chromatographic components for further identification of trace *N*-nitrosamides in human environments.

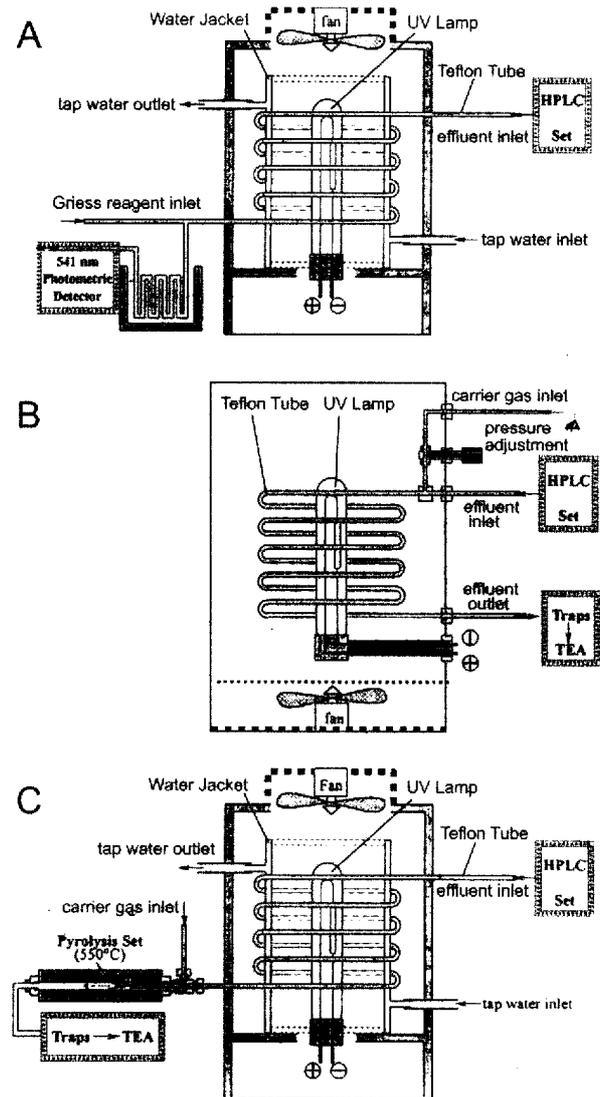


Figure 1 Diagrams of HPLC-Photolysis Device-UV Detector (A), HPLC-Photolytic Interface-TEA (B), and HPLC-Photolysis/Pyrolysis-TEA (C).

Zhang *et al*^[13] developed a method to detect the total amount of *N*-nitrosamides in biological samples with the Photolysis/Pyrolysis-TEA detector. It can be used only to roughly evaluate exposure levels of total *N*-nitrosamides, because calculation of the concentration of total *N*-nitrosamides is based on the difference in response when the UV lamp is on or off. Sample purification by extraction with organic reagents is required in the method. Therefore, *N*-nitrosamides with strong polarity in the samples will be lost during the process of extraction. Only medially polar ones can be detected by the method.

IDENTIFICATION OF *N*-NITROSAMIDES IN NATURE

There is an indispensable evidence to show existence of *N*-nitrosamides in human environments in order to prove etiological role of *N*-nitrosamides in gastric carcinogenesis. However, little is known about the detailed chemical structures of the natural *N*-nitrosamides except stre-

ptozotocin and therapeutic *N*-nitrosoureas^[14]. Establishment of above the sensitive methods to determine *N*-nitrosamides make it possible to detect the trace amounts of *N*-nitrosamides in nature.

Caffeine is a normal component of coffee. Kumar *et al*^[15] reported formation of caffeidine and caffeidine acid from pure caffeine treated under conditions similar to preparation of salted tea practised in Kashmir, which could result in two *N*-nitrosamides, dinitrosocaffeidine and *N,N'*-dimethyl-*N*-nitrosourea by nitrosation. However, there is no report to show that these *N*-nitrosamides are detectable in the nitrosated-salted tea. Mende *et al*^[16] characterized the nitrosamide precursor pyrrolidin(2)one in food and tobacco. A volatile *N*-nitrosamide, *N*-nitrosopyrrolidin(2)one, was detectable in the nitrosated precursor by gas chromatography-TEA method. They also mentioned the existence of trace amounts of the *N*-nitrosamide in natural Indian nasal snuff. But no detailed supporting materials were provided.

Fish sauce is a liquid product of small marine fish and sodium chloride (7:3). The main species of fishes used are *Sardinella aurita* (Val.) and *Decaptes maruadsi* (T & S). The fishes are completely liquidized after being fermented for 1-2 years. The product is consumed daily (about 30 mL/capita) by residents as a traditional seasoning in the Chinese southeast coast, the highest risk area for stomach cancer in China (the male standard mortality of stomach cancer in Changle County, 134.44/10⁵ in 1986-1988). Epidemiological studies showed that the intake of fish sauce is a high risk factor for gastric carcinogenesis in the local residents^[17]. It was reported that extract of fish sauce samples is markedly and directly mutagenic toward *S. typhimurium* TA100 induced high sister chromatid exchanges and micronucleus in Chinese hamster V79 cells after nitrosation with sodium nitrite under the simulated gastric conditions. But the extract of non-nitrosated samples had no such effect. The nitrosated fish sauce also induced SOS in *E. coli* PQ37 and alkylation of calf thymus DNA. The potency of nitrosated fish sauce to induce unscheduled DNA synthesis in human normal gastric mucosal cells was increased about fivefold compared with fish sauce. When the extract of nitrosated fish sauce was given to newborn rats by gavage, dysplasia and adenocarcinoma were induced in the glandular stomach in the 4th and 16th experimental week, respectively^[13,18,19]. Because of the high exposure level of nitrosating agent nitrite for the local residents^[20], dietary fish sauce may contribute to the causes of the high mortality due to stomach cancer in the areas. It is necessary to identify chemical carcinogens in the nitrosated fish sauce.

Chen *et al*^[21] reported that 100% ($n = 21$) of fish sauce samples contained some kinds of volatile *N*-nitrosamines by HPLC-TEA and gas chromatography-TEA. Concentrations of total NOC in 49 fish sauce samples ranged from 0.2 to 16 $\mu\text{mol/L}$ and rose by up to 4800-

and 100-fold after being nitrosated at pH 2 and pH 7, respectively^[19]. Deng *et al*^[7] characterized a chromatographic component of thermal-unstable *N*-nitroso compounds in nitrosated fish sauce by above HPLC-Photolysis/Pyrolysis-TEA method. A strong chromatographic peak with the same retention times as that for authentic NMU, was obtained under two different liquid chromatographic conditions after the sample was nitrosated by 5 mmol/L of sodium nitrite (final concentration) at 37 °C and pH 2.0 for 1 h. Like NMU, the chemical could not be detected by the method when UV lamp in the photolysis device was turned off. In a confirmation study, the chemical structure of the component was compared with authentic NMU by HPLC-electric spray ionization-mass spectrometer and HPLC-UV diode array detector. The chemical showed the same mass spectrum (m/z values 64, 102, 145) and spectrum of ultraviolet-absorbency ($\lambda_{\text{max}} = 230 \text{ nm}$) as those of NMU^[8]. These results indicated that the component was NMU. This was the first study reporting that there is *N*-nitrosamide, NMU, in nitrosated food. In addition, NMU could also be detected in the nitrosated human gastric juice sample spiked with fish sauce^[7]. The formation of NMU in the sample was pH- and nitrite-dependent (Table 1). These results provide direct evidences that NMU formation could occur in human gastric juice samples spiked by fish sauce during nitrosation under simulated gastric conditions *in vitro*. Another *N*-nitrosamide, one of *N*-nitrosodipeptides, was separated recently and confirmed in our laboratory.

Table 1 Comparison of formation of NMU at various doses of NaNO₂ in gastric lumen of pig model *in vivo* and control experiments *in vitro*^[9,22]

Amount of NaNO ₂ (μmol)	Formation of NMU			
	Concentration ($\mu\text{mol/L}$)		Total amount (μmol)	
	<i>in vivo</i>	<i>in vivo</i>	<i>in vivo</i>	<i>in vivo</i>
3480	25.40	29.20	4.27	1.75
870	7.97	6.48	1.91	0.38
220	ND	2.77	ND	0.17

ND, below detection limit; the value was the average of two independent experiments.

Furthermore, experimental mini-pig model and human volunteers were used to study the possibility of intragastric formation of NMU *in vivo*^[9,22]. Fish sauce sample (20-30 mL) and nitrite were administered into gastric lumen of experimental pigs by perfusion via pig stomach cannula, or taken orally by human volunteers. Gastric juice samples were taken out 30 min later. Concentration of NMU in condensed extracts of these samples was analyzed with HPLC-Photolysis / Pyrolysis-TEA. Results showed that NMU was formed in gastric lumens of both models *in vivo* and also that the formation of NMU was nitrite and pH-dependent (Tables 1 and 2).

Zhang *et al*^[20] reported that concentration of nitrite in

fasting gastric juice samples in China was up to 100 $\mu\text{mol/L}$. Pignatelli *et al*^[23] reported that the level of nitrite in fasting gastric juice in Columbia was up to 472 $\mu\text{mol/L}$. It was reported that NMU was still detectable in the condensed extract of 100 mL of mixture of pooled fasting human gastric juice samples and fish sauce sample (9 v:1 v) after treatment of 500 $\mu\text{mol/L}$ of nitrite *in vitro*, which is within the range reported in human gastric contents as reported by Deng *et al*^[9]. These results suggest that low micromolar amounts of *N*-nitrosoureas can be formed in the normal stomach when nitrite is consumed in amounts to which humans are commonly exposed.

Table 2 Status of gastric juice samples from four human volunteers and formation of NMU 30 min after taking 40 mL of diluted fish sauce and 500 μmol of nitrite^[9]

Sample's origin	Total volume of sample (mL)	pH of sample	Total amount of NMU detected in gastric lumen (nmol)
Male A	24	5.0	4
Male B	110	3.0	85
Female A	50	2.0	100
Female B	50	2.0	22

Fish sauce is rich in nitrosable amines, i.e. dipeptides, free amino acids, creatine, creatinine, and putrescine^[24]. It was reported that 16-31 mg/kg of methylurea could be detected in dried, salted bonito fish after nitrosation and denitrosation, though no methylurea could be detected in the fish directly^[25]. Further studies showed that methylurea was synthesized through 5-oxocreatinine 5-oxime and 1-methyl-5-oxohydrantoin 5-oxime during nitrosation of creatinine^[26,27]. NMU in the nitrosated fish sauce might be synthesized from creatinine.

POPULATION STUDY ON TOTAL *N*-NITROSAMIDES IN STOMACH

Previous knowledge on status of human exposure to *N*-nitrosamides is deduced indirectly from data obtained in studies to exposure of NOC precursors. The situation has been changed since the setup of chemical methods to detect total *N*-nitrosamides in biological specimens^[13]. To elucidate the correlation between exposure level of *N*-nitrosamides and causes of gastric carcinomas, a pilot cases-control study and a population study were reported by Zhang *et al* in 1991 and Deng *et al* in 1997^[13,28]. Total amount of natural *N*-nitrosamides was detected with the Photolysis/Pyrolysis-TEA method in fasting gastric juice samples from subjects in high and low risk areas for stomach cancer (Table 3, 4).

Gastric carcinogenesis is a multistage process. Its precancerous lesions include chronic atrophic gastritis, intestinal metaplasia, and dysplasia. In the case-control study, high levels of total *N*-nitrosamides were detected in the gastric juice samples from patients with chronic gastritis in Putian area, a county along the Chinese southeast coast (Table 3)^[13]. The positive rates and mean concentrations

Table 3 Relationship between presence of total *N*-nitrosamides in gastric juice samples from subjects with pathological changes in gastric mucosa

Gastric mucosal status	Positive rate of total <i>N</i> -nitrosamides in gastric juice samples (%)**		
	High risk areas		Low risk area
	Putian ^[13]	Linqu ^[28]	Cangshan ^[28]
*N and CSG	2/12 (16.7)	1/4 (25.0)	4/23 (17.4)
CAG	10/14 (71.4) ^b	38/76 (50.0) ^c	18/56 (32.1)
CAG and IM	12/13 (92.3)	17/56 (28.8) ^c	0/4 (0.0)
CAG and DYS	12/13 (92.3)	18/44 (40.9)	5/10 (50.0)

*N, normal mucosa; CSG, chronic superficial gastritis; CAG, chronic atrophic gastritis; IM, intestinal metaplasia; DYS, dysplasia; **sample was classified as *N*-nitrosamides-positive when more than 184 nmol/L (detection limit) of the chemicals was detected; ^bSignificantly different from N and CSG with $P < 0.01$; ^cSignificantly different from low risk area with $P < 0.05$; ^dSignificantly different from CAG with $P < 0.05$.

Table 4 Presence of total *N*-nitrosamides (NAD) in gastric juice samples (GJ) from low and high risk areas for stomach cancer

	High risk area (Linqu)	Low risk area (Chongshan)	P-value
No. of GJ samples	176	99	
Proportion, $\text{pH} \leq 3$	48.0%	84.0%	<0.01
$\text{pH} \geq 5$	45.3%	13.5%	<0.01
Positive rate, all	40.9%	30.3%	= 0.03*
GJ, $\text{pH} \leq 3$	46.2%	27.4%	<0.01
GJ, $\text{pH} \geq 5$	43.3%	53.9%	
Conc. ($\mu\text{mol/L}$)	0.91	0.73	

*after age-adjustment.

in the three groups of patients were positively correlated with the severity of pathological changes in the gastric mucosa. In the population study, the exposure status of total *N*-nitrosamides in stomach of subjects aged 35-68 years from high risk area for stomach cancer was further compared with that in low risk area under the same geographical and socioeconomic conditions in Shandong Province, China (Table 3)^[28]. Similar relationship between presence of *N*-nitrosamides and pathological changes of gastric mucosa was obtained both in Linqu and Cangshan areas. However, the percentage of *N*-nitrosamides-positive samples was decreased in subjects with chronic atrophic gastritis when intestinal metaplasia was developed. The mechanism of decrease in concentration of NOC is not clear. *N*-Nitrosamides are alkali-labile compounds. It is a common step to adjust pH of sample-extractant mixture to 5.0 with 10% sodium hydroxide in order to accelerate stratification of aqueous phase and organic extractant during extraction of NOC. During extraction of NMU in sample (aqueous solution), it was observed that addition of even one-drop (about 50 μL) of sodium hydroxide solution would destroy all NMU in the sample (Deng *et al*, unpublished data). Gastric mucosa with intestinal metaplasia secretes alkali-mucus. It is necessary to study whether the alkali-mucus catalyzes decomposition (activation) of *N*-nitrosamides and contributes to the decrease of amount of total *N*-

nitrosamides in fasting gastric juice from patients with intestinal metaplasia of gastric mucosa. In addition, development of intestinal metaplasia of gastric mucosa is not suitable for colonization of *H. pylori* in the alkali-mucus closely adjacent to the surface of gastric epithelium and finally eradicates them from there. It is interesting to study the relationship between disappearance of *H. pylori* and the decrease in *N*-nitrosamides amounts in gastric juice.

In the population study, more *N*-nitrosamides-positive samples in the high risk area (Linq) were observed than in the low risk area (Chongshan). The difference was significant after age-adjustment (Table 3)^[27].

Chemical formation of *N*-nitrosamides and other NOC occur mainly under acidic conditions. It had observed that 84% of samples was $\text{pH} \leq 3$ in Chongshan, whereas only 48% in Linq (Table 4, $P < 0.001$). That *N*-nitrosamides-positive rate is higher in samples with $\text{pH} \geq 5$ than with $\text{pH} \leq 3$ in the low risk area indicates that there are some factors which could catalyze formation of *N*-nitrosamides in the achlorhydric stomach. Colonizations of microorganisms are common in the achlorhydric stomach. Some species of bacteria in stomach contain nitrate-reductase and could lead to high concentration of nitrite in gastric juice. It is well known that microorganisms catalyze formation of *N*-nitrosamines. Pan *et al*^[29] reported that synthesis of *N*-nitrosamides, NMU, could also be accelerated by *Pseudomonas aeruginosa* at pH of 6-7, simulating achlorhydric stomach conditions. He *et al*^[30] reported further that 6 out of 46 strains of bacteria, isolated from patients with gastritis in Linq County, promoted formation of NMU at pH 6.0. Biological formation of *N*-nitrosamides mediated by microorganisms in the achlorhydric conditions might account for the high concentration of total *N*-nitrosamides in gastric juice sample with $\text{pH} \geq 5$.

High *N*-nitrosamides-positive rate was observed both in gastric juice samples with $\text{pH} \geq 5$ and $\text{pH} \leq 3$ from subjects in Linq, the high risk area. The results suggest that there is a high chemical formation of *N*-nitrosamides in the acidic stomach. It is supported by the observation that *N*-nitrosamides-positive rate in the samples with $\text{pH} \leq 3$ from Linq is higher than that from Chongshan, the low risk area (Table 4).

Above results indicate that human intragastric exposure to *N*-nitrosamides is positively correlated to risk of stomach cancer. *N*-Nitrosamides may be synthesized chemically in the acidic stomach and biologically in the achlorhydric stomach.

PROSPECTS

Establishment of sensitive methods to detect trace *N*-nitrosamides enabled identification of this sort of NOC in human environments greatly. More natural *N*-nitrosamides

need to be discovered in order to understand aetiological role of *N*-nitrosamides in gastric carcinogenesis in populations. Rediscovery of *H. pylori* is an important event in the history of oncology of stomach. It causes atrophic gastritis, gastric ulcer, and correlates with the occurrence of mucosa-associated lymphoid tissue lymphoma closely. It might also contribute to the causes of gastric carcinoma. *H. pylori* mediated gastritis induces high levels of nitrogen oxide (NO) in gastric mucosa. Tissue nitrogen oxide could damage DNA directly if it penetrates into cytoplasm during S-phase and further into nucleus. When Fe^{2+} exists in the tissue simultaneously it also nitrosates nitrogenous precursors to form NOC. It is noted that *H. pylori* infection leads to an increase in concentration of nitrite in the stomach lumen, decrease in secretion of ascorbic acid from gastric mucosa, and might promote formation of *N*-nitrosamides in achlorhydric stomachs. However, relationship between occurrence of *N*-nitrosamides and *H. pylori* is not investigated extensively. Such investigation may bring to light the mechanism of carcinogenesis caused by *H. pylori*.

Because of the instability of *N*-nitrosamides, it is almost impossible for the chemicals to exist in diet. Most of the human exposure might originate from intragastric formation. That provides a good target to prevent stomach cancer by inhibition of nitrosation and elimination of nitrite in stomach. Garlic and related components are ideal candidates for such interventional study. They inhibit proliferation of microorganisms, combine nitrite, block nitrosation, and destroy formed NOC. An interventional study with garlic oil and other chemicals has been undertaken in Linq area^[31].

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Mechanisms involved in *Helicobacter pylori* induced duodenal ulcer disease: an overview

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Subject headings *Helicobacter pylori*; *Helicobacter* infection; gastric acid; gastric mucosa; duodenal ulcer; bicarbonates; gastric metaplasia

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Duodenal ulcer (DU) can be developed via several different mechanisms. Hypersecretion of gastric acid is, however, a common denominator. A massive hypersecretion of acid can by itself evoke a DU, e.g. in the Zollinger-Ellison syndrome. Irrespective of the mechanism behind the development of a DU, powerful antisecretory treatment will heal the ulcer and prevent recurrence.

The hypersecretion of acid in DU patients is well characterized (Table 1). The maximal acid secretory capacity is increased in about half of the patients^[1], probably due to an enhanced trophic effect of gastrin on the oxyntic mucosa. Several mechanisms normally inhibiting gastric acid secretion, has been found defective in DU patients^[2-4], i.e. resulting in an increased release of gastrin^[2]. The final result is a raised and prolonged acid response to every meal^[5].

The most common cause of DU is *Helicobacter pylori* (*H. pylori*) infection. It is, however, only a minority (10% - 15%) of all *H. pylori* infected subjects, who will develop DU. The sequence of events leading to DU includes hypersecretion of acid, development of gastric metaplasia in the proximal duodenum with colonization of *H. pylori* in the duodenal bulb, progress towards a high density of virulent *H. pylori* bacteria in the bulb with a marked active and chronic inflammation, and a profoundly reduced bicarbonate secretion in the bulb. The overview is a short presentation of evidence supporting this concept.

Table 1 Gastric acid secretory characteristics of duodenal patients (all of which might be *Helicobacter pylori* induced)

Increased maximal acid secretory capacity
Increased parietal cell mass (trophic effect of gastrin?)
Increased basal acid secretion
Increased basal release of gastrin
Increased and prolonged acid response to meals
Increased release of gastrin
Defective inhibitory mechanisms
Antral acidification
Antral distension
Fat in the duodenum (<i>H. pylori</i> induced?)

H. PYLORI EFFECTS ON ACID SECRETION

A pronounced *H. pylori* induced inflammation of the antral mucosa in the presence of an intact oxyntic mucosa will result in acid hypersecretion, due to a blockade of mechanisms normally inhibiting gastric acid secretion^[6,7]. It should be emphasized that this acid hypersecretion does exist in all subjects with a *H. pylori* infection predominantly localized to the antrum, and is thus not a characteristic only for DU patients.

If, however, a pronounced *H. pylori* induced inflammation also includes the oxyntic mucosa, the acid secretion will instead be reduced due to inhibition induced by *H. pylori* inflammation on parietal cell level and a subsequent development of atrophic gastritis^[8].

Maximal acid secretory capacity

DU patients have a higher maximal acid secretory capacity than subjects without the ulcer disease, but there is a considerable overlapping between the two groups. *H. pylori* infection of the antrum results in a moderate increase of the release of gastrin from the antrum. Gastrin has a trophic effect on the acid secreting mucosa, that can produce a markedly increased maximal acid secretory capacity, e.g. in patients with the Zollinger-Ellison syndrome. Also moderately increased serum gastrin levels seem to be able to enhance the maximal acid secretory capacity^[9,10]. For instance, eradication of *H. pylori* has been followed by a reduced maximal acid secretory capacity^[9]. This conclusion is supported by the finding that resection of the antrum with retention of the whole acid secreting part of the

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stomach in DU patients markedly reduced the maximal acid secretory capacity^[11].

The substantial overlapping of the maximal acid secretory capacity between DU patients and subjects without ulcer can be explained partly by the fact that many subjects without ulcer have *H. pylori* infection and consequently an increased release of gastrin, and partly by the possibility that the balance between the gastrins having acid stimulatory effect and those having only trophic effect^[12], might vary from subject to subject.

Defective inhibitory mechanisms

In subjects with a predominantly antral *H. pylori* infection the gastrin release is increased in the fasting condition, during meals, and by experimental administration of gastrin releasing peptide (GRP)^[6,13-17], which probably is a consequence of a reduction of the somatostatin in the antrum by the *H. pylori* infection^[18,19]. Antral somatostatin acts as a physiological inhibitor of the gastrin release. After eradication of *H. pylori* the gastrin release is normalized. During i.v. infusion of GRP the gastrin release and acid secretion was significantly higher in *H. pylori* infected subjects, and these responses were normalized after eradication of *H. pylori*^[20]. Interestingly the acid response to GRP in *H. pylori* infected DU patients was twice that in *H. pylori* infected subjects without ulcer despite similar gastrin responses in both groups. Whether this experimental situation reflects physiological conditions is open to question for several reasons, but the results indicate that DU patients may have a more pronounced hypersecretion of acid than *H. pylori* infected subjects without ulcer.

Antral *H. pylori* infection gives rise also to a blockade of an inhibitory nervous reflex from the antrum to the acid secreting mucosa^[7]. In subjects without *H. pylori* infection distension of the antrum provokes an inhibition of acid secretion via a reflex pathway, and this inhibition is completely absent in subjects with *H. pylori* infection. The blockade of the inhibitory reflex is probably a result of the inflammatory process in the *H. pylori* infected antrum, since the inhibitory reflex seems to turn up again only when the inflammatory reaction has ceased after eradication of *H. pylori*.

The defective inhibitory mechanisms caused by antral *H. pylori* infection results in acid hypersecretion under physiological conditions. The gastrin release during meals is increased in *H. pylori* infected subjects^[6,13-15], and contributes to an increased and prolonged acid response to meals^[15]. The well-known inhibition of gastrin release by acidification of the antrum is markedly impaired in *H. pylori* infected subjects^[14,15], contributing to the acid hypersecretion of the

infected subjects. The hypersecretion of acid obviously results in an increased acid load on the duodenal bulb^[15] both in *H. pylori* infected subjects without ulcer and DU patients. Thus, it seems reasonable that the explanation for the fact that only a minority of all *H. pylori* infected subjects will develop DU has to be searched for in the duodenal bulb, but with acid hypersecretion as necessary prerequisite.

GASTRIC METAPLASIA

Gastric metaplasia (GM) is islands of gastric mucosa in the duodenal bulb. GM develops as a result of an increased acid load on the bulb, and is e.g. rather extensive in patients with the Zollinger-Ellison syndrome^[21]. GM has been found in 90% of *H. pylori* infected DU patients, and in about 60% of *H. pylori* infected subjects without ulcer^[22,23], in accordance with the increased duodenal acid load in both these groups. GM is a prerequisite for colonization of *H. pylori* in the duodenal bulb. It is possible that the extension of GM is facilitated by the *H. pylori* induced inflammatory process in the bulb, since a combination of *H. pylori* eradication and antisecretory treatment reduced the GM area more effectively than either treatment alone^[25,26].

Hypersecretion of acid and development of GM exists in both DU patients and *H. pylori* infected subjects without ulcer, albeit somewhat more pronounced in DU patients. It therefore seems reasonable to assume that a critical factor in the development of DU could be a large number of *H. pylori* and/or particularly virulent *H. pylori* in the duodenal bulb of DU patients. The density and virulence of *H. pylori* in DU patients has previously been determined in several studies, but in biopsies taken from the antrum. In these studies of antral *H. pylori* density and virulence, only a marginal difference was found between DU patients and *H. pylori* infected subjects without ulcer. However, findings regarding *H. pylori* in the antrum does not necessarily reflect the situation in the duodenal bulb.

GM in the duodenal bulb is found in patches that cannot be visualized at or during gastroduodenoscopy. In a recent study^[24] the extent of GM in the duodenal bulb was therefore determined more systematically by taking 2 biopsies from each quadrant of the bulb. The area of GM in the multiple biopsies was 4 times larger in the DU patients than in the *H. pylori* infected subjects without ulcer^[24]. These biopsies were also used to determine the prevalence and density of virulent *H. pylori* as well as the type (active and chronic) and degree of the inflammatory reaction in both the antrum and the duodenal bulb of DU patients ($n = 20$) and *H. pylori* infected subjects without ulcer ($n=21$).

BACTERIAL DENSITY AND *cagA* STATUS IN THE DUODENAL BULB

H. pylori colonization of GM in the duodenal bulb is very common. It was found in 95% of DU patients and in 80% of infected subjects without ulcer^[24], determined by quantitative culture^[27]. Rigorous precautions were taken at the endoscopy to avoid contamination of the duodenal biopsies with *H. pylori* from the stomach. Gastric epithelial cells express the blood group antigen Lewis-b, and *H. pylori* have the ability to bind to these antigens, thereby facilitating the colonization of the mucosa^[28]. *H. pylori* also has the ability to express various Lewis antigens, that might imply greater opportunity to escape the immune response of the host. It was recently shown^[29] that *H. pylori* expressing various Lewis antigens exist in a considerably higher frequency in the duodenal bulb of DU patients (90%) than in the bulb of infected subjects without ulcer (42%).

The density of *H. pylori* was much lower in the duodenal bulb than in the antrum, even after correction for the area of GM^[24], suggesting that the environment in the duodenal bulb is less favourable for colonization of *H. pylori*. The mean *H. pylori* density in the bulb was about 20 times higher in DU patients than in infected subjects without ulcer, despite the fact that the bacterial density in the antrum was similar for both groups^[24]. There was, however, a substantial overlap of the bacterial density in the bulb between DU patients and infected subjects without ulcer.

The clinically relevant virulence factors of *H. pylori* have not yet been identified. The presence of the *cagA* gene (cytotoxin associated gene A) is so far the best marker of virulence. *H. pylori* bacteria that are *cagA* positive can induce a more powerful release of proinflammatory cytokines with a subsequent potential of mucosal damage^[30]. The proportion of *cagA* positive bacteria might therefore be a significant factor determining the local mucosal damaging effect of colonized *H. pylori*. DU patients had a much higher prevalence of *cagA* positive bacteria in the duodenal bulb (81%) than *H. pylori* infected subjects without ulcer (30%), despite the fact that the prevalence of *cagA* positive bacteria in the antrum was of the same order for both groups (86% and 75%, respectively)^[24]. Furthermore, subjects with a predominance of *cagA* positive bacteria in the bulb had about 10 times higher bacterial density than subjects with *cagA* negative bacteria^[24].

H. PYLORI INDUCED INFLAMMATION IN THE DUODENAL BULB

The *H. pylori* bacteria are colonizing the GM in the duodenal bulb, and consequently the inflammatory cells are found in connection with the GM. The

chronic duodenitis, determined by the degree of infiltration of lymphocytes, was found more marked in DU patients than in *H. pylori* infected subjects without ulcer, but again with overlapping between the two groups^[24]. Active duodenitis, defined by the presence of neutrophil leucocytes, is an established and common finding in DU patients. Active duodenitis was found only in DU patients and almost exclusively in DU patients with *cagA* positive *H. pylori* in the bulb^[24]. Active duodenitis is thus a characteristic for DU patients. Activation of neutrophil leucocytes can result in tissue damage by release of proteolytic enzymes^[31] and by induction of reactive oxygen metabolites in the gastric epithelial cells^[32].

BICARBONATE SECRETION IN THE DUODENAL BULB

Hypersecretion of gastric acid with increased duodenal acid load can result in mucosal damage and development of ulcer. Under normal circumstances the acidification of the duodenal bulb will immediately activate bicarbonate secretion from the duodenal mucosa resulting in neutralization of the acid with the formation of carbon dioxide and water. The carbon dioxide will then stimulate the nitric oxide (NO) producing enzyme NO-synthase^[33], that has been demonstrated in the duodenal mucosa^[34]. NO will finally activate the bicarbonate secretion^[35].

DU patients have a markedly reduced bicarbonate secretion in response to acidification of the duodenal bulb^[36]. It has been shown more recently that the bicarbonate secretion is normalized after eradication of *H. pylori*^[37], implying that the reduced bicarbonate response to acidification of the bulb is a result of the *H. pylori* infection. Interestingly, the bicarbonate secretion was found normal in *H. pylori* infected subjects without ulcer^[37]. The *H. pylori* dependent reduction of the bicarbonate secretion is thus another characteristic of DU patients.

The mechanism by which the *H. pylori* infection is reducing the bicarbonate secretion has been studied recently. The bicarbonate secretion in response to acidification of the duodenal bulb in rats could be inhibited by local administration of a water extract of *H. pylori*^[38]. In these experiments a substantial increase of the NO synthase inhibitor asymmetrical dimethyl arginine (ADMA) was demonstrated in the duodenal mucosa. Separate administration of ADMA markedly reduced the bicarbonate response to acidification of the bulb. The presence of ADMA has not yet been determined in the bulb mucosa of humans. In the antrum, however, the ADMA concentration was 65 times higher in *H. pylori* infected subjects than in non-infected subjects^[39]. It is possible that *H. pylori* delivers peptides that are degraded by proteolysis in

the antrum-bulb region to for instance ADMA. It seems reasonable to assume that the high density of *cagA* positive *H. pylori* bacteria in the bulb of DU patients can produce high enough ADMA concentrations to explain the markedly reduced bicarbonate secretion that is a characteristic of DU patients.

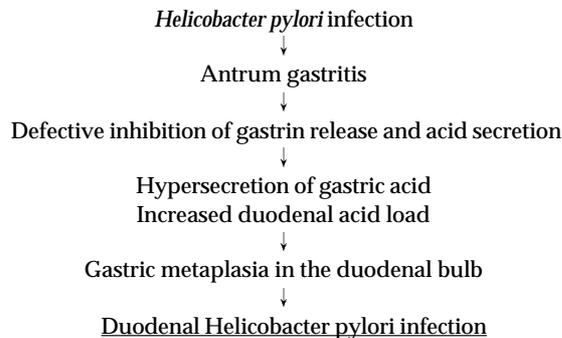


Figure 1 Successive prerequisites for the development of *Helicobacter pylori* induced duodenal ulcer.

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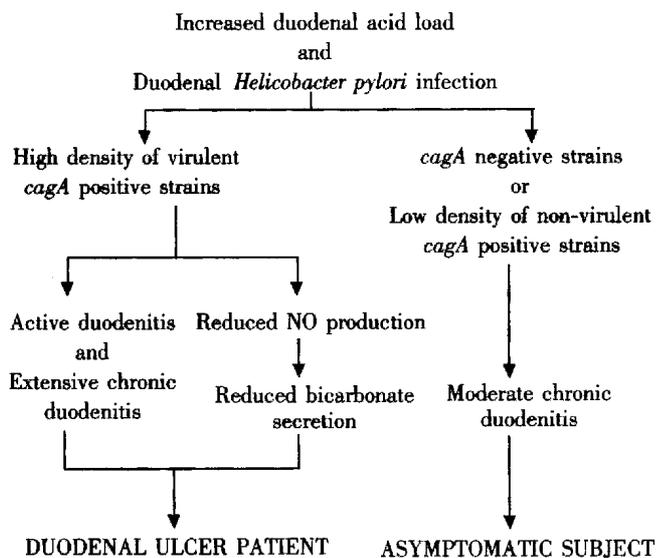


Figure 2 Conceivable sequence of events in the development of *Helicobacter pylori* induced duodenal ulcer.

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WHY DOES ONLY A MINORITY OF *H. PYLORI*/INFECTED SUBJECTS DEVELOP DUODENAL ULCER?

A special series of events seem to result in the development of DU in *H. pylori* infected subjects. The first part of this series of events should be regarded more as a pre/quisite for the development of DU, and is common for all subjects with an

antrum predominant *H. pylori* gastritis (Figure 1). The antrum gastritis results in hypersecretion of acid, the increased acid load to the duodenal bulb gives rise to formation of GM, and these islands of gastric mucosa can be colonized by *H. pylori*.

The most important factor for the development of DU seems to be a high density of virulent *H. pylori* bacteria in the duodenal bulb. When the density of virulent strains / with *cagA* positivity as a marker of virulence / reaches a high level, two effects are triggered that are characteristic for DU patients. Firstly, a high density of virulent *H. pylori* induces a pronounced release of proinflammatory cytokines, leading to a substantial chronic inflammation and an active duodenitis with its tissue damaging potential. Secondly, a high density of virulent *H. pylori* seems to produce enough amounts of NO synthase inhibitors to significantly reduce the bicarbonate secretion in response to acidification of the duodenal bulb. This entails a high acidity in the bulb leading to a potential increase of GM and tissue damage.

In summary the pivotal components in the conceptual development of DU are hypersecretion of acid and colonization of the duodenal bulb with a high density of virulent *H. pylori* strains resulting in an active duodenitis and reduced bicarbonate secretion (Figure 2).

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Genetic factors determining the host response to *Helicobacter pylori*

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INTRODUCTION

The strongest evidence that *H. pylori* infection is the cause of peptic ulcer is that treatment with antibiotics as the only regimen, is not only effective for the clearance and eradication of the infection, but more importantly for the healing of the ulcer or the remission of gastric lymphoma. However, it is still a matter of controversy and research as to why only a minority of people develop gastric ulcer, gastric lymphoma, gastric cancer or duodenal ulcer when half of the world's population is infected by *H. pylori*. Since bacteria and host form an interactive unit, the pathogenesis of gastric pathology is determined and probably follows the same principles of any infectious disease.

First, the bacteria have to undergo evolutionary changes that are needed to survive the host response. This explains to a certain extent the highly diverse virulence of *H. pylori* strains which undoubtedly contribute to determining the development of disease^[1].

Second, host factors, such as the age at which the host acquires the infection, appear to determine the outcome of the interaction between *H. pylori* and the host. It is well known that gastric and duodenal ulcer rates increase with age. The presence of a genetic predisposition is also important. The relative risk of developing gastric, duodenal ulcer or duodenitis in patients with a family history of gastric pathology, is higher than in patients without familial occurrence. More convincingly still, several studies have established that the prevalence of peptic ulcer is higher in monozygotic twins than in dizygotic twins. Also, the concordance rates for *H. pylori* infection among pairs of twins reared apart is higher for monozygotic than for dizygotic twins^[2].

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Experimental animal models have provided evidence that the host response is an important determinant in the severity of gastritis^[3].

THE BIOLOGICAL BASIS OF THE IMMUNE RESPONSE

Therefore, it is justifiable to look for the biological basis of the susceptibility to acquire the infection and which determines the pattern of disease and severity. The host response is important for the immune defense against a variety of infections and the protection of the organism against the invasion of foreign material.

It is important to recognize that immunity to infection is mediated by two systems, the innate and the acquired. Innate immunity provides a rapid antimicrobial host defense. Innate immunity is the bridge between the recognition of an invading microorganism and the mounting of an efficient acquired and adaptive immune reaction that should overcome the infection. At the first contact of bacteria and host, *Helicobacter* Lipopolysaccharide (LPS) activate cells of the innate immune system. The host defense systems are mobilized to eliminate the infection. LPS of many *H. pylori* strains express Lewis antigens (Lex, Ley, Lea, Leb) which are similar to the antigens expressed by gastric epithelial cells of the host. Recent studies in *H. pylori* strains isolated from Asian peptic ulcer patients have shown to express more Lewis antigens than strains from non-ulcer dyspepsia patients^[4]. Since it is now known that Lewis antigens are involved in adhesion and colonization the increased adherence may lead to an increased bacterial burden. This will enhance the cross talk between the *H. pylori* and the host and leads to the activation of the transcription factor NF- κ B and host signal transduction pathways. IL-8 production is enhanced and results in the characteristic neutrophil infiltration and inflammation of *H. pylori* gastritis. *H. pylori* induces also IL-12 which in turn elicits interferon-gamma IFN γ production. During the induction of cell-mediated immunity, T lymphocytes expressing IFN γ and TNF α are generated. Macrophages are recruited and become activated. It is now known that IL-12 also facilitates the development of T helper type 1 (Th1) lymphocytes required for protection against the bacteria^[5]. In principle, the Th1 response is a normal adaptive immune response, however, when this response is too strong it will produce a severe chronic inflammation. In the stomach, locally produced IL-1 β is an important mediator of hypochlorhydria

since IL-1 β inhibits gastric acid and pepsinogen secretion. The levels of IL-1 β in cultured antral biopsy specimens are significantly higher in *H. pylori* positive patients than in individuals with negative cultures for *H. pylori* with normal antral mucosa. There is evidence that other cytokines, such as TNF, are also increased in *H. pylori* positive patients.

THE GENETIC CONTROL OF THE IMMUNE RESPONSE

During the inflammatory response a multitude of new genes encoding proinflammatory cytokines such as IL-1 α , IL-1 β , TNF α , and LT α as well as other proteins with pro-or anti-inflammatory properties are induced. Genetic control of the IL-1 family of cytokines and *TNF* appears to regulate the production and transcription of the respective cytokines. In healthy individuals, significant inter-individual variations were found in *in vitro* production of IL-1 proteins. This has been interpreted as inherited inter-individual differences.

The genes controlling the innate and the acquired immune response are different and probably work in a complementary way. Little is known of the genetic control of the innate immune response. With respect to the acquired immune response it is known that the *IL-1A*, *IL-1B* and *IL-1RN* genes are clustered on the long arm of human chromosome 2. Several functional polymorphisms have been described in these genes. This strongly suggests that individuals who produce high amounts of IL-1 β and lower amounts of the IL-1ra have difficulty in controlling inflammation. Preliminary results of IL-1 gene polymorphisms in duodenal ulcer patients have shown that the simultaneous carriage of *IL-1B* + 3953 allele 2 and *IL-1RN* allele 2, is an independent factor associated with reduced risk of bleeding duodenal ulcers^[6]. In a recent study supporting the role of the interleukin-1 gene cluster in the progression to disease, El-Omar and co-workers^[7] have reported the association of specific interleukin-1 gene polymorphisms with increased risk of gastric cancer. According to these authors, carriage of *IL-1B*-31 allele 2 and *IL-1RN** 2.2 homozygosity increase both the likelihood of a chronic hypochlorhydric response to *H. pylori* infection and the risk of gastric cancer, presumably by altering *IL-1B* levels in the stomach. *IL-1B*+3953 allele 2 homozygotes seemed to play a protective role in gastric cancer, although the effect did not

reach statistical significance.

The localization of the tandemly arranged *TNF* and *LTA* genes, encoding respectively *TNF* and LT α in the central region of the MHC at the short arm of chromosome 6 (6p21.3) has prompted interest in the study of their contribution to diverse inflammatory processes. Recent studies have found that polymorphisms in the *TNF* and *LTA* genes are associated with peptic ulcers. Recently, the *TNF*-308 polymorphism located in the promoter region of the *TNFA* gene has been found associated with susceptibility to the development of duodenal ulcer^[8]. Thus, *H. pylori* positive individuals with the *TNFA*-308G/G genotype appeared to have a higher risk for the development of duodenal ulcer than individuals with the *TNFA*-308G/A or A/A genotypes.

CONCLUSION

The data suggest that the normal physiological control of the gastric secretory function and the control related to the pathogenesis of *H. pylori* associated diseases, to a certain extent are dependent on the genetic control of the inflammatory response. It is clear that more work needs to be done to clarify the role of the genetic control. Studies of the interaction between virulent *H. pylori* strains and the genetic factors as described above need to be addressed.

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Design, delivery and efficacy testing of therapeutic nucleic acids used to inhibit hepatitis C virus gene expression *in vitro* and *in vivo*

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Caselmann WH, Serwe M, Lehmann T, Ludwig J, Sproat BS, Engels JW. Design, delivery and efficacy testing of therapeutic nucleic acids used to inhibit hepatitis C virus gene expression *in vitro* and *in vivo*. *World J Gastroentero*, 2000;6(5):626-629

Despite major achievements in the treatment of chronic hepatitis C with the combination of interferons and the nucleoside analog ribavirin^[1] the majority of patients with chronic hepatitis C virus (HCV) infection cannot be treated effectively. To improve this response rate we used antisense technologies to inhibit HCV translation as possible additional option for experimental treatment. Antisense oligodeoxynucleotides (ODN) are short nucleic acid oligomers, which bind complementary to the single-stranded HCV-RNA with plus (+) strand polarity and can induce hybrid arrest at the ribosome as well as cellular RNase H activity, which cleaves the RNA template in DNA-RNA hybrids. The 5'-non-coding region (NCR) is highly conserved among the 6 main genotypes and exerts the function of an internal ribosomal entry site (IRES), which is important for translation^[2] and possibly also for viral replication (Figure 1).

Various antisense oligodeoxynucleotides varying from 8 to 23-mers and covering stem-loop structures within the 5'-NCR and the adjacent core gene were synthesized^[3]. ODN₄ comprises HCV 5'-NCR nucleotides (nts.) 326-348 including the start AUG of the polyprotein coding sequence. Since easy-to-handle and generally a available infection or replication systems for HCV are lacking. We used model systems (Figure 2), in which the HCV 5'-NCR and 66 nts. Of the core gene were fused to the firefly luciferase gene for *in vitro* transcription/

translation from the bacteriophage T7 promoter. A similar construct with the cytomegalovirus(CMV) immediate early promoter was constructed for transfection experiments of hepatoma and non-liver cell lines^[4].

As compared to mismatch or sense controls completely phosphorothioate-modified ODN 4 showed the best inhibitory effect of luciferase activity in the rabbit reticulocyte lysate, which ranged in the order 96% ± 1% at about 4μM concentration. The effectiveness of ODN 4 is possibly due to its interference with a pseudonot structure (Figure 1) in HCV RNA which is disrupted, when ODN4 binds to the target RNA. In HepG2 tissue cultures comparable specific effects were obtained. However, fluorescence labeling of this ODN demonstrated that only a small percentage of transfected cells did take up the therapeutic ODN.

In order to improve the capability of membrane permeation by variation of their lipophilicity chemically modified ODN such as methylphosphonate and benzylphosphonate ODN 4 were compared with phosphorothioate ODN 4, which had been used in completely modified form in the previously described experiments. For the following experimental setting partially modified ODN4, which carried 6 modifications over a length of 23 nts. either in terminal localization or scattered along the molecule (Table 1). Toxicological testing revealed acceptable IC₅₀ concentrations between 0.2 and 5.8μM and the best therapeutic indices of 3.8 for terminally modified benzylphosphonate (tB-) ODN 4 and terminally modified phosphorothioates (tS-) ODN4. While both scattered (s) and terminal (t) modifications inhibited HCV gene expression in case of phosphorothioate (S)-ODN 4, only terminally modified methylphosphonate (tM)-ODN 4 and benzylphosphonate (tB)-ODN 4 showed specific and effective inhibition. This is possibly correlated to an induction of RNaseH activity and the steric hindrance of this enzyme by differently modified molecules^[5,6]. In cell culture, tB-ODN 4 showed the best inhibition of about 96%, leaving, however, 4% of reporter gene expression unaffected.

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Table 1 Characterization of partially modified antisense oligodeoxynucleotides

5'-TGC TCA TGG TGC ACG GTC TACA-3'	4
5'-TGC TGA TGC TGC ACG CTC TAG GA-3'	K
5'-TCG TAG ACC GTG CAC AT GAG CA-3'	S
5'-TGC TCA TGG TGC ACG GTC TAC GA-3'	4
5'-TGC TGA TGC TGC ACG CTC TAG GA-3'	K
5'-TCG TAG ACC GTG CAC CAT GAG CA-3'	S

Site of modification in bold font.

4: ODN 4, K: mismatch control, S: sense control ODN.

Therefore, katalytically active antisense RNAs, so-called hammerhead ribozymes (RZ), which cleave at NUH recognition sites (N: any nt., U: uridine, H: any nt. except guanosine) of a RNA template, were synthesized. Taking these recognition motifs into account, one of the RZ was directed towards similar stem-loop structures as the previously tested ODN. After purification of the RZ by denaturing polyacrylamide gel electro-

phoresis and reverse phase chromatography their katalytic activity was tested with short 14-mer substrates under defined cleaving conditions. Best cleavage was obtained for RZ A328-R which cleaves between HCV nts. positions 330 and 331. Its Michaelis Menton constant K_m was 69.1 ± 19.3 nM. The katalytic constant K_{cat} was $4,3 \text{ min}^{-1}$ indicating multiple turnover. The katalytic effectiveness K_{cat}/K_m was $6.4 \times 10^7 \text{ M}^{-1} \times \text{min}^{-1}$. The cleavage with longer (452 nts.) HCV-luciferase fusion RNA substrates was best effective, if a high molar excess of RZ were used. In direct comparison with antisense ODN or not katalytically active antisense RNAs targeting exactly identical HCV-RNA sequences RZ A328-R displayed the best about 90% inhibition of luciferase activity *in vitro*.

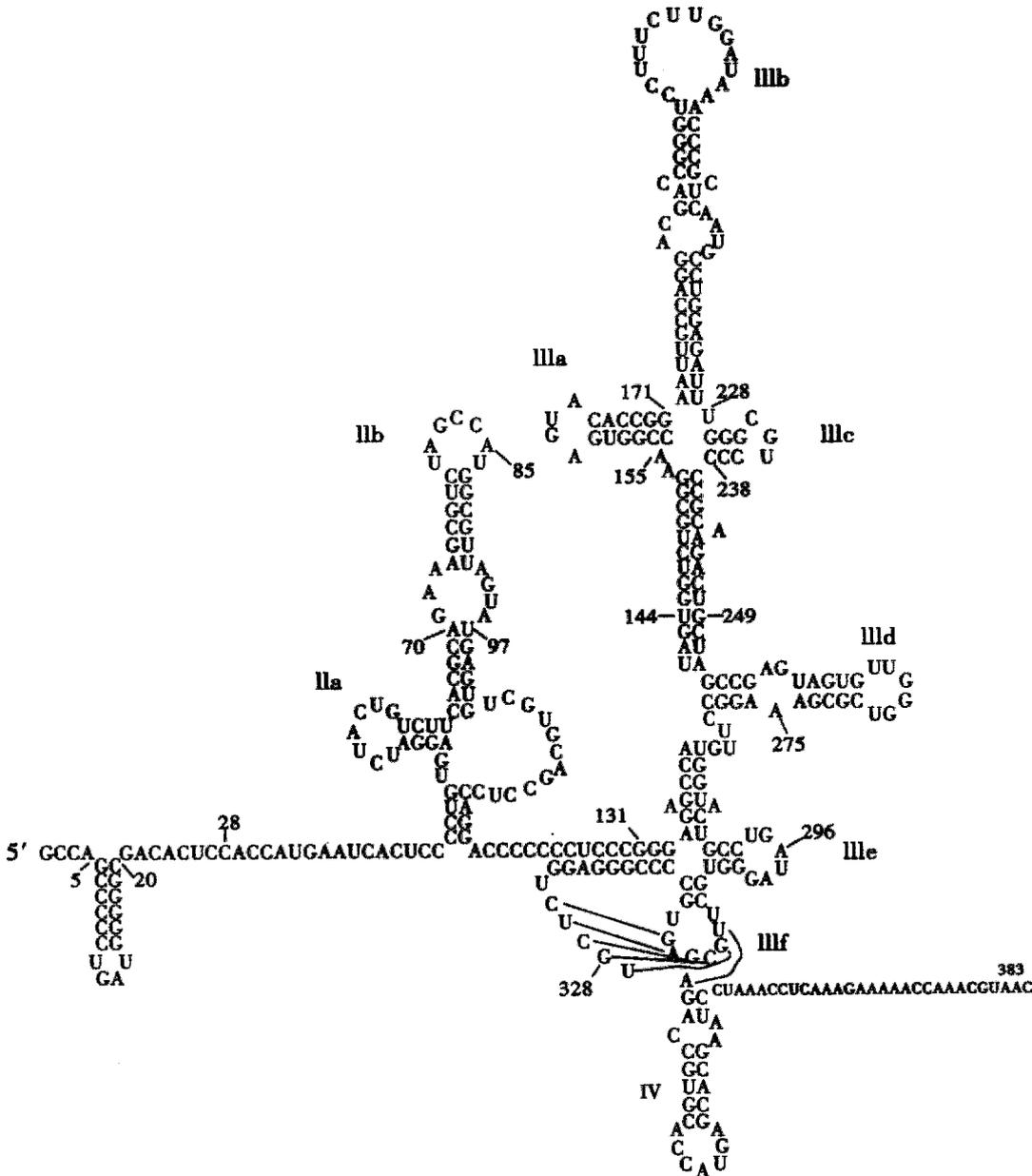


Figure 1 HCV 5'-non-coding region.

The pseudonot region and stem loop IV including the start AUG are indicated in bold font as optimum target sequences for ODN binding.

To further extend the amount of possible target regions on the HCV genome a A \geq I exchange at nt. position 15.1 was introduced (Figure 3), which reverts the polarity of an important H-bond in the hammerhead structure and allows efficient cleavage at a non-natural NCH recognition site (N: any nt., C: cytidine, H: any nt. except guanosine). For stability reasons various 2'-O-allyl or amino-ribonucleotide modifications had been introduced in these RZ^[7], which cleave between HCV nts. 346 and 347 (Figure 3). An inactive cleavage antisense RZ and an unspecific RNA completely unrelated to HCV were used as controls. Regardless of prehybridisation of the RNA molecules with their target sequence, the best inhibitions of luciferase activity were obtained with the NCH recognizing RZ. The inhibition was less pronounced, when inactive cleavage antisense RZ were used. This data could be reproduced in HCV-5'-NCR/core-luciferase-recombinant CCI13 cell lines, which stably express the HCV core-luciferase fusion protein above from the CMV immediate early promoter. *In vivo* testing using CMV-HCV 5'-NCR/core-luciferase-transgenic mice are presently going on.

Coupling of effective antisense molecules to biomolecules such as bile acids which are specifically transported into the hepatocyte by bile acid transporters are in progress (Figure 4). The size of the antisense ODN coupled to cholic acid or taurocholic acid has been optimized with respect to efficient and specific inhibition of HCV-luciferase translation *in vitro*. Experiments with cell lines overexpressing the organic anion transporting polypeptide (OATP) and the sodium taurocholate cotransporting polypeptide (NTCP) are in progress.

SUMMARY

Antisense oligodeoxynucleotides, RNAs and ribozymes can effectively inhibit HCV translation in model systems *in vitro* and *in vivo*. Chemical modifications such as phosphorothioate or benzylphosphonate modifications of oligonucleotides as well as O-allyl or amino-ribonucleotide modifications of ribozymes guarantee sufficient stability towards nuclease degradation. The specific exchange of single ribonucleotides in synthetic ribozymes permits to extend the ribozyme cleavage specificity to non-natural NCH motifs. If problems of hepatocyte-directed delivery and sufficient intracellular effector concentration can be solved, therapeutic nucleic acids may turn out to be a safe and powerful treatment option in the multimodal therapy concept of chronic hepatitis C.

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Mycobacterium avium subspecies paratuberculosis in the causation of Crohn's disease

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Mycobacterium avium subspecies paratuberculosis (MAP), originally called Johne's bacillus was first described from Germany in 1895 as the cause of a chronic inflammatory disease of the intestine in a cow. As the 20th century progressed, clinical and sub-clinical MAP infection in farm animals in Western Europe appeared to become more prevalent. Among the early reviews available are the excellent ones prepared by Doyle^[1] from the Veterinary Laboratory, Weybridge, UK, and Riemann and Abbas^[2] from the University of California, Davis. In general, the response on farms to the appearance of clinical Johne's disease was to cull infected animals. This practice over the course of the 20th century may have exerted a selection pressure on MAP favouring the emergence of strains which can infect animals for years without necessarily causing clinical disease. In the latter part of the 20th century the incidence of clinical disease due to MAP in some areas of Western Europe and North America appeared to decrease. The problem which confronts these regions now is subclinical MAP infection of domestic livestock throughout Western Europe and North America and the emergence of wildlife reservoirs including those in rabbits and their predators^[3]. In the United States and Canada the herd prevalence of MAP infection is reported in the range 21% - 54%^[4-8]. In Western Europe the herd prevalence lies in the same range, although a recent serological study of bulk-tanked milk from 900 dairy herds in Denmark reported that 70% of herds tested positive for MAP infection^[9]. What is beyond doubt is that MAP is widespread in our domestic animals.

Subclinically infected dairy cows secrete MAP in their milk. It is one of the ways the organism

passes from infected parent to offspring when the calf may be most susceptible. MAP is more robust than *M. bovis* or *M. tuberculosis* and the destruction of all viable MAP by exposure to current pasteurisation conditions of 72°C for 15 seconds is not assured. In on-going research in the Department of Food Science, University of Belfast, N. Ireland, small slow-growing, mycobactin-dependent, IS900 PCR positive colonies of MAP have been cultured from about 3% of retail units of pasteurised cows' milk, so far tested. In the U.K. what is also beyond doubt is that the human population is being exposed to MAP in retail milk supplies. These organisms accumulate particularly in the ileocolonic regions of the intestine where they may remain for years and not cause clinical disease. This situation is similar in principal to the widespread exposure of human populations in Europe, North America and elsewhere, to *M. bovis* before the introduction of milk pasteurisation and the tuberculin testing of dairy herds introduced in the middle third of the 20th century. With MAP, only those individuals with a particular inherited or acquired susceptibility may go on eventually to develop clinical disease.

Infected animals excrete MAP onto pastures. Wildlife reservoirs contribute to environmental contamination. The problems now being caused by MAP, differ from those previously caused by *M. bovis*, in that MAP can survive for long periods in the environment. Rains falling on contaminated land will wash MAP into ground and river waters. Although much research needs to be carried out in this area, it is probable that MAP in the environment is taken up into organisms such as amoebae in which they can survive. This may allow them to replicate, to increase their resistance to biocides and potentially acquire a phenotype which is more pathogenic for humans. Water abstracted from rivers and lakes contaminated with MAP may convey these organisms to human populations. MAP arriving at domestic outlets in high dilution may accumulate in biofilms lining household water systems. Either in the food chain therefore, or in water supplies, it is inevitable that humans sharing the same geographic areas with animals which are extensively infected, will be exposed to these pathogens.

The question as to whether MAP may also cause disease in humans has its origins in a proposition first published in 1913^[10]. We have recently prepared a detailed analysis of this complex issue which is in general poorly understood, even by

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medical specialists in the field of chronic inflammatory diseases of the intestine^[11]. It is now known that MAP can cause chronic inflammation of the intestine in a very broad range of animals including large and small ruminants, monogastrics such as dogs and pigs, and so far at least four types of sub-human primates. MAP shows a well defined tissue tropism and will end up causing chronic inflammation of the intestine even if administered experimentally by subcutaneous, intravenous or intraperitoneal routes. The histopathological features of MAP disease in animals ranges from one in which millions of typical ZN-positive MAP are visible microscopically in the inflamed intestine to the other extreme where no MAP can be seen at all, but there is chronic granulomatous inflammation. This is just what leprosy does in humans. One of the properties of MAP which has retarded our understanding of the problems it is causing is that it may be very difficult to culture in the laboratory. Patient work in many laboratories has, however, shown that MAP can be grown in conventional culture from about 5% of people with Crohn's disease, but not from normal people. Cultures have had to be incubated for months or years before any growth identifiable by conventional means, becomes visible.

MAP is very similar to other organisms of the *M. avium* complex (MAC) to which we are all exposed, so that immunological tests for MAP infection in humans using crude extracts of laboratory cultured organisms usually report no difference between Crohn's disease and normal people. Recent studies have however shown that if specific targets on MAP are carefully selected, highly significant differences in immune recognition can be demonstrated between Crohn's disease and normal people. An important example of this has come from recent research at UCLA which showed that the blood of 9 out of 10 people with Crohn's disease contained IgA which recognised a mycobacterial protein richly expressed on MAP called HupB^[12]. HupB is identical to the laminin receptor used by *M. leprae* to enter Schwann cells round nerves causing the neural inflammation so characteristic of leprosy^[13]. Neural inflammation is long known to be a specific feature in the inflamed gut in Crohn's disease^[14] and antibodies to the chronic inflammatory disease associated autoantigen pANCA, cross-react with HupB.

In 1990, after spending 20 months carefully optimising sample processing and experimental procedures, we began a study which revealed the presence of MAP DNA in about two thirds of people with Crohn's disease using IS900 PCR^[15]. It was also present in the intestine of 12% of normal people, which is just what would be expected to occur in a totally exposed population. Since then there have been 18 peer reviewed publications using a variety of experimental methods, 9 of which reported the

presence of MAP in CD gut some or most of the time and 9 found MAP hardly ever or not at all^[11]. Similar inconsistencies have occurred in the results of DNA tests applied to other chronic inflammatory diseases such as TB. Apart from some obvious methodological errors, the reasons for the uncertainty are the low abundance of MAP in Crohn's disease intestine and its extraordinarily tough protease-resistant phenotype. Recent research by Dr Saleh Naser and colleagues from the University of Central Florida [cited in 11], using improved liquid cultures and IS900 PCR on their centrifugal pellets, has demonstrated MAP in 86% of surgically resected Crohn's disease gut. The same authors have also demonstrated MAP in the centrifugal pellets of breast milk from each of 2 mothers with Crohn's disease who had recently given birth, but not in the breast milk of 5 normal women. Work in our own lab by Jun Cheng and Tim Bull, using much improved methods, is currently reporting Chinese MAP in 69% of Chinese surgical path blocks from Crohn's disease patients in China, and in 14% of path blocks of normal intestine from Chinese people. In our view these studies clearly demonstrate the presence of this chronic enteric pathogen in a substantial majority proportion of humans with chronic inflammation of the intestine of the Crohn's disease type. Although the incidence of Crohn's disease in China is currently much lower than in Western Europe or North America, these recent studies in humans strongly suggest that Chinese people are exposed to these pathogens and that action may need to be considered at this early stage to limit the more extensive development of disease in humans.

It has long been known that infections due to non-tuberculous mycobacteria in immunocompetent people, particularly those caused by MAC, are usually resistant to standard anti-tuberculous drugs. These organisms can prevent the drugs penetrating the microbial cell and can rapidly develop mutations which confer drug resistance. Lasting resolution of MAP infections in animals using standard anti-mycobacterial treatment has never been convincingly demonstrated, and much the same outcomes have resulted from a similar treatment approach in humans with Crohn's disease^[11]. MAP are however more susceptible to some newer drugs which are man-made chemical modifications of natural streptomycetes antibiotics such as rifabutin and clarithromycin. These agents also have the advantage of being concentrated within macrophages where MAP in infected animals and in humans almost certainly resides. Our own studies from 1992^[16], supported by the work of Dr. Tom Borody in Sydney, Dr. Ira Shafran at the University of Central Florida, and by recent work from the North of England^[17] have shown that a substantial proportion of people with active Crohn's disease will go into remission with healing of the

intestine which is sometimes lasting, when treated with combinations of these drugs. A randomised controlled trial of this treatment was initiated in Australia in September 1999.

A question which is frequently asked is how can so few MAP cause so much chronic inflammatory disease? To answer this we must allow our thinking to escape from the immobilising presumption that it must be like TB, in which a major factor in the disease process is a direct immunological response to cell wall components. MAP in animals with the paucimicrobial form of the disease and in humans, does not have a classical mycobacterial cell wall. MAP colonising immunoregulatory cells like macrophages almost certainly causes an immune dysregulation. Together with defects in the integrity of the overlying mucosa, much of the disease itself is caused by an exaggerated immunological response to leakage into the gut wall of bacteria and food residues normally confined to the lumen. Clinical improvement can be achieved by suppressing or modulating the immune system, by reducing the allergic component and altering the enteric flora with elemental diets, and by treatment with antibiotics such as ciprofloxacin and metronidazole. Without killing the underlying causative pathogens however, the benefit which follows such treatments is rarely lasting.

The present analysis of the MAP problem suggests that particularly in Western Europe and North America, we are challenged by a public health issue of substantial proportions for which a range of remedial measures are needed. These measures include conditions of pasteurisation of retail milk which do ensure the destruction of all viable MAP. We need to use the improved culture systems available together with modern molecular methods to ensure our domestic livestock are free of sub-clinical infection. We need to test water supplies to make sure they are clean. In high incidence areas, we need to make Crohn's disease reportable so we have accurate data to monitor the effect of these measures on the overall problem. We need to make a rapid increase in the volume and intensity of research in the field, to sequence the

MAP genome and to develop preventative vaccines for animals and therapeutic vaccines for humans.

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Treatment revisited and factors affecting prognosis of severe acute pancreatitis

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INTRODUCTION

As stated in the author's previous articles^[1-5], severe acute pancreatitis is a multifaceted disease with rapid and sometimes fulminating onset and may result in many serious complications or even death if treatment is improper or delayed. Therapeutic measures must be directed against these multifacets simultaneously with a view to achieve the optimum results. The following regime has been practised many years since 1990 and verified repeatedly as proper and adequate.

THERAPEUTIC REGIME OF SEVERE ACUTE PANCREATITIS

Early diagnosis is based on history, symptoms and signs, elevation of serum and/or urinary amylase and computed tomography (CT) of pancreas. Once the diagnosis is verified, treatment is immediately instituted, the treatment regime is listed in Table 1.

Octreotide (Sandostatin) and the herbal medicine, Bulpleurum Peony Cheng Qi decoction have synergistic effects, their effects are depicted in Table 2.

In Balthazar's CT grading of D and E, there are always profuse exudation peripancreatically and sometimes massive into the peritoneal cavity and retroperitoneal space, plasma is always needed to improve the hypovolemia, and human albumin for hypoalbuminemia even when hypotension or shock is absent. When the hemodynamics become stabilized, intravenous hyperalimentation should be given. Octreotide and the herbal mixture are administered simultaneously at the start, the abdominal pain and abdominal distention usually subside within 1-3 days. Usually the patients run a smooth course without much fluctuation in one to one and a half months. Another important point is that food must be restricted until one month later after abatement of all symptoms and signs.

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By the above regime, 20 cases of grade D and 10 cases of grade E recovered unequivocally with no mortality, almost no morbidity and no serious complications as ARDS, gastrointestinal failure and disseminated intravascular coagulopathy(DIC).

Table 1 Treatment regime of severe acute pancreatitis

CT grading	Treatment
Grade A-C	Octreotide 0.1mg q8h (H)×5-7d Bulpleurum Peony Cheng Qi decoction bid×5-7d
Grade D-E	Octreotide 0.1mg/25% glucose IV 0.5mg/5% glucose in saline 1000mL/24h thereafter 0.6mg/5% glucose in saline 1000mL/24h ×5-7d or more or Stilamin 6mg/5% glucose in saline 1000mL/24h×5-7d Bulpleurum Peony Cheng Qi decoction 150mL bid×5-7d Plasma 400mL-600mL stat, 200mL on 2nd and 3rd day each Human serum albumin 10-20g/d Low molecular weight dextran (-40)+Dan Shen liquid 16-20mL/ glucose water 250mL×1-2d, thereafter Dan Shen liquid in glucose in saline ×3-5d Ciprofloxacin 0.2g bid+Metronidazole 100mL bid×4 weeks or Imipenem 0.5g q8h×4 weeks Compound aminoacids 500-750mL qd, Intralipid 10% 500mL qd Fasting for food but not the herbal medicine Omeprazole 40mg IV qd×7d, and then 20mg per oral qd×2-3 weeks No atropine or gastric decompression Calcium, potassium salts supplement with occasional magnesium salt

Table 2 Effects of Octreotide and the herbal medicinals

Drug	Effects
Octreotide (Sandostatin) ^[5]	Inhibits release of CCK and pancreatic enzymes Stimulates and activates macrophages, lowering endotoxin level ^[6] Blocks release of inflammatory cytokines ^[7] Redistributes intrapancreatic blood flow Attenuates the interaction of neutrophils and endothelial cells ^[8] Inhibits eicosanoid and products as PGI ₂ , leucotrienes Relaxes Oddi's sphincter
Bulpleurum Peony Cheng Qi decoction ^[5]	Inhibits secretion and activities of pancreatic enzymes Stabilizes lysosomal membrane Inhibits inflammatory cytokines, IL-1, IL-6, IL-8, and TNF- α ; Inhibits vascular permeability ^[9] Increase peristalsis with purgating effect, expelling bacterial flora and endotoxin Protects gut mucosal barrier, preventing bacteria dislocation ^[10,11] Relaxes Oddi's sphincter Reduces urea synthesis, promotes urinary excretion of urea and creatinine Has broad antibiotic spectrum, including <i>B. Fragilis</i> Stimulates secretion of endogenous glucocorticoids which can inhibit cytokines and inflammatory mediators Decreases blood lipids, inhibits lipid peroxidation Inhibits TXA ₂ synthase, promotes PGE ₁ , PGE ₂ levels, inhibits transformation of fibrinogen to fibrin and prevents endotoxin induced DIC
Dan Shen (<i>Salvia Miltiorrhiza</i>)	Inhibits platelet aggregation, decreases blood viscosity, improves blood rheology and microcirculation Inhibits lysosomal enzymes and chemotactic neutrophils Blockage of calcium ion influx Has antioxidation effect, ameliorates inflammation and tissue damage

FACTORS AFFECTING THE PROGNOSIS OF SEVERE ACUTE PANCREATITIS

Extent of necrosis and time of treatment after onset of the disease

The extent of necrosis can be categorized into $\leq 30\%$, 30% - 50% and $>50\%$ of the surface area

of pancreas shown in CT, which is relevant to the time of treatment given. The necrotic process is dynamic in nature, if diagnosed within 24h - 48h after disease onset, the extent of necrosis is usually $\leq 30\%$, if diagnosed after 72h, it may progress to 30%-50% or even $> 50\%$ even if the treatment is proper and adequate, the pathophysiological changes can be more complicated, the rate of infection of intestinal origin is also higher with usher in of systemic inflammatory response syndrome (SIRS), then treatment can be more difficult with worsen prognosis. In America, the mortality rate of this particular disease was around 17% - 20%, because the CT report usually took three days and this delay would enable progression of necrosis to the extent of 30%-50% or even $> 50\%$ with higher mortality rate.

Proper, improper and/or inadequate treatment at the beginning

The key element in determining the prognosis of the disease is the treatment given at the start, with idiopathic severe acute pancreatitis, treatment is mainly medical, if with presence of common duct stone obstruction, emergency endoscopic or surgical removal of stone is necessary.

The comprehensive medical treatment is listed in Table 1, continuous infusion of Octreotide gives better results than 0.1mg q8h given hypodermically, in the latter, sepsis occurred in 26%, and ARDS in 37%^[12]. Adequate plasma, albumin, low molecular weight dextran and Dan Shen restore the blood volume and improve the microcirculation of pancreas and other vital organs. Concomitantly, Blupleurum-Peony-Cheng Qi decoction is given which has seven constituents, including: Bulpleurum, White peony, Scutellaria, *Unripe bitter orange*, *Magnolia bark*, *Refined mirabilite* and *Rhubarb*, each 10g. It is prepared by adding 100-150 water to the first five constituents, and heated for 20min, at the 18th min, add *Rhubarb* (wrapped by cloth), heat only for two min, then decant the liquid and dissolve the refined *Mirabilite* (Na_2SO_4) crystals in it and drink. The mixture is taken twice a day by repetition of the same procedure. This herbal medicine should be taken orally for 5-7 days. No atropine, nor gastric decompression is allowed.

Combination of these measures can ameliorate the pancreatic autodigestion caused by release of pancreatic and lysosomal enzymes and ischemia-reperfusional damage significantly, it can also halt the exudation, and avoid the complications of the kidney, lung and GI tract. This herbal mixture can inhibit the excessive stimulation of macrophages; reduce the release of inflammatory cytokines, decrease the neutrophilic infiltration and its interaction with vascular endothelial cells, maintaining the gut barrier function, preventing the second attack which leads to SIRS.

Among the destructive enzymes, phospholipase A_2 (PLA_2) and lipase are most crucial, PLA_2 can destroy the phospholipid structure of cell membranes of GI tract, lung and brain. The lipase can degradate the fat which produce toxic free fatty

acids injuring the capillaries and increase lipoperoxidation^[13]. The cytokines damaging vital organs include IL-1, IL-6, IL-8, TNF- α , INF- γ and PAF. Among these, PAF and TNF- α increase vascular permeability which not only play important pathophysiological role in the pancreas itself but also cause complications in the lungs, brain and GI tract. By given Octreotide (Sandostatin) with concomitant herbal mixture, the damage caused by the above factors can be much reduced. Sandostatin and the herbal mixture have synergistic effects on inhibition of activated pancreatic enzymic activities and their secretion, also have complementary effect on interaction of the neutrophils and endothelial cells, and inhibition of neutrophilic infiltration in the pancreas and in the lung, thus is crucial in the treatment of severe acute pancreatitis.

When given Sandostatin or oral herb mixture singly and without given plasma, human serum albumin, but infuse large volume of balance solution or glucose in saline, these not only cannot act on simultaneously, but also may lead to cardiac and pulmonary failure, this may be one of the causes of high mortality rate and high complication rate as reported in the medical literature both domestically and abroad.

Whether preventive treatment has been instituted?

During admission, Omeprazole (Losec) should be given intravenously to prevent acute gastric mucosal bleeding; ciprofloxacin and metronidazole or imipenem to prevent infection, besides, the *Rhuberb* and *Scutallaria* have also antibiotic activities. *Rhuberb* also abolishes paralytic ileus, expel the bacteria flora together with endotoxin via the anus. The antibiotics shown above have penetrating abilities into the necrotic tissue or necrotic fluid, if other antibiotics are used instead or by rotation would not only be ineffective, but also lead to superinfection in these immunocompromized patients. Once the hemodynamic changes return to normal after correction of hypovolemia, total parenteral nutrition should be instituted to maintain positive nitrogen balance, in case with SIRS, more calories should be given regularly up to 2500-3000 Kal/d or more. Regular insulin should also be given to maintain normal blood sugar level. Supplements of calcium, potassium and magnesium salts are also required for hypocalcemia and hypopotassemia. Local fluid collection is best to be left alone without aspiration, this will be absorbed during convalescence.

During the early period, one should pay attention to avoid cardiac or renal failure, also the ARDS, in the middle period, one should notice and prevent the occurrence of pancreatic encephalopathy; late in the course, one should be aware of infection and residual infection. Food fasting but allow oral herb mixture only would take one month, early feeding can give rise to recrudescence. If one is not aware of the complicated and multifaceted nature of the disease

and has not paid great attention to the preventive measures, SIRS, multiple organ dysfunction syndrome (MODS) and eventually multiple organ failure (MOF) would be unavoidable. For SIRS, short-term hemofiltration had been adopted by the surgical pancreatology group of Shanghai Ruijin Hospital to downregulate the level of inflammatory cytokines and upregulate the antiinflammatory cytokines (IL-10) level which could shorten the course and hasten recovery^[14].

Can the precipitating factors be removed or not?

The precipitating factors as microlithiasis or cholesterol crystals from the gallbladder, the high fat, high protein diet and alcohol drinking were removed after treatment and fasting, the patient usually recovered clinically in 1-1.5 months, and the pancreas showed normal texture by CT by another 1-1.5 months. But when hyperlipidemia is the inducing factor, the lipoperoxidation and pancreatic ischemia are especially severe, the reason is stated above, the course is usually fulminating, because short-acting hypolipidemic agent for intravenous use is currently unavailable, especially in those with coexistent fatty liver, the oxidation of fatty acid by the mitochondria of the liver cells cannot be augmented, the severe ischemia and lipoperoxidation would result in extensive pancreatic necrosis, and because of the persistence of free fatty acid as the stimulus, numerous complications may set in, making treatment even more difficult, fortunately this precipitating factor is rare here.

Functional status of vital organs

Aged patients usually have degeneration and deteriorating function of many vital organs, diseases as ischemic heart, hypertension, diabetes, chronic bronchitis with emphysema, chronic liver disease, impaired kidney function or even lacunar infarction of brain are common, any one or two coexisted can add further embarrassment to the management, fortunately the severe acute pancreatitis among the aged is milder than that in the middle-aged and younger patients, they are mostly of grade C and B, whereas grade D is only occasional^[15]. One must be very careful throughout the course of treatment, detailed history, physical examination, blood chemistry, blood gas analysis, electrocardiogram should be performed on admission, fluid given must be concordant with the tolerability of patient's heart and circulation, the rate of fluid infusion should be slow to avoid the unnecessary occurrence of heart failure. In case when heart failure occurs, cedilanid and dobutamine should be given to increase myocardial contractility and phentolamine to diminish the pre- and afterload. At the same time, one should also pay attention to the renal function because cardiac and renal functions are interrelated, with both cardiac and renal incompetence, the condition is usually detrimental and may be fatal.

Whether necrosectomy is timely and thorough?

With stone obstructing the pancreatico-biliary common pathway, ERCP or endoscopic sphincterotomy should be tried first, or emergency surgery to relieve obstruction with common duct T-tube drainage, but leave the pancreatic necrosis aside, operation should be as simple as possible, afterwards, comprehensive medical therapy should be instituted immediately. With nonstone obstruction where necrosis is extensive and severe, patient should be placed in ICU, given energetic medical treatment and watched carefully by dynamic CT and other measures, in case of progression of the disease, necrosectomy should be performed with thorough removal of necrotic foci. Acute peritonitis occasionally requires laparotomy for drainage, followed by short-term peritoneal lavage when there is profuse exudation and fluid collection in the peritoneal cavity and retroperitoneal space. The peritoneal exudative fluid contains PLA₂, protease, bradykinin, PAF, prostaglandins and complement component which should be evacuated. Necrosis with infection if can be controlled by antibiotics, operation may be spared, but if it is uncontrollable, surgery should be performed to remove the infected necrotic foci within the pancreas, the peritoneal cavity and retroperitoneal space thoroughly. These are the surgical viewpoints of the expertised surgeon Zhang ZT of Ruijin Hospital^[16].

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Transplantation of primary and reversibly immortalized human liver cells and other gene therapies in acute liver failure and decompensated chronic liver disease

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Subject headings liver failure, acute; liver diseases; liver transplantation; gene therapy; animals, laboratory; transferring growth factor beta

Riordan SM, Williams R. Transplantation of primary and reversibly immortalized human liver cells and other gene therapies in acute liver failure and decompensated chronic liver disease. *World J Gastroentero*, 2000;6(5):636-642

Studies performed in experimental small animals with hepatic-based metabolic disorders but no structural liver disease, including Gunn and analbuminaemic rats and rabbits with inherited low-density lipoprotein receptor deficiency, have shown that up to 95% of hepatocytes transplanted into the spleen or liver remain in these sites, with improvement in metabolic function of recipients^[1-4]. The feasibility of hepatocyte transplantation as a clinically-relevant therapeutic tool has subsequently been demonstrated in a small number of patients with disorders such as Crigler-Najjar syndrome type 1^[5], ornithine transcarbamoylase deficiency^[6] and familial hypercholesterolaemia^[7], in whom the delivery of numbers of primary hepatocytes approximately equivalent to only 5% or less of the normal liver cell mass led to satisfactory, if incomplete, correction of the metabolic defect. Hepatocyte transplantation has several real and potential advantages over conventional orthotopic liver transplantation (OLT). In addition to the relative simplicity, less invasive nature and lower associated cost of the former intervention, the ability to cryopreserve primary liver cells without substantial loss of viability or physiological function on subsequent thawing, provided that they are attached to microcarriers or gel-entrapped as spheroids^[8-10], offers the potential that, through liver cells stored in centralised banks, it will be possible one day to have them widely available for clinical use within a

few hours of a decision to institute such therapy.

A particularly important issue is whether liver cell transplantation has a role in the management of patients with severe liver damage resulting in acute liver failure (ALF), where only short-term support may be required given the potential for regeneration of the native liver^[11], or those with decompensated chronic liver disease, in whom on-going support over a longer period may be necessary. The availability of effective liver support "on demand" would constitute an important advance in the management of such patients, given the rapidity with which irreversible clinical deterioration often occurs in these settings. In this review we consider clinical experiences with hepatocyte transplantation in each of these liver failure syndromes reported to date, as well as discussing issues pertaining to the ideal cell type for clinical use. The potential applications of adjuvant and other gene therapies, as recently described experimentally, which may overcome two of the major limitations of liver cell transplantation at present, namely the relative unavailability of primary human hepatocytes and the requirement for pharmacological immunosuppression in the post-transplantation period, are also considered.

Liver cell transplantation in experimental animals with ALF due to chemically-induced hepatic necrosis or surgical models of hepatic ischaemia or resection has, to date, generally involved the use of primary hepatocytes. Such treatment has been associated with improved survival, even when small numbers of cells, in the order of only 0.5% to 3% of the normal hepatocyte mass, are used^[12-19]. These small numbers of cells may be sufficient to enhance hepatic regeneration, on which recovery ultimately depends, possibly due at least in part to reduction in levels of transforming growth factor (TGF) β 1, a potent inhibitor of this process^[20]. However, cell transplantation has been performed prior to the induction of ALF in some surgical models, a scenario clearly incongruous to the clinical setting. Furthermore, since mediators such as interleukin-6 and tumor necrosis factor- α produced by activated Kupffer cells in ALF contribute to both progressive liver damage and the development of multi-organ failure^[21-23], while the liver is also the source of TGF- β 1 and other

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inhibitors of liver regeneration^[24,25], the relevance of animal models in which the liver is removed to the clinical situation, in which it remains *in situ*, is uncertain.

Nevertheless, several preliminary studies have already been carried out on the clinical efficacy of hepatocyte transplantation in adult and paediatric patients with ALF. No randomised, controlled data is yet available and the impact of such treatment on clinical course is uncertain. Strom *et al*^[26] reported their experience at the Medical College of Virginia with transplantation of small numbers (0.1% or less of the normal hepatocyte mass) of ABO-matched, freshly isolated or cryopreserved primary human hepatocytes via injection into the splenic artery in two adults with ALF due to hepatitis B virus infection and phenytoin hepatotoxicity, respectively. Both patients were in grade IV encephalopathy prior to the treatment. Immunosuppression was with methylprednisolone and cyclosporin. Reduced blood ammonia levels along with improvement in encephalopathy grade and reversal of haemodynamic instability were noted. Other evidence of metabolic function was unimpressive, with the serum bilirubin level increasing in both patients and the prothrombin time rising in one and falling only marginally in the other. Both patients underwent OLT after three and 10 days, respectively, with subsequent full recovery. None of three control patients, for whom either fresh or frozen hepatocytes were not available or consent for hepatocyte transplantation could not be obtained, survived. Of an additional five adult patients with ALF who underwent hepatocyte transplantation in this way at the same centre, three (60%) in grade III to IV encephalopathy (due to acetaminophen toxicity: $n = 1$; hepatitis B virus infection: $n = 1$ and idiopathic: $n = 1$) were successfully "bridged" to OLT between one and five days later. Another patient in grade IV coma (due to herpes simplex virus infection and/or sodium valproate hepatotoxicity) died of sepsis-related cardiovascular instability on day 5 before OLT could be performed, while the remaining patient with acute hepatitis B virus infection recovered without OLT but had only low-grade in grade I encephalopathy prior to the procedure^[27]. Bilir *et al*^[28] from the University of Colorado subsequently transplanted by percutaneous injection larger numbers of cryopreserved primary human hepatocytes (in the order of 5% of the normal hepatocyte mass) in five adult patients with ALF and grade IV encephalopathy who were not candidates for OLT. Three (60%) patients survived more than 72 hours, with evidence of improved encephalopathy score, serum ammonia levels and prothrombin times. A delay in the order of 24 to 72 hours was apparent between hepatocyte transplantation and the first biochemical or clinical sign of improvement, possibly reflecting the time

required for effective engraftment, as demonstrated in experimental animals^[29]. However, no patient survived for more than seven weeks. The same group reported technical success in achieving engraftment via the transjugular route in an additional comatose patient with ALF, although no clinical benefit could be demonstrated^[30].

Transplantation of comparably small numbers of hepatocytes has been performed in six children with ALF, aged six months to 15 years, with aetiologies including acetaminophen hepatotoxicity ($n = 1$), other drug reactions ($n = 2$), idiopathic ($n = 2$) and sepsis/total parenteral nutrition ($n = 1$)^[26,27,31]. Five (83%) of these children were in grade IV encephalopathy at the time of treatment. Of these, four (80%) died within seven days, despite instances of reduction in serum ammonia levels and requirement for coagulation factor support, while the other patient recovered without OLT, having received multiple infusions of hepatocytes over a three day period. Another child, in whom hepatocyte transplantation was performed when in grade I encephalopathy, underwent OLT two days later.

In experimental animal models, successful hepatic engraftment of transplanted hepatocytes is associated with evidence of transient micro-circulatory damage to the host liver^[32]. This development of reversible portal hypertension precludes the transplantation of larger quantities of hepatocytes, at least in one session^[33]. However, transplantation of a relatively small number of cells can lead to substantial replacement of the recipient's liver mass in the experimental setting in the presence of various regenerative stimuli. The latter include the concurrent intravenous injection of hepatocyte growth factor (HGF)^[34], induction of ischemic atrophy of the contralateral liver lobe^[3,4] and in situations in which the recipient's own liver cells have a shortened life-span, as occurs with acute chemical injury^[35], transduction with a recombinant adenovirus vector expressing a non-secreted urokinase^[36] or anti-Fas antibody-induced apoptosis^[37].

The growth advantage of transplanted cells over native cells demonstrated in these circumstances may be maximised in the ALF setting by the use of purified hepatic stem cells able to replicate at least 100 times without loss of function or malignant transformation, as identified in adult rodent liver^[38,39]. Repopulation experiments using purified fractions of total liver cell suspensions will be required to identify any such cells in the adult human liver^[40]. Of note, a bone marrow-derived stem cell capable of repopulating the liver with mature hepatocytes following hepatic injury has recently been described in rodents. In the order of 1.0×10^6 hepatocytes (approximately 0.1% of the total hepatocyte mass) originated from transplanted

bone marrow cells by day 13 after liver injury^[41]. This finding raises the exciting possibility that bone marrow infusion may have therapeutic potential in liver failure, although, even if extrapolated from the animal to the human situation, the time required for engraftment and cell differentiation would be problematic in the acute setting.

On the premise that the fetal liver contains epithelial cells that are in different stages of lineage progression, some of which may exhibit the full regenerative potential of stem cells, transplantation of fetal hepatocytes has been suggested as the way forward, rather than use of adult cells. Clinical data so far are limited. Habibullah *et al*^[42] in India transplanted 6×10^7 blood group-matched, fetal hepatocytes per kilogram body weight by intraperitoneal injection in seven patients with ALF of unspecified aetiology and grade III to IV encephalopathy. Three (43%) transplanted patients survived. Those who survived each had a prothrombin index of 1.5 or less and were in grade III or IV a encephalopathy at the time of hepatocyte transplantation, while those who died had a more severe illness marked by prothrombin indices ranging from 2.6 to 3.0 and grade IVb encephalopathy at this time. Blood ammonia concentrations fell in 5/6 (83%) transplanted patients in whom serial levels were obtained. Survival in a control group, selected on the basis of inability to procure consent for the hepatocyte transplantation procedure, was 33%. Whether fetus-derived hepatocytes will prove superior to their adult counterparts in terms of regenerative capacity and functional characteristics, and whether any such superiority will translate into increased clinical efficacy in the ALF situation or with chronic liver damage, remain to be determined.

Irrespective of the fetal or adult nature of transplanted hepatocytes or even the use of bone marrow infusion as a source of stem cells capable of repopulating the liver, the potential role in the ALF setting of co-transplantation of non-parenchymal cells requires consideration. The co-transplantation of such cells may be advantageous to the viability and function of transplanted hepatocytes in terms of secretion of extracellular matrix, analogous to the beneficial effects in these regards documented in *ex-vivo* culture systems^[43-45]. However, the inclusion of non-parenchymal cells in suspensions for transplantation might also have deleterious effects if these cells become activated to produce cytokines such as TGF- β 1 and interleukin-1 which promote apoptosis of hepatocytes and inhibition of liver regeneration^[24,25].

Controlled studies in which liver cell transplantation in its various forms is compared to standard intensive care will be required in order to determine the efficacy or otherwise of this intervention in the ALF setting. These studies should be performed, at least in the first instance,

in the most severely affected groups fulfilling criteria for OLT but for whom this is unavailable or contraindicated by co-morbidity. Such studies will need to be conducted on a multicentre basis and using standardised outcome measures if sufficient numbers of patients are to be recruited and meaningful results obtained. If benefit is demonstrated in such groups, efficacy should then be addressed in patients with apparently lesser degrees of liver damage not fulfilling OLT criteria, as recent analyses showing poor negative predictive values of current selection criteria for OLT indicate that a substantial number of such patients nevertheless deteriorate with intensive medical care alone^[46-48].

Clinical experience to date with liver cell transplantation for decompensated chronic liver disease is similarly limited, uncontrolled and confined to the use of primary adult hepatocytes. The feasibility and apparent safety of hepatocyte transplantation in chronic liver disease was first reported by Mito and Kusano in Japan^[49], who injected small numbers of freshly isolated human cells into the spleens of 10 patients with cirrhosis or chronic viral hepatitis at laparotomy and under the influence of epidermal growth factor. Viable hepatocytes were detected in the spleen by scintigraphy in almost all patients up to 11 months later. Four patients with cirrhosis subsequently underwent hepatocyte transplantation via injection into the splenic artery at the Medical College of Virginia^[27]. One of two patients in grade IV encephalopathy survived until OLT was performed on day 2, while a third patient in grade II-III encephalopathy recovered to be discharged from hospital before relapsing and dying on day 33. Another patient in whom liver failure with grade IV encephalopathy was precipitated by trisegmentectomy died of cardiovascular instability within 2 days of the procedure. More recently, Bilir *et al*^[50] in Colorado treated five patients with Child's C cirrhosis who were not candidates for OLT with transplantation of 1×10^9 to 1×10^{10} cryopreserved primary human hepatocytes (in the order of 5% normal liver cell mass), with viability of 52% to 73% after thawing, via infusion into the splenic artery. Immunosuppression was with cyclosporin. Indications for hepatocyte transplantation were refractory encephalopathy with ascites ($n=4$) and hepatorenal syndrome ($n=1$). Improvements in encephalopathy grade and ascites and renal function, respectively were noted within days of the procedure. Improvements in serum albumin levels and prothrombin times also occurred, but only after a period of four to six months. Aside from one patient who stopped immunosuppression after five months, all patients were alive and well two to 15 months post-transplantation. There were no complications related to the procedure. In particular, no overt evidence of increased portal

venous pressure, as reported following hepatocyte transplantation in experimental animals with cirrhosis^[51], was recorded, although it is to be noted that transjugular intrahepatic portosystemic shunts had been placed prior to transplantation in the majority of patients. No instances of pulmonary vascular complications were clinically apparent, despite concerns of increased pulmonary sequestration of transplanted cells in the context of portal-systemic shunting. Results of a randomised trial of hepatocyte transplantation in cirrhosis, which is currently in progress, are awaited.

Even if the efficacy of primary human hepatocyte transplantation was to become firmly established in the ALF and decompensated chronic liver disease settings, the limited availability of these cells is likely to represent a severe, ongoing impediment to its widespread clinical application. Primary human hepatocytes are obtained from resected surgical specimens, unused segments or end-lobe wedges of donor organs for OLT and livers from apparently healthy donors which are ultimately rejected for OLT on account of overt steatosis, since these are often also fibrotic. Although approximately 10^9 hepatocytes are readily obtained from a segment of normal liver, the yield is substantially reduced when fibrosis is present^[52]. The high demand for whole organ OLT and the expertise to transplant portions of a single organ into more than one recipient mean that completely normal liver is increasingly in short supply for other uses. A satisfactory number of viable primary human hepatocytes cannot at present be obtained from needle biopsy-sized fragments of liver.

The *ex-vivo* expansion of hepatocytes recovered from surgical specimens represents a potential means of overcoming the problem of limited hepatocyte availability for transplantation. A number of proliferating human cell lines which have become immortalised by virtue of cultural conditions, without the use of oncogenes or carcinogens, have now been maintained in continuous culture for up to several years, although considerable limitations in their spectra of metabolic activity may inhibit their clinical utility were they to be used in the liver failure setting^[53-56]. An alternative approach to achieving population expansion of hepatocytes *ex-vivo*, without necessarily compromising their differentiated functional capability and the prospect of obtaining meaningful clinical support following their subsequent transplantation, is to transfect the cells with a replication-deficient retrovirus carrying a temperature-sensitive variant of the simian virus 40 large tumor (SV40 large-T) antigen gene. This gene binds to the cell cycle control protein, p53, and produces cell lines which proliferate at 33°C but cease proliferating and develop enhanced differentiated function, including upregulated synthesis of α -1-antitrypsin and inducible activity of some cytochrome P450 isoforms, at 39°C^[57,58].

Hepatocytes from Gunn rats immortalised by transfection with the SV40 large-T antigen have been used successfully for *ex-vivo* gene therapy, in which the cells were also transduced with the gene for bilirubin-uridine diphosphoglucuronate (UDP)-glucuronosyltransferase, expanded *in vitro* by culturing at 33°C and finally transplanted into syngeneic animals. Long-term reduction of serum bilirubin in association with the appearance of bilirubin glucuronides ensued^[59]. In addition, transplantation of SV40 large T-antigen-immortalised hepatocytes in porto-caval shunted rats and those with surgically-induced ALF has been shown to improve resultant hepatic encephalopathy and survival, respectively^[60,61]. Several non-clonal human hepatocyte cell lines successfully transfected with an amphotropic mouse retrovirus containing this gene have remained stable in long-term continuous culture^[58]. Nonetheless, the potential clinical applicability of such cell lines has been questioned following reports in experimental animals that hepatocellular carcinoma may develop in hepatocytes expressing the SV40 large-T antigen^[62].

Kobayashi *et al*^[63] have recently investigated the feasibility of overcoming the otherwise unacceptable risk of tumorigenicity of SV40 large-T-immortalised cells, using a technique of reversible immortalisation in which cells are initially transduced to proliferate *in vitro* prior to the excision of the T-antigen-encoding DNA sequence before transplantation. Specifically, a highly differentiated cell line, NKNT-3, was generated by retroviral transfer to primary adult human hepatocytes of the SV40 large-T gene flanked by LoxP recombination targets, with concurrent expression of a fusion protein conferring sensitivity to gancyclovir. NKNT-3 cells became immortalised without an apparent growth crisis and double in numbers every 48 hours. The immortalising and gancyclovir sensitivity-conferring genes could subsequently be completely excised *in vitro* by transient transduction with a replication-deficient recombinant adenovirus expressing the Cre recombinase, which resulted in Cre/Lox site-specific recombination. After removal of the SV40 large-T gene in this way, NKNT-3 cells stopped proliferating and appeared more differentiated, with nucleus to cytoplasm ratios and cytogranules resembling those of normal primary hepatocytes. While mRNA's for bilirubin-UDP-glucuronosyltransferase and glutamine synthetase were each detectable in NKNT-3 cells before reversal of immortalisation, levels increased significantly after excision of the SV40 large-T gene by recombination, with mRNA's for albumin and coagulation factor X becoming detectable only in this latter circumstance.

The efficacy of transplantation of NKNT-3 cells, before or after recombination, was then

assessed in a rodent model of ALF in which 5×10^7 cells, equivalent to approximately 5% of the total number of hepatocytes in an adult rat, were delivered by intrasplenic injection one day prior to 90% hepatectomy under immunosuppression with tacrolimus^[63]. Transplanted animals showed substantial improvements in total bilirubin, prothrombin time and blood ammonia levels and survival compared to controls, with a trend towards a particular survival advantage in those animals which received the reverted NKNT-3 cells rather than immortalised counterparts. Taken together, these findings demonstrate the feasibility of controlling the *ex-vivo* expansion of primary human hepatocytes by transfection with the SV40 large-T antigen gene and Cre/Lox-based reversible immortalisation, with the removal of the oncogene prior to transplantation promoting a level of differentiated function adequate to sustain short-term survival in an experimental ALF setting. Future studies should assess the *in vivo* efficacy of reverted NKNT-3 cells when attached to microcarriers or gel-entrapped as hepatocyte spheroids, modifications which would render them amenable to cryopreservation and storage in cell banks for clinical use as required.

Adjuvant genetic manipulation of transplanted human liver cells may also be used to overcome the current necessity for pharmacological immunosuppression to prevent allograft rejection^[64]. This is an important issue since, although reduction or withdrawal of pharmacological immunosuppression in the post-OLT setting has been associated with lower incidences of metabolic, infective and neoplastic complications, side effects once developed such as chronic nephrotoxicity and hypertension may be irreversible^[65-69]. One approach to preventing rejection of transplanted cells without the need for immunosuppressive drugs relates to the use of cells previously transfected with the adenoviral E3 gene, which encodes several proteins that are known to inhibit host T cell reactivity^[70,71]. Preliminary data in rodents suggests that these proteins have the capacity to protect transplanted hepatocytes from rejection. In particular, Brown-Norway hepatocytes expressing E3 proteins persisted for up to 6 weeks following transplantation into allogeneic Gunn rats, as reflected by reduced serum bilirubin levels, while no reduction in this parameter, indicative of rapid allograft rejection, occurred in control Gunn rats receiving a comparable number of untreated Brown-Norway cells^[72]. It remains to be determined whether strategies designed to induce tolerance that are currently under investigation or consideration in the post-OLT situation, such as the adjunctive infusion of donor-derived bone marrow or soluble class I antigens, the use of anti-CD4, anti-CD25 and anti-CD54 monoclonal antibodies, blockade of the CD28-B7 T cell co-stimulatory pathway with

CTLA41g, pre-treatment of the recipient with IL-10 or even the withholding of all immunosuppression for the first 24 to 48 hours^[73-80], are of relevance when hepatocytes only, rather than with additional non-parenchymal cells and donor-derived leukocytes (as with OLT), are transplanted.

Another potential clinical application of gene therapy in the liver failure setting does not involve the transplantation of liver or other cells, but rather the repeated *in vivo* transfection of skeletal muscle with the gene for human HGF. Ueki *et al*^[81] recently reported that such an approach in a rodent model of cirrhosis, induced by repeated administration of dimethylnitrosamine, led to significantly increased plasma levels of human as well as endogenous rat HGF and increased tyrosine phosphorylation of the HGF receptor. Inhibition of fibrogenesis and hepatocellular apoptosis were noted, possibly consequent to reduction in levels of TGF- β 1. Gene transfer into the rats' skeletal muscle was achieved using weekly injections of liposomes containing the haemagglutinating virus of Japan and human HGF cDNA inserted into the EcoRI and NotI sites of the pUC-SRa expression vector. Survival of the animals was significantly improved compared to that in phosphate buffered saline-treated controls, with the cirrhotic lesion completely reversed in all transfected animals within 50 days of the toxic insult. Such HGF gene therapy would seem less likely to have substantial impact in ALF, since plasma HGF levels are already substantially elevated in this circumstance^[82, 83]. The prospect of maximising liver regeneration via gene therapy in this latter situation will likely depend, in the first instance, on a more comprehensive understanding of both the expression of cell surface receptors for stimulatory and inhibitory growth factors and the integrity of downstream effector and adaptor mechanisms, allowing targeted over-expression or inhibition, respectively, of key elements in these signalling pathways.

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Metallic biliary stents for malignant obstructive jaundice: a review

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Affecting 8-10 patients per 100 000 population, pancreatic cancer is the primary cause of malignant obstructive jaundice and is the presenting feature in over three quarters of these patients^[1]. Unfortunately, using modern imaging techniques, such as endoscopic ultrasound or pancreatic protocol computed tomography with vascular reconstruction, 80% - 90% prove unresectable for cure^[2,3]. Historically, this jaundice was treated surgically with biliary bypass. Over the past 10 years, however, multiple studies have shown comparable palliation (3-6 month survival) with percutaneous or endoscopic placement of a polyethylene prostheses^[4-6]. Moreover, our group, as well as others, have shown that despite comparable survivals, resource utilization (costs) to time of death for the endoscopic group are approximately one-half of those expended in surgically treated patients^[7].

Despite this palliative advance in an often aged and infirm group of patients, however, stent occlusion has proven problematic. A consequence of bacterial biofilm development, attempts to prolong patency with chronic antibiotic therapy, ursodeoxycholic acid, change in the type of polymer used or coating the inner lining with a variety of agents to preclude bacterial colonization have all proven unsuccessful^[8]. It was with this background that the first expandable metallic stent was introduced. This review will summarize the current state of our knowledge, new developments in self-expandable metal stent (SEMS) technology, and areas in which additional studies are needed.

WHAT DO WE KNOW ABOUT EXPANDABLE STENT TECHNOLOGY IN THE BILIARY TREE?

For one, we know that the vast majority of literature has utilized the open mesh stainless-steel Wallstents^[9-16] (Microvasive Inc. Natick, MA).

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However, a number of additional SEMS (Table 1) have recently been introduced and variably studied. The latter included closed-weave prostheses fashioned from nitinol (Diamond stent, Microvasive Inc., Natick, MA; and Za stent, Wilson-Cook Inc., Winston-Salem, MA) or stainless steel (Spiral Z, Wilson-Cook Inc.)^[17-23]. They also included the Biliary Endocoil (Intratherapeutics, Eden, Prairie, MN), a tightly coiled nitinol spiral which ostensibly precludes tumor ingrowth^[24]. Not only these stents have different physical properties by virtue of wire material, gauge, and configuration, but also their delivery systems and their degree of foreshortening at time of delivery differ. For instance, neither Spiral Z nor Za stents foreshorten. Diamond and Wallstents shorten by a third and Endocoils by a half.

We also know that, to date, there has been no study which has randomized metallic stent placement against surgical bypass in the palliation of malignant obstructive jaundice. There are, however, numerous randomized and controlled studies randomizing Wallstents against plastic prostheses, all showing superior patency of the former^[4,10-13]. In a largest study, 182 patients with inoperable distal bile duct obstruction were randomized to plastic stent versus SEMS. At 30 days, one-quarter of the plastic prostheses were occluded compared with 5% of the Wallstents^[11]. Although survival was unchanged, there was a two-fold prolongation in patency rate compared to plastic stents. Comparable, albeit longer stent survival (Wallstent 273 days, plastic 126 days) was reported by Davids *et al* in a well-designed European trial^[10]. Additional, non-randomized studies have looked at the use of single or dual Wallstents in Klatskin-type tumor^[15-17]. Dual stenting was not only associated with a decreased risk of cholangitis, but a decreased need for reintervention, and, in one study, approximately a two-fold survival^[16].

Table 1 Commercially available self-expandable biliary stents for malignant obstructive jaundice

Stent	Wallstent	Endocoil	Diamond	Spiral Z	Za
Design	Mesh	Spiral coil	Mesh	Mesh	Mesh
Material	Stainless steel	Nitinol	Nitinol	Stainless steel	Nitinol
Length (cm)	4.2/6.8/8	6/7.5	4/6/8	5.7/7.5	4/6/8
Diameter (mm)	8/10	6/8	10	10	10
Stent foreshortening	Yes	Yes	Yes	No	No
Introducer diameter (Fr)	7.5/8	8/10	9	8.5	8.5

CAN THE ABOVE RESULTS BE GENERALIZED TO OTHER METALLIC PROSTHESES?

Probably not. Up to now there has been no study randomizing patients to Wallstents versus other SEMS. Dumonceau *et al*, however, reported 23 patients with malignant obstructive jaundice treated with Diamond stents and retrospectively compared them with an age and illness matched group treated with Wallstents^[18]. Technical insertion, incidence of recurrent jaundice, and life table analyses of bile duct patency were comparable with both types of prostheses. In contrast, Raijman *et al* placed Diamond stents in 21 patients noting delivery system kinking in 2 and stent distortion or displacement in 3^[22]. Moreover, mean patency was only 2.1 months compared to the 9.7 months of Wallstent patency in their historical controls. Seecoomar *et al* have also noted similar results^[23]. In one of the few comparative studies reported to date, Yoo *et al* placed Diamond stents in 75 patients, plastic prostheses in 58 and Spiral Z stents in 20. The success rate of insertion was comparable whereas the patency at 4 months was 63%, 24%, and 77%, respectively^[25]. They concluded that the patency rate of both SEMS was comparable and improved over plastic prostheses.

In addition to the above, there have been a few studies looking at biliary Endocoils but no comparative studies. In the latest series abstracted, 25 patients, including 6 with benign stenoses had Endocoil insertion and were followed for a mean of 13 months^[26]. Stents were successfully deployed in 23/25 (92%) and jaundice improved in 22/24 (92%). However, 50% of stents were dysfunctional at a mean of 7 months. Data are even sparser for the Spiral Zstent^[21] although a multi-center US trial is currently underway.

WHAT ELSE DO WE KNOW?

We recognize that in contrast to bacterial biofilm occlusion of plastic prostheses^[8], SEMS dysfunction is usually a consequence of tumor ingrowth or overgrowth or elicitation of mucosal hyperplasia at the site of individual metal stents^[27]. Whether all wire materials and gauges elicit comparable hyperplasia is unknown but common sense suggests that larger weave stents may allow more tumor ingrowth than tighter weaves. Wallstents may also become dysfunctional by local duct perforation at the proximal or distal ends, particularly if acutely angulated. Likewise, their exposed distal wires may cause prosthesis dysfunction by ulceration and impaction into the contralateral duodenal wall. Finally, Endocoils fail by virtue of migration or stent infolding. The latter may allow elicitation of granulation tissue or tumor ingrowth^[28].

Despite our knowledge about the mechanism of dysfunction, the ideal therapy of recurrent jaundice

in a patient with an imbedded SEMS remains controversial. Extraction of cholesterol and bile salt debris above a partially occluded stent is temporizing only and attempts to cauterize luminal tissue are usually unsuccessful. Most endoscopists will usually place one or two plastic stents through a SEMS although an additional SEMS may sometimes prove useful. This is currently my sole use of the biliary Endocoil^[9].

IN ADDITION TO WHAT WE DO KNOW, WHAT DO WE THINK WE KNOW?

We think that despite a 30 to 40-fold increase in cost of plastic stents as opposed to Wallstents, the latter are still cost-effective in patients with malignant obstructive jaundice^[10]. These data are derived from the incremental expense associated with repeat ERCP in patients who outlive their plastic stents. In an attempt to better define which patients with malignant jaundice would benefit from a SEMS, Prat *et al* reviewed a variety of clinical, biochemical, and imaging criteria. Patients with small tumors (<3cm) and normal albumen as well as those with good performance status were most likely to survive >6 months and were felt to benefit from a Wallstent^[13]. Those who survived <3 months were more likely to have larger tumors, metastases, low proteins, and poor performance status and should be considered for plastic prostheses. Unfortunately, this leaves a large number of patients in whom treatment remains uncertain. I would personally add that SEMS should be considered in patients who repeatedly occlude plastic prostheses or those who reside in geographically distant locations or those who do not have access to ERCP in their community.

We also think that covering a SEMS does not necessarily increase the patency rate^[29]. Not only is the migration rate increased if the stent is fully covered, but bacterial biofilms and mucosal hyperplasia are problematic in stents that are only partially covered. Recent studies, most using historical controls, have questioned this^[30-36]. For instance, Shim *et al* placed polyurethane-covered Z stents in 29 patients, following them for a mean of 15 months^[30]. Successfully inserting 32/34 (94%) prostheses, there were early complications related to sludge in 19% and tumor/tissue ingrowth or overgrowth in another 21%. Median patient survival was 15.8 months and the authors concluded that covered Z stents improved long-term palliation of malignant biliary strictures. Recent abstracts have also recently been published placing covered Wallstents and Diamond stents with variable results^[31-36].

WHAT DO WE NEED TO KNOW FOR THE FUTURE?

We desperately need comparative studies between different SEMS and need true prospective studies

randomizing covered versus uncovered SEMS. We need better algorithms using clinical data to predict survival and define who is likely to benefit from a SEMS as opposed to selecting patients who would be equally well palliated with a much cheaper plastic prosthesis. We need better knowledge about the elicitation of mucosal hyperplasia and mechanisms to prevent this. Ultimately, we will need new materials, perhaps expandable mesh plastic

polymers, cotton weaves impregnated with not only hardening agents but also antibiotics or chemotherapeutic agents, or metals that use a magnetic field or thermocouple to limit local tumor growth or treat ingrowth. If the precursor 10 years are any indication of the future 10, there will be new technologies that will be introduced and marketed before their advantages or disadvantages are fully known.

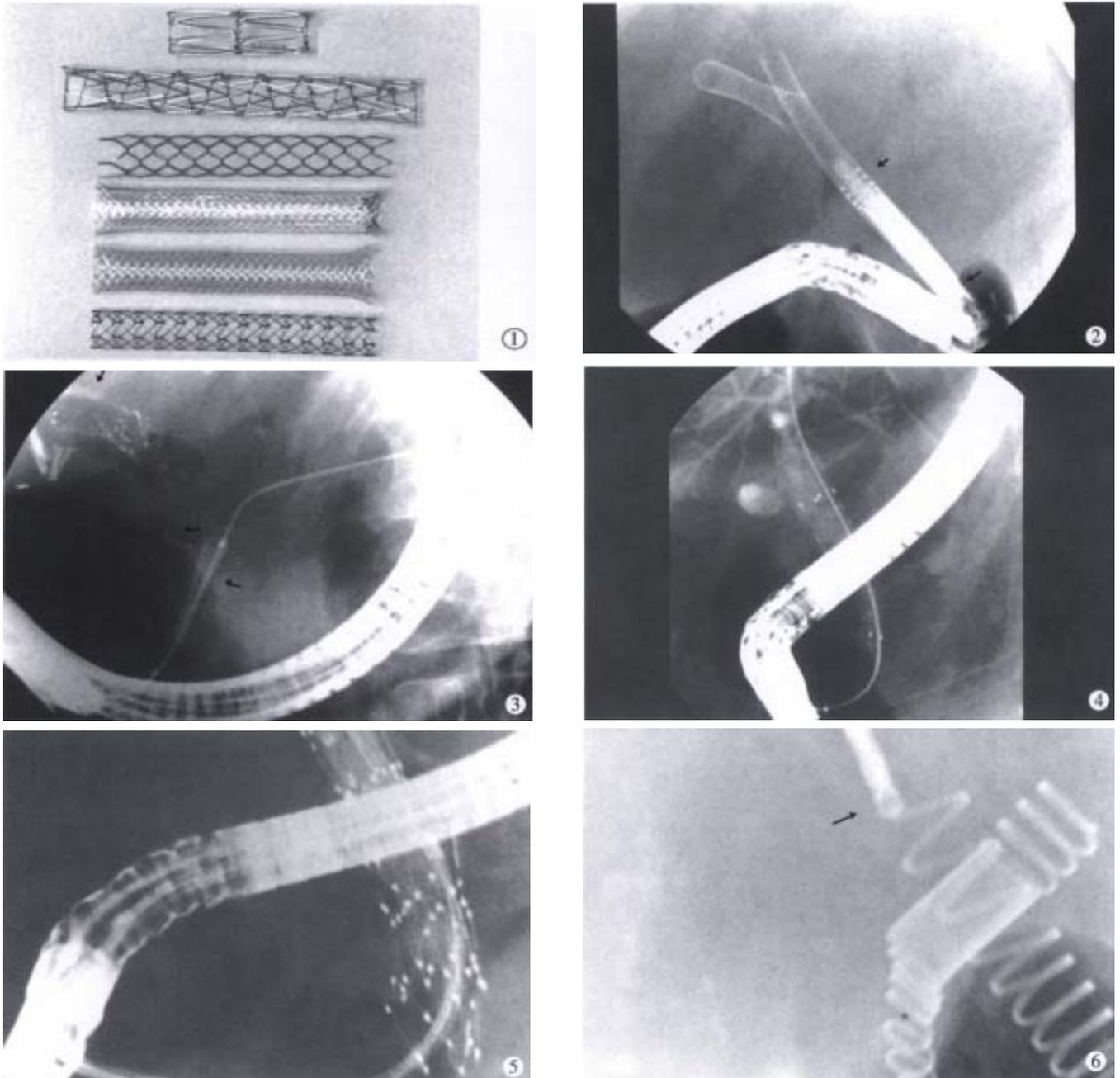


Figure 1 Currently available expandable metallic prostheses top to bottom: conventional Z, Spiral Z, Diamond, covered and uncovered Wallstent, and Za stent.

Figure 2 Dual Wallstents in patient with multiple biliary strictures from metastatic colorectal carcinoma. Arrows depict Biliary Endocoil placed for mucosal hyperplasia and recurrent jaundice.

Figure 3 Diamond stent (large arrows) placed into the biliary tree, and Wallstent (small arrow) placed into the pancreatic duct in patient with islet cell cancer and recurrent pancreaticobiliary sepsis.

Figure 4 Za stent placement in patient with distal malignant biliary stricture.

Figure 5 Spiral Z stent inserted in patient with obstructive jaundice from cholangiocarcinoma.

Figure 6 Expandable biliary endoprosthesis imbed in tissue and cannot be retrieved. The exception is the biliary endocoil which can theoretically be retrieved by grabbing the distal end with a foreign body retriever.

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Chemopreventive effect of oltipraz on AFB₁-induced hepatocarcinogenesis in tree shrew model

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Subject headings hepatocellular carcinoma; tupajidae; aflatoxin B₁; hepatitis B virus; incidence; carcinogens, environmental

Li Y, Su JJ, Qin LL, Yang C, Luo D, Ban KC, Kensler TW, Roebuck BD. Chemopreventive effect of oltipraz on AFB₁-induced hepatocarcinogenesis in tree shrew model. *World J Gastroentero*, 2000; 6(5):647-650

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major cancers in the world with a mortality of more than 250 000 cases yearly. More than 137 000 cases of HCC were diagnosed each year in China, which account approximately for more than 40 percent of the total number in the world. HCC has become the second major cause of death for cancer in China since 1990, and its annual mortality is expected to be 21.2 cases per 100 000 population in the year 2000. Even though progresses have been achieved for HCC diagnosis and treatment, its 5-year mortality is still higher than 95 percent^[1-3].

The prevalence of HCC is quite different among different areas around the world^[4,5]. It is considerably high in South-East Asia and sub-Saharan Africa, particularly in some southern and eastern regions inside China such as Fusui County in Guangxi Zhuang Autonomous Region and Qidong City in Jiangsu Province^[6-9]. The standardized incidence of HCC in these high-risk regions may exceed 100 cases per 100 000 of population^[10]. The obvious difference in geographic distribution of HCC indicates that there must be environmental factors for its pathogenesis.

Aflatoxin B₁ (AFB₁), which is produced by some strains of *Aspergillus flavus*, is a potent hepatotoxin and hepatocarcinogen^[11,12], and is considered as a major cause of HCC in some regions^[13-18]. It has also been postulated that

chronic infection with hepatitis B virus (HBV) in combination with exposure to AFB₁ in the diet may contribute to the extraordinary high risk of human HCC in some areas. Actually, two case-control studies in Shanghai have demonstrated a strong interaction between HBV and AFB₁ for risk of HCC^[14,15]. A similar chemical-viral interaction has been observed in Taiwan^[18-20]. The synergism between virus and mycotoxic carcinogen for the development of human HCC suggests that reduction in both risk factors may bring important public health consequences.

The concept of chemoprevention of cancer is over 40 years old and a number of works have been done in this field^[21,22]. Looking for effective and safe reagents against AFB₁ and/or other HCC related risk factors is one of the most important chemopreventive strategies for HCC^[23-26]. Green tea was identified years ago as one of the effective chemopreventive reagents against HCC through a series of animal experiments as well as a clinical trial^[27,28]. Recently oltipraz, another preventive agent which was previously described as a potent inhibitor of AFB₁ induced hepatocarcinogenesis in rat^[29-33], has been shown to inhibit the bioactivation of aflatoxin and enhance its detoxification in a clinical trial^[34-37] as well as in human hepatocytes in primary culture^[38]. Meanwhile, a universal vaccination program against HBV that started a decade ago now results in lower rates of HCC in children^[39]. An experimental model to test the synergistic effect of these two agents and their prevention, therefore, is needed.

RESEARCH ON TREE SHREW MODEL OF HEPATOCARCINOGENESIS

Tree shrew (*Tupaia spp.*) is a kind of small, squirrel-like mammals. Formerly it was considered to belong to the Primate order; currently it is classified into a separate order Scandentia and is supposed to be more closely related to human being than rodents^[40,41]. They have been used in biomedical researches since as early as the 1960s. Many researches have been done on its visual and nervous systems. In 1976, however, Reddy *et al*^[42] successfully induced liver cancer in tree shrew by AFB₁. Yan *et al*^[43,44] reported that tree shrews can be infected with HBV and they successfully used this HBV-infected tree shrew model for liver cancer

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research. Recently Walter *et al*^[45] reported their *in vivo* and *in vitro* study results from tree shrews infected with HBV. Yan and Li reported a significantly higher incidence of HCC in tree shrews both infected with human HBV and exposed to AFB₁ than with either agent alone^[46,47]. Thus, this tree shrew model appears to closely mirror the most common causative factors of human HCC in some prevalent regions. Furthermore, with the exception of the chimpanzee, tree shrew is the only known animal that can be infected with human HBV. Therefore, the application of tree shrew in research related to liver cancer and hepatitis is receiving increasing attentions and a number of works have been published^[48-52].

Because of the difficulties in raising tree shrews artificially, most of the tree shrews used so far for research in China are captured individually from Yunnan Province. The drawback of using tree shrews captured in the wild for animal experiments is that their age, health status and reproductive history are unknown. In an attempt to avoid this drawback, we have conducted a preliminary experiment on rearing tree shrews and a promising result was obtained^[53].

RESEARCH ON THE PREVENTIVE EFFECT OF OLTIPRAZ IN TREE SHREW

In order to study the preventive effect of oltipraz on AFB₁ by animal models other than rodents, a short-term experiment was conducted on tree shrews.

Male and female adult tree shrews (*Tupaia belangeri chinensis*) were purchased from the Kunming Institute of Zoology (Yunnan Province, P.R. China). Their body weights ranged from 100g to 160g. Upon arrival, 1mL blood was collected from each animal and tested for HBV markers (HBsAg, anti-HBsAg, anti-HBcAg) and ALT. The tree shrews that were negative for these markers of HBV infection and that had ALT value below 55 units were divided into 4 groups with 6 or 7 animals for each group. Group A: normal control; group B: AFB₁ alone; group C: AFB₁+oltipraz daily; group D: AFB₁+oltipraz weekly. All the tree shrews were allowed 1 week to acclimatize to the facilities prior to the experiment. They were housed in separate, suspended, stainless steel wire cages under controlled environmental conditions with a 12-hour light/dark photoperiod. They had free access to tap water and a natural ingredient diet.

The experimental design is presented schematically in Figure 1. Tree shrews in groups B, C and D were given AFB₁ (400μg/kg b.w./day in liquid milk) daily beginning at one week after the experiment started and continued for 4 weeks. One week before giving AFB₁, tree shrews in group C and D were respectively given oltipraz (0.5mmol/kg b.w.) daily or weekly, by gavage in a saturated solution of sucrose for 5 weeks. Blood

samples and 24-hour urine samples were collected once a week from each animal throughout the experiment. At the termination of the 9-week experiment, tree shrews were killed by cervical dislocation. Three blocks of liver tissue were taken from each animal. Serial sections from each block were stained histochemically for r-glutamyl transpeptidase (γ-GT)^[54] and HE respectively. The γ-GT positive liver cells were counted with a nest-ruler under microscope. The results were analyzed by the medical statistics analyzing software PEMS that was designed by West China University of Medical Sciences. The levels of aflatoxin-albumin adducts in serum samples were determined by radioimmune assay^[55] and the levels of Aflatoxin-N⁷-guanine adducts in urine samples were assayed by HPLC^[29].

No γ-GT positive liver cell focus, a postulated precancerous marker^[54-56] was observed in any liver of the variously treated tree shrews in our study. However, different numbers of γ-GT positive liver cells, which scattered mainly around the portal spaces, were observed in each group. Even though the distribution patterns of these cells were similar among the 4 groups, the number was quite different. Groups B and D had obviously less γ-GT positive cells than groups A and C (Table 1).

γ-GT normally exists in embryonic liver cell in human being and rat. In adult rat, it exists only in some cells around portal spaces^[57] but can be re-expressed by mature hepatocytes during the recovery process after liver damage^[58]. In this study a number of γ-GT positive hepatocytes presented in periportal regions in the normal control group. On the contrary, the number of γ-GT positive hepatocytes of the same sites was markedly reduced in the AFB₁ treated B group. This phenomenon is fairly consistent with the findings on AFB₁ induced damage in rat, in which the periportal hepatocytes are the major targets of AFB₁. As shown in the same table, the number of γ-GT positive hepatocytes in group C was strikingly similar to the normal control group. This result indicates strongly the preventive action of oltipraz against AFB₁ toxicity. The apparent ineffectiveness of oltipraz in group D is most possibly due to its inadequate dose^[59]. These results might indicate that oltipraz has the preventive dose-related effect on AFB₁.

Insufficient duration and/or insufficient dosage of AFB₁ treatment may result in that no separate focus of γ-GT positive liver cell formed at the end of this 9-week experiment^[60]. However, the decrease of γ-GT positive hepatocytes in the periportal regions may be an early marker for the damage induced by AFB₁.

The levels of both aflatoxin-albumin adducts in serum samples and aflatoxin-N⁷-guanine adducts in urine samples of the tree shrews were also significantly affected by oltipraz. Following daily

exposures to AFB₁, the levels of serum aflatoxin-albumin adducts in group B increased rapidly over 2 weeks to reach a plateau that did not diminish until cessation of AFB₁ exposure. In group C however, oltipraz attenuated the aflatoxin-albumin adducts significantly ($P < 0.05$) with a median reduction of 80%. The mean levels of aflatoxin-N⁷-guanine (ng/mg creatinine) in the urine samples collected at week 5 were 6.34 ± 2.04 and 0.47 ± 0.13 in groups B and C respectively. This 93% decrease represented a statistically significant difference ($P < 0.05$). These results were reported in detail in another article^[61]. The major mechanism of oltipraz's chemopreventive effect is probably through inducing the activities of cytochrome P450 system and phase 2 enzymes such as glutathione transferases, epoxide hydrolase, etc, as reported by Langouet *et al*^[38] and Fahey *et al*^[62].

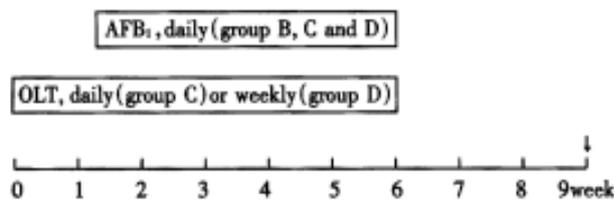


Figure 1 Experimental design for animal treatment. OLT: oltipraz; ↓: all the animals were sacrificed.

Table 1 The number and size of γ -GT positive hepatocyte-group ($\bar{x} \pm s_x$)

Group	Treatment	No./cm ²	mm ² /cm ²	mm ² /No.
A	Normal control	167.29±50.47	2.90±1.01	0.017±0.002
B	AFB ₁	71.92±42.19 ^a	1.14±0.69 ^a	0.015±0.002
C	AFB ₁ +OLT daily	167.10±45.94 ^{c,d}	2.73±0.87 ^{c,d}	0.016±0.001
D	AFB ₁ +OLT weekly	83.66±34.94 ^a	1.33±0.86 ^a	0.015±0.002 ^b

t test: ^a $P < 0.05$, ^b $P < 0.01$, vs group A; ^c $P < 0.01$, vs group B; ^d $P < 0.01$, vs group D.

OLT: oltipraz;

No./cm²: the number of γ -GT positive hepatocyte-group per cm² of liver tissue;

mm²/cm²: mm² of γ -GT positive hepatocyte-group per cm² of liver tissue;

mm²/No.: mm² of γ -GT positive hepatocyte-group per each one.

SUMMARY

Tree shrew is phylogenetically more closely related to human being than rodents. It is susceptible both to HBV infection and AFB₁ intoxication. It is a suitable experimental animal for hepatocarcinogenesis. Attempt for its rearing is promising.

Oltipraz is an effective reagent to protect AFB₁ intoxication. This effect is proved clearly not only by histological examination, but also by reduction of aflatoxin-albumin adducts in serum and aflatoxin-N⁷ guanine adducts in urine.

All of these studies mentioned above provide a foundation for further HCC chemoprevention study

by using tree shrews in the future.

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Stimulation by nizatidine, a histamine H₂-receptor antagonist, of duodenal HCO₃⁻ secretion in rats: relation to anti-cholinesterase activity

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Subject headings nizatidine; histamine H₂ receptor blockaders; duodenal HCO₃⁻ secretion; cholinesterase inhibitors; rats

Takeuchi K, Kawauchi S, Araki H, Ueki S, Furukawa O. Stimulation by nizatidine, a histamine H₂-receptor antagonist, of duodenal HCO₃⁻ secretion in rats: relation to anti-cholinesterase activity. *World J Gastroentero*, 2000;6(5):651-658

Abstract

AIM To examine whether nizatidine stimulates duodenal HCO₃⁻ secretion in rats by inhibiting AChE activity.

METHODS Under pentobarbital anesthesia, a proximal duodenal loop was perfused with saline, and the HCO₃⁻ secretion was measured at pH 7.0 using a pH-stat method and by adding 10mM HCl. Nizatidine, neostigmine, carbachol or famotidine was administered i.v. as a single injection.

RESULTS Intravenous administration of nizatidine (3 - 30mg/kg) dose-dependently increased duodenal HCO₃⁻ secretion, and the effect at 10mg/kg was equivalent to that obtained by carbachol at 0.01mg/kg. This nizatidine action was observed at the same dose range that inhibited acid secretion and enhanced gastric motility, mimicked by i.v. injection of neostigmine (0.03mg/kg), and significantly attenuated by bilateral vagotomy and prior s.c. administration of atropine but not by indomethacin, a cyclooxygenase inhibitor, or N^G-nitro-L-arginine methyl ester, a NO synthase inhibitor. The HCO₃⁻ secretory response to acetylcholine (0.001mg/kg) was significantly potentiated by the concurrent administration of nizatidine (3mg/kg, i.v.). The IC₅₀ of

nizatidine for AChE of rat erythrocytes was 1.4×10⁻⁶M, about 12 times higher than that of neostigmine. Neither famotidine (>10⁻³M, 30mg/kg, i.v.) nor cisapride (>10⁻³M, 3mg/kg, i.v.) had any influence on AChE activity or duodenal HCO₃⁻ secretion. Duodenal damage induced by acid perfusion (100mM HCl for 4h) in the presence of indomethacin was significantly prevented by nizatidine and neostigmine, at the doses that increased the HCO₃⁻ secretion.

CONCLUSION Nizatidine stimulates duodenal HCO₃⁻ secretion, in both vagal-dependent and atropine-sensitive manners, and the action is associated with the anti-AChE activity of this agent.

INTRODUCTION

Nizatidine, a histamine H₂-receptor antagonist (N-[2-[[[2-[(dimethylamino) methyl]-4-thiazolyl]-methyl]thio]ethyl]-N"-methyl-2-nitro-1,1-ethenediamine), has been shown to have a potent antisecretory action and clinically ascertained to be effective for peptic ulcers as well as gastroesophageal reflux diseases^[1-3]. Of interest, an additional effect of H₂-antagonists on gastrointestinal motility has been reported, besides the antisecretory activities^[4,5]. Indeed, some H₂-antagonists including nizatidine exhibit a potent anti-acetylcholinesterase (AChE) activity^[6-9] and, as a result of this action, facilitate gastrointestinal motor activity in experimental animals and in humans^[10,11].

On the other hand, duodenal mucosal HCO₃⁻ secretion is a key process that aids in preventing acid-peptic injury^[12,13]. The mechanisms that govern mucosal HCO₃⁻ secretion include neuro-humoral factors and luminal acid^[12-14]. The ability of the mucosa to respond to acid seems especially important in the maintenance of the surface pH gradient and in the protection of mucosa. This process is also mediated by endogenous prostaglandins (PGs) as well as neuronal factors

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including vagal-cholinergic mechanisms^[15-17]. Several investigators reported that cholinomimetic drugs increased duodenal HCO_3^- secretion, either directly or indirectly mediated by vasoactive intestinal polypeptide (VIP)^[17]. We also reported that both carbachol and bethanechol stimulated the HCO_3^- secretion, mediated by M_3 but not M_1 receptors^[18]. Because inhibition of AChE activity increases the availability of endogenous acetylcholine, it is possible that nizatidine might increase duodenal HCO_3^- secretion through inhibition of AChE activity. However, the effect of nizatidine on the HCO_3^- secretion has not been studied.

This study was undertaken to confirm the anti-AChE activity of nizatidine *in vitro*, and investigate the effect of this agent on HCO_3^- secretion in the rat duodenum, in comparison with the other H_2 -antagonist famotidine and the AChE inhibitor neostigmine. In addition, we also examined whether the doses sufficient to stimulate the HCO_3^- secretion are comparable to the gastroprokinetic and antisecretory doses.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 200g-230g (Charles River, Shizuoka, Japan), were used in all experiments. The animals kept in individual cages with raised mesh bottoms were deprived of food but allowed free access to tap water for 18h before the experiments. Studies were carried out with 4-6 rats per group under anesthetized conditions induced by pentobarbital Na (30 mg/kg, i.v.), unless otherwise specified. All experimental procedures described here were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Determination of duodenal HCO_3^- secretion

Duodenal HCO_3^- secretion was determined in the duodenal loop according to a previously published method^[16]. In brief, the abdomen was incised, and the stomach and duodenum were exposed. The duodenal loop (1.7cm) was made between the pylorus and the area just proximal to the outlet of the common bile duct, excluding the influences of bile acid and pancreatic juice. Then, the loop was perfused at a flow rate of 0.8mL/min with saline that was gassed with 100% O_2 and kept in a reservoir, and HCO_3^- secretion was measured at pH 7.0 using a pH-stat method and by adding 10mM HCl to the reservoir. Allowing 30-40min for stabilization of basal HCO_3^- secretion, the duodenal HCO_3^- secretory responses were examined for 2h after the following treatment; nizatidine (3-30 mg/kg), carbachol (0.003 mg/kg), neostigmine (0.03 mg/kg), famotidine (10 mg/kg) or

cisapride (3mg/kg). These drugs were administered i.v. as a single injection. In some cases, nizatidine (3 mg/kg) and acetylcholine (0.001 mg/kg) were administered i.v. simultaneously. In some cases, atropine (1mg/kg, s.c.), indomethacin (5 mg/kg, s.c.) or N-G-nitro-L-arginine methyl ester (L-NAME) the NO synthase inhibitor (5mg/kg, i.v.) was given 1h or 10min, respectively, before administration of nizatidine, carbachol or neostigmine. In a separate experiment, bilateral vagotomy was performed at the cervical portion 2h before administration of nizatidine, neostigmine or carbachol.

Determination of gastric acid secretion

Gastric acid secretion was measured in a chambered stomach, according to a previously published method^[19]. Briefly, the abdomen was incised, and both the stomach and duodenum were exposed. Then, the stomach was mounted in an *ex-vivo* chamber and perfused at a flow rate of 0.8mL/min with saline that was gassed with 100% O_2 , heated at 37°C and kept in a reservoir. The acid secretion was measured at pH 7.0 using a pH-stat method (Hiranuma Comtite-8, Tokyo, Japan) by adding 100mM NaOH to the reservoir. After basal acid secretion had well stabilized, the acid secretion was stimulated by continuous i.v. infusion of histamine (4mg/kg/h). Nizatidine (10 and 30mg/kg) and famotidine (10mg/kg) were administered i.v. as a single injection 1h after the onset of histamine infusion, when the acid secretory response to histamine had reached a plateau.

Measurement of gastric motility

Gastric motility was determined using a miniature balloon in conscious rats, according to a previously published method^[20]. Briefly, under ether anesthesia the balloon and the support catheter were placed in the stomach through an incision of the forestomach. The animals were kept in Bollman cages, and gastric motility was monitored on a Hitachi recorder (Model 056, Mito, Japan) using a pressure transducer (Narco Telecare, Model 151-T, Houston, TX., U.S.A.) and a polygraph device (San-ei, Model 6M-72, Tokyo, Japan) after complete recovery from anesthesia. After basal motility had well stabilized, the animals were administered i.v. with nizatidine (30 mg/kg), neostigmine (0.03mg/kg), cisapride (3mg/kg) or famotidine (10 mg/kg), and the motility was measured for 2h thereafter. In some cases, the effect of atropine (1mg/kg, s.c.) was examined on the enhanced gastric motility in response to nizatidine (30mg/kg, i.v.).

Induction of duodenal mucosal damage by acid perfusion

The increased HCO_3^- secretion caused by nizatidine might lead to decrease of the mucosal susceptibility

to acid injury. To test this possibility, we examined the effect of nizatidine on the mucosal ulcerogenic response by perfusing the duodenum with 100mM HCl at the flow rate of 1mL/h for 4h in the presence of indomethacin. The experiment was performed in the duodenal loop preparation, similar to that described for HCO₃⁻ secretion. Animals were first treated with indomethacin (5 mg/kg, s.c.), and 1h later the duodenum was perfused with acid for 4h. Nizatidine (10mg/kg) was administered i.v. 20min before the onset of acid perfusion. In comparison, the animals were treated i.v. with neostigmine (0.03mg/kg) or famotidine (10mg/kg). The duodenums were removed, inflated by injecting 0.5mL of 2% formalin, immersed in 2% formalin for 10min to fix the tissue wall, opened along the mesenteric artery, and examined for lesions under a dissecting microscope with a square grid ($\times 10$). The area (mm²) of each lesion was measured, summed per duodenum, and used as a damage score. The person measuring the lesions did not know the treatment given to the animals.

Determination of anticholinesterase activity

Erythrocyte membranes and plasma each from rats were prepared to obtain both AChE and pseudocholinesterase (PChE), according to the method described by Hansen and Bert^[6]. Before the determination of anti-ChE activity, it was confirmed that PChE was not present in the erythrocyte membranes by using the PChE inhibitor, profenamine. The anti-ChE activity of H₂-receptor antagonists and neostigmine was determined by the modified method of Ellman *et al*^[21]. In brief, a reaction mixture was prepared to contain, in a total volume of 1mL, 0.1M sodium phosphate buffer (pH 8.0), 1mM acetylcholine, 0.3mM 5,5-dithio-bis (2-nitrobenzoic acid) and 1mU ChE. The enzyme activity was determined by tracing the changes in absorbance at 412nm at 30°C on a spectrophotometer (Model 320, Hitachi, Ibaragi, Japan) for a period of 70 sec after adding acetylthiocholine. The amount of enzyme required to convert 1 μ mol of acetylthiocholine within 1min under the above conditions was taken as 1 unit. Anti-enzyme activity was measured by adding 10 μ L of each test drug solution to the reaction mixture. The concentration of test drug required to inhibit 50% of the enzyme activity (IC₅₀) was calculated from the enzyme inhibition curve.

Preparation of drugs

Drugs used were pentobarbital Na, neostigmine bromide, histamine 2HCl, carbachol, acetylcholine (Nacalai tesque, Kyoto, Japan), nizatidine (Zeria Pharm. Co., Saitama, Japan), famotidine (Gaster R, Yamanouchi Pharm. Co. Tokyo, Japan), atropine, indomethacin, NG-nitro-L-arginine methyl ester (Sigma Chemicals, Saint Louis, Mo.,

USA) and cisapride (Synthesized by Zeria Pharm. Co.). For *in vivo* experiments, each drug except indomethacin was dissolved in or diluted with saline. Indomethacin was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan). Each drug was administered i.v. in a volume of 1mL/kg or s.c. in a volume of 5mL/kg, or by i.v. infusion in a volume of 1.2mL/h. For *in vitro* experiments, each drug was prepared in purified water or equimolar hydrochloric acid solution. In all experiments, solvents alone were used as controls.

Statistics

Data are presented as the means \pm SE from 4-6 rats per group. Statistical analyses were performed using a two-tailed Dunnett's multiple comparison test, and values of $P < 0.05$ were regarded as significant.

RESULTS

Effects of nizatidine on duodenal HCO₃⁻ secretion

Under the present experimental conditions, the rat duodenum spontaneously secreted HCO₃⁻ at a steady rate of 1.0-1.2 μ Eq/15 min during a 3 h test period. Intravenous administration of nizatidine (3-30mg/kg) caused an increase of the HCO₃⁻ secretion in a dose-dependent manner (Figure 1). At 10 mg \cdot kg⁻¹, the H₂ antagonist nizatidine increased the HCO₃⁻ secretion from 1.2 μ Eq/15 min to a plateau level of 1.8-2.0 μ Eq/15 min within 30 min, remaining elevated for 2 h, the Δ HCO₃⁻ output being 5.6 ± 1.4 μ Eq/2h. Likewise, duodenal HCO₃⁻ secretion was significantly increased in response to i.v. administration of carbachol (0.01mg/kg) and neostigmine (0.03 mg/kg), the Δ HCO₃⁻ output being 4.9 ± 1.1 μ Eq/2h and 5.2 ± 0.8 μ Eq/2h, respectively, both of which were almost equivalent to that induced by nizatidine at 10mg/kg (Figure 2). By contrast, neither famotidine the H₂-antagonist (10mg/kg, i.v.) nor cisapride the gastroprokinetic drug (3mg/kg, i.v.) had any influence on basal rates of duodenal HCO₃⁻ secretion (Figures 2 and 3).

The HCO₃⁻ secretory responses induced by both nizatidine (10 mg/kg, i.v.), neostigmine (0.03mg/kg, i.v.) and carbachol (0.01 mg/kg, i.v.). Were all significantly inhibited by prior s.c. administration of atropine (1 mg/kg) (Figure 4). This agent had a minimal effect on the basal HCO₃⁻ secretion without any treatment, but almost totally attenuated the increase of HCO₃⁻ secretion induced by either nizatidine, neostigmine or carbachol; the Δ HCO₃⁻ output remained unchanged before and at all time points after administration of these drugs. Likewise, bilateral vagotomy significantly reduced the HCO₃⁻ secretory response to nizatidine and neostigmine but not carbachol. On the other hand, the pretreatment of tie animals with neither

indomethacin (5mg/kg, s.c.) nor L-NAME (5mg/kg, i.v.) significantly affected the HCO_3^- secretory response induced by nizatidine (not shown).

Effect of the combined treatment of nizatidine and acetylcholine on duodenal HCO_3^- secretion

To further investigate the relation of anti-AChE activity of nizatidine with the HCO_3^- stimulatory action, we examined whether or not the acetylcholine-induced HCO_3^- response was potentiated by co-administration of nizatidine. As shown in Figure 5, acetylcholine (0.001 mg/kg) caused a slight but significant increase in duodenal HCO_3^- secretion, while nizatidine at 3 mg·kg⁻¹ tended to increase the secretion; the ΔHCO_3^- output was $1.8 \pm 0.1 \mu\text{Eq}/2\text{h}$ and $0.7 \pm 0.6 \mu\text{Eq}/2\text{h}$, respectively. However, when nizatidine was administered together with acetylcholine, the HCO_3^- secretion was markedly increased, reaching a peak of about 150% of basal values, the ΔHCO_3^- output being $4.1 \pm 0.7 \mu\text{Eq}/2\text{h}$, which is 2.4 fold greater than that induced by acetylcholine.

Effects of nizatidine on gastric acid secretion and motility

Acid secretion Following intravenous infusion of histamine (4mg/kg/h), gastric acid secretion was increased from $18.6 \pm 3.1 \mu\text{Eq}/10 \text{ min}$ to $39.5 \pm 3.2 \mu\text{Eq}/10 \text{ min}$ -within 60min, and remained elevated during a 2h test period. The acid secretory response to histamine was significantly reduced by i.v. injection of nizatidine (10 and 30mg/kg) in a dose-dependent manner, the inhibition of total acid output for 2h being 58.9 and 86.3%, respectively (Figure 6). A potent inhibition of histamine-induced acid secretion was also observed on i.v. administration of famotidine (10mg/kg), the inhibition of total acid output for 2h being 90.4%.

Gastric motility Normal rat stomachs spontaneously contracted at a frequency of 16-20/10min with an amplitude of $18.6 \pm 3.2 \text{ cm H}_2\text{O}$. Intravenous administration of nizatidine (30mg/kg) enhanced gastric motility, which reached a plateau level (about 2.5 times greater than basal values) within 40min and remained elevated thereafter, and this action was completely inhibited by atropine (1mg/kg, s.c.) (Figure 7). Both neostigmine (0.03mg/kg) and cisapride (3 mg/kg) increased gastric motility, similar to nizatidine, while famotidine (10mg/kg) did not have any effect on spontaneous contractile activity of the stomach (not shown).

Effects of nizatidine on duodenal damage caused by acid perfusion

Perfusion of the proximal duodenum with 100mM

HCl for 4h in indomethacin-treated rats caused severe damage in the mucosa, the lesion score being $49.1 \pm 7.4 \text{ mm}^2$ (Table 1). Pretreatment of animals with nizatidine (10 mg/kg, i.v.) or neostigmine (0.03mg/kg, i.v.) was effective in significantly reducing the severity of duodenal damage in response to acid perfusion, the inhibition being 56.6% or 64.0%, respectively. Famotidine (10mg/kg, i.v.) had no effect on the development of duodenal damage induced by acid perfusion.

Table 1 Effects of nizatidine, neostigmine and famotidine on duodenal damage induced by acid in rats

Group	Dose (mg/kg)	Number of Rats	Duodenal damage (mm ²)	Inhibition (%)
Control		5	49.1±7.4	
Nizatidine	10	5	21.3±5.1 ^a	56.6
Neostigmine	0.03	5	17.7±7.3 ^a	64.0
Famotidine	10	4	40.8±4.5	16.1

Duodenal damage was induced by perfusing a duodenal loop with 100mM HCl for 4h in the presence of indomethacin (5mg/kg, s.c.). Nizatidine (10mg/kg), neostigmine (0.03mg/kg) or famotidine (10mg/kg) was administered i.v. as a single injection 20min before the onset of acid perfusion. Data are presented as the means±SE from 5 rats.

^a Significant difference from control, $P < 0.05$.

Table 2 Inhibition by nizatidine, famotidine, neostigmine and cisapride of acetylcholinesterase activity in rat erythrocyte and plasma

Drugs	IC ₅₀ (M)	
	Rat erythrocytes	Plasma
Nizatidine	1.4×10^{-6}	5.7×10^{-4}
Neostigmine	1.1×10^{-7}	3.3×10^{-6}
Famotidine	$1.0 \times 10^{-3} <$	$1.0 \times 10^{-3} <$
Cisapride	$1.0 \times 10^{-3} <$	$1.0 \times 10^{-3} <$

Values represent the IC₅₀ of AChE activity for each drug. The experiments were performed in triplicate against AChE of rat erythrocyte and plasma.

Effects of nizatidine on acetylcholinesterase activity

Table 2 summarizes the activities of nizatidine for AChE and PChE, in comparison with neostigmine and famotidine. Nizatidine inhibited the AChE activity prepared from rat erythrocytes, and the IC₅₀ was $1.4 \times 10^{-6} \text{ M}$, about 12 times greater than that ($1.1 \times 10^{-7} \text{ M}$) of neostigmine. Likewise, both nizatidine and neostigmine inhibited the PChE activity prepared from rat plasma, the IC₅₀ being $5.7 \times 10^{-4} \text{ M}$ and $3.3 \times 10^{-6} \text{ M}$, respectively. However, neither famotidine nor cisapride had any effect on AChE or PChE activities, the IC₅₀ for these drugs being over $1 \times 10^{-3} \text{ M}$.

DISCUSSION

The present study showed for the first time that nizatidine, an histamine H₂-receptor antagonist, stimulates duodenal HCO_3^- secretion in rats, in both vagal-dependent and atropine-sensitive

manners, and this action is associated with the anti-AChE activity of this agent. Furthermore, the HCO₃⁻ stimulatory property of nizatidine was observed at the dose ranges for both gastric antisecretory and prokinetic actions. Since neither famotidine nor cisapride had any effect on duodenal HCO₃⁻ secretion, it is unlikely that this action of nizatidine is simply resulted from the inhibition of gastric acid secretion due to H₂ receptor blockade or the increased luminal pressure due to enhanced duodenal motility.

It has been shown that several H₂-receptor antagonists are endowed with anti-AChE

activity^[6-10]. This action results in facilitating gastrointestinal motor activity in experimental animals and in humans^[5,11]. Ueki *et al*^[11] reported that nizatidine stimulates gastrointestinal motility and gastric emptying at antisecretory doses, mainly through its anti-AChE activity. In the present study, we confirmed that nizatidine potently inhibited both AChE and PChE activities. On the basis of the K_i values, it was also noted that the anti-AChE activity of nizatidine was much potent as compared with the other H₂-antagonist famotidine, although it was weaker than that of neostigmine, the authentic AChE inhibitor.

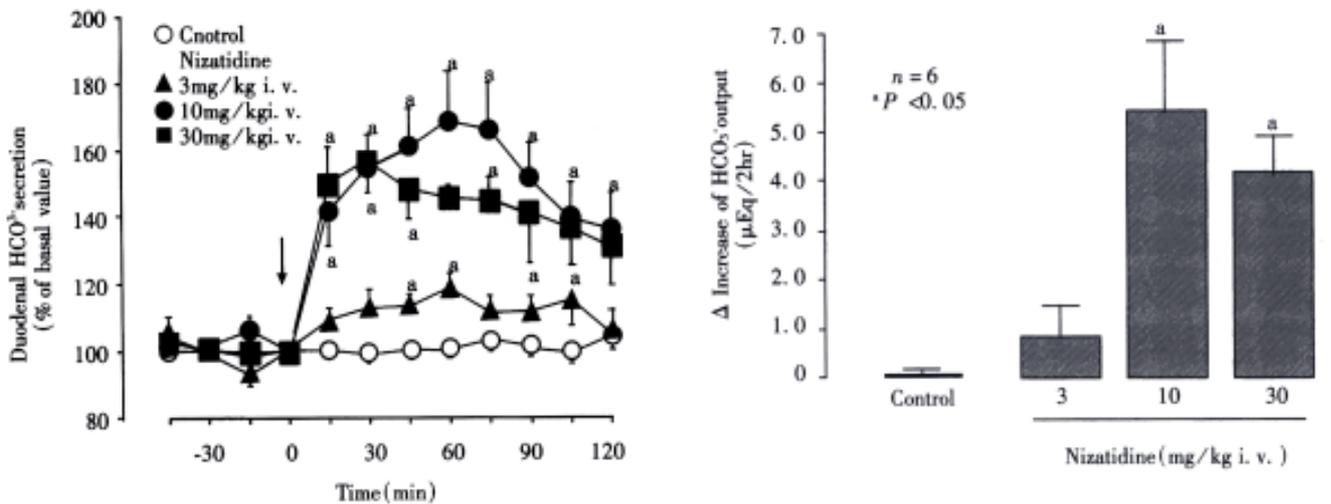


Figure 1 Effect of nizatidine on duodenal HCO₃⁻ secretion in rats. The HCO₃⁻ secretion was measured by perfusing a duodenal loop with saline and by adding 10mM HCl to the reservoir. Nizatidine (3-30mg/kg) was administered i.v. as a single injection after the basal secretion had well stabilized, and the HCO₃⁻ secretion was measured for 2h. Data are expressed as % of basal values and represent the means±SE of values determined every 15min from 6 rats. Lower panel shows Δ increase of HCO₃⁻ output for 2h. Data are presented as the means±SE from 6 rats. *Significant difference from controls, P<0.05.

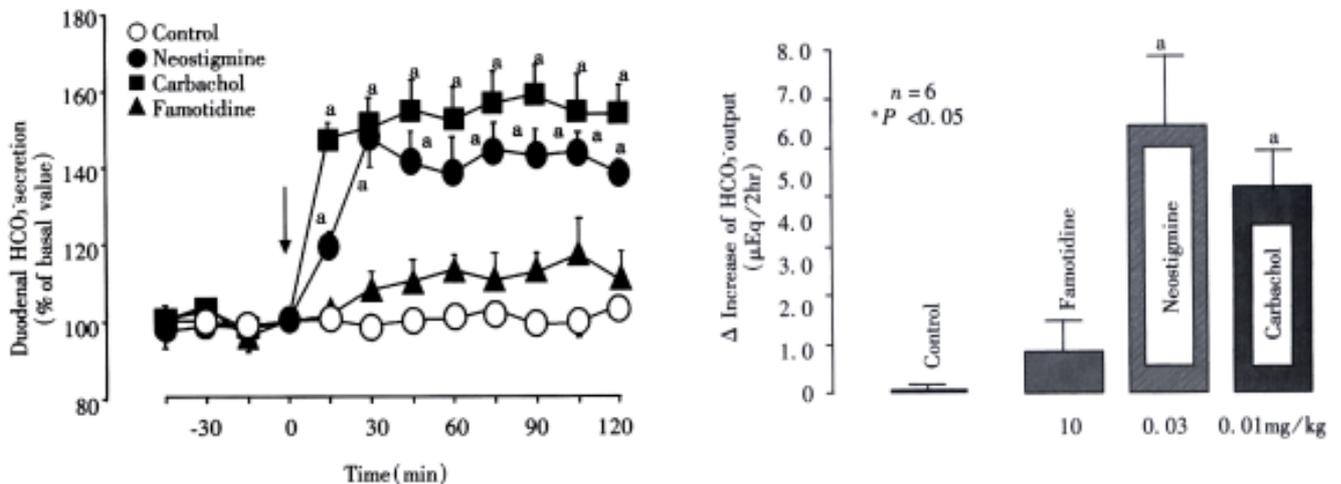


Figure 2 Effects of neostigmine, carbachol and famotidine on duodenal HCO₃⁻ secretion in rats. The HCO₃⁻ secretion was measured by perfusing a duodenal loop with saline and by adding 10mM HCl to the reservoir. Neostigmine (0.03mg/kg), carbachol (0.01mg/kg) or famotidine (10mg/kg) was administered i.v. as a single injection after the basal secretion had well stabilized, and the HCO₃⁻ secretion was measured for 2h. Data are expressed as % of basal values and represent the means±SE of values determined every 15min from 6 rats. Lower panel shows Δ increase of HCO₃⁻ output for 2h. Data are presented as the means±SE from 6 rats. *Significant difference from controls, P<0.05.

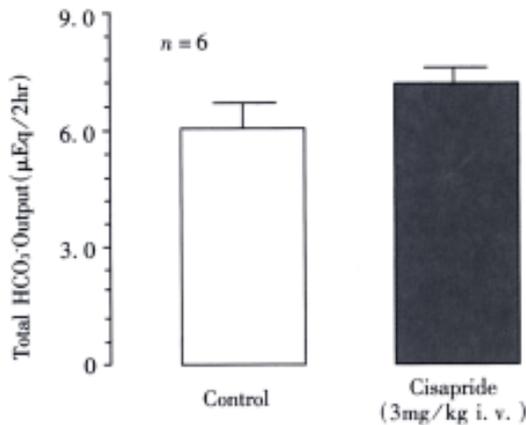


Figure 3 Effect of cisapride on duodenal HCO₃⁻ secretion in rats. The HCO₃⁻ secretion was measured by perfusing a duodenal loop with saline and by adding 10mM HCl to the reservoir. Cisapride (3 mg/kg) was administered i.v. as a single injection after the basal secretion had well stabilized, and the HCO₃⁻ secretion was measured for 2h. Data indicate total HCO₃⁻ output obtained for 2h after administration of cisapride, and represent the means±SE from 6 rats.

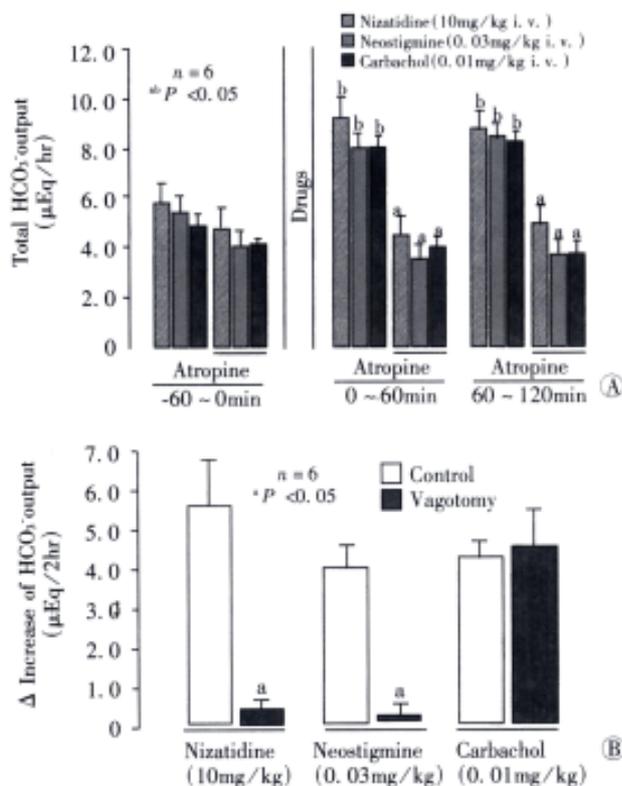


Figure 4 Effects of atropine or bilateral vagotomy on the HCO₃⁻ stimulatory action of nizatidine, neostigmine and carbachol in the rat duodenum. The HCO₃⁻ secretion was measured by perfusing a duodenal loop with saline and by adding 10mM HCl to the reservoir. Neostigmine (0.03 mg/kg), carbachol (0.01 mg/kg) or famotidine (10mg/kg) was administered i.v. as a single injection after the basal secretion had well stabilized, and the HCO₃⁻ secretion was measured for 2h. Atropine (1mg/kg) was given s.c. 1h before administration of the above agents. Bilateral vagotomy was performed acutely at the cervical portion 2h before the administration of the agents. Data are presented as the means ±SE from 6 rats. Significant difference at $P < 0.05$; ^afrom corresponding groups without atropine; ^bfrom corresponding values observed for 1h before the treatment (time: -60-0 min)

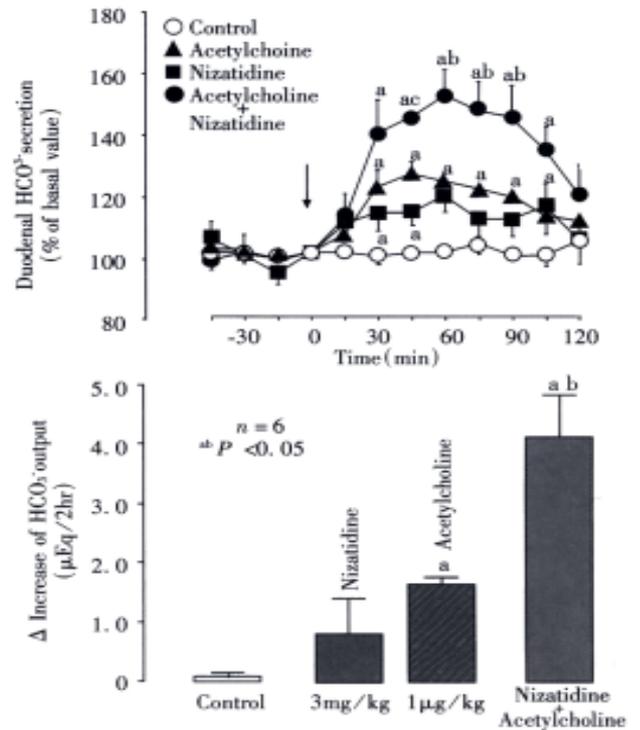


Figure 5 Effects of nizatidine and acetylcholine, either alone or in combination, on duodenal HCO₃⁻ secretion in rats. The HCO₃⁻ secretion was measured by perfusing a duodenal loop with saline and by adding 10mM HCl to the reservoir. Nizatidine (3mg/kg) and acetylcholine (0.003mg/kg), either alone or in combination, were administered i.v. after the basal secretion had well stabilized, and the HCO₃⁻ secretion was measured for 2h. Data are expressed as % of basal values and represent the means±SE of values determined every 15min from 6 rats. Lower panel shows Δ increase of HCO₃⁻ output for 2h. Data are presented as the means ± SE from 6 rats. Significant difference at $P < 0.05$ ^afrom control; ^bfrom nizatidine or acetylcholine.

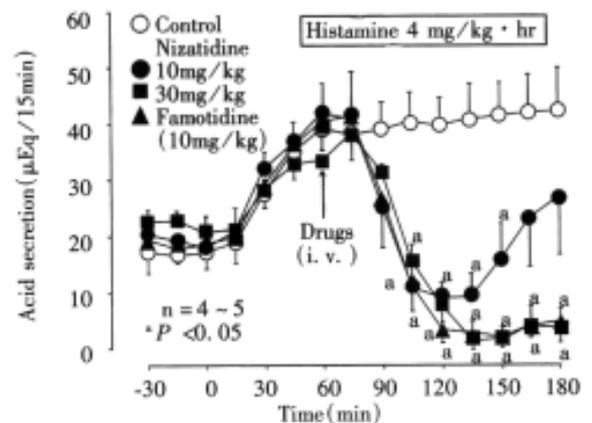


Figure 6 Effects of nizatidine and famotidine on histamine-induced gastric acid secretion in rats. A rat stomach mounted in an *ex-vivo* chamber was perfused with saline, and the acid secretion was measured by adding 100mM NaOH to the reservoir. The acid secretion was stimulated by i.v. infusion of histamine (4mg/kg/h), while nizatidine (10 and 30mg/kg) or famotidine (10mg/kg) was administered i.v. as a single injection 1h after the onset of histamine infusion, when the acid secretion had reached a plateau. Data are presented as the means±SE of values determined every 15min from 5 rats. ^aSignificant difference from controls, $P < 0.05$.

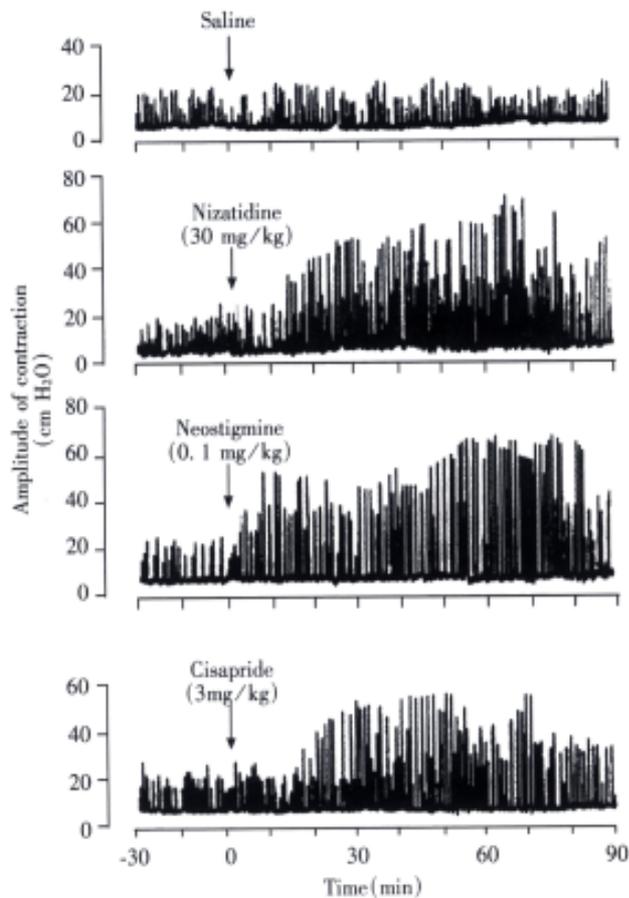


Figure 7 Representative recordings of gastric motor activity in conscious rats, before and after administration of nizatidine. gastric motor activity was monitored as intraluminal pressure recordings, by using a miniature balloon placed in the stomach. Nizatidine (30mg/kg) was administered i.v. as a single injection after the basal motility had well stabilized. Atropine (1mg/kg) was given s.c. 1h after administration of nizatidine. Note that gastric motor activity was apparently enhanced by nizatidine at the doses that stimulate HCO₃⁻ secretion in the rat duodenum, and this action was completely blocked by atropine.

It is widely accepted that acetylcholine is a transmitter released by enteric excitatory neurons to influence gastrointestinal motility. Likewise, several studies showed that duodenal HCO₃⁻ secretion was increased by the vagal-cholinergic excitation^[17,18, 22,23]. Lenz *et al*^[22] reported that the HCO₃⁻ secretion was increased in response to both central and peripheral cholinergic agents. It is also known that the vagal-cholinergic pathway plays a role in the HCO₃⁻ secretory response to acid and sham feeding^[23]. Since the electrical stimulation of the vagus nerves increased duodenal HCO₃⁻ secretion mediated by the atropine-sensitive cholinergic pathway^[24], it is expected that this process is also stimulated by peripheral cholinomimetic agents. The present study clearly showed that nizatidine increased the HCO₃⁻ secretion by stimulating cholinergic excitation

through anti-AChE activity, and this effect was mimicked by neostigmine. These results support the contention that the HCO₃⁻ stimulatory effect of nizatidine in the rat duodenum is mediated by endogenous acetylcholine released from cholinergic neurons. Indeed, this action of nizatidine on the HCO₃⁻ secretion was totally abolished by bilateral vagotomy or prior administration of atropine. Certainly, the antagonism of H₂-receptor does not account for the HCO₃⁻ stimulatory action of nizatidine, because famotidine did not have any effect on this secretion.

In the present study, the HCO₃⁻ secretion induced by nizatidine was not affected by either indomethacin or L-NAME, excluding the possibility for involvement of PG or NO in this response. We previously reported that the HCO₃⁻ response induced by cholinergic agents was not affected by indomethacin^[18]. Furukawa *et al*^[25] reported that NO stimulates the HCO₃⁻ secretion, mediated by endogenous PGs in isolated bullfrog duodenum. Thus, it is reasonable that the HCO₃⁻ response induced by nizatidine is not affected by the NO synthase inhibitor L-NAME. Segawa *et al*^[26] reported that nizatidine did not affect *in vitro* PGE₂ biosynthesis and even doses that markedly inhibit gastric acid secretion had no effect on the mucosal PGE₂ contents in rat stomachs. Hallgren *et al*^[27] found that the NO synthase inhibitors such as L-NAME caused an increase of both luminal alkalization and luminal pressure in the rat duodenum and suggested that the HCO₃⁻ stimulatory action of L-NAME is due to the neural reflex resulting from the rise in luminal pressure through mechano-receptors. The same mechanism might be applied to the HCO₃⁻ stimulatory action of nizatidine, because this agent enhanced gastrointestinal motility, leading to increase of the luminal pressure. However, cisapride at the dose that clearly enhanced gastric motility did not affect the HCO₃⁻ secretion. Thus, it is unlikely that nizatidine stimulates duodenal HCO₃⁻ secretion due to the increase of luminal pressure, resulting from the smooth muscle contraction.

The secretion of HCO₃⁻ in the duodenum is the main defense mechanism against acid. Mucus adherent to the luminal surface of the mucosa provides a zone of low turbulence, allowing the development of a gradient for HCO₃⁻ from the luminal side^[12,13,28]. Small amounts of HCO₃⁻ protect the mucosa against large amounts of acid by neutralizing H⁺ ions that diffuse back into the mucus layer^[13]. A number of studies demonstrated a close relationship between the mucosal ulcerogenic response and the duodenal HCO₃⁻ disorder^[12, 13,29]. Indeed, it is reported that patients with inactive duodenal ulcers have decreased production of HCO₃⁻ in the proximal duodenum during exposure

to acid^[30], though the exact mechanism involved remains unknown. In the present study, perfusion of the proximal duodenum with 100mM HCl for 4h produced extensive hemorrhagic damage in rats in the presence of indomethacin. However, nizatidine reduced the severity of duodenal damage at the dose which stimulated HCO₃⁻ secretion. These results confirmed that nizatidine afforded protection of the duodenal mucosa by increasing HCO₃⁻ secretion, in a PG-independent pathway.

It is known that the mucosal acidification increases the HCO₃⁻ secretion via both humoral and neural factors as well as endogenous PGs^[12,23,31]. Vasoactive intestinal polypeptide (VIP) is the most likely humoral factor mediating the HCO₃⁻ secretory response to acid, because it is a potent stimulant of duodenal HCO₃⁻ secretion and is released from nerve endings during the exposure of duodenal mucosa to acid^[17]. Odes *et al*^[32] reported that acetylcholine increases a release of VIP from enteric nerves via both muscarinic M₁ and M₃ receptors, which in turn stimulates HCO₃⁻ secretion in the duodenum. Since nizatidine significantly potentiated the HCO₃⁻ response to acetylcholine, it is possible that the acid-induced HCO₃⁻ secretion is also enhanced by the anti-AChE activity in the presence of nizatidine. Ueki *et al*^[11] demonstrated that the ED₅₀ values of nizatidine for inhibition of acid output in rats overlapped the effective doses for stimulation of gastrointestinal motility and gastric emptying. We also showed that nizatidine increased duodenal HCO₃⁻ secretion at the doses that were effective in inhibiting histamine-induced acid secretion or in stimulating gastric contractile activity. These results suggest that the HCO₃⁻ stimulatory effect of nizatidine can be expected in duodenal ulcer patients who are treated with this drug at the antisecretory dose.

In summary, the present results clearly showed that the histamine H₂-receptor antagonist nizatidine stimulates duodenal HCO₃⁻ secretion, and this action is associated with its anti-AChE activity and mediated by vagal-cholinergic mechanisms. It is assumed that the HCO₃⁻ stimulatory action of nizatidine is useful for treatment of duodenal ulcer, in addition to its anti-acid secretory and gastric prokinetic effects.

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Does flexible small-diameter colonoscope reduce insertion pain during colonoscopy ?

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Subject headings colonoscopy; abdominal pain; flexible colonoscope; insertion pressure; unfavorable factors; sigmoidoscopy

Han Y, Uno Y, Munakata A. Does flexible small-diameter colonoscope reduce insertion pain during colonoscopy. *World J Gastroentero*, 2000;6(5):659-663

Abstract

AIM It is well known that colonoscopy can be difficult due to abdominal pain induced during colonoscope insertion, if sedative agents are not given. Recently, an extra-flexible, small-diameter colonoscope (CF-SV, Olympus Inc., Japan) was developed in order to improve safety and comfort. The aim of this study was to access the usefulness of the CF-SV.

METHODS One hundred patients undergoing sigmoidoscopy were recruited and colonoscopy was performed by one experienced colonoscopist. First, a routine-type colonoscope (CF-230I) was inserted into the colon without sedation. When the patient complained of abdominal pain (even if mild), the scope was not advanced further and was withdrawn after the anatomic location of its tip was determined fluoroscopically. Then, the CF-SV was inserted until it reached the cecum or the site where abdominal pain occurred. Previous abdominal surgery and a abdominal disease were considered as unfavorable factors (UF) and the relationship between abdominal pain and UF, age and gender were investigated. Furthermore, the colonic insertion pressures in 36 patients with abdominal pain were measured with a force gauge.

RESULTS Thirty-four cases (34%) felt no pain with the CF-230I and successful pancolonoscopies to the cecum were performed. Sixty-six cases (66%) complained of abdominal pain. The procedure was painless for 47% of men and 24% of women, respectively. The CF-230I scope failed to reach the sigmoid-descending colon

junctions in 59 (89.4%) of the 66 patients complaining of abdominal pain. However, CF-SV reached proximal area in 94.9% of those who failed with CF-230I. The median pressure for pain-inducing was 700g/cm².

CONCLUSION Unsedated patients with UF were prone to complain of pain when the standard-type CF-230I scope was used. The newly developed extra -flexible CF-SV is useful for the aged and for those with UF or being prone to suffer from abdominal pain. Sedative agents may be unnecessary if this new type of colonoscope is used.

INTRODUCTION

Colonoscopy for patients who have undergone previous abdominal surgery, those with diverticular disease, women as well as the aged is technically difficult, even in expert hands^[1-8], and usually induces abdominal pain if sedative agents are not administered. One way of preventing pain during colonoscopy is to use a scope that is more flexible and thinner than the conventional scopes. It has been reported that success rate of colonoscopy could be significantly improved when a pediatric colonoscope was used instead of routine-type instrument for adults when stricture, fixation or painful looping was encountered^[9]. Kozarek *et al* achieved a similar increase in the success rate by using a small-caliber upper endoscope for colonoscopy in similar circumstance^[10]. In 1997, the softest and most flexible colonoscope (CF-SV, Olympus Inc., and 9.6mm in diameter with a 103cm working length) so far was developed^[11,12]. Moreover, a comparative study showed that fewer unsedated patients felt pain during colonoscopy with the CF-SV than with a conventional colonoscope^[11]. The aim of this self-controlled, prospective study was to evaluate whether less pain is experienced during colonoscopy with the CF-SV than with a routine-type colonoscope.

SUBJECTS AND METHODS

The study was approved by the ethics committee of Hirosaki University Hospital. One hundred consecutive patients of this hospital undergoing sigmoidoscopy over a 6-week period were recruited. For the purpose of scientific comparison all of the performance of colonoscopy was done exclusively by

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Dr. Uno who has performed over 5000 colonoscopies.

Relationship between insertion pain and unfavorable factors (UF)

Each patient was placed on the X-ray table in the left lateral position. No sedating agent was given, but the anus was treated with xylocaine jelly, and a routine-type colonoscope CF-230I (Figure 1) (Olympus Inc. 13.6mm in diameter with a 130cm working-length) was inserted. As soon as the patient complained of abdominal pain, even it was mild, the colonoscope was advanced no further, the patient was moved to supine position and the anatomic location of the tip of the scope was determined by fluoroscopy. Then, the scope was pulled out and the air in the lumen was let out. The colonoscopist knew nothing about the patients' age, past history of surgery or abdominal diseases considered as UF of the patients until the procedure had been completed.

Re-insertion of CF-SV

The patients who suffered from abdominal pain with CF-230I were checked with CF-SV five minutes after CF-230I was withdrawn. When the patient complained abdominal pain of similar severity as that with CF-230I, the proximal location of the scope tip was confirmed fluoroscopically with the patient in supine position and the procedure was discontinued. If the patient did not complain of pain, insertion of the scope was continued until up to the cecum or the 103cm working length of the shaft had been inserted. Then, the proximal location of the tip was determined fluoroscopically and the procedure was terminated.

Measurement of the pain-inducing pressure during insertion

The first consecutive 36 patients who complained of pain during colonoscopy with CF-230I were investigated. The pain inducing pressure (g/cm^2) initiated by the hand of the colonoscopist was measured with a force gauge, and the relationship between the insertion force and location of the pain was evaluated.

Statistics

All statistical analyses were performed using either Chi-squared test or differences at P values of less than 0.05 were regarded as significant.

RESULTS

Thirty-four patients (34%) were free of abdominal pain during colonoscopy with CF-230I and the scope was inserted up to the cecum. More of these pain-free subjects were male (21 cases, 61.8%) than female (13 cases, 38.2%) (Table 1). There was no significant difference ($P < 0.05$) between the average ages of the males who did not complain of

pain (60.1 years) and those who did (59.0 years), but the average ages of the corresponding female groups (55.4yr. and 60.7yr., respectively) were significantly different ($P < 0.05$), which suggests that aging females may be prone to pain during colonoscopy insertion. There was no significant difference between the average ages of the males and females who did not complain of pain.

Relationship between insertion pain and UF

Eighteen male patients had past histories of surgery or abdominal diseases: seven (38.9%) had undergone appendectomy; 3 (16.7%) had cholecystectomy; 2 (11.1%) of each had gastrectomy, Crohn's disease and ileus; one (5.6%) of each had diverticulitis, cancer of bladder, trauma, and a previous abdominal operation for an unknown reason. One of these patients had two UFs. Seventeen of these 18 patients with UF complained of abdominal pain during colonoscopy.

Thirty-four females had UF: 10 (29.4%) had cancer of the uterus; 8 (23.5%) had undergone appendectomy; 7 (20.6%) had hysterectomy for myoma uteri; 5 (14.7%) had ovarian neoplasms; 3 (8.8%) of each had undergone cholecystectomy and had peritonitis; 2 (5.9%) had carcinoma of the stomach or bladder, and one (2.9%) had ileus. Seven of these 34 patients had 2 of UFs. Thirty-one patients (91.1%) complained of no abdominal pain during colonoscopy with CF-SV. Only one (4.8%) of the 21 males who did not complain of pain had UF (Table 1). Seventeen (70.8%) of the 24 male patients with abdominal pain had UF (Table 1) and 7 (29.2%) did not. Three (23.1%) of the 13 females whose procedures were painless were UF-positive, whereas 31 (73.8%) of the 42 females with abdominal pain were UF-positive (Table 1) and 11 (26.2%) were UF-negative. There was significant difference ($P < 0.05$) between the numbers of UF-positive patients receiving painless and painful colonoscopy.

Reinsertion of CF-SV

The anatomic location of the abdominal pain induced in 61 of the 64 patients with CF-230I was the sigmoid segment. However, in 59 of these 61 (96.7%) the CF-SV was successfully inserted proximal to sigmoid segment without causing abdominal pain (Figure 2). The remaining two patients who complained of abdominal pain during re-insertion of CF-SV were female and very short in stature. Among the 59 patients in whom the CF-230I was failed to pass through the sigmoid-descending colon junction, when re-inserted with CF-SV, it was passed in 56 (94.9%); transverse colon was passed in 30 (49.2%) and the ascending colon was reached in 14 (23.7%). Only one man (8.3%) and 6 women (17.6%) complained of abdominal pain with CF-SV, which was not inserted beyond the descending colon.

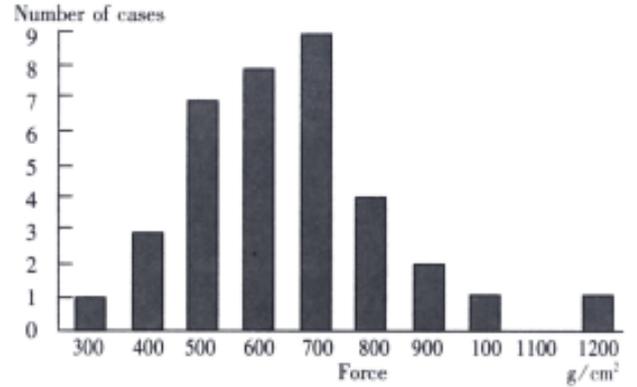


Figure 1 The standard colonoscope (CF-230 I) is shown on the top, the CF-SV is on the bottom.
 Figure 3 The pressure associated with insertion-induced abdominal pain during colonoscopy.

	Anatomic depth of penetration							Age	Unfavorable factor
	Rectum	Recto-sigmoid junction	Sigmoid colon	Sigmoid-descending junction	Descending colon	Transverse colon	Ascending colon		
Female	▲	●						36	Uterus cancer (Hysterectomy and Radiation)
	▲	●						75	Gastrectomy
	▲							69	Uterus cancer (Hysterectomy)
	▲							68	Uterus cancer (Hysterectomy and Radiation)
	▲							68	Myoma uteri (Hysterectomy) and colectomy
	▲							63	Uterus cancer (Hysterectomy)
	▲							68	Visical cancer
	▲							40	Uterus cancer (Hysterectomy)
	▲							77	Uterus cancer
	▲							85	Uterus cancer and peritonitis
	▲							65	Cholecystectomy
	▲							66	Ovarian cancer
	▲							56	None
	▲							66	None
	▲							75	Peritonitis
	▲							36	Uterus cancer (Hysterectomy and Radiation)
	▲							44	Myoma uteri (Hysterectomy)
	▲							59	None
	▲							23	Appendectomy
	▲							75	Cholecystectomy
	▲							72	Myoma uteri (Hysterectomy)
	▲							35	Ovarian tumor
	▲							85	None
	▲							50	Myoma uteri (Hysterectomy)
	▲							54	None
	▲							44	None
	▲							48	Advanced gastric cancer
	▲							51	Myoma uteri (Hysterectomy) and appendectomy
▲							59	Appendectomy	
▲							62	Myoma uteri (Hysterectomy) and appendectomy	
▲							64	Myoma uteri (Hysterectomy)	
▲							66	Uterus cancer (Hysterectomy)	
▲							68	None	
▲							44	None	
▲							72	Ovarian cancer	
▲							72	Appendectomy	
▲							50	None	
▲							68	Ovarian tumor	
▲							75	Vesical cancer	
▲							65	None	
▲							44	None	
▲							65	None	
Male	▲	●						74	Cholecystectomy and ileus
	▲							61	Appendectomy
	▲							70	Laparotomy (ileus)
	▲							45	Appendectomy
	▲							58	Appendectomy
	▲							61	Diverticulitis
	▲							65	Gastrectomy
	▲							74	Cholecystectomy
	▲							52	None
	▲							58	None
	▲							60	Appendectomy
	▲							72	Gastrectomy
	▲							77	None
	▲							18	Crohn's disease
	▲							60	Appendectomy
	▲							65	Cholecystectomy
▲							75	None	
▲							48	None	
▲							57	Traumatic injury	
▲							75	Visical cancer	
▲							24	Crohn's disease	
▲							61	None	
▲							53	Appendectomy	
▲							52	None	

Figure 2 Anatomic depths of penetration of both colonoscopes, gender, patients' ages and unfavorable factors (UF).
 ▲: The location of insertion-induced abdominal pain with CF-230 I; ●: The location of insertion-induced abdominal pain with CF-SV; ○: Proximal area reached when re-inserted with CF-SV.

Table 1 Relationship between insertion-induced pain, gender, age and UF

	Insertion induced pain	Number of cases	Age (year) $\bar{x}\pm s$	UF-positive	
				No.	%
Male	Negative	21	60.1±11.2	1	4.8
	Positive	24	59.0±14.8	17	70.8
Female	Negative	13	55.4±11.2	3	23.1
	Positive	42	60.7±13.9	31	73.8

Measurement of the pain-inducing pressure during insertion of colonoscope

The pain-inducing pressure during insertion ranged from 300 to 1200g/cm² (Figure 3) and the median was 700g/cm². In 24 of 36 patients (66.7%), the range was 500-700g/cm². Only one patient, a 56-year old woman with a history of hysterectomy and abdominal radiation therapy complained of a abdominal pain even when the insertion pressure was as low as 300g/cm², and the anatomic location of the pain was around the upper rectum with the CF-230I and the rectosigmoid junction with CF-SV. Abdominal pain was induced at a pressure of 400g/cm² only in three females: one is a 35-year-old young lady suffering from a giant ovarian tumor; the other two patients, even the CF-SV scope could not be advanced to the proximal segment (the reason remains unknown). Two patients, both were 61 years old male, did not complain of abdominal pain even when the pressure exceeded up to 1000g/cm². Interestingly, one of them was UF-negative, but the other had undergone appendectomy previously.

DISCUSSION

The Japanese Society of Gastrointestinal Endoscopy has investigated, by carrying out questionnaire surveys, and reported the incidence of complications during endoscopy every 5 years since 1983. According to their data, from 1983 to 1992, the estimated incidence of colonic perforation during diagnostic and therapeutic colonoscopy was 0.04%^[13], which is far lower than that in other countries^[14-16]. However, the incidence did not change during this period which means that if the number of colonoscopic examination increases, the absolute number of cases of perforation will inevitably goes up. In Japan, the number of colonoscopies performed each year has been estimated to be over three millions. Therefore, over 600 perforation will occur per year, 1-2 cases per day^[17]. If perforation can be prevented, colonoscopy will be performed far more frequently.

When the tip or bend of a colonoscope presses hard against the colonic mucosa, a seromuscular tear will probably occur, even when there is no muscularis injury^[18], and clinically, more damage occurs before the symptoms of perforation appear. In order to avoid such injuries, it is very important

that colonoscopists undergo special training to improve their skill. The problem of perforation might be resolved if the procedure is easy to perform and independent of experience and/or expertise.

In 1957, the "sigmoid camera" was developed in our institution (Hirosak University, Japan)^[19,20]. Examined with camera, it was not necessary to use sedation. Since then, colonoscopy without sedation had been performed till several years ago. In order to minimize abdominal pain during colonoscope insertion, the loop maneuver technique was introduced^[21]. However, in some patients with UF, although the maneuver was performed, the scope could not be inserted more proximally because of severe insertion-induced abdominal pain. Therefore, no wadays, in our institution, there are two indications for colonoscopy under sedation: when a patient complains of severe insertion-induced abdominal pain during the procedure; the patient prefers to be sedated.

In Japan, based on extensive experience, "Abdominal pain is a signal for some dangerous conditions" has become one of the most important precepts. In our study, the pressure to induce insertion-related abdominal pain was not higher than 1200g/cm². An *in vitro* experiment on perforation of the human colon^[18] showed that perforation occurred when the pressure reached up to 2-3kg/cm². Therefore, the precept of our institution is true. Recently, colonoscopy without sedation and with selective sedation has been investigated in institutes in which colonoscopy under sedation used to be a routine procedure^[22-24]. Furthermore, the cost and complications may be reduced when colonoscopy is performed without sedation. According to Rex^[25], the success rate of unsedated colonoscopy has uniformly been reported to be high in a small number of American reports, although the patients appear to have been selected. For example, in one study, attempted unsedated colonoscopy only in volunteers consisting of about 30% of consecutive patients undergoing colonoscopy. In another study, colonoscopy was performed in consecutive male veterans.

In Japan, colonoscopy without sedation has also been reported but the subjects were young males undergoing a health check-up and did not include elderly subjects or those with UF. In fact, we found that abdominal pain during colonoscope insertion usually occurred in patients who were elderly or UF-positive. The sigmoid colon was the area where pain occurred in most of the subjects undergoing colonoscopy without sedation^[26]. In patients who are very young, very old, have diverticular disease, a fixed colon due to previous abdominal surgery, underwent previous pelvic radiation, have ovarian neoplasms as well as other kinds of abdominal

pathological disorders, colonoscope advancement to the transverse colon was difficult because of making loops re-form in the sigmoid colon. This problem might be solved by introducing a stiff wire into the instrument channel to stiffen the colonoscope after it has been straightened^[27,28]. Recently, a new type of colonoscope with a shaft of variable stiffness has been developed^[29].

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Suppression of P-gp induced multiple drug resistance in a drug resistant gastric cancer cell line by overexpression of Fas

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Subject headings *fas* gene; stomach neoplasms; apoptosis; drug resistance, multiple; antineoplastic agents; immunocytochemistry/methods; gene transduction

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Abstract

AIM To observe the drug sensitizing effect and related mechanisms of *fas* gene transduction on human drug-resistant gastric cancer cell SGC7901/VCR (resistant to Vincristine).

METHODS The cell cycle alteration was observed by FACS. The sensitivity of gastric cancer cells to apoptosis was determined by *in vitro* apoptosis assay. The drug sensitization of cells to several anti-tumor drugs was observed by MTT assay. Immunocytochemical method was used to show expression of P-gp and TopoII in gastric cancer cells.

RESULTS Comparing to SGC7901 and pBK-SGC7901/VCR, *fas*-SGC7901/VCR showed decreasing G2 cells and increasing S cells, the G2 phase fraction of pBK-SGC7901/VCR was about 3.0 times that of *fas*-SGC7901/VCR, but S phase fraction of *fas*-SGC7901/VCR was about 1.9 times that of pBK-SGC7901/VCR, indicating S phase arrest of *fas*-SGC7901/VCR. FACS also suggested apoptosis of *fas*-SGC7901/VCR. *fas*-SGC7901/VCR was more sensitive to apoptosis inducing agent VM-26 than pBK-SGC7901/VCR. MTT assay showed increased sensitization of *fas*-SGC7901/VCR to DDP, MMC and 5-FU, but same sensitization to VCR according to pBK-SGC7901/VCR. SGC7901, pBK-SGC7901/VCR and *fas*-SGC7901/VCR had positively stained Topo II equally. P-gp staining in pBK-

SGC7901/VCR was stronger than in SGC7901, but there was little staining of Pgp in *fas*-SGC7901/VCR.

CONCLUSION *fas* gene transduction could reverse the MDR of human drug-resistant gastric cancer cell SGC7901/VCR to a degree, possibly because of higher sensitization to apoptosis and decreased expression of P-gp.

INTRODUCTION

Multidrug resistance (MDR), the principal mechanism by which many cancers develop resistance to chemotherapeutic drugs, is a major factor in the failure of many forms of chemotherapy^[1]. It affects patients with a variety of blood cancers and solid tumors, including breast, ovarian, lung and lower gastrointestinal tract cancers.

Tumors usually consist of mixed populations of malignant cells, some of which are drug sensitive while others are drug resistant. Chemotherapy kills drug sensitive cells, but may leave behind a high proportion of drug resistant cells. As the tumor begins to grow again, chemotherapy may fail because the remaining tumor cells are now resistant to it.

Resistance to therapy has been correlated to the presence of at least two molecular 'pumps' that actively expel chemotherapeutic drugs from the tumor cells. This action thus spares tumor cells from the effects of the drug, which has to act inside the cell at the nucleus or the cytoplasm. The two pumps commonly found to confer chemoresistance in cancer are P-glycoprotein (P-gp) and the so-called multidrug resistance-associated protein (MRP). But they are not the complete story, and can not explicate the phenomenon of MDR fully.

Recent studies indicate that some cancer cells are resistant to signals of apoptosis and so making cell life longer might be an important part of MDR mechanisms.

In this study, we assumed that inducing apoptosis in drug resistant tumors might reverse MDR. So we transfected *fas* gene, which is a key molecule in the signal transduction pathway of apoptosis, into drug resistant gastric cancer cells,

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and observed the expression of target genes in transfectants and the sensitivity of transfectants to chemotherapeutic agents in order to find the pathway of signal transduction for reversing gastric cancer MDR.

MATERIALS AND METHODS

Materials

Gastric cancer cell line SGC7901, pBK-SGC7901/VCR (SGC7901/VCR is transfected by pBK-CMV expression vectors persistently), pBK-*fas* cDNA (1.83kb full length *fas* cDNA, was inserted into pBK-CMV expression vectors), and *fas*-SGC7901/VCR cell transfectants (SGC7901/VCR is transfected by pBK-*fas*-expression vectors persistently) were kept in our department. Chemical reagents were obtained from Sigma, except as noted. Chemical reagents including PMSF, SDS, and avidin-biotin peroxidase complex (ABC) kit were obtained from SABC and Promega. P-gp mouse IgG antibody and topoisomerase II (TopoII) mouse IgG antibody were kindly given by Dr. Liu. All the antibodies and rabbit anti-human Fas polyclonal antibody were products of Dako. RPMI1640 was obtained from GIBCO, fetal bovine serum from HYCLON E, mitomycin C (MMC) from Kyowa Hakko Kogyo Co. Ltd, vincristine (VCR) and doxorubicin (Dox) from the Twelfth Shanghai Pharmaceutical Product Factory, 5-fluorouracil (5-FU) from Shanghai Xudong Pharmaceutical Company, and cisplatin (DDP) from Shandong Qilu Pharmaceutical Product Factory.

Methods

Western blot analysis Cells in log phase were harvested and washed with PBS twice, then lysed in lysis buffer (150mmol/L Tris-Cl pH 8.0; 50mmol/L NaCl; 0.2mmol/L EDTA; 0.1mmol/L PMSF; 10g/L NP-40). SDS-PAGE was performed using 100µg or 150µg total protein per lane, one electrophoresis gel was stained with commassie brilliant blue R-250, another was blotted to nitrocellular filter. After blocking with 50g/L defatted milk, the filter was incubated with rabbit anti-human Fas polyclonal antibody overnight at 4°C, then washed three times 5min each time with TBS and incubated with HRP-conjugated goat anti-rabbit IgG at 37°C for 30min. Finally, the positive bands were visualized by incubation with peroxidase substrate solution D iaminobenzidine (DAB).

Cell culture MDR gastric cancer cell subline SGC7901/VCR was induced by pulse treatment of the parent cell line SGC7901 at a single dose of Vincristine. Having been exposed to 1.0mg/L Vincristine for one hour, SGC7901 cells were replated in drug-free culture medium to allow exponential growth of cells to be recovered prior to another stimulation. Once stable resistant

phenotype was developed, the SGC7901/VCR cells were grown in drug free growth medium.

Cytotoxicity assay Dose-survival curves defining the sensitivity of gastric cancer cell line SGC7901, pBK-SGC7901/VCR and *fas*-SGC7901/VCR cell transfectants to VCR, DDP, MMC, Dox and 5-FU were obtained using MTT cytotoxic assay. Cells in log growth period $(3-4) \times 10^7/L$ diluted with 200µL RPMI 1640 supplemented with 100mL/L fetal bovine serum were seeded into 96-well plates in triplicate for each treatment, after culturing for 12h, added Dox (0.1, 1, 10 and 100µg), DDP (0.1, 1, 10 and 100µg), 5-FU (0.7, 7, 70 and 700µg), MMC (0.1, 1.0, 10 and 100µg) and VCR (0.1, 1.0, 10 and 100µg) according to the clinically established plasma peak concentration. Three days later, 20µL MTT solution (5g/L) was put into plates, and then the supernatant was discarded after 4 hours, 150µL DMSO was added to melt crystal. A value was read at 590 nm wavelength.

FACS analysis Cells $(3-4) \times 10^7/L$ were seeded in 25cm² tissue culture flasks for 36h-40h, after reaching log growth, cells were digested by 2.5g/L trypsin and harvested by centrifugation. Then the cells were fixed with ethanol and stained with propidium iodide, and subjected to fluorescence-activated cell sorting (FACS) analysis for DNA content at the Fourth Military Medical University.

In vitro apoptosis assay Cells $(3-4) \times 10^7/L$ were seeded in 25cm² tissue culture flasks and allowed to reach log growth for 36h-40h. After exposure to apoptosis inductor VM-26 for 24h at two different doses 1.25µmol/L and 2.5µmol/L, subsequently culture the cells in RPMI 1640 supplemented with 100mL/L fetal bovine serum containing no VM-26 for 48h, then harvest the cells for genomic DNA extraction, and electrophoresis in 10g/L agarose gel at 50V for 1h^[2].

Immunocytochemistry Tissue immunocytochemical staining was done with the ABC kit. Cells $(3-4) \times 10^7/L$ were seeded in a glass slide in 100g/L RPMI 1640. After the cells attached tightly to the slides, and reached log growth for 36h-48h, slides were washed with PBS for 3-5 min, then immersed in 40g/L paraformaldehyde phosphated buffer solution for 5-10 min. Slides were immersed for 45min in 3mL/L peroxide in methanol (to deplete endogenous peroxidase) and normal goat serum diluted to 1:100 in PBS for 20min. Primary antibodies against P-gp, TopoII and control normal mouse serum were used at dilution of 1:100, applied to cell attached slides and incubated in a humidified chamber at a room temperature for 30min and 4°C

overnight. The slides were then washed with PBS for 10min. Biotinylated goat anti-mouse IgG was applied on to the cell sections, and incubated at room temperature for 30min. After washing with PBS for 10min, these tissues were incubated with avidin DH-Biotinylated peroxidase for 45min. Finally, color was developed by immersion of the sections in a peroxidase substrate solution DAB.

For each slide, the extent and intensity of staining with P-gp and TopoII antibody was graded on a scale of 0-4+ by a blinded observer on two separate occasions using coded slides, and an average score was calculated. The observer assessed all tissues on the slides to assign the scores. A 4+ grade implied that all staining was maximally intense throughout the specimen, while 0 implied that staining was absent throughout the specimen.

RESULTS

Western blot

SDS-PAGE (Figure 1A) showed no significant differences between *fas*-SGC7901/VCR protein and that of pBK-SGC7901/VCR control cells. Western blot (Figure 1B) indicated a weak band of M_r 36000-40 000 in lane 3 which is consistent with M_r of Fas protein, when 150 μ g total protein of pBK-SGC7901/VCR was loaded, while lanes 4 and 5 of *fas* transfectants had strong positive band of M_r 36 000-40 000 respectively, the band of lane 4 was slightly weaker than that of lane 5 because of quantity of loading.

Cytotoxic assay

As shown in Figure 2, *fas*-SGC7901/VCR cell transfectants were more sensitive to MMC, DDP and 5-FU. The survival rates of transfectants treated with VCR and Dox were only lowered slightly, compared with non-transfectants, which showed that *fas*-SGC7901/VCR cell transfectants remained resistant to VCR and Dox.

FACS analysis

By using FCM to analyze cell cycle, the apoptotic peak appeared only in *fas*-SGC7901/VCR cell transfectants and there were no significant differences between them in G1 phase. These data show that Fas takes part in induction of apoptosis and differentiation of gastric cancer cells. Analysis of cell cycle distribution demonstrated that the fraction number of *fas*-SGC7901/VCR cells in G1/0 phase, G2 and S phase was about 0.70, 0.06 and 0.24, that of pBK-SGC7901/VCR was about 0.67, 0.17 and 0.13 respectively, and the G2 phase fraction of pBK.SGC7901/VCR was about 3.0 times that of *fas*-SGC7901/VCR, but S phase fraction of *fas*-SGC7901/VCR was about 1.9 times that of pBK-SGC7901/VCR, indicating S phase arrest of *fas*-SGC7901/VCR (Table 1).

Table 1 Cell cycle distribution of three kinds of cell lines (Number fraction)

Cell line	G1	G2	S	G1/G2
SGC7901	0.73	0.11	0.16	0.188
pBK-SGC7901/VCR	0.67	0.17	0.13	0.186
<i>fas</i> -SGC7901/VCR	0.70	0.06	0.24	0.190

In vitro apoptosis assay

As shown in Figure 3, *fas*-SGC7901/VCR cell transfectants remained sensitive to VM-26, and characteristic DNA ladder bands of apoptosis appeared.

Immunocytochemistry

In order to assess whether the overexpression of Fas in drug resistant gastric cancer cell line SGC7901/VCR involved inhibition of other MDR inducing factors except induction of apoptosis, we detected topoisomerase II (Topo II) and P-gp protein expression in these cell lines by immunocytochemistry. There was no difference in the expression of Topo II among SGC7901, pBK-SGC7901/VCR and *fas*-SGC7901/VCR. All these had positive staining of Topo II, and an average score of about 2+ grades was calculated. P-gp staining on pBK-SGC7901/VCR was stronger than that on SGC7901, and scored a grade of 4+. But there was weak staining of P-gp on *fas*-SGC7901/VCR (Figure 4). These data suggested that Fas proteins increased sensitivity of drug resistant gastric cancer cell line SGC7901/VCR to chemotherapeutic drugs may be through decreasing P-gp protein.

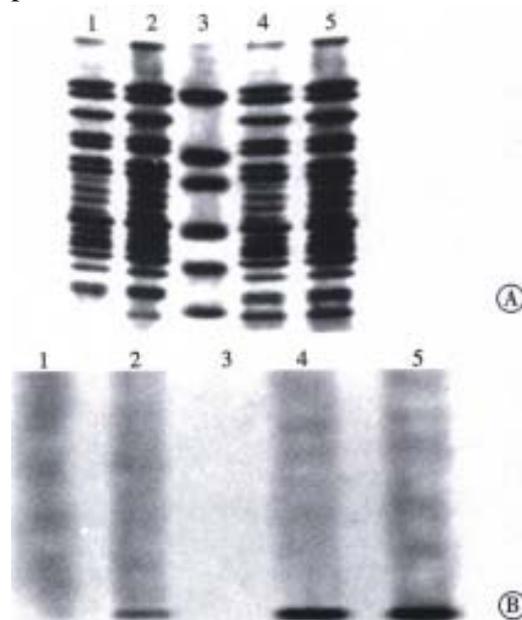


Figure 1 SDS-PAGE and Western blot of Fas in three kinds of cell lines.

A: SDS-PAGE B: Western blot

1. Total protein (100 μ g) extracted from pBK-SGC7901/VCR
2. Total protein (150 μ g) extracted from pBK-SGC7901/VCR
3. Marker ($M_r \times 10^3$)
4. Total protein (100 μ g) extracted from *fas*-SGC7901/VCR
5. Total protein (150 μ g) extracted from *fas*-SGC7901/VCR

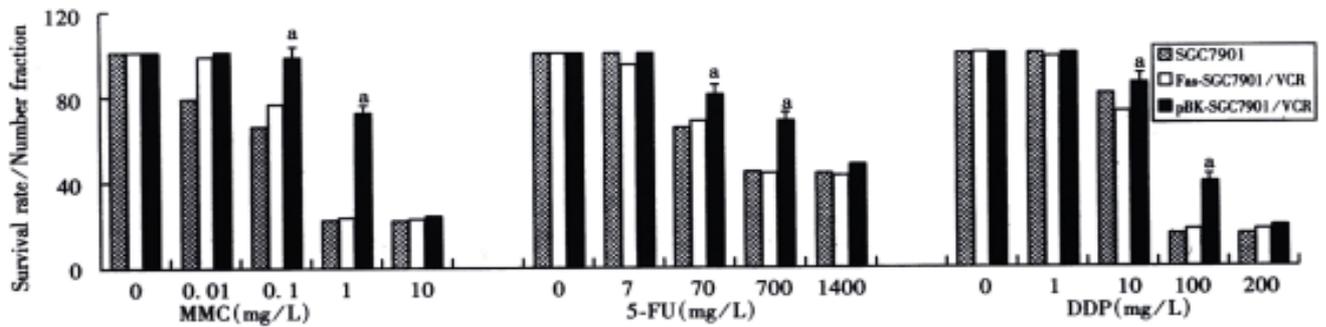


Figure 2 Survival rates of three kinds of cells treated with chemotherapeutic drugs (%).
^aP<0.05, vs fas-SGC7901/VCR



Figure 3 Agarose gel electrophoresis of apoptotic cells DNA.
 1,2: pBK-SGC7901/VCR; 3,4: fas-SGC7901/VCR; 1,3: VM-26 (1.25μmol/L); 2,4: VM-26 (2.5μmol/L)

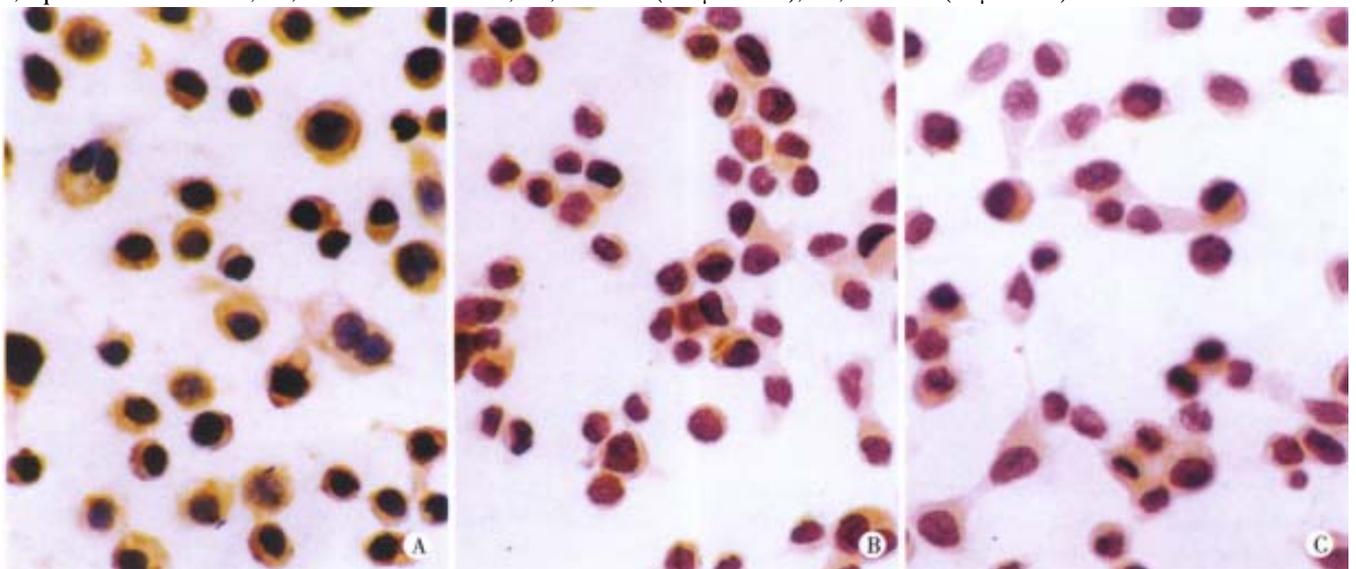


Figure 4 Detection of P-gp expression in the 3 tumor cells lines by immunocytochemical staining. ×400
 A: pBK-SGC7901/VCR; B: SGC7901; C: fas-SGC7901/VCR

DISCUSSION

Apoptosis is an important and well-controlled form of cell death that occurs under a variety of physiological and pathological conditions. This process has been recognized to be of major importance for embryonic development, tissue homeostasis, neurodegeneration, autoimmune diseases, AIDS, carcinogenesis, cancer progression,

specially for killing cancer cells induced by chemotherapeutic drugs^[3-9]. Once the apoptosis program is activated, it starts with blebbing of the membrane, followed by degradation of the chromosomal DNA by nucleases, resulting in condensation and fragmentation, finally, phagocytes remove cell fragments without causing any inflammatory reaction. Some proto-oncogenes

and tumor suppressor genes can regulate apoptosis^[10-18] and there are also accumulated evidences which show that these apoptosis regulating genes are important for the development of digestive system^[19-24].

Multidrug resistance represents a major obstacle in the successful therapy of neoplastic diseases. Studies have demonstrated that this form of drug resistance occurs both in cultured tumor cell lines as well as in human cancers. Recent studies indicate that genes that regulate apoptotic cell death might play an important role in determining the sensitivity of tumor cells to chemotherapy. Many chemotherapeutic agents are found to act through damaging DNA or regulating apoptosis related genes *p53*, *c-myc*, *bcl-2*, *bax*, *p21*, *c-H-ras* to trigger apoptosis^[25-28].

Strobel *et al*^[29] found that SW626 cells overexpressing BAX are sensitized to the cytotoxic effects of alkaloid derivatives such as paclitaxel and Vincristine, and demonstrate that these seemingly disparate effects are explained by an enhanced accumulation of paclitaxel in BAX-overexpressing cells, an effect due to diminished drug efflux. These data suggest that stable transfectants that overexpress BAX may be sensitized to apoptotic cell death through a novel mechanism involving the enhancement of intracellular levels of naturally occurring toxins. NicA *et al*^[30] used an *in vitro* model, the resistant variant, DLKP-SQ/10p derived from human lung carcinoma clonal cell line DLKP-SQ to 10 sequential pulses of pharmacologically attainable doses of doxorubicin, demonstrating that overexpression of the pro-apoptotic *bcl-xS* gene in the DLKP-SQ/10p line partially reversed resistance not only to P-gp-associated drugs, doxorubicin, but also to 5-fluorouracil, indicating that the ratio of *bcl* family members may be important in determining sensitivity to chemotherapeutic drug-induced apoptosis.

Recently mutated *N-ras* oncogene has been implicated in melanoma resistance to cisplatin, both *in vitro* and *in vivo*, and the role of two other oncogenes, *bcl-2* and *p53*, which are already involved in the chemoresistance of hematological and solid malignancies. The finding that many chemotherapeutic agents can kill susceptible cells through the apoptosis pathway provides new molecular insight into chemoresistance mechanisms and suggests that apoptosis and/or resistance to apoptosis of tumor cells should be investigated to clarify further the mechanism of tumor chemoresistance^[31].

Anti-cancer drugs and cytotoxic cytokines such as members of the TNF/Fas-ligand family play a predominant role in apoptosis induction in tumor cells including gastric cancer^[32,33]. And compared

with normal tissues, malignant tumor cells, especially metastatic tumor cells present extremely lower expression of Fas, while most benign tumors are similar to their original tissues in the expression of Fas. It is hypothesized that malignant tumors might suppress Fas expression or lose Fas molecule in order to avoid the surveillance of Fas ligand. This explains the lack of Fas expression in drug resistant cells. Our laboratory found that Fas-antigen expression in human gastric cell line SGC7901/VCR is lower than that of SGC7901, and it seems that the alteration of Fas expression following drug-resistance is not restricted to one cell type. Cai *et al*^[34] also found that after being treated by anti-Fas in the presence of IFN-gamma, human breast carcinoma cell line MCF7/Adr (resistant to adriamycin) was resistant to Fas-mediated apoptosis, and this resistance was correlated with a loss of surface Fas-protein expression^[35]. Furthermore, the down-regulation of Fas expression and subsequent resistance to anti-Fas were observed in other drug-resistant cancer cell lines including human ovarian-carcinoma IGR-OV1/VCR cells and leukemic lymphoblast CEM/VLB cells. But no evidence shows whether the down-regulation of Fas expression increases resistance to not only anti-Fas, but also to other chemotherapy drugs, and whether there is crosstalking between MDR signaling and Fas expression.

We investigated whether the CD95/CD95L system plays a role in this process. We used the human gastric cell line SGC7901, and its derivative SGC7901/VCR (induced by sequential pulses of high dose of VCR and resistant to VCR) to determine the impact of acquired chemotherapeutic drug and cytokine resistance on susceptibility to Fas-induced cytotoxicity.

CD95 (Fas/APO-1) is a death-promoting receptor that belongs to the tumor necrosis factor (TNF) receptor I family^[36,37]. Triggering of the CD95 molecule by either agonistic antibodies or the natural ligand CD95L (FasL) induces apoptosis^[38]. Ligand binding induces trimerization of CD95, and the trimerized cytoplasmic region then transduces the signal by recruiting a molecule called FADD (Fas-associating protein with death domain) or MORT1 (mediator of receptor-induced toxicity), which binds to CD95 via interaction of the death domain at its COOH terminus^[39-43]. The NH₂-terminal region of FADD is responsible for downstream signal transduction by recruitment of a protein called FLICE (FADD-like interleukin-1 β -converting enzyme [ICE]) or MACH (MORT1-associated CED-3 homologue), recently designated as caspase-8^[44-46]. The NH₂ terminus of caspase-8 binds to FADD/MORT1, while its COOH-terminal region is related to the caspase-3 (CPP32) subfamily. Therefore, FLICE/ MACH

preferentially cleaves caspase-3 substrates such as poly (ADP) ribose polymerase (PARP)^[45], and ICE proteases including CPP32^[47].

In this study, there is evidence that Fas can increase sensitivity to the cytotoxic effects of therapeutic drugs through increasing cell arrests of S phase. MTT assays show that overexpression of Fas could increase sensitivity of gastric cancer cells, to the cytotoxic effects of topoisomerase II-directed chemotherapeutic agents such as VM-26, but not doxorubin. It is suggested that transduction of pro-apoptosis Fas gene into drug resistant cells is obviously helpful in increasing the drug sensitivity.

Considerable evidence has indicated that the multidrug transporter or P-glycoprotein plays a role in the development of simultaneous resistance to multiple cytotoxic drugs in cancer cells^[48-50]. P-glycoprotein appears to act as an energy-dependent efflux pump to remove various natural product drugs from the cells before they have a chance to exert their cytotoxic effects. But expression of the MDR1 gene product has been associated with a poor prognosis in clinical studies. To obtain insight into the mechanism of drug resistance, the expression of P-170 glycoprotein as potentially able to contribute to drug resistance was investigated in these three kinds of cell lines by immunochemical technology. We found that P-glycoprotein (P-gp) is overexpressed in multidrug resistant human gastric cancer cell line SGC7901/VCR than SGC7901, but there was little expression of P-gp on fas-SGC7901/VCR.

These data suggest that stable transfectants that overexpress Fas may be sensitized to apoptotic cell death through a novel mechanism involving the enhancement of intracellular levels of naturally occurring toxins such as alkaloid derivatives.

These results revealed that fas-gene transduction could reverse the MDR of human drug-resistant gastric cancer cell SGC7901/VCR to a degree, partly because of Fas induced decreased expression of P-gp.

Other observations suggest that P-gp may play a specific role in regulating some caspase-dependent apoptotic pathways, and there is a complicated cross talking between Fas and P-gp. Researchers found that classical multidrug resistant human CEM and K562 tumor cell lines expressing high levels of P-gp were less sensitive to multiple forms of caspase-dependent cell death, including that mediated by cytotoxic drugs and ligation of Fas, and inhibition of P-gp function also enhanced drug or Fas-mediated activation of caspase-3 in drug-resistant CEM cells^[51].

In this study we have demonstrated that inducing apoptosis by *fas* transduction correlates with the development of sensitivity to MMC, DDP, 5-FU to a degree, and Fas can repress P-gp through a series of cross-linked signal transduction. Therefore, apoptosis inducing gene products may

not only increase sensitivity to the cytotoxic effects of chemotherapeutic agents by means of apoptosis, but also through other signal transduction. So gene therapy of introducing pro-apoptosis inducing gene may be a good method to overcome MDR in tumors. However, the mechanism of MDR is much complicated, and in tumors the drug resistance is multifactorial. Research to overcome this resistance should therefore be directed towards a combined treatment that eliminates all of these.

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Fish sauce and gastric cancer: an ecological study in Fujian Province, China

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Subject headings stomach neoplasm/etiology; food habits; risk factors; nitrosamines; epidemiology; fish products; fish sauce; carcinogens

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Abstract

AIM To explore the relationship between consumption of fish sauce and the risk of gastric cancer in Fujian Province.

METHODS An ecological study was carried out. A total of 11 000 subjects from 55 townships were randomly selected from 10 counties within Fujian Province. All subjects were local residents who had been living in Fujian Province for more than 20 years, within the age group of 45-74 years. Trained interviewers conducted face-to-face interviews with a standardized questionnaire, which covered the frequency and amount of food intake, dietary habit, tobacco and alcohol consumption and history of chronic gastric diseases. Univariate and multivariate analyses were performed using Epi-info and SAS statistical packages, respectively.

RESULTS A significant correlation between monthly consumption of fish sauce and mortality of gastric cancer was found. Pearson's coefficient of correlation was statistically significant with $r = 0.7356$ for males, $r = 0.5246$ for females ($P < 0.01$). In the multivariate analysis, consumption of fish sauce still showed an association with the risk of gastric cancer. No significant positive correlation between esophagus cancer, liver cancer, colon cancer and consumption of fish sauce were observed.

CONCLUSION Long-term intake of fish sauce may be related to high mortality of gastric cancer. Consumption of fish sauce might be one of important and unique etiologic factors of gastric cancer in Fujian Province. Further studies are needed to confirm this ecological study.

INTRODUCTION

Fujian Province is a high-risk area of gastric cancer in China^[1]. The standardized mortalities of gastric cancer during 1986-1988 were 37-72 per 100 000 for men and 14-90 per 100 000 for women, accounting for 25.31% of total cancer mortality. In certain areas, the annual incidence of gastric cancer is more than 50 per 100 000 persons^[2]. The health of general population in Fujian Province is most seriously threatened by gastric cancer.

A number of potential risk factors for gastric cancer have been examined in previous studies^[3]. Among these, the dietary hypotheses are of particular interest^[4,5]. N-nitroso compounds derived from the consumption of preserved food may be associated with the risk of the disease^[6].

In the investigation of dietary factors, we have reported the relationship between high salt intake and gastric cancer in the high-risk area of Fujian Province^[7-9]. The salted food includes fish sauce, which is particularly favored by Fujian local residents. Fish sauce is usually produced from several kinds of sea fishes after prolonged fermentation processes. Its mutagenicity has been reported by several experimental studies^[10-12]. However, there have been few reports from population-based epidemiological study on the relationship between fish sauce and risk of gastric cancer. Therefore, this ecological study was carried out to explore the relationship between them in the area of Fujian Province.

MATERIALS AND METHODS

Study areas, subjects and methods

Gastric cancer is the most common cancer among the major types of cancer in Fujian Province. However, even within the province the death rate distribution of gastric cancer varies greatly among different areas. This ecological study included 55 townships in 10 counties. The retrospective investigations of death cause, which was a part of a national program, had been concluded in these areas.

In reference to the national gastric cancer death rate of 17.30/100 000 (95% confidence interval: 9.15/100 000-25.45/100 000, calculated by Poisson-normal-approximation method), the death rate among these 10 counties can be divided into three groups: ① High incidence area: Changde, Putian, and Fuzhou; ② Medium incidence area: Changtai, Huian, Shaxian, Sanming, Yongding, and Zhangpu; ③ Low incidence area: Fuan (Figure 1).

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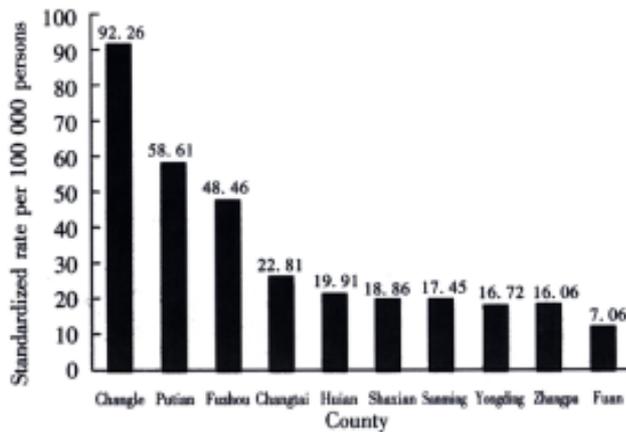
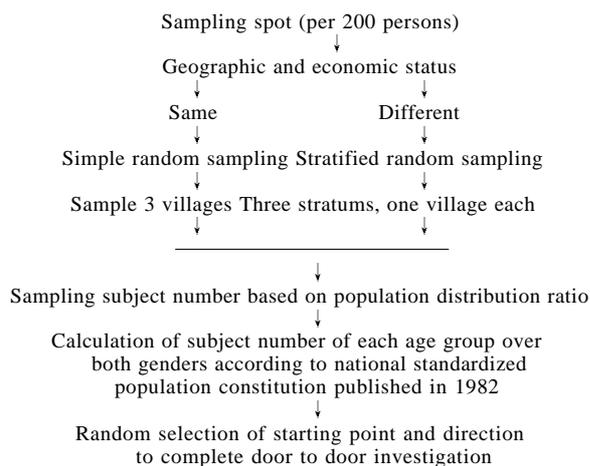


Figure 1 Gastric cancer mortality in 10 counties, Fujian, China, 1991.

A total of 200 subjects in each town (sampling spot) were interviewed. The 55 townships were randomly selected from 10 counties which include 11 000 subjects in this study.



All subjects of this study are local resident of more than 20 years, within the age of 45-74 years, who can clearly answer the questions asked by the interviewers. Specially trained interviewers conducted face-to-face interviews according to a standardized questionnaire, which covers the frequency and amount of food intake. The investigations also include demographic factors, dietary habit, smoking and alcohol consumption, and history of chronic gastric diseases.

Statistical analysis

The database was established using Epi-info, and the gastric cancer mortality for 45-74 age group was calculated based on world truncated standardized population constitution. The correlation coefficient and multiple regression analysis were carried out using SAS software.

RESULTS

Univariate analysis

The highest Pearson's correlation coefficient was found between gastric cancer mortality and average monthly consumption of uncooked fish sauce. Similar results were observed for both males and females, with Pearson's correlation coefficient for males of 0.7356 and 0.5246 for females ($P < 0.01$) (Table 1). Positive correlation were found between gastric death rate and fish sauce-related variables (Fish sauce consumption, population ratio who consume fish sauce, population ratio who consume uncooked fish sauce, and uncooked fish sauce consumption). Further, compared with other factors, larger Pearson's correlation coefficients were observed for all 4 variables ($\chi^1 - \chi^4$).

Table 1 Correlation analysis between exposure factors and standardized truncated mortality of gastric cancer (55 townships)

Factors	Pearson's coefficient of correlation	
	Male	Female
χ^1 Fish sauce average consumption (0.5kg/month)	0.5170 ^a	0.4261 ^a
χ^2 Population ratio who consume fish sauce	0.3576 ^a	0.2720 ^a
χ^3 Population ratio who consume uncooked fish sauce	0.6904 ^a	0.5086 ^a
χ^4 Uncooked Fish sauce average consumption (0.5kg/month)	0.7356 ^a	0.5246 ^a
χ^5 Salted fermented sea foods average consumption (0.5kg/year)	0.4863 ^a	0.3653 ^a
χ^6 Population ratio of crapulence	0.4887 ^a	0.3198 ^a
χ^7 Population ratio of taking moldy foods	0.5538 ^a	0.2582
χ^8 Fresh vegetables consumption (0.5kg/year)	-0.4742 ^a	-0.2307
χ^9 Fresh meat, fish, egg average consumption (0.5kg/year)	0.4254 ^a	0.1298
χ^{10} Average ratio of coarse food grain to grain ration	0.3900 ^a	0.2107
χ^{11} Prevalence of chronic gastric diseases	0.2973 ^b	0.1107
χ^{12} Average index of smoking	-0.2158	0.1737
χ^{13} Average cumulative alcohol consumption (0.05kg)	0.1703	-0.1942
χ^{14} Population ratio of taking irregular dinners	0.2172	0.1443
χ^{15} Average salted vegetables consumption (0.5kg/year)	-0.1488	-0.0079
χ^{16} average salt consumption (0.5kg/month)	-0.2455	-0.2160
χ^{17} Average fresh fruit consumption (0.5kg/year)	0.0978	-0.0581
χ^{18} Average bean products consumption (0.5kg/year)	-0.1475	-0.0422

^a $P < 0.01$, ^b $P < 0.05$.

Smoking index: (amount of smoking/day × years of smoking)/age of starting smoking.

Gastric cancer mortality for 45-74 age group was calculated based on world truncated standardized population constitution.

Multi-variables analysis

Considering the possible confounding effects among factors, multivariate regression analyses were performed on other factors ($\chi^5 - \chi^{18}$) and each of fish sauce variables ($\chi^1 - \chi^4$) respectively. MAXR method from REG of SAS package was used. The optimum subset of variables was established which also includes fish sauce as one of variables. The consumption of fish sauce still shows association with the risk of gastric cancer in analysis after controlling the possible confounding factors (Tables 2-5). The corresponding multiple correlation coefficients indicate that the goodness of fit tests was significant.

Table 2 The results of multivariate regression analysis on χ^1 , χ^5 - χ^{18} and mortality of gastric cancer for men

Factor	β	S β	Standard error	t	P
χ^1	187.363 9	0.467 8	43.167 4	4.340	0.000 1
χ^{13}	0.030 6	0.310 5	0.010 1	3.018	0.004 1
χ^{11}	861.782 4	0.571 3	241.989 1	3.561	0.000 8
χ^7	338.206 0	0.181 9	160.782 6	2.103	0.040 7
χ^{14}	421.516 6	0.332 9	132.587 1	3.179	0.002 6
χ^8	-0.804 2	-0.612 7	0.225 0	-3.574	0.000 8
χ^{17}	-2.633 9	-0.252 7	1.103 6	-2.387	0.021 0
Source of variation	DF	Sum of squares	Mean Square	F value	P
Regression	7	4 802 841.324 9	686 120.189 3	47.482	0.000 1
Error	48	693 599.320 9	14 449.985 9		
Total	55	5 496 440.645 8			

$R^2 = 0.873 8$ Adjusted $R^2 = 0.855 4$

Table 3 The results of multivariate regression analysis on χ^4 , χ^5 - χ^{18} and mortality of gastric cancer for men

Factor	β	S β	Standard error	t	P
χ^4	271.596 1	0.449 1	45.488 3	5.971	0.000 1
χ^{13}	0.024 8	0.252 1	0.009 2	2.696	0.009 6
χ^{11}	671.680 3	0.445 3	223.110 6	3.011	0.004 1
χ^7	285.953 7	0.153 8	143.870 8	1.988	0.052 6
χ^{14}	366.509 3	0.289 4	119.231 5	3.074	0.003 5
χ^8	-0.539 4	-0.410 9	0.212 4	-2.539	0.014 4
χ^{17}	-1.259 9	-0.120 9	0.856 9	-1.470	0.148 0
Source of variation	DF	Sum of squares	Mean Square	F value	P
Regression	7	4 942 225.615 6	706 052.230 8	61.149	0.000 1
Error	48	554 215.030 1	11 546.146 5		
Total	55	5 496 440.645 6			

$R^2 = 0.899 2$ Adjusted $R^2 = 0.884 5$

Table 4 The results of multivariate regression analysis on χ^1 , χ^5 - χ^{18} and mortality of gastric cancer for women

Factor	β	S β	Standard error	t	P
χ^1	81.232 7	0.502 0	24.717 9	3.286	0.001 9
χ^{11}	417.192 2	0.432 8	122.121 2	3.416	0.001 3
χ^{14}	132.182 7	0.258 2	44.083 4	2.998	0.001 5
χ^5	1.270 2	0.163 9	0.920 9	1.379	0.173 9
χ^{17}	-1.855 8	-0.404 5	0.595 8	-3.115	0.003 0
Source of variation	DF	Sum of squares	Mean Square	F value	P
Regression	5	646 897.444 8	129 379.489 0	34.871	0.000 1
Error	50	185 511.738 5	3 710.234 8		
Total	55	832 409.183 3			

$R^2 = 0.777 1$ Adjusted $R^2 = 0.754 9$

Table 5 The results of multivariate regression analysis on χ^4 , χ^5 - χ^{18} and mortality of gastric cancer for women

Factor	β	S β	Standard error	t	P
χ^4	100.238 2	0.409 0	26.013 2	3.853	0.000 3
χ^{11}	448.485 9	0.465 8	124.452 2	3.604	0.000 7
χ^{14}	153.932 8	0.300 7	43.506 8	3.538	0.000 9
χ^{15}	2.382 6	0.307 4	0.956 5	2.491	0.016 1
χ^{18}	-0.934 3	-0.407 8	0.385 3	-2.425	0.019 0
Source of variation	DF	Sum of squares	Mean Square	F value	P
Regression	5	657 252.078 6	31 450.415 7	37.52	0.000 1
Error	50	175 157.104 7	3 503.142 1		
Total	55	832 409.183 3			

$R^2 = 0.789 6$ Adjusted $R^2 = 0.768 5$

Correlation analysis between exposure factor and other digestive tract cancers

The Pearson's correlation coefficients between each factor and truncated standardized mortality for esophagus cancer, liver cancer, and colon cancer were calculated separately and results are shown in Tables 6 - 8. There are no significant positive correlation among 4 variables for fish sauce consumption and any of the mortality rates among three other digestive tract cancers. In multivariate regression analysis, the variables that reflect fish

sauce consumption were not selected into the model when analyzing relationship between fish sauce and mortality of the other three cancers.

Table 6 Correlation analysis between exposure factors and truncated standardized mortality of esophagus cancer

Factors	Male		Female	
	Pearson's r	P	Pearson's r	P
χ^1	-0.251 1	0.064 4	-0.307 9	0.022 2
χ^2	-0.253 8	0.061 5	-0.364 7	0.006 2
χ^3	-0.217 0	0.111 5	-0.251 9	0.063 6
χ^4	-0.171 4	0.210 9	-0.221 8	0.103 7
χ^5	-0.039 0	0.777 8	-0.098 8	0.473 0
χ^6	-0.071 5	0.604 2	0.036 0	0.793 9
χ^7	0.302 5	0.024 8	0.213 0	0.118 4
χ^8	-0.361 8	0.006 6	-0.227 4	0.095 0
χ^9	0.060 9	0.659 0	-0.175 1	0.201 0
χ^{10}	0.357 1	0.007 4	0.173 1	0.206 2
χ^{11}	-0.349 3	0.009 0	0.125 2	0.362 5
χ^{12}	0.533 0	0.000 1	0.059 2	0.667 7
χ^{13}	-0.068 6	0.618 7	0.372 5	0.005 1
χ^{14}	0.329 2	0.014 1	0.277 1	0.040 6
χ^{15}	0.260 6	0.054 6	0.474 0	0.000 3
χ^{16}	0.638 4	0.000 1	0.374 3	0.004 9
χ^{17}	-0.256 9	0.058 4	-0.275 9	0.041 5

Esophagus cancer mortality for 45-74 age group was calculated based on world truncated standardized population constitution.

Table 7 Correlation analysis between exposure factors and truncated standardized mortality of liver cancer

Factors	Male		Female	
	Pearson's r	P	Pearson's r	P
χ^1	-0.091 3	0.507 4	-0.140 7	0.305 5
χ^2	-0.142 0	0.301 0	-0.180 8	0.186 5
χ^3	-0.023 7	0.863 9	-0.101 7	0.459 9
χ^4	0.006 1	0.964 8	-0.097 0	0.481 3
χ^5	0.222 6	0.102 8	0.191 5	0.161 4
χ^6	0.067 9	0.622 1	0.254 6	0.060 7
χ^7	0.411 3	0.001 8	0.214 1	0.116 6
χ^8	-0.328 0	0.014 5	-0.168 1	0.219 9
χ^9	0.120 0	0.383 0	0.073 7	0.592 7
χ^{10}	0.362 7	0.006 5	0.160 9	0.240 7
χ^{11}	0.057 0	0.679 4	0.159 4	0.245 1
χ^{12}	0.135 2	0.324 9	0.059 5	0.666 1
χ^{13}	-0.012 0	0.941 2	-0.245 3	0.071 0
χ^{14}	-0.002 4	0.986 3	0.156 9	0.252 7
χ^{15}	0.063 0	0.647 7	0.293 6	0.029 6
χ^{16}	0.196 7	0.150 1	0.094 6	0.492 2
χ^{17}	-0.106 5	0.438 9	-0.005 3	0.969 3

Liver cancer mortality for 45-74 age group was calculated based on world truncated standardized population constitution.

Table 8 Correlation analysis between exposure factors and truncated standardized mortality of colon cancer

Factors	Male		Female	
	Pearson's r	P	Pearson's r	P
χ^1	0.246 1	0.070 1	0.107 7	0.434 1
χ^2	0.235 6	0.083 3	0.105 5	0.443 3
χ^3	0.242 8	0.074 1	0.053 5	0.698 2
χ^4	0.225 0	0.098 6	0.010 3	0.940 8
χ^5	0.003 5	0.979 6	-0.022 3	0.871 8
χ^6	-0.050 4	0.714 8	-0.012 7	0.926 6
χ^7	0.096 4	0.483 8	-0.124 2	0.366 2
χ^8	-0.160 5	0.241 8	0.127 4	0.353 9
χ^9	0.083 3	0.521 5	0.065 2	0.636 2
χ^{10}	-0.097 4	0.491 5	-0.248 2	0.067 7
χ^{11}	0.384 3	0.003 8	0.225 1	0.098 5
χ^{12}	-0.281 5	0.037 4	0.027 7	0.841 2
χ^{13}	-0.189 5	0.165 9	-0.181 9	0.183 9
χ^{14}	0.218 5	0.109 1	-0.061 1	0.657 9
χ^{15}	-0.289 9	0.031 8	-0.055 4	0.687 9
χ^{16}	-0.165 4	0.227 4	-0.224 7	0.099 0
χ^{17}	0.205 3	0.132 7	0.178 7	0.191 9

Colon cancer mortality for 45-74 age group was calculated based on world truncated standardized population constitution.

DISCUSSION

Gastric cancer is the second common cancer in the world today^[13]. It remains the leading cause of cancer death in China^[14]. In the studies of etiology and epidemiology on gastric cancer, the associations between gastric cancer risk and *Helicobacter pylori* (Hp) infection^[15-18], occupational exposures^[19], diet and life factors^[20-25], lack of essential trace elements^[26] have been observed. The most widely reported protective factors are dietary factor^[27-33]. It has been reported that a large amount of nitrite exists in most salted fishes and smoking meat, and may be related to the high gastric cancer incidence along coastal area and among Japanese or Chinese population.

Each local resident in Fujian Province consumed daily about 30mL fish sauce as one kind of condiments. Fish sauce is a liquid product of small marine fish and table salt (7:3). The fishes are completely liquefied after fermentation for 1-2 years. The salted fermented fish products may contain many precursors of N-nitroso compound derived from the proteins content of the fishes, which could synthesize N-nitrosamides under simulated human stomach conditions^[34]. N-nitrosamides is suggested as a major initial cause of gastric cancer^[35]. Zhang *et al*^[36] had analyzed the N-nitroso compound precursors from 49 fish sauce samples obtained from high risk area of gastric cancer in Fujian Province, and detected 630 μ M/mL of 17 free amino acids. After nitrosification the total amount of N-nitroso compound in samples was as high as 2.95 μ M/mL. The concentration of N-nitrosylamine from ethyl acetate extraction solution was 0.06 μ M/mL. Fish sauce is rich in creatinine and other nitrosamide precursors. It was mutagenic and carcinogenic for the glandular stomach of Wistar rats, after it was nitrosified under simulated human stomach condition^[37]. All these experiments have shown that fish sauce is potentially carcinogenic.

High incidence of gastric cancer may be attributed, to some extent, to some unique dietary habits in the high-risk area. This ecological study showed the positive relationship between gastric cancer mortality and fish sauce favored by Fujian residents. The relationship between gastric cancer and uncooked fish sauce intake is particularly obvious. Among four digestive tract cancers, only gastric cancer had a positive correlation with fish sauce consumption, which suggests that the association may be specific to gastric cancer. This study indicates that long-term use of fish sauce may be related to high gastric cancer mortality in the Fujian area.

This ecological comparison study may provide a possible hypothesis for the etiology of gastric cancer. Fish sauce has both N-nitroso compounds and a high salt content, which might partially

explain the high gastric cancer mortality along the Fujian coast. Further studies are needed to confirm our results. Recently, a population-based case-control study was conducted in Changle County, Fujian Province. The residents in Changle County had a high prevalence of *H. pylori* infection. However, no statistical significant difference was found in respect to presence of *H. pylori* infection between gastric cancer patients and controls. The results showed that fish sauce intake, deficiency in fresh vegetables were risk factors in gastric cancer^[38].

The development of human gastric cancer is a multistep and multifactorial process^[39]. A number of molecular events are involved in gastric carcinogenesis^[40]. Coordinate prevention and treatment measures must be taken, including changing the habit of taking fish sauce, nutrition guidance and cancer prevention education for general public. This would undoubtedly have positive effect on reducing the risk of gastric cancer in Fujian Province.

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Effects of tachyplesin on the morphology and ultrastructure of human gastric carcinoma cell line BGC-823

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Subject headings stomach neoplasms/ultrastructure; horseshoe crabs; stomach neoplasms; tachyplesin; microscopy, electron; cell morphology

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Abstract

AIM To investigate the morphological and ultrastructural changes in the human gastric carcinoma cell line BGC-823 after being treated with tachyplesin.

METHODS Tachyplesin was isolated from acid extracts of Chinese horseshoe crab (*Tachypleus tridentatus*) hemocytes. BGC-823 cells and the cells treated with 2.0mg/L tachyplesin were examined respectively under light microscope, scanning and transmission electron microscope.

RESULTS BGC-823 cells had undergone the restorational alteration in morphology and ultrastructure after tachyplesin treatment. The changes were as follows: the shape of cells was unanimous, the volume enlarged and cells turned to be flat and spread, the nucleocytoplasmic ratio lessened and nuclear shape became rather regular, the number of nucleolus reduced and its volume lessened, heterchromatin decreased while euchromatin increased in nucleus. In the cytoplasm, mitochondria grew in number with consistent structure relatively, Golgi complex turned to be typical and well-developed, rough endoplasmic reticulum increased and polyribosome decreased. The microvilli at cellular surface were rare and the filopodia reduced while lamellipodia increased at the cell edge.

CONCLUSION Tachyplesin could alter the malignant morphological and ultrastructural characteristics of human gastric carcinoma cells effectively and have a certain inducing differentiation effect on human gastric carcinoma cells.

INTRODUCTION

Horseshoe crab, a kind of marine animals, is a "live fossil" which has an unique status in evolution. It has many bioactive substances with special functions due to its primitive character. In recent years, many bioactive substances, including clotting factors, protease inhibitors, antibacterial substances, lectins and others, have been found in the hemocytes and hemolymph plasma of horseshoe crab^[1]. To seek the low molecular weight antitumor substances which can intervene in cellular signal transduction and regulate cell proliferation and differentiation^[2], we have isolated and purified a small polypeptide—tachyplesin from the blue blood of Chinese horseshoe crab (*Tachypleus tridentatus*) and appraised its antitumor activities^[3]. Then we have investigated the biological effects of tachyplesin on tumor cells systematically with the model system of human gastric carcinoma cell line BGC-823. This paper deals with the effects of tachyplesin on the morphology and ultrastructure of human gastric carcinoma cells by light microscopy and scanning and transmission electron microscopy.

MATERIALS AND METHODS

Tachyplesin isolation

Tachyplesin was isolated from acid extracts of hemocyte debris of Chinese horseshoe crab as described by Nakamura^[4] with minor modification.

Cell culture and treatment

BGC-823 cells were cultured in RPMI-1640 supplemented with 20% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/L streptomycin and 50mg/L kanamycin at 37°C, 5% CO₂ in air. Then the cells were treated with the culture medium containing 2.0 mg/L tachyplesin after being seeded for 24 hours.

Sample preparation for the light microscopy

BGC-823 cells and the cells treated with 2.0mg/L tachyplesin were seeded in little penicillin bottles with cover slip strip, and grown in the normal culture medium or in the culture medium containing 2.0mg/L tachyplesin at 37°C in 5% CO₂ atmosphere for 72 hours respectively. The cells at cover slip strips were rinsed with D-Hank's solution twice at 37°C, fixed overnight in Bouin-Hollande fixative, stained with Hematoxylin-Eosin, and

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observed under light microscope.

Sample preparation for scanning electron microscopy

BGC-823 cells culture and tachyplesin treatment were performed as the procedures for light microscopy. The cells at cover slip strips were rinsed with D-Hank's solution twice at 37°C, fixed in 2.5% glutaraldehyde for 2 hours and in 1% osmium tetroxide for 1 hour, dehydrated in ethanol, dried through the CO₂ critical point, gilded in vacuum and observed under the HITIACHS-520 scanning electron microscope.

Sample preparation for transmission electron microscopy

BGC-823 cells and the cells treated with 2.0mg/L tachyplesin were rinsed with D-Hank's solution twice, shaved into centrifuge tube with plastic scraper, followed by centrifugation at 2000rpm for 15min, and removed the supernatant. The precipitate was prefixed in 2.5% glutaraldehyde for 2h and postfixed in 1% osmium tetroxide for 2h, dehydrated in ethanol series, embedded in epoxy resin 618, stained with lead citrate and uranyl acetate, and observed under the JEM-100CXII transmission electron microscope.

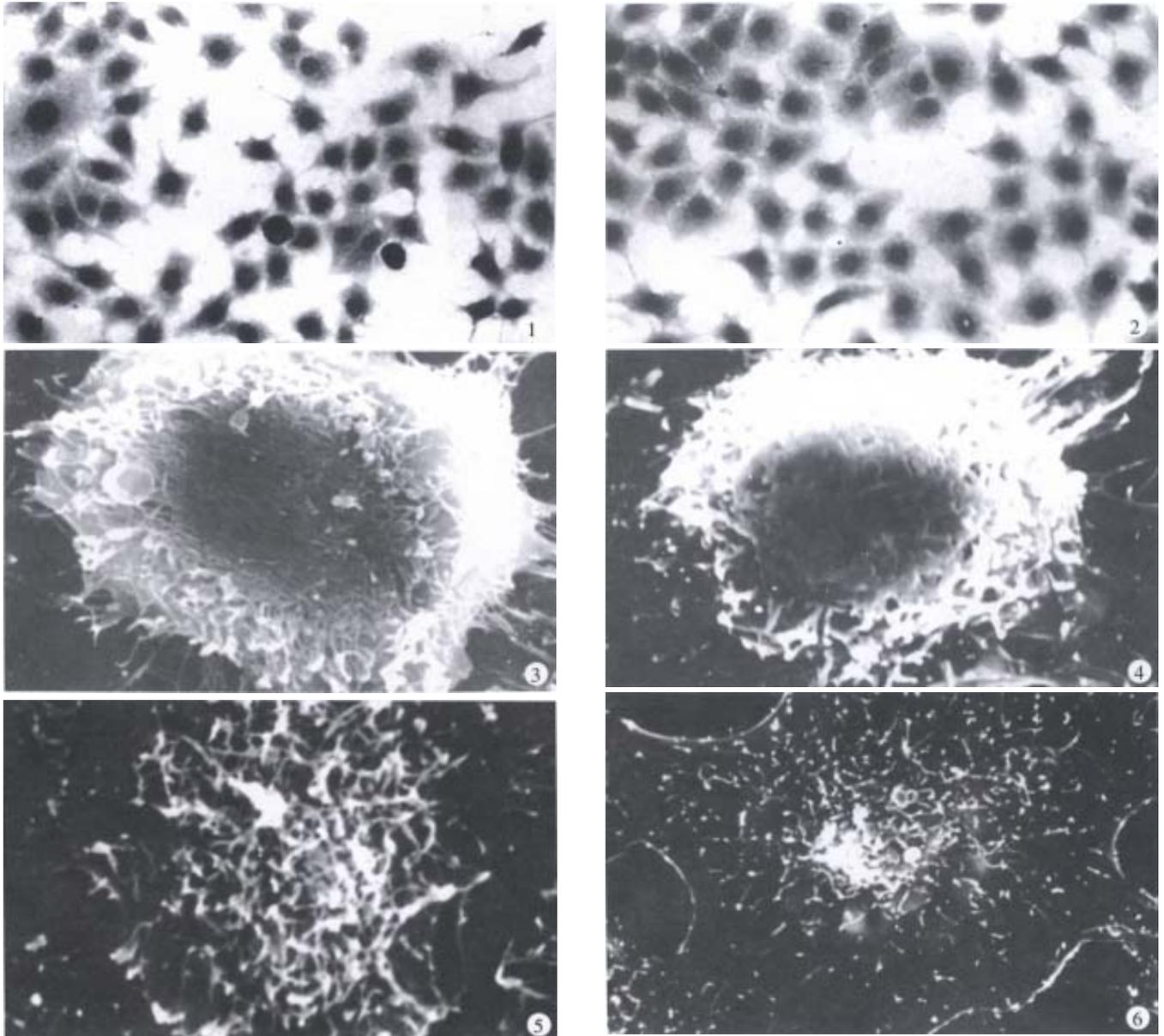


Plate I

Figure 1 BGC-823 cells. $\times 300$

Figure 2 BGC-823 cells treated with tachyplesin. $\times 300$

Figure 3 There are abundant microvilli on spherical cell surface and many filopodia emerge at the edge of BGC-823 cells. $\times 6000$

Figure 4 The microvilli on spherical cell surface disappear mainly in the cells after tachyplesin treatment. $\times 6000$

Figure 5 The microvilli are plentiful on flat and spread cell surface and many filopodia appear at the edge of BGC-823 cells. $\times 4800$

Figure 6 On the cells treated with tachyplesin the microvilli are rare, shrink and shortened on flat and spread cell surface and large lamellipodia emerge at cell edge. $\times 4200$

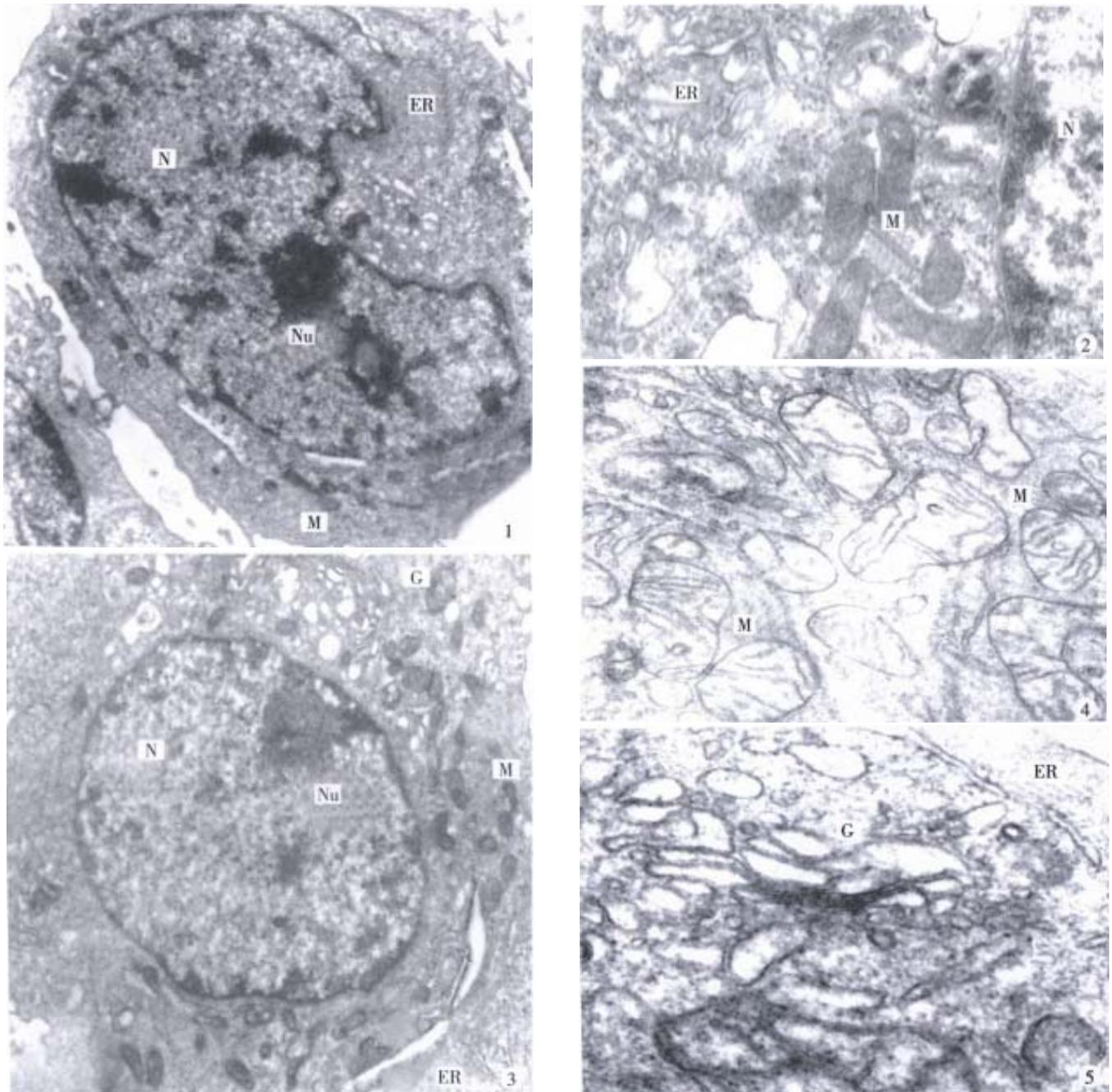


Plate II

Figure 1 The nucleo-cytoplasm ratio is large, the shape of nucleus (N) is irregular and only a few organelle in cytoplasm of BGC-823 cells (Nu nucleolus, M mitochondria, ER endoplasmic reticulum). $\times 9600$

Figure 2 The shape and structure of mitochondria (M) is non-typical and endoplasmic reticulum (ER) decrease in BGC-823 cells. $\times 40500$

Figure 3 The cellular volume enlarge, the nucleo-cytoplasm ratio decreases and the organelle increase in the cells treated with tachyplesin. $\times 10720$

Figure 4 The shape and structure of mitochondria (M) are una nimity relatively and their cristae increase and arrange rather regularly in the cells treated with tachyplesin. $\times 21000$

Figure 5 The structure of Golgi complex (G) appear typical and well-developed in the cells treated with tachyplesin. $\times 48000$

RESULTS

Light microscopic observation

There were various forms in BGC-823 cells, such as epithelioid, round, shuttle-like and irregular shapes, as well as cancerous giant cells and soon. The volume of BGC-823 cells was small relatively,

nucleus was large and irregular with several nucleoli in it, and cytoplasm was few (Plate I, Figure 1). After being treated with 2.0mg/L tachyplesin, BGC-823 cells had undergone a significant morphological change and appeared as normal differentiated epithelial cells. The cells tended to be

spread and flat apparently, their volume enlarged, cytoplasm was abundant, nucleus became relatively smaller with its shape rather regular, and the number of nucleoli was fewer. This feature was different from that of BGC-823 cells remarkably (PlateI, Figure 2).

Scanning electron microscopic observation

Under the scanning electron microscope, BGC-823 cells presented on spherical, shuttle-like and flat and spread shape. There were abundant microvilli at the surface of each type of cells. Microvilli were distributed densely at the surface of spherical cells while densely at the center and scattered at the edge of spread cells. Filopodia were abundant and arranged radiately, especially at the edge of spherical cells while there were relatively few filopodia and a little lamellipodia at the edge of spread cells (PlateI, Figures 3, 5). In contrast, the BGC-823 cells, after being treated with 2.0mg/L tachyplesin, also appeared in spherical, shuttle-like and flat and spread shapes, while the spread cells grew in number and spherical cells reduced. The cellular surface appeared rather smooth with a few small bubble and ruffled structure. The filopodia at cellular edge lessened and lamellipodia increased, especially large lamellipodia could be seen at the edge of flat and in spread cells. This surface feature was obviously different from that of BGC-823 cell (PlateI, Figures 4,6).

Transmission electron microscopic observation

It was revealed by transmission electron microscope that the necleo-cytoplasmic ratio of BGC-823 cells was relatively large, nucleus was irregular, many heterochromatins in the nucleus and nucleolus were large, varied in shape and with some nucleolus vacuoles. Meanwhile, rough endoplasmic reticulum was not well-developed, Golgi vesicle was few, arranged irregularly and Golgi cisterna swelled obviously, mitochondria were irregular and cristae within mitochondria arranged irregularly, polyribosome were abundant while free ribosome were few (PlateII, Figures 1,2). However, after 2.0mg/L tachyplesin treatment, the ultrastructure of BGC-823 cells had also undergone a significant change. The necleo-cytoplasmic ratio lessened, the nuclear shape became regular, most being round or oval, heterochromatin in nucleus decreased while euchromatin increased, the volume of nucleolus lessened, rough endoplasmic reticulum increased obviously, Golgi apparatus was well-developed and Golgi vesicle increased and arranged regularly, most of mitochondria were oval, and their cristae grew in number and arranged regularly, polyribosome reduced while free ribosome increased (PlateII, Figures 3-5). BGC-823 cells showed some ultrastructural characteristics of their normal relevant cells after being treated with tachyplesin.

DISCUSSION

There was significant difference in morphology and ultrastructure between tumor cells and their relevant normal cells. Tumor cells usually display some malignant morphological and ultrastructural characteristics such as that the nucleocytoplasmic ratio was relatively large, nucleus was large and malformed with several nucleoli in it, cell organelle were not well-developed and microvilli were abundant^[5]. Therefore, to observe and identify the changes of morphology and ultrastructure in tumor cells is important in determining the effects of exotic substances especially differentiative inducers on tumor cells^[6]. A series of research in which differentiation of gastric carcinoma, leukemia, hepatoma and lung carcinoma cells were induced by chemical inducers, have made it clear that the morphology and ultrastructure of carcinoma cells after induced treatment engendered a restorative alteration similar to those of relevant normal cells^[7-11].

BGC-823 cell is a poorly-differentiated human gastric adenocarcinoma cell line with fast-proliferation and high-malignant characteristics. Our results showed that BGC-823 cells had the typical malignant phenotypical characteristics of morphology and ultrastructure in tumor cells. However, in the BGC-823 cells after treatment with 2.0mg/L tachyplesin, it displayed the changes as follows: the cells were unanimous and tended to be spread and became flat apparently, the volume of cells enlarged, epithelioid-like cells grew in number, the necleo-cytoplasmic ratio declined, the shape of nucleus became relatively regular, the volume and number of nucleolus decreased, heterochromatin decreased while euchromatin increased, mitochondria and their cristae increased, Golgi complex was well-developed and typical, rough endoplasmic reticulum increased, polyribosome decreased while free ribosome increased, microvilli and filopodia decreased while lamellipodia increased. These changes were different from the morphology and ultrastructure of BGC-823 cells and similar to those of their relevant normal cells. It is indicated that tachyplesin could alter the malignant phenotypical characteristics of morphology and ultrastructure in gastric carcinoma cells and make them take on the morphological and ultrastructural characteristics similar to those of their normal cells.

The action of tachyplesin is identical with those of chemical inducers on gastric carcinoma cells. It was reported that the human gastric carcinoma cell line SGC-7901 treated with sodium butyrate^[12], hexamethylenemine bisacetamide (HMBA)^[13] and all-*trans* retinoic acid^[14,15]. The shape of cell was rather regular and consistent, the nuclear shape became round and nucleolus shrank, the nucleo-

cytoplasmic ratio lessened and hetrochromatin reduced, and the organelle were well-developed, and microvilli at cell surface decrease. These changes were unanimous with the changes of tachyplesin on gastric carcinoma cells in this paper. It further demonstrates that tachyplesin has the identical action with differential inducers of cancer cells, and it has a certain inducing differential effect on human gastric carcinoma cells. In this connection, it has a momentous significance for application of tachyplesin in tumor treatment and in the antitumor research of marine bioactive substance to study the acting mechanism of tachyplesin on tumor cells further.

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Effect of arsenic trioxide on human hepatoma cell line BEL-7402 cultured *in vitro*

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Subject headings arsenic trioxide; hepatoma; flow cytometry; immunohistochemistry; microscopy; electron; apoptosis; gene expression

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Abstract

AIM To study the effect of a varying concentrations of arsenic trioxide on human hepatoma cell line BEL-7402 cultured *in vitro* and its mechanism of action.

METHODS The BEL-7402 cells were treated with arsenic trioxide (at the concentrations of 0.5, 1, 2 $\mu\text{mol/L}$, respectively) for 4 successive days. The cell growth and proliferation were observed by cell counting and cell-growth curve. Morphologic changes were studied with electronmicroscopy. Flow cytometry was used to assay cell-DNA distribution and the protein expression of Bcl-2 and Bax detected by immuno cytochemical method.

RESULTS The cell growth was significantly inhibited by varying concentrations of arsenic trioxide as revealed by cell counting and cell-growth curve, which was dose- and time-dependent. Arsenic trioxide treatment at 0.5, 1 and 2 $\mu\text{mol/L}$ resulted in a sub G1 cell peak, the apoptosis rate of the control group was 9.31% and that of 0.5 $\mu\text{mol/L}$ arsenic trioxide 15.53%, no significant difference was seen between the two. The apoptosis rates of 1, 2 $\mu\text{mol/L}$ arsenic trioxide were 19.10% and 21.87% respectively, which were much higher (both $P < 0.05$). Decrease of G₀/G₁ phase cells and increase of S phase cells were observed by flow cytometry, suggesting the inhibition effect of 0.5, 1, 2 $\mu\text{mol/L}$ arsenic trioxide on BEL-7402 cell lay in

the G₀/G₁ phase. Morphologic changes such as intact cell membrane, nucleic condensation, apoptotic body formation were seen under transmission electronmicroscopy, whereas the 0.5 $\mu\text{mol/L}$ arsenic trioxide-treated BEL-7402 cells showed decrease of nucleocytoplasmic ratio, round nucleus, well-differentiated organelles in the cytoplasm. The processes and microvilli on the cell surface of the experimental groups under scanning electron microscopy were significantly decreased. High expressions of Bcl-2 and Bax were detected in 1 and 2 $\mu\text{mol/L}$ arsenic trioxide-treated cells, these were 46%, 87.33% and 83.08%, 95.83% respectively, among which that of Bax was more significant. Arsenic trioxide treatment at 0.5 $\mu\text{mol/L}$ resulted in a higher expression level of Bcl-2 and lower expression level of Bax, which were 8.81% and 3.83% respectively, as compared with that of the control group (15.33%) ($P_1 < 0.01$, $P_2 < 0.01$).

CONCLUSION Arsenic trioxide not only inhibited proliferation but also induced apoptosis of human hepatoma cell line BEL-7402. The induced-apoptosis effect of 1, 2 $\mu\text{mol/L}$ arsenic trioxide was related to the expression level of Bcl-2 and Bax.

INTRODUCTION

Arsenic trioxide is the main ingredient of traditional Chinese medicinal, pi shi. Zhang P, *et al* first reported the effect of Arsenic trioxide on promyelocytic leukemia (APL) with satisfactory results. The rate of complete remission in patients who had not received any treatment before reached 73.33%, and was 52.38% in patients with recurrence, the longest remission period was more than ten years, and intravenous route of administration was the choice, no toxic or adverse effects were seen^[2-4]. There was no cross resistance between arsenic trioxide and other chemical drugs during the course of treatment of APL^[5]. Shen^[6] concluded that arsenic trioxide treatment was effective and relatively safe in APL patients refractory to ATRA and conventional chemotherapy. Inorganic arsenic trioxide was recently shown to induce apoptosis in NB4 promyelocytic leukemic cells^[7]. The present study was so designed as to broaden the anti-tumor

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spectrum and to study the inhibitory effect of Arsenic trioxide on human hepatoma cell line and its mechanism of action, in order to provide some theoretical basis for its clinical use.

MATERIALS AND METHODS

Materials

Human hepatoma cell line BEL-7402 was purchased from the Cell Institute of Chinese Academy of Science. Arsenic trioxide was produced in Pharmaceutical Department of First Hospital of Harbin Medical University. RPMI 1640 was purchased from GIBCO. Propidium iodide and Rnase were from Sigma Chemical Co. A murine monoclonal antibody against human Bcl-2 and Bax oncoprotein and antimice rabbit polyclonal antibody were purchased from Maixin Co., Fuzhou.

Methods

Cell culturing Human hepatoma BEL-7402 cells were grown as monolayers in RPMI 1640 medium supplemented with 8% calf serum (CS), 100IU/mL penicillin and 100mg/mL streptomycin and incubated at 37°C in the humidified incubator with 5% CO₂/95% air. The exponent growing BEL-7402 cells were suspended in medium, planted 4-6×10⁵/flask or 3×10⁴/well into culture flask or 24-well plate.

Drug treatment The growing BEL-7402 cells planted into culture flasks or 24-well plates were incubated at 37°C in 5% CO₂/95% air for 24h. The medium was aspirated and replaced with medium containing arsenic trioxide (final concentration 0.5 μmol/L, 1 μmol/L, 2 μmol/L, respectively) as treatment groups and medium with non-arsenic trioxide as controls.

Determination of growth curve The human hepatoma BEL-7402 cells were digested with 0.25% trypsin, stained with 2% trypan-blue. The cell growth and proliferation of BEL-7402 cells were observed by counting the cell number every day. The non-blue-stained cells were viable cells or apoptotic cells and the blue-stained cells were necrotic cells.

Morphologic observation The BEL-7402 cells growth and morphologic changes were observed by OLYMPUS IX70 inverted phase-contrast microscopy. After incubated in medium containing 0.5 μmol/L, 1 μmol/L, 2 μmol/L arsenic trioxide (experimental groups) and non-arsenic trioxide (control groups) for 4 successive days, the cells were prefixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, dehydrated in ethanol series, and replaced in propene oxide. The cell samples attached to grids were examined with a JEM-1220 transmission electron microscope (TEM) and the cell

samples grown on covered glass-slide (made in non-enzyme digestion) were gilded in vacuum and examined with a HITACHI S-520 scanning electron microscope (SEM).

Flow-cytometry analysis^[1] After incubated in medium containing varying concentrations of arsenic trioxide and in the control groups for 4 days, the cells were digested with 0.25% trypsin, collected after centrifugation at 1000rpm for 10min, than washed two times with cold PBS by centrifugation (1000 rpm, 5 min), resuspended in 0.5mL PBS, adjusted to a cell concentration of 1×10⁶/L, and fixed with ice-cold 70% alcohol at 4°C (could be preserved for less than 2 weeks). The fixed water of single-cell suspension was discarded after centrifuged at 1000rpm for 10min, which was then washed twice with PBS (1000rpm, 5min), and adjusted to a cell concentration of 1×10⁶/L, The DNA was stained with propidium iodide at 4°C for 30 minutes, then the suspension was analyzed on the apparatus at room temperature. According to ModFit LT software, the cells were divided into four parts: subG₁ phase, G₀/G₁ phase, S phase and G₂/M phase.

Immunocytochemistry The BEL-7402 cells of experimental groups and controls were seeded into 24-well plate (with cover glass-slide). After incubated in medium with 0.5 μmol/L, 1 μmol/L, 2 μmol/L arsenic trioxide respectively and non-arsenic trioxide for 4 days, the BEL-7402 cells were fixed with pure acetone for 10min, washed three times with PBS, acted upon by 0.25% 0.5% Triton X-100 for 10min, and washed Thrice with PBS. Bcl-2 and Bax protein in the BEL-7402 cells were detected with SABC method. The cell specimens were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes to block the endogenous peroxidase activity, then washed in PBS and incubated in 10% normal goat serum for 20 minutes to reduce nonspecific antibody-binding. Specimens were then incubated with a 1:50 dilution of murine monoclonal antibody against human Bcl-2 or Bax oncoprotein overnight at 4°C, followed by washes Thrice with PBS, then incubated with biotinylated rabbit antimice polyclonal antibody at a dilution of 1:100 for 30 minutes followed by another 3 washes. Slides were then treated with streptoavidin-peroxidase reagent for 30 minutes at a dilution of 1:100 and were washed with PBS 3 times. Finally, slides were incubated in phosphate-buffered saline containing diaminobenzidine and 1% hydrogen peroxide for 10 minutes, counterstained with hematine, and mounted.

Statistics All the data were expressed as mean± standard deviation ($\bar{x}\pm s$), the differences between the rates of different groups were analysed by χ^2 test.

RESULTS

Effects of arsenic trioxide on the growth of the BEL-7402 cells

The BEL-7402 cells were incubated in medium containing 0.5 μmol/L, 1 μmol/L and 2 μmol/L arsenic trioxide respectively for 1 to 6 days. The cell-growth inhibitory effect of arsenic trioxide on cells was significant as revealed by cell counting, which was both dose-and time-dependent. During incubated in arsenic trioxide for 3 to 5 days, the speed of growth of BEL-7402 cells was remarkably slow, on the 6th day, the number of cells fell to the lowest level. The percentage of necrotic cells was less than 1% by trypan-blue staining (Figure 1).

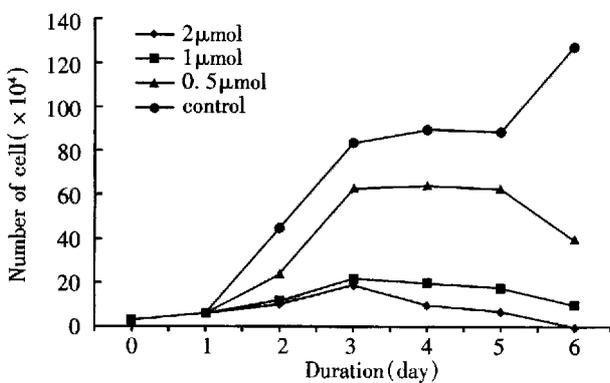


Figure 1 The effect of As₂O₃ on cell growth of BEL-7402.

Morphologic changes

By inverted phase-contrast microscopy, one could find the attaching ability of BEL-7402 cells to the flask treated with 1 μmol/L, 2 μmol/L arsenic trioxide was weaker as compared with that of the controls, and the growth markedly inhibited.

The BEL-7402 cells treated with arsenic trioxide underwent significant changes as seen in TEM, the nucleocytoplasmic ratio in BEL-7402 cells enlarged, with indentation of nuclei. The nucleocytoplasmic ratio in BEL-7402 cells treated with 0.5 μmol/L arsenic trioxide was much smaller than that in controls, and the nuclei appeared round, with loss of nuclear indentation, but with well-differentiated organelles in the cytoplasm. When treated with arsenic trioxide at 1 μmol/L, 2 μmol/L for 4 successive days, one could find intact cell membrane, nuclear condensation and apoptotic body formation, also, marked changes on the cell surface in SEM, there were abundant processes and microvilli on the surface of BEL-7402 cells in the control group, but much less in 0.5 μmol/L arsenic trioxide group. The processes and microvilli were lost when treated with 1 μmol/L and 2 μmol/L arsenic trioxide (Figures 2, 3).

Determination of sub-G₁ peak

After treated with 0.5 μmol/L, 1 μmol/L, 2 μmol/L arsenic trioxide for 4 successive days, the BEL-7402 cells were analysed by flow-cytometry. The apoptotic rate of cells in the control group was 9.31%, in 0.5 μmol/L arsenic trioxide group, 15.53%, there was no statistical difference between the two (*P*>0.05). The apoptotic rates of BEL-7402 cell in 1 μmol/L and 2 μmol/L arsenic trioxide were significantly higher than that of the control group, being 19.10% and 21.89%, respectively (*P*₁<0.05, *P*₂<0.05) (Table 1).

Analysis of cell cycle

After treated with arsenic trioxide at 0.5 μmol/L, 1 μmol/L and 2 μmol/L for 4 successive days, the DNA distribution of BEL-7402 cells showed great changes. The percentage of G₀/G₁ phase cell in the control group was 62.91%, that of S phase cell, 33.77%. The percentage of G₀/G₁ phase cell of the experimental groups decreased progressively more than that of the control groups with increase of concentration of arsenic trioxide, whereas the percentage of S phase cell was increased, but with no statistical difference between the two (*P*>0.05) (Table 1).

Protein expression of Bcl-2 and Bax

After treated with 0.5 μmol/L, 1 μmol/L, 2 μmol/L arsenic trioxide for 4 successive days, the expression of Bcl-2 and Bax protein were detected by immunocytochemistry. The positive expression rates of Bcl-2 in 1 μmol/L and 2 μmol/L arsenic trioxide were 46.00% and 83.08%, respectively, significantly higher than that in the control group (*P*<0.01). This was also higher in 0.5 μmol/L arsenic trioxide group, yet there was no statistical difference between them (*P*>0.05). The positive expression rate of Bax in 0.5 μmol/L arsenic trioxide was significantly lower than that of the control group (*P*<0.01), but it was significantly higher in 1 μmol/L, 2 μmol/L groups than that in the control group (*P*₁<0.01, *P*₂<0.01) (Table 2 and Figure 4).

Table 1 Influence of varying concentrations of arsenic trioxide on apoptotic rate and DNA distribution

Group	Apoptotic rate	DNA distribution (%)	
		G ₀ /G ₁	S
Control	9.31±0.24	62.91±1.30	33.77±0.41
0.5 μmol/L As ₂ O ₃	15.53±0.69	60.92±2.16	36.87±2.46
1 μmol/L As ₂ O ₃	19.10±1.02 ^a	57.55±2.24	40.14±2.87
2 μmol/L As ₂ O ₃	21.87±0.84 ^a	54.12±1.18	43.17±2.30

Treated group vs control group, ^a*P*<0.05.

Table 2 Influence of arsenic trioxide on expression of Bcl-2 and Bax (%)

	Control	As ₂ O ₃ concentration (μmol/L)		
		0.5	1	2
Bcl-2 expression	4.33±0.29	8.81±0.84	46.00±3.61 ^b	83.08±8.16 ^b
Bax expression	15.33±1.04	3.83±0.29 ^b	87.33±2.02 ^b	95.83±2.36 ^b

Treated group vs control group, ^b*P*<0.01.

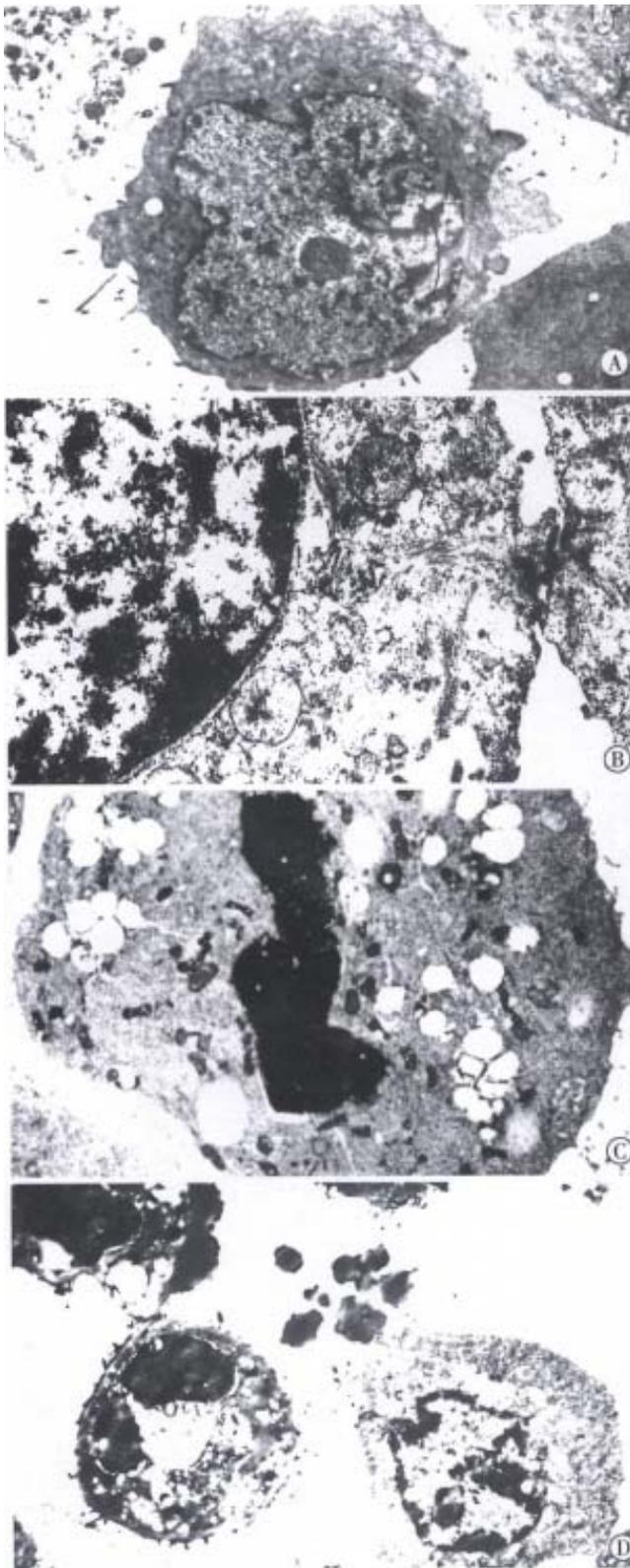


Figure 2 Transmission electronmicroscopic observati on of ultrastructural changes in human hepatoma cells treated with 0, 0.5, 1, 2 μ mol/L As₂O₃ for 96h. The nucleocytoplasmic ratio enlarged with indentation of nuclei in BEL-7402 cells of 0 μ mol/L As₂O₃ group (A). The nucleocytoplasmic ratio become smaller, nuclei appear round, but with well-differentiated organelles in the cytoplasm in 0.5 μ mol/L As₂O₃ group (B). The intact cell membrane, nuclear condensation and apoptoti c body formation in 1, 2 μ mol/L As₂O₃ group (C,D).

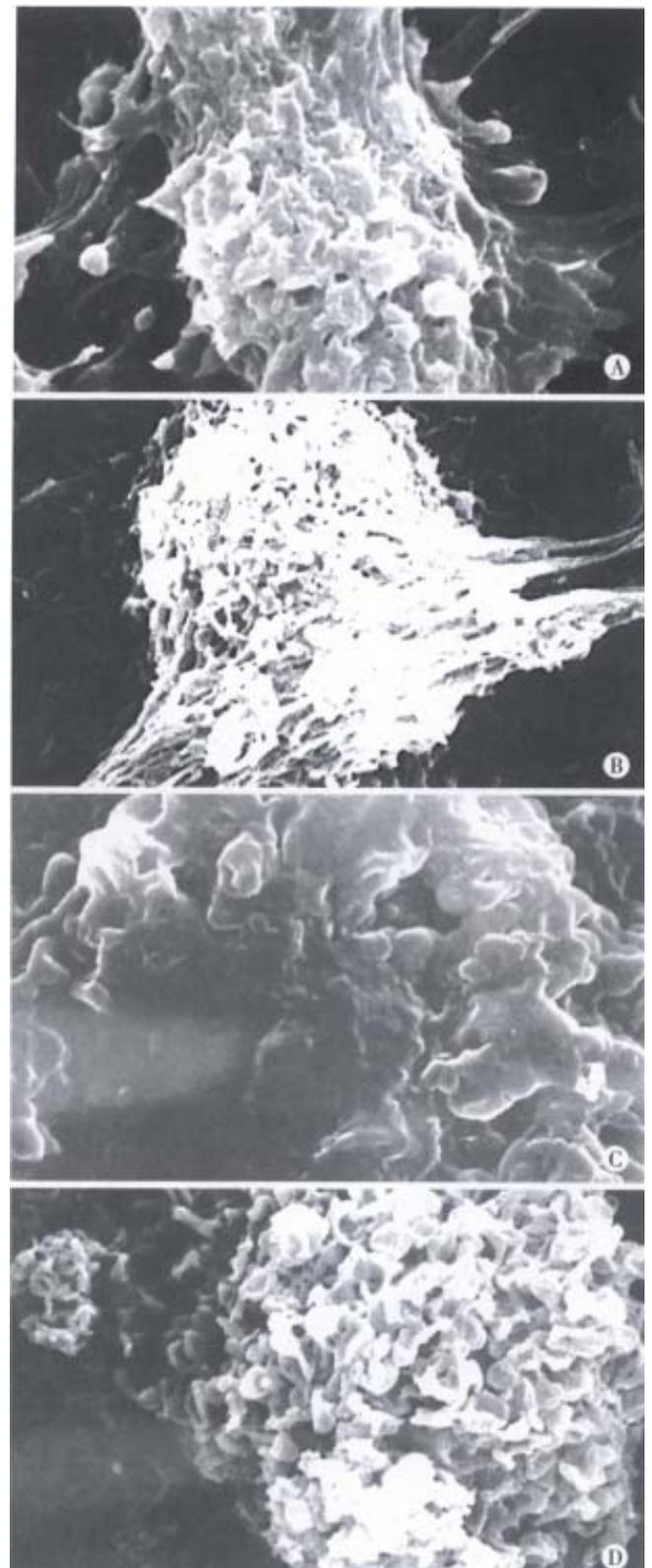


Figure 3 Scanning electronmicroscopic observation of changes in human hepatoma cell membranes treated with 0, 0.5, 1, 2 μ mol/L As₂O₃ for 96h. There were abundant processes and micro villi on the surface of BEL-7402 cells in 0 μ mol/L As₂O₃ group (A). The processes and micro villi was much less in 0.5 μ mol/L As₂O₃ group (B). The processes and micro villi were lost when treated with 1, 2 μ mol/L As₂O₃ (C,D).

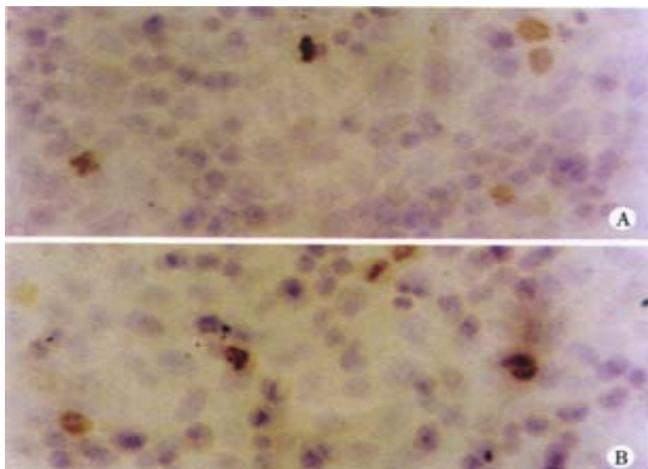


Figure 4 Bcl-2 expressions in human hepatoma cells. 0 μ mol/L As₂O₃ group (A). 0.5 μ mol/L As₂O₃ group (B).

DISCUSSION

It has been demonstrated that *in vitro* study is superior to *in vivo*, study, being also in consistency with the latter therefore, it is the main method in studying the effect of anti-tumor drugs currently^[8].

Inhibition of proliferation is the basic requirement of anticancer drugs^[9-16]. Recent clinical studies in China showed that arsenic trioxide is an effective and relatively safe drug in the treatment of acute promyelocytic leukemia. Chen^[17] found arsenic trioxide could trigger apoptosis of APL cell line NB4 cells, which was associated with downregulation of bcl-2 gene expressions and modulation of PML-RAR alpha chimeric protein. Advanced hepatocellular carcinoma has limited, treatment options and prognosis is poor. Recent studies have focused on the apoptosis either by suppression of Bcl-2, Bcl-xL or promotion of Bik, Bax, Bak^[18-24]. Our results showed the BEL-7402 cell growth was significantly inhibited by arsenic trioxide at the concentrations of 0.5 μ mol/L, 1 μ mol/L and 2 μ mol/L, and the inhibition effect was not relevant to the cytotoxicity as shown by cell counting after trypan-blue staining. When incubated with arsenic trioxide for 3 to 5 days, the speed of growth of the BEL-7402 cells was remarkably slow. Furthermore, the percentage of G₀/G₁ phase cells was lower than that of the control group, the apoptotic rate of the experimental group was significantly higher, These indicated the inhibition effect of arsenic trioxide acted mainly on the G₀/G₁ phase cells. The

percentage of S phase cells was much increased, suggesting there might be synergistic effect when arsenic trioxide used in combination with other S-phase anti-cancer drugs.

Apoptosis is a complicated physio-pathologic process involving the suicidal mechanism^[25]. Many anticancer drugs can induce apoptosis of neoplastic cells, which opens a new avenue to treatment of cancer^[26]. Modern molecular biological investigations have indicated that apoptosis is regulated by many oncogenes, such as p53, c-myc, bcl-2, bax, bad etc.^[27-32]. Bcl-2 is an apoptosis-related gene and plays an important role in regulating apoptosis^[33-38], by blocking the final pathway of apoptotic signal transmitted system^[39-44]. Bcl-2 can inhibit the apoptosis stimulated by chemotherapeutic drugs, radiotherapy, heat shock, free radicals, Ca²⁺ and TNF etc.^[45-50]. Bax is a homologous protein of Bcl-2, in the form of homopolymer or isodipolymer Bcl-2/Bax^[51-54]. The ratio of Bax and Bcl-2 protein influences the apoptotic rate of cells stimulated by several external or internal factor^[55,56]. Chen's report showed that arsenic trioxide did not influence bax, bcl-x, c-myc, or p53 gene expression, but downregulates bcl-2 gene expression at both mRNA and protein levels^[56].

Our results suggested that the proliferation inhibitory effect of arsenic trioxide at 1 μ mol/L and 2 μ mol/L was related to apoptosis induction which was possibly regulated by the expression of Bcl-2, Bax. Oltval *et al*^[29] reported the number of apoptotic cells was higher when Bax protein was predominant. In our study, the apoptotic percentage by flow-cytometry assay was concordant with the protein expression level of Bcl-2 and Bax in 1 μ mol/L and 2 μ mol/L arsenic trioxide-treated cells.

Arsenic trioxide has dual effects on APL cells: preferential apoptosis at high concentration (0.5-2 microM) and partial differentiation at low concentration (0.1-0.5 microM)^[57]. Treatment of BEL-7402 cells with 0.5 μ mol/L arsenic trioxide showed intricate results in our study. The reasons were as follows: ① The apoptotic rate of BEL-7402 cell increased after 0.5 μ mol/L arsenic trioxide treatment, but had no statistical difference as compared with that of the control. Expression of the Bcl-2 protein was increased with arsenic trioxide treatment, whereas expression of Bax protein fell. Ogasawara *et al*^[58] confirmed that Fas system played an important role in hepatocytic apoptosis induction, therefore, other apoptosis-related gene expression might be involved in that treated with 0.5 μ mol/L arsenic trioxide. ② It had been seen that the nucleocytoplasmic ratio was decreased, the nucleus became round, with loss of indentation but there were well-differentiated organelles in the cytoplasm, suggesting a possible role for

0.5 μ mol/L arsenic trioxide in the induction of differentiation.

In conclusion, our study demonstrates the proliferation inhibition and apoptosis induction effects of arsenic trioxide at the concentrations of 0.5 μ mol/L, 1 μ mol/L and 2 μ mol/L on the human hepatoma cell line BEL-7402. It seems that arsenic trioxide can be a new adjuvant drug in treatment of liver cancer. Arsenic trioxide can be introduced into human body via hepatic artery catheterization to induce cell apoptosis. The present study provides some theoretical basis for its clinical use.

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Effect of hepatocyte apoptosis induced by TNF- α on acute severe hepatitis in mouse models

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Subject headings tumor necrosis factor; hepatitis; apoptosis; alanine transaminase; aspartate transaminase; endotoxins; mice

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Abstract

AIM To study the effect of hepatocyte apoptosis and necrosis induced by TNF- α on the pathogenesis of acute severe hepatitis (ASH).

METHODS The model of ASH was prepared in D-galactosamine (GalN) sensitized BALB/c mice by injection of either endotoxin (ET) or tumor necrosis factor- α (TNF- α). Morphological changes of apoptotic hepatocytes were studied by both light and electron microscope and in site end labeling method (ISEL). Molecular biological changes of DNA ladder were observed by electrophoresis of extract from liver tissues. Biochemical changes were measured by alanine aminotransferase (ALT), aspartic aminotransferase (AST) and TNF- α . The relation between apoptosis and necrosis was evaluated simultaneously.

RESULTS The sequence of hepatocyte apoptosis, necrosis, and final death from ASH was observed both in GalN/ET and GalN/TNF- α group. Apoptosis was prominent at 3.5h and 6h after injection of inducer, while necrosis became dominant at 9h after challenge. The appearance of apoptosis was earlier in GalN/TNF- α group than that in GalN/ET group. Pretreatment of mice with antiTNF IgG1 may completely prevent the liver injury induced by GalN/ET.

CONCLUSION TNF- α can cause liver damage by inducing hepatic apoptosis and necrosis in mice with endotoxemia.

INTRODUCTION

Apoptosis is one of the cell death forms, which is quite different from cell necrosis in morphology, biochemistry and biology. Clinical studies showed that ET (Endotoxin, ET) and tumor necrosis factor- α (TNF- α) elevated obviously in the sera of patients with severe hepatitis. Recent researches showed that hepatocyte apoptosis was closely related with pathogenesis of hepatitis especially that of ASH^[1-11]. The present study deals with the effect of both apoptosis and necrosis induced by TNF- α on the pathogenesis in ASH, and their relationship.

MATERIAL AND METHODS

Reagents

Recombinant murine TNF- α and immunoglobulin (Ig) G1 fraction of anti-murine TNF- α were purchased from Pepro Tech EC LTD. Terminal-deoxynucleotidyl transferase (TdT) and Bio-11-dUTP Na salt were purchased from Dako LTD. Salmonella abortus equi endotoxin (ET) was purchased from Sigma Chemical Co. GalN was purchased from Chong Qing Medical University. Endogen mouse TNF- α ELISA Kit was provided by Endogen Inc. Unless otherwise specified, all other reagents were analytical reagents.

Animal and experimental methods

One hundred and thirty specific pathogen-free male BALB/c mice (from Shanghai Second Medical university animal breeding house) were divided into 7 groups: ① Control; ② GalN; ③ ET; ④ TNF- α ; ⑤ GalN/ET; ⑥ GalN/ TNF- α ; and ⑦ anti-TNF- α IgG1/GalN/ET.

Each group contained 20 mice except Group 7 which contained 10 mice. Both GalN (800mg/kg) and ET (2.4 μ g/kg) were injected intraperitoneally; Both TNF- α (1.0 μ g/kg) and anti-TNF- α IgG1 (100 μ g/mouse) were injected in tail vein. The control group received the same volume of normal saline.

Five mice were killed at 1.5 h, 3.5 h, 6 h, and 9 h respectively after injection. TNF- α , ALT, and AST were measured in the blood samples taken from the mouse heart. Tissue samples taken from liver were also prepared for morphological and molecular biological examinations.

The sections of liver tissue were stained with hematoxylin and eosin (HE), and ISEL^[12] for detection of apoptotic liver cells were performed, DNA was extracted from fresh liver tissue for further analysis of DNA ladder.

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RESULTS

Histological alterations of the liver in control, GalN, ET, and TNF- α groups

Hepatic lobular architecture was clear and intact without any abnormalities in the liver section of control group. Only mild swelling of hepatic cell was presented in GalN, ET and TNF- α groups. With the time prolonging from 1.5 h, 3.5 h, 6 h, to 9 h, the mild swelling become moderate degree. Neither apoptosis nor necrosis was present in HE and ISEL staining. No DNA ladder was found in any group mentioned above.

Histological alterations of the liver in GalN/ET group

No obvious liver cell alterations were present at 1.5 h on the section of HE staining and ISEL staining failed to detect positive signals of apoptosis.

Mild swelling of liver cells on HE section and a few apoptotic cells on ISEL section were present at 3.5 h (Figure 1A).

Obvious swelling of liver cells and lots of apoptotic cells were found at 6 h and mild dotted necrosis was also observed. Strong apoptotic positive signals could be detected in the cell nuclei.

Nine hours after GalN/ET injection, enormous apoptotic liver cells and pieces of hepatic necrosis with leukocytes infiltration can be seen (Figure 1B).

These data demonstrate that apoptosis occurred as an early event during the development of hepatic failure. Compared with the ET model, the majority of changes occurred earlier in the TNF model, which agrees with the phenomena that ET act on hepatocytes by inducing TNF- α .

Histological alterations of the liver in GalN/TNF- α group

Morphological changes of the liver in this group were basically the same as those in GalN/ET group but severer at 1.5 h, 3.5 h, 6 h and 9 h (Figures 2A, 2B), 1.5 h, 3 h, 6 h and 9 h after administering inducer, apoptosis positive rates of GalN/ET group were 0.0%, 0.2%, 1.21% and 3.14% respectively, while for the GalN/TNF- α group, were 0.2%, 0.5%, 2.57% and 3.19% respectively.

DNA fragment assay

DNA ladder was found at 6 h and 9 h after GalN/ET, GalN/TNF- α administration, but was not found at 1.5 h and 3.5 h after GalN/ET, GalN/TNF- α injection, and DNA ladder was not found in other groups (Figures 3A, 3B).

Electron microscopic assay

Electron microscopic study showed the chromatin condensation of apoptotic cells and near the nuclear lining (Figure 4).

Blockage of liver damage induced by GalN/ET by pretreatment of mice with anti-TNF- α IgG1

In the GalN/ET group pretreated with anti-TNF- α IgG1, only mild swelling of liver cells could be seen on the HE staining. Neither apoptosis nor necrosis could be found on ISEL staining. No DNA ladder was present on electrophoresis of agarose gel (Figure 3A).

Time course of apoptosis and necrosis

We compared the time course of typical morphology of apoptosis (chromatin condensation, apoptotic bodies) with ALT and AST (as a parameter for necrosis) in each group (Table 1). ALT and AST remained normal at 3.5 h after GalN/TNF- α challenge, at this time point, a small amount of apoptotic liver cells could be found. Apoptotic liver cells become obvious at 6 h, ALT and AST were elevated mildly at the same time. This finding indicated that apoptosis was already developed while the liver cell membrane still remained intact 6 h after challenge, suggesting that apoptosis occurred earlier than necrosis.

The prominent increase of ALT and AST occurred at 9 h, when a great number of necrotic liver cells were observed. Meanwhile, profuse apoptotic liver cells were also present even after the death of mice associated with ASH and electrophoresis of agarose gel still showed DNA ladder at the final stage.

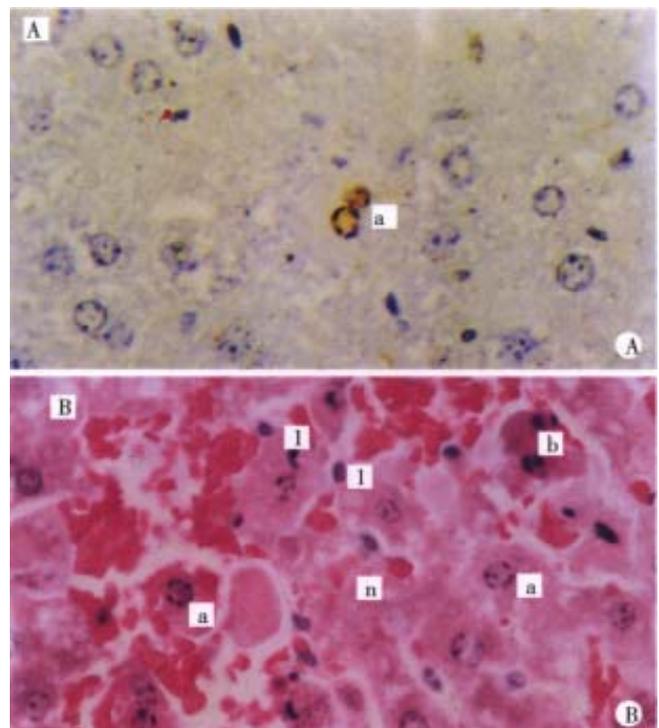


Figure 1 Liver cells apoptosis in mice induced by GalN/ET. A 3.5 hours after GalN/ET, individual apoptotic cells are visible, apoptotic positive signal mainly locates in nucleus. ISEL \times 400 B 9 hours after GalN/ET, further increase of apoptotic liver cells and pieces of liver cell necrosis and bleeding with leukocytes infiltration appear. Apoptotic cells (a), apoptotic bodies (b), leukocytes (l) and necrosis (n). HE \times 400

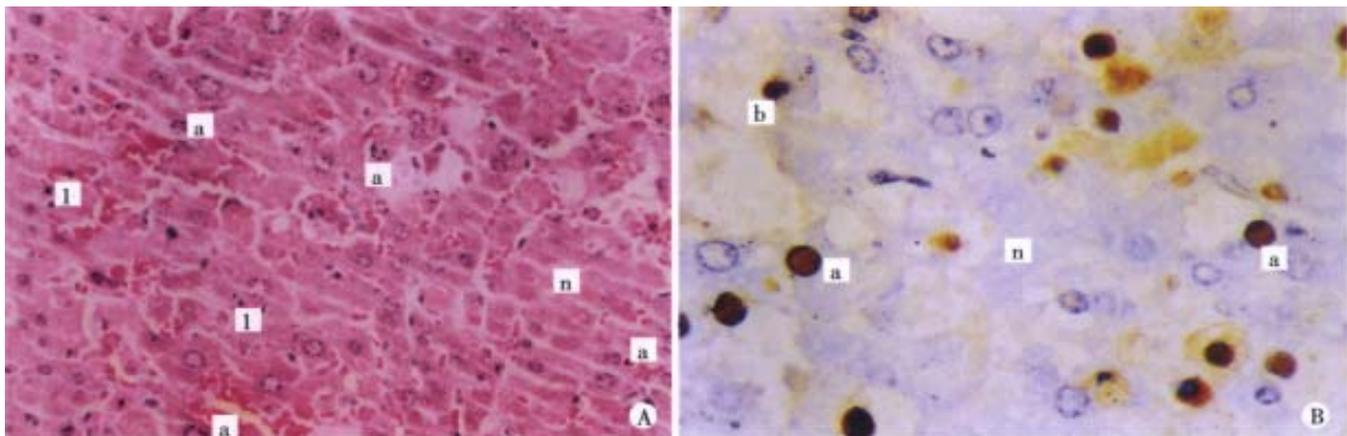


Figure 2 Liver cells apoptosis in mice induced by GalN/TNF- α .

A. 6 hours after GalN/TNF- α , a lot of apoptotic liver cells can be seen. Apoptotic bodies appear on the section, but liver necrosis is not obvious. HE \times 200

B. 6 hours after GalN/TNF- α , a great number of apoptotic liver cells are found. ISEL \times 400

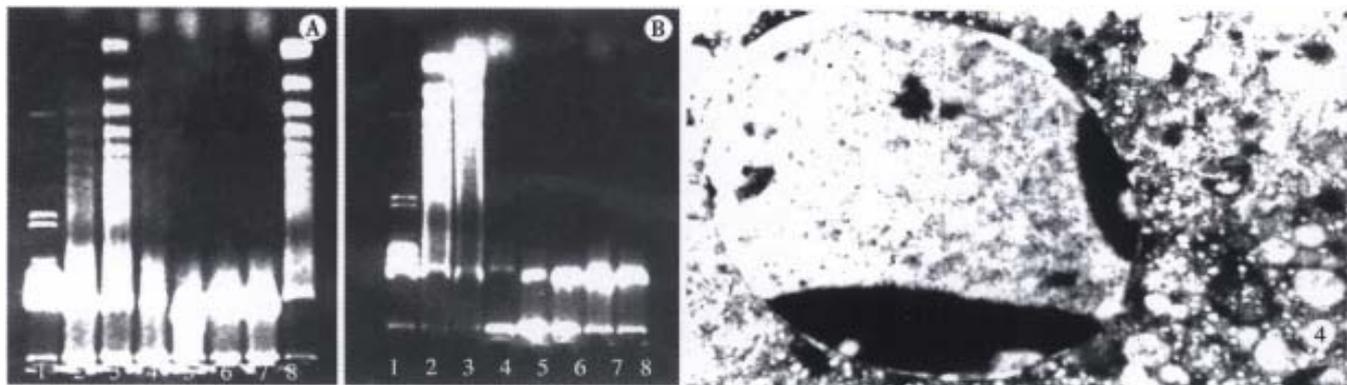


Figure 3 Analysis of DNA ladder from liver cell extract.

A. (1) Marker (λ DNA/*Hind* III); (2) GalN/ET 9h; (3) GalN/ET 6h; (4) GalN/ET 3.5h; (5) GalN 6h; (6) ET 6h; (7) Normal liver cells; (8) Dexamethasone induced apoptosis of thymus cells.

B. (1) Marker (λ DNA/*Hind*-III); (2) GalN/TNF 9h; (3) GalN/TNF 6h; (4) GalN/TNF 3.5h; (5) GalN 6h; (6) TNF 6h; (7) Normal liver cells; (8) Anti-TNF- α /GalN/ET 9h.

Figure 4 Apoptotic liver cell under electron microscope.

Chromatin condensation in apoptotic cell to form crescent moon body locating near the nucleus lining. \times 6000

Table 1 Comparison of TNF- α , ALT and AST at corresponding time in different groups ($n = 5$, $\bar{x} \pm s$)

	1.5h			3.5h			6h			9h		
	TNF	ALT	AST	TNF	ALT	AST	TNF	ALT	AST	TNF	ALT	AST
1	13.9 \pm 15.0	39.6 \pm 10.3	124.0 \pm 17.0	18.8 \pm 15.2	40.1 \pm 6.0	129.6 \pm 33.5	15.9 \pm 15.9	41.5 \pm 5.4	100.1 \pm 24.3	21.9 \pm 16.4	41.4 \pm 6.6	115.6 \pm 16.8
2	16.0 \pm 16.0	40.3 \pm 6.0	95.7 \pm 37.5	10.1 \pm 16.2	40.8 \pm 12.8	118.4 \pm 40.1	28.0 \pm 21.1	46.8 \pm 2.4	135.4 \pm 62.4	25.4 \pm 29.7	47.3 \pm 9.0	100.2 \pm 35.5
3	32.1 \pm 14.6	53.7 \pm 11.2	53.1 \pm 21.1	12.3 \pm 17.4	43.6 \pm 6.5	52.4 \pm 23.0	50.8 \pm 43.3	39.2 \pm 10.4	63.0 \pm 20.1	44.4 \pm 31.6	47.5 \pm 15.4	58.2 \pm 30.0
4	141.7 \pm 96.5 ^a	36.6 \pm 9.8	110.9 \pm 49.1	87.8 \pm 51.3	47.2 \pm 23.5	75.2 \pm 45.7	51.1 \pm 25.4	52.3 \pm 20.4	119.4 \pm 84.7	50.1 \pm 19.3	56.5 \pm 25.6	110.1 \pm 85.8
5	213.5 \pm 81.7 ^a	47.2 \pm 11.2	223.0 \pm 85.0	184.3 \pm 94.0	150.1 \pm 17.8	230.1 \pm 82.9	147.8 \pm 103.1 ^a	268.3 \pm 153.8 ^a	511.5 \pm 412.0 ^a	119.5 \pm 98.4	684.3 \pm 380.8 ^a	644.4 \pm 164.6 ^{abcd}
6	308.6 \pm 89.9 ^a	89.1 \pm 37.1	285.3 \pm 138.4	290.5 \pm 109.7 ^a	127.3 \pm 70.1 ^a	344.5 \pm 140.1 ^a	208.9 \pm 107.8 ^a	540.2 \pm 176.2 ^a	602.1 \pm 208.6 ^a	158.6 \pm 77.3 ^a	978.2 \pm 319.8 ^a	1105.7 \pm 714.5 ^{abcd}
7				32.7 \pm 18.0	46.0 \pm 13.4	108.6 \pm 36.0	36.3 \pm 27.0	48.4 \pm 14.7	129.8 \pm 50.3			

Comparing of results with controls at the corresponding time. ^a $P < 0.05$

Comparing of each other group at the different time. TNF- α : ^b $P < 0.05$; ALT: ^c $P < 0.05$; AST: ^d $P < 0.05$.

DISCUSSION

ASH may be caused by viral infection and drug intoxication. It was believed that the large amount of liver cell death was necrosis due to associated immune damage mediated by dysfunction of host immune system and TNF- α may cause liver necrosis directly^[13,14]. Recent studies have shown that besides necrosis, hepatic apoptosis induced by TNF- α plays an important role in the course of ASH^[1,15-27]. Our study showed that only mild

injury could be found by injecting ET or TNF- α alone. While the combination of GalN with either ET or TNF- α can cause ASH in mice.

Liver cells may synthesize the protecting protein after exposure to injury factors. The process needs the participation of intact cyto-metabolism and protein-synthesis mechanism. GalN may specifically deplete uridine nucleotides in liver cell and influence its metabolic course, leading to a hepatic transcriptional block and the suppression of

protecting protein synthesis and then sensitizes the liver cell to TNF- α ^[28-34].

TNF- α may induce apoptosis of liver cell which is transfected by hepatitis B virus or other virus^[35-41], suggesting the cells infected by virus involved in TNF- α sensitivity.

The results of our study showed that TNF- α was mainly produced in the early stage of endotoxemia, and decreased obviously from 6h to 9h after challenge. TNF- α combined with TNF- α receptor on the membrane of liver cells through a series signal transmission activating caspase-3 and then inducing apoptosis, and TGF- β_1 can also produce similar effect which can induce apoptosis^[42-45], delayed treatment with the caspase 3-like protease inhibitor Z-VAD attenuated apoptosis by 81% to 88% and prevented liver cell necrosis^[46]. At the same time TNF- α can activate nuclear transcription factor- κ (NF- κ) of hepatocytes^[47], Kupffer cells and endotheliocyte, which increases expression of ICAM-1, VCAM-1 and selectin, these inflammatory factors further induce the inflammatory injury of hepatocytes, and TNF- α also induce Shwartzman-like reaction in the liver^[48]. Recent study demonstrated that mitochondria may be the centre of cell apoptosis, if mitochondrial structural alterations occur without functional failure, the cell dies by apoptosis. In contrast, if the injury is severe enough to lead to mitochondrial functional failure, the cell dies by necrosis^[49,50].

In summary, our results showed that TNF- α plays an important role in the course of hepatic apoptosis and necrosis. The blockage of liver apoptotic signal transmission and caspase activation induced by TNF- α with Z-VAD, anti-ET antibody and anti-TNF monoclonal antibody can improve prognosis of fulminant hepatic failure^[2,42-46,51] and may prevent liver cell from apoptosis and necrosis and hence has an important significance in the prevention and treatment of ASH.

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Expressions of chromogranin A and cathepsin D in human primary hepatocellular carcinoma

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Abstract

AIM To determine the expression and clinical significance of chromogranin A and cathepsin D in hepatocellular carcinoma (HCC).

METHODS Double immunofluorescence staining techniques combined with laser confocal scanning microscopy (LSCM) was used to investigate chromogranin A and cathepsin D expressions in 85 HCC patients.

RESULTS Cathepsin D was expressed in 3 normal liver tissues, while in HCC the staining showed regional variation and the fraction of strongly stained cells increased as the tumors became less differentiated and usually clinically more malignant. Cells which showed strong positivity for cathepsin D were present in 71/85 (83.5%) cases. Strong expression of cathepsin D in cancer cells was related to histopathological features. They were more common in grade 3-4 (26/28, 92.9%) and grade 2 (46/53, 86.8%) tumors than in grade 1 tumors (1/4, 25.0%) ($P < 0.01$). No significant correlation was found between age and cathepsin D expression. In patients with positive cathepsin D reaction, the mean age was 52.1 ± 2.8 years (range 32-68 years) and in the group with negative reaction, the mean age was 51.3 ± 4.5 years (range 28-71 years). No obvious

relationship was observed between CgA expression in cancer cells and the histopathological features. The CgA positive rate was 75.0% (3/4) in grade 1, 71.7% (38/53) in grade 2, and 71.4% (20/28) in grade 3-4 ($P > 0.05$) tumors. The coexpression of CgA and cathepsin D was found by double labeled immunofluorescence staining techniques. The processing of cathepsin D was disturbed in HCC cells and accumulated in the cells. Cathepsin D had proteolytic activity and autocrine mitogenic effect, suggesting their functions in invasion. These findings demonstrated that the expression of cathepsin D in HCC had prognostic value.

CONCLUSION Chromogranin A and cathepsin D are expressed in a high proportion of HCC and the existence of cathepsin D in HCC might be related to processing of CgA. This is clearly a subject for further studies because of its potential clinical applications.

INTRODUCTION

The chromogranin A/secretogranin (CgA/Sg) acidic glycoprotein are widely distributed in vertebrate species. It has recently been proposed that CgA, a 50-kilodalton acidic glycoprotein, is costored and cosecreted with hormones and neurotransmitters in a variety of tissues. They thought to play a role in hormone packaging within secretory granules, in hormone secretion, and serve as prohormones for various proteolytic cleavage products^[1]. CgA can be processed to several biologically active peptides such as pancreastatin. The single copy human CgA gene was isolated from a human fetal liver gene library^[2]. The presence of CgA in hepatocellular carcinoma (HCC) was reported by Roskams *et al*^[3]. They found that occasional positive cells or clusters of weakly CgA immunopositive cells were present in HCC. Cathepsin D is a lysosomal aspartyl proteinase^[4], initially detected in breast cancer cell lines^[5], which is widely distributed in normal tissues. The proteinase cathepsin D might be related to tumor invasion and metastasis through various mechanisms associated with its proteolytic activity. It was shown to degrade *in vitro* extracellular matrix and activate

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latent precursor forms of other proteinase involved in such processes^[6]. Experimental studies have already demonstrated that invasion of HCC cells can be abrogated by proteinase inhibitors. Growing evidence indicates that lysosomal cathepsin D may promote carcinogenesis and tumor progression. The metastatic activity of cathepsin D injected into athymic mice was significantly higher than that of control groups. These results show that overexpression of cathepsin D increased the transformed phenotype of malignant cells *in vitro* and their metastatic potency *in vivo*^[7]. Higher cathepsin D serum mass concentrations were found in HCC group as compared to control patients^[8]. Plasma and ascitic fluid of rats bearing the Yoshida ascites hepatoma AH-130 were shown to contain high levels of proteolytic enzymes belonging to different classes active at neutral and acidic pH. Compared with those measured in control rat plasma, and tumor-bearing animals, the activity levels of lysosomal cathepsin D activity was about 5-fold higher in both plasma and ascitic fluid^[9]. The expression of cathepsin D was significant predictors of the prognosis^[10]. The role of cathepsin D has been studied in human breast cancer progression^[6-8], but the results were highly variable and no agreement has yet been reached on its effects on clinical behaviour and prognosis. There has been no report available on the role of cathepsin D expression in the progression of human HCC. To analyse this, the double immunofluorescence staining techniques combined with laser scanning confocal microscopy (LSCM) were used to investigate CgA and cathepsin D expression in HCC patients.

MATERIALS AND METHODS

Patients

The study group comprised 85 patients with HCC diagnosed in 117 Hospital and Xijing Hospital of the Fourth Military Medical University from 1984 to 1997. The mean age of the patients was 51.4 ± 2.5 years (range 25-77 years). The female/male ratio was 28/57. The cohort was not entirely consecutive, since adequate tumor biopsy specimens for immunohistochemistry were not available in all cases.

Histological methods

The histological samples were surgically obtained biopsy specimens from the tumors which were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 or 10 micrometer, and stained with haematoxylin and eosin. The samples were graded histologically into 1 (well differentiated, 4 cases), 2 (moderately differentiated, 53 cases), 3 (poorly differentiated, 26 cases), 4 (undifferentiated type, 2 cases) categories as described in detail before according to the Edmondson-Steiner's criteria^[11].

Immunohistochemistry

Five- or ten-micrometer sections from the primary HCC were employed in the fluorescent immunohistochemical analysis of cathepsin D protein, using rabbit anti-human cathepsin D antibody (Dako, Glostrup, Denmark) diluted 1:300 in 10mL/L bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Several dilutions of the antibody were tested to find the optimal staining concentration, before the entire series was processed. The staining procedure was carried out as our previous reports^[12], without protease treatment. Briefly, the steps included: ① the sections deparaffinized in xylene, hydrated in ethanol, and washed in 0.01mol/L PBS, then pretreated with 30mL/L normal goat serum for 40min and rinsed in 0.1mol/L PBS; ② incubation for 1h in a 1:1000 dilution of the primary monoclonal antibody of CgA and in a 1:300 dilution of rabbit anti-human cathepsin D antibody in 10mL/L BSA-PBS; ③ simultaneous incubation with 1:50 diluted secondary antibodies (biotin-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG) in 10mL/L BSA-PBS. All secondary antibodies were obtained from SABC (Luoyang, PRC); ④ incubation with 1:2000 diluted Texas-red-conjugated streptavidin (Sigma) for 30min. The sections were washed three times for 10min after incubation steps 2 to 4, respectively, and were finally mounted in 50g/L glycerin. The sections were examined with Bio-Rad 1024 LSCM. The specimens were excited with a laser beam at wavelengths of 568nm (Texas Red) and 488nm (FITC) and the emission light was focused through a pinhole aperture. The full field of view was scanned in square image formats of 512×512 pixels.

Scoring of CgA and cathepsin D protein expression

Firstly, the intensity of cytoplasmic fluorescence of the cancer cells in the entire section was scored into three categories. Cells showing no fluorescence for cathepsin D and CgA were considered as negative, cells with weak granular fluorescence (Figure 1) in the cytoplasm were scored as weak expressors, and cells with distinct cytoplasmic positivity (Figure 2) were scored as strong ones. Secondly, the fraction of cells in each of the staining categories in the entire section was also estimated in the areas with well-preserved tissue morphology. Areas with necrosis, or with distorted architecture, were excluded from the analysis. Thirdly, the presence in the invasion front of a distinct cathepsin D-positive cell zone composed of macrophage-like cells was scored positive or negative (determined in 65 cases). A breast cancer specimen showing intense uniform positivity for cathepsin D protein was used as a positive and negative control, with the expected results in all experiments. Since negative and weak

positive showed no association with other prognostic parameters, the cases were grouped into positive (strongly stained cells) or negative (negative or weakly positive cells only) for further analysis.

Controls

Primary antibodies were substituted by irrelevant antibodies and normal rabbit or goat serum as specific antibody control. PBS substituted for primary antibody as negative control. Primary antibody was omitted as blank control.

Statistical analysis

The Chi-square test was used in this study.

RESULTS

Relationship between expression of cathepsin D and histological features of HCC

Normal hepatocytes adjacent to carcinomas ($n = 3$) showed weak granular positivity for cathepsin D in the cytoplasm. Strong expression of cathepsin D in cancer cells was related to histopathological features (Table 1). Cells showing strong positivity for cathepsin D were present in 71/85 (83.5%) cases and were more common in grade 3-4 (26/28, 92.9%) and grade 2 (46/53, 86.8%) tumors than in grade 1 tumors (1/4, 25.0%, $P < 0.01$, Table 1). The positive reactivity was either granular or homogeneous in the cytoplasm (Figure 1). The positive cells distributed in disperse or patch pattern (Figures 1, 3,4).

Relationship between expression of cathepsin D and patients' age

We found no significant correlation between age and cathepsin D expression. The mean age of patients with positive cathepsin D reaction was 52.1 ± 2.8 years (range 32-68 years) and 51.3 ± 4.5 years (range 28-71 years, $P > 0.05$) in the group with negative reaction.

Relationship between expression of CgA and histological grade of HCC

The CgA positive rate was 75.0% (3/4) in grade 1, 71.7% (38/53) in grade 2, and 71.4% (20/28) in grade 3-4 tumors (Table 2). No obvious relationship was observed between expression of CgA in cancer cells and the histopathological grades of HCC ($P > 0.05$). The positive reactivity was homogeneous in the cytoplasm (Figures 2, 4, 5).

Correlation of expression of cathepsin D and CgA in HCC

Coexpression of cathepsin D and CgA was found in most of HCC (56/85, Table 3, Figures 3, 4, 6). It accounted for 91.8% of CgA-positive cases, and 78.9% (56/71) of cathepsin D-positive ones. Colocalization of cathepsin D and CgA was yellow observed by LSCM (Figures 3, 4, 6).

Table 1 Relationship between expression of cathepsin D and histological features of HCC

Histologic grade	Expression of cathepsin D	
	Positive (%)	Negative (%)
1	1/4 (25.0)	3/4 (75.0)
2	46/53 (86.8) ^a	7/53 (13.2)
3+4	26/28 (92.9) ^b	2/28 (7.1)

^a $P < 0.01$ vs Grade 1; ^b $P < 0.01$ vs Grade 2,1.

Table 2 Relationship between expression of CgA and histologic grade of HCC

Histologic grade	Expression of CgA	
	Positive (%)	Negative (%)
1	3/4 (75.0)	1/4 (25.0)
2	38/53 (71.7) ^a	17/53 (28.3)
3+4	20/28 (71.4) ^b	8/28 (28.6)

^a $P > 0.05$ vs Grade 1; ^b $P > 0.05$ vs Grade 2,1.

Table 3 Relationship between expression of cathepsin D and CgA in HCC

Expression of CgA	n	Expression of cathepsin D	
		Positive	Negative
Positive	61	56	5
Negative	24	15	9
Total	85	71	14

DISCUSSION

CgA is the major member of the granin family of acidic secretory glycoproteins that are expressed in all endocrine and neuroendocrine cells. Granins have been proposed to play multiple roles in the secretory process. Intracellularly, granins play a role in targeting peptide hormones and neurotransmitters to granules of the regulated pathway by virtue of their ability to aggregate in the low-pH, high-calcium environment of the trans-Golgi network. Extracellularly, peptides formed as a result of proteolytic processing of granins regulate hormone secretion. Some conserved features of the mature CgA protein are polyglutamic acids, calcium-binding sites, and several pairs of basic amino acids. The first two features are important for its intracellular functions, and the latter characteristic suggested that peptides could be released from the molecule by precursor processing enzymes. Several biologically active peptides encoded within the CgA molecule, such as vasostatin, beta-granin, chromostatin, pancreastatin, and parastatin act predominantly to inhibit hormone and neurotransmitter release in an autocrine or paracrine fashion. The biosynthesis of CgA is regulated by many different factors, including steroid hormones and agents that act through a variety of signalling pathways. CgA biosynthesis and that of the resident hormone or neurotransmitter can be regulated differentially. The widespread distribution of CgA has made the measurement of circulating immunoreactive CgA a valuable tool in the diagnosis of neuroendocrine neoplasia, and CgA immunohistochemistry can help identify the neuroendocrine nature of tumors. Recent molecular biological studies are identifying those elements in the CgA gene promoter responsible for its specific neuroendocrine cell expression.

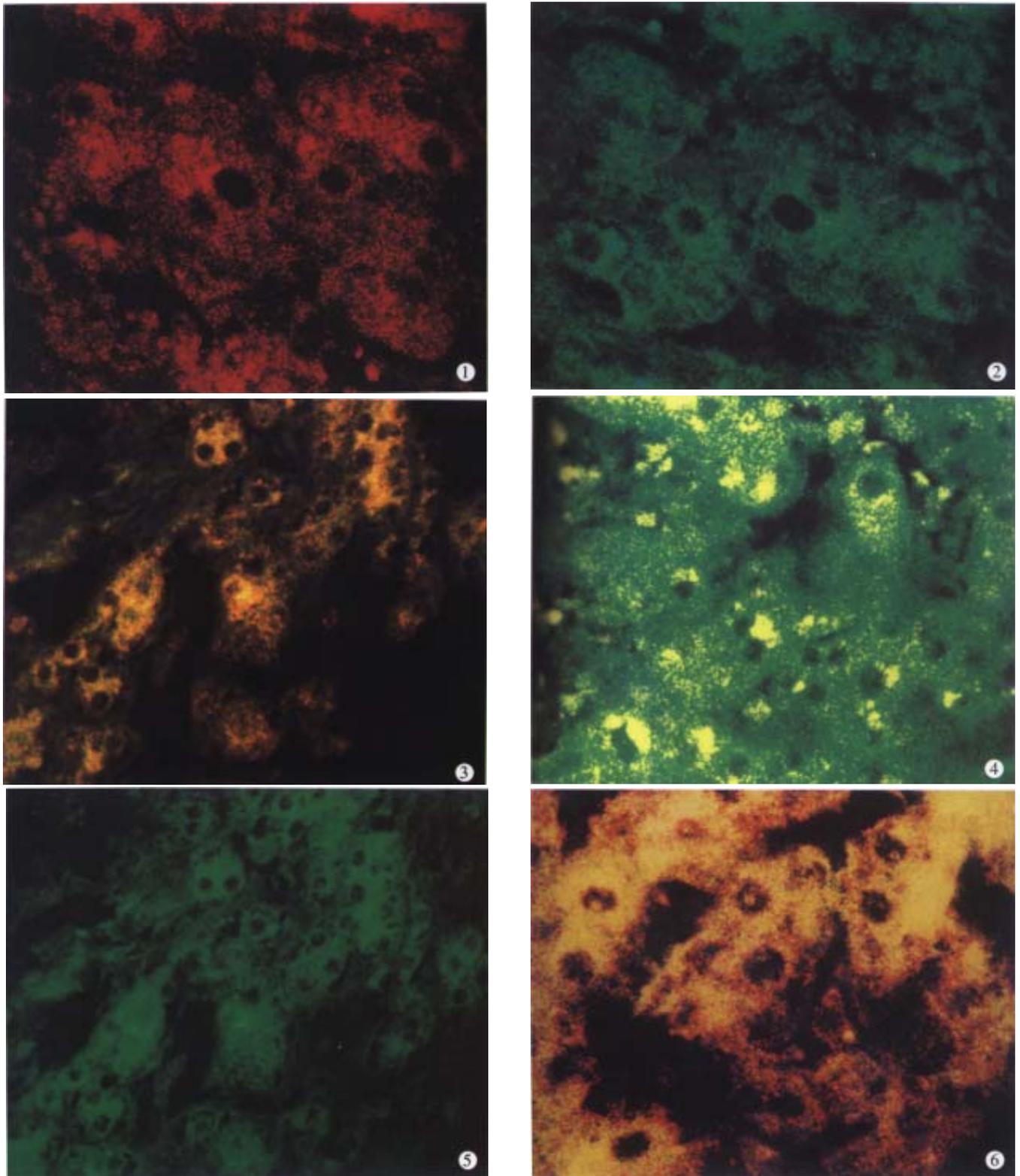


Figure 1 Distribution of cathepsin D in HCC (grade 3) The positive reactivity was granular in cytoplasm (red). TR-labelled $\times 400$

Figure 2 Localization of CgA in HCC (grade 3) The positive reactivity was homogeneous in cytoplasm (green). The positive cells distributed in patch pattern. FITC-labelled $\times 400$

Figure 3 Coexpression of cathepsin D and CgA in HCC (grade 4). The positive cells distributed in disperse (yellow). TR-labelled cathepsin D, FITC-labelled CgA $\times 200$

Figure 4 Coexpression of cathepsin D and CgA in HCC (grade 3). The positive cells distributed in disperse (yellow). TR-labelled cathepsin D, FITC-labelled CgA $\times 400$

Figure 5 Expression of CgA in HCC (grade 3) The positive cells distributed in disperse or patch pattern (green). FITC-labelled $\times 200$

Figure 6 Coexpression of cathepsin D and CgA in HCC (grade 2) The positive cells distributed in patch pattern (yellow). TR-labelled cathepsin D, FITC-labelled CgA $\times 400$

Cathepsin D was purified to apparently homogeneous form from normal human liver and hepatoma. The purified enzyme could not be distinguished between normal liver and hepatoma in terms of specific activity, subunit composition, antigenicity, amino acid composition and tryptic peptides. However, the hepatoma enzyme was treated with endo-beta-N-acetylglucosaminidase H, the acidic variant forms disappeared and were converted into forms identical to those of normal liver. The content of mannose-6-phosphate in the hepatoma enzyme was twice as much as that in the normal liver enzyme. Thus, charge heterogeneity found in hepatoma cathepsin D is ascribed to increased phosphorylation on oligosaccharides bound to the enzyme, most probably due to cancer-associated, impaired processing in carbohydrate moiety. A significant elevation of cathepsin D activity per tissue proteins was observed in hepatoma as compared to normal liver. In contrast, true specific activity per cathepsin D protein in hepatoma was significantly lower than that of normal liver. The lower true specific activity in hepatoma tissue may be attributed to an increased content in an inactive, large-molecular precursor form of the enzyme^[13].

Cathepsin D is encoded by a gene located on chromosome 11p15, but its increased expression is not usually due to gene rearrangement or amplification^[14]. Instead, it can be induced by estrogens or several other factors in the cellular micro-environment^[15,16]. Cathepsin D exists in the cell as a precursor for procathepsin D. In the normal hepatocytes, most of the procathepsin D is changed to an active form in the lysosomes, while in HCC cells the processing of procathepsin D is disturbed. Procathepsin D is accumulated in the cells, since smaller fraction is secreted than in normal liver tissues^[17]. Procathepsin D has proteolytic activity and autocrine mitogenic effect^[18], both of which suggest some functions in invasion and metastasis. Cathepsin D is a physiological regulator of other cathepsins (B and L) by activating their precursors^[19], or possibly by inactivating endogenous inhibitors of cathepsins^[20]. Cathepsin D was usually expressed in normal hepatocytes, while in malignant tumors the fluorescent staining showed regional variation and the fraction of strongly stained cells increased as the tumors became less differentiated and usually clinically more malignant. In HCC, increased expression was related to a high grade. The situation was also found in breast carcinomas^[21]. However, the dissemination of the tumor to lymph nodes and to distant sites suggested a relationship between invasive potential and the expression of cathepsin D. Strong expression of cathepsin D was associated with cell proliferation and expression of growth factor receptors^[22]. Our previous studies

also proved that the hepatoma cells expressed epithelium growth factor and its receptors^[22] and CgA^[23]. Thus, it is likely that the strong expression of cathepsin D is due to induction of cathepsin D synthesis by epithelium growth factor. The higher proliferation rate of strongly cathepsin D-positive tumors may be partly related to a mitogenic effect of cathepsin D^[18,24]. These findings, together with the clinical observations that a positive correlation between levels of expression of cathepsin D activity and malignant progression of some human neoplasms, seem to support this hypothesis^[25].

The prognostic value of cathepsin D expression in neoplasms is a matter of controversy and the results of this study suggest that the expression of cathepsin D in HCC cells has prognostic value over already established prognostic factors.

In conclusion, cathepsin D is expressed in a high proportion of HCC. This is clearly a subject for further studies because of its potential clinical applications. Chromogenic peptides act as substrates for cathepsins^[26], so the higher expression of cathepsin D, the lower expression of CgA in HCC and *vice versa*.

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Chemoprevention of tea on colorectal cancer induced by dimethylhydrazine in Wistar rats

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Subject headings colorectal neoplasms; dimethylhydrazine; tea; apoptosis; aberrant crypt foci; rats; proliferating cell nuclear antigen; chemoprevention

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Abstract

AIM To investigate the chemopreventive effects of green tea and tea pigment on 1,2-dimethylhydrazine (DMH)-induced rat colorectal carcinogenesis.

METHODS Male weaning Wistar rats were randomly allocated into four groups. Rats in the positive control group were given s.c. injection of DMH, once a week for ten weeks; rats in tea-treated groups, with the same DMH treatment as in the positive group, received 2% green tea and 0.1% tea pigments; rats in the negative control group were given s.c. injection of the same volume of saline as well as DMH in the positive group. Animals were sacrificed and necropsied at the end of week 16 and week 32.

RESULTS Aberrant cryptic foci (ACF) were formed in animals in DMH-treated groups at the end of week 16. Compared to the DMH group, green tea and tea pigments groups had less ACF (148.25 and 204.25, respectively, $P < 0.01$). At the end of week 32, all rats in DMH group developed large intestinal tumors. The results also showed that DMH increased labeling index (LI) of proliferating cell nuclear antigen (PCNA) of intestinal mucosa and the expression of ras-p21. However, in the tea-treated groups, PCNA-LI was significantly reduced as compared with the positive control group (36.63 and 40.36 in the green tea group and tea pigment group, respectively, at the end of the experiment, $P < 0.01$). ras-p21 expression was also significantly reduced (2.07 and 2.36 in the colon tumors of rats in the green tea group and tea pigments group, respectively at the end of the experiment, $P < 0.01$). Furthermore, green tea

and tea pigment inhibited the expression of Bcl-2 protein (2, 5, 1, 0 and 2, 4, 1, 0, respectively, at the end of the experiment $P < 0.01$), and induced expression of Bax protein (0, 1, 3, 4 and 0, 1, 4, 3, respectively, $P < 0.01$).

CONCLUSION Chinese green tea drinking inhibited ACF and colonic tumors formation in rats, which showed that tea had a significant chemopreventive effect on DMH-induced colorectal carcinogenesis. Such effects may be due to suppression of cell proliferation and induction of apoptosis in the intestinal crypts.

INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide^[1]. In China, the incidence and mortality rates of colorectal cancer are increasing in recent decade, being the fifth leading cause of cancer deaths^[2].

Aberrant crypt foci (ACF) in the colonic mucosa have been hypothesized to represent precursor lesions of chemically induced colon cancer. Aberrant crypts can be identified by their increased size, thicker epithelial lining, and increased pericryptal zone^[3]. Various studies have supported the concept that ACF are precancerous lesions that can be used as biologic end points in the study of modulators of colon carcinogenesis^[4-9].

Tea is one of the most popular beverages consumed worldwide. Tea polyphenols is the major constituent of green tea, whereas tea pigments, the major constituent of black tea, is a compound which is mainly composed of teaflavin and tearubigin. Many studies on the possible modifying effect of green tea and tea polyphenols on experimentally induced colorectal cancer have been carried out during the last decades^[10], but there are few reports about the effect of black tea and tea pigments.

Carcinogen-induced ACF formation in rodent colon has been used as a short-term bioassay to evaluate the role of nutritional elements and to screen potentially new chemopreventive agents^[11], however, few reports have been found regarding the early detection of ACF corresponding to the later development of tumors^[12]. The purpose of our present study is to find out the effects of green tea and tea pigments on ACF formation and colorectal cancer induced by DMH in Wistar rats, and to appraise their possible mechanisms.

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MATERIALS AND METHODS

Materials

Green tea and tea pigments were provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences. The water extract of green tea was prepared freshly everyday as follows: 2g of green tea leaves (Long Jing brand) was dissolved in 100mL boiling water, allowed to stand at room temperature for 30 minutes, and then filtered. Tea pigment solution was prepared freshly daily.

Animals and treatment

168 weaning male Wistar rats were purchased from the Animals Breeding Center of Chinese Academy of Medical Sciences, Beijing, and were randomly allocated into four groups, 42 rats in each. The animals were maintained in a controlled environment at $24^{\circ}\text{C}\pm 1^{\circ}\text{C}$ and $50\%\pm 10\%$ relative humidity with an altering 12:12-hour light-dark cycle. Rats in group 1 (positive control) were given s.c. injection of DMH-2HCl (Sigma Chemical) once weekly for 10 weeks at a dose of 20mg/kg body weight. Animals in groups 2 and 3, in addition to the same carcinogen treatment as in group 1, received 2% green tea and 0.1% tea pigments, respectively, as the sole source of drinking fluid. Animals in group 4 (negative control group) were injected s.c. with equal volumes of physiological saline. Rats in group 1 and group 4 were given tap water as drinking fluid. Body weight of all animals was recorded weekly until the last DMH injection, and then every 4 weeks until the end of the study. The daily consumption of tea and water were also recorded. Animals were killed at weeks 16 and 32. The colons were rapidly removed and opened longitudinally, cleaned with cold saline, fixed in 10% formalin, dehydrated and embedded in paraffin. Five- μm -thick sections were processed for histopathological examination for proliferating cell nuclear antigen (PCNA) analysis, and bcl-2 and bax analysis. For ras-p21 analysis, colonic mucosa were scraped with a microscope slide and stored at -80°C until used. The number of tumors were counted and the length (L), width (W) and height (H) of each tumor were measured to calculate the tumor volume ($V=L\times W\times H\times\pi/6$).

Quantification of ACF

ACF were quantified following the protocol established by McLellan and colleagues^[13]. Fixed colon specimens were stained in 0.02% methylene blue, and the number and growth of ACF were assessed under the light microscope. Criteria used to identify the ACF included: ① increased size, ② thicker epithelial cell layer, and ③ increased pericryptic zone. To determine crypt multiplicity, ACF were further categorized as small (1-3 crypts/focus), medium (4-6 crypts/focus), and large (≥ 7 crypts/focus).

Immunohistochemical analysis of PCNA

Two deparaffinized sections were used for PCNA staining by using a Straptavidin/ Peroxidase (SP) kit (ZYMED). Following the instructions, the anti-PCNA monoclonal antibody (mouse anti rat, ZYMED) was diluted with antibody-diluting buffer (1:100) and used for tissue sections.

The PCNA labeling index (LI) was determined by identifying 10 well-oriented crypts in which the base, lumen, and the apex of crypts displayed a U-shaped configuration. The PCNA-LI was calculated as the number of positive cells per crypt divided by the total number of cells per crypt multiplied by 100.

Measurement of ras-p21 expression

The expression of ras-p21 was detected by Western blot^[14]. Briefly, colonic mucosa and tumor specimens were washed in ice-cold phosphate-buffered saline (PBS) and suspended in disruption buffer. The specimens were homogenized and left on ice for 30min. The extracts were purified by centrifugation at $12\,000\times g$ for 20min at 4°C .

Clear extracts of colonic mucosa and tumors corresponding to 100 μg total protein were subjected to SDS-PAGE according to the method of Laemmli^[15]. The separated protein was then transferred to nitrocellulose membrane and detected with mouse monoclonal antibody pan-ras. Densitometric analysis of immunoblots were then performed for quantification of each band.

Immunohistochemical analysis of Bcl-2 and Bax

Bcl-2 and Bax were stained using the same method as PCNA staining except using the rabbit polyclonal Bcl-2 antibody (ZYMED) in 1:100 dilution and rabbit polyclonal Bax antibody (ZYMED) in 1:100 dilution.

Positive cells were quantified by two independent observers, expressed as the percentage of the total number of cells, and assigned to one of 4 categories: 1, 0% - 25%; 2, 25% - 50%; 3, 50% - 75%; and 4, 75%-100%.

Statistical analysis

Body weight, tea consumption, number of ACF, number of tumors, PCNA-LI and ras-p21 expression among the 4 groups were compared by Student's *t* test. Tumor volumes and the grading of Bcl-2 and Bax expression were compared by the Wilcoxon-rank test.

RESULTS

General observations

Table 1 presents the body weight of the 4 groups of animals. At the end of week 32, then compared with the negative control group, the body weights in the other three groups (DMH-treated) decreased.

However, analysis of the data revealed no significant differences between them. In addition, the quantity of tea and water consumption in 4 the groups were also not significantly different.

ACF formation

ACF was formed in DMH-treated groups (groups 1-3), but there was no ACF formation in the negative control group (group 4). Compared with the positive control group (group 1), the total number of ACF per colon in tea-treated groups 2 and 3 decreased significantly ($P < 0.01$). Small, medium and large ACF in tea-treated groups were also significantly different from group 1 (Table 2).

Cancer formation

The results of cancer formation are listed in Table 3. At the end of the experiment, all rats in the positive group developed colonic cancer, but none was developed in the negative control group. Animals that consumed 2% green tea and 0.1% tea pigments had significantly fewer cancer than that of the positive control group, and the mean volume also dramatically lower.

Immunohistochemical analysis of PCNA

Table 4 shows the immunohistochemical analysis of PCNA-LI. After 16 and 32 weeks, the PCNA-LI in DMH-treated groups increased significantly over the negative control group. Oral administration of 2% green tea and 0.1% tea pigments diminished the PCNA-LI ($P < 0.01$).

Western blot analysis of ras-p21 expression

Table 5 summarizes the results of Western blot analysis for ras-p21 expression in both colonic mucosa and the cancers. At the end of weeks 16 and 32, Green tea and tea pigments significantly suppressed the expression of ras-p21 in colonic mucosa and cancer as compared with the positive control group ($P < 0.01$).

Bcl-2 and Bax expression

The results of immunohistochemical analysis of Bcl-2 and Bax expression are shown in Tables 6 and 7. At the end of weeks 16 and 32, Bcl-2 expression was significantly suppressed in tea-treated groups as compared with the positive control group, while Bax expression was significantly induced in groups 2 and 3 ($P < 0.05$ or $P < 0.01$).

Table 1 Body weights during the study ($\bar{x} \pm s$)

Treatment groups	Week 0	Week 16	Week 32
DMH	70.14 \pm 5.92	425.69 \pm 44.06	456.25 \pm 94.36
Green tea+DMH	69.73 \pm 6.25	427.60 \pm 44.11	459.19 \pm 95.52
Tea pigments+DMH	70.23 \pm 5.35	425.39 \pm 46.99	463.24 \pm 98.50
Negative control	69.62 \pm 6.20	430.89 \pm 47.36	487.31 \pm 99.58

DMH: dimethylhydrazine; EDTA: ethylene diamine tetracetate acid.

Table 2 Effect of tea on DMH-induced ACF ($\bar{x} \pm s$)

Treatment groups	Total	Small	Medium	Large
DMH	249.88 \pm 10.891	61.50 \pm 8.07	63.75 \pm 1.85	24.62 \pm 2.87
Green tea+DMH	148.25 \pm 14.05 ^a	91.75 \pm 12.63 ^a	44.10 \pm 1.80 ^a	12.50 \pm 1.58 ^a
Tea pigments+DMH	204.25 \pm 11.94 ^a	126.50 \pm 10.37 ^a	53.00 \pm 2.24 ^a	17.38 \pm 3.04 ^a
Negative control	0	0	0	0

ACF: aberrant crypts foci.

^a $P < 0.01$, comparison with DMH group by Student's *t* test.

Table 3 Effect of tea on DMH-induced colon tumors

Treatment groups	<i>n</i> ^a	<i>n</i> ^b	Average number of tumors per rat	Mean tumor volume
DMH	20	20	2.55 \pm 1.36	294.69 \pm 614.43
Green tea+DMH	20	14	1.35 \pm 1.27 ^c	66.87 \pm 58.20 ^d
Tea pigments+DMH	20	16	1.45 \pm 1.16 ^c	94.83 \pm 120.89 ^d
Negative control	20	0	0	0

^aNo. of animals; ^bNo. of animals with tumors;

^c $P < 0.01$, comparison with DMH group by Student's *t* test.

^d $P < 0.01$, comparison with DMH group by Wilcoxon test.

Table 4 Effect of tea on PCNA-LI in colonic mucosa of DMH-treated rats ($\bar{x} \pm s$)

Treatment groups	PCNA-LI	
	Week 16 (<i>n</i> =8)	Week 32 (<i>n</i> =8)
DMH	41.75 \pm 6.01	52.53 \pm 5.40
Green tea +DMH	22.02 \pm 3.63 ^a	36.63 \pm 5.41 ^a
Tea pigments +DMH	29.66 \pm 2.65 ^a	40.36 \pm 5.64 ^a
Negative control	14.34 \pm 4.68	15.43 \pm 5.08

PCNA: proliferating cell nuclear antigen.

^a $P < 0.01$, comparison with DMH group by Student's *t* test.

Table 5 Effect of tea on expression levels of ras-p21 ($\bar{x} \pm s$)

Treatment groups	ras-p21 expression		
	Week 16 (<i>n</i> =8)	Week 32 (<i>n</i> =8)	Week 32 (<i>n</i> =8)
	colonic mucosa	colonic mucosa	colon tumors
DMH	2.03 \pm 0.35	2.26 \pm 0.28	3.16 \pm 0.32
Green tea +DMH	1.36 \pm 0.14 ^a	1.48 \pm 0.12 ^a	2.07 \pm 0.15 ^a
Tea pigments +DMH	1.51 \pm 0.19 ^a	1.72 \pm 0.15 ^a	2.36 \pm 0.16 ^a
Negative control	1	1	1

^a $P < 0.01$, comparison with DMH group by Student's *t* test.

Table 6 Effect of tea on Bcl-2 expression

Treatment groups	Week 16 (<i>n</i> =8)				Week 32 (<i>n</i> =8)			
	I	II	III	IV ^a	I	II	III	IV
DMH	1	1	3	3	0	1	2	5
Green tea +DMH	2	5	1	0 ^b	2	5	1	0 ^c
Tea pigments+DMH	1	5	1	1 ^b	2	4	2	0 ^c
Negative control	6	2	0	0	5	3	0	0

^aPositive cells were expressed as the percentage of the total number of cells, and classified into 4 categories: I, 0%-25%; II, 25%-50%; III, 50%-75%; and IV, 75%-100%.

^b $P < 0.05$, ^c $P < 0.01$, comparison with DMH group by Wilcoxon test.

Table 7 Effect of tea on Bax expression

Treatment groups	Week 16 (<i>n</i> =8)				Week 32 (<i>n</i> =8)			
	I	II	III	IV ^a	I	II	III	IV
DMH	2	5	1	0	3	5	0	0
Green tea +DMH	0	1	5	2 ^b	0	1	3	4 ^c
Tea pigments+DMH	0	3	4	1 ^b	0	1	4	3 ^c
Negative control	6	2	0	0	5	3	0	0

^aPositive cells were expressed as the percentage of the total number of cells, and classified into 4 categories: I, 0%-25%; II, 25%-50%; III, 50%-75%; and IV, 75%-100%.

^b $P < 0.05$, ^c $P < 0.01$, comparison with DMH group by Wilcoxon test.

DISCUSSION

It is recognized that colon carcinogenesis is a multistep process that includes sequential selection and propagation of preneoplastic lesions. ACF are present in carcinogen-treated rodent colons as well as in humans at high risk for colon cancer development and in patients with colon cancer^[16,17]. Several studies investigating the genotypic, morphological, and growth features of ACF have supported the contention that ACF are preneoplastic lesions^[4]. The ACF system is frequently used to identify and study the modulation of colon carcinogenesis. The results of the present study indicated drinking 2% green tea and 0.1% tea pigments significantly inhibited the formation of ACF, and decreased significantly the numbers and size of tumor. Some epidemiologic studies revealed an inhibitory effect of green tea on the incidence of colorectal cancer^[18,19]. In addition, many experimental studies have demonstrated that green tea and tea polyphenols have significant inhibitory effects on rodent colorectal carcinogenesis^[20]. Our study also demonstrated that 2% green tea significantly inhibited DMH-induced ACF formation and colorectal cancer. Black tea consists of significant amount of tea pigments including teaflavins, tearubigins, etc. Teaflavins have antioxidative and antimutagenic effects^[21]. However, very few studies on animal tumorigenesis model have been reported. Morse *et al*^[22] demonstrated that eaflavins in drinking water reduced esophageal tumor induced by N-nitrosomethylbenzylamine (NMBzA) in rats. We have observed that tea pigments have significant inhibitory effects on oral carcinogenesis^[23]. The present study demonstrated that as little as 0.1% tea pigments could significantly inhibit colorectal tumor induced by DMH in rats. It has been suggested that tea pigments may play important roles on the protective effect of black tea in chemical carcinogenesis models.

Abnormal cellular proliferation is one of crucial mechanisms in carcinogenesis^[24]. PCNA is an auxiliary protein of the DNA polymerase delta, reaching an expression peak during the S-phase of the cell cycle and playing an important role in cellular proliferation^[25,26]. PCNA-LI has been used as an intermediate biomarker in chemoprevention of colorectal cancer^[27]. Zheng *et al*^[28] observed that vitamin A significantly decreased PCNA-LI in the AOM-induced colorectal cancer animal model. Another study gave similar result^[29]. In this study, both green tea and tea pigments significantly inhibited PCNA-LI at the end of weeks 16 and 32.

Recent evidence indicates that activation of ras proto-oncogenes, coupled with the loss or inactivation of suppressor genes induces a malignant phenotype in colonic cells^[30]. The *ras* proto-oncogenes (c-Ki-ras, c-Ha-ras and N-ras)

constitute a family of highly conserved genes encoding a structurally and functionally related 21 kd protein, referred to as *ras*-p21, which is anchored to the cytoplasmic face of the plasma membrane, binds to the guanine nucleotides GTP and GDP. Ras activation represents one of the earliest and most frequently occurring genetic alterations associated with human cancers, especially in cancer of colon^[31-34]. Elevated levels of *ras*-p21 were correlated with increased cell proliferation, histological grading, nuclear anaplasia and degree of undifferentiation^[35,36]. In experiments where mutated *ras* genes are selectively inactivated, the pre-existing tumor phenotype reverts to a more normal form, indicating activated *ras* may be necessary for the maintenance of malignant behavior^[37]. Recent studies have demonstrated a strong correlation between dietary modulation of carcinogen-induced *ras* activation and consequent tumor outcome^[38]. Singh *et al*^[39] had suggested that determination of *ras*-p21 might be a useful marker to evaluate the effectiveness of tumor inhibitory properties in colon carcinogenesis. In this study, the DMH-induced expression of *ras*-p21 was significantly suppressed both in tumors and in uninvolved colonic mucosa by oral feeding of 2% green tea and 0.1% tea pigments.

Colorectal cancer is believed to result from a series of genetic alterations that destroys normal mechanisms controlling the cell growth^[30,40]. Apoptosis or programmed cell death appears to be an important mechanism in deletion of tumor cells rather than increased cell proliferation^[41-43]. The *bcl-2* proto-oncogene is a known inhibitor of apoptosis and may therefore allow an accumulation of genetic alterations that become propagated by cell division and potentially contribute to neoplastic development^[44-46]. The *bcl-2* gene encodes a 25 kd protein that localizes to the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum^[47]. Sinicrope *et al*^[48] analyzed the expression of Bcl-2 oncoprotein during colorectal tumorigenesis and concluded that abnormal activation of *bcl-2* gene appeared to be an early event on colorectal tumorigenesis that can inhibit apoptosis *in vivo* and may facilitate tumor progression. Increased *bcl-2* expression could occur in conjunction with changes in the expression of other members of the *bcl-2* family, including those counteract the antiapoptotic effects of Bcl-2. One candidate in this regard is Bax, a dominant repressor of Bcl-2 that forms heterodimers with Bcl-2 and accelerates rates of cell death^[49,50]. A recent study found that the development of IQ induced colorectal tumors was accompanied by the progressive inhibition of programmed cell death which was associated with increased expression of the antiapoptosis protein Bcl-2 and decreased expression of Bax^[51]. In our study, the expression

of Bcl-2 was inhibited significantly by oral 2% green tea and 0.1% tea pigments, while the expression of Bax was induced significantly. The results demonstrated that green tea and tea pigments induced apoptosis.

In conclusion, the present study indicated that green tea and tea pigments significantly inhibited ACF and colorectal cancer induced by DMH, and our study further supported the hypothesis that ACF are precancerous lesions of colorectal cancer and the ACF system can be used as a short-term bioassay to screen potentially new chemopreventive agents and to evaluate the effect of protective factors at a very early stage of the carcinogenic process. Although the mechanisms of the inhibitory effects of tea preparations on carcinogen-induced colorectal carcinogenesis have not been fully elucidated, our study showed that inhibition of proliferation and induction of apoptosis may be two important mechanisms.

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Ascorbic acid secretion in the human stomach and the effect of gastrin

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Subject headings gastric mucosa; gastrins; vitaminC; plasma; gastric juice; *Helicobacter pylori*

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Abstract

AIM To investigate the changes of gastric mucosal ascorbic acid secretion in patients with nonulcer dyspepsia and the effect of gastrin on it, and to relate any observed changes to *H. pylori* infection and mucosal histology.

METHODS Ascorbic acid secretions in patients were examined by collecting continuously gastric juice for one hour after having aspirated and discarded fasting gastric juice. Using the clearance rate (mL/min) of ascorbic acid from blood to gastric juice represented ascorbic acid secretion in the gastric mucosa. Ascorbic acid concentrations in plasma and juice were measured by ferric reduced method.

RESULTS Gastric ascorbic acid secretions in *H.pylori*-positive patients (1.46mL/min, range 0.27-3.78) did not significantly differ from those in *H.pylori*-negative patients (1.25mL/min, 0.47-3.14) ($P>0.05$). There were no significant differences in ascorbic acid secretions between patients with mild (1.56mL/min, 0.50-3.30), moderate (1.34mL/min, 0.27-2.93) and severe (1.36mL/min, 0.47-3.78) inflammation ($P>0.05$). There were no significant differences in ascorbic acid secretions between patients without activity (1.45mL/min, 0.27-3.14) and with mild (1.32 mL/min, 0.61-2.93), moderate (1.49mL/min, 0.50-3.78) and severe (1.43 mL/min, 0.51-3.26) activity of chronic gastritis either ($P>0.05$). Ascorbic acid secretions in patients with severe atrophy (0.56mL/min, 0.27-1.20) were markedly lower than those in patients with out atrophy (1.51mL/min, 0.59-3.30) and

with mild (1.43mL/min, 0.53-3.78) and moderate (1.31mL/min, 0.47-3.16) atrophy ($P<0.005$). There was a significant negative correlation between ascorbic acid secretion and severity of atrophy (correlation coefficient = -0.43, $P<0.005$). After administration of pentagastrin, ascorbic acid secretions were markedly elevated (from 1.39mL/min, 0.36-2.96 to 3.53mL/min, 0.84-5.91) ($P<0.001$).

CONCLUSION Ascorbic acid secretion in gastric mucosa is not affected by *H. pylori* infection. Gastric ascorbic acid secretion is markedly related to the severity of atrophy, whereas not related to the severity of inflammation and activity. Gastrin may stimulate gastric ascorbic acid secretion. A decreased ascorbic acid secretion may be an important factor in the link between atrophic gastritis and gastric carcinogenesis.

INTRODUCTION

Ascorbic acid, a powerful antioxidant, is potentially important for the prevention of gastric cancer. It may be able to protect against gastric cancer by scavenging nitrite and preventing the formation of carcinogenic N-nitroso compounds within gastric juice^[1-5]. In addition, it is capable of scavenging reactive oxygen metabolites^[6-8] that may damage gastric mucosal DNA^[7] and play a role in the development of experimental gastric carcinoma and precancerous lesions induced by N-methyl N-nitro N-nitrosoguanidine^[9] whereby it may also protect against gastric cancer. Various epidemiological studies have clearly shown that high dietary vitamin C intake may reduce the risk of gastric cancer^[10-12]. *H. pylori* infection has been associated with gastritis, peptic ulcer and an increased risk of gastric cancer^[13-17], but its precise role in gastric carcinogenesis is still unknown^[18]. Some previous studies have shown that ascorbic acid is present in the gastric juice of healthy subjects in concentrations considerably higher than those in plasma^[19-21]. This high ratio of gastric juice to plasma ascorbic acid implies active secretion of ascorbic acid by gastric mucosa. It has been recognized recently that gastric juice ascorbic acid concentrations are decreased markedly in subjects with *H. pylori* infection^[22-27] and chronic

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gastritis^[19-21,26]. This change of gastric juice ascorbic acid concentrations in *H. pylori*-infected patients may be an important factor in the link between *H. pylori* infection and gastric carcinogenesis. However, these studies only observed the changes of ascorbic acid concentrations in gastric juice. The changes of ascorbic acid secretion in gastric mucosa have rarely been studied. The purpose of this study is, therefore, to investigate the changes of ascorbic acid secretion in gastric mucosa, to assess their relationships to *H. pylori* infection and mucosal histology, and to explore the effect of gastrin on ascorbic acid secretion.

SUBJECTS AND METHODS

Subjects

Fifty-five consecutive cases shown nonulcer dyspepsia by endoscopy and type B ultrasonography were studied. None of them had undergone upper gastrointestinal surgery, nor had taken any drugs over the previous two weeks.

Methods

Collection of samples All patients were studied at the same time (6:00 am) after a 10-hour overnight fast. A 2mL sample of venous blood was withdrawn into a heparinised tube for measurement of plasma ascorbic acid concentration. Then a nasogastric tube was inserted into the patient's stomach. Gastric juice was continuously collected for one hour by a constant suction pump after having aspirated and discarded fasting gastric juice. One hour later, 20 consecutive patients of them were immediately given pentagastrin (6 μ g/kg) intramuscularly and followed by collecting gastric juice for one hour again. Each gastric sample was analyzed for volume and ascorbic acid concentration.

Ascorbic acid measurement Venous blood and gastric juice samples were immediately stored at 4°C after being collected. Ascorbic acid concentrations in the plasma and gastric juice were measured by ferric reduced method^[28] within 10 hours. This method is based on the quantitative rapid reduction of ferric to ferrous by ascorbic acid and the colorimetric measurement of the ferrous through its formation of a colored complex with bathophenanthroline. Briefly, the venous blood samples were centrifuged at 3000 \times g for 20min and gastric juice for 40min before ascorbic acid assay. Then 0.2 mL aliquots of the gastric juice and the plasma supernatants were mixed with 0.75mL of 5% trichloroacetic acid to precipitate protein, which were then removed by centrifugation at 3000 g for 20min. Subsequently, 0.5mL of the further supernatants were mixed with 1.0mL of acetate buffer, 2.0 mL of bathophenanthroline solution, 0.5mL of ferric chloride solution, and 0.2mL of phosphoric acid solution. Finally, the concentration

was determined with a spectrophotometer at 536nm against standards. Ascorbic acid secretion in gastric mucosa was estimated by clearance rate of ascorbic acid from blood to gastric juice (mL/min), which was calculated by the formula: clearance rate = GV/Bt, where V is the volume of gastric juice collected over t min (mL), G the concentration of ascorbic acid in gastric juice (μ mol/L), B the concentration of ascorbic acid in plasma (μ mol/L), t=60min.

H. pylori detection and histopathological examination

Three antral biopsies were obtained from every patient for *H. pylori* detection and histopathological examination. One biopsy from the lesser curvature was used for a rapid urease test. Other two biopsies (one from the lesser curvature and another from the greater curvature) were used for Warthin-Starry stain for *H. pylori* and hematoxylin-eosin stain for histopathological examination. Patients were considered to be *H. pylori* positive if one of the two tests was positive, whereas to be *H. pylori* negative if all negative. The severity and extent of gastric inflammation, activity and atrophy were graded on a scale of mild, moderate and severe according to the Sydney System^[29].

Statistical analysis The data in the text were expressed as median values with ranges. Statistical analysis was carried out using non-parametric Mann-Whitney U test. The Spearman rank correlation test was used to calculate correlation coefficients. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Among the 55 patients studied, 31 were *H. pylori* positive and 24 negative. According to the histological division of the Sydney System, 14 had mild inflammation, 17 moderate inflammation, and 24 severe inflammation; 16 had no activity, 11 mild activity, 14 moderate activity and 14 severe activity; 17 had no atrophy, 16 mild atrophy, 12 moderate atrophy and 10 severe atrophy.

Gastric ascorbic acid secretion and H. pylori infection

Gastric ascorbic acid secretions in *H. pylori*-positive patients (1.46mL/min, 0.27-3.78) did not differ significantly from those in *H. pylori* negative patients (1.25mL/min, 0.47-3.14) ($P > 0.05$).

Gastric ascorbic acid secretion and gastric inflammation

Gastric ascorbic acid secretions in patients with mild, moderate and severe inflammation were respectively 1.56 mL/min (0.50 - 3.30), 1.34mL/min (0.27 - 2.93) and 1.36mL/min (0.47 - 3.78). There were no significant differences between them ($P > 0.05$).

Gastric ascorbic acid secretion and activity of gastritis

Gastric ascorbic acid secretions in patients without activity and with mild, moderate and severe activity were respectively 1.45mL/min (0.27 - 3.14), 1.32 mL/min (0.61 - 2.93), 1.49 mL/min (0.50 - 3.78) and 1.43mL/min (0.51 - 3.26). There were no significant differences between them either ($P>0.05$).

Gastric ascorbic acid secretion and gastric atrophy

Gastric ascorbic acid secretions in patients without atrophy and with mild, moderate and severe atrophy were respectively 1.51 mL/min (0.59 - 3.30), 1.43 mL/min (0.53 - 3.78), 1.31 mL/min (0.47-3.16) and 0.56mL/min (0.27 - 1.20). Ascorbic acid secretions in patients with severe atrophy were significantly lower than those in patients without atrophy and with mild and moderate atrophy ($P < 0.005$). There were no significant differences between patients without atrophy and with mild and moderate atrophy ($P>0.05$). With the progress of atrophy, ascorbic acid secretion was gradually decreased, with a significant negative correlation (correlation coefficient = -0.43, $P<0.005$).

Effect of gastrin on ascorbic acid secretion

In 20 patients given pentagastrin, gastric ascorbic acid secretions rose from 1.39mL/min (0.36-2.96) to 3.53mL/min (0.84 - 5.91). There was very significant difference between them ($P<0.001$).

DISCUSSION

Some previous studies have found that gastric ascorbic acid concentrations in *H. pylori*-positive patients and patients with chronic gastritis are markedly lower than those in *H. pylori*-negative patients and healthy controls^[19-27]. However, little is known the changes of ascorbic acid secretion in the stomach. It is also uncertain whether low gastric juice ascorbic acid concentrations in *H. pylori*-infected patients are induced by impairing gastric mucosal ascorbic acid secretory capacity or other causes. Some researchers speculate that lower gastric juice ascorbic acid concentrations in *H. pylori*-infected patients are mainly related to the impaired gastric secretory capacity in the presence of gastritis induced by *H. pylori* infection. The reason for this notion is that there is a significant negative correlation between gastric juice ascorbic concentration and grading of polymorphonuclear leucocyte infiltration induced by *H. pylori* infection^[22,25]. However, some studies have shown that *H. pylori* can potentiate the polymorphonuclear leucocyte oxidative burst^[30,31], which is accompanied by a considerable production of reactive oxygen metabolites. Ascorbic acid in

gastric juice may be in itself consumed in the course of scavenging these reactive oxygen metabolites. In addition, some studies on gastric mucosal ascorbic acid levels suggest that gastric mucosal ascorbic acid concentration is not related to *H. pylori* infection^[32,33] and presence of inflammation^[34]. In order to investigate whether gastric ascorbic acid secretion is affected by *H.pylori* infection and the changes of gastric mucosal histology, we made an investigation on gastric ascorbic acid secretion in patients with *H. pylori* infection and chronic gastritis through collecting continuously gastric juice for one hour after having aspirated and discarded fasting gastric juice. We found that Gastric mucosal ascorbic acid secretions in *H. pylori*-positive patients did not significantly differ from those in *H.pylori*-negative patients. The changes of gastric ascorbic acid secretion were independent of the severity and extent of gastric inflammation and activity. However, gastric ascorbic acid secretions in patients with severe atrophy were significantly lower than those in patients without atrophy or with mild and moderate atrophy. There was a significant negative correlation between gastric ascorbic acid secretion and severity of atrophy. The results indicate that gastric ascorbic acid secretion is not influenced by *H. pylori* infection. *H. pylori* infection might lower ascorbic concentration in gastric juice through other mechanisms. A number of reasons could be responsible for low gastric juice ascorbic acid concentration induced by *H. pylori* infection. In addition to potentiating polymorphonuclear leucocyte burst described above, one study has shown that the cytochrome c-like water soluble oxidant of *H.pylori* may destroy ascorbic acid in the gastric juice of infected patients^[35]. *H. pylori* can also secrete many kinds of enzymes which have higher enzyme activity^[36]. It has been shown that *H. pylori* markedly influences the metabolism of certain endogenous organic molecules^[36-38]. These enzymes and the local biochemical alterations induced by *H. pylori* might influence the metabolism of ascorbic acid and lower the ascorbic acid concentration in gastric juice. Ascorbic acid is a powerful antioxidant and is potentially important for the prevention of gastric cancer. It may protect against gastric cancer by either preventing the formation of carcinogenic N-nitroso compounds in gastric juice^[11-51] or scavenging reactive oxygen metabolites that may damage gastric epithelium^[6-8]. Various studies have shown that ascorbic acid levels in gastric juice are related to the incidence of gastric cancer^[39-41]. Blood ascorbic acid levels in patients with gastric cancer were markedly lowered^[42-44]. These studies suggest that the decrease of ascorbic acid in gastric juice can increase the risk of gastric carcinogenesis. Chronic atrophic gastritis is an important precancerous condition and has been associated with an increased

risk of gastric carcinogenesis is^[45,46]. Previous studies have shown that an environment of hypochlorhydria in atrophic gastritis favors an overgrowth of nitrite-forming bacteria and increasing the formation of nitrite and N-nitroso compounds^[47-50]. However, as ascorbic acid is a powerful antioxidant, it may react with nitrite to form dehydroascorbic acid and nitrous oxide and prevent the formation of carcinogenic N-nitroso compounds. Only when the nitrite in gastric juice is in excess of reduced capacity of ascorbic acid in gastric juice, are carcinogenic N-nitroso compounds available. In the present study, it has been found that gastric ascorbic acid secretions in patients with severe atrophy are markedly decreased. There is a significant negative correlation between gastric ascorbic acid secretion and severity of atrophy. We speculate that this change of gastric ascorbic acid secretions in patients with atrophy may be an important factor in the link between atrophic gastritis and gastric carcinogenesis. Some studies have shown that the supplementation of ascorbic acid may elevate the ascorbic acid concentration in gastric juice^[34,51,52], so the diet rich in vitamin C may decrease the risk of gastric cancer in patients with gastric atrophy.

The mechanism whereby gastric mucosa secretes ascorbic acid is unclear. Some studies on the rats have shown that gastric ascorbic acid secretion is physiologically regulated not only by muscarinic receptor-associated cholinergic stimulation^[53] but also by CCK receptor-associated humoral stimulation^[54]. Our study found that gastric ascorbic acid secretion was markedly elevated after given pentagastrin. The result indicates that gastrin may also stimulate gastric ascorbic acid secretion. In the present study, it was observed that the changes of gastric ascorbic acid secretion were related to the severity of atrophy, whereas not related to the severity of inflammation and activity. As the histologic alteration of atrophy is the loss of specialized gland, we speculate that gastric glands may participate in the secretion of ascorbic acid. However, the detailed mechanism about gastric ascorbic acid secretion will be further investigated.

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Mechanism of exogenous nucleic acids and their precursors improving the repair of intestinal epithelium after γ -irradiation in mice

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Abstract

AIM To clone expressed genes associated with repair of irradiation-damaged mice intestinal gland cells treated by small intestinal RNA, and to explore the molecular mechanism of exogenous nucleic acids improving repair of intestinal crypt.

METHODS The animal mode of test group and control group was established, forty-five mice being irradiated by γ ray were treated with small intestinal RNA as test group, forty mice being irradiated by γ ray were treated with physiological saline as control group, five mice without irradiation were used as normal control, their jejunal specimens were collected respectively at 6h, 12h, 24h, 4d and 8d after irradiation. Then by using LD-PCR based on subtractive hybridization, these gene fragments differentially expressed between test group and control group were obtained, and then were cloned into T vectors as well as being sequenced. Obtained sequences were screened against GeneBank, if being new sequences, they were submitted to GeneBank.

RESULTS Ninety clones were associated with repair of irradiation-damaged intestinal gland cells treated by intestinal RNA. These clones from test group of 6 h, 12 h, 24 h, 4 d and 8 d were respectively 18, 22, 25, 13, 12. By

screening against GeneBank, 18 of which were new sequences, the others were dramatically similar to the known sequences, mainly similar to hsp, Nmi, Dutt1, alkaline phosphatase, homeobox, anti-CEA ScFv antibody, arginine/serine kinase and BMP-4, repA. Eighteen gene fragments were new sequences, their accept numbers in GeneBank were respectively AF240164-AF240181.

CONCLUSION Ninety clones were obtained to be associated with repair of irradiation-damaged mice intestinal gland cells treated by small intestinal RNA, which may be related to abnormal expression of genes and matched proteins of hsp, Nmi, Dutt1, Na, K-ATPase, alkaline phosphatase, glkA, single stranded replicative centromeric gene as well as 18 new sequences.

INTRODUCTION

After exposure to large dose ionizing radiations, the larger intestinal gland cell lesion is the main cause of death in humans and animals. How to enhance intestinal gland cell survival rates has become a problem to be solved. In order to increase the mouse crypt survival after irradiation, we have done a series of experiments, and finally confirmed that when a portion of the crypts was devastated by irradiation, a compensatory recovery of the intestinal epithelium by remaining crypts occurred involving three consecutive periods such as the rapid cell proliferation of the viable crypts, the fission of the proliferative crypts and the increase of crypt numbers^[1]. The nucleic acid fragment containing several hundred base pairs (bp), or even any one of the nucleic acid precursors (mononucleotides, nucleosides, and bases) can enhance the crypt survival rate by 25% or so, further confirming that the effectiveness of exogenous nucleic acids depends not upon the action exerted by their highly polymerized state, but upon their various enzymatic degradation products^[2]. Our experimental results suggest that the nucleic acids (DNA, RNA) and their precursors may be used as one of the effective measures for the treatment of intestinal radiation

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syndrome that may occur in the war time as well as in the peaceful use of atomic energy^[3-5]. However, the molecular mechanism how nuclear acids and precursors improve the repairing of irradiation-damaged intestinal gland cells is unclear. In order to clarify this molecular mechanism, we used long distance-PCR based on subtractive hybridization, isolated and cloned these genes associated with repairs of irradiation-damaged mice intestinal gland cells treated by intestinal RNA. Our studies lay foundation for further clarifying molecular mechanism of repair of radiation-damaged crypt.

MATERIALS AND METHODS

Reagents

PolyATtract[®] system 1000 kit from Promega was used for extraction of mRNA, SMART PCR cDNA synthesis kit (Clontech) for transcription of mRNA, Wizard[®] plus Minipreps DNA purification for purification of PCR production, Advantage2PCR kit (Clontech) for LD-PCR, and PE-5700 quantitative PCR cyler used for thermal cycle. PGEM-T easy vector system was purchased from Promega, γ -³²-pdATP from Beijing Fu Rei Company, and the other reagents were from Beijing Yuan Ping company.

Establishment of the model and sample collection

Ninety BALB/c male mice with body weight of 18g-22g and 10-12 weeks old were randomly assigned into two groups, *i.e.* test group and control group. They were injected with 5g·L⁻¹ barbiturate sodium 40 mg·kg⁻¹, put in organism radiation box, and then were irradiated on mice abdominal region by using ⁶⁰Co γ ray at the reagent rate of 149.47-151.13cGy·min⁻¹, and finally reached total reagent of 1150cGy. Small intestinal RNA was diluted into 100mg·mL⁻¹. Two hours after mice being irradiated, each mouse in test group was injected 0.4mL RNA liquid, and each mouse in control group was injected 0.4mL physiological saline by using local intestinal cavity expanding injection method^[6]. After that, the mice were raised according to conventional method. These mice were killed respectively at 6 h, 12 h, 24 h, 4 d and 8 d after irradiation, and jejunal tissues were quickly taken out, washed by physiological saline, and then were kept in liquid nitrogen. In order to avoid single difference, these samples were mixed together under identical condition.

Sample processing and LD-PCR based on subtractive hybridization

Extraction of mRNA was done according to manual of PolyATtract system 1000, and quantificated. mRNA transcription was done according to manual from SMART PCR cDNA synthesis kit. Subtractive hybridization between test group and control group

was done as follows: take out 0.1 μ g mRNA from the control group, add Biotinylated oligo(dT) probe (50 μ mol·L⁻¹), 70°C 5min, then add into the first strand cDNA, and hybridize for 24 h at 42°C, finally add Streptavidin magnesphere particles and mixed, magnetic steel was used to attract production of two stranded hybrids and get rid of them, and then repeat the processing twice. Take out the upper liquid, and add double volume absolute alcohol to the upper liquid to precipitate cDNAs, finally dissolve them in 10 μ L Nuclease-free water. Take 10 μ L of the first strand cDNA as template, sequentially add PCR-grade water 74 μ L, 10 \times advantage 2 PCR buffer 10 μ L, 50 \times dNTP mix-10 μ L, 50 \times advantage 2 polymerase mix 1 μ L, PCRprimer Mix 2 μ L, 50mmol·L⁻¹ MgCl₂ 3 μ L, mixed well, centrifuged for several seconds, using two steps such as 95°C 1min, 95°C 15s, 68°C 6min, 30cycles, in the end elongated at 68°C for 6min, take out 10 μ L production to run 12 g·L⁻¹ agarose gel electrophoresis.

Cloning and identification of PCR products

PCR products were purified using Wizard plus Minipreps DNA purification system, and dissolved in 10 μ L water. According to the manual, PCR products were cloned into PGEM-T easy vector, transferred into JM109, positive clone picked up, cultured overnight, plasmid extracted out, and identified by cutting with BstZ1. Products cut by BstZ1 were labelled γ -³²-pdATP at terminal, and used as probe, and hybridized with mRNA of test group and control group^[7].

Sequencing and screening against GeneBank

These obtained clones were positively sequenced by using Model 377 sequencing instrument. Obtained sequences were screened against GeneBank, if being new sequences, these sequences were submitted to GeneBank^[8].

RESULTS

LD-PCR based on subtractive hybridization

In test group of 6 h, 12 h, 24 h, 4 d and 8 d, products after subtractive hybridization were successfully amplified by LD-PCR, obtained bands centered on 1-1.5kb or so (Figure 1).

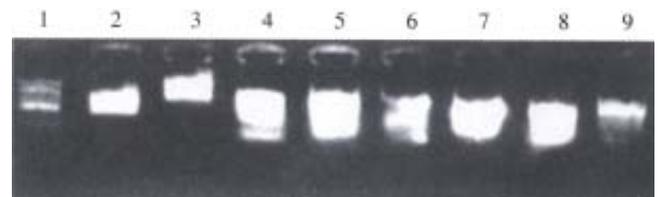


Figure 1 Electrophoresis result of production of LD-PCR. 1: Marker; 2, 6: Positive control; 3, 4, 5, 7, 8: Results of 6h,

12h, 24h, 4d and 8d; 9: Negative control

Cloning and identification of PCR products (Figure 2)

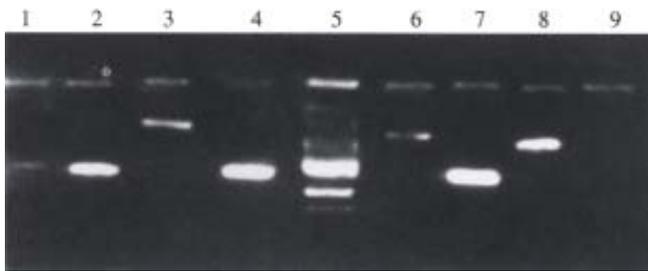


Figure 2 Results of cloning and identification of production of PCR. 1-4, 6-9: Results of part products cut by Bstz1; 5: Marker Ninety of positive clones were obtained from test group, positive clones of 6 h, 12 h, 24 h, 4 d and 8 d in test group were respectively eighteen, twenty-two, twenty-five, thirteen, twelve.

Identification of hybridization (Figure 3)

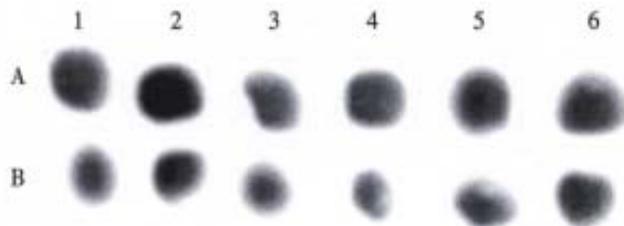


Figure 3 Part results of hybridization with RNA. A: Test group; B: Control group Dot blots confirmed that these genes were overexpressed in test group, and lower expressed in control group, that is, these genes were associated with repair of intestinal gland cells treated by RNA.

Sequencing and searching for GeneBank

These clones were sequenced according to the results of sequencing and searching against GeneBank, eighteen were new sequences, eighty-two were dramatically similar to the known sequences.

In test group of 6h, similar sequences mainly were as follows: mRNA for heat shock protein,

Nmi mRNA, Dutt1 protein, mRNA for Na, K-ATPase gamma subunit, mRNA for surface glycoprotein, Zinc finger type transcript factor, porcine growth hormone-releasing hormone gene, monocyte/macrophage Ig-related gene, telomerase-associated protein, HOX1b protein, arginine/serine kinase.

In test group of 12h, similar sequences were: Alkaline phosphatase mRNA, alkaline phosphatase 2, glkA gene, single stranded replicative centromeric gene, DMBT1, tRNA-Met gene, homeobox protein, thyroxine-binding globulin gene, alpha-a-plasmin inhibitor gene.

In test group of 24h, similar sequences were: anti-CEA ScFv antibody, anti-DNA heavy chain, mRNA for Ig kappa chain, anti BONT/A Hc ScFv antibody, mRNA for collagenase, AE0199 immunoglobulin heavy chain, Mouse Ig gamma-chain, Ig rearranged gamma-chain mRNA, anti-c-myc antibody, anti-CD30 moab ki₄ ScFv, anti-BSA antibody, D1 heavy chain, epidermal growth factor, anti-NP antibody IgH, mRNA for arginine/serine kinase.

In test group of 4d, similar sequences were: Dual specificity phosphatase, family mRNA telomerase-associated protein, anti-human erbB-2, tazarotene-induced gene2, betaine-GABA transporter gene, copy complex subunit 7a, mRNA for stress-activated protein, FK506 binding protein, calcium/calmodulin dependent gene, PEST phosphatase interactin gene, haptoglobin mRNA, acyl-ACP desaturase, mRNA for sodium channel, peroxidase, BMP-4 gene, bone morphogenetic protein.

In test group of 8d, similar sequences were: Ig variable region, DNA for mouse Ig, DNA for flexible peptide, tsr glkA, proteinase-3 neuroactin, EWS gene, repA protein.

Eighteen new sequences

No.1 sequence (AF240164)

1	ccgaattccc	gggtcgacc	acgcgtccgg	tgtatgttct	tgccaatccc	agcacagttc
60	tacaaagtaa	aatattggc	agtgaattca	tattaacaga	ggaacaaga	aattaacacg
120	ttttgcttc	gctaattcac	agttcttita	atacgtaat	tctaaatcac	ctgttctgac
180	tttgacaggc	tacagacacc	tgtttggggt	aatattccac	agctaattat	tacatgagaa
240	attcagtttc	caacaaaaga	gtttctgtgt	gaaattgcca	ttgtagttaa	caaattaata
300	tcaactatat	acaaatacat	ttctgatgtc	ttaatttaa	tacagcta	atactactca
360	tctataacta	aagaatgta	tataaaaact	aagactgcc	tcttagtgt	tgaattctgt
420	ctgtcacact	ttacaccatg	gttgactga	tcaagtcca	atgttctcc	cagtagcagt
480	aattactgt	aatactgtat	ttaaatggc	aggaaaagag	tatagaattt	tcaagtggct
540	ggaaaaattc	ctaactgtat	gttaaaaatg	actcaggaga	gttaaaaaag	aaaataactg
600	acaagagaga	gaaaaaaaaa	aaaaaggggc	ggccgtctca	gaggatccaag	cttacgtacg
660	cgtgcatgcc	gacgt				

No.2 sequence (AF240165)

1	gtgcccgacg	tcgatgctc	ccggccgcca	tggcccgggg	atztatatat	atatatatat
60	atatatatat	atatatatat	ataaatcact	agtgcggccg	cctgcaggtc	gaccatattg
120	gagagctccc	aacgcgttgg	atgcatagct	tgagtattct	atagtgctac	ctaaatagct

180	tggcgaatc	atggatcatg	ctgttcctg	tgtgaaattg	ttatccgctc	acaattccac
240	acaacatacg	agccggaagc	ataaagtgtg	aagcctgggg	tgcctaataga	gtgagctaac
300	tcacattaat	tgcgttgccg	tactgcccg	ctfccagtc	gggaaacctg	tcgtgccagc
360	tgcattaatg	aatcggccaa	cgcgcgggga	gaggcggfct	gcgtattggg	cgctctccg
420	cttctcgtc	cactgactcg	ctgctcggg	tcgttcggct	gcggcgagcg	gtatcagctc
480	actcaaggc	ggtaatacgg	ttatccacag	aatcagggga	taacgcagga	aagaacatgt
540	gagcaaaagg	ccagcaaaag	gccaggaacc	gtaaaaaggc	cgcgttgctg	gcgttttcc
600	ataggctcgg	ccccctgac	gagcatcaca	aaaatcgagc	ctcaagtcag	aggtggcgaa
660	acccgacagg	actataaaga	taccagggcg	ttccccctg	gaagctccct	ctgctcctc
720	cctgtccga	ccctgccgct	ttaccggata	cctgtccgcc	tttctct	

No.3 sequence (AF240166)

1	tgcaggtacc	ggtcgggaat	tccgggctg	accacgcgt	ccggcagtat	atgacacaaa
60	tgtataaatt	agtgttattt	ctttccaca	ctgagtcaaa	gattatttca	cagctatttc
120	aactttttag	atatcataat	tctgaccata	acactttaa	atattgtatt	tattcattca
180	aaattcattg	aaaaatcaca	tgctattata	ttaaattat	gtgattattt	tacaaaatta
240	gacttacaga	aaagaactat	ttctaccca	aaaagctgta	tgtttatatg	cagcatgttt
300	tcaaaaaaaa	aaaaaaagg				

No.4 sequence (AF240167)

1	tatgcccgc	agccggccat	ggcccagggtg	aaactgcacc	agtcaggacc	tgaagtggta
60	aagcctgggg	cttcagtga	gctgtcctgc	aaggcttcag	gctacatctt	cacaagttat
120	gatatagact	gggtgaggca	gacgcctgaa	cagggacttg	agtggattgg	atggattttt
180	cctggagagg	ggagtactga	atacaatgag	aagtcaagg	gcagggccac	actgagtgtg
240	gacaagtctc	ccagcacagc	ctatatggag	ctcactaggc	tgacatctga	ggactctgct
300	gtctatttct	gtgctagagg	ggactactat	aggcgtact	ttgactgtg	gggccaaggg
360	accacggcca	ccgtctcctc	aggtaccaag	ctggagctga	aacggggcggc	cgcaggtgcg
420	ccggtgccgt	atccgatcc	gctggaaccg	cgtgccgcat	aga	

No.5 sequence (AF240168)

1	ctttctatgc	ggcccagccg	gcatggccc	aggtgaaact	gcagcagtc	ggactgagg
60	tggtagggcc	tggggtctca	gtgaagattt	cctgcaaggg	ttccggctac	acattcactg
120	attattctat	gactggctg	aagatgaatc	atgcacagag	tctagagtgg	attggaatta
180	ttagtactta	cgatggtaat	acaactaca	accagaagtt	taagggcaag	gccactatga
240	ctgttgacaa	atcctccatt	acagcctata	tggaaactgc	cagattgaca	tctgatgatt
300	ctgccatcta	ttactgtgca	agaggggctt	actacggtag	ttttattac	tttgactact
360	ggggccaagg	gaccacggtc	accgtctcct	caggtggagg	cggttcaggc	ggaggtggct
420	ctggcgggtg	cggatcggaa	tcgagctcac	cagggggcac	caagctggaa	atcaaacggg
480	cggccgcagg	tgcgccgggtg	ccgtatccgg	atccgctgga	accgctgcc	gcatagactg

No.6 sequence (AF240169)

1	tgcggccca	gccggccatg	gcccagggtga	aactgcagca	gtcaggacct	gaactgaaga
60	agcctggaga	gacagtcagg	atctctctgca	aggcttctgg	atataccttc	acaactgctg
120	gaatgcagtg	gggtcaaaaag	atgccaggaa	agggtttgaa	gtggattggc	tggataaaca
180	cccactctgg	agtgccaaag	tatgcagaag	agttcaagg	acgctttgcc	ttctcttgg
240	aaacctctgc	cagcactgca	tatttacaga	taagcaacct	caaaaatgag	gacacggcta
300	cgtatttctg	tatgagatgg	gattacgacg	gggggtttgc	ttactggggc	caagggacca
360	cggtcaccgt	ctcctcaggt	ggaggcgggt	caggcggagg	tggtctggc	agtggcggat
420	cggacatcgt	gctcaccag	tcctcagctt	ctttggctgt	gtctctagg	cagagggcca
480	ccatctctg	cagagccagc	gaaagtgtg	ataatattgg	cattagtttt	atgaaactgg
540	tccagcagaa	accaggacag	ccaccaaac	tcctcatcta	tgctgcatcc	aagcaaggat
600	ccggggtccc	tgcaggttta	ctggcaagtg	ggtctgggac	agatttcagc	ctcaacatat
660	atcctatgga	g				

No.7 sequence (AF240170)

1	ccacgcgtcc	ggcagtatat	gacacaaatg	ttatgattag	tgttatttct	tttccacact
60	gagtcaaaaga	ttatttcaca	gctatttcaa	ctttttagat	atcataattc	tgaccataac
120	actttaaaat	attggattta	ttcattcaaa	atfcattgaa	aaatcacatg	ctattatatt
180	aaaattatgt	gattatttta	caaaattaga	cttacagaaa	agaactattt	cctacccaaa
240	aagctgtatg	tttatatgca	gcatgttttc	aaaaaaaaaa	aaaaagg	

No.8 sequence (AF240171)

1	aacagtctat	gcggcacggc	gttccagcgg	atccggatac	ggcaccggcg	cacctgcggc
60	cgcccgtttg	atttccagct	tggtgcccc	tggtgagctc	gattccgatc	cgccaccgcc

120	agagccacct	ccgcctgaac	cgccctccacc	tgaggagacg	gtgaccgtgg	tcccttggcc
180	ccagtagtca	aagtaataaa	aactaccgta	gtaagcccct	cttgcacagt	aatagatggc
240	agaatcatca	gatgtcaatc	tgcaagttc	catataggct	gtaatggagg	atgtgtcaac
300	agtcatatg	gccttgccct	taaacttctg	gtgtagtftt	gtattaccat	cgtaagtact
360	aataattcca	atccactcta	gactctgtgc	atgattcatc	ttcagccagt	gcatagaata
420	atcagtgaat	gtgtagccgg	aacccttgca	ggaaatcttc	actgagaccc	caggcctcac
480	cacctcaggt	cctgactgct	gcagtttcac	ctgggcatat	gccggctggg	ccgcatagaa
540	aggaacaact	aaaggaattg	cgaaataata	tttttcacg	ttgaaaatct	ccaaaaaaa
600	ggcttcaaaa	gcttggcgta	atcatggta	tagctgnttn	ctgtgtgaaa	ttggtattcg
660	ctcacaattt	cacacaat				

No.9 sequence(AF240172)

1	tcgatagctg	gtacgcctgc	aggtagccgt	ccggaattcc	cgggctgacc	cacgcgtccg
60	gtgtatgttc	ttgccaatcc	cagcacagtt	ctacaaagta	aaatatttgg	ccgtgaattc
120	atattaacag	atggaacatg	aaattaacac	gtttttgctt	cgctaattca	cagttctttt
180	aatacgttaa	ttctaaatca	cctgttctga	ctttgacagg	ctacagacac	ctgtttgggg
240	taataattcca	cagctaatta	ttacatgaga	aattcagttt	ccaacaaaag	agtttctgtg
300	tgaattgccc	attgtggfta	acaaattaat	atcaactata	tacaaataca	tttctgatgt
360	cttaatttaa	atacagctaa	tatactactc	atctataact	aaagaatggt	atataaaaac
420	taagactgtc	ctcttagtgt	ttgaattctg	tctgtcacac	ttacacat	ggttgactcg
480	atcaagtcat	aatgttcttc	ccagtagcag	taatttactg	taatactgta	ttttaaatgg
540	caggaaaaga	gtatagaatt	ttcaagtggc	tggaaaaatt	cctaactgta	tgtaaaaaat
600	gactcaggag	agttaaaaaa	gaaaaattct	gactggagag	agaaaaaaaa	gtagatgggc
660	ggggcgtttt	agaaggatcc	aagcttactg	ccgcgtgcat	gccaaaggcat	aactcttcta
720	tcgggcacct	aaattcaatt	ctggggccgc	ggttacacgg	ccgggactgg	ga

No.10 sequence(AF240173)

1	tctatcgggc	acgcggttcc	agcggatccg	gatacggcac	cggcgcacct	gcggccgccc
60	gtttcagctc	cagcttggtc	ccccctccga	acgtgtaagg	aacctcctta	ctttggtgac
120	agaaatacac	tgcaggatca	tcctctccca	taggatata	gttaggctg	aaatctgtcc
180	cagaccact	gccagtaaac	ctggcaggga	ccccggatcc	ttgcttgat	gcagcataga
240	tgaggagttt	gggtggctgt	cctggttct	gctggaacca	gtcataaaa	ctaagccaa
300	tattatcaac	actttcgctg	gctctgcagg	agatgggtgg	cctctgccct	agagacacag
360	ccaaagaagc	tggagactgg	gtgagcacga	tgccgatcc	gccactgcc	gagccacctc
420	cgectgaacc	gtccacctg	aggagacggt	gaccgtggtc	ccttgccca	gtaagcaaac
480	ccccgcgtg	aatcccactt	catacagaaa	tacgtagccg	tgctctcatt	ttttgaggtt
540	gcttatctgt	aaatatgcaa	gtgcctggca	gaggtttcca	aagagaaggc	aaagccgtcc
600	ctttgaactc	ttctggcata	cttttggcac	ttcagagtgg	gtngtttatt	ccagccaatc
660	cacttcaaaa	cccttttct	tgg			

No.11 sequence (AF240174)

1	taccggtccg	gaattcccgg	gtcagccac	gcgtccggca	gtatatgaca	caaatgttat
60	gattagtgtt	atttctttc	cactactgag	caaagattat	ttcacagcta	tttcaacttt
120	ttagatatca	taattctgac	cataacactt	taaaatattg	gatttatca	ttcaaaattc
180	attgaaaaat	cacatgctat	tataataaaa	ttatgtgatt	attttcaaaa	attagactta
240	cagaaaagaa	ctatttccca	cccaaaaagc	tgatgttta	tatgcagcat	gttttcaaaa
300	aaaaaaaaaa	agggcggccg	ctctagagga	tccaagctta	cgtagcgtg	catgcgacgt
360	catagctctt	ctatagtgtc	acctaaattc	aattcactgg	ccgtcgtttt	acaacgtcgt
420	gactgggaaa	accctggcgg	ttaccaact	taatcgctt	gcagcacatc	ccccttccgc
480	cagctggcgt	aatagcgaag	aggcccgcac	cgatcgcctt	tccaacagt	tgcgcagcct
540	gaatggcgaa	tgggaccgag	ccctgtagcg	gcgcattaag	ccgcggcggg	tggtgtggtt
600	acgcgcagcg	tgaccgtaca	ctggcagcg	cccctatcgg	ccgttctctt	tcgctttctt
660	tccctttcct	tttttggcca	cgttctgccc	nggttcccc	gtcaagctct	aaatccgggg
720	ggctcccttt	taagggttcc	gganttaang	gttttaccgg	nncncgac	cccg

No.12 sequence (AF240175)

1	cacagtaata	gatggcagaa	tcatcagatg	tcaatctggc	aagttccata	taggctgtaa
60	tggaggattt	gtcaacagtc	atagtggcct	tgcccttaaa	cttctggttg	tagttgtat
120	taccatcgta	agtactaata	attccaatcc	actctagact	ctgtgcatga	ttcatcttca
180	gccagtcat	agaataatca	gtgaatgtgt	agccggaacc	cttgaggaa	atcttcaactg
240	agaccccagg	cctcaccacc	tcaggtcctg	actgctgcag	ttcacctgg	gccatggccg
300	gctgggcccgc	atagaaagga	acaactaaag	gaattgcgaa	taataatttt	ttcacgttga
360	aaatctccaa	aaaaaggct	caaagcttg	gcgtaatcat	ggtcatagct	gtttcctgtg
420	tgaattgtt	atccgctcac	aattccacac	aacatacag	ccggaagcat	aaagtgtaaa

480	gcctggggtg	cctaagtgt	gagctaactc	acattaattg	cgttgcgctc	actgcccgtc
540	ttccagtcgg	gaaacctgtc	gtgccagctg	cattaatgaa	tcggncaacg	cgccggggag
600	aggcggtttg	cgatt				

No.13 sequence (AF240176)

1	tggaacattt	tatttaaatg	tcttgtgttc	cctttaaacc	aacacaaaaa	agagaaatta
60	aaattttttt	cttttttttc	ttctttttt	ttttgtctat	tccaaacagg	ggagtcgctt
120	cagtgaaggg	ttggcgagtc	tctggagctg	ggtggggagt	gtgtgcgctc	tgtccggctg
180	ggttgttctt	cccagcctgg	ccaccctggg	tagacagcca	acccgggagg	tggcttctctg
240	ggagtcctac	cctgagcaga	cctggcttcc	cctccagggga	gggttgggtt	gagggactgg
300	ctgtgactat	gggaccctgt	gttccagaga	gaaggggtag	gggagagaag	gtcagatctg
360	gaatgttcca	tgatggttgc	agggtctgga	gagtataatt	ggtagaaaaa	taaggtgctt
420	tgggaatctg	cgagctctgt	gtcctgcccgg	cccagagagag	aaaccacccc	tgtggcccaa
480	aaggcagtga	atatagaaac	aaccggcaga	gccaggaatc	ctaccacagg	agggtccggg
540	gcggaagtga	ggcaggtagc	caaacttctg	ttcctgctcg	aggttgcctg	gggctcccc
600	gttgattgtg	ggtcgggtca	gttggcatgg	cacaaaaggg	gagggggagc	cggtttgtcc
660	atttggggc	ttttgtggg	tacctggcgg	ctgcaaaaac	atggttccg	tgtgggggaa
720	caccaacca	atggaatca	atcggggccc	ccttggggag	gggcttggtc	ttagtac

No.14 sequence (AF240177)

1	ggcgaccgtg	gtcccttggc	cccagtaagc	aaacccccg	tcgtaatccc	atctcataca
60	gaaatacgtg	gccgtgtcct	catttttgag	gttgcttacc	tgtaaatatg	cagtgtggc
120	agaggtttcc	aaagagaagg	caaagcgtcc	cttgaactct	tctgcatact	ttggcactcc
180	agagtgggtg	tttatccagc	caatccactt	caaacccttt	cctggcatct	tttgaccca
240	ctgcattcca	gcagttgtga	aggtataacc	agaagccttg	caggagatcc	tgactgtctc
300	tccaggcttc	ttcagttcag	gtcctgactg	ctgcagtttc	acctggcca	tggccggctg
360	ggccgcatag	aaaggaacaa	ctaaaggaat	tgcgaataat	aatttttca	cggtgaaaat
420	ctccaaaaaa	aaggctccaa	agcttggcgt	aatcatggtc	atagctgttt	cctgtgtgaa
480	attgttatcc	gtcacaatt	ccacacaaca	tacgagccgg	aagcataaag	tgtaaagcct
540	gggggtgecta	atgagtgagc	taactacat	taattgcgtt	gogctcactg	cccgtttcc
600	agtcgggaaa	cctgtcgtgc	cagctgcatt	aatgaatcgg	ccaacgcgcg	gggagaggcg
660	gtttgcgtat	tgggcgctct	tc			

No.15 sequence (AF240178)

1	aatctgaacg	attgggcccg	acgtcgcattg	ctcccggccg	ccatggcccg	cggtatttac
60	ggctgcgaga	agacgacaga	atttttttt	ttttttttt	ttttttttt	ggagaggggg
120	gtttctctat	agccccggct	gtcctggaac	tactatgta	gaccaggctg	gcttccagct
180	cacagaaatg	cttttgctc	tgctctctga	gtgtcggggt	taagggactc	tgcgttcta
240	ccactgctta	atcactagtg	cgccgcctg	caggtcgacc	atatgggaga	gctcccaacg
300	cgttggatgc	atagcttgag	tattctatag	tgtcacctaa	atagcttggc	gtaatcatgg
360	tcatagctgt	ttcctgtgtg	aaattgttat	ccgctcacia	ttccacacia	catacgagcc
420	ggaagcataa	agtgtaaagc	ctggggtgcc	taatgatgta	gctaactcac	attaattgcg
480	ttgcgctcac	tgcccgttt	ccagtcggga	aacctgtcgt	gccagctgca	ttaatgaatc
540	ggccaacgcg	cggggagagg	cggtttgcgt	attgggcgct	cttccgcttc	g

No.16 sequence (AF240179)

1	ccggtcccga	ctcgcgtgct	cccggccgccc	atggccgccc	gattatatat	atatatatat
60	atatatatat	atatatataa	atcactagtg	cgcccgctg	caggtcgacc	atatgggaga
120	gctcccacag	cgttggatgc	atagcttgag	tattctatag	tgtaacctaa	atagcttggc
180	gtaatcatgg	tcatagctgt	ttcctgtgtg	aaattgttat	ccgctcacia	ttccacacia
240	catacgagcc	ggaagcataa	agtgtaaagc	ctggggtgcc	taatgatgta	gctaactcac
300	attaattgcg	ttgcgctcac	tgcccgttt	ccagtcggga	aacctgtcgt	gccagctgca
360	ttaatgaatc	ggccaacgcg	cggggagagg	cggtttgcgt	attgggcgct	cttccgcttc
420	ctcgcctcact	gactcgtctc	gctcggctgt	tcggctcggg	cgagcgggat	cagctcactc
480	aaaggcggta	atagcgttat	ccacagaatc	aggggataac	cgaggaaaag	aacatgtgag
540	caaaaaggcca	gcaaaaaggcc	aggaaccgta	aaaaggccgc	gtgctggcgc	ttttccata
600	gctccgcccc	ctgacgagc	atcacaataa	tccgacgctc	aagtcagagg	tggcgaacc
660	cgacaggact	ataaagatac	caagcgtttc	cccctggaag	ctccctcgtg	cgctctctg
720	ttccgacct	gcccgttacc	ggatacctgg	nccgttttc	tc	

No.17 sequence (AF240180)

1	ctggttcgcc	tgcaggtacc	ggtccggaat	tcccgggtcg	accacgcgt	ccgggacgc
60	gtgggaggac	gcgtgggaaa	agatttgtaa	gccttgtaaa	gtttcagctc	cccagttgg
120	tggaaaaacg	taaactggca	gattagattt	ttaaataaag	attgattat	aactctaaaa

180	aaaaaaaaa	aaggcggcc	gctctagagg	atccaagctt	acgtacgct	gcatgcgacg
240	tcatagctct	tctatagtgt	cacctaaatt	caattcactg	gccgtcgttt	tacaacgtcg
300	tgactgggaa	aaccctggcg	ttaccaact	taatcgctt	gcagcacatc	ccccttctgc
360	cagctggcgt	aatagcgaag	aggcccgcac	cgatcgcctt	tccaacagt	tgcgcagcct
420	gaatggcgaa	tgggacgcgc	cctgtagcgg	cgcattaagc	gcggcgggtg	tggtggttac
480	gcgcagcgtg	accgctacac	ttgcagcgc	ctagcggccg	ctccttctgc	tttcttccct
540	tcctttctcg	ccacgttcgc	cggcttccc	cgtaagctc	taaatacgggg	ggctcccttt
600	taggttcgga	tttagtctt	tacggcacct	cgaccccaaa	aaaacttgat	tagggntgat
660	ggttcacgta	attgggcat	cggccttgat	agacggtttt	ttcggccttt	gacggttgga
720	agtccccgtt	tcttaataag	nggactnctt	gttccaaacn	tggaacaaca	cgtt

No.18 sequence (AF240181)

1	gactgcgat	gtccccggcc	gccatggccc	gcgggatatc	actagtgcgg	ccgctgcag
60	gtcgaccata	tgggagagct	cccaacgcgt	tggatgcata	gcttgagtat	tctatagtgt
120	cacctaaata	gcttggcgta	atcatgttca	tagctgtttc	ctgtgtgaaa	ttgttatccg
180	ctcacaattc	cacacaacat	acgagccgga	agcataaagt	gtaaagcctg	gggtgcctaa
240	tgagtgagct	aactcacatt	aattgcgttg	cgctactgc	ccgcttcca	gtcgggaaac
300	ctgtcgtgcc	agctgcatta	atgaatcggc	caacgcgcgg	ggagagggcg	tttgcgtatt
360	gggcgctctt	ccgcttctc	gctcactgac	tcgtcgcgct	cggctgttcg	gctgcggcga
420	gcggtatcag	ctcactcaaa	ggcgtaata	cggttatcca	cagaatcagg	ggataacgca
480	ggaaagaaca	tgtgagcaaa	aggccagcaa	aggccagga	accgtaaaaa	ggccgcgttg
540	ctggcgtttt	tccataggct	ccgccccct	gacgagcatc	acaaaaattt	gctggcgttt
600	ttccatagcg	tccgcccc	tgacgagcat	cat		

DISCUSSION

After exposing to large dose ionizing radiation, intestinal crypt radiation death occurs, and no effective therapeutic measures are available to combat it. Data showed that the devastation or death of the crypt after irradiation is the crucial factor responsible for the pathogenesis. We have performed a series of experiments intending to increase the crypt survival after-irradiation in mice and confirmed that the nucleic acids (DNA, RNA) and their precursors may be used as one of the measures for the treatment of intestinal radiation syndrome that may occur in the war as well as in the peaceful uses of atomic energy^[1-5]. However, the concrete molecular and cellular mechanisms are unknown.

Human genome group comprised 100 thousand of genes, which are selectively expressed, and determined the whole life course of organism, alteration of gene expressed levels is positioned at the centre of controlling biological adjustment mechanism^[8]. Therefore, we think, after irradiation, between test group treated by RNA and the control group treated by physiological saline must exist differently expressed genes, which indicate that those genes were closely associated with intestinal crypt damage and repair. To isolate and clone these genes may not only be helpful to clarify the molecular mechanism of nuclear acids treatment, but also provide important basic theory for gene therapy of irradiation damage.

In the study, using BALB/c mice as studying target, we obtained 90 of genes associated with repair of irradiation damaged intestinal gland cells. Data confirmed that hsp was increased at mRNA level after chronic radiation, PARP, serine protease-like gene, *p53*, *bcl-2*, *bax*, arginase I, *ihsr* PB7, *Cdx1*, *NPT*, *PCNA*, *D1b-1*, *c-Ha-ras*, *c-*

myc, *c-fos* and so on were also increased at mRNA levels, which were correlated closely with drug treatment of irradiation damaged intestinal cells^[9-25]. In our experiment, such as *Nmi* mRNA, *Dutt1* protein, mRNA for Na, K-ATPase gamma subunit, mRNA for surface glycoprotein, Zinc finger type transcript factor, porcine growth hormone-releasing hormone gene, monocyte/macrophage Ig-related gene, telomerase-associated protein, *HOX1b* protein, arginine/serine kinase, alkaline phosphatase mRNA, alkaline phosphatase 2, *glkA* gene *et al* were also closely correlated with repair of irradiation damaged intestinal crypt, what especially interesting was that *RSG5* and *ODC* were identical to obtained sequences, data showed that *RSG5*, and *ODC* were overexpressed in irradiation-damaged intestinal crypt, and played an essential and positive role during DNA damage recovery and survival^[26,27], our results also fully supported the conclusion. Although their concrete mechanism is not clarified, they may increase protein products by means of increased transcript levels to improve repair of irradiation-damaged intestinal crypt, and to suppress apoptosis of crypt cells^[32].

Langberg *et al*^[28] confirmed that immunological factors participated in the course of repair of irradiation damaged intestinal crypt such as *IL-1*, *TGF-beta1*, *PDGF-AA*, *c-EGFR*, *EGF*, *TGF-beta-3*. In our experiment, anti-CEA ScFv antibody gene, anti-DNA heavy chain, mRNA for Ig kappa chain, anti-BONT/A Hc ScFv antibody gene, mRNA for ScFv collagenase, AE0199 immunoglobulin heavy chain, mouse Ig gamma-chain, Ig rearranged gamma-chain mRNA, anti-c-myc antibody gene, anti-CD30 mAb ki-4 ScFv, anti-BSA antibody gene, D1 heavy chain, epidermal growth factor, anti-NP antibody IgH, mouse Ig

gammachain and haptoglobin were likely to be correlated closely with repair of irradiation damaged intestinal crypt. What is especially interesting is several gene fragments were partly identical to sequences of ScFv genes, this point was not able to be expressed clearly. Our results support that immunological factors exert effect on the course of repair of irradiation damaged intestinal crypt^[29-45].

In our experiment, eighteen novel sequences were obtained, their concrete functions are still unclear. But we believe that these genes are closely associated with irradiation treatment, only if we clarify the function of these genes, and according to the changes of these genes, to design a controlling measure, we are likely to decrease irradiation damage, and also provide new thoughts for tumor radiation treatment^[46-64].

In summary, our results primarily demonstrate that nuclear acids are capable of improving repair of irradiation damaged intestinal crypt, its action may be closely correlated with increased mRNA levels of some genes, also with immunological factors, but the concrete molecular mechanism such as signal transduction and suppression of apoptosis still needs further studies^[65-89].

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Brief Reports

Influence of granulocyte macrophage colony stimulating factor and tumor necrosis factor on anti-hepatoma activities of human dendritic cells

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Subject headings dendritic cells; granulocyte-macrophage colony-stimulating factor; tumor necrosis factor; anti-hepatoma cell activities; *in vitro*; peripheral blood

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INTRODUCTION

Dendritic cells (DCs) play a key regulatory role in antitumor immunity, especially in its immune accessory role via MHC-I molecules^[1-5]. We have recently reported that DCs were able to enhance the killing activity of Lymphokine and PHA activated killer (LPAK) cells *in vitro*^[6-8]. In the present study, we evaluated the effects of GM-CSF and TNF upon antitumor activities of freshly isolated dendritic Cells in human peripheral blood (DC-0) and those cells cultivated for 36 hours *in vitro* (DC-36). To perform such an evaluation, we compared killing effects of LPAK cells with additional DC-0 or DC-36 on hepatoma cell line (BEL-7402) under regulation of GM-CSF or TNF. This study provided some basic data for further antitumor research.

MATERIALS AND METHODS

Tumor cell line

Human hepatoma cell line BEL-7402 was purchased from experimental center of Sun Yat-Sen University of Medical Sciences.

Isolation of DCs

According to our previous method^[9], peripheral

blood mononuclear cells (PBMNC) from healthy volunteers were prepared by using Ficoll-Hypaque ($\rho = 1077\text{g/L}$) centrifugation method. Interface cells were collected and washed three times to remove platelets. Discontinuous Percoll density gradient centrifugation (Percoll: Pharmacia, Sweden) was employed, and then interface cells between 35% and 50% were collected which were called as preliminary enrichment of DCs and divided into two shares. One share (DC-0) was panned immediately; other one (DC-36) further cultured in PRMI-1640 with 100mL/L inactivated fetal calf serum (100mL/L FCS PRMI-1640) at 37°C in a full humidified 50mL/L CO₂ atmosphere for 36 hours, and panned. The non-adherent fractions of two shares as DC-0 and DC-36 were washed and collected for the experiments.

Preparation of LPAK cells^[9]

The PBMNCs were prepared in the same procedure as above, cultured $2 \times 10^9/\text{L}$ -population with the final concentration of rhIL-2 1000ku/L and PHA 20mg/L in 100mL/L FCS PRMI-1640 at 37°C in a full humidified 50mL/L CO₂ atmosphere for 7 days. Half volume of the solution was replaced by fresh culture medium at the fourth day.

Anti-tumor experiment

The anti-tumor experiments were divided into two groups and each contained five experimental subgroups. Two ratios of effect (LPAK) to target (BEL-7402) (5:1 and 10:1) were used in all groups. ① DC-0 group: d group: BEL-7402 ($8 \times 10^7/\text{L}$) + LPAK + DC-0 ($8 \times 10^6/\text{L}$); g1 group: d group + GM-CSF (500 ku/L); g2 group: d group + GM-CSF (100ku/L); t1 group: d group + TNF (5000ku/L); t2 group: d group + TNF (500ku/L); ② DC-36 group: each experimental group was the same as that in DC-0 group except DC-36 in place of DC-0 in the same concentration. These experimental groups were called D group, G1 group, G2 group, T1 group and T2 group respectively. In addition, L groups as the corresponding control groups, BEL-7402 + LPAK, experimental control group only consisted of BEL-7402, its population was $8 \times 10^7/\text{L}$. Culture medium control group only contained 100mL/L FCS-PRMI-1640 with the supernatant of LPAK cells at a concentration of 50μL/culture well. All of these

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groups were cultured in 96-well-culture plates and each group had 3 wells at 37°C in a full humidified 50mL/L CO₂ atmosphere for 48 hours. Cytotoxicity assay was detected by using neural red uptake method.

Cytotoxicity assay (neural red uptake method)^[10]

0.1mL 0.3mL/L neural red solution was added in each well for another 1 hour of culture. Following three washings with phosphate-buffered saline (PBS), 0.1mL HCl-ethanol solution was added in each well. The absorption value (A value) of each well was immediately read by BIO-RAD 3550-UV type automatic ELISA reader at 570nm wavelength. The formula of cytotoxicity is as follows:

$$\left(1 - \frac{\text{Experimental group A} - \text{medium control group A}}{\text{Control group A} - \text{medium control group A}}\right) \times 100\%$$

The experimental results were analyzed through analysis of variance by using GB-STAT statistic software. The experiment repeated four times at the same condition.

RESULTS

Influence of DC-0 and DC-36 on cytotoxicity activity of LPAK cells

The cytotoxic activity of L group, d group and D group was enhanced when their ratios of effect to target increased ($P < 0.01$). Their cytotoxic activity were D group > d group > L group ($P < 0.01$) respectively while in the same ratio of effect to target (Figure 1).

Influence of GM-CSF on DC-0 and DC-36 in helping LPAK cells killing effect

When there were two ratios of effect to target, cytotoxic activity of g1 group and g2 group were obviously higher than d group ($P < 0.01$), meantime, cytotoxic activity of G1 group and G2 group were greatly higher than D group ($P < 0.01$). However, the difference between g1 group and g2 group was not distinct ($P > 0.05$), also there were no difference between G1 group and G2 group ($P > 0.05$). But there were obviously different between g1 group and G1 group ($P < 0.01$), at the same time, the difference between g2 group and G2 group were distinct ($P < 0.01$) (Figure 2).

Influence of TNF on DC-0 and DC-36 in helping LPAK cells killing effect

While there were two ratios of effect to target, cytotoxic activity of t1 group or t2 group was evidently higher than that of d group ($P < 0.01$), meantime, cytotoxic activity of T1 group or T2 group was markedly higher than that of D group ($P < 0.01$). However, the differences between t1 and t2 group, and between T1 and T2 group were distinct ($P < 0.01$). Furthermore, there were difference between t1 and T1 group ($P < 0.01$), at the same time, between t2 and T2 group ($P < 0.01$) (Figure 3).

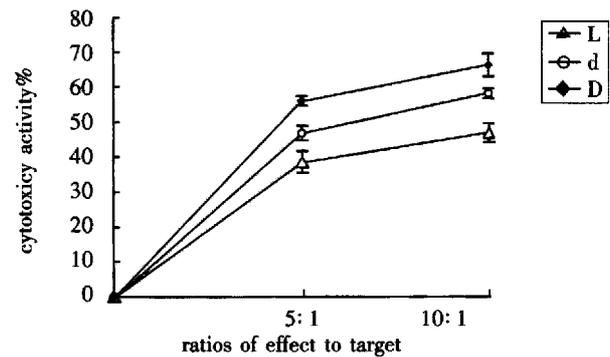


Figure 1 Influence of DC-0 and DC-36 on LPAK cells in killing BEL-7402 cells *in vitro*.

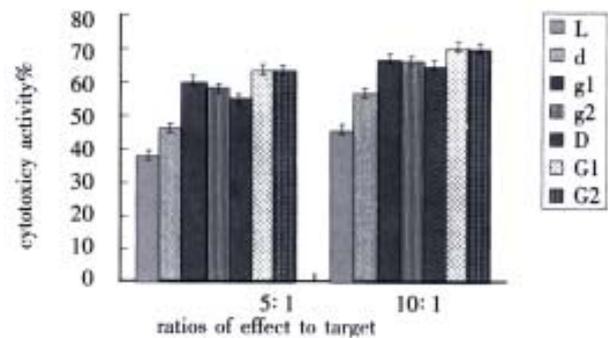


Figure 2 Influence of GM-CSF on DC-0 and DC-36 in helping LPAK cells killing activity *in vitro*.

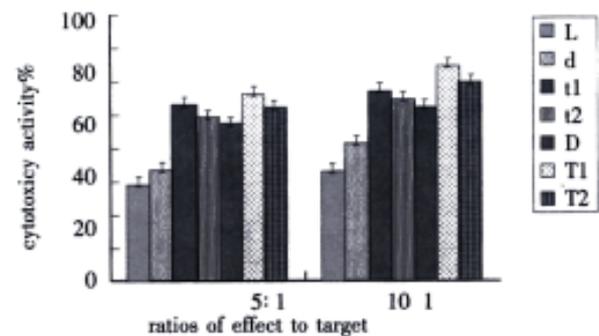


Figure 3 Influence of TNF on DC-0 and DC-36 in helping LPAK cells killing activity *in vitro*.

DISCUSSION

In recent years, it is considered that mature DCs in human peripheral blood have high stimulating function, which efficiently presents tumor-peptide epitopes leading to induce cytotoxic T lymphocytes (CTL) to produce stronger specific antitumor immune response^[11-14]. LPAK cells after 7-day induction chiefly express similar phenotype with the CD₁₆⁻, CD₈⁺, CD₃⁺ CTL subtype^[15-18]. In our experiments, cytotoxic activity in D group was obviously higher than in d group, which demonstrated that the proportion of mature DCs in DC-36 group was higher than those in DC-0 group and DC-36 group could stimulate LPAK cells to exert stronger antitumor immune response. This effect suggested that a lot of precursor cells of DCs and immature DCs in freshly isolated DCs could

differentiate into mature DCs after 36h cultivation, which coincided with Young's opinion^[19]. In human peripheral blood, however, not all the precursor cells and immature DCs are able to automatically differentiate into mature DCs *in vitro*. It is GM-CSF that promotes the differentiation, maturation and activation of DCs. GM-CSF can not only initiate and promote development of DCs from MHC II⁻, MHC II⁺ precursors and immature DCs, but also upregulated CD₈₆- expression on DCs, which make DCs to have activating and controlling antitumor immune function^[20-24]. Cytotoxic activity difference between G₂ (g₂) group and G₁ (g₁) group were not distinct, this finding illustrated that maturation and activation of DCs did not result from one factor but from combination of multiple factors. In addition, the different time required in different developing stage of DCs populations must be considered. Although developing time in DC-36 group was longer than that in DC-0 group, not all DCs in DC-36 group differentiated into mature DCs. Therefore, increasing GM-CSF concentration alone was senseless. This phenomenon may be helpful in further study of antitumor immunity and clinical research.

In the case of TNF addition, cytotoxic activity was increased greatly, this finding attributed to three roles of TNF: 1, TNF is able to serve as the first signal, which affects DCs development in their early stage or whole stage, leading to upregulate the GM-CSF receptor level of DCs^[21]. The supernatant of LPAK cells with minor quantity of cell growth factors such as TNF was added into culture medium to afford synergetic effect with GM-CSF; 2, TNF upregulates expression of CD₈₀, CD₈₃, CD₈₆ and MHC-II in a short period^[25,26]. Because these molecules are crucial for efficient antigen presenting, they promote the differentiation, development and activation of DCs. 3, TNF itself can kill tumor cells directly^[27-32]. Compared with two t groups, cytotoxic activity of T₁ and t^a-1 groups were higher than that of T₂ and t₂ groups, which showed that in DC-36 groups there was plenty of time for immature DCs to evolve into mature DCs after the addition of TNF. Furthermore, T₁ (t₁) group had higher cytotoxic activity than T₂ (t₂) group, which was further increased when TNF dosage was raised. This phenomenon may attribute to antitumor effect of TNF itself and the synergetic effect between LPAK cells and TNF.

In conclusion, as compared with uncultured DC-0, cultured DC-36 from freshly isolated DCs had greater cooperative effect with GM-CSF or TNF. Moreover, they enable DCs to fulfill stronger antitumor effect.

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Expression of regulating apoptosis gene and apoptosis index in primary liver cancer

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Subject headings liver neoplasms; apoptosis; immunohistochemistry; oncogenes; tumor markers; biological; *in situ* hybridization

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INTRODUCTION

Programed cell death plays an important role in the genesis of cancer^[1-5]. Certain cancer genes can regulate apoptosis. Recently, several proteins that are structurally related to Bcl-2, an inhibitor of apoptosis, have been identified^[6-11]. Therefore, novel strategies and agents that target specific molecular pathways, as well as triggering a process of cell death, are being evaluated in the treatment of several neoplasms. The homologous Bcl-2 and Bcl-x1 proteins can extend cell survival by suppressing apoptosis^[12,13], whereas the proapoptotic proteins (e.g. Bax, Bak, Bik) act as dominant cell death inducers when overexpressed^[14,15]. C-myc not only can promote cell growth, but also induce apoptosis. Many studies have shown that oncogene plays an important role in the growth, progression and metastasis of solid tumors. Recently, several oncogene factors have been identified. Primary liver cancer remains one of the most common malignancies worldwide, with an annual incidence of approximately 1 million cases^[16,17]. It bears the character of portal venous invasion, so metastases are often presented when the cancer is discovered. We use immunohistochemical method to detect the protein expression of Bcl-2, Bax, c-myc and use TdT-mediated dUTP nick end labeling (TUNEL) method to detect the apoptosis index. The purpose of our study was to evaluate the function of these proteins and their relationship with the clinical characteristics of hepatoma.

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MATERIALS AND METHODS

Clinical material

Twenty-two resected hepatoma specimens in our hospital from 1994 to 1998 were studied. All the specimens were confirmed by pathology. Twenty-seven were men, and 5 women, average age 50.26 years. No patient had received chemotherapy or radiation therapy before surgery. All the specimens together with some paracancerous tissues were fixed in 100mL/L formaldehyde solution and embedded in paraffin. Five μ m thick sections were cut and mounted on glass slides.

Immunohistochemical determination and in situ hybridization detection

Immunohistochemical determination of Bcl-2, Bax and c-myc

Immunohistochemical studies were performed using ABC method. The antibodies of Bcl-2, Bax and c-myc were bought from Boshide Biotechnology, Inc (Wuhan). Immunohistochemical assay was performed as described by the manufacturer. Immunohistochemical studies were performed using the streptavidin-biotin method. Sections were dewaxed in xylene, taken through ethanol, and then incubated with 3mL/L hydrogen peroxide in methanol for 30min to block endogenous peroxidase activity. Sections were then washed in phosphate-buffered saline and incubated in 100mL/L normal goat serum for 20min to reduce nonspecific antibody binding. Specimens were then incubated with a 1:50 dilution of a primary antibody overnight at 4°C, followed by three washes with PBS. Sections were then incubated with biotinylated goat antirabbit immunoglobulin G at a dilution of 1:100 for 30min followed by 3 washes slides were then treated with streptavidin-peroxidase reagent for 30min at a dilution of 1:100 and were washed with PBS 3 times. Finally, slides were incubated in PBS containing diaminobenzidine and 10mL/L hydrogen peroxide for 10min, counterstained with methyl green, and mounted. Normal rabbit immunoglobulin-G was substituted for primary antibody as the negative control. Slides were interpreted for antigen expression by two investigators without knowledge of the corresponding clinicopathologic data. Ten optical fields, about 500-1000 cells were counted in each slide under the high power ($\times 400$) microscopy. Results presented as -, indicated 25% slightly positively stained cells; +, 25%-50% positive cells; ++, 50%-75% positive cells; +++, >75% positive cells.

In site hybridization dectetion

Apoptosis was further confirmed by the TdT-mediated DUTP nick end labeling (TUNEL) method using a detection kit from Boshide Biotechnology, INC (Wuhan).

Sections were dewaxed in Xylene, taken through ethanol, and then incubated with 30mL/L hydrogen peroxide in methanol for 30min, washed with PBS, incubated in dialysate solution for 2min on ice, and incubated with TUNEL reaction mixture at 37°C for 30min. Samples were washed with PBS, incubated with converter PO D at 37°C for 1h and stained with 3, 3'-diaminobenzidine tetrahydrochloride. A negative control using all reagents except terminal transferase was performed in parallel. The nucleus of positive cells was stained brown as detected under light microscopy. Ten optical fields, about 500-1000 cells were counted in each slide under the high power ($\times 400$) microscopy. The apoptosis index is the percentage of positive cells in 1000 cells.

Statistical analysis

Statistical significance was determined by using χ^2 test or Student's *t* test.

RESULTS

The protein expressions of Bcl-2, Bax, c-myc and apoptosis

The rate of Bcl-2 protein positive expression in human hepatocellular carcinoma was 22.73% and it was 18.18% in paracancerous tissues. No difference was seen as compared with each other ($P > 0.05$). The rate of Bax positive expression was 45.45% in carcinoma tissue and 77.27% in paracancerous tissues ($P < 0.01$). The rate of c-myc protein expression was 81.82% in carcinoma tissues and 72.73% in paracancerous tissues ($P > 0.05$). The apoptosis index in hepatocellular carcinoma was 9.55% and was lower than that in paracancerous tissues ($P < 0.01$).

The relationship between the protein expressions of Bcl-2, Bax and apoptosis index in cancerous tissues and paracancerous tissues

The apoptosis index of Bcl-2 positive group was higher than that of Bcl-2 negative group, but no difference was found between them ($P > 0.05$); The apoptosis index of Bax positive group was significant higher than that of Bax negative group ($P < 0.01$) (Table 1).

Relationship between protein expressions of Bcl-2, Bax and protein expression of c-myc in cancerous tissues

In the carcinoma tissues, the expression of c-myc was not related to the expression of Bcl-2 and Bax (Table 2).

Table 1 The relationship between the protein expressions of Bcl-2, Bax and apoptosis index in cancerous tissues and paracancerous tissues

	Case	Apoptosis index %
Cancerous tissues		
Bcl-2 negative	17	9.12 \pm 5.37
Bcl-2 positive	5	11.00 \pm 5.48
Bax negative	12	5.83 \pm 1.95
Bax positive	10	14.00 \pm 4.59 ^b
Paracancerous tissues		
Bcl-2 negative	18	16.39 \pm 5.89
Bcl-2 positive	4	12.50 \pm 5.00
Bax negative	5	10.00 \pm 3.54
Bax positive	17	17.35 \pm 5.62 ^b

^b $P < 0.01$ vs compared with Bax negative group.

Table 2 The relationship between the protein expressions of Bcl-2, Bax and the protein expression of c-myc in cancerous tissues

	c-myc positive	c-myc negative
Bcl-2 positive	3	1
Bcl-2 negative	15	3
Bax positive	14	3
Bax negative	4	1

DISCUSSION

Apoptosis, a normal cellular process that provides the orderly existence and death of cells in a programmed fashion, may be positively or negatively modulated by several external or internal factors^[18-22]. Modern molecular biology investigations have indicated that proliferative inhibition of some neoplasm cells is related to apoptosis induction regulated by the oncogene expression of these cells^[23-26]. Recently, regulating apoptosis gene is divided into two groups, existence gene and death gene. Living gene includes improving cell proliferation gene, c-myc and improving cell existence gene, Bcl-2^[27-29]. Death gene includes suppressing cell proliferation gene i.e. p53 and promoting cell death gene, i.e. Bax^[30,31]. It is generally considered that Bcl-2 is an important gene for cell survival, the Bcl-2 gene can inhibit the apoptosis by blocking the last tunnel of apoptotic signal transmitted system^[32-37], C-myc is a kind of effective protein of the karyomitosis signal, which can trigger and regulate the transcription of the genes related with proliferation^[38]. Some recent study showed that Bcl-2 and Bax play important roles in regulating apoptosis in prostate cancer, mammary cancer and gastric carcinoma^[39-41]. But there has been no report on the relationship between regulating apoptosis gene and apoptosis index in human hepatocellular carcinoma.

We detected the protein expression of Bcl-2, Bax and c-myc in hepatocellular carcinoma and paracancerous tissues using immunohistochemical stain. The rate of Bcl-2 protein positive expression in human hepatocellular carcinoma was 22.73%,

and 18.18% in paracancerous tissues. No statistical difference was seen between them ($P > 0.05$). The result was similar to that of Guo *et al.*^[42]. The rate of Bax protein positive expression in hepatocellular carcinoma was 45.45%, and 77.27% in paracancerous tissues. We have found that Bcl-2 and Bax play a crucial role in the genesis of hepatoma. The higher expression of Bcl-2 and the lower expression of Bax suppressed apoptosis. These broke the balance between cell proliferation and apoptosis, and resulted in carcinogenesis. Bcl-2 protein blocks apoptosis by binding to and suppressing the action of Bax, a positive regulator of cell death. The expression of c-myc was higher in the carcinoma tissue than that in paracancerous, but no difference was seen as compared with each other. The result was similar to the report of Yang^[43]. The c-myc proto-oncogene, usually implicated in cell transformation, differentiation and cell cycle progression, also has a central role in some forms of apoptosis^[44,45]. These opposite roles of c-myc in cell growth and death require other gene products to dictate the outcome of c-myc expression on a cell. We thought that the higher expression of Bcl-2 could not effect cell proliferation, the higher expression of c-myc could improve cell proliferation. The paracancerous tissues may be in the precancer state. But the mechanism of regulating cell apoptosis is intact. The balance of cell proliferation and death was not broken, cell was still in normal state. The paracancerous tissues could become cancer following the enhancing of cell proliferation and the imbalance in regulating apoptosis. Bissonnette RP found that Chinese hamster ovary cell apoptosed due to the proliferation of c-myc gene, but it would be suppressed by Bcl-2 gene^[35], and cell would be in a balanced state of proliferation and apoptosis resulting from the appropriate expression of c-myc gene. The balance would be broken by some factors. A 'two signal' model emerges, in which c-myc can provide the first signal, leading either to apoptosis or proliferation, and certain growth factors may provide a second signal, to inhibit apoptosis and allow c-myc to drive cells into the cell cycle. Bcl-2 may substitute for the putative signal, as suggested by the abilities of Bcl-2 to delay apoptosis after growth-factor withdrawal and to cooperate with c-myc in transforming cells^[46]. In our study, no relationship was seen between the expressions of c-myc and Bcl-2 or Bax. We propose that c-myc activation affords the advantage of proliferation is essential for carcinogenesis, because the proliferative and apoptosis functions of c-myc are tightly coupled, it is impossible to select only for one without the other. In our study, the number of cases was insufficient to reflect the relationship.

Now, there are a lot of methods to detect apoptosis. But TdT-mediated dUTP nick end labeling method is the most accurate one^[47]. It could find the DNA broken in the early stage and accurate localization, so it is most accurate to reflect the status of apoptosis. Using this method, the apoptosis index of paracancerous tissue was 15.68%, which was higher than that of cancer tissues, 9.55%. There was no difference of apoptosis index between the positive and negative expression of Bcl-2. There was significant difference between the apoptosis index in Bax positive and negative group. We thought the balance of the expression of Bcl-2 and Bax may decide whether the cell is alive or dead. The isodipolymer with Bax and Bcl-2 could decide the protein molecules of active Bcl-2 or not. Bcl-2 could have different effects on inducing or inhibiting apoptosis. So isodipolymer Bcl-2/Bax is more accurate in reflecting the status of a poptosis. There were significant differences between the apoptosis index in cancer tissues and paracancerous tissues. It is valuable to study whether apoptosis index could become an index in judging canceration or not.

In conclusion, our study demonstrated that apoptosis plays an important role in the genesis of hepatoma, Bcl-2/Bax could reflect accurately the status of apoptosis. Apoptosis index was the most accurate index to reflect apoptosis, and may become an index for judging carcinogenesis. It is also valuable to study whether c-myc is affected by Bcl-2 and Bax expression.

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Transduction of primary rat hepatocytes with bicistronic retroviral vector

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Subject headings primary hepatocyte; recombinant retroviral vector; genetic markers; gene transfer; hepatocellular transplantation; polymerase chain reaction

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INTRODUCTION

Hepatocellular transplantation (HCT) could provide a therapeutic alternative to orthotopic liver transplantation (OLT) in the treatment of hepatic metabolic defects and experimental hepatic failure^[1-4]. Under appropriate conditions, the engrafted liver cells can continue to express liver-specific functions for an indefinite period of time. The major limitation of many animal studies in HCT is that, since the donor hepatocytes are often indistinguishable from those of the host, it has often been difficult to demonstrate a clear correlation between engraftment and the therapeutic effect. In order to verify engraftment dependent on the therapeutic response, a recombinant retroviral vector carrying marker genes is used to label the donor hepatocytes^[5,6]. The vector is capable of transducing hepatocytes, integrating gene stably into the genome and directing expression. Efficient retroviral-mediated gene transfer has introduced the possibility of targeting genetic markers to hepatic cells and somatic gene therapy for liver diseases^[7-11]. Stable integration and expression of retroviral genes is dependent upon active division of the infected cell^[9-13]. Although hepatocytes maintain growth potential *in vivo* and are capable of substantial regeneration following partial hepatectomy, their ability to grow in culture is quite limited.

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In the present study, we explored the optimal culture system for hepatocyte proliferation and the potential for retroviral-mediated gene transfer into primary hepatocytes. We successfully demonstrated the efficient and stable transduction of primary culture of adult rat hepatocyte by replication of defective retrovirus carrying β -gal gene and NeoR gene.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 140g - 200g were provided by Experimental Animal Center of Shanghai Second Medical University.

Sources

Hepatocyte-specific collagenase and culture medium were purchased from GIBCO-BRL (Gaithersburg, MD). Insulin, dexamethasone, transferrin, polybrene and epidermal growth factor (EGF) were Sigma Chemicals products (St. Louis, MO). 4CL-5Bt-3indolyl- β -galactoside (X-Gal) was purchased from Hua Mei Biotech Co. ³H-TdR was purchased from Neucleic Energy Institute. Tissue/cell DNA extract kit was purchased from Shanghai Hua Shun Biotechnical Limited Co. Culture plastic dishes were Nunclon Co product.

Isolation and culture of hepatocytes

Rat hepatocytes were prepared by the modified procedure of Seglen with a two-step collagenase perfusion combined with 49.5% Ficoll centrifugation^[14-15]. The dissociated cells were suspended in hormonally defined medium: M199 containing 10% fetal calf serum (FCS), 10⁻⁸M insulin, 10⁻⁶M dexamethasone and 5mg/L transferrin. They were seeded at a density of 3×10⁴ cells/cm² on a 35mm tissue culture plastic dishes, and grown at 37°C in a 5% CO₂ environment. The medium was changed 4h after seeding, and replaced by different culture mediums: group A with M199 containing 5% FCS, 10⁻⁸M insulin, 10⁻⁶M dexamethasone, 5mg/L transferrin; group B with M199 containing 5% FCS, 10⁻⁸M insulin, 10⁻⁶M dexamethasone, 5mg/L transferrin plus 10 μ g/L EGF. The medium was renewed every 24h thereafter.

Production of retroviral infected hepatocytes

PA317 cell line producing simultaneously the

recombinant retrovirus PGCEN/ β -gal expressing β -galactosidase gene (LacZ) and neomycin-resistance gene (NeoR) was a gift from Prof. Cheng Shishu (Human Genetic Therapy Research Center, SSMU). These two genes were controlled by the same promoter. Its structure is shown in Figure 1. The producer PA317 was maintained in DMEM supplemented with 10% FCS. Virus-containing medium was harvested from the producers after 16-20 hrs, filtered through a 0.45mm filter unit, and used for infecting the cultured hepatocytes. The viral titer ranged from 1 to 2×10^6 blue colony-forming unit (bcfu)/mL, when tested with NIH 3T3 cells.

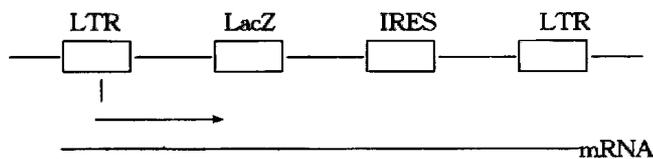


Figure 1 Structure of bicistronic retroviral vector rPGCEN/ β -gal. Arrow below vector indicates initiated site of transcription.

Hepatocytes in group B were grown for 1 to 5 days. Three dishes were selected randomly at 24, 48, 72, 96 and 120 hrs. The medium was removed and rinsed with PBS. The cells were incubated for 6 hrs with 1mL of viral supernatant plus $8 \mu\text{g}$ of polybrene per mL. Then the viral supernatant was cultured by replacing with fresh medium. Repeat the infection once a day for 24 to 96 hrs.

Detection of LacZ expression by X-Gal staining

Cells infected with PGCEN/ β -gal virus constitutively produced high levels of cytoplasmic β -galactosidase. In order to detect β -gal activity, infected hepatocytes were washed in phosphate-buffered saline (PBS) containing $\text{Ca}^{2+}/\text{Mg}^{2+}$, and fixed 5 min in 4% formaldehyde in PBS pH 7.4, rinsed again with PBS, then stained at 37°C with X-Gal (1g/L) for 2-24 hrs, as previously described^[16]. Blue precipitate in infected cells were seen under microscope. Areas of X-gal-stained rat primary hepatocyte culture dishes were quantitated for transduced cells using VIDAS computer-assisted image analysis. Three fields were randomly chosen and gene transductive efficiency was evaluated as follows:

$$\text{Gene transduction efficiency(\%)} = \frac{\text{Blue-stained cells areas}}{\text{Total cells areas}} \times 100\%$$

Measurement of hepatocyte DNA synthesis

In this assay, cells were cultured in absence or in presence of EGF for various durations. DNA synthesis was measured by ^3H -thymidine incorporation. Cultures in 35 mm-dish were treated with ^3H -thymidine ($1 \mu\text{Ci}/\text{mL}$) for 5 hrs before harvest. Cells were detached by trypsinization at

37°C , washed with PBS twice and counted. The cells were lysed with distilled water. DNA were precipitated with 5% trichloroacetic acid and absolute ethanol, and collected on glass fiber-filters. DNA were then assayed for radioactivity in a liquid scintillation counter. ^3H -thymidine incorporation in DNA was expressed as $\text{cmp}/10^5$ cells.

Glucose-6-phosphatase activity by cytochemical procedure

Glucose-6-phosphatase activity was detected by the lead phosphate enzyme cytochemical procedure^[17]. Characteristic brown/black cytoplasmic staining was seen if cultured hepatocytes expressed glucose-6-phosphatase.

Detection of NeoR expression by molecular method

DNA from cultured cells was isolated by proteinase K digestion in 10mM Tris-hydrochloric acid (pH 8.0) and 1% sodium dodecyl sulfate at 55°C for 2 hrs, followed by phenol extraction and ethanol precipitation. According to the reference^[10], the primer was synthesized by Bioengineering Research Center in Shanghai of Chinese Academy of Science. The nucleotide sequences of NeoR primers were as follows:

Sense 5'CAAGATGGATTGCACGCAGG 3',
antisense 5'CCCCTCAGAAGAAGACTCGTC3'790bp.

The total $50 \mu\text{L}$ PCR reaction system consisted of $10 \times$ amplification buffer solution $5 \mu\text{L}$, 2.5mmol/L DNTP $4 \mu\text{L}$, 25mmol/L MgCl_2 $3 \mu\text{L}$, 25 $\mu\text{mol}/\text{L}$ primer $2 \mu\text{L}$, reverse transcription product $10 \mu\text{L}$, and added ddH_2O up to $50 \mu\text{L}$ mixing together, and added to *Taq* DNA polymerase $0.5 \mu\text{L}$ after denatured for 5min. The amplification condition was pre-denatured at 94°C for 1min, 60°C for 1min, 72°C for 1.5min altogether for 30 cycles, finally, extension at 72°C for 10 min.

Ten μL PCR product ran in agarose gel (1%, containing ethidium bromide 0.5mg/L) electrophoresis at 100 V for 40min and photographed under ultraviolet lamp.

RESULTS

Isolation and culture of hepatocytes

Each rat liver weighing 150-200 g was perfused by modified two-step collagenase via portal vein. The yield of hepatocytes was $1 - 2 \times 10^8$ cells. The viability was over 95%. The cells were seeded at densities of 3×10^4 cells/ cm^2 on 35mm dish. They became attached to the dishes in 3-4 hrs. Hepatocytes became polygonal epithelium-like structure. The majority of cells were mononucleated; some were bi-or multi-nucleated. The membranes were clearly seen. Hepatocytes started to divide in aggregates a few hours after

attachment and became confluent within 3-4 days (Figure 2).

Influence of EGF on hepatocytes proliferation in vitro

In order to compare the DNA synthesis in hepatocytes, the level of ^3H -thymidine incorporation in the cell layer was measured in EGF stimulated and unstimulated primary hepatocyte cultures. After the addition of $10\mu\text{g/L}$ EGF to the cultures, the level of ^3H -thymidine incorporation began to increase at 48hr of culture, and reached the peak on the 5th day. Fifty-nine-folds increase of ^3H -TdR incorporation was found in EGF-treated cultures compared to conventional cultures. The differences between the two culture conditions were statistically significant at 48, 72, 96 and 120 hrs ($P<0.01$). The addition of $10\mu\text{g/L}$ EGF to the culture increased 50 times incorporation at 120 hrs as compared with that at 24 hrs. There was also significant difference between the two time points (Table 1).

Table 1 Effect of EGF on DNA synthesis of rat hepatocyte by ^3H -TdR incorporation [cpm/(10^5 cell·h)]

Group	Time in culture (day)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Group A	34±3	29±6	25±6	31±4	22±3
Group B	26±3 ^a	42±6 ^b	263±27 ^b	876±112 ^b	1287±215 ^{bc}

^a $P<0.05$, vs Group A; ^b $P<0.01$, vs Group A; ^c $P<0.01$, vs Day 1 of Group B.

Retrovirus transduction in cultured hepatocytes and detection of LacZ expression

Triplicate cultures of infected hepatocytes were analyzed *in situ* for retrovirus transduction and expression by cytochemical staining for β -galactosidase. Cells that expressed viral-directed β -galactosidase was exhibited specifically by this procedure. Although the proliferation of rat primary hepatocyte was limited, the highest rate of infection was obtained by adding EGF. The rate of infection was gradually increased on the first 4 days, reached the peak on the 4th day of infection, but transduction efficiency dropped gradually in cultures on the 5th day of infection. The transduction efficiency in repeated infection group was about 22% (Figure 3).

Expression of hepatocyte function

We used a liver-specific cytochemical stain to detect the functional hepatocytes. The expression of glucose-6-phosphatase was analyzed in culture at different periods by cytochemical staining. Characteristic brown/ black cytoplasmic staining was seen in >85% of the cells at 48 hr of culture. The activity was still present at a slightly diminished level in >60% of cells on the 6th day.

PCR detection of NeoR gene

Transduction with PGCEN/ β -gal was also assessed by PCR detection of NeoR gene. Analysis of PCR product showed that the amplified product with 790bp was visualized with ethidium bromide after electrophoresis in transduced rat hepatocytes, while this specific PCR product was absent in nontransduced primary rat hepatocytes (Figure 4).

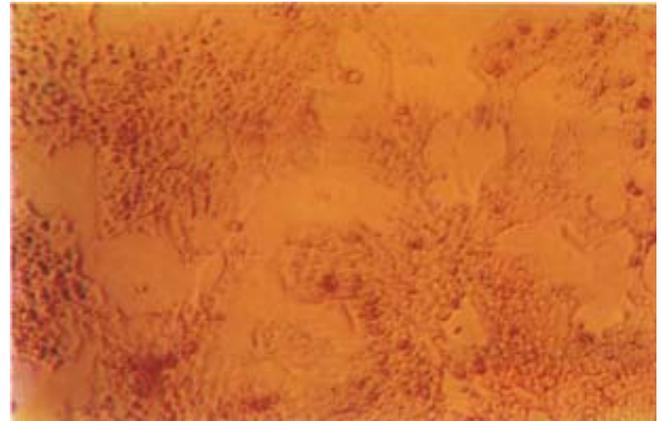


Figure 2 Morphology of hepatocytes in culture. $\times 100$ Hepatocytes became polygonal epitheliumlike structure. The majority of cells were mononucleated; Some cells were bi- or multi-nucleated. The membranes were visible. Hepatocytes became confluent at 4 days postplating.

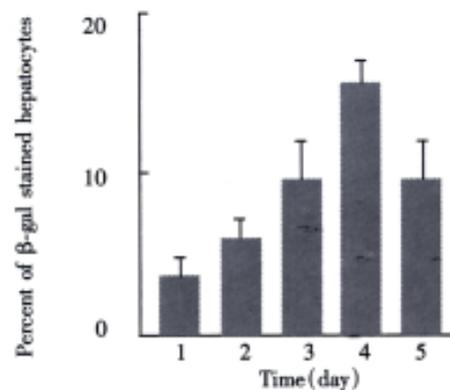


Figure 3 Transduction efficiency of hepatocytes by retroviral vector.

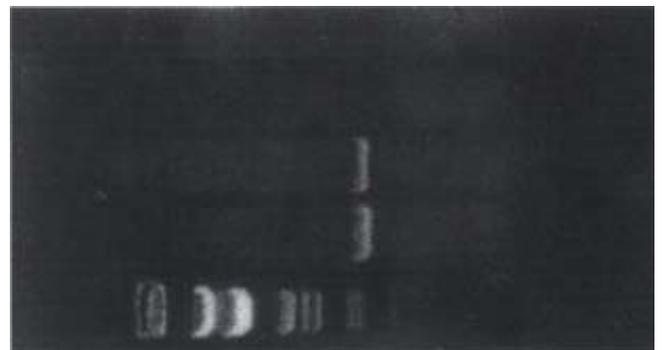


Figure 4 PCR detection of NeoR in primary rat hepatocytes transduced by PGCEN/ β -gal.

A: Size markers (Lambda DNA/EcoR+Hind III Marker)
 B: Positive template (MN45Li cell lines modified by NeoR gene)
 C: Primary rat hepatocytes transduced with retroviral vector PGCEN/ β -gal
 D: Nontransduced primary rat hepatocytes
 E: Water control

DISCUSSION

Two-step collagenase perfusion via portal vein is the conventional method for availability of hepatocytes, reports were available regarding cultured hepatocytes prepared by this method. In our present study a two-step collagenase perfusion in combination with 49.2% ficoll gradient centrifugation was used. This technique provided a higher yield of viable rat hepatocyte with a minimal nonparenchymal cells. It was helpful to culture hepatocytes *in vitro* and to perform various studies of hepatocytes, such as establishment of cell bank and retrovirus-mediated gene transfer.

It is generally considered that efficient transduction of retroviral gene is dependent upon active proliferation of infected cells^[9-13]. Although hepatocytes maintain growth potential *in vivo* and are capable of substantial regeneration following partial hepatectomy, the ability of adult hepatocytes to grow in culture without growth factors stimulation is limited^[18-25]. In this experiment, with addition of 10 μ g/L EGF to the conventional culture, the cells retained their ability to proliferate, and showed excellent hepatocyte morphology. We were able to demonstrate that these cells could divide in short-term culture, and could be infected with recombinant retrovirus.

Liver cell transplantation can support the impaired liver. If the transplanted cells exhibit a growth preponderance and specific liver functions, they will rapidly replace the patient's hepatocytes. Glucose-6-phosphatase is a well-recognized specific enzyme expressed by the hepatocytes. It can be detected in viable hepatocytes by cytochemical staining. In our experimental system, hepatocytes could be isolated and cultured under conditions that maximized the division of parenchymal cells and prolonged the expression of glucose-6-phosphatase activity more than 10 days. The labelled donor hepatocytes via transducing these cells with a recombinant retroviral vector carrying a marker gene is used for evaluating the fate and function of the transplanted cell *in vivo*. Our data demonstrate that recombinant retroviruses are efficient tools to transfer marker gene into rat primary hepatocytes. The rat hepatocytes proliferated increasingly under EGF stimulation, 20% of the cells could be transduced. Our experiments of ³H-TdR incorporation corroborate the observation of Chenoufi *et al*^[25]. We found that the cells had a high DNA synthetic rate that could be increased by adding EGF. In the first four days after plating, efficient transduction correlates positively with the state of proliferation ($r = 5.427$, $P < 0.05$). Transduction rate decreased after the fifth day. Thus, our results indicate that the susceptibility to retroviral infection of hepatocytes varies with the ability of the cell proliferation, functional status of hepatocytes such as the level of receptor expression and many other factors^[9-13].

retroviral vector was frequently used in gene therapy^[26,27]. Recombinant retroviral vector PGCEN/ β -gal used in the present study is an bicistronic retroviral vector expressing β -galactosidase gene and NeoR gene simultaneously, because these two genes were connected by the internal ribosome entry site of encephalomyocarditis virus and controlled by the same promotor^[28]. Transduction efficiency was estimated by detection of β -gal gene expression by *in situ* staining, which was shown clearly and quantitatively. NeoR gene and target gene could be detected in the integrated cells from DNA level by PCR or southern blot^[29-31]. PCR method increased the sensitivity of detection, which is helpful in tracing the life span of transplanted cell *in vivo*^[30,31]. One study indicates that primary rat hepatocytes can be efficiently transduced by a NeoR and β -gal-expressing recombinant retrovirus (PGCEN/ β -gal). This approach is now being used to determine the most efficient way of cell transplantation and to investigate the location, life span and function of the transplanted hepatocytes.

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Effects of heat shock on change of HSC70/HSP68, acid and alkaline phosphatases before and after rat partial hepatectomy

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INTRODUCTION

Only the liver has the great capability of regeneration in mammal^[1]. Few hepatocytes are in the phase of division in the normal liver of an adult mammal (including human beings)^[2-4], but the remaining hepatocytes can be induced to proliferate quickly by partial hepatectomy (PH), and, to some degree, they stop dividing and re-differentiate into cells functioning as hepatocytes^[5,6]. This shows that liver regeneration is a delicate process in which the cellular dedifferentiation, multiplication and re-differentiation are regulated accurately^[7-9]. In the past decades, the liver regeneration of mammal has been regarded as one of the best model to study the restructuring and regeneration of tissues and organs, cellular multiplication, dedifferentiation, re-differentiation, stress response and regulation of physiology and biochemistry^[10-13]. Most studies of liver regeneration focus on observing the changes in cellular morphology, structure, physiology, biochemistry, metabolism^[14-16], separation and function of liver regeneration factors^[17-20], and how to initiate the regeneration of hepatocytes. However, there are few studies focusing on some biomacromolecules, e.g. heat shock proteins, proteinases, phosphatases, peroxidases in connection with the above. How these biomacromolecules affect liver regeneration is very worth studying. This paper reports some preliminary approaches regarding these aspects.

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MATERIALS AND METHODS

Materials

Sprague-Dawley rats (Weighing 200g-250g) were provided by the experimental animal house of College of Life Science of Henan Normal University. Chemicals and reagents were of analytical grade, rat monoclonal antibody-1 HSC70/HSP68 (StressGen SPA-820) from anti-human HSC70/HSP68 combined specifically with 1-180 amino acids region of N-end of HSC70/HSP68 of humans, primates, rabbit, rat and ox; antibody-2 (Sino-American) was goat anti-mouse IgG-AP, S-P was marked by peroxidase(Sino-American).

Methods

Samples The rats were divided into groups at random (5 rats/group). In the first group (L): under ether anaesthesia, 2/3 of the liver was cut according to the method of Higgins^[21], and rats were made to recover for 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 16 h, 20 h, 24 h, 36 h, 48 h, 72 h, 96 h and 144h at room temperature. The second group (H-L): 2/3 of the liver was cut when recovered for 8h at room temperature (25°C) after heat shock (at 46°C for 30min), and then recovered as the first group. The third group (L-H): 2/3 of the liver was cut and recovered for 4h before heat shock (at 46°C for 30min), and then recovered as the first group. The rats were killed and bled by taking off the eyeballs. Liver was washed until it became white through coronary vein perfusion^[22], then put into the culture dish with icy physiological salt solution, and sheered into pieces for homogenating in the buffer (40 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5) at 4°C, and centrifugated for 10min at 12000×g. The supernatant was aliquoted and stored at -85°C.

Spectrophotometry of the activity of ACP and AKP

Homogenate 0.1mL was added to 0.4mL buffer (0.25 mol/L MgCl₂, 0.2 mol/L Tris-Cl, pH 7.5 for AKP; 0.35mol/L C₆H₇O₇Na·2H₂O NaOH-HCl, pH 5 for ACP), and 0.4mL 0.01mol/L β-glycerophosphoric acid sodium (0.4mL physiological salt solution as control), mixed and incubated for 30min at 37°C, added with 0.1mL 50% C₂O₂Cl₃ (trichloroacetic acid and homogenate

were added simultaneously for control), remixed, then added 2mL distilled water and 3mL reagent (3 mol/L H_2SO_4 : H_2O : 0.02 mol/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$: 0.6 mol/L Vit C = 1 : 2 : 1 : 1) mixed and incubated for 25min at 45°C, and cooled at room temperature. The photoabsorption value at 660nm was determined and the content of phosphate from standard curve calculated.

Qualitative analysis of HSC70/ HSP68 by stereologic method

According to Weibail^[23], five pieces of sections were counted at high power (5×10 and 5×40) for each sample, and the number of positive cells (P_{xi}) and total cell of detecting field of Vision (P_{ri}) counted separately. By the equation of $U_v = \frac{\sum P_{xi}}{\sum p_{ri}}$, the stereodensity of positive liver cells was calculated, the highest value was regarded as 100%, then statistic analysis was made.

RESULTS

Change of comparative content of HSC70/ HSP68 and activity of ACP and AKP in the first group

During the liver regeneration (0h - 144h), the activity of ACP was extremely strong at 4h and 48h and that of AKP was at 16h and the content of HSC70/HSP68 was at 96h. The change of AKP activity and heat shock protein content were almost consistent, however, the peak of ACP activity occurred ahead of 12h and 24h as compared with the two former ones correspondingly (Figure 1).

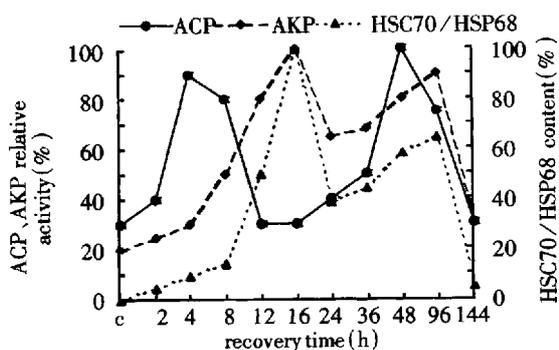


Figure 1 Change of the HSC70/HSP68 content, ACP and AKP activity in the first group.

Change of comparative content of HSC70/ HSP68 and activity of ACP and AKP in the second group

During liver regeneration after heat shock (0h-144 h), the activity of ACP and AKP changed almost consistently, which reached the peaks at 16h and 36h. Although the content of HSC70/HSP68 had three peaks (4h, 12h and 36h), the change was similar to that of ACP and AKP after 8h in liver regeneration (Figure 2).

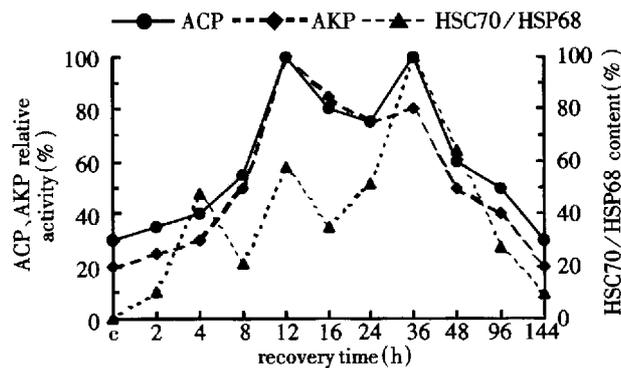


Figure 2 Change of HSC70/ HSP68 the content, ACP and AKP activity in the second group.

Change of comparative content of HSC70/ HSP68 and activity of ACP and AKP in the third group

During liver regeneration (0h - 144 h), the activity of ACP was very extremely strong at 12h and 36h, and had the small peak at 72h. But AKP had three peaks at 12h, 36h and 96h, and that of HSC70/HSP68 content appeared at 36h and 48h. The change of ACP activity was similar to that of AKP, but it was negatively relative to that of HSC70/HSP68 -content (Figure 3).

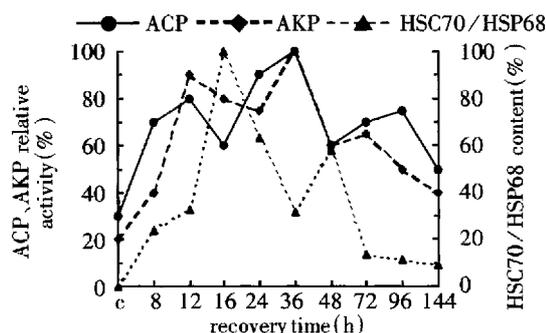


Figure 3 Change of the HSC70/HSP68 the content, AKP and ACP activity in the third group.

Change of comparative activity of ACP in the three groups

The change of ACP activity was very similar in the second group (H-L) and the third group (L-H), but being different greatly from that of the first group (Figure 4).

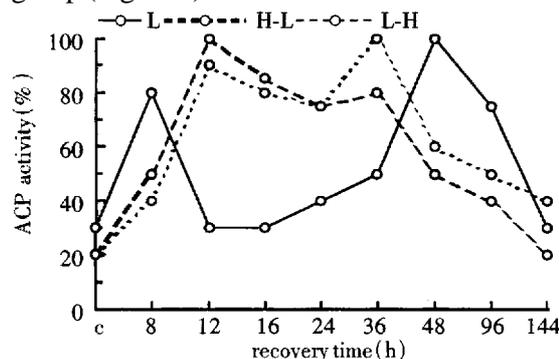


Figure 4 Change of the ACP activity in the three groups (by spectrophotometry).

Change of comparative activity of AKP in the three groups

The change of AKP activity was very complex in the three groups, from 8h to 12h, AKP activity all went up; but, at 12h-48h, that of AKP in the first group was almost inverse to that of the other two groups; at 96h, they were proportional in the first and the second group, and negatively relative to that of the third group (Figure 5).

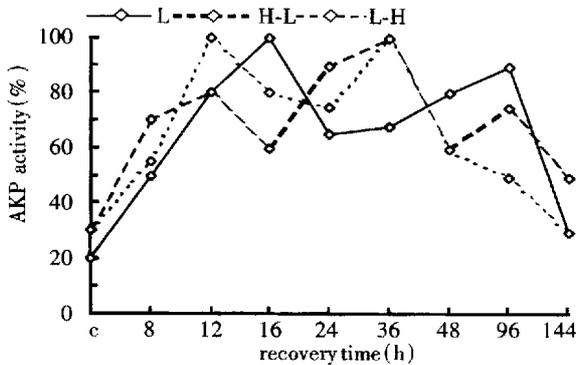


Figure 5 Change of the AKP activity in the three groups (by spectrophotometry).

Change of comparative content of HSC70/HSP68 in the three groups

The content of HSC70/HSP68 had two peaks at 16h and 96h in the first group, but at 12h and 36h in the second group, and at 16h and 48h correspondingly in the third group. Moreover, the content of HSC70/HSP68 was the lowest in the third group (Figure 6).

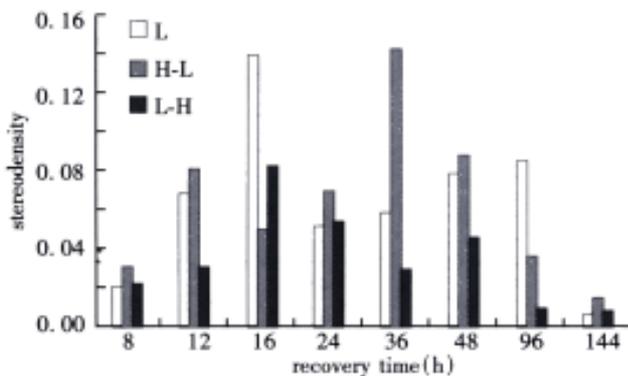


Figure 6 Change of the HSC70/HSP68 content in the three groups (by stereologic analysis).

DISCUSSION

The reversible regulation mechanism of phosphorylation and dephosphorylation and the irreversible regulation mechanism of protein degradation are regarded as two important ways which regulate the living action of cells^[24-27]. As we know, that a series of signal transductive pathways are activated and many proteins are phosphorylated and dephosphorylated are involved in promoting G₀-phase cell into cell cycle^[28-30]. In

the early phase of liver regeneration, the increase of phosphatase activities (ACP and AKP) can be related to G₀-phase cell into cell cycle. However, the peaks of ACP and AKP activity can be related to the two hepatocyte cycles (0 h - 36 h and 36 h - 96 h) and a non-hepatocyte cycle (96h - 114h)^[31] during the whole process of liver regeneration (0 h - 144h).

In the first group, the first peak of ACP and AKP activity appeared at G₁/S checkpoint and S-phase respectively. In the second and the third group, perhaps, because of heat shock, the peak of ACP was delayed, that of AKP was moved up, they all appeared at 12h, but the second peak of them were also consistent. It is supposed that heat shock can affect the synthesis of normal protein (including ACP). When cell can synthesize normal protein, some cells have entered into the first S-phase during liver regeneration, at which ACP and AKP may be synthesized simultaneously. That there were three peaks of AKP activity and the second and third peak just appeared at G₁-phase of liver regeneration are well worth further studying.

The family of HSP70 is known as molecular chaperone, which can help the new peptides to transfer, promote the new polypeptides to trim and to assemble in endoplasmic reticulum^[32-34]. Moreover, the family of HSP73 can recognize the polypeptides with KFEKQ sequence and combine them, and help the protein in cell transfer into lysosome to degrade^[35,36]. The process of liver regeneration with the synthesis and degradation of many proteins may need the family of HSP70. In recent years, the studies show that heat shock proteins play an important role in cellular differentiation, dedifferentiation and proliferation^[37,38]. The first peak of heat shock protein content reported in this paper was just at 12h-16h during liver regeneration, at which hepatocytes are in S-phase; the second peak was just the peak of non-hepatocyte in S-phase. When rats treated by heat shock before partial hepatectomy, the first peak of HSC70/HSP68 was delayed by 20h or so. These showed that heat shock may affect the expression of HSC70/HSP68. In contrast, treated rats by heat shock after partial hepatectomy, the peak of HSC70/HSP68 expression appeared at the same time as that of only partial hepatectomy group (at 16h), however, the content of HSC70/HSP68 in the former group was decreased by 40% than the latter. The results were consistent with that of two continuous treatment by heat shock done by Xu *et al*^[39].

In general, liver regeneration is a very complex physiological and physiochemical process. How on earth HSC70/HSP68, proteinase, ACP and AKP affect only and/or cooperately during liver regeneration should be further studied.

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The cloning of 3'-truncated preS/S gene from HBV genomic DNA and its expression in transgenic mice

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Subject headings hepatitis B virus; gene expression; mice, transgene; polymerase chain reaction; DNA, recombinant; hepatoma

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INTRODUCTION

Hepatitis B virus (HBV) is regarded as one of the main etiologic factors involved in the development of human hepatocellular carcinoma (HCC)^[1-20]. The open reading frame (orf) of X gene of HBV encoded a transactivating factor is the evidence that strongly supported the notion that the X gene of HBV DNA integrated in HCC genomic DNA could contribute to the carcinogenesis of liver cells by activation of some related cellular genes *in trans*^[8,9]. But it was found that the functional orf of X gene was absent in some HCCs harbouring HBV genomic DNA^[6-14]. However, the 3'-truncated preS/S sequence of HBV DNA, which also encodes a transcriptional transactivation factor, was found in all analyzed HCCs harbouring HBV genomic DNA^[20-27]. These findings indicate that transactivation of some cellular genes by the expression product of 3'-truncated preS/S sequence of HBV integrated in the genomic DNA of liver cells is a possible mechanism for HBV-associated oncogenesis^[11]. The transcriptional transactivity also can be produced in the cultured cells transfected with an artificial 3'-truncated preS/S gene of HBV genomic DNA^[11]. To explore the *in vivo* function of 3'-truncated preS/S region of HBV, we cloned the 3'-truncated preS/S region from wild-type HBV genomic DNA and constructed its expression vector for using in transgenic mice. Then, by using

pronuclear microinjection method, we obtained two transgenic mouse lines expressing 3'-truncated preS/S region from 15 new born mice. These transgenic mouse lines are helpful to identify the function of the expression product of 3'-truncated preS/S *in vivo* and the relationship between 3'-truncated preS/S and HBV-associated oncogenesis.

MATERIALS AND METHODS

Materials

Plasmids Vectors pBR322HBV carrying wild-type HBV genomic DNA and pBluscript were preserved in our laboratory. Expression vector pcDNA3.1, containing MCV promoter was provided by Dr. Yu Hong-Yu.

Cells *E. coli* DH5 α was preserved in our laboratory.

Animals C57BL/6 and BALB/c mice were preserved by our transgenic animal laboratory (SPF level). All mice were maintained on a 14:10 light-dark schedule (lights off at 10 pm, on at 8 am.).

Main reagents Restriction endonucleases, T₄ DNA ligase and DNA large fragment (klenow) polymerase were purchased from Promega company. QIA quick gene gel kit and plasmid extraction kit were from QIA gene. Anti-HBV preS1 kit was purchased from α - company.

PCR primers design and synthesis Primers were synthesized by Sangon. Positive primer: 5' GGCCAGA- GGCAAATCAGGTAGGAGG 3', Negative primer: 5' TGGGTGAGGCAGTAGTCGG- AACAGG 3'. The primers are from 1607 to 1934bp of HBVdr genomic DNA sequence, containing 327bp. We also used the T₇ primer, upstream the positive primer.

Methods

Plasmid construction A 2.0kb fragment, containing 3'-truncated preS/S of HBV genome, was cut out of pBR322HBV digested with *Xba*-I and was subcloned into pBluscript, which was named pBluscript - *Xba* 2.0. The 3'-truncated preS/S region was obtained from pBluscript - *Xba* 2.0 digested with *Bst* E II and *xba* I. Its 3'-end was filled with klenow fragment and dNTPs, and inserted into *Bam* HI site of expression vector pcDNA3.1 which also filled, named pcDNA3.1 PreS/S. Restriction endonucleases digesting and sequencing were used to identify the construction.

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Transgenic mice The pcDNA3.1-PreS/S DNA was purified and dissolved in TE buffer (10mM Tris-HCl, 0.2mM EDTA, pH 7.5) at a final concentration of 1mg/L (-2000 copies/pl). After pronuclear microinjection, the eggs were implanted into oviducts of pseudopregnant recipients to enable further development before term.

DNA isolation To isolate tail fragments from 10-day-old mice, approximately one third of the tail was cut and placed into a screw-capped 1.5mL microcentrifuge tube containing 500µL of TB buffer. The tubes containing the tail fragments were incubated overnight at 55°C. They were extracted once with 500µL of 1:1 (v/v) equilibrated phenol-chloroform, and precipitated with 2 volumes of ethanol. After centrifugation, precipitates were resuspended in 500µL water.

DNA analysis The PCR amplification conditions were used with Taq DNA polymerase. For a 50µL reaction, mix the following components: 1µg template DNA, 0.5µL dNTP 10mm, 10u-Taq, 5µL PCR Buffer (10×), 41.5µL deionized water.

We use the following cycling parameters: initial denaturation at 94°C for 5min; followed by 35 cycles at 94°C for 30s; 58°C for 30s; and 72°C for 1min; and then final extension at 72°C for 7min. The products were run on a 2% agarose gel.

Expression analysis The 100µL of blood was extracted from the mouse developed from a microinjected eggs. After centrifuged in microfuge for 5min, the supernatants were isolated and analyzed by ELISA.

RESULTS

Clone of 3'-truncated preS/S and construction of its expression vector

The approximate 2.0kb fragment containing the preS/S was cloned from HBV genomic DNA, from which 3'-truncated preS/S region was cut out and subcloned into the expression vector pcDNA3.1, and then was identified by the restrictive enzyme and sequence analysis. The results showed that the structure was identical with our design (Figures 1 and 2).

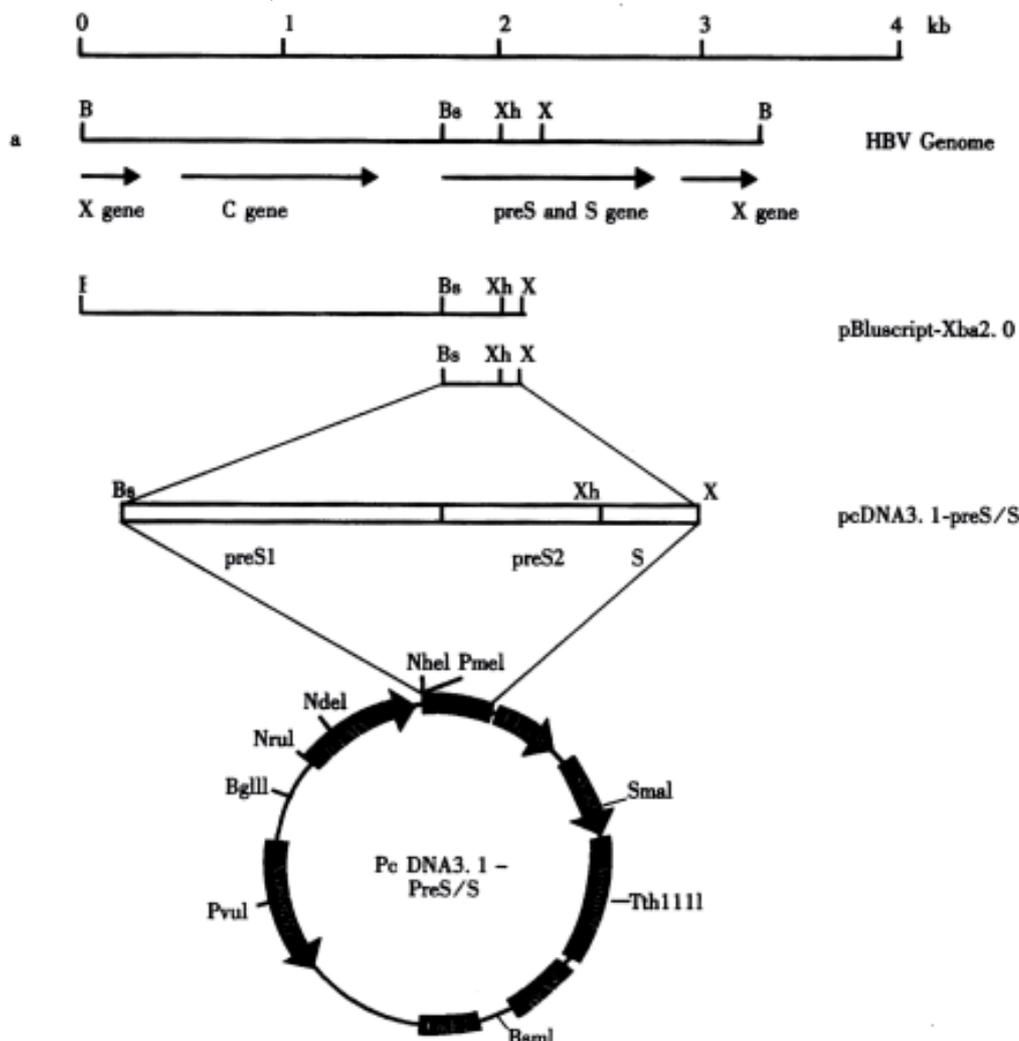
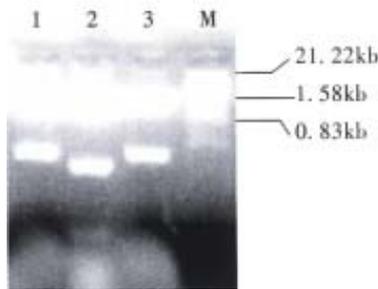
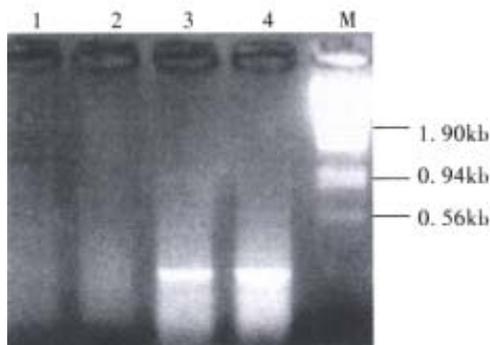


Figure 1 a: The HBV genomic sequence, cloned in pBR322. Positions of restriction site (B, *Bam* HI; Bs, *Bst* EII; X, *Xba* I; Xh, *Xho* I) b: 0.65kb fragment, containing the 3'-truncated preS/S. c: Construction of the vector (pcDNA3.1-preS/S) for expressing 3'-truncated preS/S.



Figures 2 The pcDNA3.1 digested with *Nhe*I, *Pme*I, *Xba*I and *Xho*I.

1: *Nhe*I + *Xba*I; 2: *Xho*I + *Nhe*I; 3: *Pme*I + *Xba*I; M: λ /*Eco*RI + *Hind*-III marker



Figures 3 The DNA analysis of founder mice by PCR. 324bp fragment was amplified with positive and negative primers.

1: blank control; 2: PCR for genomic DNA of normal mice; 3: PCR for genomic DNA of transgenic mice (No.1); 4: PCR for genomic DNA of transgenic mice (No.3); M: λ /*Eco*RI + *Hind* III marker

Table 1 The ELISA results of the surme of transgenic mice expressing the 3'-truncated preS/ S at different times

Serial times (week)	Positive control	Negative control	Normal mice	Transgenic mice	
				No.1	No.3
15	0.21	0.01	0.00	0.14 ^a	0.09 ^a
19	0.34	0.04	0.00	0.11 ^a	0.11 ^a
23	0.31	0.04	0.01	0.10 ^a	0.26 ^a

^apreS1 Ag-positive was defined as \geq negative control $\times 2.1$

Production of transgenic mice

The recombinant construct containing CMV promoter sequences fused to 3'-truncated preS/S region, which encodes a transcriptional transactivation factor, was microinjected into fertilized eggs from C57BL/6 mice. Of 243 microinjected eggs implanted into oviducts of 20 pseudopregnant recipient mice 15 developed to term and gave rise pups. However, only 7 of them survived and the others died within several hours after birth. DNA from the 7 mice were isolated and analyzed by PCR. It was found that 2 of them were positive for the injected transgene (Figure 3). Besides, we also noticed that the embryos from the microinjected eggs had a high miscarriage and mortality rate during the course of their development and growth in comparison with the experience in our laboratory.

Expression of 3'-truncated preS/ S gene in Transgenic mice

The serum samples were collected from the 2 mice harbouring the 3'-truncated preS/S region under the control of CMV promoter and the expression product of the recombinant gene in transgenic mice was analyzed by ELISA, in which the antibody against HBV preS1 was used as the first antibody. The results showed that 2 of them were positive for PreS1 (Table 1). Following the founders conformed, a series of expression analysis was carried out at different time points during the development. It was found that the 3'-truncated preS/S region could be stably expressed in the transgenic mice.

DISCUSSION

The full-length preS/S sequence integrated in nearly all HCCs can't show any trans-activity. However, one copy of the preS/S sequence with 3'-truncation could show a definite trans-activity^[1]. We constructed the expression vector of 3'-truncated preS/S gene, which can be expressed in cultured mammalian cells. So this vector should be very useful for exploring the biological function of expression product of 3'-truncated preS/S gene and for identifying whether 3'-truncated preS/S gene in HCCs is a causative factor of HBV-associated oncogenesis.

During the process of generating transgenic mice, the miscarriage rate and mortality rate seemed to be much higher than that in the producing transgenic mice harbouring other genes^[28-30]. This phenomenon indicates that beside the some common reasons for the death of transgenic mice there might be some other factors. It is possible that there are some effects of the trans-activation of the expression product of the 3'-truncated preS/S like those of the 3'-truncated preS/S intergrated in HCCs of human being on the development of mouse embryos and its early growing of the pups after birth.

The 2 transgenic mouse founders could express 3'-truncated preS/S sequences stably. These results indicate that the 3'-truncated preS/S is integrated in their genomic DNA, which is similar to those existing in the HCCs of human being. So we believe that the 2 transgenic mouse lines can be employed as the model for exploring the *in vivo* function of the expression product of 3'-truncated preS/S and relationship between 3'-truncated preS/S and HBV-associated oncogenesis.

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Transfusion transmitted virus infection in general populations and patients with various liver diseases in south China

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Subject headings transfusion-transmitted virus; liver disease/etiology; DNA virus; polymerase chain reaction; serodiagnosis; hepatitis viruses

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INTRODUCTION

Although several specific detecting methods had been applied to determine the hepatitis virus, there was a lot of cryptogenic hepatitis without any known hepatitis infectious marker^[1]. The prevalence of hepatitis G virus (HGV) (also known as GB-C virus) infection has been reported to be 5% -13% in patients with non-A-E hepatitis and cirrhosis, however, there is little evidence suggesting that HGV causes hepatitis in human^[2-6]. Although cryptogenic liver diseases are almost certainly related to a variety of etiologies, one or more as-yet-unidentified infectious agents are likely to account for a proportion of these cases.

In December 1997, a novel DNA virus was reported by Nishizawa *et al*^[7] to be associated with elevated aminotransferase levels in patients with post-transfusion hepatitis of unknown etiology (non-A-G hepatitis). This virus was designated transfusion transmitted virus (TTV). Then, Luo *et al*^[8] and Wei *et al*^[9] also detected TTV in the sera of patients from an outbreak of cryptogenic hepatitis in south China. And TTV was also detected in patients with post-transfusion hepatitis in China^[10]. In subsequent analyses, TTV is an un-enveloped single-stranded DNA virus for which a sequence of 3800 bases was determined^[11]. Evidence of potential hepatotropism of TTV was reported with TTV DNA detected in liver tissue^[12]. Histopathological study indicated that the characteristics of liver histology of TTV infected patients are portal inflammation and interlobular

bile duct damage^[13]. TTV was proposed as the part of causative agent of non-A to G hepatitis. Seroepidemiological studies have shown TTV to have global distribution^[12,14,15]. Although the potential association of TTV with cryptogenic hepatitis is intriguing, the pathological and clinical significance of this virus remains to be established. To assess more thoroughly the etiological role of TTV in the causation of hepatitis, we determined the frequency of TTV infections and their relationship to liver disease in several cohorts of liver diseases and rural population.

PATIENTS AND METHODS

Rural population

Nighty males and 89 females aged from 1 to 73 years were from a natural village with total population of 190 in southeast of Yunnan Province. Among them, 90 persons were of nationality of Han, others of Yi.

Patients with cryptogenic hepatitis

Forty-four patients with cryptogenic hepatitis were admitted in hospital between January 1993 and May 1999, with negative assays for sera marker of hepatitis virus A-E, and also negative assays for anti-nuclear antibody, anti-smooth muscle antibody, EB virus antibody, CMV antibody and anti-mitochondrial antibody. Part of patients were confirmed with liver biopsy suggestive of acute hepatitis.

Patients with HBV related chronic liver diseases

The prevalence of TTV infection was also determined in five cohorts of HBV related chronic liver disease: ① HBsAg asymptomatic carrier (AsC) ($n=52$); ② Chronic hepatitis B (CHB) ($n=46$); ③ Chronic hepatic failure ($n=40$); ④ Active liver cirrhosis ($n=39$); ⑤ Hepatocyte carcinoma (HCC) ($n=21$). The diagnosis accorded with diagnostic criterion of viral hepatitis in the Fifth Science Meeting of Infectious Disease and Verminosis (Beijing, 1995)^[16].

Nested PCR for the detection of TTV DNA

Evidence for TTV infection was determined by detection of TTV DNA by nested PCR. Nucleic acids were extracted from 100 μ L serum. TTV DNA was determined by PCR with nested primers described by Okamoto *et al*^[11] that sensitively detect TTV DNA, irrespective of different

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genotypes, as well as by Ampli-Taq- DNA Polymerase. In brief, the first round of PCR was performed with RD038 primer (sense: 5'-TGA CTG TGC TAA AGC CTC TA-3') and NG059 (antisense: 5'-ACA GAC AGA GGA GAA GGC AAC ATG-3') for 35 cycles (94°C, 45 seconds; 54°C, 45 seconds; 72°C, 60 seconds [additional 7 minutes for the last cycle]), and the second-round PCR was performed with NG061 (sense: 5'-GGC AAC ATG TTA TGG ATA GAC TGG-3') and NG063 (antisense: 5'-GAC CGT AAA ATG GTA AAG GTT TCA-3') for 35 cycles with the same conditions. The size of the second-round PCR was 271bp. The amplicons were electrophoresed in 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. All assays were performed in an amplicon-free work area. Positive and negative

results were confirmed with repeated assays.

Direct sequencing of the amplicons

Direct sequencing of the amplicons was carried out by Cybersyn.

Statistical analysis

Prevalence of TTV infection (as measured by TTV DNA detectable in serum by PCR) in rural population and several cohorts of liver disease was determined. Data analysis was carried out using χ^2 test. And the liver function test of chronic hepatitis B was analysed using *t* test.

RESULTS

Sequencing of the amplicons (Figure 1)

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TTV G1b: GGCAACATGTTATGGATAGACTGGCTAAGCAAAAAAACATGAACTATGACAAAGTA
SERA:
CAAAGTAAATGCTTA [A] TATCAGACCTACCTCTATGGGCAGCAGCATATGGATATGTAG
G
AATTTTGTGCAAAAAGTACAGGAGACCAAAACATACACATGAATGCC [T] GGCTACTAAT
A
AAGAAGTCCCTTTACAGACCCACA [A] CTACTAGTACACACAGACCCACAAAAGGCTT [T]
G
GTTCCCTACTCTTTAAACTTTGGAAATGGTAAATGCCAG
C

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Figure 1 Comparison of nucleic acids sequence between TTV G1b and amplicons and nucleic acids sequence nt1935-2205 of TTV G1b, sera amplicons had a homogeneity of 98.5%, suggesting the presence of TTV DNA in sera.

Prevalence of TTV infection (Table 1)

Table 1 Prevalence of TTV infection among study cohorts

Groups	TTV positive rate (n)	TTV negative rate (n)	Total
Rural population	10.61(19)	89.39(160)	179
Cryptogenic hepatitis ^a	38.63(17)	61.37(27)	44
HBsAg asymptomatic carrier	9.62 (5)	90.38(47)	52
Chronic hepatitis B ^b	15.22 (7)	84.78(39)	46
HBV related active liver cirrhosis ^c	22.5 (9)	77.5(31)	40
HBV related hepatic failure ^d	23.08 (9)	76.92(30)	39
Hepatocyte carcinoma ^e	9.52 (2)	90.48(19)	21
Total	16.15(68)	83.85(353)	421

^a $\chi^2 = 20.486$, $P < 0.005$ vs rural population; ^b $\chi^2 = 0.713$, $P > 0.25$ vs HBsAg asymptomatic carrier; ^c $\chi^2 = 0.749$, $P > 0.25$ vs chronic hepatitis B; ^d $\chi^2 = 0.853$, $P > 0.25$ vs chronic hepatitis B; ^e $\chi^2 = 0.000$, $P > 0.9$ vs HBsAg asymptomatic carrier.

Rural population

Nineteen of 179 (10.61%) unselected healthy peoples were detected TTV DNA positive. The prevalence of TTV infection was independent of sex, age and nationality (Table 2).

Table 2 The prevalence of TTV infection in a natural village in Yunnan Province, regarding of sex, nationality and age

Result	Sex ^a			Nationality ^b			Age ^c			
	Male	Female	Total	Han	Yi	Total	<14	14-55	>55	Total
TTV positive	7	12	19	8	11	19	5	13	1	19
TTV negative	83	77	160	82	78	160	47	102	11	160
Total	90	89	179	90	89	179	52	115	12	179
Prevalence(%)	7.8	13.5	10.6	8.9	12.4	10.6	9.6	11.3	8.3	10.6

^a $\chi^2 = 1.535$, $P > 0.2$; ^b $\chi^2 = 0.568$, $P > 0.4$; ^c $\chi^2 = 0.178$, $P > 0.9$.

Patients with cryptogenic hepatitis

The prevalence of TTV infection in patients with cryptogenic hepatitis was 38.63% (17 of 44). Two of the TTV-infected patients with fulminant hepatic failure, while the others with mild hepatitis.

Patients with HBV related chronic liver disease

The prevalence of TTV infection of AsC, CHB, ALC, CHF and HCC was 9.62% (5/47), 15.22% (7/39), 22.5% (9/31), 23.08% (9/30) and 9.52% (2/19), respectively. It seemed that the state of illness was related with the co-infection of TTV. However, there was no statistical difference between the prevalences (Table 1).

Effect on liver function test of TTV co-infection

While co-infected with TTV, the state of illness did not exacerbate in patients with CHB (Table 3). In patients with active liver cirrhosis and chronic hepatic failure, there were no significant differences in results of laboratory tests in patients with and without TTV DNA ($P>0.2$) whereas the co-infection of TTV increased the mortality of patients with hepatic failure ($P=0.038$).

Table 3 Effect on liver function test of TTV co-infection in patients with chronic hepatitis B

Liver function test	TTV negative	TTV positive	t value	P value
ALT(U/L)	347.0±286.5	282.3±230.2	0.523	>0.5
AST(U/L)	199.9±171.8	140.8±105.3	0.742	>0.2
Protein A/G	1.24±0.28	1.26±0.12	0.140	>0.5
TBil(μmol/L)	34.1±30.6	22.8±9.3	0.883	>0.2
Prothrombin (s)	17.7±5.5	15.6±1.53	0.783	>0.2

DISCUSSION

The recent discovery in Japan by Nishizawa *et al*^[7] of a novel parenterally transmissible, unenveloped, single-stranded DNA virus (TTV) in patients with non-A-G posttransfusion hepatitis had raised some important questions about TTV as a potential cause of liver disease.

Previous study indicated that TTV infection was common in healthy individuals, blood donors, HBsAg AsC, patients on hemodialysis and patients with various liver diseases, however played a minimal role on liver disease^[17-26]. Another paper suggested TTV may cause chronic hepatitis in a limited number of patients, but remains dormant most of the time^[27]. In our study, we found 10.61% of healthy individuals with normal liver function test were infected with TTV, and the prevalence was independent of sex, age and nationality. The result indicated TTV infection was common in healthy individuals. However, frequency of TTV infection in patients with cryptogenic hepatitis was significantly higher. As evidence of potential hepatotropism of TTV had been reported with TTV-DNA titers shown to be 10

to 100 folds greater in liver tissue than in serum^[11,12], TTV would account for part of the reason for patients with cryptogenic hepatitis. As we know that there are many HBsAg AsC, whereas HBV causes many hepatitises. This study suggested that the majority of individuals with TTV could be asymptomatic carriers, with only a small proportion of carriers actually developing hepatitis.

Generally, co-infection of hepatitis virus would lead to exacerbation of hepatitis. While co-infected with hepatitis A and B virus, patients encountered exacerbation of illness, with more severe abnormal results of laboratory tests and higher mortality^[28]. In histopathological evaluation, co-infection of hepatitis virus had no significant difference in development of liver cirrhosis^[29]. Previous study indicated that superinfection of TTV does not exert deleterious effects on the liver disease induced by HCV. Triple infection, HCV and TTV plus HBV or HGV, did not cause severe liver disease^[27]. In current study, prevalence of TTV infection in HBsAg AsC and patients with chronic hepatitis B, HBV related liver cirrhosis and chronic hepatic failure was 9.62%, 15.22%, 22.5% and 23.08% respectively. There was no significant difference among prevalence of TTV infection, suggesting the minor effect on liver disease of co-infection of TTV. The comparison of levels of ALT, AST, total bilirubin, A/G of serum protein and prothrombin time between TTV virus-positive and-negative patients did not show any differences, as accorded with another report^[30]. However, TTV co-infection increased the mortality of patients with hepatic failure.

Most cases of chronic hepatitis, cirrhosis, and HCC in developed countries are caused by HBV or HCV infections and heavy alcohol intake. A relatively small proportion of liver diseases is of unknown etiology. The recently discovered HGV seems to have no actual role in causing acute or chronic liver disease^[31], whereas the role of the more recently discovered TTV is still to be defined. To inquire the relationship between TTV infection and HCC, we investigated the prevalence of TTV infection in HBV infected patients with HCC, and compared with HBsAg AsC. The result indicated similar prevalence in both cohorts (9.52% vs 9.62%, $P>0.9$), which did not support the hypothesis of an association between TTV infection and HCC, and accorded with previous studies^[32,33]. Another report indicated that TTV was not specific for HBV-negative and HCV-negative patients with HCC. For all TTV-positive patients, the TTV genome was not integrated into host hepatocyte DNA^[34]. In conclusion, TTV was common in general population and several cohorts of liver disease. Though the majority of individuals with TTV could be asymptomatic carriers, TTV would account for part of cryptogenic hepatitis. As TTV

co-infection did not affect the state of HBV infection, further study on pathogenic effect on liver disease of TTV infection should be continued.

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A new chronic ulcerative colitis model produced by combined methods in rats

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Subject headings colitis, ulcerative; animal model; 2, 4-dinitrochlorobenzene; acetic acids; apoptosis; flow cytometry; intestinal mucosa/pathology

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INTRODUCTION

The etiopathogenesis of ulcerative colitis (UC) remains obscure, due to lack of an ideal animal model^[1-8]. With the improvement of theory and methodology in the last 30 years, people used to adopt chemicals (acetic acid, ethanol, carrageen, etc.), immunotechniques (humoral or cellular immunity, immune complex) and substance derived from UC patients to set up various kinds of UC animal model, which mimic the pathologic changes of human UC, so far these remain far from reality^[6-10]. A possible exception is the spontaneous colitis developed in the cotton-top tamarin when captured, but this animal is rarely available and expensive preventing its usage^[11]. Therefore, establishing an ideal animal model becomes the focus and key of the research study of UC. Nowadays, the models produced by 2,4-dinitrochlorobenzene (DNCB) and acetic acid (AA) came into use because of their simplicity and pathologic changes simulating those of human UC^[12-21], however, the characteristics of short course of DNCB method and absence of immunoreactivity in AA method, these two models are not ideal either. In the present study, we established a new rat UC model produced by combination of DNCB and AA, and observed the changes of general condition, the disease course, the pathology, ultrastructure, apoptosis, immunoreaction and intracolonic pressure, in order to develop a more ideal UC animal model.

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MATERIALS AND METHODS

Materials

Eighty healthy male and female adult Wistar rats weighing 250g - 350g were used in this study. They were provided by the Department of Experimental Animal of our hospital, and were raised in the SPF environment (constant temperature, humidity and sterilized water, food and padding) and acclimatized to the surrounding for 7 days prior to the experiments.

DNCB (First Reagent Factory of Shanghai), AA (Dongtai Reagent Factory), CD₄, CD₂₉ and FITC or PE conjugated monoclonal antibodies (Immunotech, Marseilles, France), PC polygraf HR multichannel recording system (CTD-SYNECTICS Ltd), scanning electron microscope (EX1200, Japan), flow cytometry FACScan (Becton Dickenson Immunocytometry System).

Methods

Animal models Eighty Wistar rats were divided randomly into 4 groups, 20 each. Group A (DNCB + AA): after the nape hair was depleted by 10% Na₂S, DNCB acetone solution (20g/L) was dropped to the nape of the rats (0.3mL for each) once daily for 14 days, on the 15th day, nylon catheter (3mm in diameter) was inserted into the colon at the site of 8cm from the anus, and 0.25mL 0.1% DNCB, 50 mL/L 0.04 mol/L solution alcohol were infused, on the 16th day, 2mL AA solution (80mL/L) were infused into the same site for 15s, then 5mL normal saline (0.9%) was used to washout AA. Group B (DNCB only): from the 1st day to the 14th day, the method was similar to that for Group A, from the 15th day to the 18th day, 0.25 mL 0.1% DNCB (50 mL/L) alcohol solution (0.04mol/L) were infused into the colon at 8cm depth by the same nylon catheter once a day for each. Group C (AA only): 2mL AA (80mL/L) solution were infused into the colon at 8 cm depth by intracolonic administration with nylon catheter (3mm in diameter) for 15s, then 5mL saline for washing the AA. Group D (saline control): equivalent volume of normal saline was given in the method similar to that for Group A.

Pathological observation After the model had been established, the rats were killed at wk1, 2, 4, 8 and 16, and the distal colon (7cm-9cm) were removed longitudinally and washed to remove the luminal contents, tissues were fixed in 10% neutral-buffered formalin, dehydrated according to the routine, embedded in paraffin wax and sectioned.

Finally, the sections were stained with HE and observed microscopically. Apoptotic cells were identified morphologically^[22], for cell shrink age, chromatin condensation, formation of apoptotic bodies. Apoptosis was calculated randomly by counting the apoptotic bodies in the lamina propria for at least 200 cells^[22].

Electron microscopy The tissues were cut into small pieces (0.5mm in diameter) and fixed first in 2.5% glutaraldehyde buffered in 0.1M PBS (pH 7.2) at 4°C for at least 2h. The tissues were washed with the same buffer and then fixed in 1% osmium tetroxide in phosphate buffer at 4°C for 2h, dehydrated in graded series of acetone and embedded in epoxy resin 812. The ultra-thin sections were observed under EX1200 electron microscope.

Measurement of CD₄⁺CD₂₉⁺ Blood CD₄⁺CD₂₉⁺ were measured using flow cytometry according to our previous article^[15,23].

Colonic pressure and motility Intracolonic pressure and motility were measured according to our previous report through pressure transducer and recorded by PC Polygraf HR multichannel recording system^[24] (Figure 1). During manometry, the catheter lumen was infused with 0.9% saline at 0.2mL/min using a miniature hydraulic infusion pump. The baseline resistance of intra colonic pressure was set at zero. The catheter (outer diameter 3mm with 4 side holes for 4 channels) was inserted into the colon at 9cm depth from the anus without laparotomy, and was withdrawn at 1mm-2mm increment, and measurement was not started until 5min after the tip of the catheter dropped out into the rectum.

Statistical analysis The data were expressed as $\bar{x} \pm s$, and analyzed, using the Student's *t* test $P < 0.05$ was considered significant.

RESULTS

General condition and disease course Anorexia, bloody diarrhea, mucus in stools were seen in all A, B and C groups after 1 to 2 weeks, which lasted for 16 weeks in group A with weight loss and 8 weeks in group C, while in group B, the bloody diarrhea and mucus in stools decreased gradually and recovered 2 weeks later. No symptoms were observed in group D.

Pathological findings Diffuse hemorrhage, edema, congestion, superficial ulceration in the mucosa with infiltration of lymphocytes, plasma cells and polymorphonuclear cells, cryptitis, crypt abscess could be observed in all A, B and C groups (Figure 2), and these characteristics lasted for 16 weeks in group A and 8 weeks in group C while only 2 weeks in group B. In group D, the bowel wall was normal by

gross and microscopic examinations.

Ultrastructural changes There were decreased number of cells, shortened microvilli, swollen mitochondria with depleted ridge, maldevelopment of goblet cell and increased number of lysosomes during the acute phase (Figure 3), which recovered to normal gradually (Figure 4).

Apoptosis The apoptosis indexes, 9.9 ± 3.8 in group A, 8.6 ± 3.5 in group B, 8.1 ± 2.9 in group C, were significantly higher than those in group D (4.0 ± 2.1 , $P < 0.05$). Under scanning electronmicroscope, shrinkage of cells, condensation and margination of chromatin could be seen (Figure 5).

Immunoreactivity The changes of CD₄⁺CD₂₉⁺ in ulcerative colitis models were shown in Table 1.

CD₄⁺CD₂₉⁺ increased significantly ($P < 0.01$) in group A and B but not in group C and D 1 week after set up of model ($P > 0.05$).

Table 1 Changes of CD₄⁺CD₂₉⁺ in ulcerative colitis models (% , $\bar{x} \pm s$)

Group	n	Prior to set up of model	1 week after set up of model
A	20	5.01±2.01	11.17±2.18 ^b
B	20	4.95±1.87	10.98±2.87 ^b
C	20	4.93±1.96	5.06±2.03
D	20	4.76±1.56	4.91±1.93

^b $P < 0.01$ vs prior to set up of model.

Colonic pressure and motility One week after set up of the model in group A, the basal intracolonic pressure was apparently lower than that of the pre-model (proximal pressure: $0.78 \text{ kPa} \pm 0.13 \text{ kPa}$ vs $0.88 \text{ kPa} \pm 0.14 \text{ kPa}$, distal pressure $0.76 \text{ kPa} \pm 0.11 \text{ kPa}$ vs $0.89 \text{ kPa} \pm 0.15 \text{ kPa}$, $P < 0.05$). The frequency of migrating motor complex waves *in vivo* were significantly faster 1 week after set up of the model as compared with that prior to ($1.59/\text{min} \pm 0.27/\text{min}$ vs $0.60/\text{min} \pm 0.12/\text{min}$, $P < 0.05$) in 7 rats of group A, this belonged to the pathologic colon of high dysrhythmia. The amplitude of migrating motor complex waves decreased markedly in 10 rats of group A after 1 week of post-model than that of pre-model (proximal pressure: $0.64 \text{ kPa} \pm 0.24 \text{ kPa}$ vs $1.98 \text{ kPa} \pm 0.38 \text{ kPa}$, distal pressure: $0.92 \text{ kPa} \pm 0.37 \text{ kPa}$ vs $2.45 \text{ kPa} \pm 0.63 \text{ kPa}$, $P < 0.01$), which belonged to asthenia colon. In the other 3 rats, while the proximal amplitude of migrating motor complex waves were lowered significantly ($0.96 \text{ kPa} \pm 0.31 \text{ kPa}$), the distal amplitude of migrating motor complex waves remained still higher ($2.35 \text{ kPa} \pm 0.50 \text{ kPa}$).

Success rate The ulceration pattern was present in all 20 rats of group A, the success rate therefore, could be considered 100%.

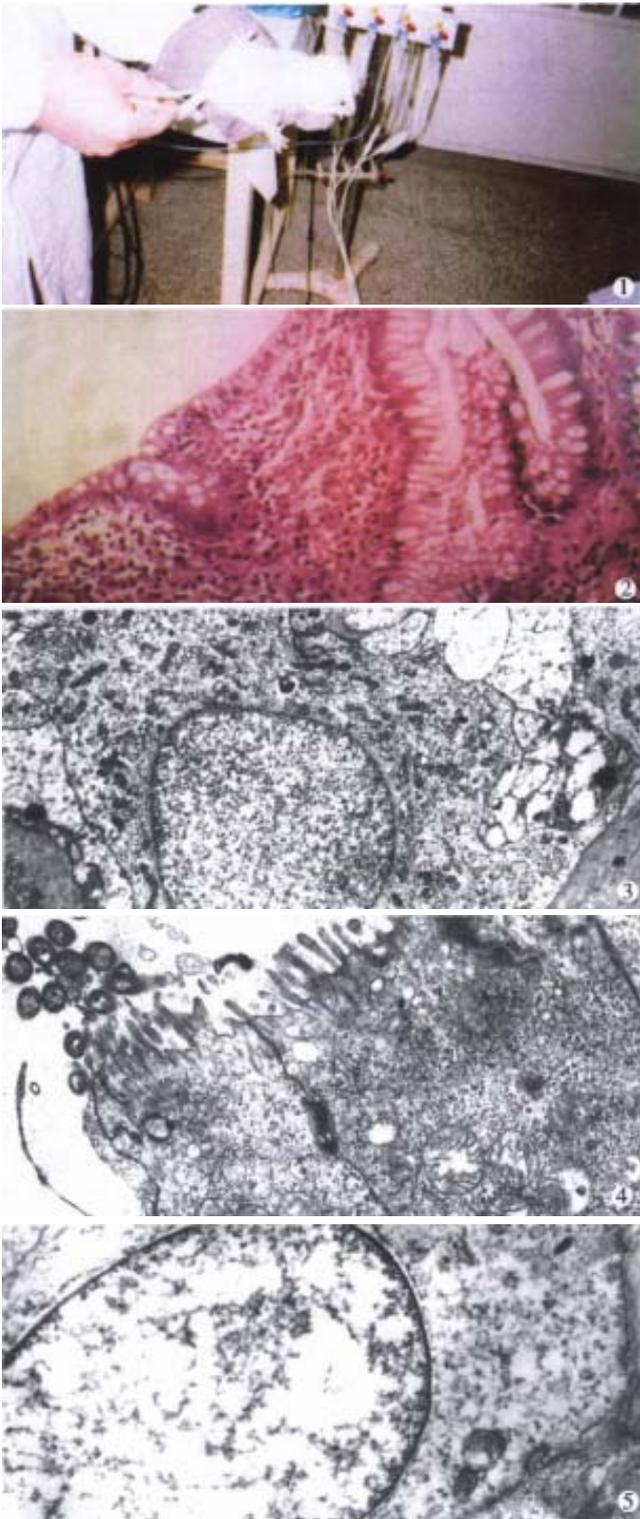


Figure 1 Measurement of intracolonic pressure and motility in UC rats without laparotomy.

Figure 2 Pathological features of ulcers and crypt abscess. HE×25

Figure 3 Swelling of mitochondria with ridge depletion in goblet cell. ×4000

Figure 4 In healing phase, the decreased number of the cell count and swelling of mitochondria recovered to normal gradually. ×10K

Figure 5 Apoptosis of epithelia cell with condensation and margination of chromatin. ×12K

DISCUSSION

So far, the precise etiopathogenesis of UC remained unelucidated^[1,25-40], though experimental colitis had been produced by various methods mimicking human UC somehow, up to the present there is not one ideal animal model which conforms with human UC in pathogenesis, pathology and biologic behavior^[6-21,41,42]. An ideal animal model should fulfill the following requirements^[15,24]: ① it should reflect the histological characteristics of the diseases; ② it should be autoimmune in nature; ③ it should have the similar clinical manifestations as in human UC; ④ it should be simple and reproducible. In this study, the UC model produced by AA method was related to its chemical stimulation to the colonic mucosa^[12-14], which led to increment of vascular permeability and activate inflammation mediators, resulting in bloody diarrhea, mucus in stools and histological features as diffuse edema, congestion and ulceration, crypt abscess and mucosal infiltration of inflammation cells. It can be used to study the inflammatory mechanism and anti-inflammatory drugs^[43-45], but it lacked the immune response which is a drawback.

DNCB is a hapten, when bound with tissue proteins will be able to elicit immunologic response and induce colitis^[18-21]. The clinical symptoms and histological features, in particular CD₄⁺CD₂₉⁺ cells are similar to those of human UC, but the self-limited course of 2 weeks, is too short to be utilized as an ideal model.

In order to overcome these shortcomings we establish a new chronic UC model by using DNCB and AA in combination which has the following advantages^[15-17]. ① Clinically it manifests mucus in stools, bloody diarrhea and weight loss, just like those occur in human UC. ② It can reflect the pathologic characteristics of UC, such as continuous superficial colonic inflammation. Microscopically there exist mucosal edema and congestion, infiltration of lymphocytes, plasma cells and polymorphonuclear cells, crypt abscesses and ulceration. ③ It is an immune response model, immunology is well studied in UC^[1,46-49]. One of the important immunoregulatory abnormality in UC is related to the T cell response^[23,50]. CD₄ interacts with HLA class II molecules, CD₄ positive T cells can be divided into Th1 and Th2 cells whereas CD₂₉ reacts with 130 KD integrin β_1 subunit which is expressed as a heterodimeric complex with one of six α subunits, forming the very late activation antigen (VLA) subfamily of adhesion receptors. The β_1 subunit has a broad distribution, and is expressed on lymphocytes, monocytes but weakly on granulocytes. These receptors are involved in a variety of cell-cell and cell-matrix interactions. Co-expression of CD₄⁺ and CD₂₉⁺ can be used as a marker to identify the Th1 cells subgroup, whose main function is to help B lymphocyte to induce

antibody production and cell-mediated dissolution^[15,23]. The increase of CD₄⁺CD₂₉⁺ can lead to highly activated B lymphocyte and immunoregulatory abnormality. In this test, CD₄⁺CD₂₉⁺ cells are significantly higher after the set up of models by combination of DNCB and AA, which is in accord with the requirement of immune response similar to that in human UC. ④ It has a long disease course, if can last at least for 16 weeks with chronic damage of the bowel hence, eligible for the assessment of the drug effects. ⑤ The ultrastructural features are similar to those of human UC. The decreased number of epithelial cells and shortened microvilli, swelling of mitochondria with depletion of ridges can lead to impairment of water absorption of colon resulting in diarrhea. The maldevelopment of goblet cell, may lead to mucus in stools. ⑥ The above findings are not only consistent with the changes in human UC^[51-59], but also concordant with other reports^[60,61]. The model produced by combination of DNCB and AA is a more ideal animal model of UC. Apoptosis was first described by Kerr^[62] and is referred to as programmed cell death, which is genetically controlled. In active UC, injury is common. Did apoptotic cells increase in colonic epithelia which led to ulceration This hypothesis had been studied since 1996^[63-67]. Apoptosis of the normal colon was localized in the superficial epithelium, far less than one apoptotic body per crypt. In active UC, the loss of epithelial cells occurred mainly by apoptosis in crypts of involved and uninvolved adjacent areas, resulting in impairment of protective mucosal barrier. The mediators of apoptosis are partly related to the Fas/Fas-L interaction and/or changes in Bcl-2 expression. Our previous study demonstrated the number of apoptotic cells of colonic mucosal epithelial cells in human UC increased as shown by flow cytometry^[68]. This led to the damage of the barrier resulting in ulceration. Under scanning electron microscope, shrinkage of cells, condensation and margination of chromatin could all be seen.

The symptoms of diarrhea and abdominal pain are partly related with colonic motility disturbance^[69-75]. In this study, we first used PC Polygraf HR multichannel recording system to measure the intracolonic pressure and motility in physiological and pathological conditions from multiple sites over prolonged periods. The resultant signals were digitized, analyzed and displayed in a readily interpretable manner, and could be easily subjected to a variety of statistical manipulations, as the colon was situated within the abdominal cavity, the temperature, humidity and pH were maintained by the rats themselves, and not interfered by operative maneuver. In this study, UC rats are characterized by decreased intracolonic basal pressure and disturbance of frequency and amplitude

of migrating motor complex which may lead to the symptoms of urgency, diarrhea and abdominal pain. In conclusion, the new chronic UC rat model produced by combination of DNCB and AA is similar to human UC in clinical manifestations, histology, ultrastructural changes, immune response, apoptosis and colonic motility, and it is simple, inexpensive and reproducible with high successful rate.

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Inhibitory effects of EGFR antisense oligodeoxynucleotide in human colorectal cancer cell line

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Subject headings colorectal neoplasms; oligonucleotides, antisense; epidermal growth factor receptor; gene therapy; liposomes; flow cytometry

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INTRODUCTION

Epidermal-growth-factor receptor^[1] (EGFR) is a polypeptide with 1186 amino acids, which binds to EGF family growth factors. Two major natural ligands in the family interact with EGFR: one is EGF, the other is transforming growth factor- α (TGF- α)^[2]. When EGF or TGF- α , binds to EGFR, tyrosine kinase activity is induced which in turn triggers a series of events regulating the cell growth^[3-8]. The importance of EGFR in growth regulating pathways was confirmed by the fact that enhanced expression of this receptor was found in brain glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas^[9,10]. Elevated EGFR levels correlated with poor prognosis in human tumors^[11-17], for this reason, it seemed to be that EGFR would be a logical target for cancer therapy.

Previous reports had shown that monoclonal antibodies to EGFR were effective in the treatment of many human carcinoma cells^[18-20]. Other drug therapies which targeted the EGFR had also been successful. Kunkel had shown a drug that inhibited EGFR tyrosine kinase activity could inhibit the growth of A431 cells in nude mice^[10]. Yoneda also reported that selective inhibitors of EGFR tyrosine kinase activity, such as tyrphostins, could inhibit the growth of squamous carcinoma in nude mice^[21].

Antisense oligodeoxynucleotides inhibit gene expression on a highly selective and target sequence in a specific manner^[22-25]. Specific oligonucleotides hybridize to complementary mRNA and decrease protein expression^[26-29]. Antisense oligonucleotides against proto-oncogenes of growth factors had already been shown to be successful in cell

lines^[18,30]. For example, an antisense oligonucleotide to the erbB2 gene product had been shown to inhibit protein production in a breast cancer cell line^[31]. Akino reported inhibition of *in vivo* growth and metastases in malignant pituitary tumors with an antisense compound to the PTHrp (parathyroid hormone-related peptide)^[32]. An oligonucleotide to the c-myc gene inhibited the growth of thyroid carcinoma cell lines^[33]. Phosphorothioate antisense oligodeoxynucleotides targeted against human c-raf-1 kinase producing potent antiproliferative effects on cell culture and *in vivo* antitumor effects against a variety of tumor types^[34].

The co-expression of EGFR along with TGF- α in human colon cancer cell lines, also in colon carcinoma tissue, had led to the suggestion that the autocrine stimulation of EGFR by its ligands could be a mechanism for tumor cells to escape from normal growth controls^[35]. Previous studies in our laboratory confirmed over-expression of EGFR in HR8348 cells^[36]. In this investigation, we hypothesized that growth and proliferation of HR8348 could be inhibited by EGFR ASODN. In this report 15-mer EGFR ASODN was synthesized and the effects of EGFR ASODN on cell proliferation and tumorigenic rate of HR8348 cells were observed.

MATERIALS AND METHODS

Cell line

The liver metastasis of human colorectal cancer cell line HR8348 was developed by Zhang *et al*^[37] and cultured at 37°C in a humidified atmosphere containing 50mL/L CO₂ in RPMI1640 medium (Gibco) supplemented with 100mL/L heat-inactivated fetal calf serum (FCS), penicillin (50× 10³ units/ mL), and streptomycin sulfate (50mg/L) unless otherwise specified.

Synthesis of oligonucleotides

The AEGFR oligonucleotide sequence, 5'-CCGTGGTCATGCTCC-3' is complementary to EGFR cDNA 3811-3825, which contains the opal translation termination codon at residues 3817-3819. The control oligonucleotide sequence, a randomized phosphodiester 15-mer oligonucleotide with the sequence 5'-GCTGACGCACTGACT-3' (RC 15) is not complementary to any cDNA. Oligodeoxynucleotides were synthesized on an automated DNA synthesizer.

Formation of the lipid-ODN complex

ODN and liposome, Lipofectamine (Gibco-BRL), were each diluted to 0.1mL with RPMI1640 (serum and antibiotic free) and then mixed together, following the manufacturer's protocols. The lipid-

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ODN complexes were used in gene transfection immediately after its formation.

Treatment of cells

To determine the effect of anti-EGFR oligonucleotides on HR8348 cell proliferation, MTT method was adopted. Forty μL HR8348 cells (1×10^4) in 96-hole culture dishes were treated at 37°C for 5h with either free or lipid-ODN mixture and then added 200 μL fresh medium with 100 mL/L fetal calf serum for a further 48h. At this point, the cells were washed twice with (serum free) RPMI1640, and RPMI1640 200 μL , added MTT (5g/L) 20 μL and the cells were incubated at 37°C for 4h, then added and quantified the DMSO.

Flow cytometry analysis

Cells of 0.8mL (1.5×10^6) were plated in 35mm tissue culture plates and added 0.2mL of the lipid-ODN mixture. The cells were incubated for 5h at 37°C , and then 4mL of RPMI1640 medium with 100mL fetal bovine serum was added for 48h, then cells were harvested, and analyzed for cell-cycle distribution by a FACScan flow cytometer.

Assay for tumorigenicity in nude mice

HR8348 cells (1×10^7) treated with or without ODN were injected subcutaneously in 6-week-old nude mice (Swiss nu/nu). The animals were monitored for tumor formation every week.

RESULTS

Antiproliferative activity of AEGFR on HR8348 cell line

A short exposure of HR8348 cells (5h) to the oligonucleotides was followed by an additional 3-day growth in maintenance medium with 10% FCS. MTT assay showed that treatment of HR8348 cells with liposome encapsulated AEGFR resulted in a 82.5% reduction in proliferation as compared with untreated cells, whereas RC15 group resulted in a 12.6% reduction in proliferation compared with untreated cells (Table 1).

Cell cycle assay

The HR8348 cells treated with AEGFR displayed an increased percentage of cells in the G_1/G_0 phase and a decreased percentage of cells in the S phase (Table 2).

Decreased tumorigenicity in AEGFR-treated HR8348 cells

The AEGFR cells displayed a marked inhibition on tumorigenicity rate in nude mice as compared with control cells (Table 3).

Table 1 Inhibitory effects of liposome-ODN

Group	48h		72h	
	MTT value	Inhibition rate	MTT value	Inhibition rate
	($\bar{x} \pm s$)	(%)	($\bar{x} \pm s$)	(%)
Control	0.445 \pm 0.016		0.337 \pm 0.003	
Liposome-RC15	0.389 \pm 0.015	12.6	0.091 \pm 0.008	12.2
Liposome-AEGFR	0.078 \pm 0.022	82.5	0.079 \pm 0.005	76.6

Table 2 Cell cycle assay

Group	G_0/G_2 (%)	S(%)	G_2+M (%)	PI
Control	21.6	59.9	18.5	0.784
Liposome-RC15	29.5	54.9	15.6	0.705
Liposome-AEGFR	57.1	32.5	10.1	0.329

Table 3 Inhibition of subcutaneous HR8348 adenocarcinoma growth by ASODN

Group	Diameter of tumor (cm)	Rate of tumorigenicity (%)
Control	1.00 \pm 0.08	100(10/10)
Liposome-RC15	0.95 \pm 0.07	100(10/10)
Liposome-AEGFR	0.80	20(2/10)

DISCUSSION

Colorectal carcinomas generally show a poor response to conventional chemotherapeutics^[38]. Several growth factors are involved in the control of colon carcinoma cell proliferation. In particular, the epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha) and their receptor EGFR which are frequently overexpressed. EGF and TGF- α are structurally related peptides that stimulate DNA synthesis and cell growth. Both EGF and TGF- α recognize and compete the same cell membrane receptor (EGFR) through which they mediate their biological action. It has been recently demonstrated that autocrine secretion also exists in the human colon carcinoma tissue and which enables uncontrollable growth of the tumor cells. In the autocrine hypothesis^[39], the increased EGFR activity during tumorigenesis is attributed to autocrine stimulation by TGF- α . TGF- α produced by transformed cells acts on the cell surface EGFR to promote unrestrained cell proliferation. Being consistently amplified in human tumors of ectodermal origin, EGFR is shown to be an active factor in development and proliferation of neoplasia. Over-expression of EGFR can promote transformation of cells.

In this study, EGFR ASODN could inhibit the proliferation of human colorectal carcinoma cell lines. The antisense compound could also inhibit significantly the cell growth *in vivo* when compared to a scrambled oligonucleotide control. This inhibition is specific since the control oligonucleotide had no effect on cell proliferation and cell growth *in vivo*.

At this moment, we do not know the precise mechanism of inhibition. It could simply be that interfering with the initial step in the cell cycle or mRNA expression, disrupting the cascade of events leading to cell proliferation. The mechanism, however, could be much more complicated. The antisense compounds could decrease levels of cyclin-dependent kinases (CDK) which are essential to cell cycle progression. Treatment of human prostatic and colon carcinoma cells with EGFR antibody could decrease CDK levels resulting in G_1 arrest^[11,40]. Another possibility is that treatment with the EGFR antisense oligonucleotides induces apoptosis, a phenomenon observed in human colon carcinoma cells when the EGFR was blocked by its monoclonal antibody^[11,40].

Antisense oligodeoxyribonucleotides have

shown great efficacy in the selective inhibition of gene expression. In fact, antisense oligonucleotides directed against TGF- α , EGFR, are able to inhibit growth and transformation of several human carcinoma cell lines. These data suggest that the EGF-like growth factors and their receptors offer potential usefulness as targets for experimental therapy of human colon carcinoma. In our study, both cell growth and DNA synthesis of human colorectal carcinoma cell line HR8348 could be inhibited by EGFR ASODN, perhaps due to blockage of autocrine stimulation cycle of EGFR by its ligands. Cell cycle analysis indicated that with AEGFR-treatment, the proportion of cells in G₀/G₁ increased, and proliferation index (PI) was lower than that in the control group. This inhibition effect of cell proliferation by specifically repressing growth-receptor productions strongly indicated the presence of this autocrine hypothesis of tumor-cell growth.

Lipofectamine^[41] was cationic liposomes. Cationic liposomes represent synthetic genetic delivery systems that avoid the potential infectious complications of viral vectors. Due to its absence of cellular toxicity, convenience and high efficiency, liposome is used widely in gene transfection. It can provide a complex with a net positive charge that can associate with the negatively charged surface of the cell and it can be taken up by cells, and this method is useful for introducing large DNA molecules, oligonucleotides, and RNAs into mammalian cells. *In vivo* gene transfer with DNA-cationic liposome complexes has been proven to be safe to the host, low in cost and relatively easy in preparation, if can be used in the treatment of cancer.

This study demonstrates that it is possible to identify oligonucleotides which selectively inhibit the expression of EGFR by an antisense mechanism, provided that a careful search for optimal target sites on the mRNA is conducted and delivery of the oligonucleotides to cells in culture is facilitated by the use of cationic liposomes. These results warrant further assessment of the compounds in inhibition of cell growth *in vivo* and in their possible use as therapeutic agents in the treatment of human cancer.

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Infrequent *p53* gene mutation and expression of the cardia adenocarcinomas from a high-incidence area of Southwest China

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INTRODUCTION

Adenocarcinomas of the cardia are the lesions arising from the proximal stomach or within 3cm of the gastroesophageal junction^[1,2]. These cancers tended to be advanced at the time of presentation, usually with poor prognosis^[2]. In recent decade, the incidence of adenocarcinoma of gastric cardia and esophagus are increasing steadily, while there has been a decrease in the proportion of the cancers arising from the distal stomach^[3-8]. The biological and epidemiological features of the cardia adenocarcinoma were distinct from those arising from the distal stomach^[9,10]. The specific etiologic factors involved in the increasing incidence remained unresolved^[1,5-10].

Significantly higher levels of DNA aneuloidy^[11,12], *p53* protein expression^[1,12-17] and mutations^[1,18] have been reported in gastric cardia tumors, compared with tumors arising in the gastric antrum. *p53* expression may be used as a marker in the early detection of cardia adenocarcinoma^[2], mutations of *p53* gene at specific codons in human cancers may be an indication of specific exposure or genomic susceptibility^[14,15,19-22], and may serve as

a reporter gene reflecting exposure to specific carcinogen^[14,21-23]. The previous studies^[1,11-23] suggested that the mutation and functional inactivation of *p53* gene might play some important roles in the carcinogenesis of cardia adenocarcinomas etiologically.

In this study, the *p53* gene mutation spectrum and protein overexpression were investigated in the cardia adenocarcinoma from a high-incidence area of Southwest China, comparison of mutation spectra was made with those in cardia adenocarcinomas from other regions, some clues may be drawn to the etiology and carcinogenesis of cardia adenocarcinomas in the local area.

MATERIALS AND METHODS

Patients and samples

All 20 cases of cardia adenocarcinoma specimens were collected from patients surgically treated in Yanting County Cancer Hospital of Sichuan Province in Southwest China. Of all 20 patients, 17 (85%) were male, 3 (15%) were female, age ranged from 46 to 69 years (mean, 54.9 years). None had received any treatment before the operation; all patients were diagnosed as having cardia adenocarcinoma arising within the gastroesophageal junction^[2]. None was associated with Barrett's esophageal epithelium. All tissue specimens were routinely processed, formalin-fixed, and paraffin-embedded. Paraffin sections were stained with hematoxylin and eosin (HE) for histopathological examination, and were used for DNA isolation and detection of *p53* protein by immunohistochemical staining.

DNA isolation

The paraffin embedded blocks were sectioned at 10 μ m, attached to glass slides and used for genomic DNA isolation by means of Pinpoint Slide DNA Isolation System (Zymo Research, USA), according to the manufacturer's instructions. Briefly, a 1mm² area of cancer tissue with few other cells except cancer cells was localized on HE staining slides under microscopy in each case; the 10 μ m slides were de-paraffinized, rehydrated through graded alcohols and air dried. With reference to the HE staining slides of the same case, the PP solution was applied to the localized area of each case. After air dry for 30-45 min, the PP solution embedded tissues were peeled and removed from the glass slides, and added into the tubes

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containing 50 μ L extraction buffer and 5 μ L proteinase K solution, then incubated at 55°C for 4 hours, heated at 95°C for 10min. The isolated DNA was further purified with DNA binding buffer and mini-spin column and PP wash buffer supplied by the manufacturer.

PCR and asymmetric PCR amplification

To amplify the DNA segments of exons 5-8 of p53, 100ng-200ng aliquots of template DNA were added in a total volume of 25 μ L- PCR reaction mixture containing 1 \times PCR buffer, 0.2mM of each dNTP (PE Applied Biosystems), 1 μ M of each primer and 2.0 units of AmpliTaq Gold DNA polymerase (Perkin Elmer). Initial denaturation at 94 was performed for 9min, followed by 50 cycles of 30 sec at 94°C, 30 sec at 52°C or 58°C (depending on the primers), 45 sec at 72°C and a final extension of 7min at 72°C. The primers used in this study covered the portions of exons 5 - 8 of the human p53 gene as described before^[24].

Twenty microliters of PCR products were run on a 2.0% agarose gel for examining the specificity of PCR reaction and DNA recovery, the specific bands were cut, recovered by Sprec-DNA Recovery Filter Tubes (Takara Biomedical, Japan), and purified according to the manufacturer's instruction. The recovered DNA fragments were used as templates for asymmetric PCR amplification. The two single-stranded DNA fragment of each exon were amplified with asymmetric PCR by using only one primer, respectively, except of the doubled primer, the conditions of asymmetric PCR were the same as those of the first PCR amplification.

DNA sequencing

Asymmetric PCR products were purified by alcohol precipitation, one fourth of purified product was used for DNA sequencing. DNA sequencing was performed by dideoxynucleotide method using the Thermo Sequenase cycle sequencing kit (Amersham, Life Science) and γ^{32} P-ATP (Amersham, Pharmacia Biotech). The sequence ladder was resolved in 7.0M urea and 6% polyacrylamide gel. After electrophoresis, the gel was dried in the Bio-Rad gel dryer before exposure to X-ray film for 6-12 hours at -70°C. When base changes were identified repeated sequencing of the same or complementary DNA strand was performed for confirmation^[19].

Immunohistochemical analysis of p53 proteins

P53 protein was detected immunohistochemically with the avidin-biotin-peroxidase method^[25]. Briefly, tissue sections were de-paraffinized and rehydrated through graded alcohols. Antigen retrieval was performed by microwave oven heating in 0.1mM citrate buffer (pH 6). Then, endogenous peroxidase activity was blocked with 3% H₂O₂, and after treatment with normal serum, the sections were incubated with CM1 polyclonal rabbit p53 antibody (Novocastra, Newcastle upon Tyne, UK) at

a dilution of 1:500, overnight at 4°C, with biotylated second antibody 20min, and with avidin-biotin-peroxidase complex 20min at room temperature. Subsequently, the sections were subjected to color reaction with 0.02% 3, 3 diaminobenzidine tetrahydrochloride containing 0.005% H₂O₂ in PBS (pH 7.4), and were counterstained with hematoxylin lightly. In each staining run, a known p53 positive sample of colon cancer was added as a positive control, and a section of the same colon cancer tissue incubated in PBS instead of CM1 was included as a negative control.

Approximate percentage of p53 positive tumor cell nuclei in carcinoma were assessed and scored as follows: negative (-), <10% (+), 10% - 50% (++) , >50% (+++); The intensity of staining in p53 positive cell nuclei was compared with the negative control and scored as follows: negative (-), weak (+), moderate (++) , strong (+++)^[25].

RESULTS

p53 mutations in cardia adenocarcinoma

In all 20 cases of cardia adenocarcinomas, only 2 mutations (10%, 2/20) of p53 gene were detected in 2 cases. There were ATC-ACC, T to C transition in one occurred at codon 195 in exon 6 and CGC-AGC, C to A transversion in another at codon 283 in exon 8. Two were missense mutations. A representative mutation is shown in Figure 1.

p53 overexpression in cardia adenocarcinomas

All immunostainings for p53 protein were confined to the cell nuclei (Figure 2), the positive cell number varied from 10% to more than 50%; The intensity of staining ranged from moderate to strong. Seven out of 20 (35%, 7/20) were detected with p53 protein overexpression.

In addition, in 2 cases with p53 gene mutations, one was p53 protein-positive (96 - 236, codon 195: ATC-ACC); one was negative for p53 protein (96-237, codon 283:CGC-AGC). All paracancerous normal tissues were p53-negative. In 5 cancers with lymph node metastasis, 2 were p53-positive of both cancer and lymph node, and 3 were p53-negative in both tissues. The positive cases with p53 gene mutations and/or p53 protein overexpression are summarized in Table 1.

Table 1 The positive cases with p53 gene mutation and/or p53 protein overexpression in cardia adenocarcinomas

Patient No.	Exon	Codon	Nucleotide change	Amino acid change	p53protein expression
96-114	-	-	-	-	+++
96-123	-	-	-	-	+++
96-166	-	-	-	-	++
96-185	-	-	-	-	++
96-236	6	195	ATC-ACC	Ile-Thr	+++
96-237	8	283	CGC-AGC	Arg-Ser	-
96-270	-	-	-	-	++
96-432	-	-	-	-	++

The negative cases without p53 gene mutations and protein expression were not listed.

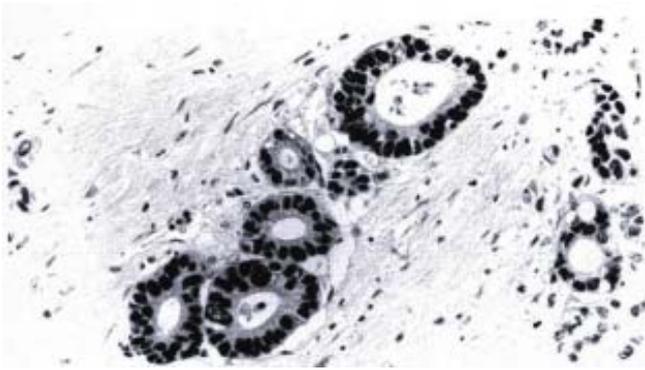


Figure 1 Results of sequence analysis of the p53 gene in the cardia adenocarcinomas from a high incidence area of Southwest China, case 96-236 showed a ATC-to ACC mutation at codon 195 of exon 6.

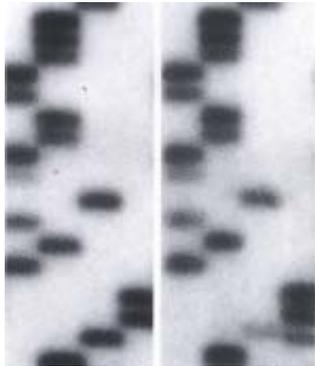


Figure 2 Immunoreactivity of p53 protein of cardia adenocarcinoma, p53 protein was located in the nuclei of cancer cells, most cancer cells were positive for p53 protein. ABC $\times 20$

DISCUSSION

The present study investigated the p53 gene mutation and protein expression in a series of 20 cases of cardia adenocarcinomas from a high incidence area of Southwest China, the rates of p53 mutation and protein expression were lower than those reported previously.

There were less reports of p53 mutation and protein expression in cardia adenocarcinomas than those in other cancers (<http://www.irac.fr/p53/homepage.htm>)^[26], such as, hepatocellular carcinoma^[27-29], esophageal cancer and gastric cancer^[14,20-22]. Li *et al*^[30] detected p53 mutations in 3 out of 4 cardia adenocarcinomas (3/4), 6 mutations were identified in exons 5-8 of p53 gene in 6 out of 14 cardia adenocarcinomas (6/14, 42.9%) from Linxian, China, 3 were missense mutations, and 3 were non-sense mutations^[18]. Recently, a high p53 mutation rate (63%, 26/41) in cardia adenocarcinomas from North Ireland was reported^[1]. As for the p53 protein expression, the positive rates reported in cardia adenocarcinoma were 59% (24/41)^[1], 44% (11/25)^[2], 56% (20/36)^[12], and 76.5% (13/17)^[13], respectively. These results suggested that p53 mutation and expression played an important role in carcinogenesis of cardia adenocarcinoma, and p53 gene mutations reflected exposures to specific carcinogens in the

environment^[14,19,21-23].

Conversely, in our series, only 2 cases of p53 gene mutation (10%) and 7 cases of p53 protein expression (35%) were detected in 20 cardia adenocarcinomas from a high incidence area of China. It had been proposed that the comparison of p53 gene mutation spectra in tumors of different origin might reveal similarities or differences concerning the endogenous and exogenous molecular processes contributing to tumor development^[24], and might reflect site-specific difference (cardia or antrum) or regional exposure to particular environmental agents^[31]. The results in this study suggested that p53 gene mutation might play some role in some subset of cardia adenocarcinomas, but, it is not the major causation in carcinogenesis of gastric cardia in local area, or the risk factors in the local environment are different from those in other regions.

To the best of our knowledge^[1,18,26,30], the two mutations detected in the present study were rare in cardia adenocarcinomas. Mutation of p53 gene at codon 195 from ATC to ACC was reported in ovarian cancer^[32], brain tumors^[33], lung cancers^[34,35], and adenocarcinoma of Barrett's esophagus^[36], etc.^[26]; Mutation at codon 283 was of polymorphism in previous studies: It was from CGC to TGC in hepatocellular carcinoma^[37] and colorectal cancers^[38], CGC to CTC in ovarian tumor^[39], CGC to GGC in lung cancer^[40,41], etc.^[26]. But, in this series, the mutation at codon 283 was from CGC to AGC, C:G-A:T transversion, no such a mutation was reported previously. It was further suggested that the environmental risk factors and their mechanism of actions in local area were different from those in other regions^[1,18,30].

The discordance between p53 protein expression and p53 gene mutation was noted in many other studies^[1,25,35], one case with p53 gene mutation was negative for p53 protein immunohistochemical staining in this study, since the frameshifts or non-sense mutations in the coding sequences of the gene might result in truncated or unstable forms, even non-expression^[25]. No mutations in exons 5-8 were detected in other 6 cases with positive p53 protein, the possibilities to account for this apparent discordance might be: Firstly, the missense mutation lay in the region of the gene not screened in this study. Although p53 gene mutations occurred frequently in the so-called 'hot-spot' region of exons 5-8^[35], a review of 50 studies that carried out sequencing of the entire coding region of p53 gene reported that 13% up to 30% of missense mutations were located outside exons 5-8^[23]; Another possibility was that the mechanism other than mutations resulted in inactivation and stabilization of p53 protein. Binding to viral oncoproteins, e.g. HPV E6, and cellular oncoproteins, e.g. mdm2, had been shown to result in p53 stabilization and in the inactivation

of wild-type function^[41,42]. Our previous studies indicated that there was a high prevalence of HPV16 of esophageal cancer patients and health population in this high incidence area^[43,44]. In these instances, p53 overexpression represented the functional, but not the structural, inactivation of p53 gene^[1].

We conducted a preliminary investigation of p53 gene mutation and p53 protein expression in 20 cases of cardia adenocarcinomas from a high incidence area in Southwest China, to elucidate the etiology and carcinogenesis of cardia adenocarcinoma in the local area. The low p53 gene mutation and expression rates, and different mutation spectrum suggested that p53 gene mutation and functional inactivation might play some roles in the subset of cardia adenocarcinomas in this high incidence area. Besides p53 gene abnormalities, there might be some other environmental risk factors and other genetic abnormalities contributing to the high incidence of cardia adenocarcinomas in this area, which deserves further investigation.

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Gene expression profiles in gastric mucosa of sleep deprivation rats

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Subject headings gene expression; mucosa, gastric; stress ulcer; GI tract; sleep deprivation; cDNA; ethanol

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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, night shift workers and medical professionals. It imposes stress on the body, and produces a variety of health problems^[10-14]. Sleep deprivation may affect the epithelium linings of the gastrointestinal tract, because stress been demonstrated to produce gastric mucosal lesions in rats^[15,16]. Although various factors has been proposed to account for this process, the precise mechanism of how sleep deprivation to affect the gastric mucosa barrier, especially at the molecular level, still remains unclear. In this project we observed the effect of sleep deprivation on the defensive factors of gastric mucosa, and used cDNA expression arrays to identify genes expressed abnormally in gastric mucosa of sleep deprivation rats.

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°C + 1°C) and humidity (65% -

70%) controlled room with a day night cycle of 12 hours. The rats were given standard laboratory diet (Ralston Purina Co. Chicago, IL) and tap water *ad libitum*. Rats were starved for 24 hours and water withdrawn 1 hours prior to any oral or intragastric administration of agents in order to obtain an 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 5min intervals and was programmed to switch off for 1h every day at 1:00 p.m. to allow for an hour of undisturbed sleep. Sleep disturbance was continued for 1wk before the animals were killed and the organ weight was determined. Daily water and food consumption as well as the body weight was recorded throughout the whole experimental period.

Ethanol-induced gastric mucosal damage

Rats were starved for 24h before 1mL of 500mL/L ethanol was administered orally to induce acute gastric mucosal damage^[17]. 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 (mm²) was traced onto a glass plate and subsequently measured on a graph paper with 1mm² divisions. The total lesion lengths divided by the number of rats in each group was expressed as the mean ulcer index^[18].

Cold-restraint-induced gastric mucosal damage

Rats were put inside the close-fitting tubular wire-mesh cages and restrained inside a cold room for 2h. At the end of the experimental period they were killed and stomachs prepared for ulcer measurement as described previously^[18].

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

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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 buffered formalin for 24h. It was cut into sections of 5 μ m and then used in histological examination. 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°C until assayed.

cDNA expression arrays^[19-24]

Total RNA for cDNA expression arrays was isolated from the gastric mucosa of rats by using the Atlas™ Pure Total RNA Isolation Kit (CLONTECH Laboratories, Inc. CA. Cat.#: K1038-1). cDNA was synthesized and radioactive labeled using 5 μ g total RNA from sleep deprivation rat and normal control rat according to standardized protocols (CLONTECH Laboratories, Inc. CA. PT3140-1). Atlas™ cDNA Expression Arrays (CLONTECH Laboratories, Inc. CA.) was used for differential expression screening. Each Atlas Array includes 588 of cDNA spotted in duplicate on a positively charged nylon membrane. Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several house keeping cDNA as positive controls for normalizing mRNA abundance. After a high-stringency wash, the membranes were exposed to X-ray film (Kodak BioMax MS film Cat.# 118 8077) at -70°C with an intensifying screen for 3d. The gene expression pattern of 588 genes in gastric mucosa of normal and sleep deprivation rats was analyzed and compared on a computerized densitometer. Signals that genes were absent or present on one of the two membranes were identified visually.

Statistics

The data were statistically analyzed with the unpaired two-tailed Student's *t* test.

RESULTS

Effect of sleep deprivation on body weight

Sleep disturbed rats had the same water and food consumption when compared with the control however there was a slower increase in the percentage (%) of body weight among the sleep disturbed animals (Table 1). The decrease in % body weight gain was observed as early as 2d after sleep disturbance, and after that they gain in weight; although slower; but in a parallel fashion to that of the control (Table 1, all values at $P < 0.05$). Sleep disturbance induced a significant increase in adrenal weight (240 μ g/g \pm 8 μ g/g body weight) when compared with the control (215 μ g/g \pm 6 μ g/g body weight). There was no difference in weights for the thymus and spleen between the two groups.

Effect of sleep deprivation on cold-restraint stress induced gastric ulceration

After rats were restrained in 4°C for 2h, the ulcer index in gastric mucosa of sleep deprivation rats (41.7mm² \pm 8.3mm²) was significantly higher ($P < 0.01$) than it in control rats (Figure 1). The results indicated that the sleep disturbance aggravated cold-restraint stress induced gastric ulceration.

Effect of sleep deprivation on ethanol induced gastric ulceration

After the 500mL/L ethanol challenge, the ulcer area found in the rats with 7d sleep deprivation (19.15mm² \pm 4.2mm²) was significantly lower ($P < 0.01$) than the corresponding control (53.7mm² \pm 8.13mm²), as shown in Figure 2.

cDNA expression array

Figure 3 shows the result obtained by hybridizing two cDNA array membranes with radioactive-labeled cDNA from gastric mucosa of control normal rat and from gastric mucosa of 7d sleep deprivation rat. More than 10 differentially expressed genes were found in total 588 genes, most of them were digestive enzyme related genes, one of the overexpression gene in the gastric mucosa of sleep deprivation rat was identical to that of inducible heat shock protein 70.

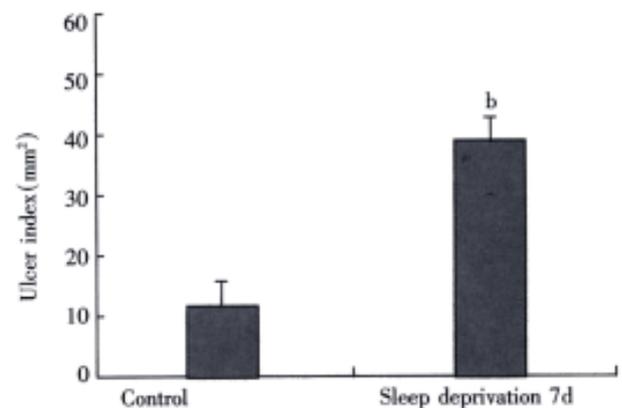


Figure 1 Effect of sleep disturbance on cold-restraint stress induced (4°C for 2h) gastric ulceration in rats. Values are means \pm SEM of 12 rats in each group; ^b $P < 0.01$, vs control group.

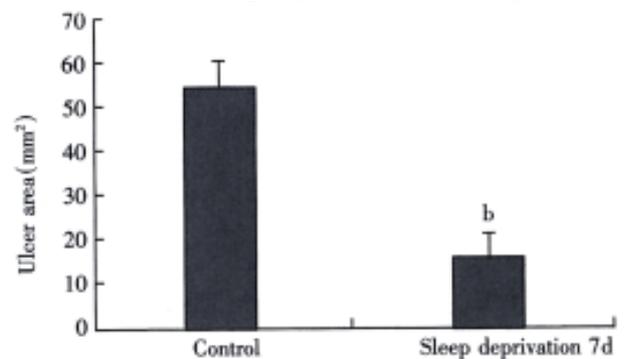


Figure 2 Effect of sleep deprivation on ethanol induced (500mL/L ethanol 1mL p.o. for 2h) gastric ulceration in rats. Error bars represent SEM, $n = 10$ for each group. ^b $P < 0.01$, vs control group.

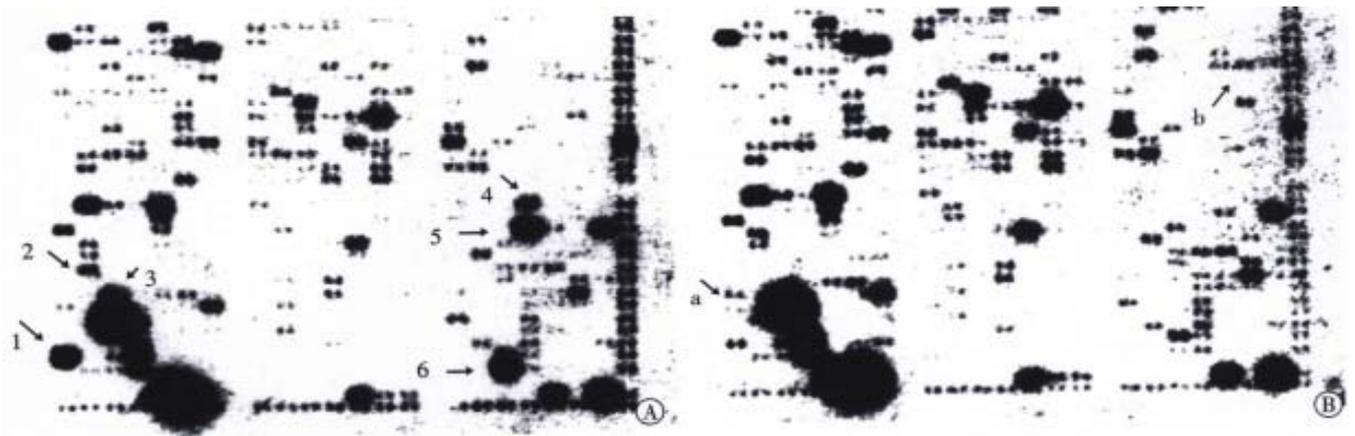


Figure 3 cDNA array: Differential gene expression in gastric mucosa of normal control rat (A) and sleep deprivation rat (B). Sleep deprivation may decrease the following genes expression (Arrow 1-6) 1: bile-salt-stimulated lipase; 2: pancreatic lipase related protein 2 precursor; 3: triacylglycerol lipase precursor; 4: elastase 2 precursor; 5: trypsinogen II; 6: chymotrypsinogen B precursor, and increase the following genes expression (Arrow a-c) a: low-density lipoprotein receptor precursor; b: glucose transporter type 1; c: transferrin receptor protein. The dot for over expressed inducible heat shock protein 70 gene is circled.

Table 1 Effect of sleep disturbance on the percentage gain in body weight

Groups	Percentage gain in body weight						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	7.2±2.6	14.4±2.2	18.3±2.4	23.9±2.2	28.5±3.1	33.1±3.6	37.7±3.2
Sleep disturbed	4.8±2.5	7.7±2.3 ^a	10.5±2.3 ^a	16.2±2.5 ^a	19.9±2.4 ^a	24.6±2.7 ^a	27.2±3.1 ^a

Values are means±SEM of 12 rats in each group; ^aP<0.05, vs control group.

DISCUSSION

In this experiment a rotating drum was used to produce sleep disturbance in rats and it was found to be an effective model for stress induction. Sleep disturbed rats had a smaller percentage gain in body weight and this was observed 2d after sleep disturbance. This suppressive effect was not intensified after d2 and the sleep deprived rats gained in weight in a fashion paralleled to that of the controls. The statistical differences between all values of percent age gain in body weight were maintained at $P<0.05$ level (Table 1). The slowing of weight gain was not due to a reduction in food intake as there was no difference in food and water consumption between the two groups. This may be the result of an increase in catabolic process that was generally observed in sleep deprived animals^[25-27].

It has been found that sleep disturbance aggravated cold-restraint stress induced gastric ulceration (Figure 1). The aggravation may be the result of lowered effectiveness of the mucosal barrier. Cold-restraint stress has been shown to produce blood stagnation in the gastric mucosa^[25], and sleep disturbance imposed psychological stress on the rats as was demonstrated by an increase in the adrenal weight. The increase in adrenal weight could be due to the activation of the hypothalamus-pituitary-adrenal axis producing an overstimulation on the adrenal glands^[28-31]. All these factors may

lead to the decrease in basal gastric mucosal blood flow and affect the defensive function of gastric mucosa. Results of this experiment indicated that the sleep deprivation did not aggravate the 500mL/L ethanol induced gastric ulceration as expected, on the contrary, it did show some protective effect for ethanol challenge.

The human genome project's large-scale sequencing efforts have generated partial sequence data for thousands of genes. Although many of these genes have been assigned to functional classes, the roles they play in various biological processes have yet to be elucidated. An important step toward understanding these roles is defining gene expression profiles, i.e. comparing patterns of expression in different tissues and developmental stages, in normal and disease states, or in distinct *in vitro* cell conditions. This can be accomplished using RT-PCR, RNase protection assays, Northern blot analysis, *in situ* hybridization, immunohistochemistry or Western blotting^[32-44], but these methods focus on only a few genes at a time. A more promising approach for analyzing multiple genes simultaneously is the hybridization of entire cDNA population to nucleic acid arrays. This technology has a wide range of application, including investigating normal biological and disease processes, profiling differential gene expression, and discovering potential therapeutic and diagnostic drug targets^[19-24]. In this experiment, for further

investigation the reason why sleep deprivation protect gastric mucosa from ethanol insult, 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 (Figure 3), most of these were digestive enzyme related genes, one of the overexpression gene was inducible heat shock protein 70 gene. 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^[45-47]. Substantial evidence showed that heat shock is capable of protecting cells, tissues, organs, and animals from a subsequent, normally lethal heating, as well as from other types of noxious condition^[48]. 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^[49-51]. The overexpression of heat shock protein 70 was reported to protect guinea pig gastric mucosal cells from ethanol damage^[52]. In conclusion, sleep disturbance imposed psychological stress on the rats as was demonstrated by decreasing body weight gain and increasing in the adrenal weight. Defensive function of the gastric mucosa was weakened by sleep deprivation thus predisposing it to ulcer formation induced by cold-restraint stress. On other hand, sleep deprivation decreased ethanol induced mucosa damage. This protective effect may be mediated by over expression of inducible heat shock protein 70 in gastric mucosa. Our experiment also showed that the cDNA arrays are the powerful approach to rapidly identify the gene expression profiles.

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Seroepidemiology of *Helicobacter pylori* infection among asymptomatic Chinese children

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Subject headings *Helicobacter* infection/infancy and children; *Helicobacter pylori*; seroepidemiology; gastritis; stomach neoplasms; gastric mucosa; family

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INTRODUCTION

Increasing data has demonstrated that *Helicobacter pylori* (*H. pylori*), a spiral gram negative bacterium, colonized in human stomach, can cause type B gastritis^[1-3], is strongly associated with gastric and duodenal ulceration^[4-6], and has been implicated in the causation of gastric carcinoma^[7-9] and mucosa-associated lymphoid tissue (MALT) lymphomas^[10-11]. It has been reported that there is relationship between *Helicobacter pylori* infection and children's gastroenterologic disease, the most convincing evidence of the role of *H. pylori* in peptic ulcer disease is provided by studies showing that recurrence rate of ulcer is markedly reduced by eradication of the bacteria from the gastric antrum^[12-15].

Recent partly retrospective epidemiologic study in adults suggested that *H. pylori* infection mainly occurred during childhood^[16-18], and its actual colonization in gastric mucosa would be determined by many factors during childhood^[19-21]. In the previous studies, we have demonstrated that the prevalence of *H. pylori* infection of children with gastrointestinal disease is high^[22]. Therefore, children are considered to be the highest risk population.

This study was undertaken to determine the prevalence of *H. pylori* infection, among asymptomatic Chinese children, and its relation with the socioeconomic status of their families.

MATERIAL AND METHOD

Study subjects

Of 1119 children aged 7 - 14 years from three primary schools (one public school in rural area, one private school and another public school in urban area) in Shanghai, China, 568 are male and 551 are female.

Sample collection and methods

A questionnaire was given to the parents of all children. The questionnaire gathered sociodemographic information, parent occupation and life habit including feeding habit for children, and information on household density (number of adults, children and rooms, not including bathrooms, toilets, and small kitchens).

A serum sample (1.5mL) was obtained from each subject and stored at -20°C and assayed in batches for *H. pylori* IgG antibodies using a commercial enzyme linked immunoadsorbent assay (ELISA), Helico Bio-Rad GAP IgG kit. Using these criteria, the sensitivity of the ELISA is 100% with specificity of 92%.

Statistical analysis

Data were analyzed using the statistical package of SAS. The prevalence of *H. pylori* infection in different age was assessed using the χ^2 test. Differences with *P* values lower than 0.05 were considered statistically significant.

RESULTS

H. pylori seroprevalence in asymptomatic children

Questionnaires were returned from 1119 subjects, aged 7-14 years. Serum *H. pylori*-IgG antibodies of 1119 children were measured by ELISA method and showed that 458 children were *H. pylori* seropositive (40.93%). Two hundred and fifty-eight of 568 boys were positive (45.42%) and 200 of 551 girls were positive (36.38%). Seroprevalence of *H. pylori* for boys was significantly higher than that of girls (*P*=0.002).

The age and seroprevalence rates of *H. pylori* infection are summarized in Table 1. From Table 1 we can see that seroprevalence of *H. pylori* increased significantly with age, the mean increasing rate being 3.55% per year from 7-11 years of age.

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Table 1 Prevalence of *H. pylori* infection of children aged 7-12 years

Age (year)	No. of samples	No. of positive samples	Rate(%)
7	165	51	30.91
8	146	51	34.93
9	167	65	38.92
10	167	77	46.11
11	226	110	48.67
12	167	79	47.30
Total	1119	458	40.93

***H. pylori* prevalence in rural and urban area**

One hundred and seventy-one of 543 children living in urban area, and 287 of 576 living in rural area were positive. Seroprevalence rate with reference to age and distribution (rural and urban) are shown in Table 2. The overall prevalence in rural area was significantly higher than that in urban area.

***H. pylori* prevalence in different families**

Prevalence of *H. pylori* in children lived in different families is shown in Tables 3 and 4. These results showed that *H. pylori* seropositivity rate was significantly higher in children whose parents were engaged in manual occupations than in children whose parents with non-manual occupations.

Table 2 Comparison of community distribution in asymptomatic children with *H. pylori* infectionV

Age (year)	Urban			Rural			P value
	No. of samples	No. of positive samples	%	No. of samples	No. of positive samples	%	
7	88	21	23.86	77	30	38.95	0.036
8	69	11	15.94	77	40	51.95	0.001
9	90	33	36.67	77	32	41.56	0.518
10	83	32	38.55	84	45	53.57	0.052
11	92	40	43.48	134	70	52.54	0.195
12	40	9	22.50	127	70	55.12	0.001
13	39	12	30.77				
14	42	13	30.95				
Total	543	171	31.95	576	287	49.85	0.001

Notice: Children aged 7-11 years are from public schools and of 12-14 years are from private schools.

Table 3 Prevalence of *H. pylori* of children lived in different families

Group	No. of samples	No. of positive samples	Prevalence (%)
Peasant	41	18	43.90
Worker (including peasant worker)	580	278	47.93
Intellectual	452	148	32.74 ^a
Other	46	14	30.43 ^a

^aP<0.005 intellectual and other families vs worker's and peasant's families.

Table 4 *H. pylori* infection rate of children aged 7-14 years in different families

Age	Peasant		Worker		Intellectual		Other	
	n	No. of positive (%)	n	No. of positive (%)	n	No. of positive (%)	n	No. of positive (%)
7	9	3 (33.33)	83	29 (34.94)	64	17 (26.56)	9	2 (22.22)
8	8	4 (50.00)	85	39 (45.83)	44	7 (15.91)	9	1 (11.11)
9	6	3 (50.00)	95	42 (44.21)	59	19 (32.20)	7	1 (14.29)
10	6	3 (50.00)	89	46 (46.46)	56	26 (46.43)	6	2 (33.33)
11	6	2 (33.33)	121	62 (51.24)	93	40 (43.01)	6	6 (100.00)
12	6	3 (50.00)	95	59 (62.11)	58	15 (25.86)	8	2 (25.00)
13	0	0 (0.00)	2	1 (50.00)	36	11 (30.56)	1	0 (0.00)
14	0	0 (0.00)	0	0 (0.00)	42	13 (30.95)	0	0 (0.00)

DISCUSSION

The epidemiology of *H. pylori* infection in human is interesting, particularly in regard to gastroduodenal disease associated with gastric colonization by the organism. But most epidemiologic studies of *H. pylori* infection have been performed in adults^[7,18]. Data on the incidence of *H. pylori* infection in children are limited^[23-26]. These factors such as age, socioeconomic level and living conditions, especially during childhood, have been considered to be important determinants in the acquisition of the microorganism. In developed countries the prevalence of *H. pylori* infection among children is low, in contrast to what is observed in developing countries, where most children are infected by the age of ten. In seroprevalence studies from western

Australia^[27] and Canada^[28], *H. pylori* was predominantly acquired in childhood. In addition, individuals born in an earlier birth year had a higher risk of acquiring *H. pylori* than those born in a later one. In a study from Toronto *H. pylori*-specific IgG serum antibodies were identified in 74% of parents and 82% of siblings of children with culture-proved infection^[29]. In the same study, seroprevalence in the control group was 24% and 13% for parents and children, respectively. Clustering of *H. pylori* infection among families suggested that acquisition of the infection in childhood might be due to the transmission from person to person.

The present study has shown that *H. pylori* infection rate of Chinese asymptomatic children is high. The mean infection rate of *H. pylori* of 1119

subjects aged 7-14 years is 40.93% and 45.42% among boys and 36.38% among girls. Infection rate in boy is significantly higher than that in girls ($P=0.002$). This result is different from the data of small sample that we reported previously (no difference in sex). Our results showed that seroprevalence of *H. pylori* increased significantly with age in asymptomatic children, *H. pylori* infection rate is 30.90% in children aged 7 years; 34.93%, aged 8 years; 38.92%, aged 9 years; 46.11%, aged 10 years; 48.67% aged 11 years; and 47.30% aged 12 years. The prevalence rates were similar to that reported by Pelsler^[30].

Our results also showed that *H. pylori* infection of Chinese children is influenced by many factors such as socioeconomic status, hygienic condition, cultural level, life habit particularly non-separate feeding, etc. The prevalence of *H. pylori* in rural area is higher than that in urban area (49.83% vs 31.48%, $P=0.001$). There is an inverse relationship with the socioeconomic status of the family. *H. pylori* infection rate of children is not the same in different families. It was 47.93% in worker's family, 43.90% in peasant's family, 32.74% in intellectual family, and 30.43% in other families with higher income. Therefore *H. pylori* prevalence of children from non-manual families is lower than that of children from manual families (30.42% - 32.74% vs 43.90% - 47.93%, $P<0.005$). We also found that there is an association between high prevalence and household density, close personal contact, particularly common use of the same bowl. These may provide the opportunity for transmission of infection from parents to children.

CONCLUSION

H. pylori prevalence among Chinese children is high and it increases with age. It has an inverse relationship with the socioeconomic status of the children's family.

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Epidermal growth factor prevents gut atrophy and maintains intestinal integrity in rats with acute pancreatitis

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INTRODUCTION

There is abundant evidence that stressful insults such as acute pancreatitis may significantly alter the metabolism of the gut mucosa and therefore its barrier integrity, resulting in an increase in mucosal permeability and subsequent trans location of enteric bacteria and their endotoxins^[1-9]. The fact that most bacteria associated with acute pancreatic and peripancreatic infections are of enteric origin implies that the gut plays a major role in the pathogenesis of pancreatic infection^[10-16]. Thus various therapeutic modalities have been undertaken to maintain gut mucosal metabolism and function as well as to reduce the bacterial translocation during acute pancreatitis.

In recent years, much attention has been focused on hormonal regulation as one of the effective therapeutic strategies. Epidermal growth factor (EGF), which presents in large amount in the salivary and Brunner's gland, and in a variety of secretions including saliva and milk, is a potent mitogen for small intestinal cells both *in vivo* and *in vivo*. Parenteral nutrition with administration of exogenous EGF has been shown to increase DNA and protein content in the small intestine^[17-20]. EGF can also regulate intestinal brush border enzymes functionally^[21,22].

The aim of this study is to evaluate the protective effects of EGF on intestinal barrier function in rats with acute pancreatitis under total

parenteral nutrition (TPN).

MATERIALS AND METHODS

Materials

Forty-one male Sprague Dawley rats, each weighing approximately 210g, were purchased from the Experimental Animals Center of Fourth Military Medical University. The rats were given water *ad libitum* and a standard rat food diet. They were subjected to alternate 12h periods of darkness and light. After overnight fasting, the rats underwent placement of a central venous catheter through a right external jugular vein under sodium pentobarbital anesthesia (40mg·kg⁻¹, intraperitoneally). The central venous catheters were tunneled subcutaneously and attached to a spring coil/brass swivel mechanism, which allowed for free movement of the animals in cages. Acute pancreatitis was induced by intraductal infusion of 35g·L⁻¹ sodium taurocholate solution (1.0mL·kg⁻¹) after clamping the proximal end of the common bile duct and puncture through the duodenum into the biliary-pancreatic duct^[23-27]. On the day of cannulation (d0), rats were randomly divided into one of the two groups. The control group (n=21) was fed a conventional parenteral nutrition solution; the EGF group (n=20) was fed besides the identical parenteral nutrition formula as in the control group, EGF (0.1mg·kg⁻¹) was injected subcutaneously twice daily.

The TPN solutions were prepared in a laminar flow hood and were sterilized by membrane filtration. The composition of these solutions is shown in Table 1^[28].

Methods

On d1 and d5 after induced acute pancreatitis, every 8 animals in each group were anesthetized with 40mg·kg⁻¹ sodium pentobarbital respectively. A midline abdominal incision was made and a 60cm length of small intestine, 20cm distal from the ligament of Treitz, was ligated at both ends. Then, 1.0mL fluorescein isothiocyanate (FITC)-dextran 4000 (25g·L⁻¹) solution was injected into the lumen of this ligated segment. A blood sample was withdrawn from the superior mesenteric vein 30min later for the analysis of plasma FITC-dextran with a fluorescence spectrophotometer at an excitation wave length of 480nm, an emission wave length of

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530nm, and expressed as mg of FITC-dextran per L of plasma^[29-31].

For histologic evaluation, 2cm of proximal jejunal segment was fixed in 100mL·L⁻¹(V·V⁻¹) formalin, embedded in paraffin, and stained with hematoxylin-eosin. Three paraffin sections were prepared from each fixed tissue sample, and each slide was analyzed. Villus height and area were measured in 10 well orientated villi, giving a total of 30 villis for each jejunal segment. Measurements were made in a blind fashion on coded slides, and mean values were obtained^[32,33].

Samples of jejunal mucosa were scraped and used for the measurement of mass and enzyme activity. The activities of sucrase and maltase were determined by the method of Dahlqvist^[34]. Myeloperoxidase (MPO) activity was determined by the method of Bradley *et al*^[35]. The protein content in each sample was estimated according to the method of Read *et al*^[36].

Statistical analysis

Data were expressed as $\bar{x} \pm s$ as indicated in each table. The significance of any difference between the two groups was determined with the Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Mortality rate

During the 5d of TPN after induced acute pancreatitis, the mortality rate was similar in the two groups. Specifically, 4.8% (1/21), 23.8% (4/21) in the control group and 5.0% (1/20), 20.0% (3/20) in EGF group on d1 and d5 respectively. This did not reach statistical significance.

Changes in body mass

The initial body mass in the two groups was similar (213g±8g in the EGF group vs 210g±6g in the control group) and there was no significant change on d1 (214g±9g in the EGF group vs 211g±6g in the control group). But the final body mass gain was significantly greater in the EGF group than in the control group on d5 (15g±2g in the EGF group vs 4g±1g in the control group, $P < 0.01$).

Changes in mucosal wet mass, villus height and area

Mucosal wet mass, villus height and area in the control group decreased significantly as compared with the EGF group on d5 ($P < 0.01$, Table 2).

Changes in intestinal permeability

Plasma FITC-dextran level in the control group increased significantly as compared with the EGF group on d5 ($P < 0.01$, Table 3).

Changes in activities of sucrase, maltase and MPO

Activities of sucrase and maltase in the control group decreased significantly as compared with those in the EGF group on d5 ($P < 0.05$, $P < 0.01$, respectively). However, MPO activity in the control group increased significantly as compared with that in the EGF group on d5 ($P < 0.01$, Table 4).

Table 1 Composition of TPN solution (mL)

Composition	Volume
50% glucose	40
7% vamin*	16
20% intralipid*	11
Addamel*	0.3
Soluvit N*	0.3
Vitalipid N adult*	0.3
Heparin 60 U	

*Products from Kabi Vitrum, Sweden.

Table 2 Changes in mucosal wet mass, villus height and area ($\bar{x} \pm s$, $n=8$)

t/d	Group	m (wet mucosa) /mg·cm ⁻¹	h(villus) /μm ²	a (villus) /μm
1	Control	35±3	385±32	40872±5194
	EGF	35±4	394±37	41328±4901
5	Control	30±2	272±21	18658±2469
	EGF	44±3 ^b	409±32 ^b	43227±5340 ^b

^b $P < 0.01$, vs control group at same time.

Table 3 Changes in plasma FITC-dextran ($\bar{x} \pm s$, $n=8$, mg·L⁻¹)

t/d	Group	FITC-dextran
1	Control	1.2±0.5
	EGF	1.1±0.4
5	Control	7.5±0.7
	EGF	3.3±0.7 ^b

^b $P < 0.01$, vs control group at same time.

Table 4 Changes in activities of MPO, sucrase and maltase ($\bar{x} \pm s$, $n=8$)

t/d	Group	z/m (MPO) /nkat·g ⁻¹	z/m (sucrase) /nkat·g ⁻¹	z/m (maltase) /nkat·g ⁻¹
1	Control	143.34±111.67	0.54±0.11	1.75±0.32
	EGF	231.19±8.34	0.49±0.02	1.82±0.21
5	Control	96.68±13.35	0.19±0.05	0.84±0.16
	EGF	66.71±13.20 ^b	0.28±0.08 ^a	1.23±0.24 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control group at same time.

DISCUSSION

The effects of EGF on intestinal integrity were investigated in an experimental acute pancreatitis model in rats. TPN was used in both groups to mimic the clinical setting in as much as acute pancreatitis patients are often nourished by TPN. Acute pancreatitis can lead to ischemic damage of intestinal mucosa. Administration of TPN even to healthy experimental animals is associated with progressive intestinal atrophy, which is characterized by reduction of mucosal mass, villus

height and area, mucosal wall thickness, etc.^[37-41], so that combination of acute pancreatitis and TPN might lead to more damage to the gut mucosa than TPN or acute pancreatitis alone. EGF was selected because it has been suggested to be a potent mitogen for small intestinal cells both *in vitro* and *in vivo*^[42-44]. A previous study by our group also demonstrated that EGF could increase DNA and protein content in the small intestine^[45].

In this study, the body mass gain in the EGF group was significantly greater than in the control group on d5. This may be contributed to an anabolic effect of EGF^[44]. On d5, the significantly increased mucosal mass, villus height and area in jejunum were also found in the EGF group as compared with the control group. This is because that EGF can enhance intestinal glutamine influx and supply more energy for mucosal regeneration so as to attenuate in testinal atrophy. And it is also related to the increased mucosal protein and DN A content in small intestine^[46,47]. The results suggest that the administration of exogenous EGF may prevent intestinal atrophy in rats with acute pancreatitis under TPN.

For the assessment of barrier function of intestinal mucosa, a permeability test can be a suitable method. Pantzar *et al*^[31] suggested that nondegradable dextrans could be used as permeability markers and reflected the proteoly sis-independent passage of proteins through the small intestinal epithelia. Because there may be a paracellular route through the tight junctions for the markers with M_r below 30000 instead of a transcellular route as suggested for the larger molecules. In the present study, permeability of the small intestine to FITC-dextran 4000 (mean M_r , 4000), through the tight junctions of the intestinal epithelia, increased significantly in the control group as compared with the EGF group on d 5. The results indicate that EGF may prevent an increase in permeability of the small intestine to FITC-dextran 4000 in rats with acute pancreatitis under TPN.

Tissue damage can be caused either directly or indirectly by the oxidative metabolism of the infiltrating polymorphonuclear leukocytes (PMNs). It is believed that after specific membrane perturbation by stimuli, PMNs may exhibit a burst in oxygen consumption and start to generate active oxygen metabolites, which may lead to oxidative stress in tissues. So, the accumulation of PMNs in affected organs is considered to be one of the causative factors of multiple organ failure (MOF). MPO is an essential enzyme for PMNs function and a useful indicator of its infiltration. Evidence indicates that normal small intestine bears a low background of MPO activity, and the enzyme activity increased significantly in ischemic small intestine followed by PMNs infiltration^[48,49]. MPO activity also increased in the lung of rats with acute

pancreatitis^[50,51]. It is still unclear whether PMNs infiltration may be involved in the damage of small intestine in acute pancreatitis. In the present study, MPO activity in the control group increased significantly as compared with the EGF group on d5. It indicates that EGF may reduce PMNs accumulation in intestinal mucosa, thus minimizing oxidative stress in rats with acute pancreatitis under TPN.

Sucrase and maltase, which lie in villus brush border, are two kinds of important disaccharidases. They are often used as the markers of the normal cell proliferation and digestive function in small intestine^[29,30,34]. In this study, activities of sucrase and maltase in the control group decreased significantly compared with the EGF group on d 5. Maintenance of sucrase and maltase activities indicates that EGF may alleviate damage of jejunum in rats with acute pancreatitis under TPN.

In summary, the present study demonstrated that treatment with EGF can lead to body weight gain, reduce gut atrophy and PMNs accumulation in intestinal mucosa, prevent increased intestinal permeability and maintain sucrase and maltase activities in acute pancreatitis rats under TPN.

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A study on α -keto adipic aciduria by gas chromatographic-mass spectrometry

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Subject headings L-lysine, hydroxylysine, L-tryptophan; alpha-amino adipic, alpha-keto adipic aciduria; metabolism, inborn error; mass fragmentography; chromatography, gas

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INTRODUCTION

α -keto adipate (α -KA), an intermediate in the catabolism of L-lysine, hydroxylysine, and L-tryptophan, undergoes oxidative decarboxylation to form glutaryl-CoA and then dehydrogenates to form crotonyl-CoA, the latter undergoes further degradation and enters in TCA cycle, as shown in Figure 1. α -keto adipic aciduria (Mckusick 245130) is a rare inborn error in the metabolism of α -KA to glutaryl-CoA and is characterized by the increased excretion of α -KA, α -amino adipate (α -AA) and α -hydroxy adipate (α -HAA). Since Przyrembel *et al* first described it in 1975^[1], only 13 cases of α -keto adipic aciduria have been reported over the past 25 years, including 7 symptomatic, and 6 asymptomatic ones even in the symptomatic siblings with α -keto adipic aciduria^[1-10]. The clinical manifestations of this metabolic disorder showed heterogeneity. However, no follow-up study on either symptomatic or asymptomatic case has been available so far. We followed up two cases of α -keto adipic aciduria clinically and metabolically using organic solvent extraction, new urease-pretreatment and gas chromatography-mass spectrometry (GC/MS).

MATERIALS AND METHODS

Subjects

Two male children were studied. Pregnancy and

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delivery were uneventful. Their clinical data were collected after diagnosis of α -keto adipic aciduria.

Reagents

Creatinine-d₃ (methyl-d₃) was purchased from Nippon Sanso Ltd., Tokyo, Japan, and 2-amino adipate, 2-oxo adipate, 2, 2-dimethylsuccinate (DMS) and urease type C₃ were obtained from Sigma Chemical Co. St. Louis, MO, USA. All chemicals were of analytical grade.

Quantitation of α -KA, α -HAA, α -AA and glutarate

Twenty nmol of DMS was used as the analyte of internal standard for preparing standard curves of α -KA and glutarate, 50nmol of DMS was also chosen as the analyte of internal standard for making standard curves of α -AA and α -HAA. Comparing with peak area of internal standard, quantitation of urinary α -KA, α -HAA, α -AA and glutarate was performed using standard curves.

Sample preparation

Urine samples from two cases were collected at different detecting time. All samples were frozen at -20°C until analysis. Creatinine was determined and urine volumes equivalent to 1 μ mol creatinine were prepared prior to GC/MS analysis by organic solvent extraction^[11] or urease-pretreatment^[12]

Measurement

Samples were analyzed using GC/MS-computer systems of QP-5000 (Shimadzu, Japan) and HP-6890 (Hewlett Packard, USA) as well as the new diagnostic method described previously^[12,14,15]. The concentrations of these compounds were normalized to urinary creatinine and expressed as mmol per mol creatinine.

Loading tests of tryptophan and lysine (each was 100mg/kg of body weight) were made in Case 1 at the age of 1 year and 4 months.

RESULTS

The two cases were followed up clinically for a period of 15 years (8 months-15 years in Case 1) and 5 years (1 day - 5 years in Case 2), respectively. Case 1 was slightly delayed in growth after birth and CT on his head showed mild cortical atrophic change at the age of 9 months. At 1 year and 4 months, the analysis of first urine sample

revealed high levels of α -KA and α -AA using GC/MS. Loading test was performed. The values of α -KA, α -HAA, glutarate and α -AA reached 16-fold, 4-fold, 9-fold and 4.5-fold, respectively after lysine was taken orally. The concentrations of these four compounds increased 7-fold, 2-fold, 3-fold and 4-fold, respectively after tryptophan was also administered orally. Treatment was carried out with a low-lysine diet (70mg/kg daily) and a low-tryptophan diet (17mg/kg daily) after diagnosis. His CT was within normal range and his mild cortical atrophic change disappeared at the age of 4 years. The dietary treatment was discontinued due to normal development. His growth is normal at present. Case 2 developed cyanosis, clonic seizures and hypoglycemia 1 day after birth, then grew normally without low protein restriction.

Excretion of abnormal urinary metabolites profile compatible with α -ketoaciduria in two cases was continuously observed using organic solvent extraction, urease-pretreatment and GC/MS techniques (Table 1). Total ion current (TIC) chromatogram of TMS derivatives of organic acid in urine from Case 1 is shown in Figure 2. Ten

major compounds were identified. Peak 4, 6 and 7 represent glutarate, α -HAA and α -KA, respectively. Figure 3 shows the TIC of TMS derivatives of metabolites in urine from the same patient using urease-pretreatment and 9 major compounds were confirmed. Peak 2, 4, and 5 demonstrated glutarate, α -HAA and α -AA, respectively. Abnormal metabolites profile of α -KA, α -HAA, α -AA and glutarate were also detected in the urine of Case 2 with those techniques. Compared to the case with α -ketoaciduria, three abnormal peaks, including α -KA, α -HAA and glutarate after organic solvent extraction and α -AA, α -HAA and glutarate using urease-pretreatment, did not appear in the urine from healthy age-matched control at same retention time. The value of α -AA is much higher than that of α -KA, and glutarate was also detected and found increased in the urine of two cases. The concentrations of α -KA, α -HAA and glutarate ranged between 9-49 mmol/mol creatinine, 12-55 mmol/mol creatinine and 9-216 mmol/mol creatinine, respectively. The amounts of α -AA were 92-450 mmol/mol creatinine in analysis of urinary amino acid (Table 1).

Table 1 Urinary concentrations of metabolites

Cases	Detecting age	α -KA	α -AA	α -HAA	Glutarate	Total value of metabolites
1	1.4yrs	33(ND)	223(2-25)	28(ND)	29(0.04)	313
	4.2yrs	33(ND)	266(0-51)	17(ND)	24(0.04)	340
	15yrs	33(ND)	92(0-4)	21(ND)	53(ND)	199
2	13d	31(ND)	200(1-11)	12(ND)	9(0.03)	252
	29d	49(ND)	450(5-197)	17(ND)	54(0.03)	570
	5yrs	9(ND)	92(0-4)	55(ND)	216(ND)	372
Average		31.3	220.5	25	64.2	

Values are expressed as mmol/mol creatinine. ():control

α -AA: α -aminoadipate, α -KA: α -ketoaciduria, α -HAA: α -hydroxyadipate, ND: not detected; d: days, yrs: years

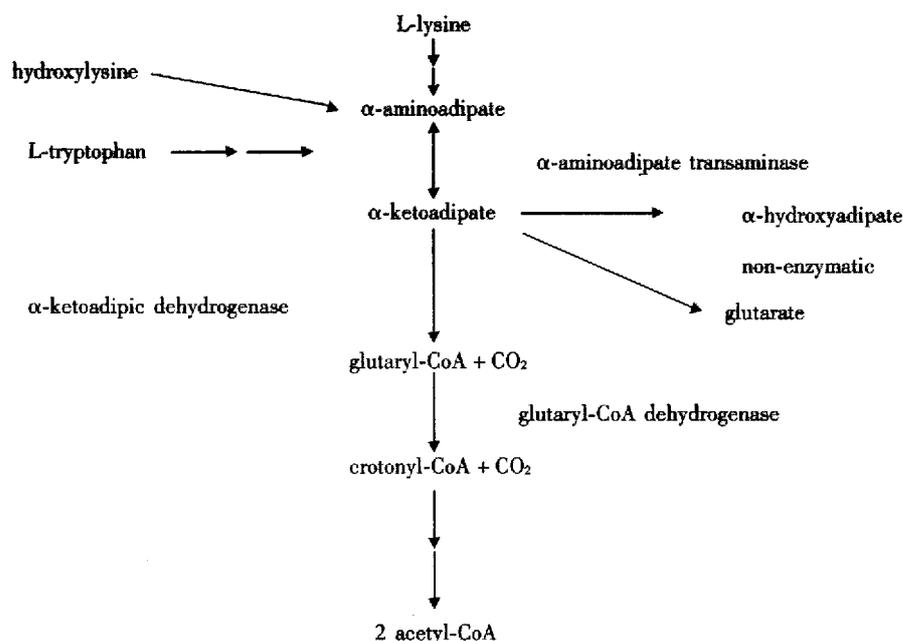


Figure 1 α -metabolic pathways of L-lysine, hydroxy-L-lysine and L-tryptophan leading to α -KA.

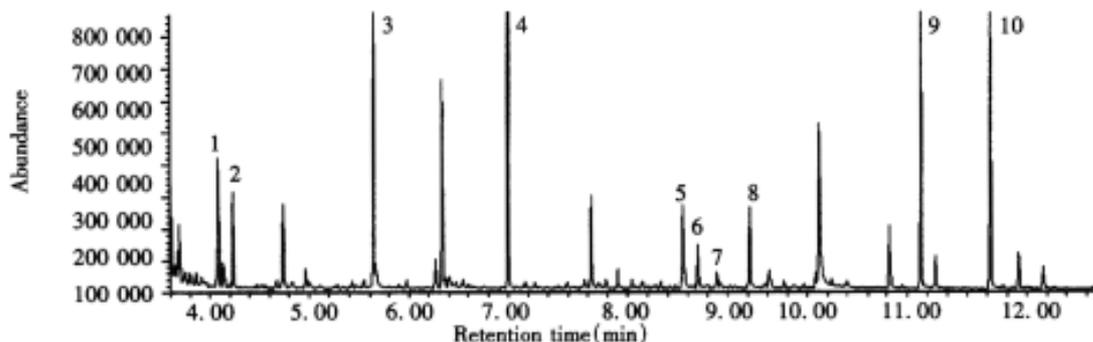


Figure 2 Total ion current (TIC) chromatogram of TMS derivatives of organic acids in urine from a patient with α -ketoadipic aciduria (case 1). Peak identities are: 1, lactate-2TMS; 2, glycolate-2TMS; 3, urea-2TMS; 4, glutarate-2TMS; 5, 4-hydroxyphenylacetate-2TMS; 6, α -hydroxyadipate-2TMS; 7, α -ketoadipate-3TMS; 8, cis-aconitate-3TMS; 9, 3-hydroxymyristate-2TMS (IS); 10, heptadecanoate-TMS (IS). IS: added as an internal standard.

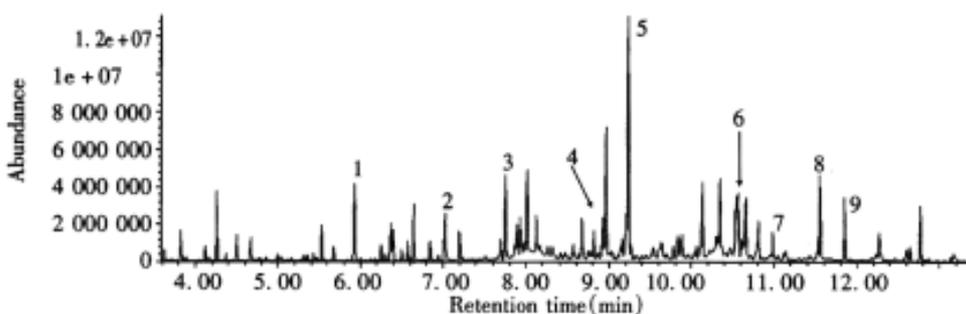


Figure 3 TIC chromatogram of TMS derivatives of metabolites in urine from a patient with α -ketoadipic aciduria (case 1). Peak identities are: 1, phosphate-3TMS; 2, glutarate-2TMS; 3, erythritol-4TMS; 4, α -hydroxyadipate-2TMS; 5, α -aminoadipate 3TMS; 6, histidine-3TMS; 7, gluconate-6TMS; 8, urate-4TMS; 9, heptadecanoate-TMS (IS). IS: added as an internal standard.

DISCUSSION

In analyzing organic acid, GC/MS has been proven by several institutions to be the most efficient method for chemical diagnosis of inborn error of metabolism.

In 1991, Shoemaker *et al* reported that urinary organic acids, amino acids and sugar could be analyzed simultaneously by GC/MS after excessive urea in the urine was degraded with urease and removed. This method, however, takes several hours, needs skillful technicians, and is not so practical. Therefore, Shoemaker's procedure was simplified for use in multiple sample analysis^[12]. As a result, rapid practical and simultaneous analysis of amino acids and organic acids became possible. Further improved-procedure was adopted, which is a stable isotope dilution method using not only d_3 -creatinine but also stable-isotope-labeled amino acids as internal standards. It only takes 1 hour for pretreatment of one sample and is a highly comprehensive diagnostic tool for a wide range of metabolic disorders^[13]. However, for detecting α -KA, the approach of organic solvent extraction is more sensitive than that of urease-pretreatment in this study.

α -ketoadipic aciduria was first described by Przyrembel *et al* in 1975 since then 7 cases have been reported^[1-3,6-9] using GC/MS or other analytical methods, and some subjects with α -ketoadipic aciduria were found by re-examination^[3]. In addition to symptomatic cases, 6

asymptomatic cases have also been reported^[2-5,10]. Up to now no follow-up study on this case with or without symptoms has been described by employing organic solvent extraction, urease-pretreatment and GC/MS techniques.

Almost all probands were identified when prominent spots of α -AA were noted on amino acid chromatography of urine, with subsequent investigations demonstrating α -aminoadipic acidemia and increased urine concentrations of α -KA and α -HAA. Different clinical symptoms were also described according to 7 affected individuals. The major manifestations include psychomotor retardation (5 cases)^[1,3,6-8], men talretardation (3 cases)^[1-3], hypotonia (3 cases)^[1,3,7] and seizures (2 cases)^[6,8]. The data indicate that the individuals with α -ketoadipic aciduria presented with nonspecific symptoms, but the central nervous system may be especially vulnerable. In this study, two cases showed symptoms at onset, including an 8-month-old boy with growth retardation (Case 1) and a boy with seizures (Case 2) as described above symptoms. In the two cases, the values of α -KA, α -AA, α -HAA and glutarate were always high at the different detecting time using present method. Meanwhile, loading tests of lysine and tryptophan were performed at 1 year and 4 months in Case 1. The concentrations of α -KA, α -AA, α -HAA and glutarate increased after lysine or tryptophan was taken orally. So the diagnosis of α -ketoadipic aciduria was confirmed. Three abnormal peaks were

still identified in recent detection as α -KA, α -HAA and glutarate after organic solvent extraction, and the three abnormal peaks of α -AA, α -HAA and glutarate also appeared by urease-pretreatment compared with healthy age-matched control.

In normal condition, α -KA and α -HAA could not be detected and only a trace of α -AA and glutarate exist in the urine, and when the patients are in the interim period, organic acid and amino acid in serum and urine are normal^[7]. In this study, abnormal excretion of these compounds was found and the value of α -AA was much higher than that of α -KA in the urine of two cases, but we still considered and diagnosed them as α -keto adipic aciduria. Based on this α -amino adipic aciduria is caused by the deficiency of mitochondrial α -amino adipic acid aminotransferase, which leads to elevated urinary excretion of α -AA without α -KA.

A small amount of glutarate was also detected in urine in the two cases, but the level was much lower than that found in glutaric aciduria type I, and 3-hydroxyglutarate was not detected, so it is almost certainly because of spontaneous decarboxylation of α -KA or artifact^[6,7].

The accumulated metabolites suggest a block in α -keto adipic acid dehydrogenase, and intact mutant fibroblasts are almost totally unable to oxidize α -amino[1-¹⁴C] adipate and α -keto[1-¹⁴C] adipate to ¹⁴CO₂, but a defect in α -keto adipic acid dehydrogenase has not been demonstrated directly. If α -keto adipic and α -ketoglutaric acid dehydrogenase are indeed the same, it is not clear a defect can produce so mild a phenotype. This may indicate that the two enzymes are different. Vallat *et al* recently reported that significant increment of α -AA occurred in the plasma and urine of 8 vigabatrin (VGB) treated children suggesting that VGB strongly inhibit α -amino adipic acid transaminase, α -keto adipic acid dehydrogenase, or glutaryl-CoA dehydrogenase^[16,17]. However, more knowledge about underlying the mechanism is required.

Although protein restriction was reported to improve clinical symptoms like Case 1 and Case 2 in which no seizures occurred without treatment. Our results and results previously reported^[3,8], suggest that the clinical course of α -keto adipic aciduria is incongruity and the condition is not apparently deleterious. Some authors investigated normal siblings of the patients, who excreted excessive amounts of α -KA and α -AA, and thought mental retardation may result from other causes^[2,3]. Others researchers consider that the analysis of mass spectrometry for this disorder would not be useful because α -keto adipic aciduria is a nondeleterious inherited metabolic defect. But up to now, the relationship between the biochemical abnormality and clinical manifestations in α -keto adipic aciduria

is still unclear.

Inheritance as an autosomal recessive trait is inferred from the pedigrees. There is no evidence so far that heterozygous carriers can be distinguished from control subjects. The incidence is not known. For these reasons, it is necessary to follow up this case with α -keto adipic aciduria using GC/MS in order to clarify the mechanism of the clinical heterogeneity of this defect.

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Case Report

Metastasis in an axillary lymph node in hepatocellular carcinoma: a case report

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Subject headings liver neoplasms; axilla; lymph nodes; lymphatic metastasis; case report

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INTRODUCTION

Although hepatocellular carcinoma often metastasizes to regional lymph nodes, spread to more distant lymph nodes is rare^[1-7]. Involvement of axillary lymph nodes by metastases appears not to have been documented. We report a patient with hepatocellular carcinoma (HCC) with a metastasis in a lymph node in the right axilla, and discuss possible routes by which such spread might occur.

CASE REPORT

M.S., a 28-year-old black African woman presented to the medical service of the Johannesburg Hospital in April 1999 with a 2-month history of worsening pain in the right upper quadrant of the abdomen, increasing abdominal girth, yellow discoloration of the sclerae, and generalized pruritus. She had been hospitalized in Zimbabwe 3 weeks earlier, when an enlarged gland in her right axilla was biopsied. This showed the histological features of a "cancer originating in the liver". She was told that there was no effective treatment for her disease and was discharged. The patient had previously been well and did not smoke cigarettes or drink alcohol. Since the birth of her only child 5 years earlier, she had been receiving intramuscular injections of Depo-Provera[®] for contraception.

Physical examination revealed a young woman who was deeply jaundiced and pale, and showed evidence of recent weight loss. Extensive tribal scarification of the skin was evident. She was afebrile. Her blood pressure was 100/70 mmHg, pulse 115/min, and respiratory rate 20/min. She had florid nasopharyngeal candidiasis and shotty

generalized lymphadenopathy. A lymph node measuring 3 by 4cm, which was firm and adherent to adjacent tissues, was present in the right axilla. The overlying skin showed a healing scar. Her liver was enlarged to 10cm below the right costal margin (total span 22cm) and was extremely tender, and the surface was smooth. A bruit could not be heard over the liver. Tense ascites was present. Splenomegaly was not obvious and distended abdominal wall veins were not seen. Air entry at both lung bases was reduced, but no adventitious sounds were heard. A grade 2 ejection systolic murmur was heard at the left sternal border. The remainder of the examination was unremarkable.

A plain X-ray of the chest revealed an abnormally raised right hemidiaphragm, but was otherwise normal. Abdominal ultrasonography confirmed the presence of ascites. The liver was enlarged with a generally coarse echogenic pattern but with areas with a mixed hyperechoic/ hypoechoic pattern. Enlarged regional or para-aortic lymph nodes were not seen and the kidneys were normal.

The serum α -fetoprotein concentration was 150 μ g/L (normal less than 20 μ g/L). The hemoglobin level was 11g/dl, total serum bilirubin 94 μ mol/L (conjugated bilirubin 49 μ mol/L), total protein 77g/L, albumin 27g/L, alkaline phosphatase 261U/L, aspartate aminotransferase 418U/L, alanine aminotransferase 65U/L, and γ -glutamyl transpeptidase 365U/L. Serosanguinous fluid was obtained on ascitic tap, but on malignant cells were seen on cytological examination.

Hepatitis B virus surface and e antigens and IgG antibody to the core antigen were present in the serum. Hepatitis C antigen and antibody were negative. The human immunodeficiency virus Elisa test and Western blot were positive. The CD4 T cell count was 398, and the CD4:CD8 ratio 0.8:1.

Fine needle aspiration of the enlarged lymph node was performed. Slides were fixed for Papanicolaou staining and air-dried for Diff-Quik staining. Microscopic examination of the slides revealed a background of blood, with moderate numbers of large neoplastic epithelial cells (Figures 1 and 2). The cells were present in both small clusters and as single cells. They were round or oval in outline, and their cytoplasmic margins well defined. The cytoplasm was dense and eosinophilic on the Papanicolaou stain, with a minor population of cells showing cytoplasmic vacuolation. Nuclear

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cytoplasmic ratios were high, and nuclei centrally located. The nuclei, generally single but very occasionally binucleate, were round, with coarse hyperchromatic chromatin, and very prominent macronuclei. In occasional cell groups, fine capillaries transected the cell aggregates. These cytological features are in keeping with those found in HCC^[8-10]. The alcohol-fixed slide was destained and used for cytochemical analysis. With good positive and negative controls, the cells were found to be negative for both cytokeratin 7 and 20. This cytokeratin profile points to carcinoma cells arising in the liver or kidneys, or to squamous cell carcinoma of the lung^[11].

On the basis of the cytomorphology, the metastasis was considered most likely to have arisen in a hepatocellular carcinoma^[8-11]. Renal carcinoma of this degree of differentiation would demonstrate eccentric nuclei in less well defined cytoplasm, with finer chromatin than noted here. Squamous cell carcinoma of bronchogenic origin would be unlikely to show the cytoplasmic vacuolation seen in some of these cell groups.

The patient was discharged on palliative treatment, and was subsequently lost to follow up.

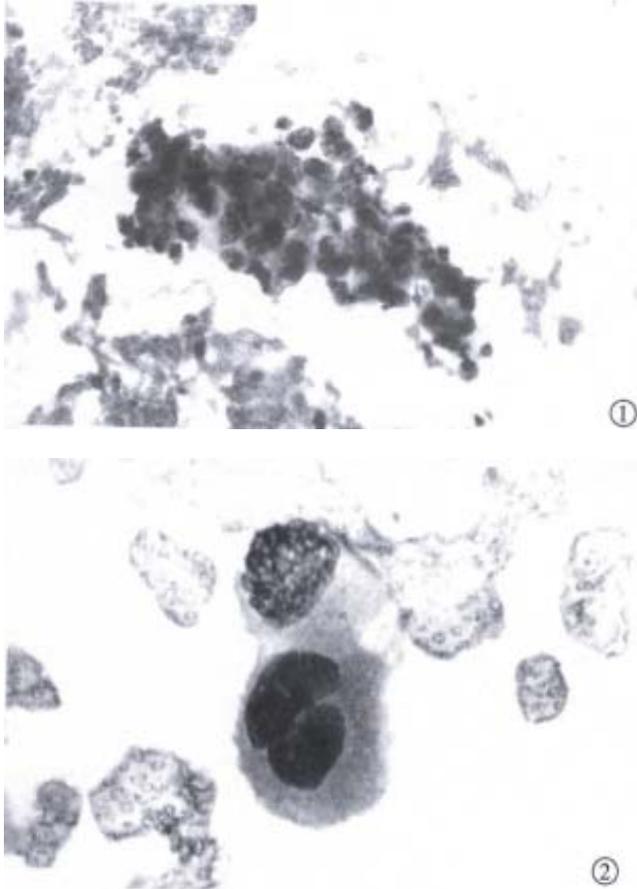


Figure 1 High power magnification of a cluster of cells from the axillary node aspirate, demonstrating pleomorphism and crowding of large poorly differentiated malignant cells. Papanicolaou stain $\times 200$

Figure 2 Single-lying cell from the axillary node aspirate, demonstrating high nuclear to cytoplasmic ratios, central nuclei, and prominent nucleoli. Diff-Quik $\times 400$

DISCUSSION

Although most patients with HCC present clinically in well defined ways, a large number and wide diversity of unusual presentations have been described^[6]. A lack of awareness of these presentations can result in the diagnosis being delayed or even missed. Because we were unaware that HCC can spread to axillary lymph nodes, this diagnosis was initially discounted even though the clinical features were typical in other respects.

A review of the literature reveals no reports of axillary lymph node metastases complicating HCC^[1-7]. In particular, four necropsy studies in populations with a high incidence of this tumor (two in Japan^[4-7], one in South Africa^[1], and one in Hong Kong^[3]) and involving more than 1000 patients do not mention this route of spread.

There are two possible routes by which HCC could spread to axillary lymph nodes. The primary tumor in our patient involved most parts of the liver. Tumors located in the upper part of the right hepatic lobe (under the bare area of the liver) could spread via lymphatic vessels to lymph nodes on the upper surface of the diaphragm and thence to either mediastinal or parasternal lymph nodes^[12,13]. Malignant cells could then track along intercostal lymphatic vessels to reach the axillary lymph nodes. Spread from mediastinal lymph nodes via intercostal lymphatic vessels is presumed to be responsible for axillary lymph node metastases in bronchogenic carcinoma^[14]. Mediastinal glands were not radiologically evident in our patient although based on the experience with bronchogenic carcinoma^[14], this does not exclude this mode of spread. Alternatively, malignant cells could spread from the portal venous system to the umbilical region via a patent umbilical vein, or could reach the umbilicus by direct spread from the anterior peritoneum or by lymphatic spread from para-aortic glands invaded by the tumor^[15], as shown by the finding of a Sister Joseph's nodule in an occasional patient with hepatocellular carcinoma^[16]. The malignant cells could then drain along subcutaneous lymphatic channels to axillary lymph nodes^[17]. The absence of an obvious Sister Joseph's nodule or even periumbilical induration in our patient makes this route less likely but does not exclude it.

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Progress in research of liver surgery in China

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Subject headings liver neoplasms/surgery; liver transplantation; liver neoplasms/diagnosis; liver neoplasms/therapy; biopsy, needle; genes, suppressor, tumor

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INTRODUCTION

Liver surgery, was started in the late 1950s in China and has developed rapidly in the past 40 years^[1]. The study on the diagnosis and treatment of primary liver cancer in China underwent four stages: ① In the 1950s, the anatomical study of the liver lay a solid foundation for liver resection^[2]. ② In the 1960s and 1970s, studies mainly focused on the detecting methods of AFP and other tumor markers, clinical and pathologic characterion of small liver cancer and epidemiology of liver cancer. ③ In the 1980s, imaging diagnostic techniques, such as CT, MRI, DSA, Doppler ultrasonography, etc., and new therapeutics^[3], such as hepatic artery chemo-embolization, percutaneous intra-tumoral ethanol injection, hepatic artery ligation with targeted chemotherapy^[4], and some new concepts such as radical regional resection, re-operation of the recurrent liver cancer, two-stage resection, the combined surgical management of liver cancer complicated with hepatic duct thrombus, splenomegaly and portal hypertension were introduced. These comprehensive treatment further improved the liver cancer surgery. ④ In the 1990s, attention was mainly focused on the biotherapy and liver transplantation. The progress in the diagnosis and treatment of primary liver cancer in recent years are summarized as follows.

EARLY DETECTION OF LIVER CANCER

The methods for early detection of liver cancer include: ① People aged more than 35 years, with a history of hepatitis, HBV or HCV infection, cirrhosis or chronic hepatitis, should be taken as a high risk population. Periodical monitoring of this population is the key step to detect early liver

cancers; ② AFP and B-US screening is, at present, the most sensitive, convenient and economical method for detecting early liver cancers; ③ for patients with low level AFP, the AFP variant detection is helpful^[5]. As to the patients with negative AFP, other liver tumor markers can also be used; ④ combined with CT, MRI, CTA or DSA, B-type ultrasonography is useful in the early diagnosis and localization of liver cancer; and ⑤ fine needle puncture for cytologic study and ethanol injection under ultrasonography is also helpful.

TREATMENT OF LIVER CANCER

At present, hepatectomy remains the treatment of choice for primary liver cancer^[6]. The efficacy of surgical intervention has been raised rapidly and significantly since 1978^[7]. The main reasons are as follows: ① the improvement of diagnostic methods for early cancer, ② renewal of surgical concepts; ③ improvement of surgical techniques and perioperative management^[8], and ④ the development of comprehensive therapy postoperatively. The pathological data from our group showed that 86.5% liver cancers were concomitant with cirrhosis or chronic hepatitis. The regular or extended hepatectomy might lead to severe decompensation of liver function. Therefore, the modality of liver resection drifted from an extended one to an irregular radical local resection^[9]. With presence of chronic hepatitis or cirrhosis, the radical local resection modality not only increases the resectability, but also significantly decreases the surgical mortality rate and attains the same long-term effect as the extended resection, or even better. The patients used to be given conservative therapy when one or more complications occurred such as jaundice, severe portal hypertension and esophageal varices with or without hemorrhage. With the accumulation of clinical experience, obstructive jaundice resulting from oppression of hepatic hilus or cancerous thrombi invading the biliary duct could be treated with hepatectomy or the removal of the biliary duct thrombi if hepatocellular jaundice and other contraindications could be excluded^[10]. Usually, the jaundice disappeared gradually after operation. In the patients with splenomegaly, hypersplenism and esophageal varices with or without hemorrhage, the hepatectomy can also be performed together with splenectomy plus ligation of varices or with splenorenal shunt. In the past, the comprehensive therapy was mainly used for advanced liver cancers

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that were unresectable^[11]. But now, this concept has been extended and includes ① the pre- and post-operative comprehensive therapy for resectable liver cancer to prevent recurrence; ② palliative removal of irresectable tumors followed by anti-cancer therapy to shrink the tumor mass and prolong the tumor bearing survival period; and ③ comprehensive therapy for temporarily non-eligible surgical patients, with the hope of performing two-stage resection and long-term tumor bearing survival period.

Comprehensive therapy includes surgical and non-surgical treatment^[12], the former including hepatectomy, hepatic artery ligation (HAL), operative hepatic artery embolization (OHAE), drug delivery system (DDS), intraoperative ethanol injection, microwave consolidation^[13], laser gasification, freezing, etc. and the latter including transcatheter arterial chemo-embolization (TACE)^[14], B-US directed percutaneous ethanol injection (PEI) or other drugs^[15], radioisotopes and bio-agents, biotherapy, radiotherapy and traditional Chinese medicine.

Rational comprehensive therapy with multimodality is superior to a single method in terms of effectiveness^[16]. The tetralogy of comprehensive surgical therapy, which is the combination of HAL, OHAE, DDS and radiotherapy performed in 603 advanced liver cancers in our hospital, showed that the rates of two-stage resection and 1, 3 and 5-year survival rates were significantly higher than that of a single procedure (HAL or OHAE). The incidence of recurrence was only 7.4% in the 27 cases treated with comprehensive immunotherapy (cytokines plus low-dose chemotherapy) after resection, whereas in the control groups, it was 32%. In 86 operated cases, DDS chemotherapy was performed and the total incidence of one-year recurrence was 34.9%, while in hepatic artery, portal vein and hepatic artery combined with portal vein groups were 33.3%, 34.6% and 23.6%, respectively. Non-surgical comprehensive therapy was eligible for all patients with unresectable liver cancer, the TACE and intra-tumoral drug injection being most popular. In a series of 8000 TACE cases, the 3-year survival rate was 13.9%. The drugs we used in the B-US directed local drug injection were absolute ethanol, ³²P radioisotope, OK432, TNF-alpha and IL-2. The 2-year survival rate in 700 patients receiving PEI was 80.0%, with a total of 3000 injections given. In another group of 113 patients receiving TACE in combination with PEI, the tumors shrank in most patients (91.2%) in varying degrees, and the total 2-year survival rate was 81.6%. Among them, 11 out of 71 patients with tremendous, solitary tumor received two-stage resection after the tumor shrinkage and the two-stage resection rate was 15.49%.

Comprehensive therapy is not simply a random combination of various methods. The design of the

protocol should be individualized and case-specific. The regimen of the comprehensive therapy is multiple in medical literature. We propose two principles: ① The two methods used should be complimentary; and ② no contradiction in their effects and the side effects should not be additive. At the same time, attention should be paid to the toxic effect of each method and not to pose damage to the liver function. Besides, special emphasis should be laid on the effectiveness and use of traditional Chinese medicine in comprehensive therapy.

In 1978, we reported that a two-stage resection of a large-sized liver cancer shrank after HAL procedure. Since then, this procedure has become a promising modality for unresectable large liver cancers. At present, the comprehensive surgical methods for massive liver cancer shrinkage comprise HAL, HOHAE and DDS, and non-surgical procedures, such as TACE, PEI, target therapy and radiotherapy. A rational combination of these methods enables some unresectable tumors to become resectable if they were sequentially employed. From 1974 to 1994, 649 patients received this therapy and 73 of them had their tumors resected with a resectability rate of 11.1% without operative death. The 5-year survival rate was 61.5% postoperatively, the longest survival being 17 years. The pathological data in this group showed that there were still some viable cancer cells remained although the tumor had shrunk due to the comprehensive therapy, it was still essential to remove the tumor remnants. At present, two-stage resectability rates are still very low, because no generally accepted criteria for tumor resectability. We propose that the two-stage resection is indicated only for those really unresectable, otherwise, the one-stage removal is of first choice; rational employment of comprehensive therapy is crucial for tumor shrinkage; and the unresectable liver cancers should be subjected to non-surgical comprehensive therapy, such as TACE, PEI and guided chemoimmunotherapy as the first choice.

BASIC RESEARCH IN LIVER CANCER

In the 1960s, the study of the liver cancer focused on the basic surgical study, including the anatomy of the liver, the postoperative hepatic metabolism and the effects of hilar occlusion on the hemodynamics and the metabolism of the liver. In that period, epidemiological study of the HBV and aflatoxin on liver cancer were completed in small samples. The first hepatoma cell line in the world was established, and the induced liver cancer models in rats were set up in many institutes. However, the study only stayed at the basic clinical level.

In the 1970s, much research work was concentrated on the relationship of AFP and the liver cancer. Various assays for AFP were set up and their sensitivity and specificity were tested. The

clinical and pathological characteristics of small liver cancer were documented. Contaminated food, water and HBV were shown as the main factors promoting liver cancer in high incidence areas as shown by epidemiological study in China. The first-line prophylactic strategy, that is, water control, fungi prophylaxis and hepatitis control was established. For the first time, liver cancer study in China appeared to have its own characteristics.

In the 1980s, liver cancer study in China reached cellular and molecular levels. The relationship between HBV and liver cancer was corroborated at the molecular level^[17]. The relationship between the DNA content of the liver cancer tissues and their biological features was documented with pathological study in liver cancer at cellular and molecular levels. Gene spectrum of liver cancer was found. HBV-DNA integration was verified as the activation factor of some oncogenes. The immunological status of liver cancer hosts and its relationship with tumor were studied^[18]. The biological significance of the agglutinin, sugar, gangliosides, serum enzymes^[19] and protein to liver cancer and also the interventional methods were observed. Monoclonal antibodies specifically targeting the membrane antigens had been generated, and their localization *in vivo* and targeted treatment were studied^[20]. A lot of work was done on the serum tumor markers.

In the 1990s, a series of oncogenes and tumor suppressive genes were found by cellular, molecular and gene-manipulating methods. The relationship of HBV, HCV, aflatoxin and other cancer-inducing factors and the mutation of oncogenes and tumor suppressive genes, and their mechanisms were elucidated. The role of the genes, such as *p16*, *p53* and *nm23-H1* and the enzymes and adhesive molecules such as metalloproteinase, CD44, ICAM-1, integrin in metastasis and recurrence of liver cancer were verified. The clonogenicity of the liver cancer was studied with DNA, oncogene and tumor suppressive gene. The mechanisms of the cell cycle control, apoptosis, senescence evading and the escape of the immunologic surveillance of liver cancer cells were studied. And in the related study of the cellular signal transduction in these processes, the negative control genes on the liver cancer growth were found. The liver cancer-related genes were found with screening of a large amount of liver cancer genome, and even in some genes, their functions and chromosome localization were also elucidated^[21]. In immunological study, many kinds of tumor vaccines have been produced. Many researches in gene therapy, induction-differentiation, prophylactic control of neoangiogenesis of liver cancer are being carried out, reaching world advanced level.

LIVER TRANSPLANTATION

It is controversial about the indications of liver

transplantation in liver cancer^[22]. In massive liver cancers, recurrence after transplantation is unavoidable due to vascular invasion and distal metastasis as well as the use of immunosuppressive agents^[23]. On the contrary, the therapeutic effects of liver transplantation on small liver cancers complicated with severe cirrhosis are corroborated. Comparing the therapeutic effects of hepatectomy and liver transplantation, Bismuth concluded that the 3-year survival rates were almost the same, while the tumor-free 3-year survival rate was higher by liver transplantation than by hepatectomy. As to the small liver cancer (mononodular or binodular, with a diameter less than 3cm), the results of liver transplantation were even better. Selby *et al* showed that the total 5-year survival rate in 105 cases of unresectable liver cancer of different stages that received liver transplantation was 36%, of whom the 5-year survival rate from stage one to three was 52.1%, while in stage four, it declined to 11%. They concluded that liver transplantation was fit for liver cancer in early stage (≤ 2 cm, no vascular invasion and no distal metastasis). Although we do not have enough data, liver transplantation should not be regarded as a routine therapeutic method because of high incidence of liver cancer, liver donation shortage and high cost in our country.

PERSPECTIVE

Further progress of liver cancer research in our country depends on the progress in multiple factors, such as further studies of the individual and circumstantial factors on the genesis of liver cancer and its mechanisms, the mechanisms of metastasis and recurrence after hepatectomy^[24], the recognition of liver cancer-specific antigens and genes and their signaling pathways, in order to find out the measures intervening the genesis and growth of the tumor. Attention should be paid to the precancerous lesions of liver. The definition of precancerous lesion at molecular level and the study on blockage of its transformation will enhance the efficacy of liver cancer treatment. In clinical practice we should stick to the principles of early diagnosis and early management, and the surgical treatment is still the treatment of choice. Pre- and post-operative TACE and other comprehensive treatment modalities should be studied to improve the tumor-free survival. The regional therapy of liver cancer, such as the intra-tumoral injection treatment and regional treatment should be generated with new techniques and new approach. The radiotherapy, biological therapy and traditional Chinese medicine are hopeful. The regimens we employ now are to be combined to fit different cases. Shrinkage of the massive liver cancer into small one and anti-recurrence therapy are the main goals of our practice. The liver transplantation should be actively applied for early liver cancer.

Due to the large number of people infected with HBV or HCV as the background of liver cancer, the liver cancer is still a major life threatening disease among Chinese people, therefore, surgical treatment should be further strengthened.

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Changing trends of surgical treatment of hilar bile duct cancer: clinical and experimental perspectives

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HISTORICAL POINT OF VIEW

Carcinoma of the confluence of the hepatic ducts has been thought to be a rare disease until recently. Carcinoma of the large hepatic ducts was rarely diagnosed correctly preoperatively. Because of its deeply seated location, resection was once thought to be impossible. In 1957, Altemeier^[1] reported 3 cases of sclerosing carcinoma of the major intrahepatic bile duct. One of the cases was operated upon for seven times within three years without a correct diagnosis until metastatic adenocarcinoma was found around the hepatic porta. However, this patient lived for three and half years after the first operation.

Klatskin^[2] was the first one who drew attention to the hilar bile duct (HBD) carcinoma as a kind of tumor with definite clinical features. His paper was entitled as "Adenocarcinoma of the Hepatic Duct at its Bifurcation within the Porta Hepatis. An unusual tumor with distinctive clinical and pathological features" was reported in the *Am J Intern Med* in 1965. Thirteen cases, were analyzed from 1947-1963. Klatskin pointed out in his paper that "The purpose of this report is to draw attention to the unusual clinical and pathological features of adenocarcinoma as that arise in the hepatic duct at its bifurcation within the porta hepatis". He laid emphasis on the fact that such type of tumor was often small-sized with a clear margin, metastasis has rarely been found, and the patient often died of prolonged biliary obstruction and liver failure

instead of tumor growth.

In our experience, slow progression of the tumor may be encountered very occasionally^[3,4]. In our series of 157 cases of HBD carcinoma, we encountered a male patient of 61 years of age, who had received operation of insertion of endoprosthesis because of jaundice about 5 years ago. After the operation, he developed repeated attacks of chills, fever and jaundice. We re-explored this patient and found that a well-localized tumor mass at the bifurcation of the hepatic duct, which was removed completely, and a cholangioenterostomy was created. Pathology of the resected tumor showed a highly differentiated adenocarcinoma of the confluence of the hepatic duct. The patient lived without recurrence for already 10 years since the last operation.

From January 1986 to January 1999, in the department of hepatobiliary surgery of our hospital 157 cases of hilar bile duct carcinoma (Table 1) were treated surgically. These experiences together with a series of experimental studies form the basis of this report^[5-7].

Table 1 Surgical treatment of cases of hilar bile duct carcinoma (1986-1999 in this hospital)

Treatment	No. of patients	%
Resection	106	67.5
Radical resection	59	37.6
Biliary drainage	51	32.5
Total	157	100.0

TUMOR EXTENSION AND STAGING

The biliary tract is a continuous structure from its beginning as Hering's tubule to the lower part of the common bile duct. The exact confinement of Klatskin tumor has not been accurately defined. Therefore, many terms have been adopted for the description of the same or not the same conditions. Carcinomas arising from the intrahepatic small bile ducts are often called cholangioma, which is one of the primary liver tumors. But, as to those tumors arising from the larger intrahepatic duct near the hilum and invading the hepatic confluence at its late stage may be difficult to be differentiated from those arising from the hilar bile duct^[8-10]. We are of the opinion that hilar bile duct cancer consisted of those cholangiocarcinomas of large bile ducts, which invaded the hepatic duct confluence^[11,12].

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There are many criteria for staging of hilar bile duct carcinoma such as those proposed by Bismuth-Corlette (1975)^[13], Gazzanign (1993)^[14], pTNM (*Am J Co Cancer*), TNM (UICC), and the T-staging classification (Blumgart 1998)^[9].

Bismuth and Corlette proposed the most widely used clinical pathological classification of hilar bile duct cancer in 1975^[13]. However, this classification is of value only in defining the origin of the tumor. Clinically, when tumor originated from one of the hepatic ducts (Bismuth-Corlette Classification Type III), jaundice will not be an early symptom, and, in fact, it may appear relatively late and clinical jaundice appeared only when tumor invaded the hepatic pedicle as the tumor advanced to the confluence of the hepatic duct to obstruct the opposite hepatic duct orifice^[15]. Therefore, we often found that the tumor was unresectable in patients suffering from jaundice only for two weeks duration. In such cases, atrophy of one lobe (either the right or the left lobe) of the liver was present^[16,17]. For staging of tumor progression, Gazzanign proposed a new staging criterion in 1993^[14]. But it is difficult to exactly define the degree of tumor progression before operation. Recently, Blumgart (1998)^[9] proposed a T-staging criteria which depends on the radiological features of tumor involvement of the portal vein and the presence of ipsilateral lobar atrophy in HBD cancer (Table 2). Since involvement of the portal vein is the chief independent risk factor in determining the resectability of bile duct cancer, therefore, it seems to be a simplified idea and is readily useful in clinical practice.

Table 2 T-staging of hilar bile duct cancer (Blumgart)^[9]

T 1	No vessel involvement or liver atrophy
T 2	With ipsilateral liver atrophy No portal vein involvement
T 3	With ipsilateral portal vein involvement With or without associated liver atrophy No main portal vein involvement
T 4	Any of the followings Bilateral 2nd order hepatic duct involvement Main portal vein involvement

THE EVER-WIDENING CURATIVE RESECTION

Since the turn of the 1980s, in order to increase the radicality of tumor resection, radical resection of hilar bile duct cancer was proposed to include skeletalization of vascular structures of the liver pedicle, the combination of hepatic lobectomy, the wide dissection of regional lymph nodes, and even the inclusion of hepatobiliary pancreaticoduodenectomy^[18-21].

The overall resectability rate of hilar bile duct cancer in China was rather low before 1985. In the Southwest Hospital, Chongqing, 60 surgical cases (1975-1985) of HBD cancer were explored with a

resection rate of 8.3%. In a nationwide survey of surgical cases of extrahepatic bile duct cancer from 1978-1988 conducted by the Chinese Surgical Society, 422 surgical cases of hilar bile duct cancer were collected, the average resectability rate was about 10% (Table 3)^[22]. This result was quite similar to the report from Bismuth (1960-1985) that in 178 cases with a resectability rate of 10%^[13].

Table 3 Resectability of bile duct cancer in China (a nationwide survey, 1978-1988)

Location	No.patients	No.resected	%
Proximal	422	44	10.0
Middle	179	41	22.9
Lower	147	50	34.0
Total	748	135	18.0

However, after the year of 1985, more enthusiastic attitude was adopted in the surgical treatment of HBD carcinoma, which resulted in increase of resectability rate, and decrease of operative mortality rate. In our hospital from 1986 to 1990, 31 cases were resected among the 50 explored cases, with a resectability rate of 62%, and no 30-day postoperative death^[23,24]. Similar trend of changes was also found in other hospitals in China^[25].

However, the resectability rate varies which was much dependent on the policy of surgical intervention toward bile duct cancer. This differences may be partly due to the awareness of the disease and early diagnosis, and, even more important, the different attitude toward the choice of operative intervention in case of hilar bile duct carcinoma. In our hospital, we found that biliary drain age and stent placement by operative means had less late complications than that obtained by transcutaneous or transendoscopic route (Table 4). This finding was also confirmed by Guthrie^[26] that operative intrahepatic biliary enterostomy of the III segmental duct carried a lower postoperative cholangitis than the percutaneously placed endoprosthesis (19% and 55%, respectively). Therefore, we adopted the surgical therapy for all HBD cancer patients who are considered to be indicated for the operation.

Table 4 Biliary drainage and postoperative cholangitis in HBD cancer

Operation	No. of patients	No cholangitis(%)	Recurrent cholangitis(%)
Resection	59	74.6	25.4
Intern.drain.	11	36.4	63.3
Extern.drain.	21	52.4	47.6

The need of preoperative percutaneous transhepatic biliary drainage (PTBD) in jaundiced patients is still a debatable subject^[27]. We used this approach in only one case when extended right

lobectomy was considered necessary. Recently, we planned a staged operation for a patient who needed a hepaticobiliary pancreaticoduodenectomy. We agreed with Nimura that preoperative biliary drainage is beneficial only in cases if extensive resection will be needed, and it should not be treated as a routine procedure^[28]. We resected the caudate lobe of the liver only when the lobe was invaded by the tumor; otherwise, we transected the caudate segmental duct. In our experience, the hepatic artery, when encased by the tumor, may be excised en masse without harmful effect. Reconstruction of the portal vein after resection was undertaken under vascular control.

OUTCOME OF SURGICAL TREATMENT

During the 6th China National Biliary Surgical Conference held in Guangzhou in 1991, 139 cases of resection of HBD carcinoma were reported with a mortality rate of 0%-22%, being 5% on the average. The surgical treatment of HBD cancer of our hospital can be divided into two periods. The first period was from 1986 to 1993, during which 31 cases were resected with no 30-day postoperative death, 4 cases were living and tumor-free for more than 5 years (Table 5). During the second period, 32 cases were resected but no 5-year survival up to present (Table 6). Totally, from 1986 to 1999, 157 cases of HBD cancer were treated surgically in the General Hospital of PLA, Beijing, 106 of them were resected with a resectability rate of 67.5%, and 37.6% being considered radical resection (resection margins free of tumor cells). The 1, 3 and 5-year survival rate in the radical resection group was 96.7%, 23.3%, and 13.3%, respectively. In the palliative resection group (tumor cells present at one of the resection margins), no patient survived longer than 3 years^[23]. But, in the recent 3 years (1996-1999), 40 (74%) of the 54 surgical cases of HBD carcinoma were resected, in which, 23 (57.5%) resections were considered radical. This represents the changing trends towards earlier operation in those patients.

Table 5 Tumor-free long-term survivals after radical resection of hilar bile duct carcinoma

Case	Sex	Age	Type	Histology	Operation	Follow-up
1	M	47	Nodular	Tubular Ca	B-D resection, lobectomy	6yr3mo
2	F	50	Papillary	Papillary Ca	B-D resection	7yr2mo
3	M	63	Nodular	Well dif.	B-D resection, Lt lobectomy	7yr10mo
4	M	53	Nodular	Well dif.	B-D resection	8yr3mo

B-D: bile duct; Ca: adenocarcinoma; Well dif.: well differentiated

Table 6 Survival rate of surgical treatment of hilar bile duct cancer (The General Hospital of PLA series, 1997)

Operation	n	1yr(%)	2(yrs)	3(yrs)	5(yrs)
Radical re	30	29(96.7)	12(40.0)	7(23.3)	4(13.3)
Palliative re	26	16(21.5)	2(7.7)	1(3.8)	0
Int. drainage	11	5(45.5)	0	0	0
Ext. drainage	23	6(26.1)	0	0	0

re: resection

At present, however, the late result of HBD cancer is still discouraging^[29]. Boerma^[30] reviewed the English literature published before 1990, resection of the tumor was obtained in 581 cases with an average mortality rate of 13%, the average survival period was 21 months, and the 1, 3 and 5-year survival rate was 67%, 22%, and 11% respectively. Recently, Nakeeb^[31], from Johns Hopkins Medical Center, reported 109 cases resected with a 1, 3 and 5-year survival rate of 68%, 30% and 11%, and compared the cases treated with hepatic resection (n=15) with the cases without hepatic resection (n=94), which showed that there was no difference in the 1, 3 and 5-year survival rate, but the 5-year survival rate was better in those with negative margin (19%) than those with positive margin (9%). Nimura from Japan reported the result of a 5-year cure rate of 31% in 127 resected cases^[28]. In the report from the Liver Transplantation Center in Pittsburg^[32,33], 28 cases of HBD carcinoma underwent extensive resection including hepatic lobectomies and vascular reconstruction, the post-operative 30 days mortality rate was 24%,! but only one patient survived 5 years (Table 7).

Table 7 Survival rate after resection of hilar bile duct cancer (reported series)

Author	n	Rx (%)	Mortality rate (%)	Survival rate (%)		
				1yr	3yrs	5yrs
Pinson(1962-1983) ^[18]	156	16.0	4.0	84	44	36
Bismuth(1969-1990) ^[19]	122	18.9	0	87	25	
Tsuzuki(1973-1986) ^[21]	50	50.0	4.0	80	29	19
Cameron(1973-1989) ^[20]	96	40.6	2.6	70	27	11
Hadjis(1977-1985) ^[29]	131	20.6	7.4	70	26	22
Nimura(1977-1993) ^[28]	127	64.6	6.1	84	50	31
Boerma(1980-1989) ^[30]	581	32.4	15.4	61	28	17
Zhou, Huang(1986-1996) ^[23]	103	34.9	0	96.7	23.3	13.3

Rx: radical resection; !:collected series

Therefore, Madariaga^[33] from the Pittsburg group raised the question that the possible beneficial effect obtained through extensive operative procedure for HBD carcinoma is outweighed by the accompanying high morbidity and high mortality rate, so that the real significance of extensive surgery is questioned. Madariaga claimed that, for HBD carcinoma, it may be useless to prosuite for a cure resection as such is rarely possible, it may be encouraging to have a palliative resection if the operative morbidity and mortality rate can be kept much lower. We reach the same contention, that is, radical cure of hilar bile duct cancer is unlikely at present and should be left to those patients who may have an early diagnosis and early operation. But for most patients, efforts should be made to obtain a good palliative outcome with less postoperative complications.

Finally, we realized that carcinoma of the hilar bile duct, which rarely ran a "benign" course as described by Altemeier and Klatskin, is not an uniform disease, and is a regional disease rather than a local affection. It can metastase along the perineural space by a "jumping" fashion and invade adjacent tissue and blood vessels and lymphatics^[34-36], therefore, surgical excision is bound to be unradical in the region of the porta hepatitis for anatomical reasons.

EXPERIMENTAL STUDIES FOR FURTHER UNDERSTANDINGS OF HEPATIC HBD CANCER

Carcinoma of the hepatic duct bifurcation was considered a slowly growing malignancy and might have a better prognosis. However, from recent experience, this conclusion has been challenged. The long-term result of surgical resection of HBD carcinoma has been far from satisfactory. The 5-year survival rate of the radical resection group was from 13.3% to 17%^[6,23]. Patients often died of local recurrence and hepatobiliary failure. Therefore, a research on the mode of recurrence in HBD carcinoma was undertaken by the author and his collaborators^[35-39].

Coordinated clinical and pathological studies showed that metastasis of HBD cancer occurred rather early in the clinical cases. Therefore, in 32 cases of resections, metastasis was evidenced in 26 (83.9%) of the cases. The mode of spread was nerve invasion in 57.7%, direct liver invasion in 42.3%, soft tissue infiltration in 42.3%, liver metastasis in 7.7%, and lymph node metastasis was only found in one case (1.8%). Histologically, 21 (65.6%) of the 32 resected specimens were well-differentiated adenocarcinoma, 6 papillary adenocarcinomas, 3 were of low differentiation, and 2 carcinoma simplex^[4].

Cholangiocarcinoma cells frequently metastasized along the perineural lymph space. In 40 resected cholangiocarcinoma specimens, perineural space infiltration index (PNI) by cancer cells in relation to the median survival time was investigated, the result showed a reverse correlation (Table 8).

Table 8 Neural infiltration index and median survival time

Differentiation of tumor cells	PNI*	Median survival time (mos)
Papillary	0.31±0.12	32
High	0.39±0.18	13.5
Moderate	0.74±0.39	10.8
Low	0.85±0.41	7.2

* PNI>7.0: severe nerve infiltration

In 78 surgical cholangiocarcinoma resection specimens (collected between 1989-1996), the significance of neural cell adhesion molecule (NCAM) in relation to clinicopathological findings was investigated, 68 of the 78 specimens showed

nerve infiltration, blood vessels infiltration in 72, and lymphatic infiltration was present in 68. In 68 cases with neural infiltration, positive expression for NCAM was found in 51 cases. Furthermore, a reverse relationship was found between the positive NCAM expression and the degree of tumor cell differentiation (Table 9)^[40,41].

Table 9 Expression of NCAM and tumor differentiation

Cell type	NCAM Expression	
	(-)	(+)
Papillary	5	3
High diff.	10	12
Moderate diff.	8	16
Low diff.	3	21

$P=0.0267$, $\chi^2=9.20$

For further demonstration of spread of cholangiocarcinoma cell along the perineural space, observations of computer assisted 3 dimensional reconstruction of the pathological sections was made in 2 cases, totally 110-200 slides were selected for reconstruction using a SHOW 3D image analysis system. The results showed that a dense network of small vascular and lymphatic channels together with a branching network of tumor infiltrates are closely related along the nerve fiber. By using the "wire framing" technique to visualize the interior structures, it was demonstrated that the tumor cells stayed in perineural space, lymphatics, and small vessels far from the primary focus of carcinoma. Probably, carcinoma cells involving a nerve at a place far from the original site must have reached there via lymphatics, vascular vessels or by direct invasion. These facts might be important in explaining the high recurrence rate of HBD carcinoma after conventional radical resection^[42].

To investigate the relationship between hepatitis B and C virus infection of the bile duct mucosa and the occurrence of bile duct carcinoma, 51 excised bile duct cancer specimens from 1995 to 1998 were taken from the Department of Pathology, General Hospital of PLA, Beijing, for histochemical and IS-PCR studies. Five (9.8%) of the 51 cases of extrahepatic bile duct cancer (EBDC) showed positive reaction for HBsAg in the tumor tissue. Positive reaction was expressed as brownish granules in the cytoplasm of cancer cells, and no case was detected as inclusion bodies, membranous type, nor granules were seen in the nucleus or the cell membrane. A positive serum HBsAg was found in 3.9% of the 51 cases. Therefore, from this study, it was evident that HBV can infect the epithelial cells of the bile duct. In China, a positive rate of serum HBsAg was expected to be about 10% of the population. It may be concluded that, from pathological studies, HBV infection may not be strongly related with the

development of EBDC. However, as to the significance of HCV infection, the result may be on the contrary. Examination of the HCV NS₅ antigen in the 51 EBDC cases, 14(27.5%) showed positive expression in the cancer tissue. The pattern of antigen distribution in the cancer cells was similar to that of HBV granules, which was brownish granules distributed in the cell cytoplasm with no nucleus or cell membrane staining. Since the positive rate of anti-HCV was 0%-2.4%^[43,44] in Chinese population, the positive rate of 27.5% found in the cancer tissue was much higher than the average, and may indicate a positive relationship between HCV infection and the development of EBDC^[45,46]. This finding was in accordance with the recent report by Yin *et al*^[47]. The mechanism of cancerous changes of HCV infected bile duct mucosal cells may be the consequence of bile duct cell destruction and hyperplastic proliferation.

Further study was made with *in situ* polymerase chain reaction (IS-PCR) in paraffin-embedded sections of 51 cases of EBDC, the results showed that 8 (15.9%) of the 51 cases was positive for HBV, the positive cells of HBV DNA were mainly clustered in the cancer tissue. In 2 cases, the brownish granules were exclusively located inside the nucleus of the cancer cells. Therefore, there is a rather high incidence of HBV infection in EBDC in spite of a negative serum test for HBsAg, and the integration of HBV DNA in the cell nucleus may play an important role in the carcinogenesis of EBDC.

Study of positive HCV RNA in the 51 cases of EBDC with IS-PCR revealed that 18 (35.4%) had detectable HCV RNA, of which, 15 were plus-strand RNA, and 3 were minus-strand RNA. Twelve detectable RNA was located in the cytoplasm, and 6 were in the nucleus. The positive cells of HCV RNA were mainly clustered in the cancer cells. Of 34 control specimens, only 2(5.9%) had detectable HCV RNA. These results suggested that HCV infection might be involved in the carcinogenesis of carcinoma of extrahepatic bile duct.

In recent years, studies of cancer pathogenesis have provided evidences that cancer is a disease developed through a process of cytogenetic disorders. It is now known that activation of proto-oncogen and inactivation of tumor suppressive gene are the most common genetic alternations in tumor, especially the loss of the function of tumor suppressive gene is considered as a key step in tumor progression. So the restoration of the function of tumor suppressive gene is a major approach for gene therapy for cancer. In recent years, attention has been focused on the *p16* gene, which resides within a locus commonly deleted in established cancer lines (almost 75%).

In a series of 51 specimens of EHBC, we detected the expression of *p16* gene so as to identify

the relationship between the expression of *p16* gene and the occurrence of metastasis and prognosis of the patients. Furthermore, we investigated the efficiency to transfer interest gene (Ad-*p16*, Ad-*p53*), cooperation of Ad-*p16* with Ad-*p53*, Ad-*p16* with CDDP and evaluated their inhibitory effect on human cholangiocarcinoma cell line QBC939^[48] *in vitro* and Ad-*p16* with CDDP *in vivo*. The results revealed that expression of *p16* analyzed with S-P immunohistochemistry method, was found in 43.14% of the 51 EHBC, but the positive rate varied significantly in the pathological grading, nerve invasion, prognosis and option of surgical procedure ($P < 0.05$). The growth rates of the Ad-*p16*-infected QBC939 cells were inhibited by 35.1% (8 days), and Ad-*p53*-infected QBC939 cells were inhibited by 24.4% (8 days). The growth rates of the Ad-*p16*-infected QBC939 cells with CDDP (0.5mg/L) were inhibited by 77.7%. The suppression effects mediated by expression of the exogenous *p16* and *p53* in tumor cell resulted mainly from apoptosis and G1 arrest, while the suppression effects mediated by CDDP in tumor cell were mainly produced by apoptosis and G2 arrest. Experimental gene therapy on the nude mice model bearing subcutaneous tumor of QBC939 cells showed that intratumor instillation of Ad-*p16* and intra-abdominal instillation of CDDP inhibited the growth of the tumors. The average size of the Ad-*p16*-treated, CDDP-treated or Ad-*p16* with CDDP-treated tumors was inhibited by 30.0%, 41.0% and 62.6% respectively, as compared with that of the tumors injected with Ad-LacZ^[49,50]. However, gene therapy of EHBC cholangiocarcinoma, as in most of the malignant tumors, still has a long way to go.

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Helicobacter pylori infection and gastrointestinal hormones: a review

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INTRODUCTION

Helicobacter pylori (Hp) infection is closely related to gastrointestinal hormones and involves the formation of gastritis, gastric carcinoma and peptic ulcer^[1-7]. Its pathogenesis relevant to pathophysiological changes of gastrointestinal hormones are described as follows according to individual hormone.

HP INFECTION AND GASTRIN

Gastrin is a basic stimulus to parietal cells in producing gastric acid, and shows two ways in acid production: one directly stimulates parietal cell and the other acts on enterochromaffin-like (ECL) cell to release histamine by which stimulates parietal cells. So the formation of gastric acid and hyperchlorhydria is one of the physiopathological implications of gastrin. Hp infection in patients leading to increased release of gastrin from antral G cells and hypergastrinemia formation is currently an interesting medical problem^[8-11].

Hypergastrinemia produced by Hp infection

It is well known that Hp infection induces hypergastrinemia. In patients with normal gastroscopic features, those infected with Hp had significantly higher basal serum gastrin levels than non-infected individuals, and were similar to DU patients who are almost entirely infected with Hp. Therefore, hypergastrinemia seems to be associated with Hp infection, and is not a distinctive feature of DU disease^[12].

Hp-infected hypergastrinemia may be produced by cytokines^[13,14]: ① TNF- α : antral G cells (both

human and canine) undergoing TNF- α - pretreatment significantly increased in both basal and bombesin-stimulated gastrin release (compared to control); TNF- α increased in Hp infection, so the Hp-infected hypergastrinemia may be due to TNF- α - stimulation to G cells; ② IL-8: IL-8 stimulated gastrin release from isolated G cells, and this effect was dose-dependent and potentiated by Hp extract products.

The density of antral G cells was evaluated by expression of gastrin mRNA. The G cell density in patients with Hp infection was significantly higher than in controls; after eradication of Hp, the density was significantly lower as compared with pre-eradication value. The study suggests that increased gastrin mRNA is directly related to Hp infection^[15].

In a study of DU and non-ulcer dyspepsia (NUD), the Hp (absence of CagA gene and presence of VacA alleles s2 and m2) was only found in NUD (not with DU), and associated with lesser extent of gastrin increase and higher value of tryptase. The CagA negative s2m2 strain of Hp may be less dangerous for the gastric mucosa than other Hp strains, since it enhances tryptase production by gastric mucosal mast cells. This enzyme is thought to stimulate tissue turnover and favour wound healing^[16]. We may predict that the development of DU or NUD would probably depend upon the strains of Hp.

Combined serum levels of gastrin and pepsinogen (PG) were used to study Hp infection^[17-19]. As a screening procedure to show the status of Hp infection, both levels significantly increased in Hp infection, and significantly decreased after Hp eradication. The high levels may indicate Hp infection and the significant decrease after treatment may indicate cure, and reappearance of high levels suggests reinfection. The level of PG-II could be more useful in this situation^[18]. To classify DU, serum PG-I concentrations reflect the chief cell mass. Blood samples were taken before and at 15, 30, and 60 min after test meal, and the serum concentrations of fasting PG-I and gastrin were measured after meal. The area, under the serum gastrin 1h curve, was taken as integrated gastrin response (IGR). The DU patients (Hp positive) were divided into two groups (hyper-IGR and normal-IGR, with significant difference). The hyper-IGR DU patients

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had higher serum PG-I concentrations, and suggest to be acid hypersecretors^[19].

Change of acid (parietal cells) sensitivity to gastrin. In a study of three groups of subjects (DU patients, *Hp*-negative and *Hp*-positive healthy volunteers), the MAO (maximal acid output), and the acid sensitivity to gastrin were measured (serum gastrin required to achieve 50% MAO during intravenous administration of gastrin). The result revealed that the *Hp* positive healthy volunteers showed significantly higher gastrin concentrations, which meant less sensitive than the other two groups. The DU group had significantly higher MAO. Conclusively, a combination of decreased sensitivity to gastrin in infected healthy volunteers and increased maximal acid secretory capacity in patients with DU underlies their different acid response to *Hp*-induced hypergastrinemia, and they are caused by the hypergastrinemia^[8].

In patients with end stage of renal disease on dialysis, the hypergastrinemia was induced by *Hp* infection in stomach, and the serum gastrin concentrations were decreased to normal range following eradication of *Hp*^[20]. Either *Hp* infection or omeprazole administration can cause hypergastrinemia, and both of them would cause severe hypergastrinemia, which may exert potential deleterious effects. Since omeprazole treatment produced a similar percentage increase in serum gastrin, it is appropriate to eradicate *Hp* prior to commencing proton pump inhibitor treatment in order to reduce the degree of hypergastrinemia^[21,22].

Evidence indicated that gastrin concentrations decreased to normal range after *Hp* eradication^[11,23-26].

***Hp* INFECTION AND SOMATOSTATIN (SS)**

***Hp* infection and D cells**

Hp infection is associated with exaggeration of gastrin release following meals or bombesin stimulation attributed to a defect of SS secretion of antral D cells^[27,28]. Mucosal cytokines (TNF- α and IL-8 are predominant ones) were increased in *Hp* infection. TNF- α could stimulate the isolated canine gastric D cells to increase SS release dose-dependently, and the stimulatory effect was potentiated by IL-8 and inhibited by octreotide. In conclusions, TNF- α can regulate SS release from cultured D cells in a divergent manner^[29].

SS inhibition on *Hp* proliferation

In *Hp* infection, SS contents in antral mucosa and gastric juice, and number of D cells were decreased, and less expressions of SS mRNA and *Hp* eradication resulted in restoration of those indices, suggesting the *Hp* inhibition on SS release. In different SS concentrations *in vitro*, SS at

$10^{-11}\text{mol}\cdot\text{L}^{-1}$ significantly suppressed the proliferation of cultured *Hp*. SS at a similar level in human gastric juice ($\approx 10^{-11}\text{mol}\cdot\text{L}^{-1}$) indicated an inhibitory effect of SS in the gastric lumen on *Hp* proliferation in humans^[30].

Lipopolysaccharide (LPS) of *Hp* and SS

LPS inhibits the binding of SS to its gastric mucosal receptors. The antiulcer agents, sucralfate, ebrotidine and sulglycotide, possess the ability to restore the receptor-SS binding by 92.5%, 94.9% and 84%, respectively. Conclusively, LPS of *Hp* inhibits the SS binding to its receptor, and results in less SS effect, more gastrin and gastric acid secretion, and ulcer formation^[31,32].

***Hp* infection and gastrin-somatostatin equilibrium^[33-41]**

SS from D cells inhibits G cells, and decreased SS results in increase in gastrin secretion; gastrin stimulates, and SS inhibits parietal cells in gastric acid release. So, gastrin and SS form the gastrin-SS equilibrium, which was even called mechanism of gastric regulatory physiology. Phenomena related to imbalance of gastrin-SS equilibrium are as follows: ① hypergastrinemia: *Hp* infection influences D cells firstly to diminish SS release, then increases release of gastrin and acid, leading to the milieu favoring ulcer formation; eradication of *Hp* gets rid of the imbalance; the large mass of parietal cells in DU patients might be due to the long-term trophic effect of gastrin on parietal cells, and 6-12 months after *Hp* eradication, the MAO (representing parietal cell mass) fell significantly; this investigation supports the viewpoint; ② atrophic pangastritis: the degree and extent of gastritis affect destructive number of cells (D, G and parietal) which lead to the released amounts of SS, gastrin and acid in severe destruction; the resultant hyperchlorhydria tends to ulcer formation, and hypochlorhydria to develop carcinoma; pangastritis involving much more D cells and parietal cells may play an important role in imbalance of gastrin-SS equilibrium; ③ increased cytokines (TNF- α and IL-1 β) in *Hp* infection lead to gastrin-SS imbalance with hyperchlorhydria or hypochlorhydria, and then forming DU or carcinoma; ④ hypergastrinemia of *Hp* (CagA) gastritis is due to decreased density of D cells; ⑤ water extract of either *Hp*(CagA and VacA) positive or *Hp*(CagA and VacA) negative delayed ulcer healing as compared with saline controls in rats, because of impairment of gastrin-SS link; ⑥ in *Hp*(+) DU patients, the amount change of T lymphocyte subsets was obviously correlated with those of gastric active inflammation, serum gastrin, and SS in gastric juice, indicating that gastrin and SS played a role in immune regulation. Gastrin-SS equilibrium restores after *Hp*

eradication. SS may be the most widely effective gastrointestinal hormone in human body, and it is worthy of further studies^[42].

***Hp* INFECTION AND GASTRIN RELEASING PEPTIDE (GRP)**

GRP can stimulate gastric acid secretion, and is particularly valuable in detecting disturbances of gastric secretory function of patients with DU and *Hp* infection. Its attractiveness lies in the fact that it simultaneously activates many physiological control processes, both stimulatory and inhibitory. This facilitates the detection of a defect in any of the controls involved in regulating biological function. Other gastrointestinal functions such as gallbladder contraction, pancreatic secretion and gastroesophageal motility are subject to complex regulatory controls, and GRP may also be of value in investigating disturbances of these processes^[43].

GRP effect on gastrin and SS mRNAs in humans infected with Hp

GRP stimulates gastrin secretion, but also inhibits its release via SS. Exogenous GRP stimulates a greater increase in plasma gastrin concentrations in *Hp* infected patients than in uninfected controls. This is due to less inhibition on gastrin mRNA in *Hp* infection, probably because of low stimulated SS levels in *Hp* infection^[44].

PAO from GRP in DU patients before and after Hp eradication

In a study of gastric acid increment in DU patients, the GRP stimulated PAO (PAOGRP) and pentagastrin stimulated PAO (PAOPG) were measured in *Hp* positive DU patients and in *Hp* negative as controls. This study has shown that BAO, PAOGRP and PAOPG are significantly higher in *Hp* positive DU than in *Hp* negative controls. All decreased significantly six months after *Hp* eradication to fall within the range of controls. These results are compatible with a hypothesis that acid hypersecretion in DU is caused by *Hp* infection^[45]. Peterson's investigation showed a similar result that GRP stimulated hypergastrinemia and hyperchlorhydria which were lowered to normal range after *Hp* eradication^[46].

HP INFECTION AND GROWTH FACTORS

***Hp* infection and epidermal growth factor (EGF)**

EGF was found in at least seven individuals, and four of them were expressed in gastrointestinal tract. They were EGF, transforming growth factor alpha (TGF- α), amphiregulin (AR), and heparin binding-epidermal growth factor (HB-EGF). EGF and TGF α have the same receptor, EGFr. EGFs combined to their receptors which widely existed

over cellular membrane to regulate cell growth and play biological roles. EGF serves gastrointestinal tract with cell growth, ulcer healing and suppression of acid secretion^[47].

Acute exposure to *Hp* caused cell damage and impaired the processes of cell migration and proliferation in cultured gastric mucosal cells *in vitro*. EGF-related growth factors play a major role in protecting gastric mucosa against injury, and are involved in the process of gastric mucosal healing. In the study of Romano *et al*, using MKN 28 gastric mucosal cells (derived from gastric adenocarcinoma), *Hp* increased mucosal (MKN 28 cells) generation of EGF-related peptides; the inhibitory effect of *Hp* on the reparative events mediated by EGF-related growth factors might play a role in the pathogenesis of *Hp*-induced gastrointestinal injury^[48].

Hp inhibits both EGF binding to its receptors and EGF-stimulated gastric cell proliferation, and they are the mechanisms of peptic ulcer formation and its difficulty in healing^[49-52]. In a study of gastric luminal release of EGF, the stomach was capable of secreting large amounts of EGF, and pentagastrin appeared to be a potent stimulus to gastric EGF release; the *Hp* infection reduced the release of gastric EGF, and eradication of *Hp* resulted in the augmentation of basal and pentagastrin-induced EGF release into the stomach. Since the eradication of *Hp* infection in DU patients resulted in DU healing which was accompanied by an increase in EGF release, conclusively, EGF plays a crucial role in DU healing^[53,54]. Accordingly, it also plays a major role in ulcer formation.

***Hp* infection and hyperproliferation of gastric mucosal epithelium**

The overexpression of C-myc gene protein and EGFr (receptor of EGF) may be the molecular basis for hyperproliferation of gastric mucosal epithelium in *Hp* infection^[55].

***Hp* infection and growth factors in gastric juice**

The EGF concentrations in gastric juice were affected by *Hp* and pH. There were four situations in NUD patients studied: ① EGF was 80% lower in *Hp*(+) than in *Hp*(-); ② those with pH<4.0 in gastric juice had significantly lower EGF concentrations; ③ those with gastric juice pH>4 showed similar concentrations of EGF in both *Hp*(+) and *Hp*(-) groups; ④ those with both *Hp*(+) and pH<4.0 had further reduction in EGF concentrations. These results suggested that *Hp* may elaborate factors that accelerate its proteolytic degradation or inhibit its rate of synthesis and/or secretion; pH reduction (<4) may increase EGF degradation; the diminished content of EGF at low pH, especially in *Hp*-positive patients, may

facilitate the development of mucosal damage. The TGF- α concentrations in gastric juice remained unaffected by *Hp* or pH^[56].

The cytoprotective effect of sulglycotide

In studying *Hp* protease activity and its suppression by sulglycotide, it was found that the *Hp* protease evoked a 61.7% degradation of PDGF (platelet derived growth factor) and a 62.3% degradation of TGF β ; introduction of sulglycotide to the reaction assay system caused a dose-dependent inhibition in PDGF and TGF β proteolysis by the *Hp* protease; the maximal inhibitory effect was obtained with sulglycotide at 100mg·L⁻¹, at which dose an 84.4% decrease in PDGF and 88.3% decrease in TGF β degradation were achieved; the results provide a strong evidence for the effectiveness of sulglycotide in the protection of gastric mucosal growth factors against degradation by *Hp*^[57].

Eradication of Hp related to EGF and TGF

EGF and TGF- α are potent gastric secretory inhibitors, mitogens, and mucosal protectors, and their gastric mucosal expression and luminal contents are closely related to *Hp* infection. A study of DU and NUD patients showed that chronic *Hp* infection and resulting antral gastritis were associated with increased plasma gastrin and increased mucosal cell proliferation, probably due to enhanced expression of EGF and TGF- α ; the *Hp* eradication decreased plasma gastrin, but the increase in gastric EGF and TGF- α contents was sustained, suggesting that they may be involved in ulcer healing^[58].

Effect of *Hp* infection on gastric mucosal expression of EGF, TGF- α , and EGFr. In a study of Konturek *et al*, the DU patients with *Hp*(+) were accompanied by increased mucosal expression and contents of TGF- α , EGF, and EGFr, and eradication of *Hp* infection enhanced all of them and contributed to ulcer healing^[59]. Russo *et al* also indicated that *Hp* eradication resulted in a significant increase in expression of EGF and TGF- α ; *Hp* possibly inhibited the mucosal expression of EGF and TGF- α ^[60]. The results from different authors seem not wholly consistent. It is appropriate to study further.

Implications of Hp, mucosal growth factors and gastric acid in pathogenesis of DU and gastric carcinoma

It has been revealed that patients with DU and gastric carcinoma possessed 3 common features which contributed to the pathogenesis of the two diseases: they were *Hp* infection, increase in gastric mucosal and luminal growth factors (EGF, TGF- α), and hypergastrinemia. They remained different in hyperchlorhydria in DU patients, and in patients

with gastric carcinoma. These changes returned to normal values two years after *Hp* eradication in DU patients. The hypochlorhydria was possibly due to atrophy of oxyntic mucosa and overexpression of growth factors in gastric mucosa may be implicated in the pathogenesis of both DU and gastric cancer. Hypergastrinemia, hypochlorhydria, and increase in mucosal growth factors are predisposing to gastric cancer^[61].

Hp infection and insulin-like growth factor-1 (IGF-I)

Taha *et al* measured gastric and fasting serum concentrations of IGF-I in patients with and without *Hp* infection. As a result, IGF-I was detected at very low concentrations in gastric juice and in mucosal incubates. The median serum IGF-I concentration was 88 μ g·L⁻¹ in the patients infected with *Hp* compared with 90 μ g·L⁻¹ in the non-infected controls; IGF-I concentrations significantly dropped to 77 μ g·L⁻¹ following eradication therapy. Conclusively, the similarity in baseline IGF-I concentrations in the presence and absence of *Hp* suggests that their subsequent drop after treatment is more likely to be produced by the treatment^[62].

HP INFECTION AND OTHER GASTROINTESTINAL HORMONES

Hp infection and insulin

It is true that *Hp* gastritis resulted in increased secretion of basal and meal-stimulated gastrin, which is also a physiologic amplifier of insulin release. In order to confirm whether *Hp* gastritis may enhance nutrient-stimulated insulin secretion, both glucose and a mixed meal stimulated insulin response were investigated in *Hp* positive gastritis and *Hp* negative control subjects. The areas under the curve (AUC) for serum insulin following both oral glucose and a mixed meal in the patients with *Hp* gastritis were significantly higher than those of non-*Hp* controls. After *Hp* eradication, the AUC for serum insulin following oral glucose and mixed meal decreased significantly, and serum basal and meal-stimulated gastrin levels also obviously decreased. These results suggest that *Hp* gastritis enhances glucose and meal-stimulated insulin release probably by increasing gastrin secretion^[63].

Hp infection and glicentin

Glicentin seems to promote intestinal metaplasia (IM) in the gastric mucosa. In order to clarify whether *Hp* infection accelerates glicentin gene expression, glicentin mRNA was investigated using gastric biopsies. The results disclosed that glicentin mRNA was significantly correlated with histological IM and was positively correlated with *Hp* infection. Conclusion is that *Hp* infection is associated with

the induction of glicentin in the gastric mucosa, thus supporting the hypothesis that *Hp* infection accelerates IM of the stomach^[64].

Hp infection and cholecystokinin (CCK)

In healthy subjects, CCK has the feedback control of postprandial gastrin release and gastric acid secretion, but in *Hp*-positive DU patients the CCK loses its control effect. Eradication of *Hp* restores the inhibitory effect of CCK on postprandial gastrin release and gastric acid secretion. This suggests that *Hp* infection eliminates or lessens the inhibitory effect of CCK on gastrin release^[65].

Hp infection and gastric inhibitory polypeptide (GIP)

There was no significant difference of serum GIP levels among groups of gastrectomy (total, subtotal), healthy subjects, *Hp* infection, age, gender, body mass index, smoking. Only the elapsed time since operation in patients following total gastrectomy exhibited a significant positive correlation with their GIP levels ($r = 0.89$, $P < 0.05$). Hence GIP is less important in mediating gastric acid secretion, and *Hp* does not influence its levels^[66].

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Long term omeprazole therapy for reflux esophagitis: follow-up in serum gastrin levels, EC cell hyperplasia and neoplasia

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Subject heading gastroesophageal reflux; proton pump inhibitors; enterochromaffin cell; hypergastrinemia; carcinoid tumor; biopsy

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Abstract

AIM To evaluate the long-term safety of omeprazole in patients of gastroesophageal reflux disease resistant to treatment with H₂ receptor antagonist.

METHODS We prospectively followed 33 patients on omeprazole therapy for severe erosive esophagitis for 5-8 years, with periodic gastrin levels, *H. pylori* infection, gastric biopsies for incidence of ECL cell hyperplasia, carcinoids, gastric atrophy and neoplasia. A total 185 patient follow-up years and 137 gastric biopsies were done.

RESULTS Among the 33 patients, 36% reached their peak gastrin levels in an average of 8 months to one year, then drifted Down slowly over 1-2 year period to just above their baseline level, 24% of the patients had a peak gastrin level above 400ng·L⁻¹ and one patient had a peak level above 1000ng·L⁻¹. One patient had a mild ECL cell hyperplasia which was self limiting and did not show any dysplastic changes. Eighteen percent of patients were positive for *H. pylori* infection. The gastric biopsies did not show gastric atrophy, intestinal metaplasia or neoplastic changes.

CONCLUSION In a series of 33 patients followed for 5-8 years on omeprazole therapy

for severe reflux esophagitis, we did not observe any evidence of significant ECL cell hyperplasia, gastric atrophy, intestinal metaplasia, dysplasia or neoplastic changes.

INTRODUCTION

Acid suppression therapy plays a pivotal role in the medical management of reflux esophagitis. The most recently developed acid suppressive agents, the proton pump inhibitors (PPIs) directly inhibit hydrogen ion exchange and inhibit acid secretion in response to all stimulatory agents^[1-5]. The PPIs are benzimidazole derivatives, which are converted to active metabolites within the acidic confines of the secretory canaliculi of the gastric parietal cells. They promote oxidation of sulphhydryl components of the proton pump, leading to irreversible inactivation of the enzyme^[1]. Recovery of acid secretion requires synthesis of new proton pumps. There is now a definite evidence that PPIs are more effective than H₂ receptor antagonists in treating esophagitis, severe refractory reflux disease and non-healing esophageal ulcers, providing faster healing and symptomatic relief^[6-9]. Esophagitis is however a chronic problem, as early relapses have been observed after cessation of H₂ blockers and omeprazole^[7,8]. Therefore, maintenance treatment is often required in treating the reflux esophagitis. H₂ blocker has failed, as an effective maintenance agent in preventing the relapses^[9,10]. There is enough evidence to support the role of PPIs as an effective agent in maintaining healing of erosive esophagitis^[8,11-16]. However, questions regarding the long-term safety of the proton pump inhibitors have been raised. The resulting hypo-/achlorhydria and resulting hypergastrinemia have been implicated in the development of enterochromaffin cell hyperplasia and gastric carcinoids^[17-20]. The epidemiological evidence of increased incidence of gastric fundal carcinoma has further raised concerns regarding, gastric atrophy and the long-term use of PPIs in acid related peptic diseases, especially in patients with *H. pylori*

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infection^[21].

Considering the efficacy of PPIs and possibility of their role in development of EC cell hyperplasia, it was logical to study their safety profile as long term maintenance therapeutic agents in esophagitis.

MATERIALS AND METHODS

At Gastroenterology Department, Long Island Jewish Medical Center 33 patients with severe reflux esophagitis of grade 2 and above, whose symptoms were not responding to H2 blockers and motility agents were enrolled in the study. Patients were started on omeprazole (PPI) either daily or on an alternate day regimen and were followed periodically for symptom relief, endoscopic healing, plasma gastrin level, *H. pylori* infection and gastric biopsies for occurrence of neoplasia. Esophagitis was graded endoscopically using the following scale: grade 0, normal appearing; grade 1, mucosal edema, hyperemia and/or friability; grade 2, one or more erosions/ulcerations involving <10% of the distal five cm of the esophagus; grade 3, erosions/ulceration's involving 10% to 50% of the distal 5cm of the esophagus or an ulcer 3mm-5mm in diameter. In cases of Barrett's esophagus, the area 5cm proximal to the squamo-columnar junction was evaluated; grade 4, multiple erosions involving 50% of the distal 5cm of the esophagus or a single ulcer >5mm in diameter. Whenever clinically feasible, all endoscopies for a particular patient were performed by the same endoscopist. Complete healing of erosive reflux esophagitis was defined as the return of esophageal mucosal inflammation to grade 0 or grade 1.

Periodic plasma gastrin levels were measured after an 8 hours fast prior to endoscopy. Four gastric biopsy specimens of full thickness of the mucosa (two fundal, two antrum) were obtained at the screening visit and at the end of 8-12 weeks to assess healing. At intervals of 6 to 12 months periodic upper endoscopies were performed to monitor for carcinoids, gastric atrophy and gastric neoplasia. Bouin's fixed, paraffin-embedded, 3 microns hematoxylin and eosin-stained sections of each biopsy specimen were evaluated and graded for active and chronic inflammation. The presence of intestinal metaplasia, atrophy, dysplasia and neoplasia was evaluated. The enterochromaffin like cells of the oxyntic gastric mucosa were assessed using Grimelius stain sections and Solcia's scale of gastric endocrine growth. Two independent pathologists who were blinded to each other's assessments of the biopsy specimens reviewed gastric biopsies.

H. pylori infection was assessed by identifying the organisms and chronic inflammation in the biopsy specimens, serum *H. pylori* IgG antibodies or by CLO test. Out of the 33 patients, 22(66%) were male and 11(34%) were female (Table 1).

The mean age of the patients was 76 years with a range of 34 to 86 years (Figure 1). Twenty-six (78%) patients were on omeprazole daily 10mg-20mg or 10mg-20mg twice daily. Seven (21%) patients were on alternate day 10mg-20mg omeprazole therapy for severe reflux esophagitis (Table 1). The average period of follow-up was for 6 years, with a range of 3 to 8 years, a total of 185 patient years of follow up (Figure 2). The total number of biopsies done was 137, averaging 4, with a range of 2 to 13.

Table 1 Type of treatment and sex distribution (n=33)

Group	Number
Alternate day omeprazole therapy	7
Daily omeprazole therapy	26
Male patients	22
Female patients	11

RESULTS

Twelve (36%) of the patients reached their peak plasma gastrin levels in one year, and then drifted to a level above their baseline levels in 1 to 2 years. Three (9%) reached their peak in 2 years, 5 (15%) in 3 years, 3 (9%) in 4 years, 5 (15%) in 5 years, 2 (6%) in 7 years, 1 (3%) in 8 years (Figure 3). Eleven (33%) of patients had plasma gastrin levels below 100pmol·L⁻¹, 24% of patients above 400pmol·L⁻¹ and in one patient it was above 1000 pmol·L⁻¹. Gastric biopsies showed normal mucosa on initial biopsy in 26 and gastritis in 7. On repeat biopsy 7 changed from normal to gastritis and 4 from gastritis to normal. Nineteen were normal at all times. Of the 6 *Hp*+ (*H. pylori* positive) patients, 1 was normal on all occasions, 2 showed gastritis, 2 varied from normal to gastritis and 1 from gastritis to normal. Of the 27 *Hp* negative patients, 18 were normal at all times, 1 showed gastritis, 5 varied from normal to mild gastritis and 3 from gastritis to normal. No atrophy was diagnosed at any time over the 5-8 years, on any of the 137 biopsy specimens.

EC cell hyperplasia was seen in one of the biopsies. Neither fundal gastric neoplasia, nor carcinoids were seen (Table 2).

Table 2 Results of gastric biopsies in-patients on long term omeprazole therapy for reflux esophagitis

Pathology	Number
Normal throughout the treatment	19
Normal at onset, later gastritis changes	7
Initial fundal gastritis	7
Initial gastritis changed to normal	4
ECL cell hyperplasia	1
Barretts esophagus	4
<i>H. pylori</i> infection +ve	6
<i>H. pylori</i> infection -ve	27

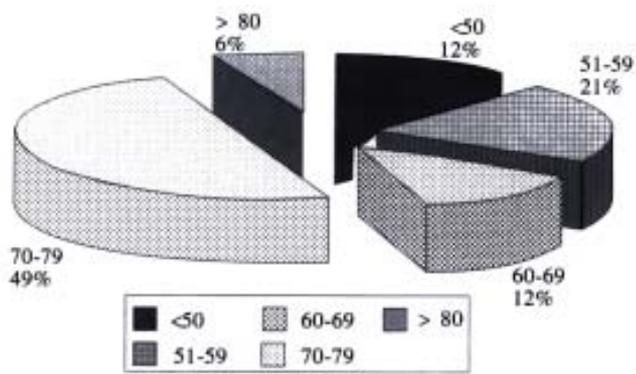


Figure 1 Age distribution of patients on long-term omeprazole treatment for reflux esophagitis.

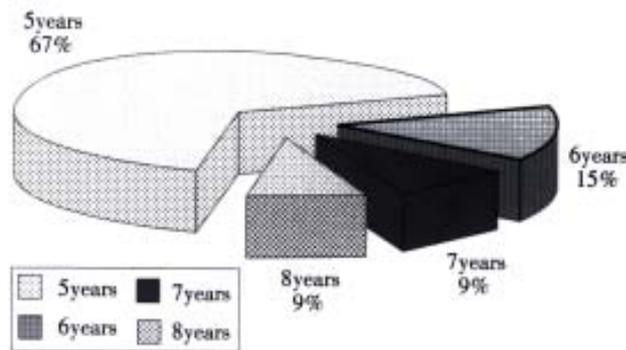


Figure 2 Distribution of follow-up years of patients on long-term omeprazole treatment for reflux esophagitis.

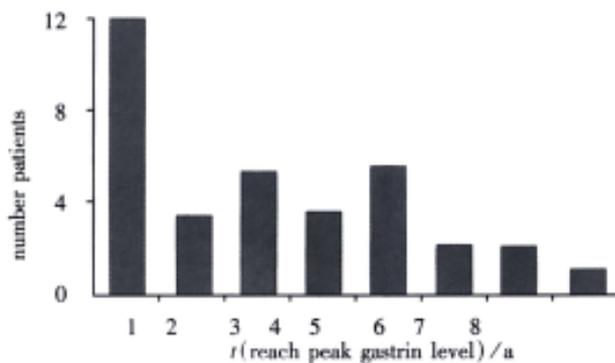


Figure 3 Time to reach maximum plasma gastrin levels in patients treated with long term omeprazole.

DISCUSSION

Esophagitis is a chronic problem with frequent early relapses^[7,8]. Therefore, it is not only important to treat the reflux disease but also to prevent relapses. It is now known that PPIs are more effective than H-2 receptor antagonist in treating refractory reflux disease^[1,6,12-16] providing faster healing and symptomatic and in preventing early relapses^[9,10]. Concerns were raised regarding the safety of PPI as a long-term maintenance agent^[17-19]. In our study, we found omeprazole to be a safe drug even when used over a long period. Hypochlorhydria,

encountered with PPIs has strong association with hypergastrinemia^[28]. Hypochlorhydria leads to fall in the secretion of somatostatin from antral D cells. Somatostatin is a major negative feedback mediator of gastrin release and its absence leads to persistent gastrin release^[22-25]. Because PPI's are better inhibitors of gastric acid secretion than H-2 receptor antagonist is, they are associated with higher gastrin levels. Freston *et al* and other studies showed that plasma gastrin levels generally peak in the first four months of treatment with PPIs and stabilize without further increase thereafter^[26,27]. In our studies, the gastrin level peaked on an average in initial 8-12 months of treatment. The early peak in other studies could have been because of development of gastric atrophy in some of their patients^[26,27]. The same reason was postulated for the eventual decline in gastrin level. Although we also observed the decline in gastrin level after a period of 1-2 years, but we did not observe gastric atrophy in any of our patients. It is not yet clear why after some time the gastrin levels came down. The consequences of hypergastrinemia have aroused interest because gastrin has a trophic effect on gastrin cell, especially ECL cells^[26,27]. Rat model studies, have revealed that sustained hypergastrinemia secondary to PPIs are associated with increased ECL cell hyperplasia and carcinoid tumor^[26-28]. These changes regress on bringing back the gastrin level to previously normal levels. In our study, we did not observe the similar relation between gastrin and ECL cell hyperplasia and carcinoid. This is in concordance with other studies done on long term safety profile of PPIs^[8,11-16,29]. However, it would be in appropriate to associate similar relations in humans, given that rats have a higher density of gastrin ECL cells and a greater gastric response to hypochlorhydria than humans^[21,30]. This is further supported by the fact that, PPIs when used in other animals (e.g. dogs, guinea pigs, hamsters, mice), do not cause ECL cell carcinoids.

Borch *et al*^[31] identified ECL cell carcinoids in approximately 4% of patients with pernicious anemia, thus raising the concern that this may be of relevance to use of PPIs. This seems unlikely as patients who developed carcinoid in his study had gastrin level 10-20 times normal level versus 2-4 folds during omeprazole treatment. The carcinoids were observed on an average after a period of 19 years of persistent severe hypergastrinemia^[31]. The development of carcinoid has also been observed in patients with hypergastrinemia secondary to Zollinger Ellison syndrome. All of these cases had coexisting familial MEN-I α -syndrome^[28]. This combined group of ZES and MEN-I α comprises 25% of all Zollinger Ellison syndromes and the fact that all the cases of carcinoid has been observed only in this subgroup suggests that familial factor also plays an important role in carcinoidogenesis^[28].

It would appear from available information that severe and sustained hypergastrinemia is required to produce ECL cell hyperplasia. When coupled with other factors, such as genetic trait of MEN-1 syndrome, it may lead to carcinoid formation.

In conclusion, our results show that omeprazole is a safe drug, when used for a long-term maintenance treatment in healing reflux disease.

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Detection of hepatitis C virus NS5 protein and genome in Chinese carcinoma of the extrahepatic bile duct and its significance

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Subject headings hepatitis C virus; bile duct neoplasm; polymerase chain reaction; immunohistochemistry; risk factors; genes, suppressor, tumor; transfection; genome

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Abstract

AIM To investigate the hepatitis C virus (HCV) infection in the tissues of carcinoma of extrahepatic bile duct and study their correlation.

METHODS HCV NS5 protein and HCV RNA were detected by labeled streptavidin biotin (LSAB) method and *in situ* reverse transcription polymerase chain reaction (IS-RT-PCR) in sections of 51 cases of carcinoma of extrahepatic bile duct and 34 cases of control group (without malignant biliary disease).

RESULTS In 51 cases of carcinoma of extrahepatic bile duct, HCV NS5 protein was detected in 14 (27.5%), which was clearly stained in the cytoplasm of cancer cell but not in the nucleus or cell membrane. HCV RNA was detected in 18 (35.4%), which was located in the nucleus of cancer cell in 12 cases and in the cytoplasm in 6 cases. HCV NS5 protein and RNA coexistence was found in 2 cases. In 34 cases of control group, HCV RNA was detected in 2 (5.9%). HCV NS5 protein and RNA positive cells were found either scattered or in clusters.

CONCLUSION The prevalence of hepatitis C

viral infection in the tissues of carcinoma of extrahepatic bile duct was significantly higher than in control group ($\chi^2=9.808$, $P=0.002$). The findings suggest a correlation between HCV infection and carcinoma of extrahepatic bile duct, which is different from the traditional viewpoint. HCV infection might be involved in the development of carcinoma of extrahepatic bile duct.

INTRODUCTION

Cancer of bile duct arises from malignant transformation of the epithelia of bile duct. It is even less common than gallbladder carcinoma, and seen in 0.01%-0.46% of all autopsies^[1] and its pathogenesis has not been fully understood. Cancer of bile duct is different from hepatocellular carcinoma (HCC) in etiologic factors, the former is not correlated to HBV or HCV infection and liver cirrhosis, traditionally^[2]. It has been reported that only 10%-20% patients with bile duct cancer accompany liver cirrhosis, but 70%-90% patients with HCC are associated with liver cirrhosis^[3-5]. There is a high incidence of bile duct cancer in the southeast Asia, and liver fluke infection due to clonorchis sinensis and opisthorchis viverrini is the most frequently cited cause of bile duct cancer^[6,7]. Now the development of bile duct cancer has been linked to hepatolithiasis, clonorchis sinensis, congenital dilatation of bile duct, and chronic inflammatory bowel disease^[8-11]. In China, 0.33%-9.7%, patients with hepatolithiasis^[12,13], 2.1%-21% with the choledochal cyst^[14-17] and 0.22% with clonorchis sinensis infestation^[18] are simultaneously complicated with bile duct cancer. Bile duct cancer simultaneously complicated with gallstone, choledochal cyst, and clonorchis sinensis infestation accounts for 6.15% - 16.9%^[19-22], 7%^[15] and 6.4%^[23] in total bile duct cancer of the corresponding period, respectively. But the incidence of bile duct cancer complicated with ulcerative colitis simultaneously is not estimated in literature in China now. These investigations indicated, therefore, that Chinese patients with bile duct cancer suffered from above-mentioned diseases before only account for one third or a half of the

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total patients with bile duct cancer. In extrahepatic biliary carcinoma in China, carcinoma of extrahepatic bile duct covered 75.2%^[24], the incidence of carcinoma of extrahepatic bile duct tends to increase over the past decade, but its cause is still unclear^[24].

Hepatitis C virus (HCV) is a RNA virus with a genomic size of 9.6kb, and now known to be the chief cause of transfusion-associated non-A, non-B hepatitis, which has been reported to occur in 7%-10% patients who received transfusion^[25,26]. More than 50% of individuals exposed to HCV develop chronic infection. Of those chronically infected individuals, about 20%-30% will develop liver cirrhosis and/or HCC when followed for 20 or 30 years^[27]. In China, the prevalence of HCV antibodies in blood donors as measured by the second or third generation assays is about 0%-4.6%^[28,29], and in a rural population the HCV infection rate is up to 15.3%^[30], which indicates that China is a relatively high incidence area of HCV infection. *In situ* reverse-transcription polymerase chain (IS-RT-PCR) has been successfully applied to the detection and localization of HCV RNA in formalin-fixed paraffin-embedded liver sections^[31,32], indicating that it becomes easy to detect the low level of HCV RNA. This study aims to investigate the HCV infection in the tissues of carcinoma of extrahepatic bile duct (51 cases) and control group specimens (34 cases) by detecting HCV NS5 protein and RNA using labeled streptavidin biotin (LSAB) method and IS-RT-PCR, and to determine their correlation.

MATERIALS AND METHODS

Carcinoma of extrahepatic bile duct tissues

Fifty-one cases of carcinoma of extrahepatic bile duct, and 34 specimens as control group (including 10 cases of choledochal cyst, 8 cases of hepatolithiasis, 2 cases of congenital dilatation of the intrahepatic bile duct, 2 cases of cystadenoma and 2 cases of adenoma of common bile duct, and 10 cases of wall of extrahepatic bile duct near gallstone) were collected from Department of Hepatobiliary Surgery, General Hospital of People's Liberation Army (PLA). All specimens were resected from 1995 to 1998, and fixed and embedded routinely. All carcinomas of extrahepatic bile duct were diagnosed as adenocarcinoma by the Department of Pathology, General Hospital of PLA. Five-micrometer thick for malin-fixed paraffin-embedded sections were prepared.

Primers and probe preparation

Primers and probe were all located at the highly conserved 5' non-coding region of the HCV genome. The oligonucleotide primers and probe were synthesized, and the probe was labeled with biotin (Sangon Co.Ltd). The sequences of outer primers are: sense, 5'-GGCGACACTCCACCATAGATC 3' (1-

21nt), antisense, 5' GGTGCACGGTC-TACGAGACCT 3' (304-324nt). The sequences of inner primers are: sense, 5' CTGTGAGGAAC-TACTGTCTTC 3' (28-48nt), antisense, 5' CCC-TATCAGGCAGTACCACAA 3' (264-284nt). Probe sequence is: 5' ACACCGGAATTGC-CAGGACGACCGGGTCTTTCTTG 3' (142-177nt).

HCV NS5 protein detection by LSAB method

The sections were dewaxed and rehydrated routinely, and then treated for 5min with 0.03% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. Antigen of the tissues was prepared in microwave oven. Sections were incubated with normal goat serum for 5min, and then incubated with anti-HCV-NS5 IgG (diluted 1:50) at 4°C overnight. After washed with phosphate buffered saline (PBS), sections were incubated with biotin-labeled goat anti-mouse IgG (diluted 1:200) for 45 min at 37°C. Sections were then incubated with horseradish peroxidase streptavidin (S-A/HRP) for 45min at 37°C. After washed with PBS, peroxidase activity was developed using 3,3'-diaminobenzidine (DAB, 0.3g/L) for 15min. The DBA detection method yields a yellow precipitate. Sections were subsequently counterstained with hematoxylin.

Negative control groups

PBS substituting for anti-HCV-NS5 IgG, biotin-labeled goat anti-mouse IgG, S-A/HRP, and omitting DBA in procedure served as negative controls, respectively.

HCV RNA detection by is RT-PCR method

The sections were deparaffinized with fresh xylene and graded alcohols, followed by PBS for 5min. The tissues were digested with proteinase K (30mg/L, 37°C, Sigma) for 15min and rinsed with DEPC-treated PBS three times, and then treated with Rnase-free Dnase I(700U/mL, Promega) at room temperature overnight or 37°C 2h in a humidified chamber. The sections were then fixed twice in 95% and 100% alcohol each for 3min. RT was achieved with 30µL- RT solution for each section (1×RT buffer, dNTP 250µM each, antisense of outer primer 1µM, Rnasin 1U/µL, AMV reverse transcriptase 0.4u/µL Promega) in a humidified chamber at 42°C for 60min. The reaction solution was dripped away and was hed with DEPC-treated PBS twice each for 5min, then fixed twice in 95% and 100% alcohol each for 2min, and the 50µL PCR solution for each section was applied which consisted of MgCl₂ 2.5mM, 1×PCR buffer, each primer 1µM, dNTP 250µM, *Taq* DNA polymerase 4U/50µL and BSA 3g/L. The "hot-start" approach was employed during which *Taq* DNA polymerase was added at 80°C. The *In situ* amplification of target sequences was performed in a

thermal cycler (GeneAmp *In situ* PCR System 1000 [Perkin Elmer]), using two primer pairs. The cycling conditions used were: the initial denaturation step at 94°C for 4min followed by 20 cycles of denaturation at 94°C for 2min, annealing at 55°C for 1.5min, and final extension of 72°C for 3min. The cover slip was removed. The sections were washed with PBS for 5min and fixed in 100% alcohol for 10min, then second PCR amplification was made. Except inner primers substituted for outer primers, the remaining steps were the same as the initial PCR amplification. The cover slip was removed. The sections were washed with PBS, and fixed in 100% alcohol for 10min. Sections in PBS were heated at 80°C for 10min, and put on ice. Then hybridization solution (probe 2.5mg/L, 50% deionized formamide, 5×SSC, 1×Denhardt's solution, sssDNA 100mg/L) was added on the slides at 37°C overnight. The section was washed with serial SSC, and covered with 10% normal sheep serum. The specimens covered with S-A/HRP at 37°C for 45min. DAB solution was added in slides at 37°C for 15min. The sections were incubated in the dark and checked at a 5min interval. The DBA detection method yields a yellow precipitate. After development, the sections were counterstained with hematoxylin. Positive cells and their histological distribution were examined.

Negative control groups

Hepatitis B liver tissues; HCV RNA positive specimens digested by Rnase (10g/L) at 37°C for 1h; HCV RNA positive specimens omitted AMV reverse transcription; HCV RNA positive specimens omitted Taq polymerase; and no probe control.

RESULTS

In 51 cases of carcinoma of extrahepatic bile duct, HCV NS5 protein was detected in 14 (27.5%), which was clearly stained in the cytoplasm of cancer cell but not in the nucleus or cell membrane. The positive signals of NS5 protein were distributed diffusely in the cytoplasm (Figure 1). The positive immunochemical reaction was not obtained in the same section when PBS substituted for anti-HCV-NS5 IgG, as did omission of the primary antibody from the staining procedure.

HCV RNA sequence was detected in 18 (35.4%) of 51 cases of carcinoma of extrahepatic bile duct. HCV RNA was located in the nucleus in 12 cases (Figure 2), and in the cytoplasm in 6 cases (Figure 3). In 34 cases of control group, HCV RNA sequences were detected in 2 (5.9%) (Figure 4). The HCV RNA positive signal was located occasionally in the mononuclear cells. After treated by Rnase or omitting AMV, Taq polymerase and probe in the procedure, no sections showed HCV RNA positive signal.

HCV NS5 protein and RNA coexistence were found in 2 cases. HCV NS5 protein and RNA

positive cells were found to be either scattered or in clusters. In the cytoplasm, some positive signals of HCV NS5 protein and RNA were so strong that it might be difficult in determining nucleic positive.

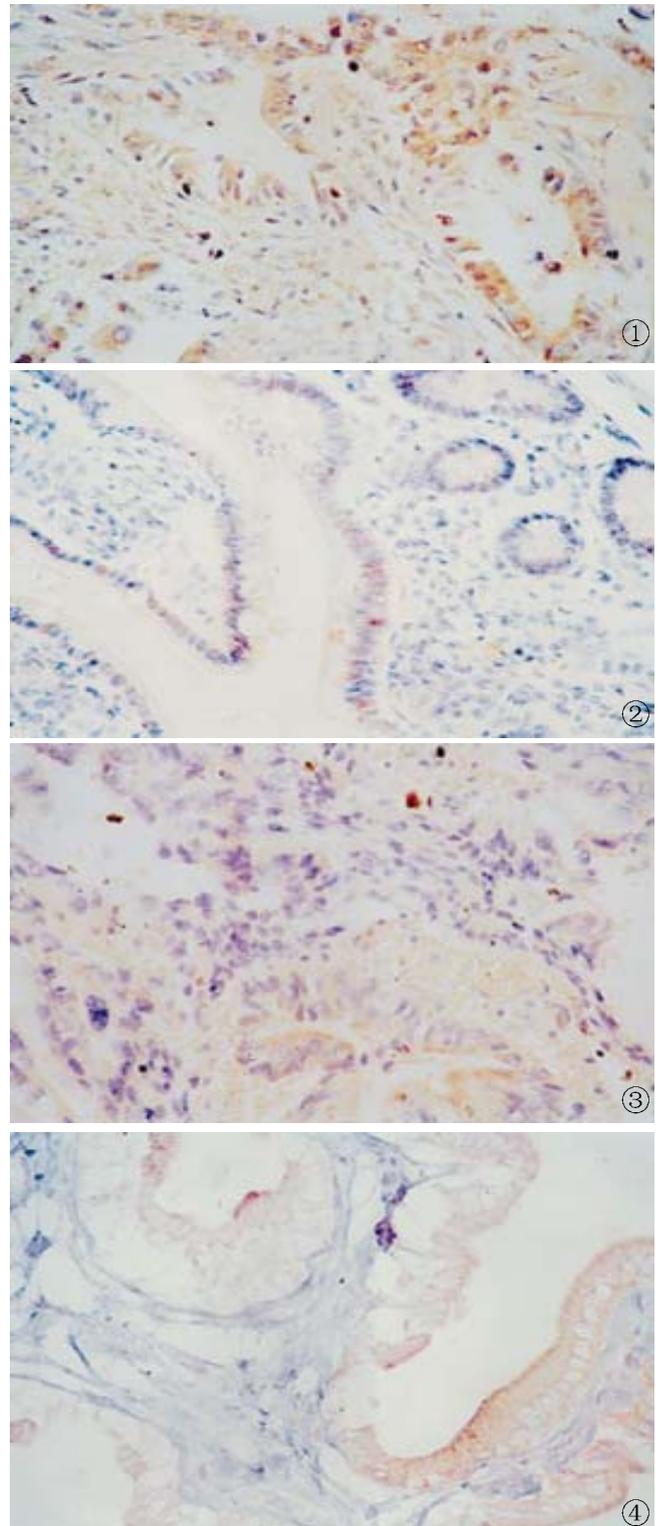


Figure 1 HCV NS5 protein located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. LSAB×400

Figure 2 HCV RNA located in the nucleus of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR×400

Figure 3 HCV RNA located in the cytoplasm of cells of carcinoma of extrahepatic bile duct. IS-RT-PCR×400

Figure 4 HCV RNA located in the cytoplasm of cells of choledochal cyst. IS-RT-PCR×400

DISCUSSION

Although HCV is considered essentially to be hepatotropic, some studies indicate that it can also be found in the extrahepatic tissue, such as peripheral blood mononuclear cells^[33-37], kidney tissue and salivary glands^[38]. In the present study, HCV NS5 protein and RNA were found in cells of carcinoma of extrahepatic bile duct, which further demonstrates that HCV has wide host cells, and the main nucleic localization of HCV RNA in cells of carcinoma of extrahepatic bile duct was resembled the localization of HCV RNA in HCC^[39]. The incidence of HCV infection in the tissues of carcinoma of extrahepatic bile duct is significantly higher than in control group ($\chi^2 = 9.808$, $P=0.002$). This study indicates a correlation between HCV infection and carcinoma of extrahepatic bile duct, which is different from the traditional viewpoint-HCV infection is not correlated to bile duct cancer^[2]. It is inferred that HCV infection, being similar to hepatolithiasis, choledochal cyst, etc., may be one of the risk factors involved in the development of carcinoma of extrahepatic bile duct.

HCV is a plus-strand RNA virus^[40]. HCV RNA and proteins can be detected in cells of HCC^[41-46], and infection with the HCV is now known to be a major risk factor for the development of HCC^[47-51]. But HCV genome does not integrate into the genome of infected cells^[52,53]. For hepatitis B virus (HBV), integration of HBV DNA into host chromosomes raises the possibility of a direct carcinogenic effect of HBV through interaction with oncogenes, growth factors, or tumor suppressor genes^[54-58]. The mechanism of carcinogenesis of HCV is not fully understood now, which may be involved in proteins HCV gene encoding. It has been noted recently that the HCV core protein demonstrates diverse biological functions, including the regulation of cellular and unrelated viral genes at the transcriptional level, and has some potential direct carcinogenic effects *in vitro*. HCV core protein could activate human c-myc, early promoter of SV10, Rous sarcoma virus LTR and HIV-1 LTR^[59], inhibit cisplatin-mediated apoptosis in human cervical epithelial cells and apoptosis induced by the overexpression of c-myc in Chinese hamster ovarian cells^[60], and repress transcriptional activity of P53 promoter^[61]. Translocated expression of HCV core protein may also inhibit apoptosis in the tissue of HCC^[62]. REF cells cotransfected with HCV core and H-ras genes became transformed and exhibited rapid proliferation, anchor-independent growth, and tumor formation in athymic nude mice^[63]. Transformation of NIH3T3 cells to the tumorigenic phenotype by the nonstructural protein NS3 of HCV was demonstrated and the proteinase activity associated with this protein was suggested as the

cause of transformation^[64]. HCV NS3 protein may exert its hepatocarcinogenic effect in early stage on host cells by endogenous pathway which may bring about mutation of p53 gene and transformation of hepatocytes^[65]. NS5 protein from HCV-1b ORF includes NS5A and NS5B. Recently, NS5A protein is reported to be a potent transcriptional activator^[66], and can repress the interferon-induced protein kinase through direct interaction with each other^[67]. The experimental data suggest that HCV gene products have a function of gene regulation, and can modulate cell growth and differentiation, and may be directly involved in the malignant transformation of HCV-infecting cells. But how HCV infection is involved in the development of carcinoma of extrahepatic bile duct needs further research.

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Hepatitis C virus may infect extrahepatic tissues in patients with hepatitis C

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Abstract

AIM To explore the status of extrahepatic hepatitis C virus (HCV) infection and replication in hepatitis C patients, and its potential implication in HCV infection and pathogenicity.

METHODS By reverse-transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH) and immunohistochemistry, HCV RNA, HCV replicative intermediate (minus-strand of HCV RNA), and HCV antigens were detected in 38 autopsy extrahepatic tissue specimens (including 9 kidneys, 9 hearts, 9 pancreas, 5 intestines, 2 adrenal glands, 2 spleens, 1 lymph node, and 1 gallbladder) from 9 hepatitis C patients, respectively; and the status of HCV replication in extrahepatic tissues was studied.

RESULTS By RT-PCR, all 9 patients were positive for HCV RNA in kidney, heart, pancreas, and intestine, but only 6 (66.7%) patients were positive for HCV replicative intermediate. HCV RNA and HCV antigens were detected in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder in 5 (55.6%) and 6 (66.7%) patients by ISH and immunohistochemistry, respectively. HCV RNA

and HCV antigens were not detected in these extrahepatic organs in 3 (33.3%) patients, although their livers were positive for HCV. HCV replicative intermediate detected by RT-PCR was consistent with HCV RNA and HCV antigens detected by ISH and immunohistochemistry ($Kappa = 0.42-0.75$). HCV RNA and HCV antigens were detected in myocardial cells, epithelial cells of intestinal glandular, interstitial cells of kidney, epithelial cells of tubules and glomerulus, pancreas acinar cells and epithelial cells of pancreatic duct, epithelial cells of mucous membrane sinus of gallbladder, cortex and medulla cells in adrenal gland, and mononuclear cells in lymph node. HCV RNA was also detected in bile duct epithelial cells, sinusoidal cells, and mononuclear cells in liver tissues by ISH.

CONCLUSION HCV can infect extrahepatic tissues, and many various tissue cells may support HCV replication; extrahepatic HCV infection and replication may be of "concomitant state" in most of patients with hepatitis C. The infected extrahepatic tissues might act as a reservoir for HCV, and play a role in both HCV persistence and reactivation of infection. HCV as an etiologic agent replicating and expressing viral proteins in extrahepatic tissues itself contributes to extrahepatic syndrome associated-HCV infection in a few patients with chronic HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) was discovered in 1989^[1], and is in a separate genus of the virus family *Flaviviridae*. Some aspects of cell tropism of *Flaviviridae*, such as the yellow fever virus and the dengue fever virus, have been clarified by *in situ* staining technique^[2]. Different members of the *Flaviviridae* family infect a distinct, wide array of cells, resulting in multi-faceted disease expression. It should be noted that even a single amino acid change in the envelope molecule may modulate or even change the cell tropism and virulence of flaviviruses^[3,4]. It may only be inferred that HCV may gain access to and replicate in extrahepatic tissues when compared with other flaviviruses. In

1985, the report of Hellings *et al* showed that HCV, which was previously known as the non-A, non-B hepatitis virus, was successfully transmitted via the infusion of PBMC (peripheral blood mononuclear cells) purified from the patients; and then, Nouri Aria *et al* reported that HCV RNA and HCV replicative intermediate (minus-strand of HCV RNA) were detected in the cytoplasm and nuclei of mononuclear and biliary epithelial cells in liver tissue by ISH. Other studies also indicated that HCV can transmit from mother to infant, possibly occurring in utero^[5,6]. These results suggested that HCV might replicate in PBMC and nonhepatic cells. In recent years, studies in extrahepatic HCV infection focused on readily obtainable tissue and body fluid samples such as PBMC^[7-10], saliva, semen, urine, ascites, and biliary juices^[11,12]. Subsequently, HCV replication in cells of the hematopoietic lineage has been demonstrated^[8-10,13]. The results showed that it might be of pathological significance in HCV infection^[8,10,14,15]. Recently, HCV RNA and HCV replicative intermediate in lymph node, pancreas, adrenal gland, thyroid, spleen, ovary, and uterus were detected by RT-PCR^[16,17], but there was lack of *in situ* detection of HCV RNA in these extrahepatic tissues.

During HCV infection, the viral burden and subsequent tissue damage were mainly confined to the liver^[18-22]. However, several extrahepatic syndromes associated with HCV infection have been reported^[23-26], which include mixed cryoglobulinaemia, glomerulonephritis, lymphoma, and other extrahepatic diseases such as porphyria cutanea tarda^[27]. Up to now, little is known about the mechanisms and the role of HCV in the development of these extra hepatic syndromes; and the extrahepatic tissue cells that support viral replication have not been identified. Accordingly, we conducted reverse-transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH), and immunohistochemistry for HCV RNA, HCV replicative intermediate, and HCV antigens in several extrahepatic tissues from 9 hepatitis C patients in an attempt to explore the status of extrahepatic HCV infection and replication, and its potential implication in HCV infection and pathogenicity. We believe that the information so derived could lead to a better understanding of the mechanisms of both HCV persistence and reactivation of infection, and the role of HCV in the development of extrahepatic syndromes associated with HCV infection.

MATERIALS AND METHODS

Patients

Nine patients with severe viral hepatitis (1986-1994, diagnosed by autopsy, according to the Diagnostic Criteria of Viral Hepatitis Prevention

and Cure Guideline, China'95), 8 male, 1 female, aged 25-48 years, Han nationality, were included in this study. All the patients were positive for HCV antigens and HCV RNA in livers, and their sera were positive for HBV markers. Thirty-eight paraffin-embedded autopsy extrahepatic tissue specimens (including 9 kidneys, 9 hearts, 9 pancreas, 5 intestines, 2 adrenal glands, 2 spleens, 1 lymph node, and 1 gallbladder) were provided by the Department of Pathology and Anatomy, Third Military Medical University, China.

Main reagents

Mouse monoclonal anti-HCV NS3, NS5 and CP10^[28]. A digoxigenin-labeled cDNA antisense probe to the 5'-non-coding region of the HCV genome was 145bp in length (provided by the Division of Clinical Immunology, Tongji Medical University). HCV genome primers were derived from the highly conserved 5'-non-coding region of the HCV genome, and were synthesized by Gibco BRL; outer primers: sense 5'-ACTCCACCA TAGATCATCCC 3', antisense 5'-AACACTACTCGGCTAGCAGT-3'; inner primers: sense 5'-TTCACGCAGAAAGCGTCTAG-3', antisense 5'-GTTGATCC AAGAAAGGACCC-3'.

RT-PCR

RNA was extracted from 2 to 3 pieces of 5µm thick paraffin-embedded extrahepatic tissue sections (31 specimens, including 9 kidneys, 9 hearts, 9 pancreas, and 4 intestines) cut from the same pathological blocks as used for ISH and immunohistochemistry with TRIzol (Gibco BRL).

RT-PCR was performed as previously described^[29,30]. The expected size of the amplified product was 145bp. The PCR products were analyzed by agarose gel electrophoresis. The sera which were negative for HCV RNA and HCV replicative intermediate and the distilled water were used as negative control.

Immunohistochemistry and ISH

Immunohistochemistry and ISH were performed as previously described^[28,31]. Liver specimens-positive for HCV RNA and HCV antigens were used as positive control. Ten autopsy extrahepatic tissue specimens from viral hepatitis patients, whose livers were negative for HCV antigens and HCV RNA, and 10 autopsy extrahepatic tissue specimens from patients with rabies were used as negative control. Furthermore, control slides were treated with ribonucleases (RNase) and deoxyribonuclease (DNase) before hybridization; and the digoxigenin-labeled probe was substituted with non-labeled HCV cDNA probe, which was used as control in ISH as well.

RESULTS

Detection rates of HCV RNA and HCV antigen in

the tissues

By RT-PCR, all 9 (positive in 20 specimens, 64.5%) patients were positive for HCV RNA in kidney, heart, pancreas, and intestine, but only 6 (66.7%), (positive in 11 specimens, 35.5%) of 9 patients were positive for HCV replicative intermediate (Figure 1). By ISH and immunohistochemistry, positive staining for HCV RNA and HCV antigens in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder were found in 5 (55.6%), (positive in 11 specimens, 28.9%) of 9, and 6 (66.7%), (positive in 23 specimens, 60.5%) of 9 patients, respectively. Positive rates of HCV RNA and HCV antigens in single extrahepatic organ are shown in Table 1. Three (33.3%) of 9 patients were negative for HCV RNA and HCV antigens in these extrahepatic organs, although their livers were positive for HCV.

Table 1 Results of HCV RNA and HCV antigens detection in extrahepatic organs and livers

Tissue	Cases	HCV RNA (+)			HCV antigens (+)		
		RT-PCR	ISH	NS3	NS5	CP10	
Kidney	9	7	4*	3	6	5	4
Heart	9	5	3*	2	5	4	4
Pancreas	9	5	3*	2	6	6	5
Intestine	5	3/4	1/4*	1	3	2	0
Adrenal gland	2			1	1	1	0
Spleen	2			0	0	0	0
Lymph node	1			1	1	1	1
Gallbladder	1			1	1	0	1
Liver	9	9	7*	9	8	7	7

Note: *Minus-strand RNA detected.

The positivity of minus-strand RNA detected by RT-PCR was consistent with that of HCV RNA and HCV antigens detected by ISH and immunohistochemistry ($Kappa = 0.42-0.75$).

Expression of HCV RNA and HCV antigens in the tissues

HCV antigen staining was only seen within the

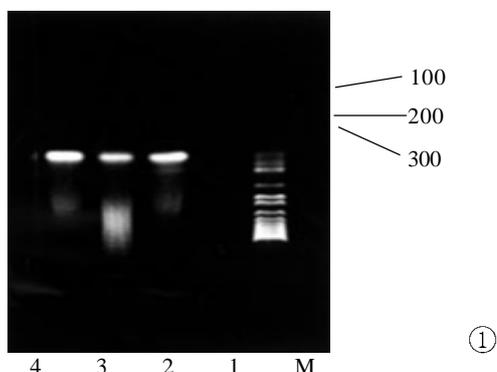


Figure 1 RT-PCR amplification result of minus-strand RNA in extrahepatic tissues. M: markers; 1: negative control; 2-4: amplification results of kidney, heart, and pancreas. The expected size of the amplification product is 145bp

cytoplasm with homogenous, inclusive or submembranous distribution. The hybridization signal was observed in both cytoplasm and nuclei, with a greater proportion of cytoplasm signal.

HCV existed in these extrahepatic organs except 2 spleens. There were HCV RNA and HCV antigen positive expressions in myocardial cells, epithelial cells of intestinal glandular, interstitial cells of kidney, epithelial cells of tubules and glomerulus, pancreas acinar cells and epithelial cells of pancreatic duct, epithelial cells of mucous membrane sinus of gallbladder, cortex and medulla cells in adrenal gland, and mononuclear cells in lymph node (Figures 2-10). The amount of HCV positive cells in extrahepatic tissues was obviously less than that in liver tissues. In addition, there were some differences in HCV expression in various cells among different extrahepatic tissues, e.g. the amount of positive cells in heart tissue was less than that in kidney, pancreas, and intestine. In liver tissues, HCV antigens were only detected in hepatocytes, but hybridization signal of HCV RNA was seen in not only hepatocytes, but also bile duct epithelial cells, sinusoidal cells, and mononuclear cells (Figures 10 - 12). The amount of HCV positive cells in liver tissues was lower than 1%^[28], but obviously higher than those in extrahepatic tissues.

Negative control

The samples (such as extrahepatic tissues, sera, and the distilled water) from the subjects of control groups were negative for HCV RNA and HCV antigens detected by RT-PCR, ISH, and immunohistochemistry, respectively. Using RNase treatment of the extrahepatic tissues before hybridization, the HCV RNA staining in these tissue cells faded. DNase treatment did not reduce HCV RNA signal in these tissue cells. Using non-labeled cDNA probe, HCV RNA hybridization signal was not observed in these tissues.

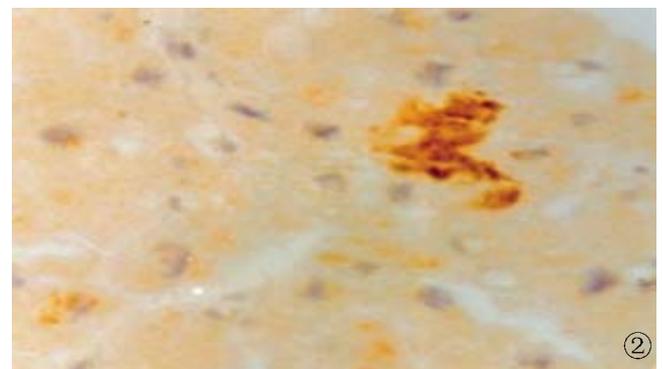


Figure 2 The expression of HCV NS3 in myocardial cells, showing brown yellow. S-P (DAB)×400

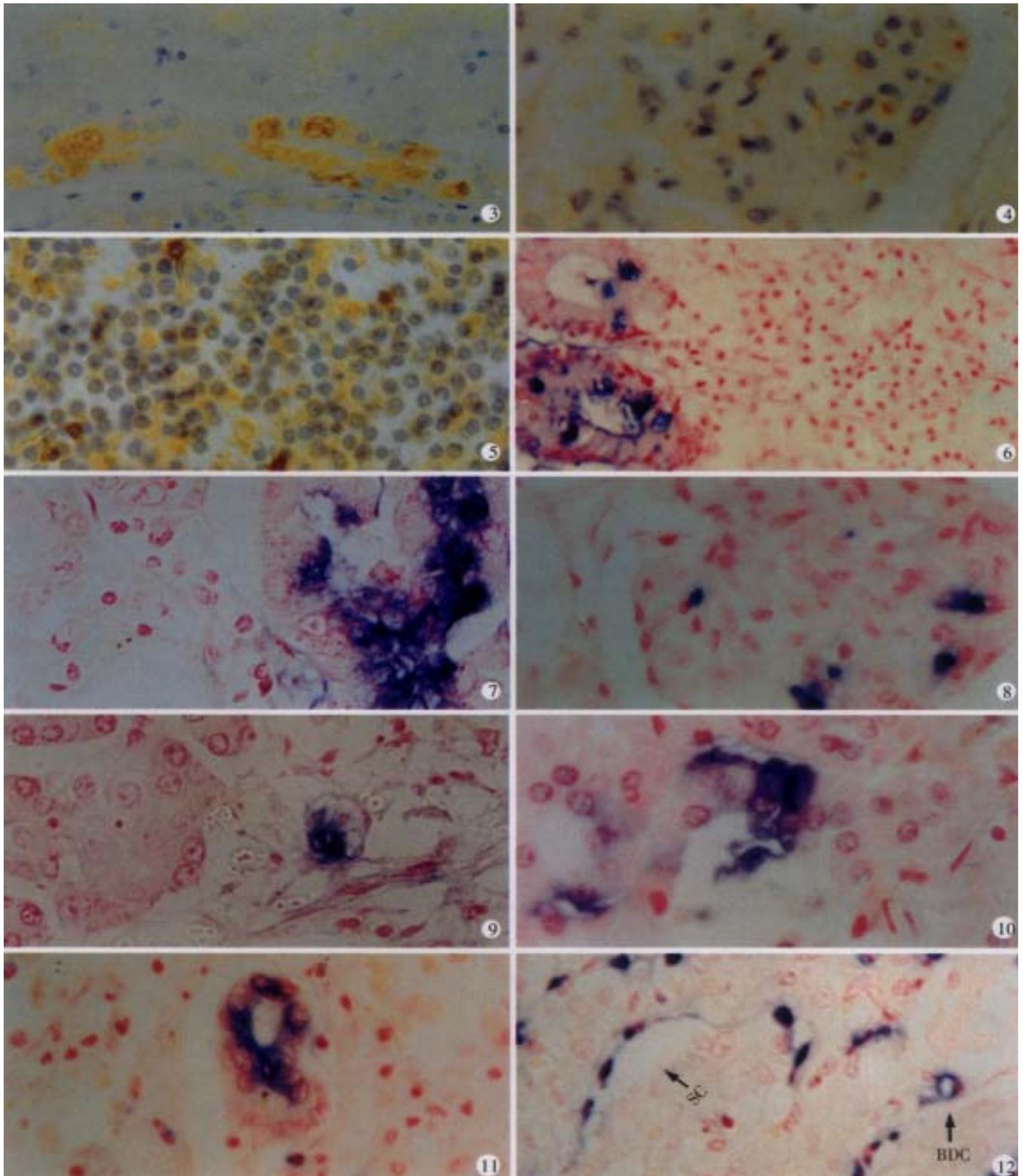


Figure 3 The expression of HCV NS5 in epithelial cells of tubules, showing brown yellow. S-P (DAB)×400

Figure 4 The expression of HCV NS5 in the glomerulus, showing brown yellow. S-P (DAB)×400

Figure 5 The expression of HCV NS3 in mononuclear cells in lymph node, showing brown yellow. S-P (DAB)×400

Figure 6 The expression of HCV RNA in epithelial cells of mucous membrane sinus of gallbladder, showing purple blue. ISH×200

Figure 7 The expression of HCV RNA in epithelial cells of intestinal gladular, showing purple blue. ISH×400

Figure 8 The expression of HCV RNA in the glomerulus, showing purple blue. ISH×400

Figure 9 The expression of HCV RNA in the pancreas acinar cells, showing purple blue. ISH×400

Figure 10 The expression of HCV RNA in cortex cells in adrenal gland, showing purple blue. ISH×400

Figure 11 The expression of HCV RNA in bile duct epithelial cells in liver, showing purple blue. ISH×400

Figure 12 The expression of HCV RNA in bile duct cells (BDC), sinusoidal cells (SC) in liver, showing purple blue. ISH×400

DISCUSSION

Extrahepatic HCV infection was found in hepatitis C patients, recently. But as RT-PCR is fraught with problems^[32,33], contradictory data related to extrahepatic HCV infection have been reported^[16,17,34-36], and there have been few *in situ* detection of HCV in extrahepatic tissues, the identification of extrahepatic HCV infection and replication has been controversial.

In this study, HCV RNA and HCV replicative intermediate in kidney, heart, pancreas, and intestine were detected by RT-PCR, but more importantly, the localization of HCV RNA and HCV antigens (NS3, NS5 and CP10) in kidney, heart, pancreas, intestine, adrenal gland, lymph node, and gallbladder was demonstrated convincingly by ISH and immunohistochemistry, and various tissue cell types harboring HCV such as pancreas acinar cells were also identified. Moreover, the positivity of HCV replicative intermediate detected by RT-PCR was consistent with that of HCV RNA and HCV antigens in these tissue cells detected by ISH and immunohistochemistry ($Kappa=0.42-0.75$). More recently, we used a digoxigenin-labeled HCV oligonucleotide sense probe and detected HCV replicative intermediate in the extrahepatic tissues-positive for HCV RNA and HCV antigens by ISH (data being summarized). These results showed that HCV could infect extrahepatic tissues, and various tissue cells might support viral replication. Recently, Lorient *et al.*^[37] reported that gallbladder epithelial cells from HCV-negative subjects were successfully infected by HCV *in vitro*, and ISH and immunohistochemical studies identified pancreas and gastric mucosa as the sites of HCV infection^[38,39]. These reports support the results of our studies.

So far, there have been few reports on the status of extrahepatic HCV infection and replication in hepatitis C patients. In order to explore this issue, we compared with the status of HCV expression between extrahepatic tissue and liver, and also among different extrahepatic tissues, the results indicated that the detection rates of HCV in extrahepatic tissues were low^[40], and the amount of HCV positive staining cells in extrahepatic tissues was also obviously lower than that in livers; and there were differences in HCV expression in various cells among different extrahepatic tissues. These results suggested that the levels of extrahepatic HCV infection and replication were relatively low as compared with those of livers; and there were differences in the status of HCV replication among different extrahepatic tissues. Whether HCV can bring about cell injury of infected extrahepatic tissues is not well understood because of the lack of extrahepatic histopathological observation. Recently, some reports showed that HCV is an causal agent in the pathogenesis of hypertrophic

cardiomyopathy and chronic myocarditis^[41,42], and that the patients' gastroduodenal mucosal lesions might be associated with HCV infection^[43]. However, most of the patients with hepatitis C did not have extrahepatic clinical manifestations^[44]. In our previous studies, we found no relationship between the HCV expression in extrahepatic tissue cells and tissue lesions^[45]. We speculate that extrahepatic HCV infection and replication may be of "concomitant state" in most of patients with hepatitis C, while liver was a key organ in HCV infection and pathogenicity^[18,21,31,45], i.e. HCV may replicate in various tissue cells, but viral replication may not cause obvious cell injury of these infected tissues.

Zignego *et al.*^[46] reported that the infected PBMC might act as a reservoir for HCV. In this study, various tissue cells may support viral replication. These extrahepatic tissue and PBMC reservoirs are considered to provide the source of HCV for the patient's liver reinfection and recurrence of infection after liver transplantation for hepatitis C. Once the immunologically privileged sites are infected, HCV is more difficultly eliminated by the host and/or antiviral therapy, which might play a role in both HCV persistence and reactivation of infection after interferon therapy^[48,47].

At present, little is known of the mechanisms and the HCV role in the development of HCV-related extrahepatic syndromes. Some reports showed that the vasculitic and kidney damage in mixed cryoglobulinemia and glomerulonephritis associated with HCV infection might be mediated by immune mechanisms^[48,49]. However, in this study, HCV may replicate and express viral proteins in many tissue cells such as interstitial cells of kidney, epithelial cells of tubules, and glomerulus. It is noticeable that some of the cell types found to harbor HCV are also associated with HCV-related extrahepatic syndromes such as glomerulonephritis. In addition, HCV antigens were also found in mesangial and paramesangial cells and epithelial cells of tubules in glomerulonephritis kidney tissues^[24,48], and in lymphoid cells in hyperplastic reactive lymphadenopathy^[50]; HCV RNA and/or HCV replicative intermediate were detected in residual parotid epithelial cells in the parotid non-Hodgkin's lymphoma lesion by ISH^[51], and in epineurial cells in mixed cryoglobulinemia-associated neuropathy by *in situ* RT-PCR^[52]. HCV as an etiologic agent replicating and expressing viral proteins in extrahepatic tissues itself contributes to extrahepatic syndrome associated-HCV infection in a few patients with chronic HCV infection. For example, a pathogenetic role of HCV as an exogenous trigger might be hypothesized in the parotid non-Hodgkin's lymphoma^[51], or in hypertrophic cardiomyopathy^[41]. In hepatitis B

patients, several extrahepatic syndromes associated-HBV infection have been considered to be mediated by immune complexes or other immunological mechanisms, but various vasculitic and other HBV-related skin lesions are associated with local HBV replication. So Mason *et al*^[53] suggested that once the HBV-related immune complexes are captured by the vascular endothelium, the vasculitic damage might also be mediated by viral replication and immune mechanisms. We speculate that HCV might use a similar mechanism to bring about the vasculitic and kidney damage in the development of mixed cryoglobulinemia and glomerulonephritis associated-HCV infection.

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Effect of *Boschniakia rossica* on expression of GST-P, p53 and p21^{ras} proteins in early stage of chemical hepatocarcinogenesis and its anti-inflammatory activities in rats

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Abstract

AIM To investigate the effect of *Boschniakia rossica* (BR) extract on expression of GST-P, p53 and p21^{ras} proteins in early stage of chemical hepatocarcinogenesis in rats and its anti-inflammatory activities.

METHODS The expression of tumor marker-placental form glutathione S-transferase (GST-P), p53 and p21^{ras} proteins were investigated by immunohistochemical techniques and ABC method. Anti-inflammatory activities of BR were studied by xylene and croton oil-induced mouse ear edema, carrageenin, histamine and hot scald-induced rat paw edema, adjuvant-induced rat arthritis and cotton pellet-induced mouse granuloma formation methods.

RESULTS The 500mg/kg of BR-H₂O extract fractionated from BR-Methanol extract had inhibitory effect on the formation of DEN-induced GST-P-positive foci in rat liver (GST-P staining was 78% positive in DEN+AAF group vs 20% positive in DEN+AAF+BR group, $P < 0.05$) and the expression of mutant p53 and p21^{ras} protein was lower than that of hepatic preneoplastic lesions (33% and 22% positive respectively in DEN+AAF group vs negative in DEN+AAF+BR group). Both CH₂Cl₂ and H₂O extracts from BR had anti-inflammatory effect in xylene and croton oil-induced mouse ear edema (inhibitory rates were 26%-29% and 35%-59%, respectively).

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BR-H₂O extract exhibited inhibitory effect in carrageenin, histamine and hot scald-induced hind paw edema and adjuvant-induced arthritis in rats and cotton pellet-induced granuloma formation in mice.

CONCLUSION BR extract exhibited inhibitory effect on formation of preneoplastic hepatic foci in early stage of rat chemical hepatocarcinogenesis. Both CH₂Cl₂ and H₂O extracts from BR exerted anti-inflammatory effect in rats and mice.

INTRODUCTION

Boschniakia rossica (BR) Fedtsch. et Flerov is a parasitic plant growing on the root *Alnus* plants (Betulaceae)^[1]. It is one of the valuable medicinal plants growing mostly on the Changbai Mountain at 1450-1800 meters above sea level, Jilin, China. It is also distributed in the Democratic People's Republic of Korea (DPRK), Japan and Russia. *Boschniakia rossica* is named "Bu Lao Cao" (antisenile plant), because it has effects of tonifying the Kidney and strengthening Yang, and has been used as a tonic or invigorating medicine in China. Yin ZZ *et al* isolated four iridoid compounds from *Boschniakia rossica* of the Changbai Mountain by chromatographic techniques. Their structure was determined by means of the spectra of nuclear magnetic resonance (NMR) and mass spectra^[2]. We discovered that Methanol extract of *Boschniakia rossica* exerted inhibitory effect on the formation of diethylnitrosamine (DEN) induced GST-P-positive foci in the liver of F344 rats^[3,4] and BR also has antioxidative activities^[5,6]. In the present study, we report the inhibitory effect of BR-water extract fractionated from BR-Methanol extract on the expression of GST-P, p53 and p21^{ras} proteins in early stage of rat chemical hepatocarcinogenesis and its anti-inflammatory activities in rats and mice.

MATERIALS AND METHODS

Chemicals

Diethylnitrosamine (DEN), 2-Acetylaminofluorene (AAF), determination kit for GGT and histamine

were purchased from Sigma Chemical Co.(USA). Vectastain ABC kit (pk 4001) was obtained from Vector Laboratories Inc. (USA); anti-GST-P antibody was kindly supplied by Professor Shigeki Tsuchida, Second Department of Biochemistry, Hirosaki University School of Medicine, Japan. p53 (DO-1) and pan ras (F-132) monoclonal antibody were purchased from Santa Cruz Biotechnology.

Preparation of the extract of *Boschniakia rossica*

Boschniakia rossica harvested from the Changbai Mountain area was used and the plants were identified by the authors. They were dried, cut, made into powder and extracted for overnight with Methanol five times. The Methanol extract was fractioned with CH₂Cl₂ and H₂O, and H₂O extract was vacuum-concentrated. The extract was dried by speed vacuum.

Animals and treatment

Male Wistar rats, aged 6 weeks and weighing 160g-180g were used in the experiments of hepatocarcinogenesis. The male Wistar rats (180g-200g) and Kunming strain mice (20g-22g) were used in the anti-inflammatory experiment. Animals were housed in groups of 5 animals in plastic cages with stainless-steel grid tops at room temperature with a 12h light/dark cycle.

Induction of preneoplastic hepatic foci^[7-11]

Enzyme-altered hepatic foci and hyperplastic nodules were induced by the modified protocol of Solt and Farber. The animals were divided into 3 groups. The rats in groups B and C were given a single i. p. injection of DEN (200mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks on basal diet, the rats received 0.004% 2-AAF in the diet for the following 6 weeks. Group C, after 2 weeks of injection of DEN, was given the diet containing 0.004% 2-AAF+500mg/kg BR for the following 6 weeks as a BR treatment group. Group A, as a control group, was intraperitoneally injected with the saline instead of DEN and then maintained on basal diet for 8 weeks. All rats of experimental and control groups were subjected to two-thirds partial hepatectomy (PH) at the 3rd week. Rats in each group were killed for examination at the 8th week.

Immunohistochemical staining for GST-P, p53 and p21^{ras}^[12-16]

Rat liver slices were fixed with ice-cold acetone and embedded in paraffin. Immunohistochemical staining for GST-P was performed by ABC method using anti-GST-P antibody; immunohistochemical staining for p53 and p21^{ras} proteins was performed using p53 (DO-1) and pan ras (F-132) monoclonal antibody, respectively.

Quantitative analysis

The number and the area of GST-P-positive hepatic foci larger than 0.1mm in diameter were analyzed using the microscopic quantitative analyzer (OC.M 19m/m Square 10/10×10, Tokyo, Japan).

Investigation of anti-inflammatory activities of BR extract^[17-19]

Xylen or croton oil-induced mouse ear edema An edema was induced on the right ear by topical application of xylen in mice 30 minutes after oral administration of 500mg/kg-1000mg/kg BR-H₂O extract or BR-CH₂Cl₂ extract. The left ear was controlled. Ear edema was measured by comparing the difference in weight (mg) between the same size of left and right ears 30 minutes after xylen-induction and 4h after croton oil-induction of inflammation and swelling degree and inhibition rate were calculated.

Carrageenin-induced rat paw edema An edema was induced on the rat right hind paw by aponeurosis injection of 0.15mL of 1% carrageenin in 0.9% saline. Test drug (500mg/kg-1000mg/kg of BR-H₂O extract) was given orally 30 minutes before the injection of carrageenin. The volume of the right paw was measured before injection and at 1, 2, 3, 4, 6 and 24h after induction of inflammation. The edema was expressed as an increase in paw volume due to carrageenin injection. The results were obtained by measuring the volume difference before and after injection of the right paw. The swelling degree of paw and inhibition rate of edema were calculated.

Histamine-induced rat paw edema An edema was induced on the right hind paw of rat by subplantar injection of 200µg/0.1mL of histamine. Test drug (500mg/kg of BR-H₂O extract) was given 30 minutes before the injection of histamine. The volume of the right paw was measured before injection and 0.5, 1, 2, 3 and 4h after induction of inflammation. The swelling degree of paw and inhibition rate of edema were calculated.

Hot scald-induced rat paw edema Edema was induced on the right hind paw of rat by hot scald. The right hind paw of rat soaked in thermostate water bath maintained at 53°C±0.5°C and cut-off time was 14 sec and test drug (500mg/kg of BR-H₂O extract) was given 30 minutes before the hot scald test. The volume of the right paw was measured before test and 1, 2, 3, 4, 5, 6 and 24 h after induction of inflammation. The swelling degree of paw and inhibition rate of edema were calculated.

Adjuvant-induced arthritis in rats The Arthritis was induced by injection of 0.1mL complete Freund's adjuvant into the subplantar region of the right hind paw of rats. Five hundred mg/kg of BR-H₂O

extract was orally administrated 30 minutes before the injection of adjuvant and the BR extract was given daily for 3 days after induction of inflammation. From the 8th day the BR extract was given daily for 7 days more. The volume of the right paw was measured before injection and at 18h, and on day 3, 6, 9, 12, 15, 18, 21 and 24 after induction of inflammation. The swelling degree of paw and inhibition rate of edema were calculated.

Cotton pellet-induced granuloma formation Pellets of surgical aseptic cotton weighing 15mg were implanted in both scapular regions in mice. The test drug (250mg/kg-500mg/kg) was administered daily for 7 days, and on the 8th day, the granulomatous tissues were removed. The pellets were dried overnight at 60°C and weighed. The dry weight was considered the weight of the granuloma. The results of this subacute inflammation were compared with the control group.

Statistical analysis Statistical analysis was made using the χ^2 test and the Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

Effect of *Boschniakia rossica* extract on expression of GST-P, p53 and p21^{ras} proteins during chemical hepatocarcinogenesis in rats Immunohistochemical investigation of expression of

GST-P, p53 and p21 protein in DEN-induced preneoplastic hepatic foci (group B), in administration of BR extract in the Solt-Farber protocol of rats (group C) and control (group A) are summarized in Table 1. GST-P staining was 78% positive in group B and 20% positive in group C, while in group A it was negative (B vs C, $P < 0.05$). Expression of oncogene products p53 and p21^{ras} protein in group B was 33% and 22% positive, while in groups A and C it was negative. The number (no/cm²) and area (mm²/cm²) of GST-P-positive hepatic foci in group C given DEN-AAF+BR was significantly decreased as compared with the values of group B given DEN-AAF (B vs C, $P < 0.001$ and $P < 0.05$) and these quantitative values are shown in Table 1 and Figure 1.

Effect of extract from BR on the anti-inflammatory activities in rats and mice

Both CH₂Cl₂ and water extract from BR have inhibitory effect in the xylene and croton oil-induced mouse ear edema, its inhibitory rate was 26%-29% and 35%-59% respectively (Tables 2 and 3) and exert inhibitory effect in the cotton pellet-induced granuloma formation in mice (Tables 8 and 9). BR-H₂O extract fractionated from BR-Methanol extract exhibited inhibitory effect in carrageenin, hot scald and histamine-induced rats hind paw edema (Tables 4, 6 and 7) and adjuvant-induced arthritis in rats (Table 5).

Table 1 Effect of *Boschniakia rossica* on the expression of GST-P, p53 and p21 protein in early stages of rat chemical hepatocarcinogenesis

Groups	Treatment (8 weeks)	n	GST-P positive(%)	No.of foci (No/cm ²) [*]	Area of foci (mm ² /cm ²) [*]	p53 positive (%)	p21 positive (%)
A	Saline-BD-PH	10	Negative	0	0	Negative	Negative
B	DEN-AAF-PH	9	7(77.8)	18.9±1.54 [△]	0.27±0.32	3(33.3)	2(22.2)
C	DEN-AAF-BR-PH	10	2(20.0) ^a	0.30±0.67 ^b	0.03±0.07 ^a	Negative	Negative

^a $P < 0.05$, vs B(χ^2 test); $P < 0.05$, ^b $P < 0.01$, vs B(*t* test).

^{*}Foci more than 0.1nm in diameter were quantified; [△]Values $\bar{x} \pm s$

Table 2 Effect of BR-extract on xylene-induced mouse ear edema

Groups	Dose (mg/kg)	Mouse (n)		BR-H ₂ O fraction (I)		BR-CH ₂ Cl ₂ fraction (II)	
		I	II	Edema degree(mg)	Inhibitory rate(%)	Edema degree(mg)	Inhibitory rate(%)
NS(A)	0.85%	10	20	19.1±3.6 [*]		11.2±4.0	
Ind(B)	20	10	20	15.5±1.8 ^a	18.8	8.8±2.6 ^a	21.5
BR(C)	500	10	20	17.4±3.1 ^b	8.9	8.3±3.9 ^a	26.2
BR(D)	1000	10	20	13.6±3.1 ^a	28.8	8.1±3.7 ^a	27.4

^{*} $\bar{x} \pm s$, ^a $P < 0.05$; ^b $P < 0.01$, vs NS (*t* test);

NS: normal saline; Ind:indomethacin; BR: *Boschniakia rossica*

Table 3 Effect of BR-extract on Croton oil-induced mouse ear edema

Groups (mg/kg)	Dose	Mouse (n)		BR-H ₂ O fraction (I)		BR-CH ₂ Cl ₂ fraction (II)	
		I	II	Edema degree(mg)	Inhibitory rate(%)	Edema degree(mg)	Inhibitory rate(%)
NS(A)	0.85%	10	12	8.6±2.5 [*]		7.6±3.3	
Ind(B)	20	10	12	6.6±4.0 ^a	23.3	5.0±2.1 ^a	33.5
BR(C)	500	10	12	5.4±3.4 ^b	45.3	4.9±1.7 ^a	35.1
BR(D)	1000	10	12	4.7±1.7 ^c	59.3	4.5±2.8 ^a	40.0

^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$; vs A (*t* test);

NS: normal saline; Ind:indomethacin; BR: *Boschniakia rossica*

Table 4 Inhibitory effect of BR-H₂O extract on carrageenin-induced paw edema in rats ($\bar{x}\pm s$)

Groups	Dose (mg/kg)	(n) Rats	Swelling degree (inhibitory rate)					
			1h	2h	3h	4h	6h	24h
NS		9	39.5±16.4	57.1±19.3	63.6±26.1	67.9±22.6	67.5±19.5	28.2±13.0
Ind	20	9	25.9±13.8 (34.2)	30.6±19.2 ^a (46.5)	32.6±18.6 ^a (47.2)	38.6±22.1 ^a (43.1)	42.2±34.6 (37.5)	16.9±12.3 (39.9)
BR	500	9	30.6±13.8 (22.7)	28.7±8.3 ^b (49.8)	39.4±12.2 ^a (38.0)	40.1±14.2 ^a (36.2)	39.5±20.0 ^a (41.5)	9.6±8.5 ^b (65.8)
BR	1000	9	31.8±15.7 (19.6)	26.3±18.9 ^b (54.0)	36.5±19.9 ^a (40.6)	37.0±28.8 ^a (45.5)	23.5±16.4 ^c (65.2)	6.8±10.4 ^b (76.5)

^a*P*<0.05; ^b*P*<0.01; ^c*P*<0.001, vs NS(*t* test)NS: normal saline; Ind:indomethacin; BR: *Boschniakia rossica***Table 5** Effect of BR-H₂O extract on adjuvant arthritis in rats

Groups	Dose (mg/kg)	Rats (n)	Swelling degree (inhibitory rate)								
			18h	3d	6d	9d	12d	15d	18d	21d	24d
NS		6	108.2 ±43.8	74.7 ±33.0	66.9 ±47.9	58.0 ±39.0	77.8 ±39.6	77.1 ±28.0	99.6 ±34.9	78.6 ±31.3	71.5 ±28.7
BR	500	6	79.1 ±22.3 ^b (26.9)	33.9 ±17.2 ^a (54.7)	51.8 ±25.0 (22.6)	52.6 ±23.7 (9.3)	67.5 ±21.1 (13.2)	75.7 ±23.7 (1.8)	61.7 ±19.0 ^a (38.1)	53.4 ±14.9 ^b (32.1)	46.5 ±17.2 ^b (35.0)

^a*P*<0.05; ^b*P*<0.01, vs NS (*t* test)NS: normal saline; Ind: indomethacin; BR: *Boschniakia rossica***Table 6** Effect of BR-H₂O extract on hot scald-induced paw edema in rats

Groups	Dose (mg/kg)	Rats (n)	Swelling degree (inhibitory rate)							
			1h	2h	3h	4h	5h	6h	24h	
NS		8	67.2±4.7	59.5±3.9	74.2±6.5	81.5±23.7	77.7±17.1	69.8±19.1	63.7±14.5	
BR	500	8	40.4±11.9 ^b (39.9)	23.3±10.1 ^b (60.8)	33.5±16.8 ^b (68.6)	39.7±10.5 ^a (51.3)	24.8±8.2 ^b (68.1)	18.0±16.1 ^b (74.2)	23.4±7.0 ^b (63.3)	

^a*P*<0.05; ^b*P*<0.01, vs NS (*t* test) NS: normal saline; Ind: indomethacin; BR: *Boschniakia rossica***Table 7** Effect of BR-H₂O extract on histamine-induced rat hind paw edema

Groups	Dose (mg/kg)	Rats (n)	Swelling degree (inhibitory rate)				
			30min	1h	2h	3h	4h
NS		9	48.0±11.1	34.8±12.6	22.9±8.1	18.3±3.8	12.7±5.9
BR	500	9	28.5±7.5 ^a (36.2)	18.4±7.5 ^a (31.1)	9.0±6.5 ^a (31.7)	9.6±6.7 ^a (36.8)	2.3±3.9 ^b (71.3)

^a*P*<0.05; ^b*P*<0.01, vs NS (*t* test)**Table 8** Effect of BR-HO extract on proliferation of granuloma caused by cotton pellet in mice

Groups	Dose Mouse (mg/kg)	(n)	Weight of granuloma		Inhibitory rate (%)	
			Wet w.(mg)	Dry w.(mg)	Wet w.(mg)	Dry w.(mg)
NS(A)		10	615.2±119.1	152.3±54.8		
BR(C)	250	10	507.0±41.1 ^a	103.7±14.4 ^a	17.6	31.9
BR(D)	500	10	463.5±49.6 ^b	101.0±15.1 ^a	24.7	33.7

^a*P*<0.05; ^b*P*<0.01, vs NS (*t* test)**Table 9** Effect of BR-CH₂Cl₂ extract on proliferation of granuloma caused by cotton pellet in mice

Groups	Dose (mg/kg)	Mouse (n)	Weight of granuloma		Inhibitory rate (%)	
			Wet w.(mg)	Dry w.(mg)	Wet w.(mg)	Dry w.(mg)
NS(A)		10	613.4±160.4	130.3±42.0		
BR(C)	250	10	445.8±37.0 ^b	97.9±12.8 ^a	27.3	24.9
BR(D)	500	10	422.8±33.2 ^b	84.8±7.8 ^a	31.1	34.9

^a*P*<0.05; ^b*P*<0.01, vs NS (*t* test)

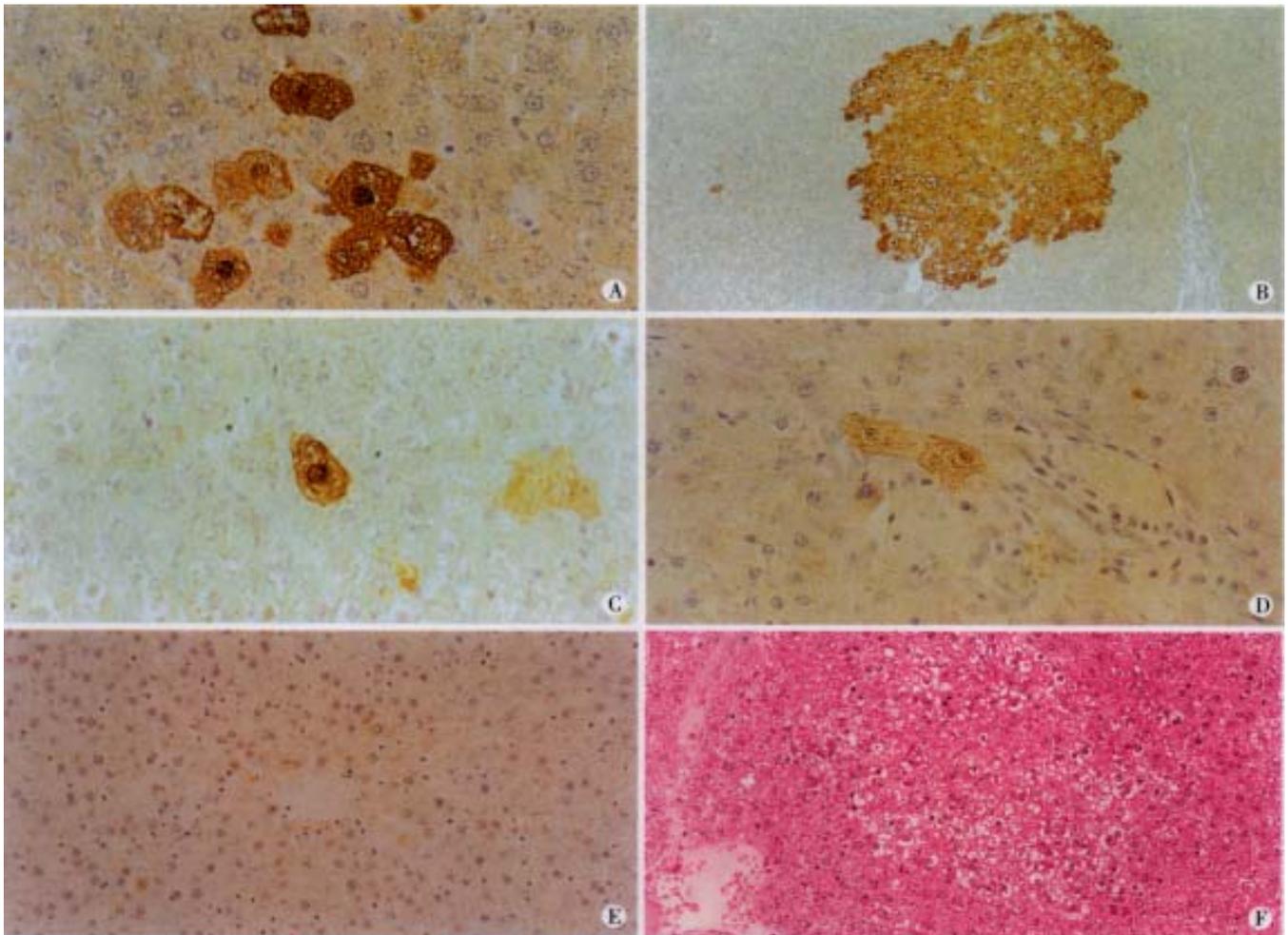


Figure 1 Immunohistochemical staining for GST-P in rat hepatic preneoplastic lesions induced by Solt-Farber protocol (A and B), in rat liver treated with DEN and AAF plus BR for 6 weeks (C and D), and in normal rat liver (E).

A, GST-P-strongly positive minifoci in group B $\times 400$; B, GST-P-strongly positive large foci in group B, $\times 100$; C, GST-P-positive single cell in group C, $\times 400$; D, GST-P-weakly positive two cells in group D, $\times 400$; E, GST-P was negative in group A, normal rat liver, $\times 200$; F, Hematoxylin and eosin staining for preneoplastic hepatic foci in group B, $\times 200$.

DISCUSSION

Changes in GST-P, p53 and p21^{ras} proteins during chemical hepatocarcinogenesis in rats

Placental form of glutathione S-transferase (GST-P) was first isolated from rat placenta as a sensitive marker enzyme in chemical hepatocarcinogenesis of rat by Sato *et al* in 1984^[20-24]. GST-P is considered to be an accurate marker for very early "initiated cells"^[21,24]. GST- π , purified from human term placenta, is related to rat GST-P in many properties and is grouped into the class Pi. It is of important clinical value. GST- π is also a useful human tumor marker for hepatoma, esophageal, gastric and colonic carcinomas and its preneoplastic lesions^[12-15,25-32]. Thereafter, using DEN as a initiator and AAF as a promoter, modified system based on Solt-Farber method was designed to screen the medium-term bioassay of chemical-induced carcinogenesis by Ito *et al*^[33]. This screening system was used in this study and successfully induced the preneoplastic GST-P-positive hepatic foci and nodules. Recently Tsuda H *et al* developed a trial

for an initiation bioassay system. Initiation potential was assayed on the basis of significant increase in values of preneoplastic GST-P positive foci. This protocol may be useful for detection of the initiation potential of carcinogens irrespective of their mutagenicity^[34].

We investigated the effect of *Boschniokia rossica* on formation of GST-P positive preneoplastic hepatic foci during chemical hepatocarcinogenesis. The results demonstrated that BR-H₂O extract fractionated from BR-Methanol extract with CH₂Cl₂ and H₂O has inhibitory effect on DEN-induced GST-P positive preneoplastic hepatic foci in early stage of rat chemical hepatocarcinogenesis. We consider that it is related to its antioxidative action of *Boschniokia rossica*^[4]. Recently there are reports that GST-P is as a sensitive marker for preneoplastic hepatic foci during chemical hepatocarcinogenesis in rats^[35-40]. It indicated that GST-P and GST- π are very sensitive tumor markers for basic research, prevention and cure of cancer.

Rat chemical hepatocarcinogenesis can be

divided into several steps: initiation, promotion, and progression stages. During the initiation stage, alterations of specific genes and in particular the activation of cellular proto-oncogenes occurs. During the promotion stage, groups of preneoplastic cells have been observed in many organs prior to appearance of malignant cancers, and in rat chemical hepatocarcinogenesis enzyme-altered foci and hyperplastic nodules have been excited^[21]. Recently many scientists reported expression of ras, jun oncogene^[42,47-49], p53 tumor suppressor gene^[41,43,45,52-54], and nm23 tumor metastasis suppressor gene^[44,46,50,51] in hepatocellular carcinoma, esophageal, gastric, and colonic carcinomas. Smith *et al*^[41] reported a p53 gene mutation occurring in foci of enzym-altered hepatocytes induced by diethylnitrosamine in F 344 rats. We observed the relation to the expression of GST-P, ras gene product p21^{ras} protein and suppressor gene product p53 protein in preneoplastic hepatic foci of rat liver. The results suggest that one-third of GST-P positive foci were positive for p53 protein and approximately one-fourth of GST-P positive foci were also positive for p21^{ras} protein in group B, while in rat liver of group C treated with DEN and AAF plus BR for 6 weeks p53 protein and p21^{ras} were not immunohistochemically detectable. Our result is similar to the report by Smith *et al*, and also is similar to that GST-P appearing at an early stage of chemical hepatocarcinogenesis, when oncogene product c-jun was not immunohistochemically detectable, reported by Suzuki *et al*^[42]. These results indicate that BR-H₂O extract fractionated from BR-Methanol extract exhibited an inhibitory effect on DEN-induced preneoplastic hepatic foci in rats administered with BR for 6 weeks during chemical hepatocarcinogenesis.

Anti-inflammatory activities of *Boschniakia rossica*

Results of the present study demonstrated that BR-extract exerted significant anti-inflammatory activities. An inhibitory effect of H₂O extract and CH₂Cl₂ extract from BR was observed in the acute inflammatory process, such as xylene and croton oil-induced mouse ear edema, carrageenin induced rat hind paw edema and inflammatory factors such as histamine and hot scald-induced rat hind paw edema. Anti-inflammatory action of BR was also observed in the chronic inflammation process, such as cotton pellet-induced granuloma formation in mice and immune inflammation process, such as adjuvant-induced arthritis in rats. The experimental model of inflammation induced by carrageenin is highly sensitive to non-steroidal anti-inflammatory drugs, and it has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs. The oral administration of the BR-H₂O extract (500mg/kg) inhibited the edema

formation by 49.8 % and 65.8% in 2 and 24 hours, respectively, after the administration of carrageenin in rats. Indomethacin, the standard anti-inflammatory drug used in this experiment, inhibited the edema by 46.5% and 40% in 2 and 24 hours. The anti-inflammatory effect of extract from BR was also observed in the cotton pellet-induced granuloma formation in mice. The daily oral administration of 250mg/kg and 500mg/kg of BR-CH₂Cl₂ extract, using this model, showed on the eighth day an inhibitory effect of 25% and 35%, and 250mg/kg and 500mg/kg of BR-H₂O extract 35% and 34%, respectively. The mechanism of anti-inflammatory action of *Boschniakia rossica* can be related to the chemical structure of BR. Recio *et al*^[55] reported that Iridoids is an anti-inflammatory agent. The results obtained in present study suggest that CH₂Cl₂ extract and H₂O extract from BR have anti-inflammatory effects. We isolated four iridoid compounds, a group of cyclopentano[c]pyran monoterpenoids, from *Boschniakia rossica*, and among them 8-epideoxyloganic acid was shown to exhibit a strong anti-inflammatory activity. Based on the results obtained in this study, we conclude that extract from BR exerted an anti-inflammatory effect.

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Effects of retinoic acid on proliferation, phenotype and expression of cyclin-dependent kinase inhibitors in TGF- β 1-stimulated rat hepatic stellate cells

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Subject headings retinoic acid; cyclin-dependent kinase inhibitor; hepatic stellate cell; cell culture; transforming growth factor beta 1; liver fibrosis

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Abstract

AIM To study the molecular mechanisms of retinoic acid (RA) on proliferation and expression of cyclin-dependent kinase inhibitors (CKI), i.e. p16, p21 and p27 in cultured rat hepatic stellate cells (HSC) stimulated with transforming growth factor beta 1 (TGF- β 1). **METHODS** HSC were isolated from healthy rat livers and cultured. After stimulated with 1mg/L TGF- β 1, subcultured HSC were treated with or without 1nmol/L RA. **MTT assay, immunocytochemistry (ICC) for p16, p21, p27 and β -smooth muscle actin (β -SMA) protein, *in situ* hybridization (ISH) for retinoic acid receptor beta 2 (RAR- β 2) and p16, p21 and p27 mRNA and quantitative image analysis (partially) were performed.** **RESULTS** RA inhibited HSC proliferation (41.50%, $P < 0.05$), decreased the protein level of β -SMA (55.09%, $P < 0.05$), and induced HSC to express RAR- β 2 mRNA. In addition, RA increased the protein level of p16 (218.75%, $P < 0.05$) and induced p21 protein expression; meanwhile, p27 was undetectable by ICC in both control and RA-treated HSC. However, RA had no influence on the mRNA levels of p16, p21 or p27 as determined by ISH.

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CONCLUSION Up-regulation of p16 and p21 on post-transcriptional level may contribute, in part, to RA inhibition of TGF- β 1 initiated rat HSC activation *in vitro*.

INTRODUCTION

Hepatic stellate cells (HSC) play crucial roles in the development of liver fibrosis^[1-6]. Stimulated HSC transform from vitamin A-rich quiescent cells to myofibroblast-like cells characterized by the expression of α -smooth muscle actin (α -SMA), loss of retinoids and diminished retinoid signaling^[4-15]. Exogenous retinoids such as retinoic acid (RA) may recover the contents of retinoids and nuclear retinoic acid receptors (RAR) in HSC and therefore suppress hepatic fibrogenesis, but the mechanisms of RA on HSC inhibition were not well understood^[3,16-27]. Recent studies on other cell types have shown that modulation of cell cycle regulatory proteins might contribute to RA-induced inhibition of cell proliferation and differentiation^[28-37] and Kawada *et al*^[38] reported that expression of G1 cyclin was involved in cell cycle transition of HSC from G1 to S. The present study was designed to investigate the effects of RA on negative cell cycle regulators cyclin-dependent kinase inhibitors (CKI) in cultured rat HSC stimulated with transforming growth factor beta-1 (TGF- α 1). The results showed that RA inhibited HSC activation may be in part due to post-transcriptional up-modulation of p16 and p21.

MATERIALS AND METHODS

Reagents

Collagenase IV, pronase E, Nycodenz, RA and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide or tetrazolium (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human TGF- β 1 was from Oncogene Science (Uniondale, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was Gibco/BRL- product (Life Technologies, Inc. Grand Island, NY, USA). Newborn calf serum, plastic tissue culture flasks and multi-plates were from Corning Incorporated (Corning, NY, USA). Polyclone anti- α -SMA antibody was purchased from Dako A/S (Glostrup, Denmark). Antibodies to p16, p21 and p27 were from Santa Cruz

Biotechnology, Inc. (Santa Cruz, CA, USA). ABC kit and DAB were from R & D Systems (Minneapolis, MN, USA). DIG Nucleic Acid Label and Detect Kit and Taq DNA polymerase were from Roche Diagnostics GmbH (Mannheim, Germany).

Isolation and culture of HSC

Cells were isolated from healthy Sprague-Dawley male rats (weighing 400g-450g) as described by Weiner *et al*^[26] with minor modifications by the laboratory^[39], seeded onto 25cm² plastic tissue culture flasks and incubated at 37°C in a humidified 5% CO₂/95% air. The medium was replaced 24h after seeding and every 48h thereafter. After they reached confluence (10d after planting), activated HSC were subcultured onto plastic tissue culture multi-plates with or without coverslips.

Experiments were performed on cells between serial passage 1 and 3 using three independent cell lines.

Cell treatments

Activated HSC were depleted of serum for 48h, followed by incubation with 1mg/L TGF- α 1 for another 48h, and then the medium was removed and cells maintained in DMEM with or without 1nmol/L RA for 48h. Preliminary dose dependence experiments indicated that 1mg/L TGF- β 1 or 1nmol/L RA had significant influence on HSC proliferation.

Proliferation assay

Cell proliferation was measured by MTT assay as previously described^[40] with minor modifications. Briefly, during the last 4h of incubation the cells were loaded with 10 μ L of freshly prepared and filtered MTT (5g/L in PBS) per well. The medium was then replaced with 100 μ L absolute ethanol and the cells were left for 30min for color development, followed by reading on Vmax[®]R Kinetic Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA) at 570nm wavelength.

Immunocytochemistry (ICC)

Cells grown on coverslips were fixed, permeabilized, blocked with 1% serum in PBS, and then incubated with primary antibodies to either α -SMA, p16, p21 or p27. ABC assay and DAB system were used to detect the proteins^[41] and photomicrographs were taken with an Olympus microphoto-microscope (Olympus Optical Co. LTD., Shinjuku-ku, Tokyo, Japan).

In situ hybridization (ISH)

cDNA probes for human RAR- β 2 and p16 were gifts from the Department of Biochemistry, School of Basic Medical Sciences, Fudan University; and cDNA fragments for rat p21 and p27 were presented as gifts by Dr. Chen Guang-Ping. Fragments were labeled with digoxigenin using random priming assay.

ISH was performed as previously described^[39] with immunohistochemical detection using an alkaline phosphatase (AKP) conjugated anti-digoxigenin monoclonal antibody. Hybridization signal was visualized through the substrates of AKP (NBT and BCIP). Photomicrographs were taken with an Olympus microphoto-microscope again.

Image analysis

Quantitative analysis of protein and mRNA were performed by scanning using KS 400 Imaging System 3.0 (Carl Zeiss Vision GmbH, Germany) and means of density values were determined.

Statistical analysis

Data were presented as mean values \pm S.D. and statistical significance was assessed by Student's *t* test.

RESULTS

RA inhibited HSC proliferation and α -SMA expression

As shown in Figure 1, there were fewer (41.50%, $P < 0.05$) HSC in RA-treated cells compared with control cells. In addition, RA decreased expression of α -SMA (55.09%, $P < 0.05$; Figure 2 and Table 1).

RA induced RAR- β 2 mRNA

To evaluate retinoid signaling, ISH was performed to determine RAR- β 2 gene expression. No mRNA was detected in control cells, but HSC treated with RA did express RAR- β 2, indicate RA induced expression of RAR- β 2 in HSC (Figure 3), and therefore enhanced retinoid signaling.

Expression of CKI

To further clarify the mechanisms of RA on cell cycle regulation in HSC, protein and mRNA levels of CKI were determined. As shown in Figure 4, p27 was undetectable by ICC in both control and RA-treated HSC. In addition, RA increased the protein levels of p16 (218.75%, $P < 0.05$) and p21 protein was detected in HSC treated with RA (Figure 4 and Table 1).

ISH results showed that the mRNA level of p16, p21 or p27 was not influenced by RA (Figure 5 and Table 1).

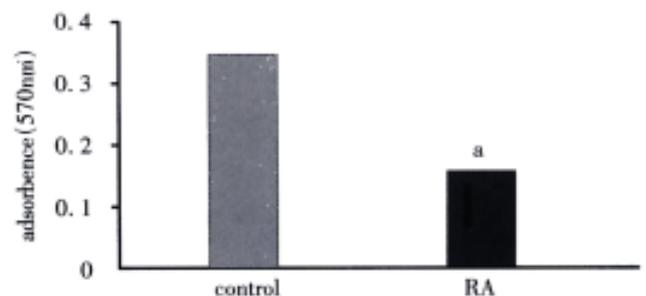
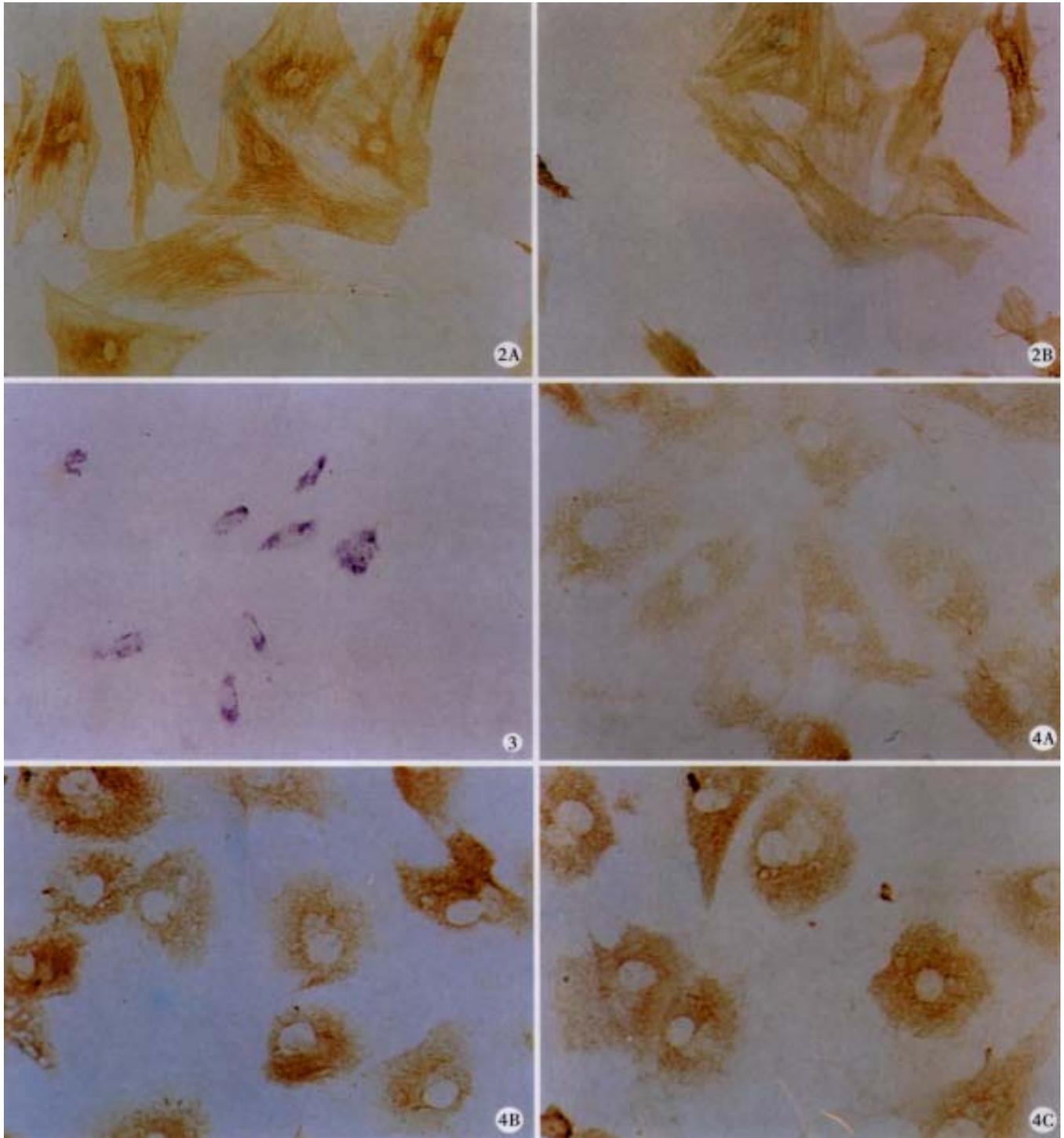


Figure 1 RA inhibited HSC proliferation. TGF- β 1-stimulated rat HSC were cultured and treated with or without 1nmol/L RA for 48h, followed by MTT assay as described in MATERIALS AND METHODS. ^a $P < 0.05$ vs control.

Table 1 Effects of RA on α -SMA and CKI expression in HSC

Group	α -SMA (protein)	RAR- β 2 (mRNA)	p16		P21		p27	
			Protein	mRNA	Protein	mRNA	Protein	mRNA
Control	0.285 \pm 0.050	ND	0.160 \pm 0.024	0.377 \pm 0.043	ND	0.285 \pm 0.043	ND	0.165 \pm 0.021
RA	0.157 \pm 0.042 ^a	0.227 \pm 0.24	0.350 \pm 0.029 ^a	0.353 \pm 0.023	0.0339 \pm 0.034	0.277 \pm 0.027	ND	0.179 \pm 0.023

ND: not determined; ^a P <0.05 vs control



- Figure 2** RA decreased the protein level of α -SMA. TGF- β 1-stimulated HSC were treated with (A) as described in Figure 1, and then immunocytochemistry was performed to detect α -SMA protein. ABC \times 200 (B) or without RA
- Figure 3** Expression of RAR- β 2 in RA-treated HSC. *In situ* hybridization with DIG-labeled RAR- β 2 cDNA probe was used to determine RAR- β 2 mRNA expression in HSC. NBT/BCIP \times 200
- Figure 4** Expression of CKI protein. Immunocytochemical study was performed to detect CKI, i.e. p16 (A), p21 (B). ABC \times 200 (C) expression in control (A) or RA-treated HSC

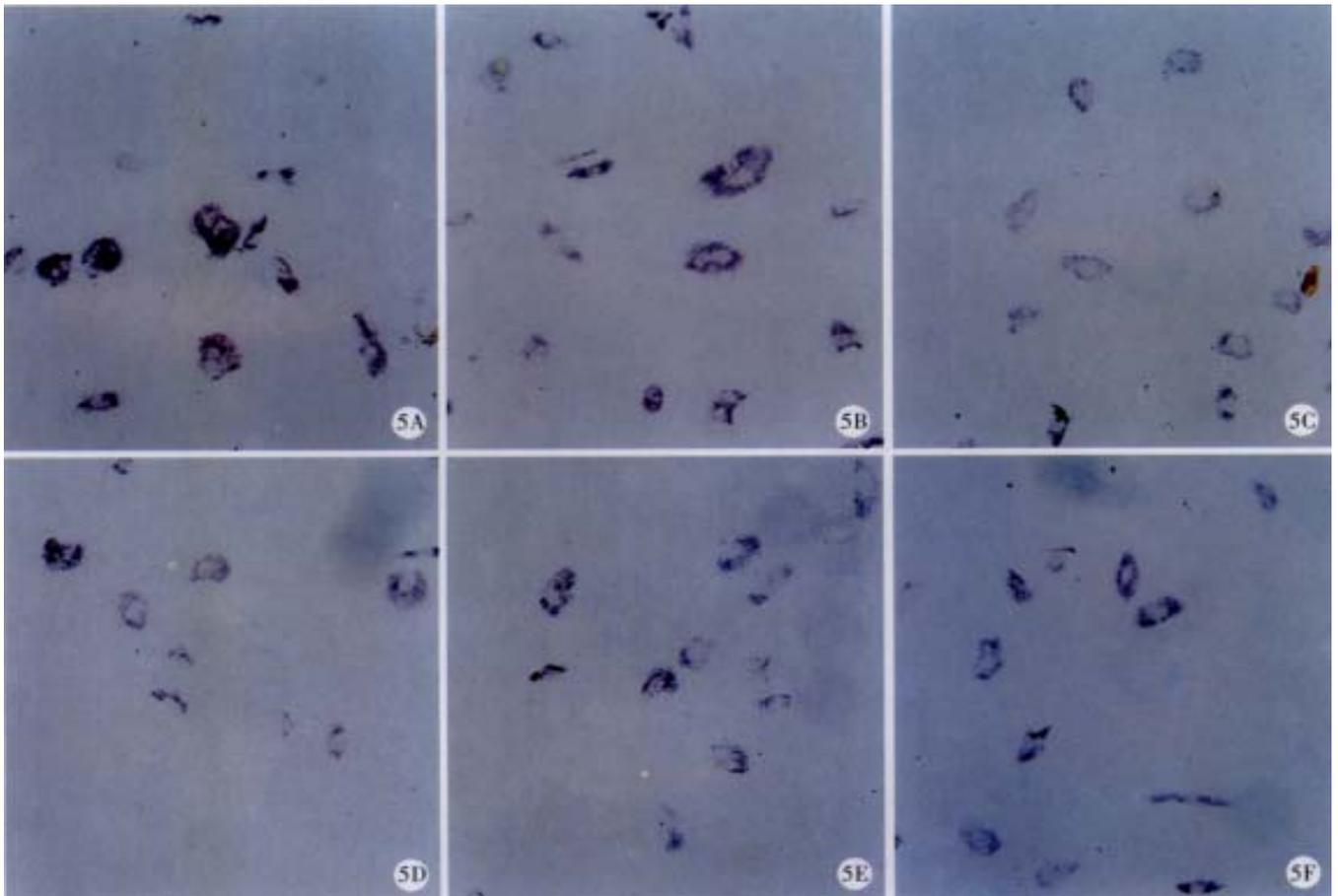


Figure 5 mRNA expression of CKI. mRNA of p16 (A.), p21 (B.) (C.) or p27 (D.) was determined with ISH in control (E.) or RA-treated HSC (F.). NBT/BCIP \times 200

DISCUSSION

TGF- β 1 is one of the most fibrogenetic cytokines on HSC, which initiates HSC activation characterized by loss of retinoids, proliferation, and expression of α -SMA and extracellular matrix^[2,6,42-45]. Our results showed that even 48h depletion of serum could not completely suppress the expression of α -SMA, implying that serum depletion can not reversibly suppress TGF- β 1-initiated activation of rat HSC in culture.

RA may modulate cell growth and differentiation through retinoid signaling^[30,46-49], mainly by nuclear retinoid X receptors and RAR. Present study showed that RA inhibited HSC proliferation and down-regulated α -SMA protein, demonstrating that RA may suppress HSC activation induced by TGF- β 1. Our results showed that RA induced RAR- β 2 mRNA, which may then modulate expression of some other genes including CKI^[28-37,50-52]. In addition, cells in controls displayed no RAR- β 2 mRNA, agreeing with its insufficient to completely suppress HSC activation again.

The protein level of p16 was increased in RA-treated HSC with detectable p21 protein, while RA had no influence on those mRNA levels, suggesting

RA may up-regulate p16 and p21 gene expression on the post-transcriptional level. p16 or p21 can inhibit cyclin-CDK complexes and then prevent G1 transition^[53-58]; therefore, our study indicates that RA induced inhibition of TGF- β 1-initiated HSC activation may be in part due to up-modulation of p16 and p21 on the post-transcriptional level, and reveals a new mechanism of RA induced HSC inhibition.

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The regulatory role of AT 1 receptor on activated HSCs in hepatic fibrogenesis: effects of RAS inhibitors on hepatic fibrosis induced by CCl₄

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Subject headings renin-angiotensin system; liver cirrhosis; enalapril; extracellular matrix; immunohistochemistry; losartan; liver/injuries

Wei HS, Lu HM, Li DG, Zhan YT, Wang ZR, Huang X, Cheng JL, Xu QF. The regulatory role of AT 1 receptor on activated HSCs in hepatic fibrogenesis: effects of RAS inhibitors on hepatic fibrosis induced by CCl₄. *World J Gastroentero*, 2000;6(6):824-828

Abstract

AIM To assess the effect of ACE inhibitor and AngII type 1 (AT1) receptor antagonist in preventing hepatic fibrosis caused by CCl₄ administration in rats; to investigate whether or not there are expression of AT 1 receptors on hepatic stellate cells; and to observe the effect of AngII on proliferation and ECM synthesis of cultured HSCs.

METHODS Studies were conducted in male Sprague-Dawley rats. Except for the hepatofibrotic model group and the control group, in three treated groups, either enalapril (5mg/kg), or losartan (10mg/kg), or enalapril + losartan were given to the fibrotic rats by daily gavage, and saline vehicle was given to model and normal control rats. After 6 weeks, liver fibrosis was assessed directly by hepatic morphometric analysis, which has been considered the gold standard for the quantification of fibrosis. The expressions of AT 1 receptors and (α -smooth muscle actin, α -SMA) in liver tissue or isolated hepatic stellate cells (HSCs) were detected by immunohistochemical techniques. The effect of AngII on HSC proliferation was determined by MTT method. Effect of AngII on collagen synthesis of HSCs was determined by ³H-proline incorporation. **RESULTS** Contrasted to the fibrosis in rats of the model group, groups of rats treated with

either enalapril or losartan, or a combination of two drugs showed a limited expansion of the interstitium (4.23 ± 3.70 vs 11.22 ± 4.79 , $P < 0.05$), but no difference was observed among three treated groups (5.38 ± 3.43 , 4.96 ± 2.96 , 4.23 ± 2.70 , $P > 0.05$). Expression of AT 1 receptors was found in fibrotic interstitium of fibrotic rats, whereas in normal control rats they were limited to vasculature only to a very slight degree. AT 1 receptors were also expressed on activated HSCs in the culture. At concentrations from 10^{-9} to 10^{-5} mol/L, AngII stimulated HSC proliferation in culture in a dose-dependent manner. Increasing AngII concentrations produced corresponding increases in ³H-proline incorporation. Differences among groups were significant.

CONCLUSION Angiotensin-converting enzyme inhibitors and AT 1 blocker may slow the progression of hepatic fibrosis; activated HSCs express AT 1 receptors, and AngII can stimulate the proliferation and collagen synthesis of HSCs in a dose-dependent manner; and activation of RAS may be related to hepatic fibrogenesis induced by CCl₄.

INTRODUCTION

Liver fibrosis is a consequence of chronic liver injury from different causes, including alcohol, toxins, chronic viral infections, metabolic disease, etc. In liver fibrogenesis there is an increased deposition of extracellular matrix (ECM) in the perisinusoidal and periportal spaces. The activated hepatic stellate cells (HSCs), have now been identified as the primary source of ECM synthesis in liver fibrogenesis^[1-3]. The pathogenetic significance of HSC relies on their ability to be activated into myofibroblast-like cells with enhanced production of ECM^[4,5]. Proliferative cytokine like platelet-derived growth factor and fibrogenetic cytokines like transforming growth factor- β (TGF- β) are major cytokines involved in the activation process, causing enhanced proliferation of HSC and matrix synthesis^[6,7].

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Researches increasingly show that locally synthesized angiotensin II (Ang II), as a fibrogenetic factor, is involved in cardiac fibrosis^[8,9], renal interstitial fibrosis^[10,11], and pulmonary fibrosis^[12,13]. Thus, treatment with angiotensin-converting enzyme (ACE) inhibitor or angiotensin II type 1 (AT1) receptor antagonist may attenuate the cardiac fibrosis that occurs in experimental myocardial infarction^[8], and may also retard the progression of renal glomerulosclerosis and interstitial fibrosis^[14,15] and pulmonary fibrosis^[16].

It is believed that Ang II has a direct fibrogenetic effect, independent of its systemic hemodynamic effect. The evidence supporting this notion comes from *in vitro* studies showing that the AT1 receptor is present on interstitial fibroblasts, such as cardiac fibroblasts^[17], renal interstitial fibroblasts^[18], and that Ang II directly increases ECM synthesis in cultured fibroblasts.

We hypothesized that the activation of local RAS might also be related to hepatic fibrogenesis. Thus, it is important to ascertain the existence of expression AT1 receptor on HSCs in the process of hepatic fibrosis. If this hypothesis is true, new preventive and therapeutic approaches may be provided to hepatic fibrosis. This study was designed: to assess effects of ACE inhibitor, enalapril, and AT1 receptor antagonist, losartan, on hepatic fibrosis induced by CCl₄; and to investigate whether or not there is expression of AT1 receptors on HSCs.

METHODS

Animals and therapeutic regimen

Adult male Sprague-Dawley rats (Animal Centre of Shanghai Medical University, China) weighing 180g-200g were housed in temperature and light-controlled room. The rats were divided into five groups ($n=10$ in each group): the control group, model group and three treated groups. Except for rats of the control group, all rats were given subcutaneous injection of 40% CCl₄ (CCl₄:olive oil 2:3), 0.3mL/100g, every 3 days for 6 weeks). Simultaneous treatments with enalapril (Changzhou Pharmacy, China) ($5\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$), losartan (Merk Co. England) ($10\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$), or enalapril + losartan administration (once a day by gavage for 6 weeks) were withdrawn a day before the rats were killed for study.

Serum analysis

After sacrificing the rats, blood samples were immediately taken and centrifuged at 4°C, and plasma were kept at -20°C for assays. The aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined by

the standard enzymatic methods. The serum levels of angiotensin II and renin activity were determined by radioimmunoassays (kit purchased from Northern Biot Co, China).

Image analysis of liver fibrosis

Liver fibrosis was assessed directly by hepatic morphometric analysis, which has been considered to be the gold standard for the quantification of fibrosis. For image analysis, three liver fragments (> 10mm²) were randomly taken in the right, median, and left lobes of each rat liver. The liver sections were fixed in a 10% solution of formaldehyde in 0.1mol/L phosphate-buffered saline (pH 7.4), and embedded in paraffin. Five-micrometer slides were prepared. Collagen expression was detected with standard van Gieson staining. The histomorphometric analysis was performed on a KS400 image analysis system (German). The liver slides were placed on the X-Y motorized stage of microscope after equalization of light intensity. The percentage of fibrosis or area of fibrosis could be obtained in microscopic fields. Total liver area of fibrosis was expressed as the mean of the percentage of fibrosis in the three liver fragments.

Expression of α -SMA and AT1 receptor in liver tissue

Immunohistochemical methods were used to detect the expressions of α -SMA and AT1 (anti-rat rabbit polyclonal AT1 antibody was the product of Santa Cruz Biot Co, USA) receptors in liver tissue.

Cells isolation and culture

HSCs were isolated from normal male Sprague-Dawley rat (500g-600g body weight) liver by a combination of pronase-collagenase perfusion and density gradient centrifugation, as previously described^[19], and identified by positive ultraviolet autofluorescence. Primary HSCs were cultured in 24-well plastic culture plate at a density of 5×10^5 cells/L, and maintained in Dulbecco's modified Eagle medium (DMEM) with 15% fetal calf serum (FCS) (Sigma) and penicillin/ streptomycin. After seven days, the cells were washed with PBS, and the expression of α -SMA (Monoclonal antibody purchased from Maixin Biot Co. China) was determined by immunohistochemical method. The expression of AT1 receptor was detected by immunofluorescence staining with anti-rat rabbit polyclonal antibody (Santa Cruz Biot) and labeled with fluorescein-isothiocyanate (FITC, Sigma).

Effect of AngII on HSCs proliferation

The effect of AngII on HSC proliferation was determined by MTT method. After being cultured

for 4 days, HSCs were digested with 0.25% trypsin and then cultured in serum-free medium in 96-well culture plate (200 μ L per well), to which was added AngII (to make a series of final concentrations: 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} mol/L). Each concentration included six wells, while the serum-free medium served as a control. After being cultured for 48 hours, HSCs were supplemented with 20 μ L MTT (5g/L) (Fluka Co. Product) and incubated for another 6 hours. Then the supernatant was discarded by aspiration and the HSC preparation was shaken with 200 μ L DMSO for 10 minutes, before the OD value was measured at 490nm.

Effect of AngII on collagen synthesis of HSCs

Collagen synthesis was determined by 3 H-proline incorporation. After cultured for 48 hours, HSCs were further cultured for additional 48 hours in serum-free medium containing 2 μ L 3 H-proline in various concentrations of Ang II (the final concentrations being 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} mol/L). Subsequently, HSCs were digested with 0.25% trypsin and collected on glass fiber membrane, then washed with PBS. After being dried, HSCs were suspended in 10mL scintillation solution for 12 hours. The radioactivity was determined by liquid scintillation and expressed as cpm / 2.5×10^5 cells.

Statistical analysis

Data were presented as mean \pm SEM. Comparisons among three or more groups were made by one-way ANOVA followed by Dunnett's *t* test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of RAS inhibitors on rat serum levels of ALT and AST Contrasted with rats of the control group, as shown in Table 1, rats receiving CCl_4 had increased serum ALT and AST significantly. However, chronic administration of enalapril and losartan prevented the increase in ALT and AST ($P < 0.01$, Table 1).

Serum renin activity and Ang II levels Serum renin activity ($\mu\text{g}\cdot\text{L}^{-1}/\text{h}$) of rats in the model group was three times higher than that of the control group ($P < 0.01$). Serum Ang II (ng/L) levels of model group also increased two times than that of control group ($P < 0.01$). Treatment with RAS inhibitors significantly decreased the serum renin activity and

serum levels of Ang II ($P < 0.05$, Table 2).

Fibrosis quantification As expected, there was an increase in the area of fibrosis in rats of the model group compared with that of the control group. There was a significant decrease of fibrosis in all three treated groups ($P < 0.05$), but no significantly difference was observed between the enalapril and losartan treated groups ($P > 0.05$, Table 3).

Table 1 Effects of RAS inhibitors on rat serum levels of ALT and AST

	<i>n</i>	ALT(U/L)	AST(U/L)
Control	10	99.50 \pm 18.78	244.50 \pm 46.52
Model	7	1863.29 \pm 893.68 ^a	2680.86 \pm 1039.12 ^a
Enalapril	7	466.14 \pm 132.64 ^b	785.29 \pm 262.40 ^b
Losartan	8	535.25 \pm 200.78 ^b	777.50 \pm 270.32 ^b
Enalapril+losartan	8	863.87 \pm 345.75 ^c	449.60 \pm 130.36 ^b

^a $P < 0.01$ vs control; ^b $P < 0.01$ vs Model; ^c $P < 0.05$ vs Model.

Table 2 Serum renin activity and Ang II levels

	<i>n</i>	Renin(mg·L ⁻¹ /h)	Ang II(ng/L)
Control	10	4.63 \pm 2.87	495.78 \pm 248.96
Model	7	17.70 \pm 12.13 ^a	1505.46 \pm 849.72 ^a
Enalapril	7	6.85 \pm 4.59 ^b	951.78 \pm 451.67
Losartan	8	10.92 \pm 4.97	747.62 \pm 316.34 ^b
Enalapril+losartan	8	7.84 \pm 5.78	643.82 \pm 261.47 ^b

^a $P < 0.01$ vs Control; ^b $P < 0.05$ vs Model.

Immunohistochemistry Expressions of AT1 receptors and α -SMA in liver tissue were detected by immunohistochemical methods. AT1 receptors mainly localized in the vasculature of liver tissue in the normal control rats. In rats of the model group, the expression of AT1 receptors mainly localized in the fibrotic areas, correlated with the expression of α -SMA. Enalapril, losartan, and combined treated groups showed a reduced AT1 receptor staining.

After culturing HSCs for seven days, the expression of AT1 receptors in HSC could be detected by FITC-immunofluorescence, together with the expression of α -SMA (Figure 1A-1D).

Effect of AngII on HSCs proliferation and collagen synthesis

Over a wide range of concentrations from 10^{-9} to 10^{-5} mol/L, AngII stimulated cultured HSCs proliferation in a dose-dependent manner (Figure 2). Increasing AngII concentration produced an increase in 3 H-proline incorporation (Figure 3). Differences among groups were significant ($P < 0.05$).

Table 3 Fibrosis measurements

	Control	Model	Enalapril	Losartan	Enalapril+losartan
Area of fibrosis	0.94 \pm 0.33	11.22 \pm 4.79 ^a	5.38 \pm 3.43 ^b	4.96 \pm 2.96 ^b	4.23 \pm 3.70 ^b

^a $P < 0.01$ vs Control; ^b $P < 0.05$ vs Model.

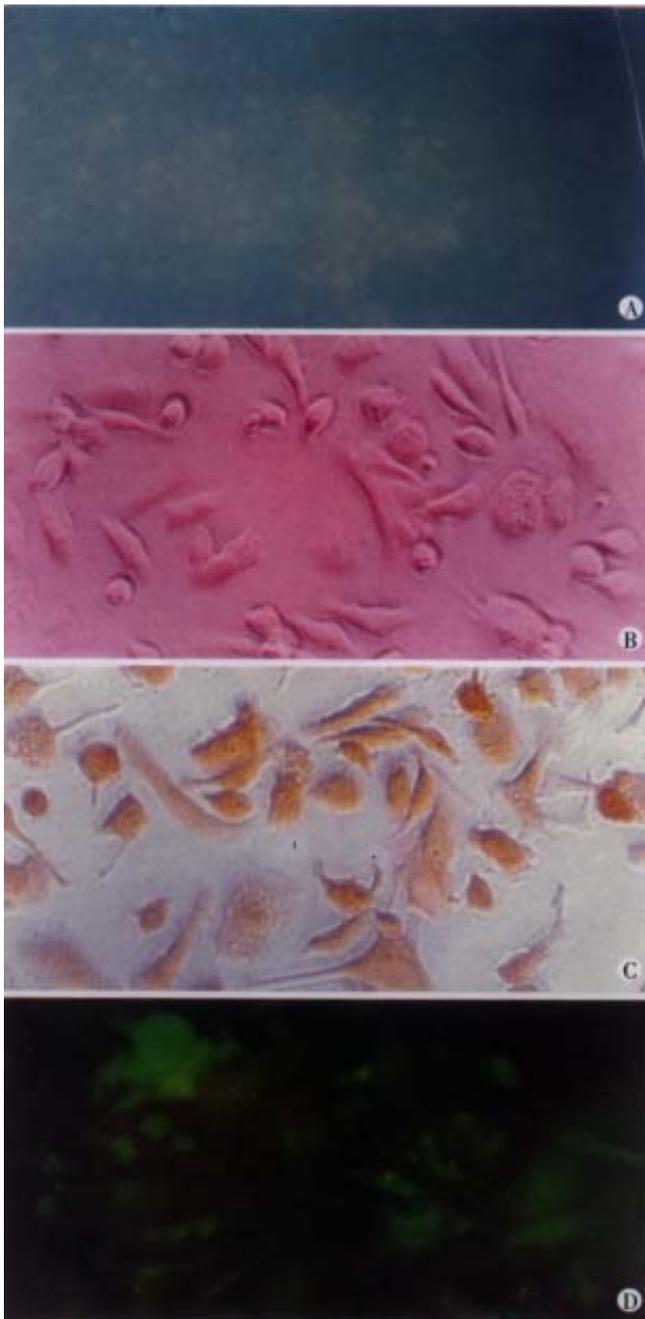


Figure 1A-1D A: Under the 328nm ultraviolet, HSCs had an autofluorescence. ($\times 400$ times) B: After cultured five days, HSCs transformed into myofibroblast-like cells. ($\times 400$ times) and expressed α -SMA (C, staining with DAB), labeled with FITC, expression of AT1 receptor was observed (D).

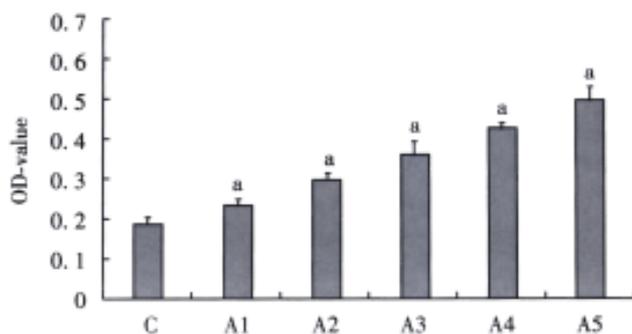


Figure 2 Effect of Ang II on HSC proliferation.

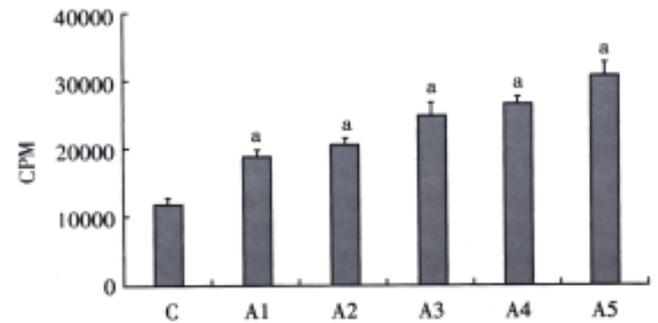


Figure 3 Effect of Ang II on collagen synthesis of HSC.

DISCUSSION

The present study demonstrates for the first time the expression of AT1 receptor subtype in fibrotic liver tissue and in primary cultured rat HSC.

In recent years, a series of investigations demonstrated that fibrogenesis was related to the activation of local RAS^[20-24]. Expression of AT1 receptors in several types of interstitial cells, such as cardiac fibroblasts^[17], renal interstitial fibroblasts^[18] and lung fibroblasts^[25]. *In vitro* study suggested that Ang II induced a net stimulation of collagen synthesis and expression of TGF- β via AT1 receptors in a dose-dependent manner^[26-30]. Blockade of AT1 receptors has been shown to inhibit DNA and collagen syntheses in interstitial cells after myocardial infarction^[31,32], renal and pulmonary injury^[15,33], and retarded the progression of cardiac, renal and pulmonary interstitial fibrosis^[34-41].

It is believed that the activation and proliferation of HSCs are major processes in hepatic fibrosis, although its pathogenesis has not been fully clarified^[42-45]. The expression of AT1 receptors in HSCs and fibrotic liver tissue supports the notion that activation of local RAS might be related to hepatic fibrogenesis induced by CCl₄. Our results showed that in rat livers, the expression of AT1 receptors in fibrotic areas correlated with the expression of α -SMA. In primary cultured HSCs, the expression of AT1 receptors also correlated with α -SMA. These findings indicated that, as a growth factor of interstitial cells, Ang II might prompt activation of HSCs in the process of hepatic fibrosis. Although both ACE inhibitor and AT1 receptor antagonist could alleviate hepatic fibrosis, as our results showed, a combination of these two gave no added effects of slowing the progression of hepatic fibrosis.

Another interesting finding in our study was that ACE inhibitor and AT1 receptor antagonist could attenuate the hepatocytic injury induced by CCl₄, suggesting their cytoprotective effects by virtue of antifibrogenesis.

As the principal effector molecule of RAS, the production of a profibrogenic cytokine, TGF- β was

enhanced by Ang II in progressive fibrosis of heart and kidney. Recently, Powell *et al*^[46] demonstrated that a statistically significant relationship was observed between inheritance of high TGF- α and angiotensinogen-producing genotype and the development of progressive hepatic fibrosis. Patients with chronic hepatitis C virus infection who inherited neither of the profibrogenic genotype had no or only minimal fibrosis. The documentation of a significant relationship between angiotensinogen genotype and fibrosis also suggested that Ang II might be another mediator of ECM production in the liver.

In conclusion, our investigation demonstrated that: ① ACE inhibitor and AT1 receptor antagonist might slow the progression of hepatic fibrosis induced by CCl₄, but a combination of the two gave no additive effect; ② activated HSCs expressed AT1 receptors, and Ang II could stimulate the proliferation and collagen synthesis of HSCs in a dose-dependent manner; and ③ local activation of RAS in liver tissue might be related to hepatic fibrogenesis induced by CCl₄.

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H2 strain attenuated live hepatitis A vaccines: protective efficacy in a hepatitis A outbreak

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Abstract

AIM To investigate the protective efficacy of H2 strain attenuated live hepatitis A vaccines (H2-strain vaccines) in hepatitis A (HA) outbreaks.

METHODS With the permission of their parents, 5551 pre-school and grade 1-3 primary school children were inoculated with 1 dose ($10^{6.5}$ TCID₅₀) of H2-strain vaccines in a nonrandomized, controlled trial conducted in Fucheng County, Hebei Province in May 1997. Another 6485 children in the same grades and compatible in gender and age were enrolled as controls. Epidemiological and serological survey was conducted to evaluate the protective efficacy of the vaccines. ELISA was used to detect serum IgM anti-HAV.

RESULTS HA outbreak started in early May 1998, peaked in the middle of the same month, and lasted about 80 days. Overall 302 HA cases were found, 192(63.58%) were 5-9 years old. One vaccinee and 25 control cases were found to have hepatitis A, which account for 0.28% (1/356) and 5.92% (25/422) of all vaccinees and controls in the 14 villages, respectively. The

protective efficacy of vaccines was 95.27% (95% CI: 85.83%-104.72%). In subjects tested for anti-HAV IgM from 13 villages, 1(0.40%) overt and 11(4.06%) asymptomatic HAV cases were found in 271 vaccinees, but 21(6.69%) of overt and asymptomatic ones were found in 314 controls.

CONCLUSION H2 strain vaccines were excellent in preventing overt hepatitis A, but not so effective in preventing asymptomatic hepatitis A virus infection. A booster dose might be needed to get permanent reliable immunity.

INTRODUCTION

Hepatitis A (HA) is the most common infectious diseases in China. Its notified incidence rates in recent years were 10-20 per 100 000. As severe under-reporting existed, the real incidence rates were possibly 10-fold higher than the notified rates. Community-wide outbreaks occurred at intervals of 5-10 years. The development of HA vaccine made this disease preventable. Two kinds of HA vaccines are available now in China: inactivated HA vaccine and live attenuated HA vaccine. Inactivated HA vaccine, with good tolerance, stability and immunogenicity, proved to be a good selection in developed countries^[1-3]. But most Chinese people refuse them for its considerably high cost. Live attenuated HA vaccine, with only about 1/10 expenses of the inactivated ones, proved to be the best alternatives. China-made H2 strain live attenuated HA vaccine (H2-strain vaccine), has been studied and on trial for more than 10 years, now is the best available one in China. Previous studies showed that it was safe, immunogenetic and had satisfactory preventive effect on sporadic HA^[4,5].

In May 1997, a controlled trial was performed in Fucheng County of Hebei Province, China, 5551 children were inoculated with H2 strain vaccine. Another 6485 children were set as controls. One year later, an outbreak of HA occurred in this county. We conducted an epidemiological and serological survey on this outbreak to evaluate the preventive effect of H2 strain vaccine on HA outbreaks.

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METHODS

Vaccination

The H2 strain HA vaccine ($10^{6.5}$ TCID₅₀, 1mL) used were produced by Zhejiang Academy of Medical Sciences. Subjects were children from pre-school class and grade 1-3 primary school who had no history of viral hepatitis or HA vaccination. One dose of vaccine was inoculated to each of the 5551 children with the permission of their parents. Another 6485 children who were in the same grade but refused to accept the vaccines were enrolled as controls. The two groups were compatible in gender and age. The vaccinations were finished in May 1997.

Criteria of diagnosis

Overt HAV infection or HA ① Those with symptoms and signs of acute hepatitis (fever, jaundice, anorexia, vomiting, abdominal discomfort, etc.), elevated serum alanine aminotransferase (ALT) levels and a positive test for serum anti-HAV IgM; ② with negative anti-HAV IgM for untimely serum collection, with significant epidemiological contact history to HA and without positive test results of hepatitis B, diagnosed by town clinics or higher level hospitals.

Asymptomatic or subclinical HAV infection Those who were seropositive for anti-HAV IgM but had no symptoms of hepatitis.

Novel HAV infection Those who were diagnosed as overt or asymptomatic HA cases.

Collection of HA cases

In order to avoid under-reporting, special persons were designated to be in charge of reporting HA cases in each village, school or town clinic, on the basis of previously existed infectious diseases reporting system. HA cases were collected mainly through the following ways: ① routine infectious disease notification; ② periodical review the results of outpatient and laboratory examinations in local hospitals; ③ periodical meeting of designated physicians in the town or village; and ④ direct visit to villages and schools where HA cases were found by trained specialists.

Interview of HA cases

All the HA cases from the trial population and some others from HA amassed villages were interviewed by specialists. A questionnaire was filled out for each case and his/her parents. Three mL serum sample was drawn from each of them for the detection of anti-HAV IgM.

Serological study

A serological survey was performed on trial populations from 13 villages (including 10 of the 14 villages with HA cases and 3 other villages). Serum

samples were obtained from 80.2% (271/338) of the vaccinees and 83.3% (314/377) of the controls. Serum anti-HAV IgM was detected by ELISA kits made by Beijing Wantai Bio-medical Limited Company, batch number: 8080104.

Analysis

All data from epidemiological survey or serological test were input into FoxBASE database. ANOVA (variances to be homogeneous) or Kruskal-Wallis one way analysis (variances to be different) was used to compare the means. Chi square test was used to compare the rates by more than two. Mantel-Haenszel (M-H) test or Fisher exact 1-tailed test was used to compare two rates. All these analyses were done by EPI INFO 6.0 software. Calculation of vaccines' protective efficacy (VE): $VE = (P_1 - P_2) / P_1 \times 100$. The 95% confidence intervals (95% CI) of $VE = VE(\%) \times 1.96 \times (1/P_1^2 \times P_2 Q_2 / N_2 + P_2^2 / P_1^2 \times P_1 Q_1 / N_1)^{1/2}$. P_1 and N_1 are the morbidity rate and sample number of control group, $Q_1 = (1 - P_1)$; P_2 and N_2 are the morbidity rate and sample number of vaccine group, $Q_2 = (1 - P_2)$.

RESULTS

Status of vaccination

With 307 003 population, Fucheng county was divided into 10 administrative towns, or further, 615 administrative villages. The distribution of vaccine group and control group was shown in Table 1. There were 5551 subjects in the vaccine group, which account for 1.8% of the whole population or 7.74% of the 1-15 year-old children in the county.

Table 1 Status of H2 strain vaccines vaccination in Fucheng county in May 1997

Towns	No. of villages	Whole population	Population aged 1-15	No. of trial villages	Vaccine group	Control group
Fucheng	79	50847	11815	28	1224	1457
Manhe	38	26958	6563	31	560	920
Gucheng	76	40083	8375	44	694	1087
Cuimiao	84	40818	8931	40	1155	1133
Matou	82	37005	8817	32	653	352
Xiakou	56	29030	7123	14	265	297
Wangji	58	21468	5607	32	431	525
Dabai	46	19154	4369	16	201	344
Jianqiao	39	17440	4437	8	196	109
Jiangfang	57	24200	5717	10	172	260
Total	615	307003	71754	255	5551	6485

Features of the outbreak

Cases of HA found during the first 9 months of 1998 are shown in Table 2. The incidence rates remained in sporadic level during the first 4 months (<10 cases each month) and an increasing tendency could be noticed in April. The morbidity began to rise significantly in the first ten days of May (46 cases), peaked on the next ten days (154 cases), lowered to sporadic level on the last ten days of June, and another small peak appeared on the second ten days

in July. The outbreak lasted about 80 days with 302 HA cases found. The incidence rate was 98.37/100 000. Two hundred and eighty-seven cases (95.03%) were found in the main outbreak period about 50 days (from the beginning of May to the second ten days of June), nine cases found in the second ten days of July might be secondary cases.

Most (255, 84.44%) HA cases were found in Gucheng town where the incidence rate reached 638.18/ 100 000. Others were found in Jianqiao (19 cases), Jiangfang (19 cases), Fucheng (6 cases), Manhe (2 cases) and Wangji (1 case) towns. The incidence rates in the 5 towns were 108.95, 78.51, 11.80, 7.42 and 4.66 per 100 000, respectively. No case was found in the other 4 towns.

The mean age of all HA cases was 8.81 years±6.87 years(ranged 1 year-63 years,median 7 years, most cases (252, 83.44%) aged 3 years-11 years and 192(63.58%) aged 5 years-9 years. More male cases were found than female ones (1.65:1) Table 3.

We tested 127 1-15 year old HA cases and 112 (88.19%) were anti-HAV IgM positive. The results are shown in Table 4. The mean ages of the 4 groups were not statistically different ($F=2.03$, $P>0.05$, ANOVA). Following the prolonging of the interval between HA attack and interview, decreasing trends were observed in anti-HAV IgM positive rate ($r=-0.7811$, $P>0.05$, linear correlation) and S/CO of anti-HAV IgM ($r=-0.9948$, $P<0.01$, linear correlation), the former being not statistically significant.

Table 2 HA cases found during the first 9 months in 1998

Months	First ten days	Second ten days	Last ten days	Total
January	1	0	0	1
February	1	3	3	7
March	2	3	0	5
April	5	0	4	9
May	46	154	48	248
June	26	13	3	42
July	3	9	3	15
August	3	3	2	8
September	0	1	0	1

Table 3 Age and sex distribution of HA cases found in Fucheng County during the HA outbreak in 1998

Age	Male	Female	Total
1	2	1	3
2	2	1	3
3	7	4	11
4	12	3	15
5	20	16	36
6	31	19	50
7	28	17	45
8	20	10	30
9	18	13	31
10	12	6	18
11	9	7	16
12-14	13	4	17
15-24	10	7	17
25-34	2	3	5
35-63	2	3	5
Total	188	114	302

Table 4 Results of serum IgM anti-HAV in 138 1-15 year old HA cases found in the HA outbreak in Fucheng, 1998

Days between HA attack and interview	No. of tested	Age (mean±SD)	Positive		
			No	%	S/CO(mean±SD)
1-29	12	6.33±2.57	12	100.00	10.33±1.22
30-59	46	8.11±2.39	44	95.65	7.12±2.50
60-89	61	7.36±2.52	49	80.33	5.09±2.71
90-119	8	7.13±1.25	7	87.50	2.80±2.24
Total	127	7.52±2.45	112	88.19	6.31±3.04

Protective efficacy of vaccines (VE)

In the HA outbreak, twenty-six HA cases were found in trial population, one in vaccine group and 25 in control group. They were distributed in 14 trial villages. The incidence rates of vaccine group and control group in the 14 villa ges were 0.28%(1/356) and 5.92%(25/422), respectively. The VE was 95.27% (95% CI: 85.83%-104.72%), Table 5.

Table 5 Incidence rates of the trial population in the 14 villages invested

Villages	Vaccine group			Control group		
	No. of Subjects	No. of HA cases	Incidence rate	No. of Subjects	No. of HA cases	Incidence rate
Dongni	17	0	0.00	29	1	3.45
Dongyi	32	0	0.00	34	3	8.82
Fuzhuang	0	0	0.00	9	1	11.11
Gengzhuang	5	0	0.00	10	3	30.00
Gucheng	48	0	0.00	62	3	4.84
Haitun	19	0	0.00	20	1	5.00
Houxong	32	1	3.13	26	2	7.69
Lihui	35	0	0.00	27	1	3.70
Liqiao	48	0	0.00	23	2	8.70
Liuhui	16	0	0.00	26	1	3.85
Liunan	35	0	0.00	40	2	5.00
Mengzhuang	4	0	0.00	49	3	6.12
Qiansong	40	0	0.00	42	1	2.38
Dainan	25	0	0.00	22	1	4.55
Total	356	1	0.28	422	25	5.92

We tested 80.2%(271/338) subjects of vaccine group and 83.3%(314/377) subjects of control group in 13 villages. The sampling rates of the two groups were not significantly different ($\chi^2=1.16$, $P>0.28$, M-H test). Results are shown in Table 6.

Table 6 Comparison between vaccine group and control group on HAV infection features

Groups	No. of Subjects	IgM anti-HAV(+)		Clinical HA cases		Subclinical infection		Novel infection		Clinical/subclinical ratio
		No.	%	No.	%	No.	%	No.	%	
Vaccine	271	12	4.4	1	0.4	11	4.1	12	4.4	1:11
Control	314	39	12.4	21	6.7	21	6.7	42	13.4	1:1
Relative risk		2.8		18.1		1.7		3.0		6.0
95%CI		1.5-5.3		2.5-133.9		0.8-3.4		1.6-5.6		0.9-40.2
χ^2		11.7		16.0		1.9		13.9		
P value		<0.001		<0.001		>0.5		<0.001		<0.01
Statistical method		M-H		M-H		M-H		M-H		Fisher

From Table 6 we found that anti-HAV IgM positive rate, HA incidence rate, HAV novel infection rate and ratio between HAV overt infection and asymptomatic infection were all significantly higher in control group than in vaccine group. HAV asymptomatic infection rate in control group was also higher than in vaccine group, but not statistically significant.

DISCUSSION

H2-strain vaccine was developed by Zhejiang Academy of Medical Sciences and proved to be safe and immunogenic^[6,7]. Its VE on sporadic HA was studied in a few trials. The results showed that it was dose-dependent. In early years, the titre of live HA vaccines was considerably low ($10^{5.0-5.5}$ -TCID₅₀) and the VE was only 79% - 90%^[8]. Researchers recommended $\geq 10^{6.5}$ TCID₅₀ as a standard dose in recent years. Large-scale trial showed that a dose of H2-strain vaccine $10^{7.0}$ TCID₅₀ induced 94.84% anti-HAV seroconversion and 100% VE^[5]. Our study showed that a dose of H2-strain vaccine ($10^{6.5}$ TCID₅₀) could induce >95% VE in HA outbreak. The result was comparably satisfactory.

Although only 1 HA case was found in vaccine group, 11 subclinical HAV infection cases were observed. This was understandable considering the low geometric mean titre (GMT, about 100mIU/mL)^[5] of serum anti-HAV after inoculation of live vaccines. We concluded that the vaccine was not so effective in preventing HAV infection as in preventing overt HA. The natural HAV infection 1 year after vaccination might act as a natural booster dose and induce life-long immunity. This was an ideal result. But the result might be different if the outbreak comes a few years later. The necessity of a booster dose, therefore, remains to be further studied.

The occurrence of the HA outbreak showed that 7.74% coverage of HA vaccine in children aged 1-15 year's was not enough to prevent HA outbreaks. Since the beginning of reporting viral hepatitis (HV) by type in 1990, only 1 HA outbreak

(1992, the incidence rate was 176/ 100 000) was observed in Fucheng County. The HA incidence rates remained at sporadic level between 1993 and 1997 (4.89-44.63/100 000). The accumulation of susceptible subjects might be the basic reason for the HA outbreak in 1998.

Although HA cases were found in 6 of the 10 towns in the county, most cases were found in Gucheng Town. Another HA outbreak will probably occur in the other 9 towns in a few years. So HA vaccination was still needed in Fucheng County. As the outbreak occurred in summer and most cases were less than 10 years of age, we suggested that another vaccination program be performed among children aged below 10 in next spring in the county except Gucheng town.

The fairly high positive rate (88.19%) of serum anti-HAV IgM in cases tested within 4 months after the attack showed that it was feasible to use anti-HAV IgM as an index in the retrospective study of HA outbreaks which occurred in recent 4 months.

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A high frequency of GBV - C/HGV coinfection in hepatitis C patients in Germany

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Subject headings GB Virus C; hepatitis G virus; hepatitis C virus; coinfection; polymerase chain reaction; sequencing; dominant viral subtype, Germany

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Abstract

AIM To detect infection rate of GBV-C/HGV in hepatitis C patients, to determine the methods of higher sensitivity and the primers of higher efficiency for GBV-C/HGV RNA detection and to study the dominant subtype and mutation of GBV-C/HGV.

METHODS Quantitative RT-PCR for detection of HCV RNA concentration in serum samples, RT-nested PCR with two sets of primers for detection of GBV-C RNA, RT-PCR ELISA with two sets of primers for detection of HGV RNA, nucleotide sequence and putative amino acid sequence analysis.

RESULTS The positive rates of GBV-C RNA at the 5'-NCR and NS3 region in 211 serum samples from the patients with HCV infection were 31.8% and 22.8% respectively. The positive rates of HGV RNA at the 5'-NCR and NS5 region in the same samples were 47.9% and 31.8% respectively. The total positive rate of GBV-C/HGV RNA was as high as 55.5%. HCV copy numbers in the patients without GBV-C/HGV coinfection were statistically higher than that in the patients with GBV-C/HGV coinfection ($P < 0.01$). Frequent mutation of nucleotide residue was present in the amplification products. Frameshift mutation was found in two samples with GBV-C NS3 region nucleotide sequences. All nucleotide sequences from amplification products showed higher homology to HGV genome than to GBV-C genome even

though part of the sequences were amplified with GBV-C primers.

CONCLUSION A high frequency of GBV-C/HGV coinfection existed in the hepatitis C patients. RT-PCR ELISA was more sensitive than RT-nested PCR for detection of GBV-C/HGV RNA. The primers derived from the 5'-NCR was more efficient than those derived from the NS3 and NS5 regions. A reverse relationship was found to exist between HCV RNA concentration and GBV-C/HGV infection frequency. HGV was the dominant subtype of the virus in the local area. The major mutations of GBV-C/HGV genomes were random mutation of nucleotide residue.

INTRODUCTION

Hepatitis G virus C (GBV-C) and hepatitis G virus (HGV) were recently identified as novel member of Flaviviridae family associated with human non A-E hepatitis^[1-5]. The two viral agents are different isolates of the same virus because of their high homology in nucleotide sequences and putative amino acid sequences^[6,7]. GBV-C/HGV can be transmitted parenterally through transfusions of blood, blood products, intravenous drug user, hemodialysis and vertical transmission^[8-19]. Although GBV-C/HGV were considered to be the major causative agent of human non A-E hepatitis, many later investigative data revealed that GBV-C/HGV infection rates in non A-E hepatitis patients were lower than 15%^[20-25]. GBV-C genome does not have the gene responsible for encoding core protein^[26,27]. HGV capsid protein is absent or defective and the capsid may be provided by another virus^[2,28]. Therefore, coinfection of GBV-C/HGV with other viruses is an interesting and important subject for investigation. The reported GBV-C/GV infection rates in hepatitis C patients were approximate 20%^[29-32] but a quite lower coinfection rate of HGV and HCV (5.6%) was also reported^[33]. Contradictory data about the clinical importance of GBV-C/HGV infection and nucleotide sequence mutation of the viral isolates indicated that the pathogenes is and variability of the virus are not fully understood^[34-44].

In this study, GBV-C/HGV RNAs in the 211 serum samples of hepatitis C patients were detected by RT-nested PCR and RT-PCR ELISA with four different sets of primers. The GBV-C/HGV RNA

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positive amplification products from part of the serum samples were cloned and then sequenced. The results of this study may contribute to the determination of GBV-C/HGV coinfection frequency in hepatitis C patients, choice of the methods with high sensitivity and the primers with high efficiency for GBV-C/HGV RNA detection and understanding the mutation of GBV-C/HGV genomes.

MATERIALS AND METHODS

Materials

211 serum samples of hepatitis C patients were obtained from the hospitals in Leubeck and in Koln of Germany. The 211 samples were confirmed to be HCV RNA positive by using HCV Monitor-TM Test Kit (Hoffmann LaRoche). This test is a routine work in our laboratory for quantitative detection of HCV RNA. HCV RNA concentration in the 211 samples ranged from 200 to 4 166 000 copies per milliliter of serum. All serum samples were HBV negative by using EIA and PCR. T-A Cloning Kit was purchased from Invitrogen. All other materials used in this study were purchased from Boehringer Mannheim.

Methods

Total serum RNA isolation Highly Pure RNA Isolation Kit was used to prepare total RNA from the samples according to the manufacturer's instruction.

Reverse transcription (RT) reaction Ten μ L total RNA preparation was mixed with 10 μ L RT master mixture containing 0.2 mmol/L dNTP, 50 nmol/L hexanucleotide, 20 U M-MuLV-reverse transcriptase, 20 U RNA inhibitor and 4 μ L 5 \times RT buffer (pH 8.3), and incubated at 37 $^{\circ}$ C for 45 min.

RT-nested PCR for GBV-C RNA detection Two sets of primers derived from GBV-C 5'-NCR and NS3 region were used in the RT-nested PCR^[45]. 5'-NCR external primers: 5'-ATGACAGGGTTGGTAG GTCGTAAATC-3' (sense), 5'-CCCCACTGGTC CTTGTCAACTCGCCG-3' (antisense). 5'-NCR internal primers: 5'-TGGTAGCCACTATAGGTGG-GTCTTAA-3' (sense), 5'-ACATTGAAGGGCGACG-TGGACCGTAC-3' (antisense). NS3 region external primers: 5'-GCT CGCCTATGACTCAGCAT-3' (sense), 5'-GTCACCTCAACGACCTCCTC-3' (antisense). NS3 region internal primers: 5'-ATCCATAATTGAGAC AAAGCTGGA-3' (sense), 5'-CCACCAACCCACAGTCGGTG-3' (antisense). For the first PCR round, 10 μ L- RT product was mixed with PCR master mixture containing 20 pmol/L primers, 0.2 mmol/L dNTP, 3 U *Tag* polymerase and 10 μ L 10 \times PCR buffer (pH 9.1) and 25 mmol/L MgCl₂. For the second PCR round, 5 μ L product from the first PCR round was used as template and the other reaction reagents were the same as that in the first PCR round except the

primers. The volume per reaction in the two rounds was 100 μ L. The PCR parameters were described as the following: 94 $^{\circ}$ C 3 min (\times 1); 94 $^{\circ}$ C 30 s, 56 $^{\circ}$ C 30 s, 72 $^{\circ}$ C 30 s, (\times 10); 94 $^{\circ}$ C 30 s, 56 $^{\circ}$ C 30 s, 72 $^{\circ}$ C 35 s, (\times 25, 5 s in addition for each of the following cycle); and 72 $^{\circ}$ C 7 min (\times 1). The products of RT-nested PCR were detected by 2% ethidium bromide stained agarose gel electrophoresis.

RT-PCR ELISA for HGV RNA detection HGV RNA in the samples was detected by Hepatitis G Virus Primer and Capture Probe Set Kit according to the manufacturer's instruction. This kit contains two sets of primers for detection of HGV 5'-NCR and NS5 RNA and two sets of capture probes for detecting PCR products. 5'-NCR primers: 5'-CGGCCAAAAGGTGGTGGATG-3' (sense), 5'-CGACGAGCCTGACGTCGGG-3' (antisense). NS5 region primers: 5'-CTCTTTGTGGTAGTAGC-CGAGAGAT-3' (sense), 5'-CGAATGAGTCAG-AGGACG GGGTAT-3' (antisense). 5'-NCR capture probe: 5'-Biotin-GGTAGCCA CTATAGGTGGG-3'. NS5 region capture probe: 5'-Biotin GTTACTGAGAGCAGCTCAGAT-3'. *Taq* and two polymerase mix in this kit was used in PCR to allow DIG-11-dUTP incorporating the products during amplification process. The PCR parameters were described as the following: 94 $^{\circ}$ C 3 min (1 \times); 94 $^{\circ}$ C 30 s, 55 $^{\circ}$ C 30 s, 72 $^{\circ}$ C 30 s (10 \times); 94 $^{\circ}$ C 30 s, 55 $^{\circ}$ C 30 s, 72 $^{\circ}$ C 35 s (5 s in addition for each of the following cycle, 30 \times); 72 $^{\circ}$ C 7 min (1 \times).

The RT-PCR products were detected by using PCR ELISA DIG Detection Kit according to the manufacturer's instruction. The PCR ELISA is a liquid phase hybridization/DIG detection system. It was found that DNA fragments amplified from HGV RNA could also be seen in 2% ethidium bromide stained agarose gel if the OD value at 405 nm by RT-PCR ELISA was higher than 1.0.

Analysis of nucleotide sequences and putative amino acid sequences The target DNA fragments in GBV-C RT-nested PCR products or HGV RT-PCR products (DIG-11-dUTP replaced by dTTP) were cloned into PCR2.1 plasmid by using T-A Cloning Kit according to the manufacturer's instruction. The plasmid was amplified in *E. coli* and then recovered by the Sambrook's method^[46]. The inserted sequences were analyzed by MWG-BIOTECH. The homology of the nucleotide sequences and putative amino acid sequences was compared with the those thow reported^[1,2].

RESULTS

GBV-C and HGV RNA detection rates Seventy-four of the 211 serum samples (35.1%) were GBV-C RNA positive, 41 of the 74 samples (55.4%) were GBV-C RNA positive for both the 5'-NCR and NS3 region, 26 of the 74 samples (35.1%) were only 5'-NCR RNA positive and only 7 of the 74 samples (9.5%) detected NS3 region RNA. A statistically

significant difference ($\chi^2=4.32, P<0.05$) was found to exist between the positive rates of GBV-C 5'-NCR (67/211) and those of NS3 region (48/211).

One hundred and five (49.8%) of the 211 serum samples were HCV RNA positive, 63 of 105 samples (60.0%) were HGV RNA positive for both the 5'-NCR and NS5 regions, 38 of the 105 samples (36.2%) were only 5'-NCR RNA positive and 4 of the 105 samples (3.8%) were only detectable for NS5 region RNA. A statistically significant difference ($\chi^2=11.43, P<0.01$) was also found between the positive rates of HGV 5'-NCR (101/211) and NS5 region (67/211). The respective target fragments amplified from GBV-C 5'-NCR, NS3 region and HGV 5'-NCR, NS5 region are shown in Figures 1, 2.

Total positive rate of GBV-C/HGV and distribution of the positive samples One hundred and seventeen (55.5%) of the 211 serum samples were GBV-C and/or HGV RNA positive and 94 of the 211 samples (44.5%) were negative for both viral RNAs, 62, 12 and 43 of the 117 samples were positive for both viral RNAs, GBV-C RNA positive and detectable HGV RNA respectively. The distribution of the 117 samples is shown in Table 1.

Relationship between HCV RNA concentration and GBV-C/HGV infection frequency The range of HCV RNA concentrations of the 211 serum samples was 200-4 166 000 copies/mL. A statistically significant

difference ($t'=2.559, P<0.01$) was present in the HCV RNA copy numbers of the group with GBV-C and/or HGV RNA positive samples (mean =344 000- HCV RNA copies/mL) and the group with both viral RNA negative samples (mean=556000 HCV RNA copies/mL). In addition, HCV RNA concentration in each of 21 cases of the 211 samples was higher than 1 000 000 copies/mL. Six of the 21 cases distributed in GBV-C and/or HGV RNA positive group and the other 15 cases belonged to the negative group. In comparison with the two percentages (6/211 and 15/211), a statistically significant difference was also found ($\chi^2=4.06, P<0.05$).

Table 1 Distribution of 117 GBV-C and/or HGV positive samples

GBV-C		HGV		Positive cases	Positive rate (%)
5'-NCR	NS5	5'-NCR	NS5		
+	*	+	+	26	12.3
+	+	+	**	7	3.3
+	+	-	+	3	1.4
+	-	+	+	9	4.3
-	+	+	+	3	1.4
+	-	+	-	11	5.2
-	+	+	-	3	1.4
+	+	-	-	5	2.4
-	-	+	+	25	11.9
+	-	-	-	6	2.8
-	-	+	-	17	8.1
-	+	-	-	1	0.5
-	-	-	+	1	0.5
Total				117	55.5

* +: the viral RNA was detectable. ** -: the viral RNA was undetectable.

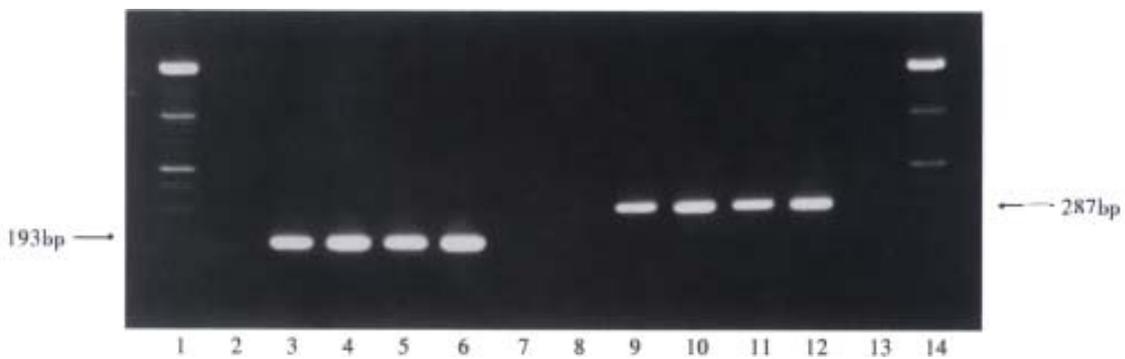


Figure 1 Target amplification fragments from GBV-C 5'-NCR and NS3 region. (1 and 14: markers; 2 and 8: negative serum samples; 7 and 13: blanks; 3-6: four GBV-C 5'-NCR RNA positive serum samples; 9-12: four GBV-C NS3 region RNA positive serum samples)

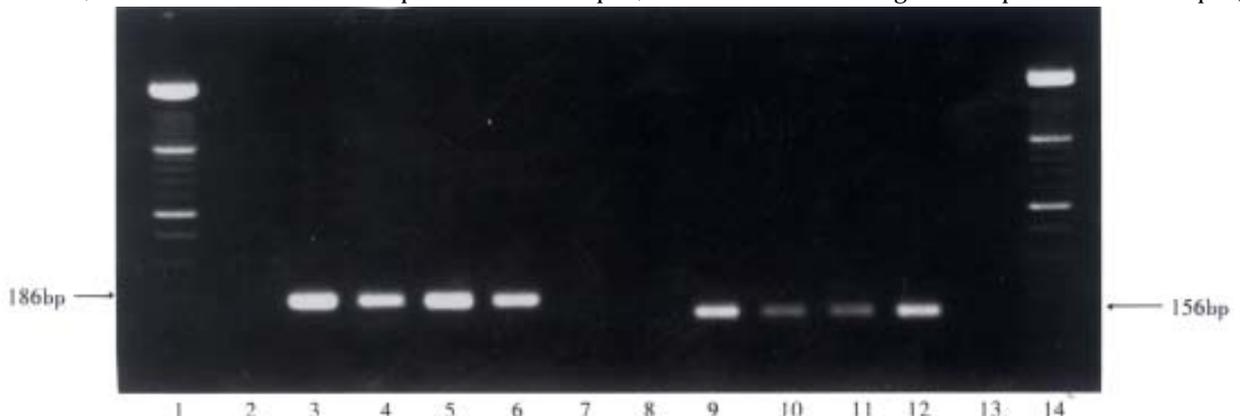


Figure 2 Target amplification fragments from HGV 5'-NCR and NS5 region. (1 and 14: markers; 2 and 8: negative serum samples; 7 and 13: blanks; 3-6: four HGV 5'-NCR RNA positive serum samples; 9-12: four HGV NS5 region RNA positive serum samples)

Nucleotide sequence analysis The homology of the nucleotide sequences of GBV-C 5'-NCR RT-nested PCR products from 3 serum samples compared with the reported GBV-C sequence^[1] and the reported HGV sequence^[2] was 81.8%, 81.1%, 82.5% and 97.6%, 97.2%, 99.3% respectively. The homology of the two reported sequences at the 5'-NCR was 81.8%. The homology of the nucleotide sequences of GBV-C NS3 region RT-nested PCR products from 6 samples compared with the reported GBV-C sequence^[1] and the reported HGV sequence^[2] was 80.2%, 82.3%, 81.5%, 84.4%, 85.6%, 85.2% and 86.8%, 88.1%, 88.1%, 88.1%, 86.8%, 87.7% respectively. The homology of the two reported nucleotide sequences at the NS3 region was 84.4%. Although the primers used in the RT-nested PCR derived from GBV-C genome, each of the 9 nucleotide sequences showed a higher homology to HGV than to GBV-C. These nucleotide sequences are shown in Figures 3, 4.

The homology of the nucleotide sequences of HGV 5'-NCR RT-PCR products from 3 samples compared with the reported HGV sequence^[2] and

the reported GB V-C sequence^[1] was 95.2%, 99.3%, 98.6% and 86.4%, 87.1%, 87.8% respectively. The homology of the two reported sequences at the 5'-NCR was 86.4%, whereas the homology of the nucleotide sequences of HGV NS5 region RT-P CR products from 3 serum samples compared with the reported HGV^[2] sequence and the reported GBV-C sequence^[1] was 95.3%, 96.3%, 96.3% and 94.4%, 94.4%, 93.4% respectively. The homology of the two reported sequences at the NS5 region was 93.4%. These nucleotide sequences are shown in Figures 5, 6.

The homology in comparison of these sequences mentioned above did not contain the primer sequences.

The putative amino acid sequence analysis Putative amino acid sequences translated from the 6 nucleotide sequences from GBV-C NS3 region and the 3 nucleotide sequences from HGV NS5 region were respectively compared with the reported HGV and GBV-C sequences^[1,2]. These putative amino acid sequences were shown in Figures 7, 8.

```

(1) 1  TGGTAGCCACTATAGGTGGGTCTTAAGGGGAGGCTACGGTCCCTCTTGCG
(2) 1  .....A.A...T..A.A.T.....T.
(3) 1  .....A.A...T..A.A.T.....T.
(4) 1  .....A.A...T..A.A.T.....T.
(5) 1  .....A.A...T..A.A.T.....T.

(1) 51  CATATGGAGGAAAAGCGCACGGT CCACAGGTGTTGGTCCTACCGGTGT
(2) 51  .C.GC..C.AG.CC.....C.....GG
(3) 51  .G.GC..G.AG.CC.....C.....G
(4) 51  .C.GC..C.AC.CC.....C.....G
(5) 51  .C.GC..C.AG.CC.....C.....G

(1) 99  AATAAGGACCCGGCGCTAGGCACGCCGTTAAACCGAGCCCGTTACTCCCC
(2) 100 .....G...A..TC...T..T.....C.A..
(3) 101 .....G...A..TC...T..T.....A..C.A..
(4) 100 .....G...A..TC...AC.T.....C.....C.A..
(5) 100 .....G...A..TC...T..T.....C.A..

(1) 149  TGGGCAAACGACGCCACGTACGGTCCACGTCGCCCTTCAATGT
(2) 150 .....
(3) 151 .....
(4) 150 .....T.....
(5) 150 .....

```

Figure 3 Homology of the nucleotide sequences from GBV-C 5'-NCR RT-nested PCR products from 3 serum samples as compared with the reported sequences.

(1) the reported GBV-C 5'-NCR sequence, (2) the reported HGV 5'-NCR sequence, (3)-(5) the sequences of GBV-C 5'-NCR RT-nested PCR product from 3 samples. Underlined areas indicated the primers' position.

```

(1) 1  ATCCATAATTGAGACAAAGCTGGACGTTGGTGAGATCCCCTTTTATGGGC
(2) 1  T.....AT.....G..C.....T.....
(3) 1  .....T..G..A.....
(4) 1  .....T..G..A.....
(5) 1  .....T..G..A.....
(6) 1  .....T..G.....
(7) 1  .....T..G.....C.....
(8) 1  .....T..G.....C.....

(1) 51  ATGGTATCCCCCTCGAGCGTATGAGGACTGGTCGCCACCTTGTATTCTGC
(2) 51  ...A..A.....G...C..A..C..AA.G....C..G.....
(3) 51  ...C..A.....T....G...C..A..C..GA.G..T..CA.....
(4) 51  ...C..A.....T....G...C..A..C..GA.G..T..CA.....
(5) 51  ...C..A.....T....G...C..A..C..GA.G..T..CA.....
(6) 51  ...C..A.....G....G...C...C..AA.G..T..C..G.....
(7) 51  .....A.....T....A...C...C..AA.G..T..C.....
(8) 51  ...C..A.....G...C..C..C..CA.G....C..G.....

(1) 101  CATTCCAAGGCGGAGTGCGAGAGATTGGCCGGCCAGTTCTCCGCGCGGGG
(2) 101  ....T.....T.....C..CC..T..T.....TA....
(3) 101  ....T.....T.....T...C..CC..T.....T..TA....
(4) 101  ....T.....T.....T...C..CC..T.....T..TA....
(5) 101  ....T.....T.....T...C..CC..T.....T..TA....
(6) 101  ..C..A.....T...C..CC.....T....T..T..CA....
(7) 101  .....T.....C..GC..A....T....T..T..CA....
(8) 101  ..C.....T...C..TC..T..T.....T..T..TA....

(1) 151  GGTTAATGCCATCGCCTATTATAGGGGTAAGGACAGTTCATCA TCAA
(2) 151  ...C.....T.....A.....T....G
(3) 151  ...G.....T..T.....C....C..A.....T..GTG
(4) 151  ...GG....T..T.....C....C..A.....C..TGTG
(5) 151  ...G.....T..T.....C....C..A.....GTG
(6) 151  ...A..C....T.....A....T..G
(7) 151  ...G.....T.....G..A.....T....G
(8) 151  .....T.....G.....G

(1) 200  GACGGAGACCTGGTGGTTTGTGCGACAGACGCGCTCTCTACCGGATACAC
(2) 200  ..T..G.....C.....T.....T..C..T.....
(3) 201  .....A.....G.....T.....A..C..T.....
(4) 201  .....G.....T.....A..C..T.....
(5) 200  .....A.....G.....A..C..T.....
(6) 200  .....G.....T.....A..C.....
(7) 200  .....G..C.....A..C.....
(8) 200  .....G..C..T.....A..C.....

(1) 250  AGGAAACTTCGATTCTGTCACCGACTGTGGGTGGTGG
(2) 250  T.....T.....C..C.....A..A....
(3) 251  T..G..T.....
(4) 251  T..GCCT.....
(5) 250  T..G..T.....
(6) 250  T.....T.....C.....
(7) 250  T..G.....
(8) 250  T..T.....
    
```

Figure 4 Homology of the sequences from GBV-C NS3 RT-nested PCR products from 6 serum samples compared with the reported sequences.

(1) the reported GBV-C NS3 region sequence, (2) the reported HGV NS3 region sequence, (3)-(8) the sequences from GBV-C NS3 region RT-nested PCR products from 6 serum samples. Underlined areas indicated the primers' position.

```

(1) 1  CGGCCAAAAGGTGGTGGATGGGTGATGACAGGGTTGGTAGGTCGTAAATC
(2) 1  .....
(3) 1  .....
(4) 1  .....
(5) 1  .....

(1) 51  CCGGTCACCTTGGTAGCCACTATAGGTGGGTCTTAAGAGAAGGTTAAGAT
(2) 51  .....T.C.....G.G...C..C.G.
(3) 51  .....T.C.....G.....
(4) 51  .....G.....
(5) 51  .....

(1) 101 TCCTCTTGTGCCTGCGGCGAGACCGCGCACGGTCCACAGGTGTTGGCCCT
(2) 101 C.....C..A.AT..A.GA.AA.....T...
(3) 101 .....TG.....A.....C.....
(4) 101 .....
(5) 101 .....T.....A.....

(1) 151 ACCGGTGGGAATAAGGGCCCGACGTCAGGCTCGTCCG
(2) 151 .....T.....A....G..CT....A..C..
(3) 151 .....T.....
(4) 151 .....T.....
(5) 151 .....T.....

```

Figure 5 Homology of the nucleotide sequences of HGVC 5'-NCR RT-PCR products from 3 serum samples compared to the reported sequences.

(1) the reported HGVC 5'-NCR sequence, (2) the reported GBV-C 5'-NCR sequence, (3)-(5) the sequences of HGVC 5'-NCR RT-PCR products from 3 samples. Underlined indicated the primers' position.

```

(1) 1  CTCTTTGTGGTAGTAGCCGAGAGATGCCTGTATGGGGAGAAGACATCCCC
(2) 1  ....C.....G.....A...
(3) 1  .....G.....
(4) 1  .....G.....
(5) 1  .....G.....

(1) 51  CGTACTCCATCGCCAGCACTTATCTCGGTTACTGAGAGCAGCTCAGATGA
(2) 51  ..C.....T.....G.....
(3) 51  ..C.....A.....C.....
(4) 51  ..C.....A.....
(5) 51  ..C.....G.....

(1) 101 GAAGACCCCGTCGGTGTCTCCTCGCAGGAGGATACCCCGTCCTCTGACT
(2) 101 .....T.....A.....C.....A....
(3) 101 .....
(4) 101 .....
(5) 101 .....

(1) 151 CATTCG
(2) 151 ....T.
(3) 151 .....
(4) 151 .....
(5) 151 .....

```

Figure 6 Homology of the nucleotide sequences from HGVC NS5 RT-PCR products from 3 serum samples compared to the reported sequences.

(1) the reported HGVC NS5 region sequence, (2) the reported GBV-C NS5 region sequence, (3)-(5) the sequences from HGVC NS5 region RT-PCR products from 3 serum samples. Underlined areas indicated the primers' position.

```

(1) 1  SLIETKLDVGEIPFYGHGIPLERMRTGRHLVFCSKAECERLAGQFSARG
(2) 1  .....
(3) 1  .....I.....
(4) 1  .....I.....
(5) 1  .....I.....
(6) 1  .....
(7) 1  .....
(8) 1  .....T.....

(1) 51  VNAIAYYRGKDSSI IKDGLVVCATDALSTGYTGNFDSVTDCGLV
(2) 51  .....
(3) 51  .....RGRRPSGVCYRRAIHWVHWEFRFCHRLWVG
(4) 51  .D.....TCGRRPGGVCYRRAIHWVHWAFRFCHRLWVG
(5) 51  .....V.....
(6) 51  .....
(7) 51  .....
(8) 51  .....
    
```

Figure 7 Comparison of the putative amino acid sequences from GBV-C NS3 RT-nested PCR products from 9 serum samples with the reported sequences.

(1) the reported GBV-C sequence, (2) the reported HGV sequence, (3)-(8) putative amino acid sequences translated from the 6 nucleotide sequences from GBV-C NS3 region. Underlined are indicated the primers' position.

```

(1) 1  LCGSSREMPVWGEDIPRTPSPALISVTESSSDEKTPSVSSSQEDTPSSDS
(2) 1  .....L..T.....
(3) 1  .....
(4) 1  .....
(5) 1  .....A.....
    
```

Figure 8 Comparison of the putative amino acid sequences from HGV NS5 RT-PCR products from 3 serum samples with the reported sequences.

(1) the reported HGV sequence, (2) the reported GBV-C sequence, (3)-(5) putative amino acid sequences translated from the 3 nucleotide sequences from HGV NS5 region. Underlined areas indicated the primers' position.

DISCUSSION

As mentioned in the introduction, the reported GBV-C/HGV infection rates in non A-E hepatitis patients were lower than 15% and the reported GBV-C/HGV and HCV coinfection rates were approximate 20%. In this study, a high total positive rate (55.5%) of GBV-C and/or HGV RNA in hepatitis C patients was found. Such a high frequency of GBV-C and/or HGV and HCV coinfection including most of the reported coinfection rates, suggested that GBV-C/HGV coinfecting with HCV may be one of the features of its prevalence.

GBV-C RNA positive rate in the 211 samples by using RT-nested PCR was 35.1%. HGV RNA positive rate in the same samples by RT-PCR ELISA was as high as 49.8%. Statistical analysis of the two positive rates indicated that RT-PCR ELISA is more sensitive than RT-nested PCR ($\chi^2 = 9.32, P < 0.01$).

In the 211 serum samples, the positive rate

(31.8%) of GBV-C 5'-NCR was higher than that (22.8%) of GBV-C NS3 region ($\chi^2 = 4.32, P < 0.05$) and the positive rate (47.9%) of HGV 5'-NCR was also higher than that (31.8%) of HGV NS5 region ($\chi^2 = 11.43, P < 0.01$). These data led to the conclusion that the primers derived from the 5'-NCRs were more efficient than those derived from the NS regions^[47]. According to the results of nucleotide sequence analysis, this efficiency difference of primers was probably due to the higher conservation of sequences within the 5'-NCRs. In addition, only 26 of the 117 GBV-C and/or HGV RNA positive samples were positive for all the four viral genomic regions. It suggested that application of multiple primers is helpful to increase positive rate of GBV-C/HGV RNA detection.

A reverse relationship of HCV RNA concentration and GBV-C/HGV infection frequency was found in this study. This finding suggested that GBV-C/HGV and HCV might suppress each other *in vivo*. This reverse

relationship seemed to be contradictory to the high GBV-C/HGV and HCV coinfection rate. However, this kind of situation of cocurrence and competition is a normal phenomenon among microorganisms. For example, HBV and HCV coinfection is frequent in Asia but HCV was found to suppress the replication of HBV and vice versa to a lesser extent [48-50].

In this study, the homology of nucleotide sequences in the products amplified with the primers derived from HGV genome was higher in the reported HGV sequence than in the reported GBV-C sequence. Such results could be expected, however, all nucleotide sequences in the products amplified with the primers derived from GBV-C genome also showed higher homology with the reported HGV sequence. These data reveal the fact that: HGV and not the GBV-C, is the dominant subtype in the local area. Besides, these existed random mutations in a large number of nucleotide residue in all amplification products and two showed frameshift mutation which indicate that mutation of GBV-C/HGV genomic sequences occurs easily at the subtype level.

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Identification, localization and morphology of APUD cells in gastroenteropancreatic system of stomach-containing teleosts

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Subject headings stomach-containing teleosts; gastroenteropancreatic system; APUD cells; immunocytochemistry

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Abstract

AIM To identify the type localization and morphology of APUD endocrine cells in the gastroenteropancreatic (GEP) system of stomach-containing teleosts, and study APUD endocrine system in the stomach, intestine and pancreas of fish species.

METHODS Two kinds of immunocytochemical (ICC) techniques of the streptavidin biotin-peroxidase complex (SABC) and streptavidin-peroxidase (S-P) method were used. The identification, localization and morphology of APUD endocrine cells scattered in the mucosa of digestive tract, intermuscular nerve plexus and glandular body of northern snakehead (*Channa argus*), ricefield eel (*Monopterus albus*), yellow catfish (*Pelteobagrus fulvidraco*), mandarin fish (*Siniperca chuatsi*), largemouth bass (*Micropterus salmoides*), oriental sheatfish (*Silurus asotus*), freshwater pomfret (*Colossoma brachypomum*) and Nile tilapia (*Tilapia nilotica*) were investigated with 8 kinds of antisera.

RESULTS The positive reaction of 5-hydroxytryptamine (5-HT) immunoreactive endocrine (IRE) cells was found in the digestive

tract and glandular body of 8 fish species in different degree. Only a few gastrin (GAS)-IRE cells were seen in *C. argus*, *M. albus* and *P. fulvidraco*. Glucagon (GLU)-IRE cells were not found in the digestive tract and glandular body but existed in pancreatic island of most fish species. The positive reaction of growth hormone (GH)-IRE cells was found only in pancreatic island of *S. chuatsi* and *S. asotus*, no positive reaction in the other 6 fish species. *Somatostatin (SOM)*, calcitonin (CAL), neurofilament (NF) and insulin (INS)-IRE cells in the stomach, intestine and pancreas of 8 kinds of fish were different in distribution and types. The distribution of all 8 APUD cells was the most in gastrointestinal epithelium mucosa and then in digestive glands. The positive reaction of SOM-and 5-HT-IRE cells was found in intermuscular nerve plexus of intestine of *P. fulvidraco* and *S. chuatsi*. Only GH-IRE cells were densely scattered in the pancreatic islands of *S. chuatsi* and *S. asotus*, and odd distribution in the pancreas of *S. asotus*. SOM-IRE cells were distributed in the pancreatic islands of *S. asotus*, *C. Brachypomum* and *T. nilotica*. There were INS-IRE cells in the pancreatic islands of *S. chuatsi* and *S. asotus*. Eight kinds of APUD cells had longer cell body and cytoplasmic process when they were located in the gastrointestinal epithelium, and had shorter cell body and cytoplasmic process in the gastric gland, and irregular shape in the esophagus and pancreatic island.

CONCLUSION Eight kinds of IRE cells were identified in the GEP system of stomach-containing teleosts. These endocrine cells were scattered in gastrointestinal mucosa, intermuscular nerve plexus, gland body, pancreatic gland and islands under APUD system. CAL-and GH-IRE cells in the pancreatic islands of fishes showed functional diversity for these two hormones. Their morphological feature provides evidence of endocrine-paracrine and endocrine-exocrine acting mode. This research can morphologically prove that the GEP endocrine system of fish (the lowest vertebrate) is almost the same as of mammal and human.

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INTRODUCTION

In recent years, APUD (amine precursor uptake and decarboxylation) cells in the digestive tract attracted worldwide attention. It was a research domain developed quickly in recent years, especially gastrointestinal hormones^[1-7]. Gastrointestinal tract was not only the digestive organ but also the biggest and most complex endocrine organ in animal^[8]. It has been proved that gastrointestinal hormones were not only related with feeding behavior but also with nutrition of gastrointestinal tract. At present it has been applied to clinical practice^[9-24]. The endocrine tumor of digestive tract could occur in gastrointestinal tract and liver or in pancreatic gland^[25,26], even over ninety per cent of carcinoid occurred in gastrointestinal^[27-40]. What is the hormone's effect on it So it is of special significance to study endocrine cells in digestive system. Up till now, researches about digestive tract in animal have been reported^[41], but the research data about fish having close relations with human food are scarce. The distribution of APUD cells in the gut of 8 stomachless teleosts was reported^[42]. In the present paper, ICC studies on APUD cells in the GEP system of stomach-containing teleosts were reported further, delving into difference of gastrointestinal hormone types, secretory way, distribution and cell's shapes among fish, mammal and human, in order to provide basic materials for studying gastrointestinal endocrinology, gastroenterology, origin and prevention of disease in digestive system and its treatment.

MATERIALS AND METHODS

Specimens and section

Ricefield eel (*M. albus*), northern snakehead (*C. argus*), oriental sheatfish (*S. asotus*), mandarin fish (*S. chuatsi*), and yellow catfish (*P. fulvidraco*) were bought separately from the aquatic products market in Wuhan; freshwater pomfret (*C. brachyomum*) and Nile tilapia (*T. nilotica*) were bought from The Second Fish and Stock Farm in Hongshan District of Wuhan; largemouth bass (*M. Salmoides*) was bought from Nanhu Fish Farm. We reared above 8 kinds of fish temporarily in fresh water for 24 hours, then according to the methods in references^[43-46], and collected the samples and made the sections. Monoclonal antibody of GH was used only in SABC ICC stain method^[46], S-P ICC stain method was used in the other 7 kinds of antibody^[43].

Reagents and antisera

The details of the different antisera, the working dilutions and main reagents used in this study are listed in Table 1.

ICC staining steps and control

The steps of S-P ICC stain and control references and

the SABC ICC staining steps and control followed references^[43,46].

Observation, photomicrograph and count

Five fishes used for each species in all 8 kinds of teleost studied, 7 specimens of each fish were observed and photomicrographed under the Olympus (BH-2) photomicroscope. The dark brown positive cells on section were counted under 10×20 times field. The average number of positive cells from 10 fields selected randomly in each specimen part was the IRE cell number of this part in each fish. The average number of 5 fishes of each species was quantified IRE cell's distribution density in every part. The distribution density was showed with five grades.

RESULTS

Types and distribution of APUD cells

APUD cell types, distribution and density in different parts of GEP system of 8 kinds of stomach-containing teleosts are listed in Table 2. It can be seen from Table 2 that IRE cell types and distribution quantity were the least in the esophageal epithelium, only GAS, SOM and CAL-IRE cells were found in the esophageal epithelium mucosae of *M.albus* (Figure 1). IRE cells were the most common types and the highest distribution density in the gastric epithelium and glands of 8 kinds of fish (Figures 3, 5, 7, 9, 10, 12); then in the intestine (Figures 2, 4, 8, 11, 13, 14). GLU- and INS-IRE cells were mostly located in every specimen of pancreatic island. SOM- and 5-HT-IRE cells were found in the gastrointestinal intermuscular nerve plexus of *P.fulvidraco* and *S.chuatsi* separately (Figure 8, Table 2). No GAS, 5-HT, CAL- and NF-IRE cells were seen in the pancreatic island of all 8 kinds of teleosts. There were 5-HT-IRE cells in the digestive tract of all fish species; only a few GAS-IRE cells were distributed in the esophagus and stomach of *C. argus*, *M.albus* and *P.fulvidraco*; GH-IRE cells were distributed in a small amount in the pancreas of *S.asotus*, but were scattered in the pancreatic islands of *S.chuatsi* and *S.asotus*; and were not seen in the gastrointestinal tract of 8 kinds of fish species. In gastrointestinal tract, APUD cells were distributed between epithelium mucosa and glandular epithelium (Figures 1-14). Negative reaction was found in all controls.

Morphological feature of APUD cells

According to the distribution of APUD cells, their morphology was of great diversity. APUD cells located in stratified squamous epithelium of esophagus were scattered or piled in distribution, and irregular in shape, and had a shorter cytoplasmic process (Figure 1). While the APUD cells distributed between gastrointestinal columnar epithelium had a longer cell body, an apical

cytoplasmic process extended to the gastrointestinal lumen or a basal process extended to the basement membrane (Figures 2, 4, 9, 11-14). The APUD cells located in gastric glands were mainly pyramid-shaped (Figures 5-7,10), their cytoplasmic process extended to the gastrointestinal lumen. In the pancreatic islands of *S.chuatsi* and *S.asotus*, GLU-IRE cells were located in the edge of

pancreatic islands (Figures 15, 18) and SOM, INS- and GH-IRE cells scattered in the whole pancreatic islands (Figures 16, 17, 19). Their shapes were irregular, some had a longer cytoplasmic process (Figure 18), the secretory granules were clear (Figure 19) in some of IRE cells. There were a few scattered GH-IRE cells in the pancreatic exocrine area of *S.asotus* (Figure 19).

Table 1 Details of antisera and main reagents used

Antisera & reagents	Working dilution	Specificity	Source
Human CAL	1:400		ZYMED Lab. Inc.,USA
Grass carp GH	1:600		Yangtze River Fisheries Institute, Chinese Academy of Fishery Sciences
Human NF	1:400		ZYMED Lab. Inc., USA
Human INS	1:1000		ZYMED Lab. Inc., USA
Synthetic human GAS	1:5000	No cross reaction with Cholecystokinin-8	Dr. N Yanaihara & Shizuoka
Procine GLU	1:1000	Wholly cross react with Pancreatic & intestinal glucagon	Amersham International pl.
Synthetic human SOM	1:3000		Dr. S Ito Niigata
5-HT	1:10000		Immunonuclear Corp., Stillwater
S-P Kit	1:100		ZYMED Lab. Inc., USA
SABC Kit(mouse IgG)	1:100		Boster Biotechnology Co.
SABC Kit(rabbit IgG)	1:100		LTD., Wuhan
DAB	1:2000		Dr.Kinji INOUE

Table 2 APUD cells types and distribution feature and density i n GEP system of 8 kinds of stomach-containing teleosts

Fish species	GAS	SOM	5-HT	GLU	CAL	GH	NF	INS
Northern snakehead (<i>C. argus</i>)	3 +++/+ 4 +/		5 +++/	ND	2 +/ 3 ++++/ 4 +++/		1-6 ND	1-6ND
Ricefield eel (<i>M. albus</i>)	1 +++/+	1 +++/+ 3 +++/++	4 +++/	ND	1 ++++/ 3 +/ 4 +/		4 /++	4/+
Yellow catfish (<i>P. fulvidraco</i>)	3 /++	3 /++++ 4 /++++ 5 /++	3 ++/ 5 ++/	7 +++/ 2 +/ 3 ++/ 4 +++/ 5 +/	1 +/		3 ++++/	2 /++ 4 / +
Mandarinfish (<i>S. chuatsi</i>)		2 +++/++ 4 ++/++	3 +++/++ 5 ++/ 6 ++	7 +++/		7 +++/	1-6 ND	1-6 ND 7 +++/
Largemouth Bass (<i>M. salmoides</i>)			3 +/ 4 +/ 5 +++/	7 ++++/	7 +++/		1-6 ND	1-6 ND
Oriental Sheatfish (<i>S. asotus</i>)		7 +++/	3 ++/ 4 ++/ 5 ++++/	7 ++++/	2 +/ 3 /++ 4 /++ 5 +++/ 7 +++/	7 ++++/ +	1-6ND	1-6ND 7 ++/
Freshwater Pomfret (<i>C. brachypomum</i>)		4 +/ 7 ++/	2 ++/+ 3 ++/+ 4 +++/+ 5 +/	7 ++++/	1 +/		1-7ND	1-7ND
Nile tilapia (<i>T. nilotica</i>)		3 +++/ 4 ++/ 5 +/ 7 ++/	2 +++/+ 3 +++/+ 4 +++/+ 5 +/	7 ++++/			1-7ND	1-7ND

Notes: 1=esophageal epithelium/esophageal gland; 2=cardiac epithelium/cardiac gland; 3=gastric epithelium/fundus gland; 4=pyloric epithelium/pyloric gland; 5=intestinal epithelium/ intestinal gland; 6=intermuscular nerve plexus of digestive tract; 7=pancreatic island/pancreas; ++++=above 30 IRE cells in one field 10×20; +++=20-29 cells; ++=10-19 cells; +=below 10 cells; No IRE cell was found if the parts of sample were not listed in table; ND=not detected.

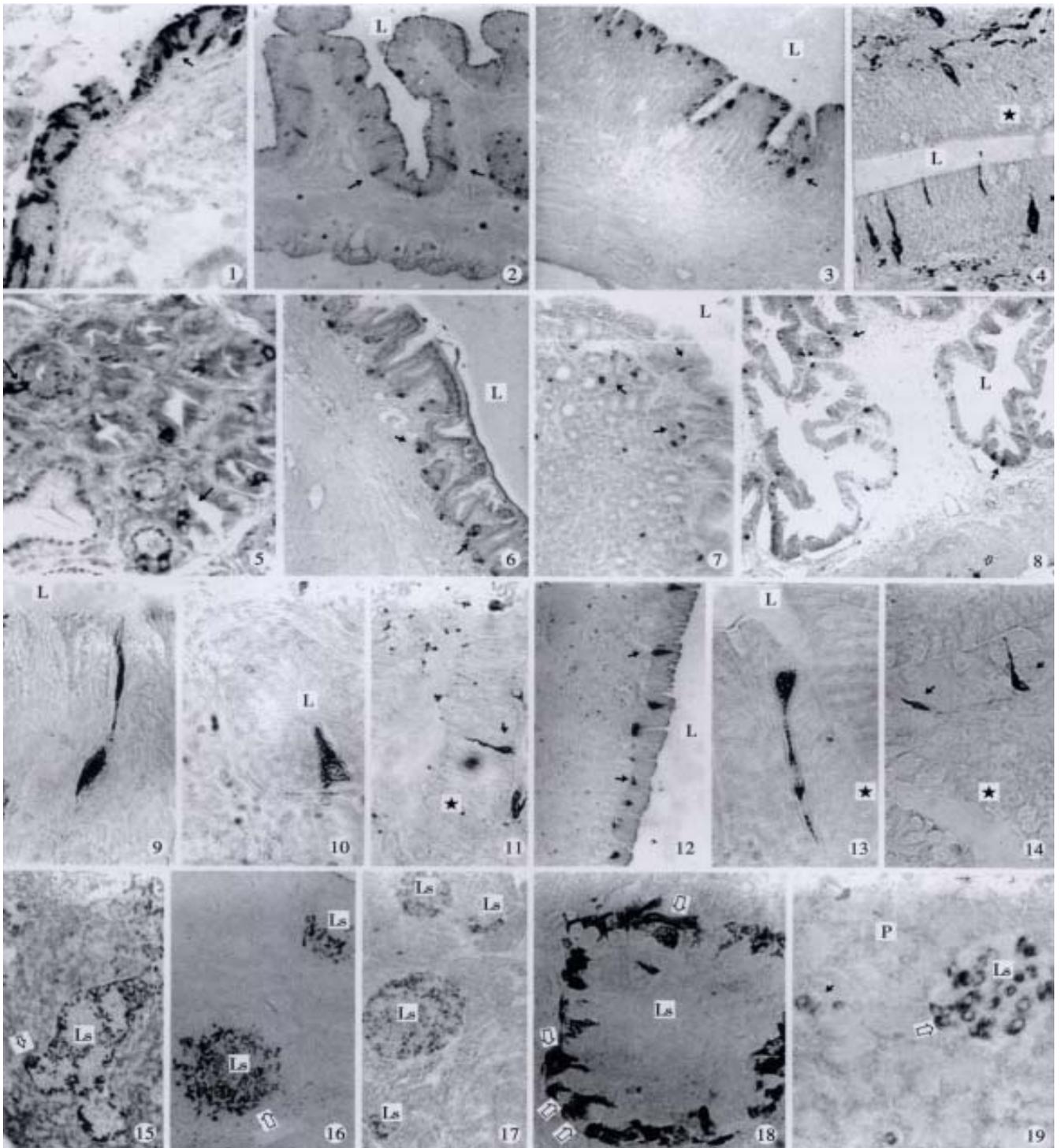


Figure 1 Distribution of CAL-IRE cells (♣) in esophageal epithelium of *M. albus*. ×66

Figure 2 SOM-IRE cells (♣) in intestinal epithelium of *T. nilotica*. ×33

Figure 3 GAS-IRE cells (♣) in gastric epithelium of *C. argus*. ×33

Figure 4 5-HT-IRE cells (♣) in intestinal epithelium of *C. argus*. ×100

Figure 5 SOM-IRE cells (♣) in gastric gland of *P. fulvidraco*. ×152

Figure 6 SOM-IRE cells (♣) in pyloric gland of *P. fulvidraco*. ×33

Figure 7 SOM-IRE cells (♣) in cardiac epithelium and gland of *S. chuatsi*. ×66

Figure 8 5-HT-IRE cells in intestinal epithelium (♣) and intermuscular nerve plexus (♣) of *S. chuatsi*. ×33

Figures 9-10 Shape of 5-HT-IRE cells (♣) in gastric epithelium and glands of *M. salmoides*. ×200

Figure 11 5-HT-IRE cells (♣) in intestinal epithelium of *M. salmoides*. ×100

Figure 12 5-HT-IRE cells (♣) in gastric epithelium of *S. asotus*. ×33

Figure 13 Shape of 5-HT-IRE cells (♣) in intestinal epithelium of *S. asotus*. ×200

Figure 14 5-HT-IRE cells (♣) in intestinal epithelium of *C. brachyomum*. ×200

Figure 15 GLU-IRE cells (♣) in pancreatic islets of *S. chuatsi*. ×33

Figure 16 SOM-IRE cells (♣) in pancreatic islets of *S. asotus*. ×33

Figure 17 INS-IRE cells (♣) in pancreatic islets of *S. chuatsi*. ×33

Figure 18 Shape of GLU-IRE cells (♣) in pancreatic islets *S. asotus*. ×268

Figure 19 GH-IRE cells in pancreatic islets (♣) and pancreas (♣) of *S. asotus*. ×200

Note: ★: goblet cell; L: gastrointestinal lumen; Is: pancreatic islet; P: pancreas

DISCUSSION

The distribution of APUD cells in the GEP system of stomach-containing teleosts and in the digestive system of human and stomachless teleosts is quite different. GAS-IRE cells existed in human GEP system^[8,14-16] and in the intestine of 7 kinds of stomachless teleosts^[42], but at present study, GAS-IRE cells were not seen in the digestive system of 7 fish species except in the esophagus of *M. albus* and in the stomach of *C. argus* and *P. fulvidraco*. After ICC identification of APUD cells in the gut of 7 stomachless teleosts, the 5-HT-IRE cells were not found in the gut^[42]; but in the intestine of 7 stomach-containing teleosts except *M. albus*. Rombout and Reinecke^[47] found SOM-IRE cells only existing in the stomach of stomach-containing teleosts; but at present studies, SOM-IRE cells were seen in the esophagus of *M. albus*, the gastric epithelium and intermuscular nerve plexus of *P. fulvidraco*, the gastric epithelium of *S. chuatsi* and *T. nilotica*. GLU-IRE cells existed universally in the gut of stomachless teleosts^[42]; but in this study they were not found in the gastrointestinal tract of 8 stomach-containing teleosts.

It was reported that there were 4 APUD cell types: B cell (secreting INS), PP cell (secreting pancreatic polypeptide), A cell (secreting GLU) and D cell (secreting SOM) in the pancreatic island of fish species^[48]. In our studies, CAL-IRE cells were found in the pancreatic island of *S. asotus* and *M. salmoides*. Calcitonin was a hormone secreted by ultimobranchial gland of fish species^[49], and also was found in the secretory cells of prolactin hormone of rat hypophysis^[50]. But up to now, no report showed that CAL-IRE cells exist in the pancreatic island of fish species. CAL-IRE cells in the pancreatic island of *S. asotus* and *M. salmoides* were morphologically long shuttle-shaped which was different from other endocrine cells in the pancreatic island which had the shorter cytoplasmic process and irregular shape. This specific morphological feature provides evidences that CAL-IRE cells in the pancreatic island of *S. asotus* and *M. salmoides* may adapt to some specific physiological functions and conduct endocrine paracrine action^[8].

It is a common knowledge that there are GH-IRE cells in the meso-adenohypophysis of fish species and can promote the growth of them. Our studies demonstrated that there was no GH-IRE cell in the gastrointestinal tract of fish species^[46], and GH was not gastrointestinal hormone. However our studies found for the first time that GH-IRE cells in the pancreatic islands of *S. chuatsi* and *S. asotus*, and in the exocrine pancreas of *S. asotus* had scattering distribution. It is evidenced that GH-IRE cells were not only located in the endocrine organ-pancreatic island but in the digestive gland in a small amount. It shows morphological evidence of brain-

gutpeptide with an endocrine-exocrine mode of action^[51]. It suggests that the GH-IRE cells existed simultaneously in the hypophysis, pancreatic island and pancreas, and the other brain-gut peptide or brain-pancreas peptide varied in pattern of distribution, and the diversity of physiological function of GH-IRE cells based on their morphology.

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Multiple genetic alterations and behavior of cellular biology in gastric cancer and other gastric mucosal lesions: *H. pylori* infection, histological types and staging

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Abstract

AIM To investigate the expression of multiple genes and the behavior of cellular biology in gastric cancer (GC) and other gastric mucosal lesions and their relations to *Helicobacter pylori* (*H. pylori*) infection, tumor staging and histological subtypes.

METHODS Three hundred and twenty-seven specimens of gastric mucosa obtained via endoscopy or surgical resection, and ABC immunohistochemical staining were used to detect the expression of p53, p16, Bcl-2 and COX-2 proteins. *H. pylori* was determined by rapid urea test combined with pathological staining or ¹⁴C urea breath test. Cellular image

analysis was performed in 66 patients with intestinal metaplasia (IM) and/or dysplasia (Dys). In 30 of them, both cancer and the paracancerous tissues were obtained at the time of surgery. Histological pattern, tumor staging, lymph node metastasis, grading of differentiation and other clinical data were studied in the medical records.

RESULTS p16 expression of IM or Dys was significantly lower in positive *H. pylori* chronic atrophic gastritis (CAG) than those with negative *H. pylori* (CAG: 54.8% vs 88.0%, IM:34.4% vs 69.6%, Dys: 23.8% vs 53.6%, all $P<0.05$), Bcl-2 or COX-2 expression of IM or Dys in positive *H. pylori* cases was significantly higher than that without *H. pylori* (Bcl-2: 68.8% vs 23.9%, 90.5% vs 60.7%; COX-2: 50.0% vs 10.8%, 61.8% vs 17.8%; all $P<0.05$). The mean number of most parameters of cellular image analysis in positive *H. pylori* group was significantly higher than that in negative *H. pylori* group (Ellipser: 53±14, 40±12 μm, Area 1: 748±572, 302±202 μm², Area-2: 3050±1661, 1681±1990 μm², all $P<0.05$; Ellipseb: 79±23, 58±15 μm, Ratio 1: 22%±5%, 13%±4%, Ratio-2: 79%±17%, 53%±20%, all $P<0.01$). There was significant correlation between Bcl-2 and histologic pattern of gastric carcinoma, and between COX-2 and tumor staging or lymph node metastasis (Bcl-2: 75.0% vs 16.7%; COX-2: 76.0% vs 20.0%, 79.2% vs 16.7%; all $P<0.05$).

CONCLUSION p16, Bcl-2, and COX-2 but not p53 gene may play a role in the early genesis/progression of gastric carcinoma and are associated with *H. pylori* infection. p53 gene is relatively late event in gastric tumorigenesis and mainly relates to its progression. There is more cellular-biological behavior of malignant tumor in gastric mucosal lesions with *H. pylori* infection. Aberrant Bcl-2 protein expression appears to be preferentially associated with the intestinal type cancer. COX-2 seems to be related to tumor staging and lymph node metastasis.

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INTRODUCTION

Gastric cancer (GC) is a worldwide disease with a dismal prognosis. A better understanding of its pathogenesis and biological features is crucial in improving the diagnosis and treatment^[1]. Tumorigenesis of GC is attributable to the interaction of environmental and genetic factors^[2-12]. In addition to dietary factors, *Helicobacter pylori* (*H. pylori*) infection has been regarded recently as a presumed environmental factor contributing to the tumorigenesis of the disease based on the theory that *H. pylori* infection may enhance cellular proliferation and therefore lead to somatic mutations of critical genes^[13-24]. However, the increase of *H. pylori* in the gene alterations as p53, p16, Bcl-2, and COX-2 remain unclear^[25-30].

GC consists of two distinct histological types: intestinal and diffuse types^[31]. They differ not only in morphology but also in their clinical and epidemiological characteristics. A different spectrum of genetic changes is believed to be involved in the intestinal type rather than in the diffuse type^[32]. Whether such genes as p53, p16, Bcl-2, and COX-2 play different roles in these two different types is not known^[33-38].

Little is known about the sequential genetic changes associated with the progression of GC as compared with colon cancer. In terms of its natural history, GC may be divided into early and advanced stages according to the invasiveness of tumor cells. Many reports have described genetic alterations in GC, but few have compared these alterations between the early and advanced stages^[35-37, 39,40].

It is necessary to shed further light on gastric tumorigenesis, the loss of p16 and the overexpression of mutant p53, Bcl-2, and COX-2 were investigated in patients with GC and other gastric mucosal lesions. The status of *H. pylori* infection, tumor staging, and histological types were correlated with alterations of these four genes and behavior of cell biology.

PATIENTS AND METHODS

Patients and samples

A total of 327 patients with histologically confirmed GC (60 cases) and other gastric mucosal lesions (chronic superficial gastritis, CSG; chronic atrophic gastritis, CAG; intestinal metaplasia, IM; dysplasia, Dys; 84, 56, 78, and 49 cases, respectively) were enrolled in the study. Those cancers were resected in the first affiliated hospital of Nanjing Medical University between 1996 and 1998. Both cancer and the neighboring nontumorous tissue of 30 patients were obtained at the time of surgery. The histological slides were independently reviewed by one pathologist, who was unaware of the parameters to be investigated. Tumors were classified as intestinal or diffuse types according to the Lauren's criteria. The extent of tumor invasion

was further divided into early or advanced GC according to the criteria proposed by the Japanese Research Society for Gastric Cancer. It is defined as an early GC if the tumor is limited in the mucosa and submucosa, and advanced GC if the tumor invades the muscularis propria. Lymph node metastasis, grading of differentiation and other clinical data were obtained from medical records, and the status of *H. pylori* infection was determined by a rapid urease test and a histological examination (Giemsa stain)/ ¹⁴C urea breath test.

Immunohistochemistry

The specimens in paraffin blocks were sectioned into 4µm in thickness. The first section was routinely stained with HE for histological diagnosis, and additional sequential sections were retained for immunohistochemistry. Immunostaining for p53, p16, Bcl-2, and COX-2 was performed by a standard avidin-biotin-peroxidase complex detection system. Mouse monoclonal antihuman p53 (diluted with PBS to 1:50; Oncogene Science, Inc, USA), rabbit polyclonal anti-p16 (diluted with PBS to 1:80, Dako, USA), mouse monoclonal antihuman Bcl-2 (diluted with PBS to 1:100; Dako, USA), and rabbit polyclonal anti-COX-2 (diluted with PBS to 1:50; Gene Company Limited) were used as the primary antibodies. Tissue sections were dewaxed, microwaved, and rehydrated. The sections were boiled for 30 minutes in 10mM/L citrate buffer solution (pH 6.0) using a microwave heater for antigen retrieval. Endogenous peroxidase activity and nonspecific binding were blocked by incubation with 30mL/L hydrogen peroxide (H₂O₂) and nonimmune serum, respectively. The slides were then incubated sequentially with primary mouse monoclonal or polyclonal antibodies overnight at 4°C, with a biotinylated goat anti-mouse secondary antibody (diluted with PBS to 1:200) for 30min, with peroxidase-conjugated asreptavidin for 10min, and finally, with 3.3'-diaminobenzidine -tetrachloride (0.25mg dissolved in 1mL 0.2mL/L hydrogen peroxide) chromogen substrate for 10min so that demonstration of binding sites with peroxidase reaction was obtained. Negative control sections were prepared by substituted primary antibody with buffered saline, and positive control sections were obtained from known positive sections. The percentage of positively stained cells was evaluated for each tissue section after counting 1000 cells at high power field. Tissues were classified as being immunohistochemically positive if ≥5% of cells stained and showed distinct nuclear staining for p53 dominant nuclear staining and a little cytoplasmic staining for p16, nuclear membrane or cytoplasmic staining for Bcl-2 and cytoplasmic staining for COX-2. The staining intensity was expressed as weakly positive (+), moderately positive (++) and strongly positive (+++).

Cellular image analysis (CIA)

CIA was performed in 66 patients (*H. pylori* positive 33, *H. pylori* negative 33) with intestinal metaplasia (IM) and/or dysplasia (Dys). CIA (Vidas, Opton, Germany) supported by the Department of Cellular and Molecular Biology, Shanghai Second Medical University, which was used to study the following parameters: Fshape (shape factor), Fllipsep (long axle, μm), Fllipseb (short axle, μm), Ratio₁ (nucleus to cytoplasm ratio, %), Ratio₂ (structure atypical index, %), Area₁ (area of nuclear, μm^2), Area₂ (area of cytoplasm, μm^2), taking the mean value of the parameters for analysis.

Statistical analysis

Statistical analysis system (SAS) software package for *t* test, χ^2 test, and Wilcoxon Scores (Rank Sums) test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Expressions of p53, p16, Bcl-2, and COX-2 proteins

The positive rates of p53, p16, Bcl-2, and COX-2 in GC were 46.7%, 38.3%, 68.3%, and 75.0% respectively. The positive rate of p53 gene in GC was significantly higher than that in DYS ($P < 0.05$); p16 gene expression in CSG or CAG was significantly higher than that in GC ($P < 0.01$) or Dys ($P < 0.05$). As to Bcl-2 expression, there was no significant difference between Dys and GC ($P > 0.05$), the positive rate of Bcl-2 expression in DYS or GC was significantly higher than that in CSG, CAG or IM ($P < 0.01$, $P < 0.05$). In

addition, COX-2 expression in GC was also significantly higher than that in Dys or IM ($P < 0.05$, Table 1).

Table 1 Expressions of p53, p16, Bcl-2, and COX-2 proteins in gastric mucosal lesions (n,%)

Lesion	n	p53	p16	Bcl-2	COX-2
GC	60	28(46.7) ^a	23(38.3)	41(68.3) ^{ba}	45(75.0) ^a
Dys	49	10(20.4)	20(40.8)	36(73.5) ^{ba}	18(36.7)
IM	78	1(1.3)	43(55.1)	33(42.3)	21(26.9)
CAG	56	0(0)	39(69.6) ^a	9(16.1)	5(8.9)
CSG	84	0(0)	67(79.8) ^b	7(8.3)	0(0)

p53:^a $P < 0.05$ GC-Dys; p16: ^b $P < 0.01$ CSG- Dys or GC, ^a $P < 0.05$ CAG-Dys or GC; Bcl-2: ^b $P < 0.01$ Dys (GC)-CSG or CAG; ^a $P < 0.05$ Dys (GC)-IM; COX-2:^a $P < 0.05$ GC-Dys (IM).

The expression of p53, p16, Bcl-2 and COX-2 proteins in gastric mucosal lesions with *H. pylori* infection

No significant difference for the positive rate in p53 expression of GC or Dys between positive *H. pylori* and negative *H. pylori* group ($P > 0.05$). There was also no significant difference for p16, Bcl-2, or COX-2 expression in GC between positive *H. pylori* and negative *H. pylori* group ($P > 0.05$). However, the positive rate and staining intensity of p16 expressions of CAG, IM or Dys with *H. pylori* infection were significantly lower than those without *H. pylori* infection ($P < 0.05$, $P < 0.01$). On the contrary, Bcl-2 and COX-2 expressions of IM or Dys with positive *H. pylori* were significantly higher than those with *H. pylori* ($P < 0.05$, Tables 2,3).

Table 2 Expression rates of p53, p16, Bcl-2, and COX-2 proteins in gastric mucosal lesions with *H. pylori* infection (n,%)

Protein	CSG (84)		CAG (56)		IM (78)		Dys (49)		GC (60)	
	Hp+(49)	Hp-(35)	Hp+(31)	Hp-(25)	Hp+(32)	Hp-(46)	Hp+(21)	Hp-(28)	Hp+(33)	Hp-(27)
p53	0	0	0	0	1	0	6(28.6)	4(14.3)	17(51.5)	11(40.7)
p16	36(73.5)	31(88.6)	17(54.8)	22(88.0) ^a	11(34.4)	32(69.6) ^a	5(23.8)	15(53.6) ^a	11(33.3)	12(44.4)
Bcl-2	4(8.2)	3(8.6)	6(19.4)	3(12.0)	22(68.8) ^a	11(23.9)	19(90.5) ^a	17(60.7)	25(75.6)	16(59.3)
COX-2	0	0	3(9.7)	2(8.0)	16(50.0) ^a	5(10.8)	13(61.8) ^a	5(17.8)	26(78.7)	19(70.4)

^a $P < 0.05$ p16: (CAG, IM or Dys) Hp+-Hp-; Bcl-2 or COX-2: (IM or Dys) Hp+-Hp-.

Table 3 The expression intensity of p53, p16, Bcl-2, and COX-2 proteins in gastric mucosal lesions with *H. pylori* infection (n)

Lesion	Hp	p53				p16				Bcl-2				COX-2				
		-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++	
CSG	+				13	17	13	6	45	3	1							
	-				4	13	15	3	32	2	1							
CAG	+				14 ^b	12	5	5	25	2	4			28	3			
	-				3	5	9	8	22	2	1			23	2			
IM	+		1		21 ^b	7	4		10 ^a	4	10		8	16 ^a	5	11		
	-				14	4	15	13	35	6	5			41	4	1		
Dys	+	15	4	2	16 ^a	2	3		2 ^a	2	11		6	8 ^a	3	10		
	-	24	2	2	13	2	8	5	11	9	5		3	23	3	2		
GC	+	16	6	8	3	22	4	5	2	8	6		11	8	7	2	17	7
	-	16	4	6	1	15	5	6	1	11	5		9	2	8	4	12	3

^b $P < 0.01$ p16 (CAG or IM) Hp+-Hp-; ^a $P < 0.05$ p16 (Dys) Hp+-Hp-; Bcl-2 or COX-2 (IM or Dys) Hp+-Hp-.

Cellular image analysis of gastric mucosal lesions with *H. pylori* infection

Cellular image analysis (CIA) was performed in 66 patients with IM and/or Dys, the mean value of most parameters of CIA in the positive *H. pylori* group was significantly higher than that in the negative *H. pylori* group ($P < 0.05$, $P < 0.01$, Table 4).

Table 4 Comparisons of each parameter of CIA between *H. pylori* positive and *H. pylori* negative

parameters	Hp+ (33)	Hp- (33)
Ellipser (μm)	79 \pm 23	58 \pm 15 ^b
Ellipseb (μm)	53 \pm 14	40 \pm 12 ^a
Ratio ₁ (%)	22 \pm 5	13 \pm 4 ^b
Ratio ₂ (%)	79 \pm 17	53 \pm 20 ^b
Area ₁ (μm^2)	748 \pm 572	301 \pm 202 ^a
Area ₂ (μm^2)	3050 \pm 1661	1681 \pm 1990 ^a
Fshape	0.67 \pm 0.09	0.71 \pm 1.10

^a $P < 0.05$, Ellipseb, Area₁ or Area₂: Hp+-Hp-; ^b $P < 0.01$, Ellipser, Ratio₁ or Ratio₂: Hp+-Hp-.

Expressions of *p53*, *p16*, *Bcl-2*, and *COX-2* proteins in GC related to tumor staging and histological types

No correlation between *p53*, *p16*, or *Bcl-2* expression and pathologic staging; grading of differentiation, or status of lymph node metastasis was observed ($P > 0.05$), there was significant correlation between *Bcl-2* and types of GC ($P < 0.05$). However, there was significant correlation between *COX-2* and pathologic staging or lymph node metastasis (all $P < 0.05$), no correlation between *COX-2* expression and type or grading of differentiation (all $P > 0.05$, Table 5).

Table 5 Expressions of *p53*, *p16*, *Bcl-2*, and *COX-2* proteins in GC related to tumor staging and histological types (n,%)

Lesion	n	<i>p53</i>	<i>p16</i>	<i>Bcl-2</i>	<i>COX-2</i>
Type					
Intestinal	24	14(58.3)	9(37.5)	18(75.0) ^a	16(66.7)
Diffuse	6	3(30.0)	2(33.3)	1(16.7)	4(66.7)
Stage of GC					
Early	5	2(40.0)	2(40.0)	4(80.0)	1(20.0)
Advanced	25	15(60.0)	9(36.0)	15(60.0)	19(76.0) ^a
Grade of differentiation					
High or middle	14	6(42.9)	6(42.9)	12(85.7)	8(57.1)
Low or not	10	8(80.0)	3(30.0)	7(70.0)	8(80.0)
Lymph node metastasis					
No	4	2(50.0)	2(50.0)	3(75.0)	1(16.7)
Yes	26	15(57.7)	9(34.6)	16(61.5)	19(79.2) ^a

^a $P < 0.05$ *Bcl-2*: Intestinal-Diffuse; *COX-2*: Advanced-Early, Lymph node metastasis Yes-No.

DISCUSSION

GC remains a common disease with a dismal prognosis in China and other Asian countries. Both genetic and environmental factors, such as *H. pylori* infection and dietary carcinogens, are crucial in cancer development and progression. The role of genetic changes in the pathogenesis of GC has recently received considerable attention^[26-28]. The development of GC is a multistep process with

accumulation of multiple oncogene activations and inactivation of tumor suppressor gene.

A number of molecular events are being recognized as implicating a part in the gastric carcinogenesis^[41]. Among these, mutation of the tumor suppressor gene *p53* is well described in gastric adenocarcinomas^[42] as is loss of heterozygosity on chromosome 17p, the locus of *p53*^[43]. The wild type acts functionally as a tumor suppressor gene^[44] and may also have a role in preventing replication of the damaged DNA^[45] while failure of this function in mutant *p53* may lead to instability of the genome and predisposed to the development of aneuploidy^[46]. The missense mutations of *p53* genes have frequently been associated with the progression of GC^[34,47].

Progression of cells through the different phases of the cell cycle is closely regulated by phase-specific activators and inhibitors. A novel cell cycle control gene, the *p16* gene, also referred to as *CDKN2*, *MTS1*, or *INK4A*, which encodes components of cell cycle checkpoints, has been cloned and characterized. *p16* gene is located on chromosome 9p21 and has been described as one of the principal negative regulators of the early G₁ phase. Changes in this gene could thus lead to uncontrolled cell growth and contribute to tumorigenesis^[35,48]. Recent studies have shown that the *p16* gene is indeed inactivated in a wide range of cancer cell lines and primary tumors^[48,49]. *p16* gene may correlate with tumorigenesis and tumor expansion due to decrease or loss of gene products in gastric cancer^[50,51]. However, studies on changes in *p16* genes in GC remain scanty^[38].

Recently, emphasis has been placed on the role of apoptosis and its regulation in carcinogenesis. In the gastrointestinal tract, apoptosis has been demonstrated to play an active role in the maintenance of the mucosa. Apoptosis may play a role in selection of clonal subpopulations with high growth potential resulting in malignant transformation^[52]. The *Bcl-2* protooncogene, located on chromosome 18, codes for a 26Kd protein involved in inhibiting apoptosis^[53]. *Bcl-2* protein is believed to play a role in the gastric carcinogenic sequence where it has been demonstrated in dysplastic epithelium^[54]. Aberrant *Bcl-2* protein expression has been noted in gastric epithelial Dys and in CAG^[36].

In addition, there was no expression of a novel isoenzyme of cyclooxygenase, cyclooxygenase-2 (*COX-2*) in normal tissue, however, large expression occurred in inflammatory sites. Recently, some researches show that there is an increasing expression of *COX-2* in gastrointestinal tumors^[37,55]. Although the potential role of the four genes' protein expression have been recognized to be related to the carcinogenic sequence, that the role of *H. pylori* infection may promote changes of

gene remains unclear^[26,27,29,56]. Moreover, the relationship between these gene alterations and histological types, tumor staging, lymph node metastasis, grading of differentiation remains controversial^[34,35,37,40]. Hence, we undertook the present study to investigate the effect of *H. pylori* infection on changes in the four genes and to further study their relation to types of GC and tumor progression.

H. pylori infection has been documented as an important risk factor for GC^[57]. Chronic inflammation due to persistent *H. pylori* infection would be expected to cause repetitive degeneration and regeneration of the mucosal epithelium that could facilitate malignant transformation. While epithelial cell proliferation is not carcinogenic in itself, it is likely to promote neoplastic transformation in combination with additional factors, such as genetic alterations and oncoprotein overexpression. In the current study, we noted that the positive rate of *p53*, *p16*, *Bcl-2*, and *COX-2* in GC was 46.7%, 38.3%, 68.3%, and 75.0% respectively and four genetic changes were not different between positive *H. pylori* and negative *H. pylori* GC. However, the positive rate and staining intensity of *p16* expression of CAG, IM or Dys with positive *H. pylori* were significantly lower than those with negative *H. pylori*. On the contrary, *Bcl-2* expression of IM or DYS with positive *H. pylori* was significantly higher than that with negative *H. pylori*. *p16* or *Bcl-2* gene expression alteration might play a role in the early development/promotion of gastric carcinoma and was associated with *H. pylori* infection^[50,51,58]. It seemed that *H. pylori* infection was not related to *p53* gene alteration^[30,59,60] because *p53* gene overexpression was closely associated with the potential for advancement of tumor and a poorer prognosis in patients with GC^[25,41,58,59,61,62] (Tables 1-3). The result was different from that reported by Chang *et al*^[63]. In the early stage of *H. pylori* infection, it not induces apoptosis in the gastric epithelium, at least in part due to downregulation of antiapoptotic *Bcl-2*^[26] but promotes proliferation due to activation of oncogene^[14]. However, persistent *H. pylori* infection would cause deletion of *p16* gene and overexpression of *Bcl-2*^[27], leading to overproliferation of gastric epithelium. In addition, inducible *COX-2* is an important regulator of mucosal inflammation and epithelial cell growth. The expression of *COX-2* gene is the result of direct response to *H. pylori* infection^[28,29]. Our study found that *COX-2* expression of IM or Dys with positive *H. pylori* was significantly higher than that with negative *H. pylori*. It is possible that the induced *COX-2* in positive *H. pylori* gastric mucosal lesions plays a role in gastric carcinogenesis. Moreover, we found that the mean number of parameters of CIA except Fshape in the

positive *H. pylori* group was significantly higher than that in the negative *H. pylori* group. It confirms that there are more cellular-biological behaviors of malignant tumor in gastric mucosal lesions with *H. pylori* infection.

Histologically, GC can be classified into intestinal and diffuse types based on the differences between precancerous lesions and glandular formation^[31]. The intestinal type GC predominates in the elderly and has a similar histological appearance to colonic cancer, while the diffuse type GC occurs more commonly in younger patients with scattered tumor cells. The possible alterations of *p53* suppressor gene were even in samples of gastric cancer and non-tumoral mucosa. Mutation of *p53* suppressor gene was frequent in gastric carcinoma^[64]. Overexpression of *p53* was more frequent in early intestinal than early diffuse GC^[59]. In the current study, *p53* was not different between the two types^[33,65]. Although not statistically significant^[65], differences in *p53* immunopositivity between the various growth patterns were observed, with tumors invading the submucosa tending to show a higher frequency of staining than mucosal tumors (60.0% vs 40.0%). Tumors with lymph node metastases showed higher frequency of *p53* staining (57.7%). We also found immunopositivity for *p53* in 80.0% GC of low and undifferentiated tumor cells. These observations support the suggestion that overexpression of *p53* is associated with tumor progression in gastric carcinogenesis and may be related to prognosis^[34,39,40,47]. Although the *Bcl-2* proto-oncogene is important in determining tumor cell susceptibility to apoptosis, data about its clinical importance in GC are not available^[66]. A significantly higher expression of *Bcl-2* protein was found in the intestinal type than in the diffuse type of GC (75.0% vs 16.7%, $P < 0.05$) in our studies^[36,67]. No correlation was also found between *Bcl-2* expression and the prognostic parameters, depth of invasion, differentiation, and lymph node invasion. Although not statistically significant, well and moderately differentiated tumors were more often *Bcl-2*-positive than poorly differentiated tumors, and lymph node negative tumors were more often *Bcl-2*-positive than nodal positive tumors. *Bcl-2* expression has no prognostic impact on GC^[36], which was different from the report of Inada *et al*^[66]. Little is known about the role of *p16* gene alterations in the genesis of GC^[68,69]. This result suggested that no correlation between *p16* expression and grade of pathologic staging, grading of differentiation, or lymph node status was observed. *p16* gene played a limited role in tumor progression^[25,68], which was discordant with some other reports^[38]. Finally, no correlation was present between *COX-2* expression and type or grading of differentiation (all $P > 0.05$), however, there was significant correlation between *COX-2*

and pathologic staging or lymph node metastasis (all $P < 0.05$). It was proposed that COX-2 played an important role in the development of GC^[37,55,70].

In conclusion, prolonged and persistent *H. pylori* infection may promote Bcl-2 and COX-2 protein overexpression, but suppress p16 protein expression in gastric precancerous lesions. It seems that *H. pylori* infection is not related to p53 gene alteration because the p53 gene overexpression is relatively a late event in gastric tumorigenesis and is mainly relates to progression. However, p16, Bcl-2, and COX-2 gene expression alterations might play a role in the early development/ promotion of gastric carcinoma but not in tumor progression and aberrant Bcl-2 protein expression appears to be preferentially associated with the intestinal type tumors. Moreover, there is significant correlation between COX-2 and pathologic staging or lymph node metastasis. p53, p16, Bcl-2, and COX-2 not only play independent but also synergistic role in the development and progression of gastric cancer^[25,71].

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The therapeutic effects of recombinant adenovirus RA538 on human gastric carcinoma cells *in vitro* and *in vivo*

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Subject headings stomach neoplasms/therapy; adenoviridae; apoptosis; gene therapy; polymerase chain reaction; genes MYC; flow cytometry

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Abstract

AIM To evaluate the potential of RA-538 gene therapy for gastric carcinoma.

METHODS Human gastric carcinoma cell line SGC7901 treated with Ad-RA538 or Ad-LacZ were analysed by X-gal stain, MTT, DNA ladder, Tunel, flow cytometric analysis, PCR, and Western Blot *in vitro*. The tumorigenicity and experimental therapy in nude mice model were assessed *in vivo*.

RESULTS Ad-LacZ could efficiently transfer the LacZ gene into SGC7901 cells. X-gal-positive cells at MOI 25, 50, 100, and 200 were 90%, 100%, 100%, and 100% respectively. Ad RA538 could strongly inhibit cell growth and induced apoptosis in SGC7901 cells. The proliferation of the Ad-RA538-infected SGC7901 cells was reduced by 76.3%. The mechanism of killing of gastric carcinoma cells by Ad-RA538 was found to be apoptosis by DNA ladder, Tunel and flow cytometric analysis. The tumorigenicity in nude mice using Ad-RA538 showed that all three mice failed to form tumor from 7 to 30 days compared with Ad-LacZ and parent SGC7901 cells. Experimental therapy on the nude mice model bearing subcutaneous tumor of SGC7901 cells

showed that intratumor instillation of Ad-RA538 inhibited the growth of the tumors. Ad-RA538-treated tumors were inhibited by 60.66%, compared with that of the tumor injected with Ad-LacZ and mock.

CONCLUSION The expression of Ad-RA538 can inhibit growth and induce apoptosis of gastric cancer cell *in vitro* and *in vivo*. Ad-RA538 can be used potentially in gene therapy for gastric carcinoma.

INTRODUCTION

Gastric carcinoma is one of the most common malignant tumors in the world. It is treatable by surgical resection in the early stages, but advanced gastric carcinoma does not usually respond to conventional therapy^[1-6]. Therefore gene therapy represents an attractive alternative for the treatment of gastric carcinoma. Present studies, suggest that overexpression of oncogenes, with or without functional loss of tumor suppressor genes, is responsible for the progression of human malignancies through multistep processes^[7-13]. On the basis of this multiple hit model of carcinogenesis, cancer gene therapy has rapidly developed as alternative to conventional cancer therapy. It is widely accepted that c-myc gene plays a pivotal role in regulating cell proliferation and differentiation. Some studies indicated that c-myc gene has a close relationship with carcinogenesis^[14-16]. Using subtractive hybridization strategy, RA538 was isolated, a cDNA clone from a human esophageal cancer cell line before and after RA-treatment^[17]. It is proved that RA538 can induce differentiation and apoptosis of tumor cell and down-regulate c-myc gene expression^[18-21]. In the present study, we treated a human gastric carcinoma cell line with adenovirus-recombinants carrying RA538 or LacZ gene.

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MATERIALS AND METHODS

Cell lines and culture

Human gastric carcinoma cell line (SGC 7901) was obtained from the Academy of Military Medical Sciences, China. Human embryonic kidney cell line (293) was kindly provided by Professor Zhan Qi-Ming (Academy of Sciences, China). SGC 7901 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Two hundred and ninety-three cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Recombinant adenovirus production

The recombinant, replication-deficient type 5 adenoviruses that have been developed for gene therapy contained deletions of the E1 and E3 regions. The vector was constructed by homologous recombination in 293 cells of the PJM17 plasmid containing the right end of Ad5 (Microbix Biosystems Inc., Canada) and a plasmid containing 0-16 m.u. of left end of pAd CMV (a gift from Zhang Wei-Wei). A recombination adenovirus vector, called Ad-RA538 containing the human RA-5 38 cDNA fragment, the total 3.8kb of RA538 suppressor gene was constructed. Ad-RA538 and Ad-LacZ were constructed by National Laboratory of Molecular Oncology, Department of Cell Biology, Chinese Academy of Medical Sciences. The resulting vector was plaque-purified twice on 293 cells and propagated. Virus stocks were titered by plaque-forming assay on 293 cells. Ad-RA538 and Ad-LacZ were identified by polymerase chain reaction (PCR).

Recombinant adenovirus infections of the cell lines were carried out by dilution of viral stock to certain concentrations, addition of viral solution to cell monolayers (0.5mL per 6cm dish), and incubation at room temperature for 30min with agitation every 10min. This was followed by addition of culture medium and the return of the infected cells to the 37°C incubator.

Adenovirus transduction efficiency

Gastric carcinoma cells were seeded in 6cm culture plates at a density of 1×10^6 cells /dish and cultured 12 hours. Cells were infected with Ad-LacZ at a multiplicity of infection with 25, 50, 100 and 200 (MOI). After 48 hours, the cells were washed with phosphate-buffered saline (PBS), treated with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). X-gal-positive cells were revealed by a blue precipitate in the cell.

Assay of cell growth

Cells were seeded at 8×10^3 cells/ well in 96-well plates and were infected with either Ad-RA538, Ad-LacZ and PBS (Mock) at 25, 50, 100 and 200 MOI within 12-24 hours. At different time after

adenoviral infection, 3-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Cell viability is proportional to the absorbance at the test wave length (525nm).

DNA extraction and gel electrophoresis^[22]

Cells were lysed in guanidine-isothiocyanate and DNA was extracted by benzyl chloride using Herrmann M'ethord^[5]. RNA was digested with 1 μ g/mL RNase A 50 μ g of each DNA sample was loaded on to a 18g/L agarose gel.

TUNEL assay

Air dried cell samples were fixed with paraformaldehyde solution for 30min. Slides were rinsed with PBS and incubated with blocking solution (0.3% H₂O₂ in methanol) for 30min. Slides were rinsed with PBS and incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2min in ice. TUNEL reaction mixture were added with 50 μ L in sample. Slides were incubated in a humidified chamber for 60min at 37°C, and analysed under a fluorescence microscope. Converter-POD were added with 50 μ L in sample. Slides were incubate in a humidified chamber for 30min at 37°C. Slides were rinsed with PBS for three times. DAB substrate solution were added with 50 μ L-100 μ L for 15min. Slides were rinsed with PBS for three times and were stained with hemotoxin. Slides were mounted under glass coverslip and analysed under light microscope.

Flow cytometry

The cells were fixed in 70% ethanol, treated with 0.1g/L Rnase A, and with 100mg/L propidium iodide. Cell cycle phase distribution were analysed by flow cytometry (Coulter Epice, ELITE, ESP, USA).

Polymerase chain reaction

The polymerase chain reaction (PCR) was designed to amplify a 800bp fragment of the adenovirus gene using primer (5'-primer TCGTTTCTCAGCAGCTGTTG; 3'- primer CATCT-GAACTCAA GCGTGG) and a 300bp fragment of the RA538 gene using primer (5' primerATGGGT-GAACA ACAGAAGAG-3'primer TTAGA-GATTGAGATTTGGCT). Hot-start PCR amplification was performed. In a Pektin-Elmer 2400 thermocycler using the following program: 1 \times 95°C for 5min; 30 \times 95°C for 1min; 59°C for 50sec; 72°C for 50sec; and 1 \times 72°C for 7min. The final concentration for all PCR components in a 25 μ L volume was as follows: 100 μ M of each of extracted genomic DNA and 1 unit of Taq polymerase in 1 \times Taq polymerase buffer. PCR products were run on 1% agarose gels.

Western blot analysis

Total cell lysates were prepared by lysing the cell

with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer after rinsing the cells with PBS. For the SDS-PAGE analysis, each lane was loaded with cell lysates equivalent to 6×10^4 cells (30 μ L). The protein in the gel were transferred to nitrocellulose filter (NC filter, Bio-Rad Company, California, USA). The membranes were blocked with 0.5% dry milk in PBS. The primary antibodies used were mouse anti-human c-myc or β -actin monoclonal antibody (Santa Cruz, California, USA), and the secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham, Uppsala, Sweden). The membranes were hybridized according to the Amersham's ECL protocol. The membrane was exposed to X-ray film.

Tumorigenicity assay of SGC7901 cell growth after treatment with Ad-RA538

The gastric cancer cells were infected with Ad-RA538 and Ad-LacZ at a dose of 100 MOI. An equal number of cells was treated with PBS and mock infection. Twenty-four hours after infection, the treated cells were harvested and rinsed with PBS. For each treatment, 10^6 cells in 0.1 mL were injected to BALB/c male nude mice. Tumor formation was evaluated after 4 weeks.

Adenovirus treatment in vivo

Mice (BALB/c nude mice aged 5 weeks) were inoculated with 10^6 SGC 7901 cells into the flank. Tumors were allowed to grow to 5 mm in diameter. The animals were divided into three groups: Ad-RA538 injection; Ad-LacZ injection and PBS injection. There were five nude mice in each group. Ad-RA538 or Ad-LacZ (1×10^9 pfu/each/100 μ L) or PBS/100 μ L were directly injected into the tumor centre of each nude mouse at d1, d3 and d5. Tumor sizes were observed 4 weeks after the injection, and estimated with calipers.

Statistics

Data are presented as means \pm standard errors of the means. Comparisons among different groups of samples were made by two-tailed *t* test and χ^2 test.

RESULTS

Recombinant adenovirus prediction

Ad-RA538 and Ad-LacZ were propagated on 293 cells. Their titers were 3.0×10^9 pfu/mL and 3.0×10^{11} pfu/mL respectively. Ad-RA538 and Ad-LacZ were identified by PCR. Ad-RA538 showed expression of RA538 gene. Ad-LacZ showed expression of adenovirus gene.

Adenovirus transfection efficiency in SGC7901 cell line

The time course of β -galactosidase (β -Gal) expression was first determined by counting the

percentage of X-Gal-positive cells at 48h after infection with Ad-LacZ. Ad-LacZ could efficiently transfer the LacZ gene into SGC7901 cells. And X-gal-positive cells at MOI 25, 50, 100 and 200 were 90%, 100% 100% and 100% respectively.

Inhibition of SGC 7901 cells growth

The degree of growth inhibition was measured by MTT assay. The growth rates of Ad-RA538 infected SGC 7901 cells were inhibited by 76.3 % (MOI 200, 8 day) as compared to Ad-LacZ and Mock ($P > 0.01$) (Table 1). Ad-RA538 induced apoptosis in SGC 7901 cells.

DNA fragmentation

SGC7901 cells were treated with MOI 100 Ad-RA538 for 2, 4 and 6 days. Figure 1 shows that the electrophoresis pattern after treatment with Ad-RA538. DNA fragmentation became apparent at d2, d4 and d6. The peak of ladder pattern was detected at d6. Ladder pattern was not detected in Ad-LacZ treated cells.

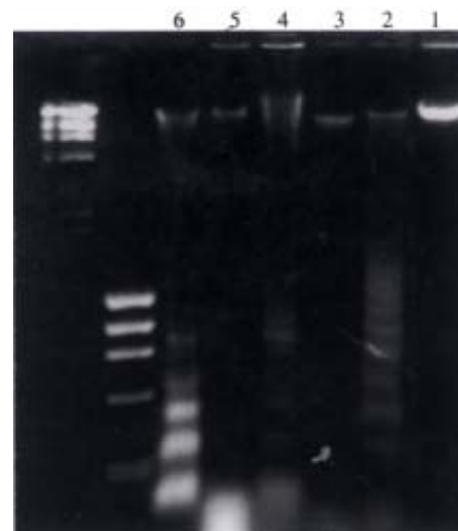


Figure 1 DNA ladder of Ad-RA538 on SGC7901 cells.

lane 1: Ad-lac Z (2d); lane 2: Ad-RA538 (2d);
lane 3: Ad-lacZ (4d); lane 4: Ad RA538 (4d);
lane 5: Ad-lacZ (6d); lane 6: Ad-RA538 (6d).

TUNEL assay

SGC 7901 cells treated with Ad-RA538 were assayed for apoptosis by TUNEL. SGC 7901 cells could induce apoptosis. The abnormal chromatin clumps, nuclear membrane wrinkling, nuclear collapse, cytoplasm bubble and cytomembrane wrinkling had appeared after treatment with Ad-RA538. Apoptotic cells were not shown by TUNEL assay in Ad-LacZ treated cells. In this kit terminal deoxynucleotidyl transferase, which catalyzes polymerization of nucleotides to free 3'-OH DNA ends in a template-independent manner, was used to

label DNA strand breaks. After substrate reaction, stained cells can be analyzed under light microscope (Figure 2).

Flow cytometric analysis

A flow cytometric analysis was performed on SGC 7901 cells infected with Ad-RA 538 and Ad-LacZ. Apoptotic cells of Ad-RA538 infected cells played a peak in the flow cytometry histogram. Apoptotic peak was 34.2% at d2. The cell cycle G-2M arrest were shown at d2, d4 and d6. The apoptosis peak and cell cycle arrest were not found in Ad-LacZ treated cells (Table 2).

c-myc expression in Ad-RA538 infected cells

The expression of c-myc protein in SGC 7901 cells was detected by western blot analysis after Ad-

RA538 infection for 1, 3, 5 or 7 days. Ad-RA538 may down-regulate expression of c-myc gene (Figure 3).

Inhibition of tumor growth in vivo

The tumorigenicity in nude mice of using Ad-RA538 showed that three of these mice failed to form tumor from d7 to d30 compared with Ad-LacZ and parent SGC 7901 cells ($P>0.01$) (Figure 4). The tumorigenicity in nude mice using Ad-LacZ and parent SGC 7901 cells was 100%.

Experimental therapy on the nude mice model bearing subcutaneous tumor of SGC 7901 cell showed that intratumor instillation of Ad-RA538-treated tumor were inhibited by 60.66%, compared with that of the tumor injected with Ad-lacZ and mock (Figures 5,6).

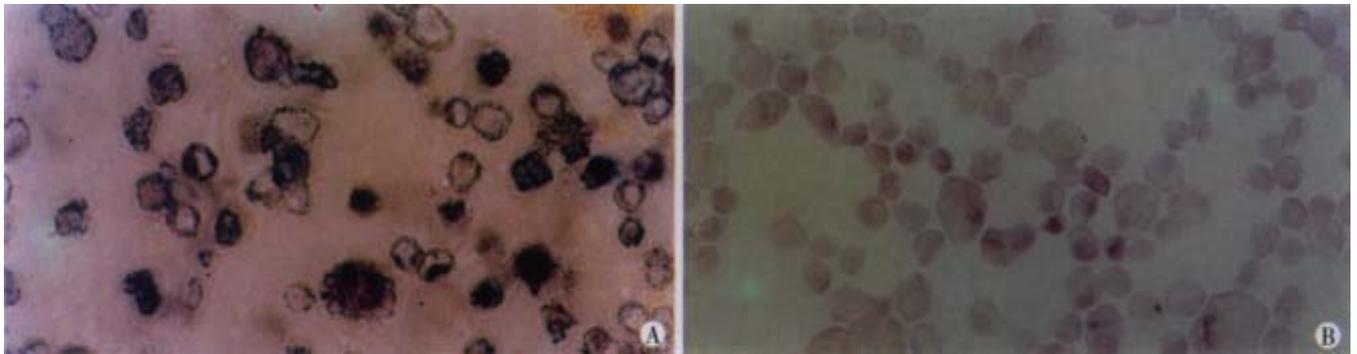


Figure 2 TUNEL assay of Ad-RA538 and Ad-LacZ on SGC 7901 cells.

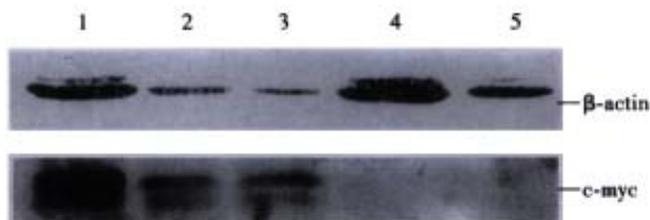


Figure 3 Western blot analysis c-myc and β -actin expression of SGC7901 cell infected with Ad-RA538. Lane 1: control, Lane 2-5: 1d, 3d, 5d, 7d

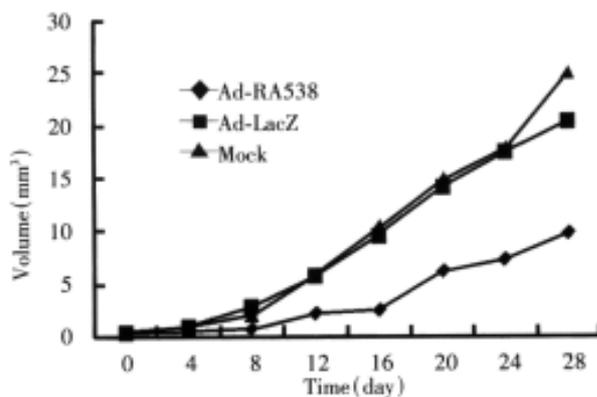


Figure 5 *In vivo* therapy of Ad-RA538 on SGC7901 tumors in nude mice.



Figure 4 Tumorigenicity assay in nude mice follow AdRA538.



Figure 6 Experimental therapy on the nude mice model of Ad-RA538.

Table 1 Anti-proliferative effect of Ad-RA538 on SGC7901 cells (8d, $x \pm s$)

MOI	Ad-RA538		Ad-LacZ		Mock	
	A	Survival(%)	A	Survival(%)	A	Survival(%)
25	1.062±0.05	95.0±4.5	1.120±0.077	100±6.9	1.31±0.065	100±6.3
50	0.941±0.067	84.0±6.0	0.984±0.024	87.5±2.1	1.29±0.034	100±5.2
100	0.534±0.091	47.4±8.1 ^a	1.195±0.095	100±8.5	1.28±0.041	99.8±7.0
200	0.265±0.090	23.7±8.0 ^b	1.241±0.091	100±8.0	1.23±0.057	98.0±4.5

^a $P > 0.05$ vs Ad-LacZ or Mock; ^b $P > 0.01$ vs Ad-LacZ or Mock.

Table 2 Flow cytometry analysis of cell cycle effects of Ad-RA538 on SGC7901 (%)

Type of adenovirus	12h			1d			2d			4d			6d		
	G1	G2M	S	G1	G2M	S	G1	G2M	S	G1	G2M	S	G1	G2M	S
Ad-RA538	74.6	13.0	12.4	55.7	28.2	17.2	69.9	14.0 ^a	16.1	39.7	26.6 ^b	33.8	44.4	20.9 ^c	34.7
Ad-LacZ	66.5	15.3	18.2	59.2	18.1	22.7	59.5	22.8	17.3	71.0	12.4	16.6	73.3	8.4	18.3

^a $P > 0.01$ vs Ad-LacZ; ^b $P > 0.01$ vs Ad-LacZ; ^c $P > 0.01$ vs Ad-LacZ.

DISCUSSION

The ability to infect numerous different cell types and the absence of requirement for dividing cells make adenovirus an attractive candidate for *in vivo* gene therapy^[23]. They can be grown to a very high titer, and can be easily concentrated to reach titers of 10^{13} - 10^{14} particles per milliliter. Adenoviral vectors are very effective agents for gene transfer with extremely high transduction efficiency for a wide variety of cell types. In this study, adenovirus vector has high transduction efficiency for SGC 7901 cells, and can mediate a high level of expression of interested gene in transduced cells. The percentage of X-gal staining positive SGC 7901 cells 48h after infection with Ad-LacZ was 90% at MOI 25. Ad-RA538 could strongly inhibit growth and induce apoptosis of SGC 7901 cells. It may be related to high transduction efficiency of adenovirus for SGC 7901 cells.

It is known that activation of proto-oncogene and inactivation of tumor suppressive gene are the most common genetic alternation in tumor^[24-33]. Using subtractive hybridization strategy, RA538 was isolated, a cDNA clone from a human esophageal cancer cell line before and after retinoic acid (RA) treatment. RA can induce differentiation and apoptosis of tumor cell^[34-37]. It is proved that RA538 can induce differentiation and apoptosis of tumor cell and down-regulate c-myc gene expression^[17,18,38-41]. Reducing the expression of c-myc may effectively suppress the proliferation and malignant phenotype of cancer cells^[42-48] and to counter c-myc gene amplification in gastric carcinoma cells^[49]. The effects of Ad-RA538 on human gastric carcinoma cell line were examined both *in vitro* and *in vivo*. Ad-RA538, containing the human RA538 cDNA fragment, was constructed. Firstly, Ad-LacZ could transfer the LacZ gene into more than 90% of gastric carcinoma cells. Ad-RA538 could successfully inhibit expression of c-myc protein. These data showed the

capability of adenovirus to transfer exogenous genes efficiently into gastric carcinoma cell line. Secondly, the growth inhibitory effect of Ad-RA538 was then examined. In c-myc gene amplification on gastric carcinoma cell line SGC7901, the growth inhibition by infection with Ad-RA538 correlated with both transduction efficiency and adenovirus dose. The growth rates of Ad-RA538 infected SGC 7901 cells were inhibited by 76.3%. DNA fragmentation, TUNEL and flow cytometric analysis suggested that Ad-RA538 could strongly induce apoptosis of gastric carcinoma cells. Some reported that tumor suppressive gene BCL-XS could inhibit the proliferation and malignant phenotype of cancer cells^[50], Bouillelet^[51] reported that strA6, a retinoic acid-responsive gene could induce apoptosis of tumor cells. These are the same with our studies about Ad-RA538. Our studies with the tumorigenicity in nude mice and experimental therapy on the nude mice model using Ad-RA538 also supported these findings and showed that Ad-RA538 may be useful to inhibit the growth of gastric tumors. Our studies suggested that adenovirus-mediated RA538 overexpression may result in the elimination of tumor cells by apoptosis, and inhibition of proliferation, thus reducing the tumor burden. In conclusion, these findings indicated that the Ad-RA538 might promote efficient tumor cell death and inhibit tumor cell proliferation. The use of these vectors may be a potential tool for reducing tumor growth *in vivo* and treatment of gastric carcinoma.

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Morphological study on colonic pathology in ulcerative colitis treated by moxibustion

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Subject headings colitis, ulcerative/therapy; moxibustion; intestinal mucosa; morphological study

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Abstract

AIM To observe the therapeutic effect of moxibustion on ulcerative colitis and its influence on the colonic mucosal morphology.

METHODS Forty-six patients with ulcerative colitis were randomly divided into the moxibustion with herbal medicine underneath group and the western medicine group. Thirty patients were treated with the above moxibustion and 16 patients with Salicylate fapyridine(SASP). The colonic mucosa of 13 patients in the moxibustion group was observed by colonoscopy before and after the treatment. Mucin was also analyzed by H.E and AB-PAS staining.

RESULTS Seventeen patients were clinically cured, 12 were improved and 1 unchanged in the moxibustion group. In the control group, 5 patients were clinically cured, 7 improved and 4 unchanged. Thirteen patients with active UC were taken as the subjects for histopathologic analysis in this study. The colonic mucosal lesions were remarkably improved and the characteristic of the mucin also changed. In most sections, the chronic inflammation of mucosa was greatly ameliorated ($P<0.01$). The inflammatory cell infiltration much decreased

and neutrophils, disappeared in most sections ($P<0.001$). The goblet cells significantly increased ($P<0.001$); crypt paracrypt abscess or mucosal ulceration was seen ($P<0.001$).

CONCLUSION The rate of cure of ulcerative colitis by moxibustion with herbal medicine beneath is superior to that by SASP. This sort of moxibustion can effectively improve the colonic mucosal lesions and restore the proportion of mucoprotein to near normal.

INTRODUCTION

Chronic ulcerative colitis (UC) is autoimmune in nature^[1-3]. Aminosalicylic acid and corticosteroids are the drugs most commonly used in treatment of UC^[4-19], but long-term use may give rise to adverse effects. At present, many of them can be treated by combined western medicine and TCM^[20-28], or Chinese herbs^[29-41] or acupuncture^[42,43]. The present study is primarily aimed at the effectiveness of moxibustion on the morphological study of colonic mucosa in ulcerative colitis.

SUBJECTS AND METHODS

Subjects

Forty-six cases of UC were out-patients and in-patients who were diagnosed according to the diagnostic criteria established by National Academic Conference on Digestive System Diseases held in Taiyuan, 1993^[3], and were randomly divided into two groups: moxibustion with herbal medicine underneath (designated below as moxibustion) group and the SASP group. Differentiation of symptoms and signs by TCM was classified according to the criteria described in "Internal Medicine" edited by Shanghai College of TCM. The moxibustion group was made up of 30 cases, including 16 males and 14 females, mean age of 38.75 years (25-63). The course of illness ranged from 5 months to 18 years. The control group comprised 16 cases, including 9 males and 7 females with a mean age of 37 years (27-69), the course of illness ranged from 4 months to 17 years.

Methods of treatment

Method in the moxibustion group Selection of

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acupoints: The main acupoints of the two groups: Zhongwan (RN 12), Qihai (RN 6) and Zusanli (ST 36 Bilateral); Dachangshu (BL 25 bilateral), Tianshu (ST 25, bilateral), and Shangjuxu (ST37, bilateral) were used alternately. Auxiliary acupoints was added to different clinical types: Pishu (BL 20) for deficiency of Spleen and Stomach; Shuifen (RN 9) for accumulation of damp-heat Ganshu and Pishu for stagnancy of Liver-qi and deficiency of Spleen; Guanyuan (RN 4) for deficiency of both Spleen-yang and the Kidney-yang, Zhongzhu (KI 15) for constipation and Yinbai (SP 1) for pus and bloody stool.

Preparation of medicinal pads for moxibustion: *Radix Aconiti Praeparata*, *Cortex Cinnamomi*, *Radix Salviae Miltiorrhizae*, *Flos Carthami*, *Radix Aucklandiae*, *Rhizoma Coptidis*, etc were respectively ground into fine powder and stored for use. *Rhizoma Coptidis*, *Radix Salviae Miltiorrhizae* and *Flos Carthami* were used as main ingredients for the type of accumulation of damp-heat, supplemented with an appropriate amount of *Radix Aucklandiae*; *Radix Aconiti Praeparata* was used as the main ingredient for other types of the syndrome, supplemented with a suitable amount of *Cortex Cinnamomi*, *Flos Carthami*, *Radix Salviae Miltiorrhizae* and *Radix Aucklandiae*. Each medicinal pad contained herbal powder 2.5g which was mixed up with rice wine 3g to make paste. The paste was then made into pads of 2.3cm in diameter and 0.5cm in thickness.

Method of moxibustion Mugwort floss was made into moxacones, each being 2.1cm in base diameter, 2cm in height, and about 2g in weight, which were then placed on the medicinal pads and ignited. For deficiency of spleen and stomach, 3 moxa cones were used for each acupoint; for the type of accumulation of damp-heat, 2 cones were used for Dachangshu (BL 25), Tianshu (ST 25), Zhongwan (CV 12), Qihai (CV 6) and 4-7 cones for Zusanli (ST 36) and Shangjuxu (ST 37) until a strong warm sensation was felt by the patient; for stagnancy of liver-qi and deficiency of spleen, 3 cones were needed; for deficiency of both spleen-yang and kidney-yang, 3 cones were needed for the main acupoints and 4-7 cones for the auxiliary acupoints; for constipation, 2 cones were used on Zhongzhu (KI 3) and one cone was used on Tianshu (ST 25); and for serious pus and bloody stool, 4-7 moxacones were needed on Yinbai (SP 1). The treatment was given once daily, 12 treatments constituting a therapeutic course with an interval of 3 days between each two courses. After 5 courses of treatment the results were analyzed.

Method in the control group Patients were given Salicylazosulfapyridine 1g each time, 4 times a day at the beginning, and 0.5g each time, 4 times daily

in the convalescent period. After 3 months of treatment the results were analyzed.

Criteria for therapeutic effects

Clinically cured: clinical symptoms and signs disappeared, and colomoscopy showed disappearance of ulceration of colonic mucosa without recurrence after follow-up for 6 months. Improved: symptoms and signs ameliorated significantly, colonoscopy indicated amelioration of mucosal pathological changes. Ineffective: Clinical symptoms, signs and colonoscopy showed no significant difference before and after treatment.

Indexes and methods of observation

Pathological observation of colonic mucosa All specimens were taken from the most masked region or the edge of mucosal ulceration during colonoscopy. Three specimens were obtained in each patient, fixed with formalin, embedded in paraffin and sectioned, followed by H.E staining and pathologic observation.

Analysis of the mucin in colonic mucosa

AB-HID and AB-PAS methods were used for the staining.

RESULTS

Analysis of therapeutic effects

Seventeen patients were clinically cured, 12 were improved and one was ineffective, which made up 56.66%, 40.00%, and 3.33% respectively in the moxibustion group. In the control group, 5 patients were clinically cured, 7 improved and 4 ineffective, which accounted for 31.25%, 43.75%, 25.00% respectively. The cure rate in the moxibustion group was superior to that in the control group ($P < 0.01$).

Changes of colonic mucosal histopathology and mucin

Thirteen patients with active UC were taken as the subjects for histopathologic analysis in this study.

Pathological changes before treatment The mucosa showed non-specific inflammation. The lamina propria showed hyperemia and edema with a great number of plasma cells, lymphocytes, mononuclear cells, neutrophils and eosinophils infiltration. The goblet cells in the body of the gland reduced in various extent, even disappeared, and replaced by columnar cells (Figures 1-3). Vacuoles, could be seen in the distal region of the nucleus (Figure 2). No mucin was present in the vacuoles. The lumen of gland became dilated, irregular or branched in some lesions, some regions were neplaced by immature glandulas body of smaller volume. The neutrophilic infiltration was present in the inter-epithelial cells

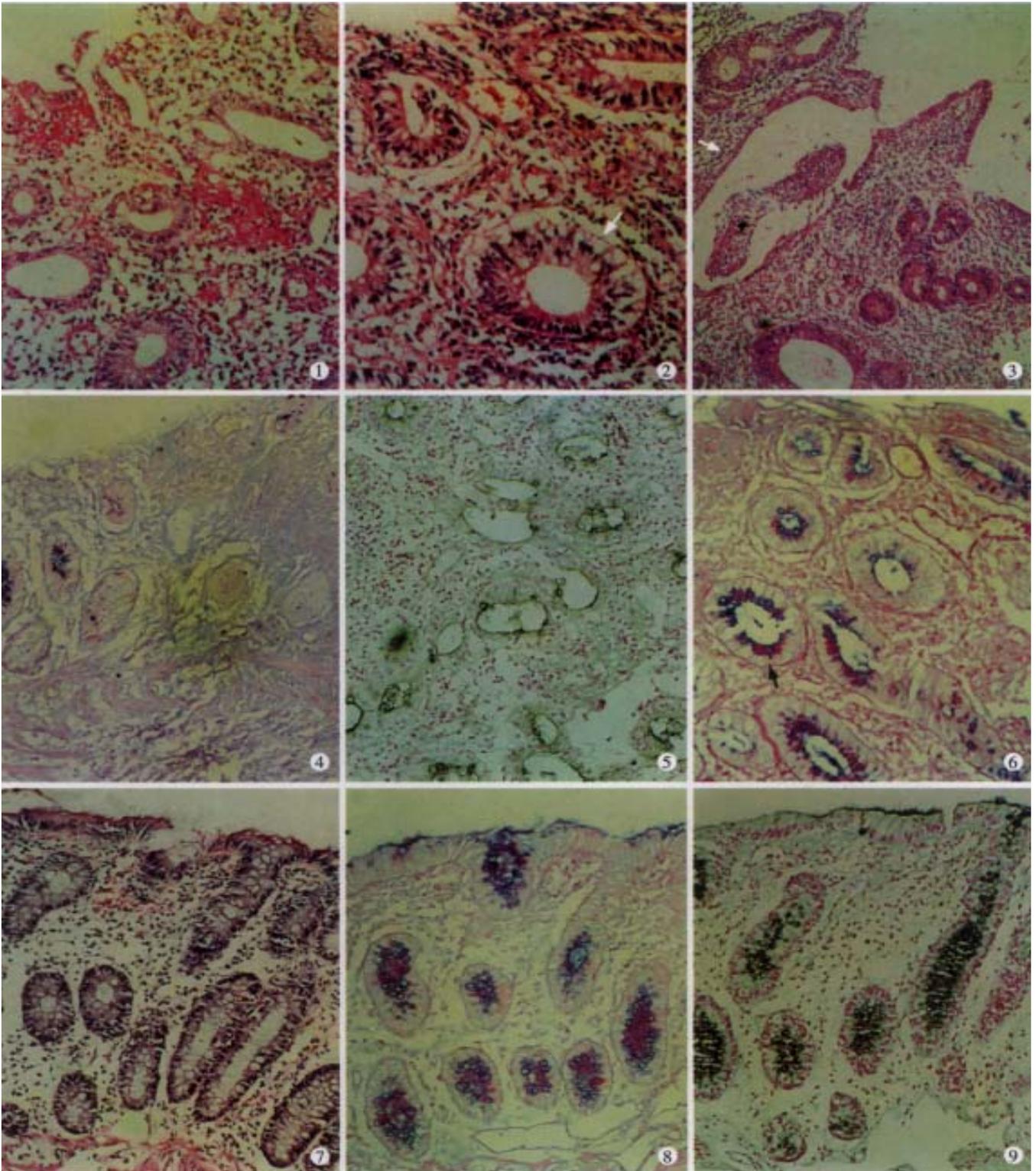


Figure 1 The colonic mucosa shows hyperemia, edema and inflammatory cell infiltration. The goblet cells in the body of gland decrease remarkably, which are composed of columnar cells.

Figure 2 The lesion is the same as above. The arrowhead shows an infranuclear vacuole.

Figure 3 The lesion is the same as above. The arrowhead shows a crypt abscess. H.E.×100

Figure 4 The majority of acid mucin in the body of gland disappear. AB-PAS staining ×100

Figure 5 The majority of mucin sulfate in the body of gland disappears. HID-AB staining ×100

Figure 6 The mucin in the body of gland decreases. The amount of neutral mucin relatively increases (arrowhead). AB-PAS staining ×100

Figure 7 The colonic mucosa shows mild hyperemia, with less mucosal inflammatory cell infiltration. The goblet cells in the body of gland show no reduction. H.E. ×100

Figure 8 The reduction of acid mucin is not obvious. AB-PAS staining ×100

Figure 9 No reduction of mucin sulfate occurs. HID-AB staining ×100

Table 1a The histologic changes of 13 cases with UC

	n	Chronic mucosal inflammation				Neutrophil infiltration reduction				Decrease of goblet cells			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Before treatment	13	0	0	4	9	0	1	5	7	0	1	5	7
After treatment	13	1	11	1	0	10	3	0	0	7	4	2	0

χ^2 test: $\chi^2=20.69$, $P<0.001$; $\chi^2=21.09$, $P<0.001$; $\chi^2=16.71$, $P<0.001$

Table 1b Histologic changes of 13 cases with UC

	n	Neutrophil infiltration in the interepithilium				Crypt abscess, abscess near crypt and ulceration of mucosa			
		-	+	++	+++	-	+	++	+++
Before treatment	13	3	5	5	0	1	2	6	4
After treatment	13	10	2	1	0	13	0	0	0

χ^2 test: $\chi^2=7.07$, $P<0.01$; $\chi^2=18.78$, $P<0.001$.

in majority of sections. Most of sections showed crypt abscesses (Figure 3), abscesses near crypts and ulceration of the mucosa. The amount of mucin in the body of the gland of the colonic mucosa in all patients decreased or decreased remarkably. As to the change of the mucin characteristic, most of the acid mucins in the body of gland disappeared (Figure 4). Most of AB-PAS staining was negative, mucin sulfate in the body of gland disappeared (Figure 5). HID-AB staining became hypochromatic. As the mucin in the body of gland decreased, the amount of neutral mucin increased relatively (Figure 6).

Changes after moxibustion treatment The mucosal inflammatory lesions in the majority of sections from the 13 patients with active UC abated remarkably. No hyperemia and edema were seen. Inflammatory cell infiltration decreased. In the majority of sections, neutrophil infiltration disappeared. Crypt abscesses, abscesses near crypts and ulceration of the mucosa were not seen. The reduction of the goblet cells in the body of gland was not obvious (Figure 7). The amount of mucin in the body of gland was almost normal. AB-PAS staining showed no obvious reduction in acid mucin (Figure 8). HID-AB staining showed no reduction in mucin sulfate (Figure 9).

The occurrence and severity of the main pathologic lesions before and after treatment (Table 1a,ab).

DISCUSSION

The clinical manifestations of UC is non-specific, and the disease is usually diagnosed by fibercolonoscopy and biopsies^[44-51]. All sections were examined and reexamined by the same pathologist. The sections showed typical pathologic features of UC, such as chronic mucosal inflammation, neutrophil infiltration, reduced goblet cells, crypt abscesses, mucosal ulceration.

In recent years, many studies on the change of colonic mucus were carried out. It was suggested that it might be the prodromal change of carcinoma of large intestine. As we all know, UC is a precancerous lesion of colon carcinoma. In this group, most mucin sulfate in glandular body disappeared whereas neutral mucin was relatively increased. We also found that the reduction of mucin was associated with the degree of local inflammation, the more severe the inflammation, the more obvious the reduction of mucin. The mucosa in this region also showed certain histologic characteristics: The goblet cells in the glandular body decreased remarkably or disappeared. With appearance of simple columnar cells which were lack of mucin section. The lumen of some mucosal glands became dilated, branched or irregularly arranged.

According to TCM, chronic non-specific ulcerative colitis belongs to the category of "Changpi" (bloody stool) and "Xiuxie" (diarrhea), and results from deficiency and hypofunction of spleen and stomach, accumulation of damp-heat, stagnancy of the liver-qi and deficiency of spleen, or insufficiency of spleen-yang and kidney-yang. In this study, good curative effect was achieved by the treatment of the moxibustion. The results showed that this moxibustion has the function of warming yang, promoting flow of qi and blood, improving the lesional blood circulation, and is helpful to hemostasis and the absorption of inflammatory products, and eventually attains the goal of the neogenesis of granulated tissue in the region of ulceration, and the repair of mucosal epithelium.

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KAI1 gene is differently expressed in papillary and pancreatic cancer: influence on metastasis

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Subject headings pancreatic neoplasms; papillary neoplasms; KAI1 gene; immunohistochemistry; *in situ* hybridization; blotting, northern

Guo XZ, Friess H, Shao XD, Liu MP, Xia YT, Xu JH, Buchler MW. KAI1 gene is differently expressed in papillary and pancreatic cancer: influence on metastasis. World J Gastroentero, 2000;6(6):866-871

Abstract

AIM To compare KAI1 in cancer of papilla of Vater and pancreas to evaluate whether there are differences in biologic behavior which might account for prognosis.

METHODS We compared the expression in 24 papillary and 29 pancreatic cancers using Northern blot analysis, immunochemical assay and *in situ* hybridization, and investigated whether early diagnosis or molecular differences predict the outcome in these tumor entities.

RESULTS By Northern blot analysis there is no statistical difference of KAI1 levels in normal and cancerous papilla. No association between KAI1 mRNA expression and tumor stage or tumor differentiation was found in the tumors. By immunohistochemical assay, KAI1 staining in cytoplasm of papillary cancer cells was similar to that of normal papillary cells. By *in situ* hybridization, the results of KAI1 mRNA expression in normal and cancerous papilla were similar to those with immunohistochemical assay. The normal and cancerous pancreas tissues were also analyzed by the methods used in papillary samples.

CONCLUSION Although the biologic roles of KAI1 have not been clarified, our results suggest that KAI1 may restrict the progression of malignant papillary cancer, but its expression might not have any effect on the characteristics of papillary tumor, whereas by the analysis of

KAI1 gene, its reduced expression is closely related to the progression and metastases of pancreatic cancer.

INTRODUCTION

Metastasis is a complex process, involving local invasion, inward and outward infiltration of tumor cells, and decreased host immunological responses^[1-4]. In carcinoma of papilla of Vater, lymph node metastases are present at the time of diagnosis in 31%-52% of the patients and by radical tumor resection, 5 year-survival rates of reached 21%-61%^[3]. In contrast to cancers of the papilla of Vater, cancer of pancreas has a dismal prognosis. Most pancreatic cancers have already had local or distant metastasis which restricts palliative surgical procedures. Therefore, median survival period of 4-6 months in most patients with pancreatic cancer remains in reality. The aggressive growth behaviour of pancreatic cancer results in a death/incidence ratio of approximately 0.99 in the United States and also in most European countries^[4,5].

The reason why pancreatic cancer has a prognosis different from that of the papilla of Vater is not known. It was postulated that earlier diagnosis due to jaundice accounts mainly for the better prognosis of papilla of Vater cancer patients. However, it is not known whether differences in tumor biologic behavior play any role in the difference in prognosis.

KAI1 has been identified to influence the metastatic ability of a various gastrointestinal cancer cells or other tumors^[6-22]. The gene is located on human chromosome 11p 11.2^[23]. Recently, it was reported that decreased KAI1 mRNA expression correlated with the metastases of pancreatic cancer^[24]. After transfer of the KAI1 gene into highly metastatic prostatic cancer cells, the metastatic ability was suppressed, whereas their primary tumor is not affected^[23]. These results suggested that decreased KAI1 expression is involved in the progression to metastatic cancers. However, whether changes in expression of tumor metastases influencing gene account for the better prognosis of papilla of Vater cancers is not known. Currently, it is believed that the better prognosis of

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papilla of Vater cancer patients compared with pancreatic cancer patients caused no differences in tumor growth and metastasis formation but from the earlier establishment of the diagnosis. Therefore, in the present studies, we compared KAI1 in papilla of Vater and pancreatic cancer patients to evaluate whether there exist differences in tumor biological behavior which might account for the differences in prognosis.

MATERIALS AND METHODS

Patients

Nine normal human papilla of Vater tissue specimens (4 females, 5 males, mean age \pm SD: 35.7 \pm 6.5 years, ranging 23-43); and 16 normal human pancreatic tissue specimens (6 females, 10 males; mean age \pm SD: 36.6 \pm 10.7 years, ranging 10-47) were obtained through a multiorgan donor program in 9 cases. The whole pancreas was obtained with the duodenum and the papilla of Vater was completely resected. Tissue specimens from 24 patients with carcinoma of the papilla of Vater (9 females and 15 males, mean age \pm SD: 58.4 \pm 14.1 years, ranging 16-81) were obtained following a Whipple's operation. The diagnosis of cancer of the papilla of Vater was confirmed by histopathological analysis. According to the TNM classification^[25] there were 2 stage I, 9 stage II, 11 stage III and 2 stage IV tumors. Pancreatic cancer tissues were obtained from 14 female and 15 male patients after operation. The median age of the pancreatic cancer patients was 64 years, ranging 37-78. The partial duodenopancreatectomy (Whipple's operation) and distal pancreatectomy was performed in 26 and 3 patients, respectively. According to the TNM classification of the International Union Against Cancer^[25], the patients were in stage I, 3 cases, stage II, 10 cases and stage III 16 cases; their gradings were well differentiated in 7, moderately in 17 and poorly differentiated in 5.

Tissue sampling

For RNA extraction and Northern blot analysis, normal and tumor specimens were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C until use. Additionally, freshly removed normal and cancerous tissue samples were immediately fixed in formaldehyde solution for 12h-24h and paraffin-embedded for *in situ* hybridization and immunohistochemical assay.

Northern blot analysis

Total RNA was extracted by the single-step guanidinium isothiocyanate method^[26] size-fractionated on 1.2% agarose 1.8mol/L formaldehyde gels^[26], and stained with ethidium bromide for verification of RNA integrity and loading equivalency. The RNA was electro-transferred onto nylon membranes (Gene Screen,

Du Pont International, Regensdorf, Switzerland) and cross-linked by UV irradiation. For hybridization a digoxigenin-(DIG) labeled KAI1 cRNA probe and ³²P-labeled 7S cDNA probe were used.

Prehybridization for KAI1 was performed for two hours at 65°C in a buffer containing 50% formamide, 5 \times SSC (sodium chloride/ sodium citrate buffer), 2% blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.1% Nauroyl sarcosine. After adding the DIG-labeled KAI1 antisense probe, hybridization was carried out at 65°C for 18 hours. The filters were washed afterwards for 5min, in 2 \times SSC, and 0.1% SDS. At room temperature, followed by two washes at 68°C for 15min each in 0.1 \times SSC and 0.1% SDS. The filters were then incubated in 20mL blocking buffer (1% blocking reagent in 100 mmol/L maleic-acid, 150mmol/L sodium chloride and 175mmol/L sodium hydroxide) containing 1 μ L anti-DIG alkaline phosphatase antibodies (Boehringer Mannheim) for 30min, washed with blocking buffer for 15min, and incubated with 4iL CDP-Star (25mmol; Boehringer Mannheim). The membranes were then exposed to X-ray films for 15sec. At room temperature as previously reported^[27,28]. In order to assess equivalent RNA loading, the membranes were rehybridized with the ³²P- labeled mouse 7S cDNA probe that cross-hybridizes with human 7S RNA^[28-30] to verify equivalent RNA loading. The membranes were prehybridized for 4-8 hours at 42°C in a buffer that contained 50% formamide, 1% sodium dodecyl sulfate, 0.75mol/L NaCl, 5mmol/L EDTA, 5 \times Denhardt's solution, 100mg/L salmon sperm DNA, 10% Dextran sulfate, and 50mmol/L sodium phosphate (pH 7.4). The hybridization was carried out at 42°C for 18 hours by adding the labeled cDNA probe 1 \times 10⁵ cpm /mL. The blots were rinsed twice in 2 \times SSC at room temperature and washed three times at 55°C in 0.2 \times SSC and 2% SDS.

The blots were then exposed at -80°C to Fuji X-ray films with intensifying screens for 24-48 hours. The intensity of the KAI1 and 7S signals was quantified by video densitometric analysis (Biorad 620, New York, USA) as previously reported^[10,26]. The ratio between the KAI1 signal and the corresponding 7S signal was calculated for each sample.

Immunohistochemistry

From each normal and cancer tissue sample, three tissue sections were examined. After deparaffinizing and hydrating, tissue sections were submerged for 15min in Tris-buffered saline (10mmol/L Tris HCl, 0.85% NaCl, pH 7.4) containing 0.1% (vol/vol) Triton X-100 and briefly rinsed 3 times for 1min-2min in TBS solution. Following incubation in methanol containing 0.6% hydrogen peroxide for 30min to block endogenous peroxidase activity, the

slides were covered with 10% normal goat serum at 23°C for 30min then incubated overnight with mouse monoclonal anti-human KAI1 antibody (antibody C33, kindly supplied by Dr.J.C.Barrett, Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA). After washing with TBS buffer, biotinylated goat anti-mouse immunoglobulin and streptavidin-peroxidase complex (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added at 23°C for 45 and 30min, respectively, followed by incubation with a 3, 3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide mixture. The slides were counterstained with Mayer's-hematoxylin.

In situ hybridization

In situ hybridization was performed as reported previously in detail using DIG-labeled cRNA probes^[31]. Tissue sections of normal and cancerous samples were processed always simultaneously. In addition, the consecutive tissue slides were processed, one slide each was incubated with the sense probe, a antisense probe. The prehybridization, hybridization and washing conditions were the same for pancreatic and papilla of Vater tissue samples. Four μm tissue sections were deparaffinized, rehydrated, and incubated in 0.2mol/L HCl for 20min. After washed with 2 \times SSC, the tissues were permeabilized with proteinase K at a concentration of 35mg/L for 15min at 37°C. After post-fixation with 4% paraformaldehyde in saline phosphate buffer (5min) and washing in 2 \times SSC, the sections were prehybridized for 1h at 60°C, in a buffer containing 50% formamide (v/v), 4 \times SSC, 2 \times Denhardt's reagent and 250 μg RNA/mL. Hybridization was performed overnight at the same temperature in 50% (v/v) formamide, 4 \times SSC, 2 \times Denhardt's reagent, 500 μg RNA/mL and 10% dextran sulfate (v/v). The final concentration of the DIG-labeled KAI1 probes (antisense or sense) was approximately 0.5ng/ μL . After hybridization, excess probe was removed by washing in 2 \times SSC, and by RNase treatment: 100U/mL RNase TI and 0.2U/mL RNase DNase-free (Boehringer Mannheim) at 37°C for 30min. Washings were performed at 60°C for pancreatic tissue slides and 63°C in 2 \times SSC (10min), and twice in 0.2 \times SSC (10min each). Afterward the sections were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim). For the color reaction 5-bromo-4-chloro-3-indolyl phosphatase and nitro blue tetrazolium (Sigma, Buchs, Switzerland) were used.

Pretreatment of the slides with RNase abolished the hybridization signals, and hybridization with the sense probes corresponding to the antisense probes failed to produce an *in situ* hybridization signal.

The *in situ* hybridization signals were semiquantitatively evaluated by two independent observers blind to patient status followed by resolution of any differences by joint review and consultation with a third observer. The *in situ* hybridization results were scored as previously described^[32]; (-) no detectable signal; (+) weak detectable signal; (++) moderate detectable signal; and (+++) strong detectable signal.

Preparation of KAI1 sense and antisense cRNA probes

To prepare digoxigenin-labeled KAI1 cRNA probes for Northern blot analysis and *in situ* hybridization, a 500bp fragment of human KAI1 cDNA was subcloned into the pCR-II vector (Invitrogen, San Diego, USA), which contains promoters for DNA-dependent SP6 and T7 RNA polymerases. After linearization of the plasmid, the antisense KAI1 probes were transcribed using SP6 polymerase and the Ribomax System (Promega Biotechnology, Madison, WI, USA). A DIG-labeled KAI1 cRNA probe was used for Northern blot analysis and *in situ* hybridization. To evaluate the specificity of the *in situ* hybridization reaction, DIG-labeled sense probes of KAI1 were generated after linearization of the plasmid with Bam-HI and Hind-III, respectively and *in vitro* transcription with T7 polymerase and the Ribomax System (Promega Biotechnology, Madison, WI, USA)^[33]. For the *in situ* hybridization experiments, the KAI1 antisense and sense probes were shortened to a length of approximately 150 bases^[27].

Preparation of 7S cDNA probe

To verify equivalent RNA loading on Northern blot membranes, all filters were rehybridized with a murine 190bp Bam HI fragment or 7S cDNA which cross-hybridizes with human 7S RNA as previously reported^[28,29,34]. The 7S cDNA probe was radiolabeled with [α -³²P]dCTP (3000Ci/mmol; DuPont, Boston, USA) using a random primer labeling system (Pharmacia Biotech AG, Dubendorf, Switzerland)^[28,29].

Statistical analysis

Results were expressed as median and range or as mean \pm SD. For statistical analysis the Mann-Whitney U test and the Chi-square test were used. Significance was defined as $P < 0.05$.

RESULTS

KAI1 mRNA expression by Northern blot analysis of papillary samples Measurable KAI1 mRNA signal was detected in 67% of the normal papillary tissue samples. In papilla of Vater cancer samples KAI1 mRNA expression was present in 46%. Densitometric analysis of the expression signals revealed a 1.07 fold (not significant) increase in

KAI1 mRNA levels in papillary cancer compared with the normal controls when all cancerous tissue samples were included. When only cancer samples with increased KAI1 mRNA expression level were statistically analyzed, the increase was 1.60-fold (not significant). The papillary cancer samples with lymph node metastases present at the time of tumor resection were compared with papillary tumor samples in lymph node-free metastases, no difference ($P>0.05$, Table 1) in KAI1 mRNA levels was found.

Pancreas The expression signals by densitometric analysis exhibited 2.2-fold increase ($P<0.05$) in KAI1 levels in pancreatic cancer compared with the normal controls when all cancerous tissue samples were included. When only-cancer samples with increased KAI1 expression levels were statistically analyzed, the increase was 2.8-fold ($P<0.05$). There was a significant negative correlation between KAI1 ($r=0.59$) mRNA levels and the tumor staging ($P<0.0007$, Table 1). Primary pancreatic cancer samples in which lymph node metastasis were present at the time of tumor resection (stage III) exhibited significantly lower KAI1 mRNA levels compared with primary tumor samples without lymph node metastasis at the time of tumor resection (stage I/II; Table 1, $P<0.005$).

Comparison between papillary and pancreatic cancer samples The mRNA levels of KAI1 gene in pancreatic cancers were higher than that in papillary cancers ($P=0.03$). Statistical analysis of the densitometric data revealed that these differences were statistically significant.

Immunohistochemistry of KAI1

Papilla of Vater In the normal samples of papilla of Vater, moderate to strong KAI1 immunoreactivity was present in the cytoplasm of epithelial cells. In addition, strong membranous immunostaining was found for KAI1 in the normal samples of papilla of Vater. Papillary cancer cells exhibited a similar staining like normal samples of papilla of Vater. However, only a few cancer cells showed membranous KAI1 immunoreactivity in tumors with or without metastases.

Pancreas KAI1 immunoreactivity was weakly detectable in the normal pancreas in a few acinar and ductal cells and strong KAI1 immunostaining was present in all pancreatic islets. Other results were similar to that seen with *in situ* hybridization.

KAI1 mRNA expression by *in situ* hybridization

To localize the exact site and cellular distribution of KAI1 mRNA expression, *in situ* hybridization was performed in normal and cancerous tissue samples.

Papilla of Vater In the normal papilla of Vater,

moderate to strong KAI1 mRNA staining was present in the cytoplasm of most epithelial cells. Lymphocytes in the submucosal areas of the normal papilla of Vater exhibited weak or moderate expression of KAI1 mRNA. In the papilla of Vater cancer samples, a similar intensity of KAI1 mRNA staining was present in the cytoplasm of cancer cells compared with normal epithelial cells. Fibroblasts of the connective tissue surrounding papillary cancer cells showed weak to moderate KAI1 mRNA staining. The intensity of KAI1 mRNA signals in samples of primary papilla of Vater cancer with or without metastases was not different.

Pancreas In normal pancreatic tissue samples only very faint KAI1 mRNA staining was found in a focal pattern in a few acinar and ductal cells of the normal pancreas. In contrast, pancreatic cancer cells demonstrated moderate to strong cytoplasmic KAI1 mRNA staining. However, the staining intensity for KAI1 mRNA in the pancreatic cancer cells was dependent on the tumor staging. Cancer cells of tumors without lymph node or distant metastases (Stage I /II) exhibited stronger KAI1 mRNA staining than primary tumor samples in which lymph node metastases were present (stage III). The stroma cells surrounding the pancreatic cancer lesions exhibited low levels of KAI1 mRNA expression. Similarly, only low KAI1 mRNA level was found in lymphocytes infiltrated in the cancer samples.

Table 1 KAI1 gene expression in papillary and pancreatic cancer

Carcinoma of papilla of Vater			Pancreatic cancer		
Clinical data	Total No.	P	Clinical data	Total No.	P
Sex					
Femal	9		Femal	14	
Male	15	NS	Male	15	NS
Age (years)					
<60	10		<60	8	
>60	14	NS	>60	21	NS
Tumor-staging					
T1-T2	11		T1-T2	13	
T3-T4	13	NS	T3-T4	16	0.001
Differentiation					
			G1-G2	24	
			G3-G4	5	NS

NS: no significance

DISCUSSION

Patients with carcinoma of papilla of Vater have the best prognosis in all patients with periampullary carcinomas^[35]. The most possible reason for it is the aggressive surgery carried out in early tumor stage or the tumor possessed a different biological local growth behavior and spreading characteristic. In contrast to cancer of papilla of Vater, pancreatic head carcinoma has a dismal prognosis and it was believed previously to be due to late diagnosis^[36]. It

is very difficult to distinguish patient with jaundice between papillary cancer and pancreatic cancer. Hence, the search for sensitive and reliable prognostic factor is of primary importance. The level of KAI1 mRNA expression were associated with clinical parameters influencing metastasis in gastrointestinal cancers in different ways^[24,28,29]. Tumor invasion and metastasis might be contributed by the down-regulation of KAI1^[24], whereas no significant KAI1 mRNA expression was found in gastric cancer and esophageal cancer with poor prognosis and metastases^[29]. These findings suggest that the effects of KAI1 on metastasis depend on the underlying malignancy.

In papillary cancer, no information about KAI1 has been obtained recently. By Northern blot analysis our results showed that the KAI1 mRNA level was not different in primary papillary carcinoma with or without lymph node metastases.

KAI1 immunoreactivity was mainly located in the membrane and/or cytoplasm of normal and/or cancerous papillary epithelia cells. By *in situ* hybridization, the results of KAI1 mRNA expression in normal and cancerous papilla were similar to those with immunohistochemical assay. Our findings indicate that KAI1 in papillary cancers exhibits different KAI1 mRNA expression patterns from prostate and pancreatic cancers^[23,29]. This suggests that KAI1 may limit the progression only in malignant papillary cancers, and this expression might have no effect on the characteristics of papillary tumors. Furthermore, the divergent expression patterns of KAI1 in the investigated cancer tissues show that it plays a role in the formation and metastases of these malignancies, that are different from those in previously analyzed tumors of the prostate, pancreas, breast, or the lung^[23-25,35]. However, by immunohistochemical assay and *in situ* hybridization, there was some heterogeneity of KAI1 mRNA expression levels in the cancers of papilla.

Our results showed that reduced expression of KAI1 mRNA might be regarded as the potential candidates for predicting tumor metastasis and invasion in pancreatic cancer. A few prospective randomized trials were reported that decreased expression of KAI1 gene was associated with metastasis in pancreatic cancer^[24]. By contrast to Northern blot analysis, similar expressions of KAI1 mRNA in immunohistochemistry and *in situ* hybridization were found. Down-regulation expression was only present in the patients with lymph nodes and distant metastases. We suggested that the expression of KAI1 gene could predict the prognosis of patients with pancreatic cancer. It is well known that the accumulation of genetic alterations causes the progression of tumors^[37]. But there are very few reports on its relationship with the mechanism of metastases in pancreatic cancer. K-ras, P16, P53, DPC4 and BRCA2 gene

influenced the tumor-suppressive pathways in pancreatic cancer. Underscoring the multigenic nature of cancer, and tumor PX101, having alterations identified in the five genes exemplified the extent of accumulation of genetic alterations^[38].

Cancer is a disease of gene alterations accumulated in several genes resulting in the development of the tumor^[39-50]. Multiple genetic lesions with either activating dominant oncogenes or inactivating tumor-suppressor genes have been recognized in human pancreatic cancer^[38]. Although our results suggest that expression of KAI1 gene is not associated with papillary cancer, it is closely related to metastases and prognosis of pancreatic cancer, and serves as a biological marker beneficial to diagnosis and treatment of pancreatic cancer. By further study on the pathogenesis of metastasis, the problem of how to prevent the early metastasis of pancreatic cancer will be solved.

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Brief Reports

DSA analysis of hepatic arteriovenous fistula concurrent with hepatic cancer and its clinical significance

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Subject headings liver neoplasms; hypertension, portal; hepatic arteriovenous fistula; angiography, digital subtraction

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INTRODUCTION

Intervention therapy has become one of the main therapies of hepatic cancer^[41,43,46,50]. The introduction of hepatic arterial perfusion and embolization has provided opportunities for a secondary operation on patients with intermediate and advanced cancer^[42,51-53], thus prolonging patients' life and improving their life quality^[1]. However, intractable ascites and hemorrhage of upper digestive tract caused by portal hypertension still greatly threatened the patients' life^[2,7-9,11,14,17,19-25,39]. So far there has been little report on the incidence rate of hepatic arteriovenous fistula (HA-V)^[4] and the relationship between its typing and hepatic cancer as well as portal hypertension^[3,6,12,18,26-28,31,32,34,54,59]. In the present study, a series of observation and analysis were made for 110 cases of hepatic arteriovenous fistula concurrent with intermediate and advanced hepatic cancer.

MATERIALS AND METHODS

Clinical data

One hundred and ten cases were selected from the 583 cases of hepatic cancer, which were clinically verified by radiological images and admitted to our department from 1989 to 1999. The cases were diagnosed as hepatic arteriovenous fistula by

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Seldinger technique with DSA, among which, 102 were males and 8 females aged between 27 years and 70 years (average 48.5 years). One hundred and three cases were primary and 7 were secondary hepatic cancer. The latter include 3 cases of primary carcinoma of colon, 2 carcinoma of ampulla, 1 carcinoma of pancreas and 1 cardiac cancer. Among the 110 cases, there were 58 (52.72%) cases of concurrent ascites and 31 (28.18%) hemorrhage of upper digestive tract.

Examination

Examination of hepatic arteriovenous fistula All the cases underwent percutaneous femoral puncture by Seldinger technique. Guided by X-ray, the head of the conduct cannula was placed in the celiac artery and upper mesenteric artery. Through the cannula, 38% cardiografin or Ultravis t of 20mL-25mL at a time and at a rate of 4mL/S-5mL/S was perfused with high pressure while the arteries were photographed continuously at 2 frames per second until the pyloric stem was developed or the photography lasted up to 20 seconds. The DSA results indicate that portal vein, hepatic vein or inferior caval vein in hepatic arterial phase or hepatic parenchymic phase developed ahead of the expected time could be diagnosed as concurrent hepatic arteriovenous fistula. The 110 cases of hepatic arteriovenous fistula received DSA examination for a total of 273 times, for some cases once at least and some 9 times at most.

Barium meal examination of upper digestive tract

Thirty cases received routine pneumobarium double contrast examination.

Gastrofiberscopy Thirty-three cases took routine gastrofiber scopy.

Statistical analysis χ^2 test and U test were performed according to NoSA software package.

RESULTS

Among the 583 patients with hepatic cancer, 110 were found to have hepatic arteriovenous fistula, which indicated an average incidence rate of 18.86%. From 1989 to 1999, the annual incidence

rate of hepatic arteriovenous fistula ranged from 2.38% to 47.72%, as shown in Figure 1.

Of the 110 cases of hepatic arteriovenous fistula, 57 had their focuses in the right lobe (51.81%), 13 in the left lobe (11.81%) and 40 involved both (36.36%). Seventy-four were massive (67.27%), 21 nodular (19.09%) and 15 diffuse (13.63%). Eleven were aberrant in their hepatic arterial origins (10.00%), of which 7 were of the right vagohepatic artery, 1 of the common vagohepatic artery, 1 of the right vagoaccessary hepatic artery (all the above-mentioned 9 aberrant cases originated in the superior mesenteric artery), and the others, 1 left vagohepatic artery originated in the left arterial gastric and 1 common vagohepatic artery in the right diaphragmatic artery. DSA detected sufficient blood supply in 89 cases (89.90%), moderate blood supply 14 (12.72%) and poor supply 7 (6.36%).

According to the locations of the fistula or the abnormal split-flow, the 110 cases can be divided into two main types: 95 peripheral (86.36%) and 15 central (13.63%). Hepatic arteriovenous fistula usually occurs in the cases with sufficient blood supply and massive hepatic cancer (Table 1) There were 103 simple cases (93.63%) and 7 complicated cases (6.36%) (Table 2).

Table 1 The relationship between locations of hepatic arteriovenous fistula and the typing as well as neoplastic blood supply

Location	n ^a	Typing ^b			Neoplastic blood supply		
		Massive	Nodular	Diffuse	Sufficient	Moderate	Poor
Peripheral	95	67	18	10	79	9	7
Central	15	7	3	5	10	5	0
N=110		74	21	15	89	14	7

^aPeripheral cases significantly outnumbered central ones ($P < 0.05$, $\chi^2 = 6.05$).

^bMassive cases significantly outnumbered diffuse ones ($P < 0.05$, $\chi^2 = 6.09$). There is no difference between massive ones and nodular ones, or nodular ones and diffuse ones ($P > 0.05$).

Peripheral ones outnumbered central ones in blood supply ($P < 0.05$, $\chi^2 = 7.34$). Sufficient blood supply cases significantly outnumbered cases of moderate blood supply ($P < 0.05$, $\chi^2 = 5.83$). There is no difference between those with sufficient and poor supply, or with moderate and poor supply ($P > 0.05$).

One hundred and three cases (93.63%) are simple ones which indicate hepatic arterioportal vein fistula in 97 cases (88.18%) (Figure 2), hepatic arteriovenous fistula in 4 cases (Figure 3), hepatoinferior vena cava fistula in 2 cases (Figure 4). Seven cases are complicated ones which indicate both HA-IV fistula and HA-PV fistula in 3 cases, both HA-IV fistula and HA-V fistula in 1 case, first HA-V and then HA-IV fistula in 1 case, HA-IV fistula first and then HA-PV fistula in 1 case, three HA-PV fistulas in 1 case (Figures 5-7). The simple ones are more than complicate ones significantly (Table 2).

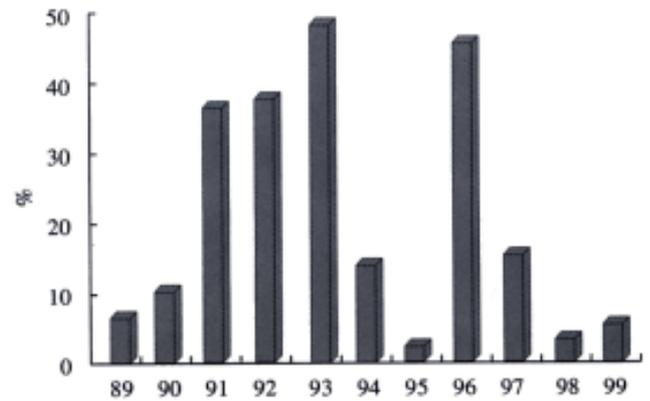


Figure 1 Annual incidence rate of hepatic arteriovenous fistula from 1989 to 1999.

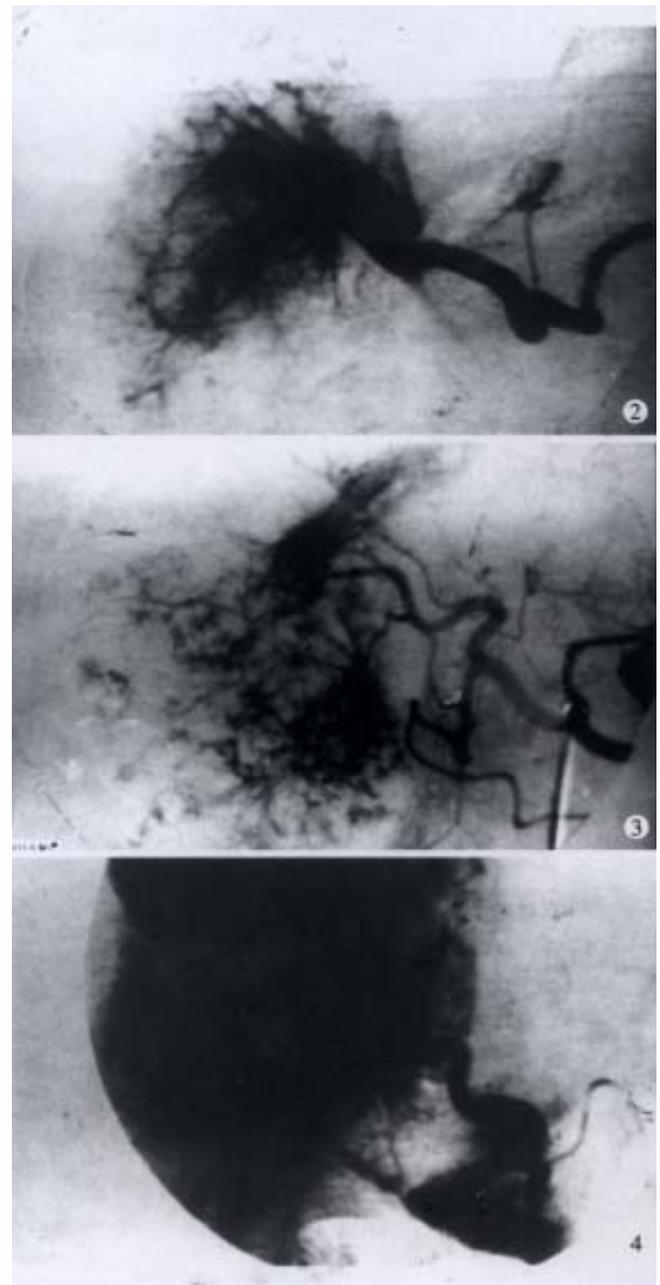


Figure 2 The hepatic arterioportal vein fistula (central cases).
Figure 3 The hepatic arteriovenous fistula (peripheral cases).
Figure 4 The hepatoinferior vena cava fistula (peripheral cases).

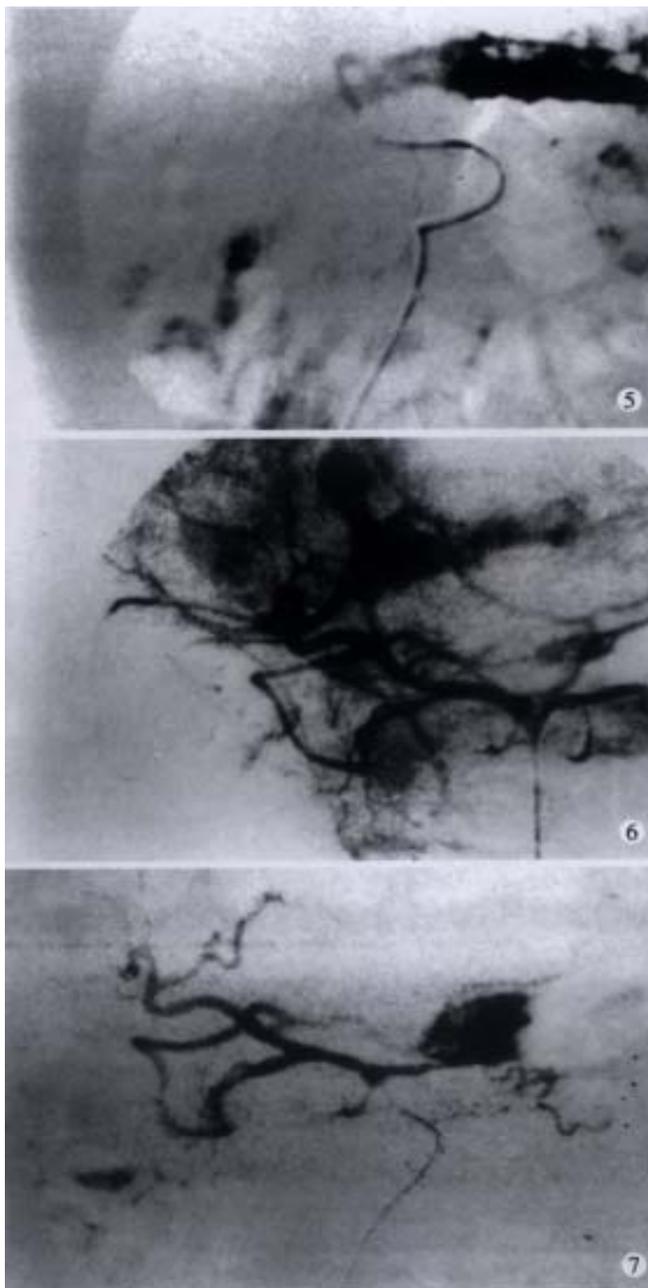


Figure 5 The left vagoaccessory hepatic arterioportal vein fistula (central cases)

Figure 6 The right hepatic arterioportal vein fistula (peripheral cases): the superior mesenteric vein retrogradely developed.

Figure 7 The left hepatoportal vein fistula (central cases).

Table 2 Typing of hepatic arteriovenous fistula

Typing	n	HA-PV	Others	P
Simple	103	97	6	<0.01
Complicated	7	0	7	$\chi^2=55.78$

Portal hypertension is clinically characterized by ascites and hemorrhage of the upper digestive tract. Barium meal examination and gastrofiberscopy of upper digestive tract of the

portal hypertension cases indicated that the positive rate of varices of esophagus detected by gastrofiberscopy was significantly higher than that by barium meal examination. The positive rate of ascites by gastro fiberscopy was 100% whereas that by barium meal check up was only 56.66% ($P<0.01$, $U=4.08$). The positive rate of hemorrhage of upper digestive tract detected by gastrofiberscopy was 96.96% whereas that by barium meal checkup was 53.33% ($P<0.01$, $U=3.77$). It is worth notice that among the 52 patients with concurrent hepatic arteriovenous fistula who received neither gastrofiberscopy nor barium meal exam, the incidence rates of ascites and hemorrhage of upper digestive tract were 46.15% and 19.23% respectively, from which the possible existence of esophageal varices could not be excluded.

DISCUSSION

In the present study, we analyzed the relationship between the incidence rate and typing of hepatic arteriovenous fistula and the typing and blood supply of hepatic cancer, compared and analyzed ascites and hemorrhage of upper digestive tract by gastrofiberscopy^[57,60-62], barium meal checkup and DSA. According to the results of these investigations, we have come to the following conclusions.

Hepatic arteriovenous fistula claims a relatively high incidence rate^[3,5,35-40], among hepatic cancer cases, with an average of 18.86% and every 4 or 5 years as a cycle of change. Primary hepatic cancer accounts for 93.63% of concurrent hepatic arteriovenous fistula cases.

Hepatic arteriovenous fistula is usually found in cases of sufficient blood supply and massive type hepatic cancer, which usually indicates the severity of lesion.

The incidence rate of hepatic arterio-pylic fistula (HA-PV) is significantly higher than that of other types of abnormal hepatic arteriovenous split flow, which indicates that hepatic arteriovenous fistula is an important factor that leads to concurrent portal hypertension of moderate and advanced hepatic cancers^[39].

Portal hypertension caused by hepatic arteriovenous fistula is clinically and chiefly characterized by ascites (52.72%) and secondarily by hemorrhage of upper digestive tract (28.18%)^[8,11,30].

Barium meal checkup and gastrofiberscopy of upper digestive tract of concurrent portal hypertension cases indicate that the positive rate of esophageal varices detected by gastrofiberscopy was much higher than that by barium meal checkup^[44,48,49]. Ascites and hemorrhage accounted for 46.15% and 19.23% of the 52 patients with hepatic arteriovenous fistula who did not receive barium meal checkup and gastrofiberscopy^[19,29]. It

is thus suggested by the authors that gastrofiberscopy should be performed in patients with moderate and advanced cancers no matter whether they have concurrent ascites and hemorrhage of upper digestive tract or not. Gastrofiberscopy should be included in the routine examinations^[10,13], especially for patients with concurrent ascites and hemorrhage of upper digestive tract so as to detect esophageal varices as early as possible and treat it timely^[15,17,18].

Hepatic arteriovenous fistula is usually found in hepatic cancers of the peripheral type^[47], which indicates that embolisation of hepatic artery can block the neoplastic blood supply and inhibit neoplastic growth^[48]. In the meantime, the embolisation can also block the abnormal split-flow between hepatic arteries and veins, relieve ascites and hemorrhage of upper digestive tract caused by portal hypertension as well as the distant metastasis caused by venous shunt.

The current therapies applied to the treatment of hepatic cancer mainly include hepatic arterial perfusion, embolisation, local injection of absolute alcohol guided by ultrasonography, radio frequency therapy, etc^[45,55,58]. However, it is difficult for ultrasonography and CT to preliminarily detect hepatic arteriovenous fistula which is highly incidental to primary hepatic cancer of massive and sufficient blood supply types. Besides, the abnormal split-flow between hepatic arteries and veins may inhibit the effect of these therapies and even give rise to some complications^[12,26,31,56].

Therefore, we conclude that DSA should be included as an important part of the routine examination for the treatment of moderate and advanced hepatic cancer. In order to detect hepatic arteriovenous fistula early and treat the patients with embolisation timely, DSA should be given especially to hepatic cancer patients with concurrent ascites and hemorrhage of upper digestive tract^[5,6,16,17,33,34].

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Effect of anti-fibrosis compound on collagen expression of hepatic cells in experimental liver fibrosis of rats

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Subject headings liver cirrhosis; collagen; hepatic stellate cells; immunohistochemistry; anti-fibrosis complex prescription; rats

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INTRODUCTION

Liver fibrosis is mainly characterized by the excessive synthesis and decreased degradation of extracellular matrix (ECM), especially the synthesis and deposition of collagen. Almost all kinds of cells in the liver have participated in the production of collagen. The most important ones are hepatic stellate cells (HSC) and hepatocytes^[1-3]. We have observed the collagen expression of normal and fibrotic rat liver, compared its expression in passaged HSC of the normal liver and in the primary HSC of the fibrotic liver. Serum pharmacological method was used to study the influence of anti-fibrosis compound prescription (ACP) on the production of collagen in hepatocytes and HSC of rats.

MATERIALS AND METHODS

Experimental animal

Wistar male rats of 250g-300g were used to separate the hepatocytes, of 300g-350g were used to separate HSC. The rats were provided by Shanghai Centre of Experimental Animals. Animal model of liver fibrosis was formed by intraperitoneal injection of DMN (10mg/kg) 3 times a week for three weeks.

Drugs and pharmacological serum preparation

ACP (composed of *Radix Astragali seu Hedysari*, *Herba Leonuri Radix Curcum ae*, etc.), prepared by the Pharmaceutical Department of Shuguang Hospital, contained 2g/mL crude drugs. Colchicine (Supplied by Serva Company) was prepared at the concentration of 0.3g/L. ACP and

colchicine were instilled into the stomach of the rats once a day for 1 week. One hour after the last time of instillation, blood was drawn in aseptic condition and serum was separated. After 30 minutes of inactivation at 56°C, it was packed and refrigerated separately.

Separation and culture of rat hepatocytes

After anesthesia of the rats, a tube was inserted through the portal vein to infuse collagenase perfusion (0.05% of collagenase IV, 0.007% trypsin inhibitor magnesium-free Hank's medium, pH 7.4) to digest the liver and disperse the liver cells. Density gradient centrifugation was done with 49.2% (v/v) lymphocytic separation fluid to purify the liver cells. Then culture medium 199 with 10% calf serum (added with 10⁻⁸mol/L of insulin and 10⁻⁸mol/L of dexamethasone) was used to suspend the cells to 5×10⁵/mL and was inoculated to the 60mm culture plate and stored in the incubator with 5% CO₂ and 37°C. After 4 hours, it was replaced by 5% calf serum in 199 medium for primary culture.

Separation and culture of the HSC

After anesthesia of the rats, a tube was inserted through the portal vein to infuse D-Hank's liquid to cleanse the blood in liver. Then the perfusion medium with enzyme (Hank's medium containing 0.05% of collagenase IV and 0.1% Pronase E) for cyclic infusion. The liver was cut into pieces, and re-digested. After centrifugation with 18% (w/v) Nycondenz density gradient centrifugation, the purified HSC was suspended in DMEM containing 20% calf serum attending 5×10⁵/mL for inoculation and culture. After full growth, the normal HSC was passaged while the HSC from the rat with fibrotic liver were processed in primary culture.

Assay of immunohistochemistry

The hepatocytes or HSC were inoculated in the plate covered with a piece of glass with a density of 5×10⁵/mL. The hepatocytes after cultured for 2 days, 5% medicated serum or controlled serum was used to incubate for 48 hours. After 5 days, the HSC adhered to the plate were incubated in 10% medicated serum or controlled serum for 72 hours. The cells were then divided into ACP group with medicated serum of ACP, colchicine group with

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medicated serum of colchicine and control group with serum from normal rats. After culture, the glass slides were taken out to detect type I, III and IV collagen by ABC method of immunohistochemistry. After staining, the light density of the cells in each group was tested with the computer image analysis system. The higher the density, the deeper the positive staining.

RESULTS

Comparison of the production of collagen between normal and fibrotic hepatocytes

The immunocytochemical staining indicated that the collagen of the normal hepatocytes was negative, the light density of the collagen type I, III and IV was 3.80, 4.30 and 3.90 respectively, the staining of those three kinds of collagen in cytoplasm of the fibrotic hepatocytes was significantly increased. The staining of collagen type I was the deepest one, the next was type III. The light density of these three kinds of collagen was 42.40, 32.10 and 27.40 respectively. There was significant difference in the cells between the two groups ($P < 0.01$).

Influence of the ACP on the collagen expression of type I, III and IV in hepatocytes from the fibrotic rat

The staining of the type I, III and IV collagen was positive in cytoplasm of hepatocytes from the fibrotic liver. After treatment with serum processed by the ACP, the staining obviously turned lighter as compared with the control group, indicating that the collagen expression of the type I, III and IV of the cells was reduced. The inhibition of colchicine serum group on collagen type I was close to the group of ACP, while the effect on expression of the collagen type III and IV was weaker (Table 1) than those of the ACP group.

Comparison of the collagen expression between passage HSC and fibrotic HSC

The immunochemical staining indicated that positive staining of the type I, III and IV collagen appeared in both of the normal passage HSC and the fibrotic HSC. The collagen expression of the cells in both groups was nearly similar. But the correlation analysis on type I and III showed that the collagen expression of the type III in passage HSC was significantly increased, type I/type III was 0.91. However, the collagen expression of type I in fibrotic HSC increased more significantly as compared with that of type III. Type I/ type III was 1.22 (Figure 1).

Influence of the ACP on the collagen expression of type I, III and IV fibrotic HSC

ACP medicated serum could significantly reduce the staining of collagen type I, III and IV in fibrotic HSC and could remarkably inhibit the collagen type

IV and I. The effect was better than that of the colchicine group. Compared with the control group, the positive staining of the HSC in the colchicine group became lighter. However, its inhibition on the expression of collagen type III was close to that of ACP (Table 2).

Table 1 Influence of ACP on the collagen expression of the fibrotic hepatocytes (density value, $n=20$, $\bar{x} \pm s$)

	Inhibiting rate (%)					
	Col (I)		Col(III)		Col(IV)	
Control	45.6±2.29		38.0±2.76		27.9±2.39	
Colchicine	19.2±2.52 ^a	25.29	15.2±1.75 ^{ab}	20.36	13.4±1.56 ^{ab}	11.87
ACP	18.3±2.61 ^a	26.15	10.7±1.68 ^a	24.37	7.9±2.55 ^a	16.38

^a $P < 0.01$ vs control group; ^b $P < 0.01$ vs ACP.

Table 2 Influence of the ACP on the collagen expression of fibrotic HSC (density value, $n=20$, $\bar{x} \pm s$)

	Inhibiting rate (%)					
	Col (I)		Col(III)		Col(IV)	
Control	26.8±1.99		21.9±1.97		33.3±2.19	
Colchicine	12.2±2.18 ^{ab}	11.85	9.0±2.00 ^a	10.07	9.5±2.06 ^{ab}	20.39
ACP	8.4±1.50 ^a	14.93	7.9±1.87 ^a	10.93	5.8±1.40 ^a	23.56

^a $P < 0.01$ vs control serum; ^b $P < 0.01$ vs ACP.

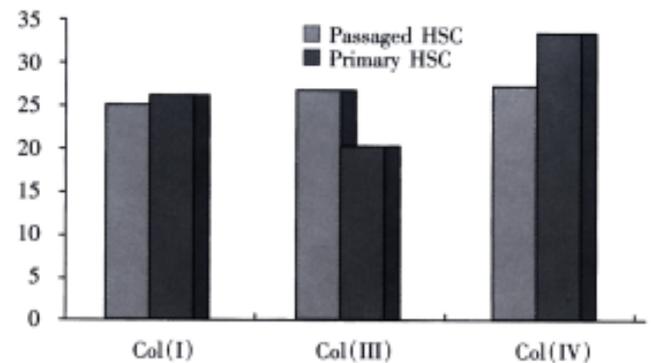


Figure 1 Comparison of the collagen expression between the passaged HSC and the fibrotic HSC.

DISCUSSION

The cells responsible for the production of collagen in the liver have aroused many scholars' attention. Recent studies have confirmed that HSCs are the key cells to produce collagen in the liver. The activated HSC can excessively synthesize collagen^[4-6]. Through automatic secretion and multiplication, they maintain a constant progression of fibrosis and play very important roles in the course of liver fibrosis^[7-10]. There are different ideas as to whether the hepatocytes can synthesize collagen. There are contradictory reports about whether there exists collagen mRNA or not in the hepatocytes^[11-14]. The latest studies have proved that the hepatocytes can synthesize collagen^[15-18]. Immunohistochemical studies have discovered that there is no collagen of the type III in the hepatocytes of normal liver. However, small quantity of it was found in the hepatocytes of fibrotic liver^[19]. It can

be inferred that the excessive synthesis of collagen which may lead to liver fibrosis, is related to the constant abnormal regeneration of hepatocytes. Through *in situ* hybridization, we have discovered that the fibrotic rat liver induced by CCl₄ can express procollagen mRNA $\alpha 1$ (I) and $\alpha 1$ (III) in the hepatocytes of early, middle and advanced stages. This shows that the hepatocytes can synthesize the collagen type I and III, and also participate in the course of liver fibrosis^[19,20].

The hepatocytes can synthesize at least the collagen of type I, III, IV and V while in the normal liver, collagen synthesis in hepatocytes is quite weak or even inhibited. In the process of liver fibrosis, the synthetic activity of collagen was remarkably increased^[21]. This study indicates that in normal liver, hepatocytes do not express or seldom express collagen and that in the fibrotic liver the expression of collagen type I, III and IV in hepatocytosol is more significant. When the hepatocytes are impaired or under the stimulation of certain factors, the capacity of the hepatocytes to synthesize collagen will be activated^[22,23]. As to a single liver cell, its capacity to synthesize collagen is much lower than that of the HSC. However an absolute majority of the cells in the liver are hepatocytes, and their capacity to synthesize collagen should not be overlooked. And in chronic liver disease, the degeneration and necrosis of the hepatocytes can directly or indirectly stimulate non-parenchymal cells like HSC to increase collagen production, thus exerting great effort on liver fibrosis^[24-27].

At the early stage of liver fibrosis, collagen type IV increases first and then gradually decreases, while the collagen type III increases and expresses predominantly. In the developing and advanced stages, the collagen type I gradually increased, and collagen of both type I and III significantly increased, especially the type I^[11,28,29]. After passage culture of the HSC from normal liver, a series of changes have been observed in their features and the production of collagen and the expression scale is evidently increased. So HSC is regarded as *in vitro* cell model of fibrosis and is extensively used in the *in vitro* experiment of liver fibrosis^[30-33]. In order to be close to the collagen expression of the *in vitro* HSC during the fibrosis of liver, the experiment was also made using the HSC of the primary culture from the fibrotic liver. The collagen expression of the HSC from passage culture and HSC from primary culture of the fibrotic liver was tested by immunocytochemical staining. Positive staining was observed in the collagen type I, III and IV.

When fibrosis occurs in the liver, the total amount and varieties of the collagen produced by HSC in the process of liver fibrosis are

changed^[34-36]. Such change is related to the course of liver fibrosis. Maker^[37] has discovered that in the culture of HSC from normal SD rats, the gene expression of type I collagen is the weakest, while the gene expression of the collagen type III or IV is comparatively stronger. However, The mRNA of type I collagen is 30 times that of normal and type III is 5 times that of normal due to difference in culture time and in the expression of procollagen mRNA in the HSC from both the normal liver and fibrotic liver. At the early stage of fibrosis, the expression of the collagen type III is more significant, while at the advanced stage the expression of the collagen I type is more remarkable. The passage culture for the HSC separated from the normal liver is about 2 weeks, while the time for *in vivo* modeling of the HSC in the fibrotic liver is 4 weeks. The experimental results show that the collagen expression of type III in passage HSC is significantly increased, while the increase of collagen expression of type I in passage HSC is more significant. It seems to show that these two kinds of cells bear the features of the HSC at the early and advanced stages of fibrosis.

Seropharmacological studies indicate that ACP can significantly inhibit the excessive synthesis of collagen by the hepatocytes and HSC under the condition of liver fibrosis^[38]. *In vitro* experiment, medicated serum can eliminate the effect of impurity and acidity in the Chinese pharmaceutical preparation on the growth of cells, making it more closely mimic the absorption and metabolism of drugs *in vivo*^[39,40], and bringing the pharmaceutical effect into full play.

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Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression in fibrotic rat liver

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INTRODUCTION

Liver fibrosis is an excessive deposition of extracellular matrix (ECM) resulted from both increased synthesis and decreased degradation^[1-3]. Matrix metalloproteinases (MMPs) represent a group of neutral proteinases with variable substrate spectra. Their activity may be regulated at the level of gene transcription, proenzyme activation and inhibition of active enzyme by specific inhibitors such as the tissue inhibitor of metalloproteinases (TIMPs). The remodeling of extracellular matrix during chronic liver disease may partially be attributed to the altered activity of matrix metalloproteinases and their tissue inhibitors (such as TIMPs)^[4,5]. Hepatic stellate cell (HSC) (fat storing cell, Ito cell, lipocyte) is the main source of ECM production in liver fibrosis^[6-13], which also can express and secrete matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in the primary culture^[14-16]. The alteration of MMP-2 and TIMP-1 expression may be implicated in the hepatic fibrogenesis.

In this study, we examined the expression of MMP-2 and TIMP-1 in liver tissue and HSC isolated from normal and CCl₄ induced fibrotic rat respectively, in order to explore the role of MMP-2 and TIMP-1 during hepatic fibrogenesis.

MATERIALS AND METHODS

Materials

Animals Male Wistar rats, body weight exceeding

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300g ($n=24$), purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences, were randomly divided into test group and control group.

Reagents Dulbecco's Modified Eagle Medium (No. 69K3042), TRIZOL reagent (No. 15596026) and SUPERSRIPT™ preamplification system for first strand cDNA synthesis (No. JDQ702) were purchased from GIBCO Co. Nycodenz (No. 16H0162), Pronase E, DNase and collagenase I were from Sigma Co; 10×PCR buffer, 25mmol/L MgCl₂, 10×dNTP, *Taq* DNA polymerase (No. 6923214, 65595010), RNasin and pGEM-72-f (+)/*Hae*-III markers were obtained from Promega Co. Primers of MMP-2 and TIMP-1 and β-actin were synthesized by Tumor Molecular Biology Institute, Chinese Academy of Sciences. β-actin: (+) 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3', (-) 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3'; primer of MMP-2: (+) 5'-GTG CTG AAG GAC ACA CTA AAG AAG A-3', (-) 5'-TTG CCA TCC TTC TCA AAG TTG TAG G-3'; primer of TIMP-1: (+) 5'-GAC CTG GTC ATA AGG GCT AAA-3', (-) 5'-GCC CGT GAT GAG AAA CTC TTC ACT-3'.

Methods

Development of a CCl₄-induced fibrotic rat model

Test group rats ($n=12$) received subcutaneous injection of 40% CCl₄ diluted with olive oil at a dose of 0.3mL/100g of body weight twice a week for 6-8 weeks. Hepatic fibrosis was induced by CCl₄^[17].

Separation, purification and identification of rat HSC

We modified the procedures of Friedman's methods^[18] to improve the yield and viability of HSC. HSC was isolated from control and experimental animals by collagenase perfusion and density gradient centrifugation^[19-21]. Rats were anesthetized with 1% sodium pentobarbital 0.2mL/kg of body weight. The liver was perfused through the portal vein *in situ* with heparinized calcium-free solution (142mmol/L NaCl, 6.7mmol/L KCl, 10mmol/L HEPES, 5.5mmol/L NaOH, pH 7.4) at 37°C for 10min at a flow rate of 30-40 mL/min and the inferior vena cava cut at the same time. Until the perfusion solution from inferior vena cava turned colorless and the liver became white, the liver was carefully excised and placed on a special appliance, and then

perfused with 0.1% Pronase E and 0.05% collagenase added enzymatic solution (67mmol/L NaCl, 6.7mmol/L KCl, 100mmol/L HEPES, 66mmol/L NaOH and 5mmol/L CaCl₂) at 37°C for 20min at the same flow rate. After the liver turned yellow and became soft, it was cut into pieces and incubated in 30mL enzymatic solution with 0.05% DNase on a magnetic stirrer at 37°C for 30min. At the end of the incubation period, the suspension was filtered through screen mesh and the filtrate in Hank's solution was centrifuged at 450×g for 7min. The cell pellets were repeatedly resuspended in Hank's solution and centrifuged for three times to further dissociate the cells. The cells were separated by density gradient centrifugation with 1:2 v/v 18% Nycodenz at 1450 ×g for 17min at 4°C. HSC, which remained at the upper and middle interface, was collected and washed twice in DME followed by centrifugation at 450×g for 8min to remove hepatocyte debris. The pellet was resuspended in a small amount of DME and used for further studies. Cell viability was evaluated from the capacity of the cells to exclude trypan blue. Desmin positive cell was identified by immunohistochemistry.

Total RNA isolation Total RNA was extracted from liver tissue of the control and fibrotic rats using TRIZOL reagent. HSC was pelleted by centrifugation, lysed with TRIZOL reagent by repetitive pipetting and diluted as 1mL of the reagent per 5×10⁶-6×10⁶ of HSC. Incubate the homogenized samples for 5min to permit the complete dissociation of nucleoprotein complexes, add 0.2mL of chloroform per 1mL of TRIZOL reagent, cap sample tubes securely, shake tubes vigorously by hand for 15s and incubate for 2-3 min at room temperature. Centrifuge the samples at 12 000×g for 15min at 4°C. After centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Transfer the aqueous phase to another tube, add 0.5mL of isopropanol per 1mL of TRIZOL reagent according to the initial homogenization, incubate samples for 10min at room temperature and centrifuge at 12 000×g for 10min at 4°C. Remove the supernatant, wash the RNA pellet once with 75% ethanol, add at least 1mL of 75% ethanol/mL of TRIZOL reagent according to the initial homogenization. Mix the sample by vortexing and centrifuge at 7 500×g for 5min at 4°C, remove the supernatant, and briefly vacuum-dry the RNA pellet. The RNA precipitate can be stored in RNase-free water at -70°C. Dissolve 2μL RNA sample (1:50) for measuring OD₂₆₀ and OD₂₈₀ by UV spectrophotometer. Three μL RNA sample was electrophoresed through a 1.1% denaturing agarose gel with ethidium bromide.

RT-PCR Four μg of total RNA in 1μL water was

reversely transcribed. The RNA sample was incubated at 70°C for 10 min with 1μL Oligo (dT)₁₂₋₁₈ (0.5μg/μL) and 10μL 0.1% DEPC, then placed on ice bath for at least 1min. Then the reagents including 2μL of 10×PCR buffer; 2μL of 25mmol/L MgCl₂; 1μL of 10mmol/L dNTP mix; 2μL of 0.1mmol/L DTT and 0.5μL of 40U RNasin were added to each RNA/primer mixture. After mixing, the tubes were incubated at 42°C for 5 min. One μL (200U) of SUPERScript II RT was added to each tube and incubated at 42°C for 50min and 70°C for 15min. The sample was chilled on ice bath. One μL RNase H was added and incubated at 37°C for 20min. Then PCR was made or stored at -20°C. The following reagents were added to a 500μL polypropylene microcentrifuge tube: ① 5μL of 10×PCR buffer; ② 3μL of 25mmol/L MgCl₂; ③ 5μL of 10×dNTP; ④ 0.5μL of 5U/μL TaqE; ⑤ two pairs primer mixture (β-actin primer was used for internal control); ⑥ 2μL room temperature sample and ⑦ water to 50μL. The components were concentrated at the bottom of the tube by centrifugation and covered with mineral oil. PCR incubation was carried out in a programmable thermal controller. During each PCR cycle (40 cycles), the samples were heated to denature template complexes (94°C 180s initially and 60s during all subsequent cycles), cooled to 56°C to anneal template and primers (60s) each cycle and heated to 72°C to extend for 60s. The final 72°C incubation was extended for additional 10min to maximize strand completion. The samples were then rapidly cooled to 4°C and kept on ice bath or frozen until analysis. After amplification, 10μL of each PCR reaction mixture was electrophoresed through a 2.0% agarose gel with ethidium bromide (0.1%). The size of the markers are 1000bp, 750bp, 500bp and 300bp. The gel was photographed over UV light at the same exposure and development time for all gels photographed. The bands on the film were scanned by densitometry for quantitation. The PCR products were electrophoresed in the same gel and ratios for MMP-2/β-actin and TIMP-1/β-actin were determined to eliminate gel-to-gel or film-to-film variance.

Statistical analysis Data were expressed as mean ± SD ($\bar{x} \pm s$)^[22].

RESULTS

Expressions of MMP-2 and TIMP-1 in HSC

MMP-2 mRNA was undetectable in HSC isolated from normal rat liver, but it was detected in HSC from fibrotic rat liver. TIMP-1 mRNA was detected both in HSC from normal and fibrotic rat liver (0.25±0.16 and 0.56±0.09), and the expression was increased markedly in liver fibrosis ($P < 0.02$).

Levels of MMP-2 and TIMP-1 mRNA in liver tissue
MMP-2 mRNA was detected both in normal and fibrotic rat liver tissue (0.86 ± 0.09 and 0.99 ± 0.05). Although the level of MMP-2 mRNA was increased in fibrotic liver tissue, there were no significant differences between them ($P > 0.05$). TIMP-1 mRNA was detected in normal and fibrotic liver tissue (0.46 ± 0.03 and 1.36 ± 0.62). TIMP-1 expression was enhanced remarkably in fibrotic liver tissue ($P < 0.05$).

DISCUSSION

MMP-2 degrades collagen IV, V, VII and X, as well as elastic, fibronectin, and denatured collagen type I^[23]. Collagen type IV is the primary component of basement membrane. Therefore, the increment of MMP-2 can result in the damage of membrane in the space of Disse, which will activate the perisinusoid cells including HSC and promote hepatic fibrogenesis. Our results showed that the MMP-2 mRNA was only detected in HSC isolated from fibrotic liver, not in HSC from normal rat liver. This provided evidence for MMP-2 expression involving in liver fibrosis^[24]. Comparing with cell culture, isolating HSC from liver and extracting RNA directly to determinate the level of MMP-2 mRNA can more exactly reflect the situation *in vivo*, because the HSC has been activated in the process of culture *in vitro*. The MMP-2 mRNA was detectable in normal liver tissue. It indicated that there were sources other than HSC. In consistency with our findings, *in situ* hybridization showed low levels of MMP-2 gene transcripts in some mesenchymal cells of portal tracts, central veins and sinusoids of normal human liver tissue^[25]. Although the level of MMP-2 mRNA was increased in fibrotic liver tissue^[26], there were no significant differences between fibrotic and normal liver tissue ($P > 0.05$). We inferred that the increased MMP-2 mRNA was mainly from activated HSC and exerted effect at local area. This suggested that the expression of MMP-2 by HSC might be important in liver fibrogenesis^[24,26].

Liver fibrosis is associated with excessive accumulation of extracellular matrix, particular collagen type I and III^[27-29]. Collagen type I may comprise over 70% of extracellular matrix as compared to 40%-50% in normal liver. Besides an increase of collagen synthesis, decrease of fibrolysis may be an important factor responsible for the preferential accumulation of interstitial collagens^[30]. MMP-1 has a substrate specificity for native type I and III collagens. Reduced MMP-1 activity may therefore contribute to the patterns of extracellular matrix constituents in fibrotic liver. TIMP-1 is a major inhibitor of MMP-1 by combining with the active enzymes^[31-34]. TIMP-1 expression was enhanced markedly both in liver tissue and HSC

during liver fibrogenesis. It indicated that the increase of TIMP-1 expression is a main cause possibly for the reduced collagen degradation^[35-40].

In conclusion, there were no detectable gene transcripts of MMP-2 in HSC isolated from normal rat liver, and it was expressed by HSC during liver fibrogenesis. This suggested that the expression of MMP-2 is determined by the state of activation of HSC. The marked increase of TIMP-1 expression may result in the prominent deposition of interstitial collagens by inhibition of matrix metalloproteinases (especially MMP-1). Both MMP-2 and TIMP-1 make contributions to the liver remodeling and the progression of liver fibrosis.

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CYP11B2 expression in HSCs and its effect on hepatic fibrogenesis

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Subject headings hepatic fibrogenesis; hepatic stellate cells; *In situ*; hybridization; renin-angiotensin system; aldosterone; polymerase chain reaction; liver cirrhosis/prevention and control

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INTRODUCTION

It has been reported that renin-angiotensin system exists in tissue^[1] and aldosterone can be synthesized in extra-adrenal tissue including heart, blood vessels^[2] and brain^[3]. Recent studies have brought rich evidences in favour of aldosterone as a strong stimulator of fibrogenesis and mitogenesis^[4-9]. Recently Wu PS reported that aldosterone synthase gene -CYP11B2 can be expressed in hepatic stellate cells (HSCs) of liver^[10]. As the activation of HSCs is the central event in fibrogenesis of liver^[11-26], we undertook the present study to investigate the relationship between hepatic fibrogenesis and locally produced aldosterone in liver.

MATERIALS AND METHODS

The establishment of animal model

Male Wistar rats (250g-280g, $n=48$, purchased from Animal Center of the First Military Medical University) were divided into 2 groups randomly. Model group ($n=24$): the rats were injected with 40% CCl₄ (the mixture of CCl₄ and olive oil) 0.25mL/100g subcutaneously three times a week. Control group ($n=24$): the rats were injected with olive oil only.

Histology

At the end of the week 4, 6, 8, and 10, 6 rats in

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each group were sacrificed. The rat liver was regularly fixed, embedded, sliced and stained with VG and HE. Cryosections of liver tissue were rinsed in 0.2g/L gold chloride solution for 6h at room temperature, then the sections were placed in 50g/L sodium thiosulphate for 5min and mounted with resin.

Extraction of total RNA

The tissue of liver was promptly frozen in liquid nitrogen and stored at -70°C prior to use. Total RNA was extracted with GTC solution (6M guanidinium thiocyanate, 5mM sodium citrate, 5g/L sodium sarcosyl, 0.1M beta. mercaptoethanol). The amount of total RNA was measured at 260nm using an ultraviolet spectrophotometer.

RT-PCR for CYP11B2 mRNA

One microgram of total RNA was incubated at 25°C for 30min in 20μL reverse-transcriptase buffer containing 20u AMV reverse transcriptase and 2μL random primer p(dN)6(Boehringer Mannheim, Germany). Reverse transcription was terminated by heating at 99°C for 5min. The polymerase chain reactions were performed in 50μL PCR buffer containing 0.32μg of each primer and 2.5 u Taq DNA polymerase (Promega). Samples were subjected to 30 cycles of PCR amplification. Each cycle includes denaturation at 94°C for 1min, annealing at 56°C for 1min, and primer extension at 72°C for 2min. The rat CYP11B2 PCR primers were 5'-ACCATGGATGTCCAGCAA-3' and 5'-GAGAGCTGCCG AGTCTGA-3', synthesized by Shanghai Cell Institute according to the published sequences of Oaks and Raff^[27], corresponding to positions 657-954 of the gene that does not cross-react with the CYP11B1 gene. As control, β-actin primers were used and had the following sequences: 5'-TTTCTGGCAAGTTAGGTTTTGTCAA-3' and 5'-CCTAGCACCATGAAGATCAA-3'^[28]. Each 6μL amplification mixture was subjected to electrophoresis on 15g/L agarose gel, and DNA was visualized by ethidium bromide staining. The signal intensity was quantified by a computerized medical image-processing system (GDS-7500, UVP, England). Six separate gels were run for the enzyme, and the data were then averaged. The ratio of CYP11B2 to β-actin was used to express relative mRNA levels.

In situ hybridization

The Wistar rats (model group, 6th week, $n=6$; control group, $n=6$) were anesthetized by intraperitoneal injection of pentobarbital, and perfused with 9g/L NaCl and 40g/L paraformaldehyde solution buffer at pH 7.4 in 0.1M phosphate buffered saline (PBS). Each specimen was divided into halves, immersed in the same fixative solution for 4h at 4°C. After the tissue samples were rinsed in PBS containing 300g/L sucrose, cryosections 10µm thick were cut and mounted on polylysine-coated slides. *In situ* hybridization^[29], slides were washed in 0.1M PBS, treated with 3g/L triton X-100 for 20min, washed in 0.1M glycine for 5min; and incubated in 2µg/mL proteinase K at 37°C for 30min, fixed in buffered 40g/L paraformaldehyde at RT for 10min, washed in PBS for 20 min; immersed in 2.5g/L acetic anhydride in 0.1M triethanolamine solution at RT for 10min, washed in 2×SSC for 15min; incubated in a prehybridization mixture at RT for 8h. After blotting the solution, the slides were incubated in a hybridization solution consisting of 500g/L formamide, 5×SSC, 20g/L SDS, 1×Denhardt's solution, 100mg/L salmon sperm DNA, 100g/L dextran sulfate, 2mg/L Digoxigenin labeling probe (Boehringer Mannheim, Germany) at 42°C for 24h. After hybridization, the slides were washed in 4×SSC, 2×SSC, 1×SSC, 0.5×SSC and 0.05M PBS at 37°C for 20min respectively. The detection procedure was performed according to the maneuver of DIG DNA labeling and detection kit (Boehringer Mannheim, Germany). The slide exposure duration was 12 hours at 4°C. The slides were dehydrated in ethanol and mounted with resin. A negative control was prepared for each sample using a hybridization solution without CYP11B2 probe. We selected 7 high power fields randomly and counted the positive cells per high power field.

Statistics

Analysis of data was performed with one way ANOVA (SPSS 7.5) and rank sum test. Results were expressed as mean ± SD, a value of $P<0.05$ was regarded as statistical significance.

RESULTS

Morphological changes

Observed with naked eyes, at the end of week 4, the liver surface of model group presented tiny particle-like changes. After that, the tissue became hard and shrank progressively.

Microscopically, at the end of week 4, fibroblasts proliferated obviously in the portal tracts of model group, and collagen invaded into the

hepatic lobules along with the injured limiting laminae, but the collagen was not completely connected with each other. At the end of week 6, most rats developed cirrhosis in model group.

RT-PCR

The expressions of CYP11B2 mRNA in model group were significantly up-regulated compared to those in control group ($P<0.01$). The levels of CYP11B2 mRNA in model group (week 8) were higher than those in model group (week 4) ($P<0.05$), without significant difference between the two groups ($P>0.05$) (Figures 1,2).

In situ hybridization

Duck purple precipitate was localized in the endoplasm of HSCs corresponding to the gold chloride stained section. The number of positive cells per high power field in model group (4.5) was higher than that in control group (0.55) ($P<0.05$).

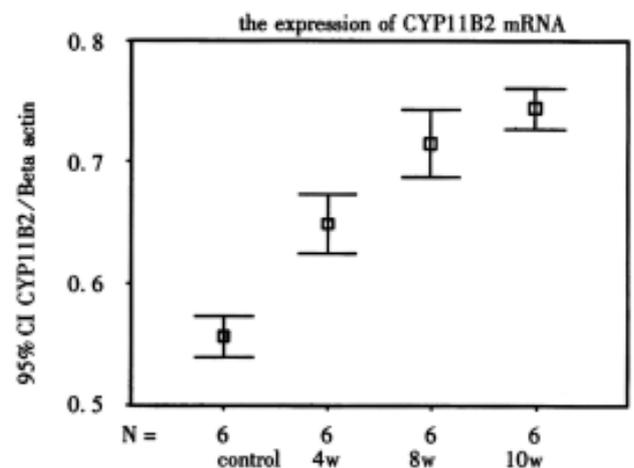


Figure 1 (PDF) The ratio of CYP11B2/ β -action presents the relative level of CYP11B2.

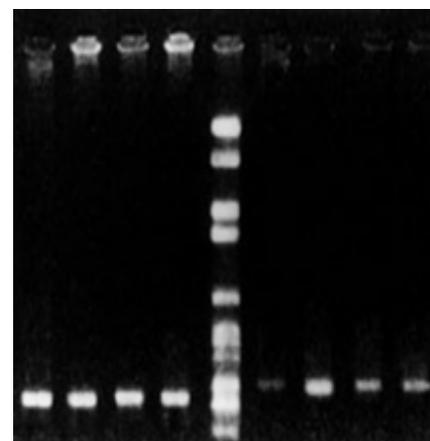


Figure 2 Lanes 1: model group (week 4) β -actin; Lanes 2: model group (week 4) CYP11B2; Lanes 3: model group (week 8) β -actin; Lanes 4: model group (week 8) CYP11B2; Lanes 5: model group (week 10) CYP11B2; Lanes 6: control group β -actin; Lanes 7: control group CYP11B2; Lanes 8: Makers; Lanes 9: Makers

DISCUSSION

It has been reported that renin-angiotensin system exists in tissues. Aldosterone can be synthesized in extra-adrenal tissue including blood vessels and brain. Aldosterone is a strong stimulator of fibrogenesis and mitogenesis which exerts a marked effect on stimulating the proliferation of myofibroblasts and collagen production.

There are two genes encoding the enzymes of aldosterone biosynthesis^[30,31]. One is P450 11 β (CYP11B1) that is responsible for the early steps of aldosterone biosynthesis. The other is P450 Aldo (CYP11B2) that encodes the key enzyme for the final steps of its biosynthesis.

HSCs are mesenchymal cells located in the space of Disse. They demonstrated synthetic activity of collagen and other extracellular matrix proteins involved in hepatic fibrosis. As a source of cytokines, prostaglandins and other bioactive substances, they play a crucial role in the mechanisms of liver injury, regeneration and fibrosis. Now, fat-storing cell function is expanding from a retinol (fat)-storing site to a center of extracellular matrix metabolism and mediator production in the liver. Transition of the stellate cells from the vitamin A-storing phenotype to "activated" or "myofibroblastic" cells is the central pathobiochemical event for liver fibrosis^[32-36].

Using RT-PCR, we clearly provided direct evidence for the expression of CYP11B2 mRNA in liver. The expression was up-regulated when fibrogenesis occurred. *In situ* hybridization further revealed that the expression of CYP11B2 mRNA located in the endoplasm of HSCs increased in fibrotic liver.

In conclusion, based on present study, we first proposed that there is a positive acting relationship between the expression of CYP11B2 in HSCs and fibrogenesis of liver. Locally produced aldosterone in liver is likely to promote the process of fibrogenesis. The present study provides a new way to investigate the mechanism and treatment of hepatic fibrosis.

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Follow-up study of hepatitis C virus infection in uremic patients on maintenance hemodialysis for 30 months

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Subject headings hepatitis C virus; hemodialysis; blood transfusion; cross infection; polymerase chain reaction; risk factors; follow-up studies

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INTRODUCTION

A high prevalence of antibodies to hepatitis C virus (HCV) (range from 3.3% - 80%) has been reported in hemodialysis (HD) patients^[1-20], and worrisome as it often becomes chronic and induces chronic liver disease^[7,14,15], therefore the nephrologists face a major challenge of how to prevent it. The main route of HCV transmission is parenteral, and most cases of HCV infection are thought to be related to blood transfusion, intravenous drug addiction, or HIV infection^[1,6,9-10,21-24], but an increased prevalence of anti-HCV has also been reported in non-transfused HD patients without identifiable risk factors^[1,2,5,6,10-12,15,18,25,26]. In fact, some recent reports and our report outline that HCV-negative patients who shared dialysis machines with HCV-positive patients were seroconverted within HD period^[1,5,8,12-16,25-35]. We undertook a prospective study to assess the incidence and the risk factors for HCV infection in HD patients.

MATERIALS AND METHODS

Subjects

A total of 346 patients at our HD center followed up from June 1, 1995 to December 1, 1997, and 63 patients in June 1995 were included in the study.

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They were dialyzed for 1 to 92 (mean 23.4) months. Their age ranged from 20-76 years (mean 44.7 years).

From June 1995 to November 1995, December 1995 to May 1996, June 1996 to November 1996, December 1996 to May 1997, June 1997 to November 1997, additional 42, 39, 45, 65 and 71 new patients were included, respectively. Of these 262 patients, 226 had recently started HD, 15 resumed HD after a transplantation failure and 21 had been transferred from other units. Their age range was similar to that of previously included patients. In December 1997, 21 patients who recently started HD, were included, their age range was similar to that of previously included patients.

In June 1996, the sera of 62 patients were tested for anti-HCVIgM, anti-HCVIgG and HCV RNA. Among them, 38 were males and 24 females, their age ranged from 16-71 years (mean 44.6 years), mean duration of HD was 16.1±7.6 months (1-96).

Anti-HCV antibodies

All patients at our HD center were tested every 3 months for the presence of anti-HCV antibodies during the follow-up period. All blood samples were obtained before HD, and were centrifuged, serum stored at -80°C until testing. The kits for examining anti-HCVIgM and anti-HCVIgG were purchased from Shanghai Chang Zhang Chemical Reagent Company, and were tested by the Laboratory of Microbiology, The Second Military Medical University.

HCV RNA

Primers were designed from the conserved 5'-noncoding (5'-NC) region of the HCV genome. S1: 5'-CACTCCACCATGAATCA CTC-3', AS1: GGTG-CACGGTCTACGAGACC-3', S2: 5'-ACTCCCCTG-TGAGAAGCTAC-3', AS2: 5'-CTCGCAAGCACCC-TATCAGG -3'. A RT-PCR assay procedure was performed as previously described^[1].

Risk factors for seroconversion (SC) for HCV

All data of risk factors for HCV were collected by using special questionnaire, including the sex, age, renal function, HBV marker, EPO, infection at hospital, history of CAPD and kidney transplantation, duration of dialysis, history of transfusion, ALT abnormality, dialyzer reused.

Dialysis and disinfection procedure

HD was performed using dialysis machines from Baxter, Fresenius. We used bicarbonate dialysis fluid (flow 500mL/min). Water quality and bacterial count were monitored regularly. HD patients have been dialyzed with Cuprophan, polysulfone or hemophan dialyzer. A strict chemical disinfection using Citrosteril, run at 85°C for 35min after each dialysis session, was adopted before using the machine for the next patient. It was hoped that both the high temperature bath and the low pH offered by Citrosteril would be enough to combat HCV. But our patients shared machines indiscriminately.

Statistical analysis

Results are expressed as mean±SEM as indicated. Unpaired Student's *t* test, χ^2 and Fisher's exact test were used. *P* values <0.05 were considered as significant.

RESULTS

Prevalence of HCV marker in June 1996

In June 1996, the total positivity of HCV marker (HCVM) was 59.7% (37/62), 27 patients (43.6%) were anti-HCVIgM positive, 29 (46.8%) anti-HCVIgG positive, 34 (54.8%) HCV RNA positive, 22 (35.5%) anti-HCVIgM, anti-HCVIgG and HCV RNA positive, 27 were anti-HCVIgM and HCV RNA positive, 22 were anti-HCVIgM and anti-HCVIgG positive, 26 anti-HCVIgG and HCV RNA positive, 34 patients at least one of anti-HCVIgM and anti-HCVIgG positive, 3 each were anti-HCVIgG positive or HCV RNA positive only.

Sixty-two patients were divided into two groups, GI: 43 HD patients with histories of transfusion, ALT abnormality, kidney transplantation and HBV markers positive; GII: 19 HD patients without the history and abnormality as those of GI. Comparison of detection of HCVM in GI and GII is shown in Table 1.

When comparing the clinical manifestation between HCVM positive and negative groups, no significant differences were found with respect to the sex, age, renal function, HBV marker, EPO and history of CAPD; but there were significant differences with regard to the duration of dialysis, history of transfusion, ALT abnormality and kidney transplantation (Table 2).

Relationship between dialysis times and HCV markers is shown in Table 3. The risk of HCV marker positivity increased significantly as the duration on HD increased ($\chi^2=9.23$, *P*<0.01). Seven patients were dialyzed for more than 3 years (mean 5 years) and 100% HCVM positive.

Relationship between number of transfusion and HCV markers is shown in Table 4. The risk of HCV marker positivity increased significantly as the number of transfusion increased ($\chi^2=21.14$, *P*<0.01). There were 8 patients with positive HCV

markers in 22 patients without histories of transfusion. Among them, 6 (27.3%) were anti-HCVIgM positive, 7 anti-HCVIgG positive, and 6 HCV RNA positive. When comparing the clinical manifestation between HCVM positive and HCVM negative groups in 22 patients without transfusion, no significant differences were found with respect to the sex, age, renal function, HBV marker, EPO and history of CAPD and kidney transplantation; but there were significant differences in the duration of dialysis and ALT abnormality (Table 5).

Positive prevalence of anti-HCV antibodies in consecutive six-month period

Positive prevalence of anti-HCV antibodies was found in six consecutive period of 6 months as shown in Table 6.

Positive prevalence of anti-HCV antibodies was found in uremic patients when they entered into our HD center as shown in Table 7. Among the 283 new patients admitted to our HD center, 11 (3.9%) were positive for anti-HCV. Of the 11 patients, 6 had histories of kidney transplantation, and 3 were transferred from other units, they all had histories of transfusion, only 2 patients with positive anti-HCV antibody started HD recently.

Adequacy of serologic follow-up

Out of 346 patients followed up from June 1995 to December 1997, 48 did not complete the study as a result of death (*n*=22), kidney transplantation (*n*=24), transfer to other dialysis units (*n*=1), and transfer to peritoneal dialysis (*n*=1).

Incidence of SC for HCV

During the follow-up period (1-30 months), 80 patients had seroconversion (SC) for anti-HCV positive; 298, 167, 87, 48 and 11 were followed up for 6, 12, 18, 24, and 30 months; and their positive seroconversion rates were 6.4%, 11.9%, 20.7%, 35.4% and 54.5%, respectively. Of the 80 seroconverted patients, 57 patients had histories of transfusion, mean number of transfusion being 12.5U±6.2U.

ALT level in seroconverted patients

ALT determinations obtained every one month from the onset of HD were reviewed in 23 (28.8%) patients with SC. SC was preceded (1 to 6 months) by an unexplained, sustained (5 cases) or interrupted (18 cases) elevation of ALT level. This rise was not accounted for by hepatitis B virus infection or hepatotoxic drugs, and was noted for the first time since the initiation of HD. During the follow-up period, 4 patients had liver cirrhosis, 8, 3, 4 and 5 months after HD, respectively, 1 died after SC for 10 months, others remained in our HD center.

Serologic follow-up of seroconverted patients

All 80 seroconverted patients remained positive throughout the follow-up.

Table 1 Comparison of detection of HCVM in GI and GII

Parameter	GI(%) (n=43)	GII(%) (n=19)	P value
anti-HCVIgM (+)	21 (48.8)	6 (31.6)	>0.05
anti-HCVIgG (+)	25 (58.1)	4 (21.1)	<0.05
HCV RNA (+)	27 (62.8)	7 (36.8)	>0.05
All three (+)	18 (41.9)	4 (21.1)	>0.05
At least one (+)	29 (67.4)	8 (42.2)	>0.05

Table 2 Comparison of clinical manifestation in HCV positive and negative group of HD patients

Clinical manifestation	HCV positive group (n=37)	HCV negative group (n=25)	P value
M/F	22/15	16/9	>0.05
Mean age (years)	49.6±16.8	48.4±14.4	>0.05
Range	16-71	26-67	
Mean duration of HD (months)	31.2±8.6	8.1±4.7	<0.01
Range	2-96	1-24	
History of transfusion	29 (78.4%)	11 (44.0%)	<0.01
EPO	12 (32.4%)	8 (32.0%)	>0.05
History of kidney transplantation(n=6)	6 (16.2%)	0 (0.0%)	<0.05
History of CAPD (n=8)	6 (16.2%)	2 (8.0%)	>0.05
HBVM positive	18 (48.6%)	10 (40.0%)	<0.01
ALT abnormality	10 (27.0%)	1 (4.0%)	<0.01
BUN (mmol/L)	24.6±8.6	28.4±10.2	>0.05
Cr (μmol/L)	1154.4±402.6	1164.8±468.5	>0.05

Table 3 Relationship between dialysis times and HCV markers

HD time (Year)	n	IgM		IgG		HCV RNA		Only one marker positive	
		n	%	n	%	n	%	n	%
<1	32	9	28.1	8	25.0	12	37.5	14	43.8
1-2	18	9	50.0	10	55.6	11	61.1	12	66.7
2-3	5	3	60.0	4	80.0	4	80.0	4	80.0
>3	7	6	85.7	7	100.0	7	100.0	7	100.0

Table 4 Relationship between number of transfusion and HCV markers

Transfusion (U)	n	IgM		IgG		HCV RNA		Only one marker positive	
		n	%	n	%	n	%	n	%
0	22	6	27.3	7	31.8	6	27.3	8	36.4
1-5	8	2	25.0	2	25.0	4	50.0	4	50.0
6-10	16	7	43.8	6	37.5	10	62.5	11	68.8
11-20	6	4	66.7	5	83.3	5	83.3	5	83.3
>30	10	8	80.0	9	90.0	9	90.0	9	90.0

Table 5 Comparison the clinical manifestation between HCVM positive and negative group in 22 patients without transfusion

Clinical manifestation	Positive group	Negative group	P value
Cases	8	14	
M/F	5/3	8/6	>0.05
Mean age (Years)	53.1±14.2	49.6±12.5	>0.05
Range	30-71	30-67	
Mean duration of HD (months)	24.2±6.4	3.4±1.6	<0.01
Range	4-36	1-6	
History of kidney transplantation	0	0	
History of CAPD	0	0	
EPO	3	5	>0.05
ALT abnormality	3	0	<0.01
BUN (mmol/L)	25.7±7.8	26.4±6.8	>0.05
Cr (μmol/L)	1132.6±482.6	1182.4±464.3	>0.05

Table 6 Positive prevalence of anti-HCV antibodies in six consecutive periods of 6 months

Time	Cases	Positive (%)	Negative (%)
1995.6	63	37 (58.7)	26 (41.3)
1995.12	67	36 (53.7)	31 (46.3)
1996.6	62	34 (54.8)	28 (45.2)
1996.12	64	32 (50.0)	32 (50.0)
1997.6	76	40 (52.6)	36 (47.4)
1997.12	83	44 (53.0)	39 (47.0)

Table 7 Positive prevalence of anti-HCV antibodies in uremic patients when they were admitted to our HD center

Time	New patient	Positive (%)	Negative (%)
1995.6-1995.11	42	2 (4.8)	40 (95.2)
1995.12-1996.5	39	1 (2.6)	38 (97.4)
1996.6-1996.11	45	3 (6.7)	42 (93.3)
1996.12-1997.5	65	3 (4.6)	62 (95.4)
1997.6-1997.11	71	2 (2.8)	20 (97.2)
1997.12	21	0 (0.0)	21 (100.0)
Total	283	11 (3.9)	273 (96.1)

DISCUSSION

Non-A, non-B hepatitis is a major worldwide health problem. It accounts for more than 90% of transfusion associated hepatitis cases^[21], and was associated with a high incidence of chronic carrier state and subsequently progressive liver disease^[7,21]. In 1989, HCV was isolated from most cases of blood-borne non-A, non-B hepatitis by Choo *et al*^[36], the HCV was considered as the major cause of such disease, and HCV has evoked great interest, a plethora of reports have appeared in the literature for HD patients. Different prevalence rates of anti-HCV have been reported from different countries and the reported rates varied from as low as 3.3% in Newzland^[14], 39% in South America^[6], 44%-60% in the Far-Eastern countries^[29] to as high as 80.0% in Egypt^[16]. By contrast, the country-wide anti-HCV prevalence among volunteer blood donors is 0.86%^[31]. Our results showed that the positivity of anti-HCVIgM was 43.6% (27/62), anti-HCVIgG 46.8% (29/62) and HCV RNA 54.8% (34/62), the total positivity was 59.7% (37/62). By excluding the HD patients with histories of transfusion, ALT abnormality, history of kidney transplantation and positive HBV markers, the positivity of HCVM was 42.2% (8/19). So HCV infection in our HD center is a very serious problem.

Our findings that 3 HD patients had detectable serum HCV RNA despite their anti-HCV negativity confirms previous observations, although figures vary considerably, ranging from 1% to 15%^[3,4,37]. Fernandez *et al*^[37] reported that 53 anti-HCV negative blood donors were examined for HCV RNA, and within the study group 4 patients (12.9%) were HCV RNA positive. The existence of HCV viremia without specific antibody expression in HD patients differs significantly when compared with blood donors who represent a control population. One explanation to account for the difference is that HCV RNA positivity precedes anti-HCV SC in an acute infection^[3]. Another possible explanation is that the immunosuppression

that characterizes HD patients may be responsible for the inability to express detectable amount of serum anti-HCV in the same way as is observed in kidney and liver transplantation recipients^[19,38]. Therefore, detection of HCV RNA can improve positivity of HCV infection, as the compensation of inadequacy of anti-HCV.

Prospective studies of HCV infection in HD patients were fewer and the conclusions were not consistent^[8,22, 28,29,32-34,39]. Dentico *et al*^[11] using a less sensitive ELISA I test reported in 115 Italian HD patients, a yearly incidence of SC for HCV falling from 6.1% to 2.2% between 1984 and 1990. Two small prospective studies relying on the ELISA I test in 35 and 62 HD patients followed for 12 and 18 months, reported a yearly incidence of 11.4%^[23] and 6%^[40], respectively. Chan *et al*^[4] using ELISA II test prospectively reported a yearly incidence of 4.9% in 39 HD patients followed for 19 months. Jadoul *et al*^[28] reported prospectively the SC for HCV in 401 HD patients, the SC rate averages 1.7% per year. More recently, in a prospective study relying on the ELISA II test in 187 HD patients followed for 36mo, a yearly incidence of SC for HCV was 22.6%^[29]. Our study defined prospectively the SC using the sensitive ELISA II in a large series of 346 HD patients. During the 1-30 months follow-up period, a total of 80 patients had SC for anti-HCV positive; 298, 167, 87, 48 and 11 patients were followed up for 6, 12, 18, 24, and 30 months; their positive SC was 6.4%, 11.9%, 20.7%, 35.4% and 54.5%, respectively.

Our study confirms that blood transfusion is an important risk factor for the transmission of HCV in HD patients. We have demonstrated a positive correlation between the two which increased with the increase in the number of transfusion units. Similar results have been reported by many other investigators^[1,2,8,9,12,15,19,21-23,31]. In addition, the introduction of screening of blood products for anti-HCV has led to a decline in the incidence of post-transfusion hepatitis^[24].

However, factors other than transfusion contribute to the transfection of HCV as demonstrated by the absence of blood transfusion in 23 of the 80 patients with SC. Interestingly, other studies have detected HCV antibodies in up to 19% -39% of HD patients who never received blood transfusion^[9,25,41]. The span of dialysis was significantly longer in anti-HCV positive patients than in anti-HCV negative patients^[1,5,12,19,25,31]. The risk of acquiring HCV infection on HD has been estimated to be 10% per year^[29]. These facts raised the possibility of nosocomial transmission. This hypothesis is supported by our observations that patients dialyzed in a bed adjacent to that of an anti-HCV positive patients had significantly higher risk of SC than the others in our unit^[1,8,12,15,23,31]. But until recently, the route of nosocomial transmission is not very clear. Transmission of

infection to dialysis staff by needle-stick injury^[1,25,26], breakdown in standard infection control practices^[1,23,26,28,42,43], physical proximity to an infected patient^[1,8,25,28], through dialysis machines^[1,25, 26], dialyzer membranes^[44], hemodialysis ultrafiltrate^[13,44] and reprocessing of dialyzers^[14,19] implicated a variety of potential modes of HCV transmission.

Several studies advocated the segregation of HCV (+) from HCV (-) patients to prevent nosocomial transmission^[9,22,25,29]. Implementation of a rigorous separation between patients according to their HCV status is rather cumbersome. Also in treating HBV positive patients, this would imply up to four separate facilities (B+C+, B+C-, B-C+ and B-C-). However, there are strong arguments against a policy of isolating anti-HCV positive patients because: HCV is not as infective as HBV, circulates in low titers in infected serum and is rapidly degraded at room temperature^[45]; and Currently licenced anti-HCV test detects non-neutralizing antibodies, does not distinguish between current and post infection, and a negative test does not exclude HCV infection^[42]; although isolation may protect uninfected patients, it might also increase the risk of superinfection in patients originally infected with a single strain^[46]. Infection with two or more different HCV genotypes has been observed in HD patients and 13% of patients referred for renal transplantation^[47]. Grouping of anti-HCV positive patients in dialysis units might thus increase their risk acquiring multiple HCV strains.

In view of the above debate, the centers for Disease Control and Prevention in the U.S. (CDC) recommend dedicated machines, patient isolation or a ban on reuse in HD patients with HCV infection^[48,49]. Such measures are detailed in the universal precautions for prevention of transmission of blood borne pathogens in health care settings^[47,49] and recommended precautions for patients undergoing hemodialysis who have AIDS or non-A non-B hepatitis delineated by the CDC^[48]. They include cleaning and disinfection of instruments, machines and environmental surfaces that are routinely touched, avoidance of sharing of articles between patients, frequent handwashing and use of glove.

In conclusion, the status of HCV infection in HD patients was a very serious problem, contamination appears to be both transfusion and nosocomial. Strict adherence to the 'CDC guidelines' and isolation of HCV positive patients during dialysis sessions are recommended. Further long-term studies are needed to confirm these conclusions.

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Supra-angular biopsy is more reliable for atrophy recognition: analysis of 1598 cases for gastric mucosal histological examination

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Subject headings *Helicobacter pylori*; gastric mucosa/pathology; biopsy; gastroscopy; gastritis, atrophic/pathology; metaplasia

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INTRODUCTION

Chronic gastritis might be a disease of the highest morbidity in the world. Since Warren and Marshall successfully isolated and cultured *Helicobacter pylori* (*H. pylori*) from a gastric antrum biopsy^[1], intensive researches produced a historic change in the etiology and treatment of gastroduodenal diseases^[2-14]. Stimulated by this momentous discovery, a group of gastroenterologists mainly from Europe and pathologists presented a novel classification of gastritis (so-called the Sydney system) at the 9th World Congress of Gastroenterology in Sydney, Australia, in 1990^[15]. In the Sydney system, attempts were made to incorporate etiologic, topographic, and morphologic criteria into a clinically relevant scheme. It usually involves the histopathological analysis of the biopsy specimens obtained from the arbitrary sites in the antrum or corpus. In September 1994, a group of gastric pathologists from various parts of the world gathered in Houston, Texas, USA, to reprove the Sydney system 4 years after its introduction^[16]. One of the most controversial issues at the Houston Workshop was the concept of atrophy. Since the relationship of *H. pylori* with gastric adenocarcinoma rests on the natural history of atrophical gastritis induced by the bacterial infection^[17-23], it is very important to identify the histological lesions. According to the Sydney system, *H. pylori*, chronic and active inflammations were usually recognized and scored

with an agreement of degree of accuracy, but the judgments of the atrophy were often poor^[24-31]. Although many factors are involved in the failure of responsible detection of the atrophy, the biopsy sites in gastric mucosa may be one of most important factors for this lack of concordance. In this study, we collected biopsy specimens from the antrum, corpus and angularis simultaneously to compare the differences among the biopsy sites for the evaluation of mucosal atrophic inflammation.

MATERIAL AND METHODS

Patients

A total of 1598 cases underwent endoscopic and histological examinations. Among them, 1047 cases were male and 551 females (a male:female ratio of 1.9:1) with an average age of 53.2 years (ranged 11 to 94 years). All cases were diagnosed by endoscopy, which consisted of 76 normal subjects, 85 chronic superficial gastritis, 116 atrophic gastritis, 297 erosive gastritis (173 flat-erosive type and 124 elevated erosive type), 467 gastric ulcer, 175 duodenal ulcer, 77 gastroduodenal ulcer, 194 hyperplastic polyp, 23 adenoma, 74 carcinoma, and 14 submucosal tumors. The atrophic change in gastric mucosa by endoscopy was evaluated and scored as “-, -/+, +, ++, +++” by observing the location of the atrophic border in gastric supra-angulus on the mucosal changes, such as fine transparent capillaries in the pale colored and rather thin mucosa. Biopsies were obtained from the three fixed sites (3-points biopsy): the greater curvature of the lower antrum, the greater curvature of the corpus and the supra-angulus. All biopsies were taken from an area of intact mucosa at a distance from any focal lesion, such as an ulcer or erosion.

Assessment of *H. pylori* infection and mucosal inflammation

Biopsy specimens for histological examination were fixed in 10% formalin and processed routinely to paraffin and 3µm sections. *H. pylori* were identified as curved, rod or coccoid by toluidine blue and immunostaining according to our previous report^[32-34]. The biopsy sections were stained with haematoxylin-eosin. The histological chronic inflammation and activity were assessed and scored according to the Sydney system. Lymphocytes and plasmocytes were responded for chronic

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inflammation and PMN for activity. It was scored based on the density of inflammatory cells in both lamina proprias and glandular epithelium. The histological atrophy was identified when the gastric glands were correspondingly shortened and widely separated^[15]. In order to avoid the variation, only the cases with muscularia mucosa were judged for histological atrophy, in which, the lower layer of glands almost touch the muscularia mucosa in normal mucosa.

Statistical analysis

The data were analyzed by the Student's *t* test and the *Chi*-square test. *P* values less than 0.05 were considered significant.

RESULTS

The infective rate of *Hp* evaluated by biopsy in different sites

By means of toluidine blue and *H. pylori* antibody staining, *H. pylori* was easily identified in the lower third of the superficial mucous layer and in the gastric pits. The prevalence of *H. pylori* evaluated by different biopsy specimens was not obviously different (Table 1). The positive cases of *H. pylori* infection were 983 (61.5%), 1196 (74.8%) and 994(62.2%), respectively in antrum, corpus and angulus. No significant difference was found in the detective rates among the different site biopsies.

Table 1 Infective rate of *Hp* evaluated by different site biopsy

Cases	<i>Hp</i> infective rate (%)		
	Antrum	Corpus	Angulus
Normal	76 30(39.5)	35(46.1)	31(40.8)
Superficial gastritis	85 34(40.0)	42(49.0)	37(43.5)
Erosive gastritis	297 160(53.9)	164(55.2)	156(52.5)
Atrophic gastritis	116 90(77.6)	102(87.9)	79(68.1)
Gastric ulcer	467 314(67.2)	368(78.8)	326(69.8)
Gastric carcinoma	74 38(51.4)	43(58.1)	

Though the detective rate for *H. pylori* infection was slightly improved by combining the biopsy in the three sites of gastric mucosa (Table 2), the difference was not statistically remarkable among one point biopsy in the antrum and two-points in the antrum-corpus, or three points in the antrum-corpus-angulus ($P > 0.05$).

Table 2 Infective rate of *Hp* evaluated by combined biopsies

Cases	One point (Antrum)	Two points (Antr.corp.)	Three point (Antr.corp.angu)
Normal	76 39(51.3)	46(60.5)	47(61.8)
Superficial gastritis	85 34(40.0)	46(54.1)	48(56.5)
Erosive gastritis	297 160(57.9)	190(64.0)	195(65.7)
Atrophic gastritis	116 90(77.6)	107(92.2)	107(92.2)
Gastric ulcer	467 314(67.2)	399(85.4)	406(86.9)
Gastric carcinoma	74 38(51.4)	61(82.4)	64(86.5)

Mucosal inflammation and atrophy identified in the biopsy specimens from different sites

In the 1598 biopsy cases, the histological changes of gastric mucosa were evaluated by combining the results of observation in three different biopsy sites. It was found that there were 1413(88.4%) cases with mucosal chronic inflammation, in which, lymphocytes and plasmocytes were observed in both lamina proprias and glandular epithelium. PMN infiltration, which was responsible for the activity of chronic inflammation, was found in 1287 (80.5%) cases and intestinal metaplasia in 773 (48.8%) cases. A total of 1292 cases with muscularia mucosa met the standard for atrophy evaluation, histological atrophy was found in 489 (37.8%) cases.

By comparing the results of different sites biopsies, it was surprised to find that the mucosal inflammation and activity were in concordance evaluated among the antrum, corpus or angulus, but the detective rates for atrophy and intestinal metaplasia were remarkably higher in angulus. In the antrum biopsy specimen, 26.6% and 26.1% showed mucosal atrophy and intestinal metaplasia respectively, however, 65.4% and 31.8% were identified in angularis biopsy (Table 3) with significant difference ($P < 0.05$) compared with those in antrum and corpus.

Table 3 Histological lesions identified in different biopsy sites

	Lesion identified		
	Antrum	Corpus	Angulus
Inflammation	1268(72.9)	1290(74.1)	1249(71.8)
Activity	1039(59.7)	1148(65.9)	1071(61.6)
Atrophy*	130(26.6)	96(19.6)	320(65.4) ^a
Intest.metaplasia	455(26.1)	137(17.7)	554 (31.8) ^a

* only 1292 cases in all three points judgable included.

^a $P < 0.05$, vs the results of antrum and corpus.

The endoscopical atrophy and histological confirmation

Cases (1290) with muscularia mucosa examination were evaluated histologically and compared with the results of the judgment of endoscopy (Table 4). Among them, mucosal atrophy could be judged in 487 cases, 106 were negative and 697 cases were suspected. The biopsy specimens were further examined based on the correspondingly shortened and widely separated glands. Angular biopsy was found more available for the atrophical identification, in which, 48.3% endoscopical atrophy was confirmed, but in antrum or corpus biopsy, only 22.2% or 15.8% cases were verified. Better agreement of mucosal atrophy was reached in the cases with the 3+ score of endoscopical atrophy. Although the confirmation for endoscopical atrophy was slightly improved when the evaluation was made based on the different points biopsy, the difference was not statistically significant ($P < 0.05$).

Table 4 The accuracy of endoscopic atrophy

Endoscopy atrophy	Cases	Evaluation by separate point		
		Antrum	Corpus	Angulus
Negative	106	10(9.4)	9(8.5)	15(14.2)
Suspected	697	124(17.8)	47(6.7)	171(24.5)
Positive	487	108(22.2)	77(15.8)	235(48.3)
+	252	47(18.7)	36(14.3)	103(40.9)
++	201	46(22.9)	35(17.4)	107(53.2)
+++	36	15(41.7)	6(16.7)	27(69.2)

Mucosal atrophy identified in different diseases

Histological atrophy not only occurred in the atrophic gastritis, but also in different gastric lesions, even in normal subjects (Table 5). The confirmation of mucosal atrophy in different diseases also varied with the biopsy specimens from different sites. In angular biopsy, the histological atrophy was much easier to be identified than in antrum. By the evaluation from the angular specimens, the occurrence of mucosal atrophy ranked the highest in the atrophic gastritis (82.2%), then in the carcinoma and adenoma and gastric ulcer. Though the occurrence of atrophy was lower in the endoscopic normal mucosa, superficial and chronic gastritis, there were still about 13.6% to 35.1% cases with the change of histological atrophy.

Table 5 The occurrence of histological atrophy in different diseases

Endoscopic diagnosis	Cases	Histological atrophy (%)		
		Antrum	Corpus	Angulus
Normal	69	6(8.7)	5(7.2)	15(21.7)
Superficial gastritis	79	15(18.9)	8(10.1)	23(29.1)
Flat erosion	152	26(17.1)	5(3.3)	33(21.7)
Elevated erosion	119	29(24.4)	7(5.9)	39(32.7)
Atrophic gastritis	107	59(55.1)	35(32.7)	88(82.2)
Gastric ulcer	387	134(34.6)	39(10.1)	175(45.2)
Duodenal ulcer	135	36(26.7)	8(5.9)	47(34.8)
Gastroduodenal ulcer	64	21(32.8)	5(7.8)	28(43.8)
Hyperplastic polyp	111	14(12.6)	8(7.2)	33(29.7)
Adenoma	11	3(27.3)	3(27.3)	6(54.5)
Carcinoma	46	22(47.8)	6(13.4)	27(58.7)
Submucosal tumors	12	3(25.0)	2(16.7)	3(25.0)

DISCUSSION

Since Warren and Marshall successfully isolated and cultured *H. pylori* from gastric antrum biopsy, the intensive researches into *H. pylori* infection during the past decade have provided important insights into the pathophysiology of gastric diseases^[1-16]. It was suggested that over the past years there was a slow progression of chronic gastritis with atrophy and intestinal metaplasia developing, and that the proportion of the population affected increased with age, and was high in geographical areas with a high risk of cancer^[35-45]. It is now generally supposed that *H. pylori* is one of the important factors in the etiology of chronic gastritis. Recently, a positive

relationship between *H. pylori* and gastric cancer was reported simultaneously by several authors^[45,46]. In light of these observations, it is important to determine the prevalence of atrophic gastritis and intestinal metaplasia since both of these lesions are closely related to the gastric cancer. There have been many reports as to the distribution of *H. pylori* colonization and atrophic gastritis, but usually only involve in small group of cases, and most researchers have not taken into consideration the effect of biopsy from the different sites of gastric mucosa on the atrophic evaluation^[47-52]. Though the Sydney system, a novel classification of gastritis usually based on the biopsy from antrum or corpus for histological analysis, can incorporate etiologic, topographic and morphologic criteria, the agreement for atrophic assessment is often poor^[15, 16]. Since many factors are involved in the failure of detection of the atrophy, the biopsy sites in gastric mucosa may be one of the most important factors responsible for this lack of concordance. In this study, we collected biopsy specimens from the antrum, corpus and angulus simultaneously in 1598 cases to compare the differences among the biopsy sites for the evaluation of mucosal atrophic inflammation.

As to the distribution of *H. pylori* and inflammation in the stomach, Genta *et al* reported that *H. pylori* was distributed evenly throughout the stomach^[26,27]. In this study, we found that the prevalence of *H. pylori* infection evaluated by different biopsy specimens was not obviously different. The positive cases of *H. pylori* infection were 61.5%, 74.8%, and 62.2%, respectively in antrum, corpus and angulus. This finding corresponds closely to Genta's. Since no significant difference was found in the *H. pylori* detective rates among the different site biopsies, it was suggested that one of the biopsies from antrum, corpus or angulus was enough for the evaluation of bacterial infection.

It is interesting to find that mucosal inflammation and atrophy identified in the biopsy specimens from different sites were varied. The evaluation for mucosal inflammation and activity was in concordance among the antrum, corpus or angulus, but the detective rates for atrophy and intestinal metaplasia were remarkably higher in angularis. In the antrum biopsy specimen, only 26.6% and 26.1% showed mucosal atrophy and intestinal metaplasia respectively, however, 65.4% and 31.8% were identified in angularis biopsy.

The endoscopic atrophy or metaplasia was suggested by a group of researchers for the chronological spread of atrophic gastritis and the evaluation of the entire stomach^[36,40]. A border between the normal mucosa and that showing atrophic gastritis was recognized by endoscopy, and this was designated as the endoscopic atrophic border. In this study, 1292 cases with muscularia

mucosa identification were evaluated histologically based on the correspondingly shortened and widely separated glands for the atrophical identification. The histological atrophy was only observed in half of the cases of the endoscopical atrophy, and in the cases without endoscopical atrophy, 20.8% cases still showed histological atrophy, though better agreement of mucosal atrophy was reached in the cases with the 3+ score of endoscopical atrophy. If biopsy was only taken from the antrum or corpus, the concordance of endoscopical atrophy with the histological atrophy was poor.

It is believed for a long time that the atrophic gastritis extends from the antrum to the body with age. Satoh *et al* indicated that *H.pylori* infection was usually associated with antral atrophic gastritis and intestinal metaplasia^[36]. In contrast to this concept, we found by analysis of 1598 cases that both atrophy and intestinal metaplasia were much more commonly identified in angularis than in antrum, no matter of the different number of *H.pylori* colonization or the different diseases. Although it remains to be further confirmed whether the real histological origin of atrophy or intestinal metaplasia developed first from the angulus, we should take this fact into consideration in evaluation on biopsy. Histological atrophy not only occurred in the atrophic gastritis, but also in different gastric lesions, even in normal subjects. The confirmation of mucosal atrophy in different diseases also varied with the biopsy specimens from different sites. In angular biopsy, the histological atrophy was much easier to be identified than in antrum. By the evaluation of the angular specimens, the occurrence of mucosal atrophy ranked the highest in the atrophic gastritis (82.2%), then in the carcinoma and adenoma and gastric ulcer. Although the occurrence of atrophy was lower in the endoscopical normal mucosa, superficial and chronic gastritis, still about 13.6% -35.1% cases had the change of histological atrophy.

In conclusion, our results based on the analysis of 1598 cases of gastric mucosal histology indicate that antrum biopsy is suitable for *H.pylori* evaluation, but supra-angular biopsy is more reliable for atrophy and intestinal metaplasia observation.

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Early apoptosis in intestinal and diffuse gastric carcinomas

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Subject headings stomach neoplasms/pathology; apoptosis; flow cytometry; Bcl-2 protein; phosphatidylserines

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INTRODUCTION

Apoptosis, described by Kerr^[1] in 1972, plays a key role in all types of regulated cellular processes in multicellular organisms. It is defined as a morphologic change, including fragmentation of the DNA, cell shrinkage, dilation of the endoplasmic reticulum, cell fragmentation and formation of apoptotic bodies^[2,3]. One of the abilities of apoptotic cell is to trigger its own engulfment by phagocytic cells prior to cell lysis, which is crucial to the avoidance of the tissue damage and inflammation associated with necrosis^[4]. Apoptotic cells lose membrane phospholipid asymmetry and expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane, macrophages then phagocytose apoptotic cells after specific recognition of the exposed PS^[5]. Annexin V (AV), having a high affinity for PS in the presence of Ca²⁺ ions^[6] and inhibition to PS-dependent procoagulant reactions^[7], is proved to be a very useful general probe for early apoptosis-associated membrane change on live cells before the nuclear condensation events and fragmentation of the DNA^[8].

Gastric carcinoma is estimated to be one of the most frequent *Cancers* in the world. According to Lauren's classification^[9], gastric carcinoma can be divided into adenocarcinomas of diffuse and intestinal type, which differ in growth pattern, morphology, and some phenotypic markers. Recently, apoptotic index and apoptotic genes have been found different in both types^[10,11]. In this study, we investigated the quantitations of early

apoptosis in both intestinal and diffuse gastric carcinoma and their adjacent non-neoplastic tissues with regard to classification and differentiation, and whether the exposure of PS could be inhibited by Bcl-2 protein through various pathways.

MATERIALS AND METHODS

Patients

The 27 surgically resected specimens used for this study were obtained from consecutive patients with gastric Cancer in the Department of General Surgery, Affiliated Hospital of Xuzhou Medical College during the period from April to September 1999. The patients received neither chemotherapy nor radio therapy before undergoing gastrectomy. After tissue sections of 0.5cm³ were excised from the carcinomatous and adjacent non-neoplastic tissues, they were embedded in ice box, and brought back to FCM laboratory. In addition, formalin fixed and paraffin embedded tissue specimens were used for pathological diagnosis according to the Lanren's classification. All cases had adequate clinical information.

Preparation of single-cell suspension

The tissues (carcinomatous and adjacent non-neoplastic tissues) were thoroughly washed with an excess of ice-cold phosphate buffered saline (PBS), immersed in PBS and minced quickly to approximately 0.5mm³ pieces using a pair of sharp scissors. After two more washings with PBS to remove red blood cells, these pieces were dispersed into single cells through an nylon screen (300 holes/cm³) by the press of an glass rod.

Detection of early apoptosis

The cell samples were washed with ice-cold PBS after centrifugation at 500×g for 5 minutes at 4°C. Supernatant was discarded, and finally the cell pellets were resuspended in ice-cold, diluted bind buffer to 2×10⁶ cells/mL. Five μL AV FITC (Immunotech, Cat. No.2375) solution and 5μL propidium iodide (PI, Immunotech, Cat. No.2375) were added to 490μL of the prepared cell suspension. And then the mixture was mixed gently and kept on ice and incubated for 10min in the dark. Aliquots were directly aspirated into a FACSCalibur flow cytometer (USA BD Company) for analysis with simultaneous monitoring of green fluorescence for AV-FITC and red fluorescence for PI. The FCM software (Cell Quest) was used to

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calculate and analyze the AV+/PI- cells^[12].

Statistical analysis

To analyse the correlation among early apoptosis, necrosis, viable cells in gastric carcinomatous and adjacent non-neoplastic tissues, the Student's *t* test and the Pearson's correlation coefficient were performed with State statistical software.

RESULTS

Clinical and pathological information

The 27 patients with gastric carcinomas (16 male and 11 female), including 9 intestinal and 18 diffuse tumors, with a mean age at operation of 57.4±13.1 years (ranging from 31 to 88 years). There were 7 cases of early (restricted to mucosa and submucosa) and 20 cases of advanced gastric carcinomas, respectively. The mean tumor size was 4.4cm ± 2.0cm (ranging from 1.0cm - 8.0cm). Lymph node metastasis was found in 16 cases.

Detection of early apoptosis with AV and PI

Figure 1 shows typical flow cytometer histograms for gastric carcinoma and adjacent non-neoplastic tissues. AV was an able to bind to viable cells of normal or abnormal tissues as it can not penetrate the phospholipid bilayer and PS did not expose to the outer leaflet. In apoptotic cells, however, the bilayer lose its symmetry when PS was flipflopped from the inner to outer leaflet. Once the cells were dead, the integrity of the plasma membrane was lost and the penetration was enhanced promptly. The bivariate AV/PI analysis by cytogram showed that viable cells were negative for both AV and PI (LL), apoptotic cells were AV positive (LR), while dead cells were positive for both AV and PI (UR).

Early apoptosis in various histological types

By bivariate AV/PI analysis using flow cytometer, the percentages of early apoptosis of carcinomatous or non-plastic tissues were 16.7%±5.2% ($\bar{x}\pm s$) and 9.3% ± 3.9%, respectively. The apoptotic index of carcinoma was significantly higher than that of the adjacent non-neoplastic tissue, the difference being statistically significant (Table 1). However, the same result only appeared in carcinomatous tissues of diffuse and intestinal carcinomas, whereas no difference was found in adjacent non-neoplastic tissues (Figure 2).

Table 1 Percentages of three subpopulations in gastric carcinomas

	AV+/PI-	AV+/PI+	AV-/PI-
Normal	9.3±3.9	4.1±2.5	85.7±5.6
Vicious	16.7±5.2 ^a	6.1±2.7 ^b	76.7±8.1 ^b

^a*P*<0.001, ^b*P*<0.01 vs normal (adjacent non-plastic tissues).

Correlation between early apoptosis and necrosis

The early apoptosis did not demonstrate a dose correlation with the necrosis, i.e. the correlation coefficients between AV+/PI- and AV+/PI+ in tumors or normal tissues were 0.3650 and 0.3877 respectively. And the early apoptosis was not correlated with sex, age, tumor size, lymph nodes metastasis, whereas closely correlated with depth of invasion.

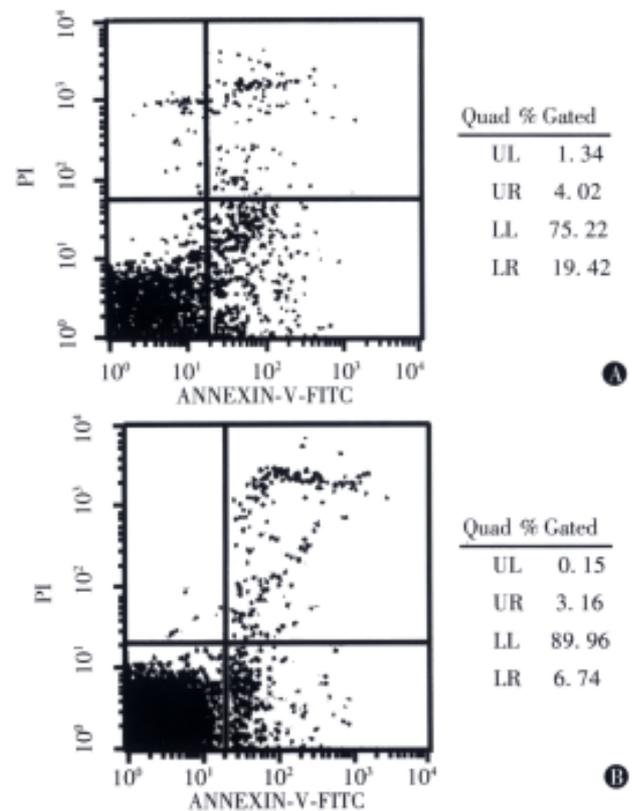


Figure 1 Bivariate AV/PI analysis of the gastric carcinoma (A) and adjacent non-neoplastic (B) tissues. The different labeling patterns in this assay identify the different cell subpopulations. i.e. region LL, viable cells (AV-/PI-), region LR, apoptotic cells (AV+/PI-), region UR, dead cells (AV+/PI+).

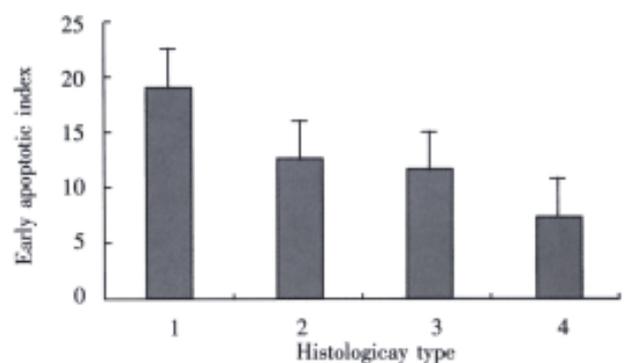


Figure 2 Early apoptosis in diffuse and intestinal gastric carcinomas. 1 and 2 were EAIs in carcinomatous tissues of diffuse and intestinal tumor, 19.0% ± 3.9% and 12.0% ± 4.3% (*P*=0.0002); 3 and 4 were in adjacent non-neoplastic tissues, 10.8% ± 3.3% and 7.3% ± 4.2% (*P*=0.0516).

DISCUSSION

In this report, we investigated the early apoptosis in diffuse and intestinal gastric carcinomatous and adjacent non-neoplastic tissues by AV/PI bivariate flow cytometry. Apoptosis, found in the normal tissues and in virtually all malignant tumors^[13], is defined morphologically as the double-strand cleavage leading to the formation of fragments that are detected usually by agarose gel electrophoresis (Ladder) or terminal deoxynucleotidyl transferase mediated dUTP-digoxigenin nick end labelling technique (TUNEL). The results, however, derived from the Ladder or TUNEL methods only presents the latter process of apoptosis and can not be analyzed quantitatively^[14-16].

With the development of the study on the role and basic mechanisms underlying apoptosis, the externalization of phosphatidylserine (PS), a kind of plasma membrane lipid, which results in the loss of phospholipid asymmetry, is a downstream event of early caspase activation^[8,17] and recognized as an early and ubiquitous phenomenon event in apoptosis^[5]. Based on the abilities of annexin V (AV) to bind to PS with high affinity and of propidium iodide (PI) to permeate the membrane losing integrity to stain DNA, AV and PI can be added simultaneously to the cell suspension to discriminate among viable, apoptotic and dead cells^[18]. Viable cells with its membrane's integrity and asymmetry were kept, and AV negative/PI negative were manifested (Figure 1, LL). After PS was exposed to the surface of membrane, apoptotic cells were presented with AV positive/PI negative (Figure 1, LR), whereas the necrotic cells with AV positive/PI positive (Figure 1, UR) were indicated when the membrane loses its integrity. In our study on fresh specimens, we found that the suspension from gastric carcinomatous and adjacent non-neoplastic tissues was identified easily to three subpopulations, among which the EAIs were different statistically. The percentages of apoptosis (AV positive/PI negative) were $16.7\% \pm 5.2\%$ and $9.3\% \pm 3.9\%$, respectively, being higher than those reported in literature^[19]. The reason might be that the experiments method are different and the sensitivity and specificity of AV/PI bivariate FCM are high, we therefore think that the results from our study can reflect objectively the spontaneous occurrence of apoptosis.

The externalization of phosphatidylserine(PS) plays a potential role in cellular kinetic significance. It has been shown that tumorigenic cells expressed relatively larger amounts than that of normal keratinocytes by semiquantitative analysis^[20] of PS in the outer leaflet of the cells. The same results were seen in our study. At the execution phase of apoptosis, PS was displayed at the outer membrane by flippases, scramblase and other proteinases. Possibly, with the participation of autologous cytophilic antibodies, components of the clotting

cascade, and special phospholipases, macrophages discriminate between "self" and altered "self" by recognizing, phagocytosing, and disposing of effete or tumorigenic cells. Furthermore, it was found in cells that the binding of PS and AV could suppress phagocytosis^[21]. That is, the expression of PS on the outer membrane leaflet of cells serves as a recognition moiety for macrophages.

There are various reports regarding the relationship between apoptotic indices and histological type in gastric carcinomas^[22,23], the significance of apoptosis in human gastric carcinomas suggested that the higher occurrence of apoptosis in well differentiated carcinomas reflects their slow-growing nature, and poorly differentiated carcinomas escape from this process. However, our results differed from the reports above. According to Lauren's classification, gastric carcinomas can be divided into adenocarcinomas of diffuse and intestinal type. One of the main difference between them is the degree of infiltration by macrocytes histologically, i.e. intestinal carcinomas grow with no or little infiltration whereas diffuse carcinomas do inversely. It might be that the heterogeneity of diffuse carcinomas is higher than that of intestinal carcinomas, the cells are prone to be initiated by multiple triggers of apoptosis, such as DNA damage, Fas ligand binding or withdrawal of growth factors^[24-27].

In recent studies, at the trigger and execution phase of apoptosis, the expression of bcl-2, bax^[28-30] was demonstrated to play important roles in cell life. Bcl-2 and Bax are members of the group proteins that regulate the apoptotic pathway, that is, Bcl-2 acts as an inhibitor of apoptosis, opposing Bax effects on cell life. Bcl-2 could protect cells from apoptosis in several ways, such as inhibition from transmembrane streaming of Ca^{2+} ion, blockage in releasing of cytochrome C, formation of heterodimers with Bax. In addition, Bcl-2 may regulate special PS-sensitive signal transduction pathway by protecting lipids from peroxidation^[31,32]. Some clinicopathological studies demonstrated that Bcl-2 appears to be preferentially associated with the intestinal type carcinomas and more prevalent in poorly differentiated ones^[33-35].

In conclusion, the early apoptosis has been investigated in gastric carcinomatous and adjacent non-neoplastic tissues by AV/PI bivariate flow cytometry. The early apoptosis is clearly related to tumor and its differentiation and depth of invasion.

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Microsatellite instability, MMR gene expression and proliferation kinetics in colorectal cancer with familial predisposition

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INTRODUCTION

Genetic instability is a common property of many human cancers^[1], including those of HNPCC^[2,3]. A novel form of genetic instability involving somatic alterations, such as deletions and insertions in simple repeated sequences^[4], has been found. Microsatellites are relatively short runs of tandemly repeated sequences scattered throughout the genome^[5,6]. Ubiquitous alterations in these sequences were initially detected by unbiased DNA fingerprinting in a subset of colorectal cancer^[7,8], implying the presence of genome-wide genetic instability. Subsequently, amplification by polymerase chain reaction (PCR) of a few microsatellite loci should be used to reveal this microsatellite instability (MSI) in colorectal cancers^[9,10] and other malignancies^[11-14].

MSI was observed to be a common feature of HNPCC^[15]. Physical mapping and finding of mutations in HNPCC patients revealed that a human homologues of the *Escherichia coli* DNA mismatch repair (MMR) enzyme MutS was one of the candidate genes for HNPCC and was named hMSH2^[16]. Human homologues of the *E.coli* and yeast mismatch repair enzymes hMLH1, hPMS1 and hPMS2 have also been associated with HNPCC^[17]. Defects of these mismatch repair genes have been reported to produce MSI in bacteria and yeast, and a high mutation rate of microsatellites has been

observed in HNPCC patients and in cell lines established from HNPCC tumors^[18]. Therefore, MSI in HNPCC is one of the results of mutations in these mismatch repair genes. In the present study, we analyzed MSI, expression of MMR genes and cell proliferation activity in colorectal cancer (CRC) patients with familial predisposition, to reveal the characteristics of these patients' genetic defects and to afford the biological basis for screening high-risk relatives of CRC.

MATERIALS AND METHODS

Patients

Forty-six colorectal cancer patients who underwent surgical resection between 1993 and 1995 at Nanfang Hospital in Guangzhou, China, were analyzed. These included 26 patients with familial predisposition (Group A) by summarizing clinic archives and surveying their pedigrees, and 20 randomly selected colorectal cancer patients without familial predisposition (Group B). In Group A, 4 patients were eligible HNPCC according to the Amsterdam criteria^[15].

PCR

Genomic DNA was extracted from formalin fixed, paraffin embedded tumor tissues and corresponding normal tissues, respectively, using a modification of the method reported previously^[19-21]. About 100ng of genomic DNA was used for PCR amplification of microsatellite sequences.

Microsatellite instability

Four microsatellite loci, D2S119, D2S123, D5S107 and D17S250 were analyzed in all patients, using silver staining polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique^[22,23]. We classified patients as positive for MSI when the PCR product using tumor DNA revealed the presence of extra bands or shifting bands that were not visible in the PCR product of the corresponding normal tissue DNA, and these changes were found in at least two microsatellite loci, called MSI-H^[24,25].

Immunohistochemical staining of hMLH1, hMSH2 and proliferation cell nucleus antigen (PCNA)

hMLH1, hMSH2 enzyme was detected using a rabbit polyclonal antibody, and PCNA, a

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monoclonal antibody (Dako Co). By streptavidin-peroxidase (SP) immunohistochemical method^[26], we defined positive cases as those of hMLH1, hMSH2 that showed staining of tissues (tumor or normal) cell nuclei or cytoplasm in clusters^[27], and PCNA that showed staining of tumor cell nuclei in clusters^[28,29]. The label index (LI) of PCNA was analyzed.

Flow cytometry for DNA analysis

Using Flow Cytometry (Type of EPICS ELITE), the DNA index (DI), heteroploid rate, proliferation index (PI) and S-phase percentage were assayed in tumor cells. We defined heteroploid cells as that DI was not between 0.90 and 1.10.

Statistical analysis

Two-tailed Fisher's and χ^2 exact tests were used to analyze the significance of differences between the groups. The significance level was set at $P<0.05$.

RESULTS

All patients were detected for MSI in four microsatellite loci (Figure 1). MSI was detected in 20 (76.9%) out of 26 patients in Group A. The MSI-positive (MSI-H) proportion in Group A was 46.2% (12/26), significantly higher than 10% (2/20) in Group B ($P<0.05$). Three of 4 HNPCC patients in Group A were MSI-positive.

We examined the association of MSI with clinicopathologic characteristics of CRC patients with familial predisposition, and elucidated the following features: ① An early age of onset (Table 1). Patients positive for MSI in Group A tended to have a younger mean age at onset than patients in Group B ($P<0.01$) and patients negative for MSI in Group A. ② A preponderance of tumors in the proximal colon (Table 2). Proximal colon includes cecum, ascending and transverse colon^[30]. Among patients positive for MSI, 9 (64.3%) of 14 tumors were located in the proximal colon. In contrast, among patients negative for MSI, 9 (28.1%) of 32 tumors were located in the proximal colon ($P<0.05$). In MSI-negative patients, tumors in Group A also had a proclivity for the proximal colon (5/9) but tumors in Group B did not (4/14). ③ Correlation with a poorly differentiated phenotype (Table 2). Among patients positive for MSI, 11 (78.6%) of 14 tumors were poorly differentiated adenocarcinomas. It was significantly higher than that among MSI-negative patients ($P<0.05$). Also in tumors of Group A, the poor differentiation rate positive MSI was higher than that of negative MSI. ④ Proclivity for extracolorectal malignancy. Among patients in Group A, 6 cancers were associated with cancers in other organs (2 in uterus, 2 in stomach, 1 in bladder, and 1 in bile duct), and 5 of these were positive for MSI. In contrast, there was no patient with cancers in other organs in Group B.

Immunohistochemically, contrast with Group B, patients in Group A tended to be negative for hMLH1 protein staining in tumor tissues, and hMLH1, hMSH2 protein staining in normal tissues (Figure 2, Table 3). Among patients positive for MSI, 6 of 14 colorectal tumors were negative for hMLH1 together with hMSH2 protein, whereas 4 of 32 tumors negative for MSI ($P<0.05$). The LI of PCNA staining (Figure 3) in tumors of Group A was 0.54 ± 0.10 , which was lower than that of Group B (0.62 ± 0.07), $P<0.01$, meanwhile in cancer tissues with MSI-positive, the LI was 0.53 ± 0.10 , which was lower than that of negative MSI (0.59 ± 0.08), $P<0.05$.

According to flow cytometry (Table 4), the heteroploid rate of DNA in tumors of Group A was 23.1%, PI and S-phase percentage in tumors with positive MSI were 14.34 ± 5.49 and 8.18 ± 2.55 . In contrast with that of Group B and negative MSI respectively, they decreased obviously ($P<0.05$).

Table 1 MSI and mean age at onset of colorectal cancer

Group	MSI	n	Age($\bar{x}\pm s$)
HNPCC		4	41.8 \pm 7.7 ^b
A	+	12	45.4 \pm 8.3 ^b
	-	14	49.6 \pm 9.7
B	+	2	57.5 \pm 9.2
	-	18	58.9 \pm 11.0

^b $P<0.01$ vs Group B; Fisher's test.

Table 2 MSI and location, histologic differentiation of colorectal cancer

Group	MSI	Location n (%)		Differentiation n (%)	
		Pr	Di	PD	WD
A	+	8(66.7)	4(33.3)	9(75.0) ^b	3(25.0)
	-	5(35.7)	9(64.3)	4(28.6)	10(71.4)
B	+	1(50.0)	1(50.0)	2	0
	-	4(22.2)	14(77.8)	5(27.8)	13(72.2)
Total	+	9(64.3) ^a	5(35.7)	11(78.6) ^a	3(21.4)
	-	9(28.1)	23(71.9)	9(28.1)	23(71.9)

^a $P<0.05$, vs patients negative for MSI; χ^2 test. ^b $P<0.05$, vs patients negative for MSI in Group A; χ^2 test.

Pr: proximal colon; Di: distal colon (descending, sigmoid colon and rectum); PD: poorly differentiated adenocarcinoma; WD: well and moderately differentiated adenocarcinoma.

Table 3 Negative for hMLH1, hMSH2 protein staining in CRC patients' tissues (n,%)

	Group A (26 cases)		Group B (20 cases)	
	Tumor	Normal	Tumor	Normal
hMLH1 -	16(61.5) ^a	17(65.4) ^b	6(30.0)	5(25.0)
hMSH2 -	14(53.9)	16(61.5) ^b	9(45.0)	6(30.0)

^a $P<0.05$, vs tumor tissues in Group B; χ^2 test.

^b $P<0.05$, vs normal tissues in Group B; χ^2 test.

Table 4 MSI and DNA analysis in CRC tumor cells by flow cytometry ($\bar{x}\pm s$)

Group	Cases	Heteroploid (n,%)	PI	S-phase
HNPCC	4	1(25.0)	14.58 \pm 3.12	8.70 \pm 1.18
A	26	6(23.1) ^a	17.57 \pm 6.51	9.47 \pm 2.85
	18	10(55.6)	17.94 \pm 7.51	10.38 \pm 3.89
MSI+	14	4(28.6)	14.34 \pm 5.49 ^b	8.18 \pm 2.55 ^b
MSI-	30	12(40.0)	19.30 \pm 6.93	10.62 \pm 3.36

^a $P<0.05$, vs tumor cells in Group B; Fisher's test.

^b $P<0.05$, vs tumor cells negative for MSI; Fisher's test.

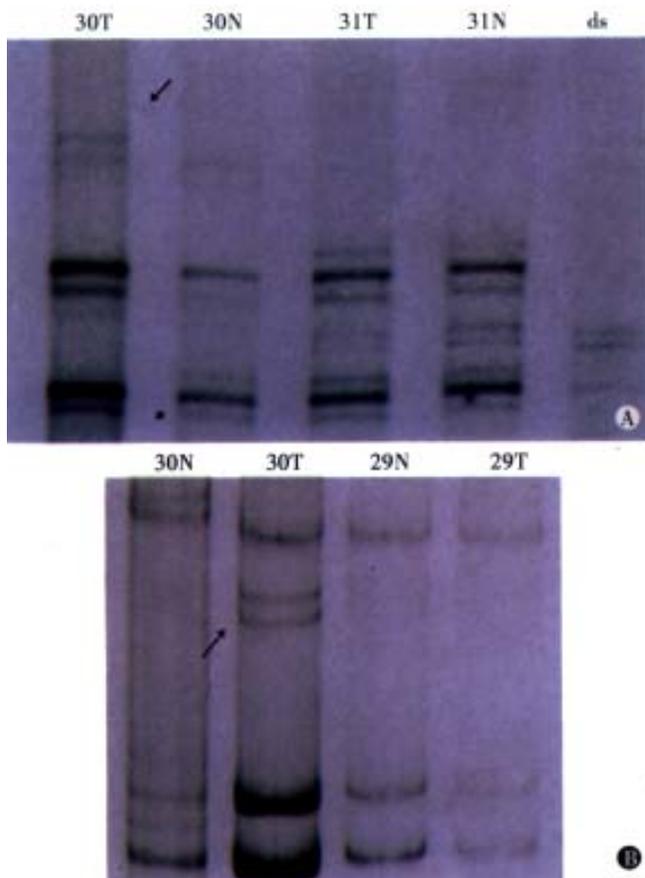


Figure 1 Patient of No.30 positive for MSI. Lane N: corresponding normal tissue, Lane T: tumor tissue, Lane ds: di-strand control. MSI is defined as showing the presence of extra or shifting bands in PCR products using tumor DNA that are not visible in the products from corresponding normal tissue. (Left)D17D250, (Right)D2S123.

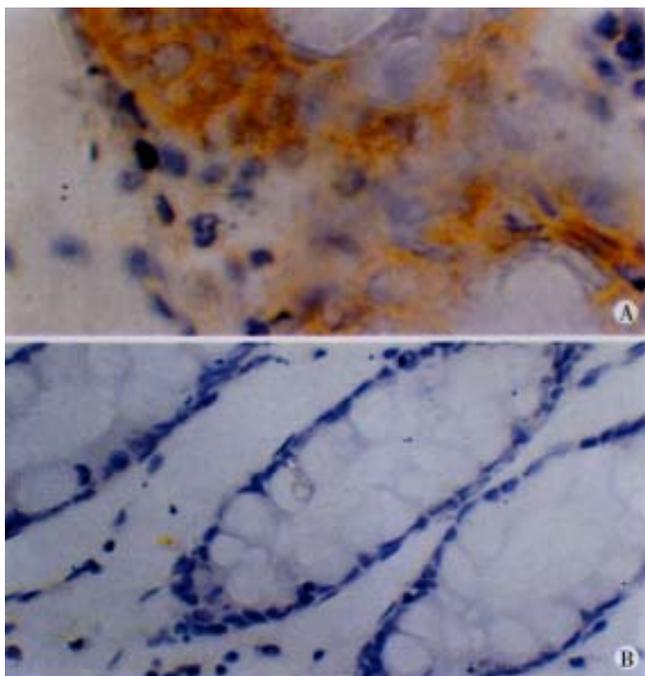


Figure 2 Immunohistochemistry of hMLH1. (Left) Patient positive for hMLH1 immunostaining. ×40. (Right) Patient negative for hMLH1 immunostaining.×20.

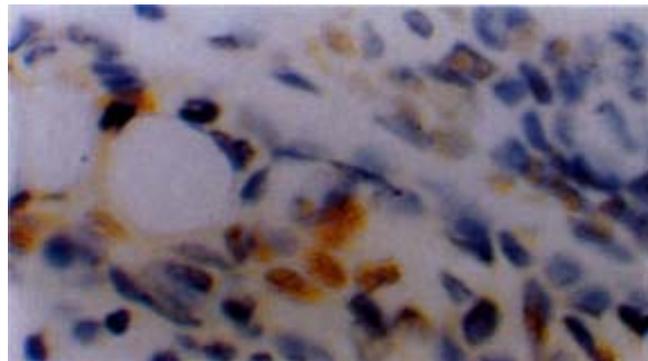


Figure 3 Immunostaining for PCNA in colorectal cancer tissue of patient with familial predisposition. ×40.

DISCUSSION

MSI is the phenotype of a profound genomic instability caused by a mutator phenotype mechanism for cancer^[31,32]. Four microsatellite loci (D2S119, D2S123, D5S107 and D17S250) used in our study resemble to the loci recommended by NCI workshop^[25]. The ratio of MSI-positive patients in Group A (46.2%) was significantly higher than that among randomly selected patients in Group B (10%). These results showed that MSI was an important contributor in CRC with familial predisposition. Colorectal tumors with MSI exhibit several clinicopathologic characteristics. In this study, we confirmed that the age at onset of cancer in Group A was younger than that in Group B, especially in HNPCC patients and patients with MSI-positive in Group A. We also confirmed that the ratio of proximal colon tumors, complication by extracolorectal malignancies and poorly differentiated carcinomas in patients with positive MSI were higher than those with negative MSI, and these characteristics were significant in Group A. These results suggested that it was possible and effective to identify high-risk relatives of CRC by applying pedigree survey together with MSI detection.

Leach *et al* established the method for detecting the products of MMR gene mutation^[33]. If the MMR genes mutate, there may be a kind of methylation on promoter in transcription. And it brings the products in C-termination which block the normal expression of MMR genes. Therefore, negative for hMLH1, hMSH2 staining means potential mutation of MMR genes in tumor or normal tissues, and positive means no mutation^[27,34]. In our study, we found that in Group A the incidence of negative hMLH1 staining in tumor tissues and hMLH1, hMSH2 staining in normal tissues was significantly high (Table 3). Among patients positive for MSI, ratio of negative hMLH1 together with hMSH2 staining in tumors was also higher than that among patients negative for MSI. It suggested that the rate of hMLH1, hMSH2 abnormality in CRC patients with familial

predisposition increased in colorectal tumors and normal tissues. The colorectal epithelium of this group could have wide gene abnormality. And these gene changes had obviously been related with MSI.

According to the previous studies, the expressions of PCNA^[35,36], DNA ploidy and proliferation phases of cells^[37,38] are the important markers for diagnosing and prognosticating cancers. We found that the LI of PCNA staining of tumor cells in Group A and MSI-positive group were lower than that in Group B and MSI-negative group respectively. According to Flow Cytometry, the heteroploid rate of DNA in tumor cells of Group A was lower than that of Group B. PI and S-phase percentage in tumor cells with positive MSI were also lower than those in negative MSI. It was reported that the metastasis and recurrence in colorectal cancer with MSI (due to replication error, RER) were less than in sporadic cancer. The low invasion of cancer could be explained that the mutation was too frequent and complicated to express the phenotype of metastasis and recurrence^[18]. Our study showed that this low invasion was also associated with the decreased proliferation activity in colorectal cancer with familial predisposition and MSI.

Studies on MSI, MMR gene expression and proliferation kinetics showed the characteristics of colorectal cancer with familial predisposition. According to this, the early diagnosis and warning system^[39] of colorectal cancer can be established. It is important to screen high-risk relatives of colorectal cancer and diagnose colorectal cancer early^[40].

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The effects of PAF antagonist on intestinal mucosal microcirculation after burn in rats

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Subject headings platelet activating factor; intestinal mucosa; intramucosal PH; burn

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INTRODUCTION

Gut originated infection (GOI) has been recognized as a potential factor for postburn irreversible shock, early sepsis and multiple system organ failure^[1-5]. The intestinal mucosal barrier injury has been implicated as the cause of postburn GOI^[6-8]. However, pathogenesis of the lesion is not well known. Platelet activating factor (PAF), an endogenous phospholipid mediator, has recently been proposed as an important mediator of postburn intestinal mucosal barrier injury and gut originated infection^[9-11]. But the mechanism of PAF is not well defined. In this study, we have evaluated sequential hemodynamic changes in the intestinal mucosa after burn injury and investigated the role of PAF by assessing whether pretreatment against intestinal mucosal hemodynamic disturbance and pathologic damage, so as to further explore the role and its mechanism of PAF in postburn intestinal mucosal barrier injury.

MATERIALS AND METHOD

Animals

Wistar rats, male or female, weighing 220 g±30 g were used. They were provided by Animal Laboratory, Institute of Burn Research, Third Military Medical University.

Experimental design

Animals were randomly divided into three groups: group 1 (*n*=10) served as a control with sham, burn injury; group 2 (*n*=40), burned rats that had

undergone 30% TBSA III° burn; group 3 (*n*=40), rats that received PAF antagonist WEB2170 (5mg/kg) by intraperitoneal injection immediately after burn and repeated every 8 hours^[12]. WEB2170 was provided by Boeringer Ingelheim Pharmaceuticals Inc, Federal Republic of Germany. The index was observed on postburn 6, 12, 24, and 48 hours.

Burn model

Rats were anesthetized intraperitoneally with 80mL/kg body weight, ketamine hydrochloride, and their backs were shaved. They were placed in a mould that left approximately 30% of their body surface area exposed. The exposed surface was immersed in 92°C water for 18s. This type of burn injury is a fullthickness burn. Animals were resuscitated with 40mL/kg of lactated Ringer's solution.

Measurement of intestinal mucosal blood flow

The intestinal mucosal blood flow was directly measured with a laser-Doppler flowmeter^[13], made by Nakai university. The unit was expressed as mv.

Measurement of intestinal intramucosal PH (PHi)^[14,15]

Rats were anesthetized after fasted overnight, a midline abdominal incision was made. Fifteen cm segments of ileum were isolated, cannulated proximally and distally, and 3mL saline was injected into the ileal segment. After 30min, 1mL intestinal perfusion and 1mL arterial blood were collected for measurement of Pco₂ in intestinal perfusion and [HCO₃⁻] in arterial blood. The intestinal mucosal PHi was calculated from the Henderson-Hasselbach equation:

$$PHi = 6.1 + \log(Pco_2 \times 0.0307)$$

Measurement of intestinal water content^[16] Five cm segments of ileum were excised and weighed for wet weight, and placed in to 140°C oven for 4 hours and weighed again for dry weight. The intestinal water content was calculated using the following formula:

$$\text{Water content} = (\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$$

Histologic examination of intestinal mucosa The ileum was fixed in 10% formalin and processed by the routine techniques. Specimens were stained with hematoxylin and eosin and examined histologically under light microscope.

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Statistical analysis

All data were expressed as $\bar{x}\pm s$, and statistical analyses were made using Student's *t* test.

RESULTS

The changes of intestinal mucosal blood flow

The intestinal mucosal blood flow began to decrease significantly on postburn 6h and became lowest on postburn 12h as compared with control group. In PAF-antagonist treatment group, the intestinal mucosal blood flow was significantly increased compared with burn group (Table 1).

The changes of intestinal mucosal PHi

The intestinal mucosal PHi in burn group was significantly lower than in control group on postburn 6h, 12h, and 24h, but not on postburn 48h. In PAF antagonist treatment group, intestinal mucosal PHi was significantly increased compared with burn group (Table 2).

The changes of intestinal water content

The changes of intestinal water content on postburn 6h and 48h were not significantly different, but on postburn 12h and 24h, it was significantly increased as against burn group. The intestinal tissue water content in treatment group on 12h and 24h was significantly lower than in the burn group (Table 3).

The histologic changes of intestinal mucosa

In the burn group, extensive vascular congestion and edema were noted in ileal mucosa. Subepithelial space at the tip of the villi was developed, and lacteals were dilated. The degenerative fragmentation and atrophy of mucosal villi were

apparent on postburn 12h and 24h. These changes were significantly reduced in PAF antagonist treatment group.

DISCUSSION

The intestinal mucosal blood flow is an important factor for maintaining the structure and function of intestinal epithelial cells^[17-19]. Some studies have indicated that gastrointestinal mucosal blood flow significantly decreased following early burn injury^[20-22]. Binnaka *et al*^[23] reported that gastric mucosal blood flow fell rapidly to 40% of normal value on postburn 2h on a rat model with 30% TBSA III° burn. Tokyay *et al*^[24] reported that intestinal blood flow decreased significantly to 25% -30% of the baseline on 2h and 4h of the early postburn phase, and during the late phase at 48h to 30% of the baseline again on a pig model with 40% TBSA III° burn. The results showed that intestinal mucosal blood flow significantly decreased to 65% of normal value on postburn 6h to 46% on postburn 12h, to 68% and 82% on postburn 24h and 48h. The intestinal mucosal ischemia following burn was proved again. The decrease in the intestinal mucosal blood flow caused a decrease in oxygen delivery (DO₂) and a marked increase in oxygen consumption (VO₂) in intestinal epithelial cells^[25,26]. When DO₂ fell below a critical level, further decrease in DO₂ can induce anaerobic metabolism and decrease in cells PH, causing the damage of intestinal epithelial cells^[27,28]. The results also showed that the intestinal mucosal PHi was significantly decreased following burn injury compared with control group. It is suggested that hypoxia in intestinal epithelial cells occurs following burn.

Table 1 Changes of the intestinal mucosal blood flow (mv, $\bar{x}\pm s$)

Groups	n		Postburn			
			6h	12h	24h	48h
Control	10	46.55±3.01				
Burn	40		30.60±3.08 ^b	21.85±2.94 ^b	31.85±2.72 ^b	58.56±3.11 ^a
Treatment	40		40.22±2.86 ^{da}	37.1±2.90 ^{db}	42.06±2.14 ^{da}	45.89±4.51 ^c

^a*P*<0.05, ^b*P*<0.01 vs Control; ^c*P*<0.05, ^d*P*<0.01 vs Burn.

Table 2 Changes of the intestinal mucosal PHi ($\bar{x}\pm s$)

Groups	n		Postburn			
			6h	12h	24h	48h
Control	10	7.419±0.058				
Burn	40		7.217±0.085 ^b	7.316±0.067 ^b	7.347±0.016 ^a	7.432±0.046
Treatment	40		7.326±0.087 ^{da}	7.406±0.113 ^c	7.374±0.148 ^c	7.435±0.055

^a*P*<0.05, ^b*P*<0.01 vs Control; ^c*P*<0.05, ^d*P*<0.01 vs Burn.

Table 3 Changes of the intestinal tissue water content (% , $\bar{x}\pm s$)

Groups	n		Postburn			
			6h	12h	24h	48h
Control	10	76.02±2.97				
Burn	40		76.21±4.16	80.43±2.78 ^a	79.89±2.60 ^a	76.97±1.91
Treatment	40		75.83±2.67	76.65±2.14 ^c	76.47±1.43 ^c	76.14±1.56

^a*P*<0.05, vs Control; ^c*P*<0.05, vs Burn.

PAF is a phospholipid mediator released from stimulated leukocyte, platelets, endothelial cells and mast cells, etc. PAF has a potent vasoactive effect^[29,30]. intravascular infusion of PAF can induce hypotension and extensive vasoconstriction in heart, lung, brain and gastrointestinal, and a marked increase of vascular permeability as well^[31,32]. PAF-induced responsiveness was significantly attenuated by PAF antagonist^[33,34]. It was reported that PAF antagonist can significantly reduce intestinal mucosal ischemia and pathological damage caused by endotoxin shock and hemorrhagic shock^[35-38]. In this study, treatment with antagonist WEB2170 for the scalded rats could significantly increase the intestinal mucosal blood flow and PHI, decrease intestinal tissue water content and alleviate the pathological damage of intestinal mucosa. Conclusion can be drawn that PAF is one of the important factors causing postburn disturbance of intestinal mucosal microcirculation.

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Growth hormone stimulates remnant small bowel epithelial cell proliferation

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Subject headings short bowel syndrome; insulin-like growth factor I; intestine, small/surgery; proliferation; growth hormone; intestinal mucosa; rats

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INTRODUCTION

Currently the major treatment choices for short bowel syndrome are parenteral nutrition and small bowel transplantation^[1]. Both therapies involve great fiscal challenge and recurring complications. Recent years have witnessed the promising experimental results of pharmacological rehabilitation of remnant small bowel^[2-10]. Preliminary clinical trial also indicates salutary effect of growth hormone on patients with short bowel syndrome^[11,12]. However, the mechanism by which growth hormone benefits the patients remains unclear.

In this animal experiment we attempted to address the possibility that administration of exogenous growth hormone (GH) after massive small bowel resection can stimulate remnant small intestinal epithelial cell proliferation.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Shanghai Laboratory Animal Center, the Chinese Academy of Sciences) were allowed to acclimate to our laboratory conditions for at least 5 days. The animals were fed standard commercial rat pellet chow, which contained crude protein 25%, fat 10%, crude fiber 50%, calcium 1.0%, phosphate 0.8% (Jiangsu Province Jiangpu Laboratory Animal Feeds Factory, Nanjing, China) and tap water ad libitum. Constant room temperature (22°C) with 12h light and dark cycles were supplied. The experiment protocols described below were

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Experimental design

After an overnight fasting, all animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (100mg/kg). Resect-control animals ($n = 10$) and growth hormone-resect (GH-resect) animals ($n=10$) underwent 80% mid-small bowel resection, leaving 7cm of proximal jejunum and 7cm of terminal ileum. Control-transect animals ($n=10$) underwent bowel transection of ileum 7cm proximal to the ileocecal valve. Postoperatively, animals were allowed to drink water libitum on the first day, and chow on the beginning of the second postoperative day. Somatropin (1IU/kg) (Saizen, Laboratories Serona S.A., 1170 Aubonne, Switzerland) was administered subcutaneously once daily at 16:00 from the first postoperative day to the day of killing. All animals were sacrificed at about 08:00 to 10:00 on the 29th postoperative day. Residual ileum from 2cm distal to the anastomosis to the ileocecal valve was excised and luminal contents were removed. In transected animals anatomically similar segments were removed. The first 1cm of the excised ileum was for histological analysis. The mucosa of the following 2cm segment was scraped for nuclear acid analysis. And the mucosa of the remaining ileum was scraped for flow cytometric analysis.

Plasma insulin-like growth factor-I (IGF-I) determination

Total plasma IGF-I concentrations were measured after acid-ethanol extraction with the use of DSL-2900 rat IGF-I radioimmunoassay kit (Diagnostic Systems Laboratories Inc., Webster, Texas, USA).

Mucosal histological image analysis and immunohistochemical assay for proliferating cell nuclear antigen (PCNA)

Fixed specimens of ileum were embedded in paraffin and oriented to provide cut sections parallel with the longitudinal axis of the bowel. Four-micrometer-thick slices were mounted and stained with hematoxylin and eosin. Villus height, crypt depth and mucosal thickness were measured using a videoassisted integrated computer program (HPIAS-1000 True Color Image Analysis System). Villi were chosen on the basis of the ability to completely visualize the central lymphatic channel and crypts to

visualize the crypt-villus junction on both sides of the crypt. At least 15 villi and crypts were counted per sample.

The immunohistochemical staining for PCNA was performed by Labeled Streptavidin-Biotin method (S-P) with staining kit (Fuzhou Maxim Biotech, Inc., Fuzhou, China) following the instructions of the manufacturer. Those cells with brown-stained nuclei were reckoned as positively stained cells. The PCNA index was measured as a percentage of the number of positively stained cells over the total number of cells in the crypt. Five high-resolution fields from each slide were randomly chosen for measurement.

Flow cytometric analysis

Freshly scraped small bowel mucosa was mechanically dispersed through wire mesh and collected into cold (4°C) phosphate buffered solution (PBS). The cell suspension was washed and filtered through 60µm nylon mesh and adjusted to a concentration of 1×10^6 cell/mL. Aliquots of 1mL mucosal cell suspension were centrifuged at $500 \times g$ for 10min and the cell pellets were subjected to DNA labeling with method described in^[13]. The stained cells suspension was analyzed using an Epics XL flow cytometer (Coulter Corp., Hialeah, FL, U.S.A.). The percentages of cells in the G₀/G₁, S, and G₂/M phases of the cell cycle were determined. The cell proliferative activity was measured by proliferative index (PI), calculated by $(S+G_2/M)/(G_0/G_1+S+G_2/M) \times 100\%$.

Semiquantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from the ileal mucosa using TRIzol reagent (GIBCO BRL, Gaithersburg, MD, U.S.A.) following the instructions of the manufacturer. Concentrations of total RNA were determined spectrophotometrically at A₂₆₀. RT reactions were carried out using 2.5µg of total rat RNA and a cDNA synthesis kit (Promega, Madison, WI, U.S.A.) in a final reaction volume of 20µL. Synthetic oligonucleotides (Sango Biotech. Corp., Shanghai, China) for cDNA amplification were as follows: 5'-gcatgaggaaccgattgccgctccaagt-3' (sense), 5'-cgcacagtctgcc ggccaataggccgct-3' (anti-sense) for *C-jun* and 5'-catttcgggtgca cgatggag-3' (sense), 5'-ggcatcctgcgtctggacctg-3' (anti-sense) for β -actin respectively. The PCR amplified products were of 459-base-pair for *C-jun* cDNA and 599-base-pair for β -actin cDNA respectively. PCR was performed using 6.0µL of reverse transcription product and a master mixture containing 10mmol/L Tris-HCl (pH 8.8), 1.5mmol/L MgCl₂, 75mmol/L KCl, 0.2mmol/L dNTPs, 5' and 3'-*C-jun*-oligonucleotides (0.25µmol/L each), 5' and 3'- β -actin oligonucleotides (0.25µmol/L each), and 2.0 unit Taq polymerase (GIBCO BRL, Gaithersburg, MD,

U.S.A.). The samples were denatured initially at 94°C for 5min. Amplification was performed on Gene Cyclor (Bio-Rad, Japan). The cycling condition was selected for β -actin as denaturing at 94°C for 1min, annealing at 58°C for 1min, and extension at 72°C for 1min for 28 cycles; for *C-jun* as denaturing at 94°C for 1min, annealing at 61°C for 1min, and extension at 72°C for 1min for 33 cycles. The final cycle was followed by an extension step at 72°C for 10min. The PCR amplifiers were subjected to electrophoresis through 1.6% agarose gel containing 0.5mg/L ethidium bromide, visualized by ultraviolet illumination. The electrophoresis gel images analyzed with Kodak Digital Science 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, U.S.A.). The expression of *C-jun* was measured as a ratio compared with β -actin.

Statistical analysis

Results were presented as mean \pm SD. Statistical analysis was performed using one-way ANOVA. Possible post hoc comparison of pairs of means was conducted by Duncan's multiple range test. A probability value of less than 0.05 was considered significant.

RESULTS

Body weight

There was no significant difference in the body weight among three groups before operation. After bowel resection, body weight decreased gradually and reached the nadir by the end of the first postoperative week. Then the body weight gradually rose at a speed slower than those undergoing bowel transection. One week after the operation, animals in control-resect group and GH-resect group had similar body weights, which were significantly lower than those in control-transect group. Growth hormone treatment resulted in significant increase in body weight gain at the end of the experiment (Table 1).

Table 1 Effect of growth hormone on body weight

	Control-transect (n=10)	Control-resect (n=10)	GH-resect (n=10)
Day 0	249 \pm 13	251 \pm 11	244 \pm 10
Day 8	287 \pm 11	205 \pm 10 ^a	211 \pm 11 ^a
Day 29	351 \pm 13	297 \pm 16 ^a	332 \pm 15 ^b

Day 0: before operation; Day 8: 1 week after operation; Day 29: 4 weeks after operation. Data are expressed as mean \pm SD;

^aP<0.05, vs control-transect group; ^bP<0.05, vs control-resect group.

Plasma IGF-I concentrations

Plasma IGF-I concentrations in both sets of resected animals (0.84mg/L \pm 0.08mg/L in control-resect; 1.03mg/L \pm 0.07mg/L in GH-resect) were lower than those in control-transect group (1.56mg/L \pm 0.14mg/L, P<0.05).

However, GH administration had significantly increased plasma IGF-I level compared with control-resect animals ($1.03\text{mg/L} \pm 0.07\text{mg/L}$ vs $0.84\text{mg/L} \pm 0.08\text{mg/L}$, $P < 0.05$) (Figure 1).

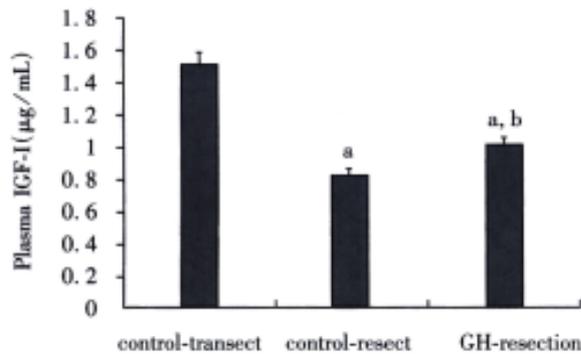


Figure 1 Plasma IGF-I concentration 28 days after 80% mid-small intestine resection in animals from three groups.

^a $P < 0.05$, vs control-transect group; ^b $P < 0.05$, vs control-resect group.

Table 2 Effect of growth hormone on small intestinal mucosal parameters

	Control-transect (n=10)	Control-resect (n=10)	GH-resect (n=10)
Villous height (µm)	180±40	270±40 ^a	340±30 ^b
Crypt depth (µm)	92±17	105±11	120±24 ^a
Mucosal thickness (µm)	320±40	510±80 ^a	520±50 ^a
PI value (%)	3.7±2.0	4.4±1.7	7.6±2.0 ^b
PCNA index (%)	1.6±0.5	3.6±0.6 ^a	8.8±1.1 ^b
<i>C-jun</i> mRNA (%)	10.7±0.7	19.5±2.5	49.2±1.0 ^b

Data are expressed as mean±SD; ^a $P < 0.05$, vs control-transect group; ^b $P < 0.05$, vs control-resect group.

Mucosal parameters

As indicated in Table 2, animals in control-transect group and control-resect group had similar cryptal depth, PI value, and *C-jun* mRNA level. Small bowel resection resulted in significant increase in villous height, mucosa thickness and PCNA index in control-resect group compared with those in control-transect group. GH treatment led to greater villus height, PI value, PCNA index, and *C-jun* mRNA level in GH treatment group than in control-resect group. The crypt depth in GH treated animals was significantly greater than that in control-transect animals.

DISCUSSION

Our animal experiment demonstrated that small bowel resection led to significant increase in villous height, mucosal thickness and PCNA index, which proved that our massive small bowel resection model had induced adaptive hyperplasia in remnant ileum. We chose to study the remnant ileum because previous studies had revealed that after massive small bowel resection the remnant ileum bore greater adaptive potential than the remnant jejunum^[14,15]. Moderate dose of rhGH

($1\text{IU}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) stimulated further hyperplasia of remnant small bowel mucosa, which was manifested by increased villous height, PI value, and PCNA index. *C-jun* encoded a member of the AP-1 complex of transcription factors that were known to increase after various mitogenic stimuli. Previous studies had demonstrated that enhanced intestinal proliferative activity was associated with augmentation of intestinal expression of *C-jun* mRNA^[16,17]. Semi-quantitative analysis of *C-jun* mRNA revealed enhanced expression of *C-jun* in GH-resect animals, which further reflected increased proliferative activity.

The result of our study was consistent with the notion that GH is an important growth factor for small intestinal mucosa. Complete GH depletion due to hypophysectomy caused pronounced hypoplasia of small intestinal mucosa with decreased villous height and reduced cryptal cell proliferation. Simple replacement of GH can restore mucosal proliferative activity^[18]. Hypophysectomy was shown to impair the adaptive hyperplasia in response to small bowel resection^[19]. Study of the transgenic mice overexpressing the bovine growth hormone demonstrated that chronic GH excess could produce hyperplasia in small intestinal mucosa whether food intake was *ad lib* or restricted^[20]. Short-term GH administration has also been shown to promote further hyperplasia of remaining small intestinal mucosa, which was not associated with appetite variation^[2].

Plasma IGF-I is primarily derived from liver and GH is the principal stimulus for IGF-I synthesis in liver as well as in peripheral tissues^[21]. Apart from GH, nutritional status is another major regulator of plasma IGF-I concentration^[22]. Both calorie restriction and protein restriction have been shown to down-regulate the plasma IGF-I level. In this study, massive small bowel resection significantly lowered the plasma IGF-I concentrations in two bowel-resected groups. This may be attributed to bowel resection-induced impairment of nutrient absorption as the mean body weights of these two groups were significantly decreased. Nevertheless, animals in GH-resect group had higher plasma IGF-I concentrations than those in control-resect group. This suggests that as a powerful stimulatory agent for IGF-I synthesis, GH is still effective in enhancing IGF-I synthesis even in condition of mild nutritional restriction.

GH and IGF-I can both exert marked anabolic effect, stimulate nitrogen accretion, and lean body weight gain even when nutrition supply is restricted mildly or moderately^[23]. Therefore, in our experiment, improved body weight gain in GH-resect group could at least partially result from the anabolic effect of GH. On the other hand, GH treatment stimulated the remnant small bowel

mucosa hyperplasia and increased the absorptive area of the remnant small bowel in GH-resect group. As adaptive enlargement of absorptive area of remnant small bowel is a major mechanism by which the absorptive capacity of the remnant small bowel can be enhanced^[24], it is reasonable to speculate that the nutrient absorption function of animals in GH-resect group was improved by GH treatment. The accelerated body weight gain in GH-resect group therefore is very likely due to the combined effect of increased nutrient absorption and improved anabolic metabolism.

The enterotrophic effect of GH observed in this study may well be mediated by GH-induced enhancement of local IGF-I production within the intestinal mucosa. Convincing evidence has shown that IGF-I is a very important intestinal growth factor. Systemic IGF-I administration increased rat small bowel mass in normal rat^[25], in parenterally fed rat^[26], in catabolic status induced by dexamethasone^[27], in compensatory intestinal mucosa hyperplastic status^[9], as well as in post-transplantational status^[28]. GH transgenic mice have increased expression of endogenous IGF-I locally within small bowel as well as increased plasma concentrations of IGF-I, whereas IGF-I transgenic mice have increased IGF-I serum level, local expression in small bowel, and secondary GH deficiency. However, the effects on small bowel growth are similar in these two transgenics^[20,29]. These observations suggest that IGF-I mediates most of the enterotrophic effects of GH. Besides, recent studies on IGF-I null mice have proved definitely that IGF-I is essential for postnatal somatic growth and mammary development in response to GH^[30,31]. These discoveries make it very possible that IGF-I mediates the enterotrophic effect of GH. In addition, recent gene targeting study has demonstrated that liver-derived IGF-I is not essential for normal postnatal growth^[32]. This discovery gives strong emphasis to the importance of IGF-I locally produced. In support, GH induced high serum IGF-I level is not necessarily associated with enhanced growth of peripheral tissues^[33,34]. Therefore, we deduce that IGF-I produced locally within intestinal mucosa mediates the enterotrophic effect of GH.

However, Vanderhoof and his colleagues have published conflicting data, which failed to prove the mitogenic effect of short-term GH administration on rat intestinal mucosa^[35,36]. Close examination of the experimental conditions revealed that in one of the two studies continuous subcutaneous infusion of rhGH at a dosage of $3\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ failed to produce significant change in serum concentrations of insulin-like growth factor (IGF) and IGF-binding protein-3 (IGFBP-3) whether the animals had undergone bowel transection or resection^[35]. On

the other hand, serum IGF-I and IGFBP-3 both are known to be up-regulated by serum GH concentration^[37]. So unaltered serum levels of IGF-I and IGFBP-3 may be taken as evidence for insufficient action of GH in their experiment. On the contrary, in our experiment rhGH at $11\text{IU}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ was sufficient to increase rat plasma IGF concentration in bowel-resected rats. In another study the authors increased the dosage to $12\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, which produced significant increase in serum IGF concentration. However, they had not observed GH mitogenic action on intestinal mucosa^[37]. To interpret these phenomena, other difference in experimental conditions should be emphasized. First, the control of food-intake, both the presence and quantity of which have been established as important regulators of postranssectional small bowel adaptive hyperplasia^[38,39]. Vanderhoof *et al* had employed pair-feeding to distinguish the effect of diet and GH, while our animals were fed *ad lib*. Though our preliminary observation indicated unremarkable difference in food-intake between the control-resect group and GH-resect group, at present, we cannot decide whether the effect of food-intake on intestinal proliferation in our experiment is involved in the effect of GH administration. Second, the ages of experimental animals were different, we and Shulmen *et al* used adult rats while Vanderhoof *et al* used young rats. Therefore it is most possible that the responsiveness of intestinal mucosa to GH stimulation is influenced by age. These apparently inconsistent observations indicate the complexity of growth regulation of intestinal mucosa and warrant further studies to explore the influence of age and food intake on the enterotrophic effects of GH.

These findings of our animal experiment certainly cannot be used directly to extrapolate the condition in human beings. However, the possibility that patients with short bowel syndrome may be benefited by the same mechanism from GH administration during the early adaptation stage is of important clinical relevance. After massive small bowel resection, the adaptation of the remnant small bowel usually takes months to 2 years^[40,41]. Augmentation or acceleration of the adaptive process means not only less hospital care and expense on parenteral nutrition but also better well-being and life quality.

In summary, we have observed that GH administration stimulates remnant small intestinal mucosa proliferation though whether it is due to direct GH action on intestinal mucosa or due to indirect effect of increased food-intake induced by GH administration has not been clearly determined in this study. Early GH treatment may benefit patients immediately after massive small bowel resection.

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Pancreaticoduodenal transplantation with portal venous and enteric drainage in rats

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Subject headings pancreaticoduodenal transplantation; kidney transplantation; portal venous drainage; enteric drainage; postoperative complications; rats

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INTRODUCTION

The use of combined pancreatic and renal transplantation in patients with diabetes and end-stage renal failure has gained widespread recognition as an acceptable treatment option. The prevailing method used for transplantation of the pancreas involves anastomosis of the graft's portal vein and the recipient's iliac vein to provide systemic venous drainage of the graft, and a duodenocystostomy to provide bladder drainage of exocrine secretions^[1]. Although graft survival rates have improved with the use of this technique, potential postoperative problems arise due to the physiologically abnormal exocrine and venous drainage of the pancreas allograft. Bladder drainage of exocrine secretions increased the loss of bicarbonate in the urine, creates electrolyte derangements, and contributes to dehydration leading to a state of metabolic acidosis^[2-4]. Pancreatic secretions drained into the bladder also provide a constant source of irritation to the bladder mucosa, accentuating the abnormalities associated with autonomic diabetic dysfunction. This environment subsequently led to recurrent hematuria, infection, and repeated episodes of graft pancreatitis^[5-7]. The method of pancreas transplantation with portal venous and enteric drainage can overcome these problems. One of the earliest series of portal pancreatic transplants was reported in 1984^[8]. Because of the complex surgical technique, big animal models were used widely in the pancreas transplantation research^[9-11], up to now, there has been only portal venous drainage in

segmental pancreatic transplantation in rats^[12]. In our study, we established an animal model of pancreaticoduodenal transplantation with portal venous drainage through the superior mesenteric vein and enteric drainage in rats in order to achieve a better understanding of the immunology and physiology of this graft.

METHODS

Inbred male Wistar rats (220g-330g body weight) were used as the recipients and the donors, the recipient weight was 50g higher or so than the donor. At the beginning, all recipients were measured for blood insulin concentrations using radioimmunoassay as previously described^[13], diabetes was induced by a single intravenous injection of streptozocin (Sigma Chemical Co, St. Louis, MO) at a dose of 55mg/kg. Rats with nonfasting plasma glucose levels of more than 22mmol/dL were used as recipients.

The donors received 5% glucose and 0.9% saline for 24 hours and the recipients received 2.5% glucose and 0.45% saline for 24 hours before surgery. They were anesthetized with an intraperitoneal injection of pentobarbital (40mg/kg) and chloral hydrate (160mg/kg i.p.) supplemented with the pentobarbital as required.

We used a modified method reported by Lee and Li^[14,15], to minimize graft trauma. Pancreaticoduodenum was harvested from the donor rats with an attached segment of donor's aorta including the superior mesenteric artery and celiac truncus as arterial inflow and the portal vein was maintained for venous outflow, and the proximal end of duodenum was ligated with 3-0 silk and the 2cm of distal duodenum was removed. The spleen arteries and veins were ligated with 5-0 silk, but did not remove the spleen. The graft was flushed with 2mL-3mL cold heparinized lactated Ringer's solution (25U/mL), removed, and stored in cold lactated Ringer's solution (4°C) until transplantation into the recipient rat. Finally, 2.5mL-3mL fresh blood was taken from the donor's abdominal aorta with 5U heparin.

All the recipients were given 8mL-12mL of 0.9% saline by subcutaneous injection and were opened via a middle incision. Segments of the recipient abdominal aorta were mobilized below the vessels to the left kidney. A modified Lee's clamp^[16] was placed across the aorta. The aorta was punctured with a 30 gauge needle and opened via a longitudinal arteriotomy. The lumen was flushed

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with heparinized lactated Ringer's solution. The superior mesenteric vein was isolated at a low level. The superior mesenteric vein was controlled proximally with a microvascular clamp and was ligated distally with 5-0 silk and the superior mesenteric artery was ligated at the same level and manner. The superior mesenteric vein was cut off between the microvascular clamp and silk ligation point as closely as possible to the latter, and the lumen was flushed with heparinized lactated Ringer's solution. The ischemia small intestine (about 30%-40%) and ileocecum were removed, an end-to-end intestinal anastomosis was performed using one layer of full thickness 7-0 nylon suture. The donor graft was removed from the ice water and wrapped by a wet gauze sponge packed with ice crystals.

The venous anastomosis was made first. After ensuring that the vein was not twisted, an end-to-end anastomosis of the donor portal vein and the recipient superior mesenteric vein was made using 10-0 nylon interrupted suture. The vein was irrigated with saline to keep the vessel walls apart during the venous anastomosis and gently teased apart before tying the sutures to avoid stricture of this anastomosis. Next, an end-to-side arterial anastomosis of the donor arterial segment and the recipient abdominal aorta was performed using continuous 9-0 nylon suture. The venous clamp was released first, followed by the arterial clamp. The arterial anastomosis was compressed lightly with a dry sponge for 1-2 minutes after reperfusion. Finally, we performed an end-to-side intestinal anastomosis of the donor distal end duodenum and the recipient jejunal side and removed the donor spleen.

Twenty milliliters of warm saline was instilled in the peritoneal cavity prior to closing the abdomen, 2.5mL-3mL donor blood was injected via the dorsal penile vein as the final procedure.

The time for the donor surgery was about 45 minutes, and for the recipient surgery was 90 minutes, the vein anastomosis was 25 minutes and the arterial anastomosis 15 minutes.

The rats were kept on a warming blanket and under a heating lamp for the first 24 hours. They usually recovered from the anesthesia within 1 hour after the operation. They were given 30mL-40mL of 2.5% glucose and 0.45% saline daily containing 150mg piperacillin sodium by subcutaneous injections for the 36-48 hours, followed by normal diet accordingly.

RESULTS

We finished 67 cases of consecutive pancreatic transplantations with portal vein drainage through superior mesenteric vein and enteric drainage. Forty-six rats survived over 7 days, the success rate was 68.6%(46/67). The most common causes of postoperative deaths within the first few hours were

venous thromboses in the anastomosis (18.9%, 12/67). Eight deaths occurred due to hypovolemic shocks within the first 48 hours, one death due to enteric leakage. Rats that survived over 48 hours generally could survive indefinitely. The nonfasting plasma glucose levels of successful rats (91.3%, 42/46) were turned normal. Though the other four cases were still hyperglycosemia, the plasma glucose levels became significantly lower than the preoperative value. All successful 46 cases were measured for insulin concentrations again, the values did not change significantly.

DISCUSSION

Most patients with a systemically draining pancreatic graft displayed both hyperinsulinemia and insulin resistance, sensitivity and responsiveness to insulin in removing glucose decreased^[17]. In this model of pancreas allograft using venous drainage into portal vein and enteric drainage rather than systemic vein and bladder exocrine drainage, it is more closely approximated to enteric drainage and venous drainage of the pancreas allograft in physiology and to avoid the impairment of the first-pass removal of insulin by the liver, otherwise it leads to hyperinsulinemia and insulin resistance^[7,17,18]. Meanwhile, we chose the venous anastomoses at lower level of superior mesenteric vein without heparinizing the recipient rather than the portal vein in order to avoid the venous thromboses at high level and prevent the whole intestinal injury. Once the venous thrombosis happened, the graft could be removed without impairment of the recipient intestine, and the manipulation was rather easy.

The venous thromboses were the most common causes of postoperative deaths, the high blood flow through superior mesenteric vein and the technique of venous anastomosis were responsible for the venous thromboses. To overcome high blood flow through superior mesenteric vein, we took the following measures: ① the recipient was 50g heavier than the donor; ② remove 2cm of donor's distal duodenum; ③ ligate the donor spleen arteries and veins at operation. The end-to-end anastomosis of the donor portal vein and the recipient mesenteric vein was likely to be twisted and narrowed, we therefore, performed the venous anastomosis first and solved these problems readily.

It has been indicated that hypovolemic shock is the most common cause of deaths occurring immediately after transplantation in the small animal. Continuous rat intravenous infusion of the tail vein and the dorsal penile vein, were adopted by many colleagues^[19]. But we gave the recipient 8mL-12mL of 0.9% saline by subcutaneous injection at the beginning and 2.5mL-3mL fresh blood via the dorsal penile vein as the final procedure^[19], instead of keeping long-time continuous intravenous infusion during the surgical

procedure. The 2.5mL-3mL donor blood could eliminate the most frequent causes hypovolemic shock of postoperative mortality after organ transplantation.

The enteric drainage of exocrine secretion can avoid the loss of bicarbonate in urine, otherwise which will result in eletrolyte derangements and dehydration, leading to a state of metabolic acidosis. These distinct advantages reduced postoperative complications markedly in rats, and provided a better understanding for the immunology and physiology of the pancreaticoduodenal transplantation. These are of important clinical significance.

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Detection of k-ras gene point mutation in fine needle aspiration and pancreatic juice by sequence special primer method and its clinical significance

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INTRODUCTION

The point mutation rate of *k-ras* gene at codon 12 in pancreatic adenocarcinoma is reported to be as high as 90%^[1-30], and with no mutations in normal pancreas tissues or other pancreatic disorders. We have detected the presence of *k-ras* gene mutation and its mutant styles since 1994 by PCR-SSP in the FNA or pancreatic juice obtained from pancreatic adenocarcinoma tissues.

MATERIALS AND METHODS

Sources of samples

Eighty-eight copies of samples were collected by fine needle aspiration preoperatively under ultrasound guidance or with direct viewing intraoperatively from January 1994 to December 1996, among which there were 35 pancreatic adenocarcinoma, 20 chronic pancreatitis, 8 ampullary carcinoma, 7 bile duct carcinoma, 6 insulinoma and 12 normal pancreas tissues. All the aspirates were routinely smeared, then mixed with 50 μ L lysis solution and stored in the Eppendorf tubes. Another 47 pancreatic juice samples were obtained by ERCP or puncturing from pancreatic duct intraoperatively or from external drainage postoperatively, including 17 pancreatic adenocarcinoma. The juice volume was more than 1.5mL. All the samples were immediately frozen in liquid nitrogen and stored at -70 $^{\circ}$ C.

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Preparation of samples

FNA samples The aspirates were quickly made into 2 - 5 pieces of smears for light microscopic observation. The other aspirates mixed with 50 μ L lysis solution were added in proteinase K, making the final concentration of 500mg/L. The mixture was then incubated at 55 $^{\circ}$ C for 2 hours and put into water bath at 95 $^{\circ}$ C for 10 minutes to inactivate proteinase K, then 15 μ L supernatant was collected after centrifugation for PCR detection.

Pancreatic juice samples Pancreatic juice was put into 1.5mL Eppendorf tube and underwent high speed centrifugation. Some parts of the sediments were used for smears (2-5 pieces), the residual parts were completely washed with PBS, centrifuged and added 50 μ L lysis buffer solution. The subsequent procedures are the same as used in FNA specimens.

PCR detection

Our primers were synthesized and supplied by Shanghai Bio-Engineering Research Center. The sequences of primers were: R1=5'GGTAGTTG-GAGCTC3', R2=5'GTAGTTGGAGCTGT3', R3=5'GTAGTTGGAGCTGA3', R4=5'CTATTGTTG-GATCAT ATTCG3'. The primers combination were R1-R4 to amplify 89bp fragment of CGT mutation and R2-R4, R3-R4 to produce 88bp fragments of GTT and GAT mutation respectively. The PCR kits were purchased from Shanghai Huamei Biological Products Corporation (PCR KitA system) and the DNA amplifier is the Perkin-Elmer 2400 model. The reaction volume was 50 μ L containing 50mmol/L KCl, 10mmol/L Tris-HCl pH = 8.5, 1.7mmol/L MgCl₂, 0.01% gelatin, 0.08% Triton-X-100, 1.0 μ mol/L of each primer, 200 μ mol/L of each dNTP and 1.5 units of Taq DNA polymerase. Three amplification reactions were performed for each sample. There were 35 circles including denaturation at 94 $^{\circ}$ C for 45 seconds, annealing at 55 $^{\circ}$ C for 45 seconds and extension at 72 $^{\circ}$ C for 45 seconds. Each reaction was set with positive and negative control, the primers and templates of positive control were included in the PCR kitA system. The template was prepared from human genome DNA with its amplification fragment of 110bp. Fifteen μ L amplifying products were loaded on 8% acrylamide gel electrophoresis under 120 volts for 50 minutes, stained with ethidium bromide

and then observed, and photographed under UV transillumination.

RESULTS

PCR findings

There were 32(91.4%) positive cases and 3(8.6%) negative ones in FNA samples of pancreatic adenocarcinoma. The mutant styles included 15 GTT, 11 GAT and 6 CGT. Among the 17 pancreatic juice specimens, 16 (94.1%) were positive and 1 (5.9%) negative with 9 cases of GTT, 4 GAT and 3 CGT. No mutations occurred in aspirates or pancreatic juice specimens of insulinoma, chronic pancreatitis, ampullary carcinoma, bile duct carcinoma, duodenal papilla carcinoma and normal pancreas tissues.

Cytological results (Table 1)

Table 1 Cytological findings of FNA and pancreatic juice in PA, AC and BDC

	FNA(No. %)				Pancreatic juice (No. %)			
	No	P	SP	N	No	P	SP	N
PA	35	20(57.1)	9(25.7)	6(17.2)	17	2(11.8)	2(11.8)	13(76.4)
AC	8	4(50.0)	2(25.0)	2(25.0)	3	0(0)	0(0)	3(100.0)
BDC	7	4(57.1)	2(28.6)	1(14.3)	3	0(0)	0(0)	3(100.0)

FNA=fine needle aspirates, PA = pancreatic adenocarcinoma, AC = ampullary carcinoma, BDC = bile duct carcinoma, P = positive, SP=suspicious positive, N = negative

DISCUSSION

Comparison of different methods for detecting k-ras gene point mutation in pancreatic adenocarcinoma

It has been reported that the k-ras gene at codon 12 had a high incidence of mutation in pancreatic adenocarcinoma, usually presented with CGT, GTT, and GAT styles, occasionally showed TGT, AGT ways. At present, the available methods for detecting its mutation include PCR-RMCA^[1,2], PCR-ASO^[3-11,24-26], PCR-DSM^[12-17], PCR-SSP^[18,31], PCR-RFLP^[17,19-27,33] and PCR-SSCP^[28-30,32], among which PCR-SSP is the simplest and quickest one. No enzyme cut, hybridization, radioactive and non-radioactive imaging technique were needed. It only took a few hours to complete the entire procedure. Tada *et al* collected 9 samples of pancreatic juice for detection by PCR-SSP in 1993^[18]. The results showed that all the 6 cases of pancreatic adenocarcinoma had positive findings and one cholelithiasis, two chronic pancreatitis had no mutation. The number of samples, however, was too small to have any statistical significance. We have used the PCR-SSP method on FNA and pancreatic juice samples of pancreatic adenocarcinoma since 1994. The point mutation rate of k-ras gene was 91.4% and 94.1% respectively without false positive.

Comparison of FNA and pancreatic juice cytological results with PCR-SSP findings

Currently, the accuracy rate for diagnosis of pancreatic neoplasms by FNA technique is about 58%-83%, and the positive rate of pancreatic juice cytology is less than 10%. Our research in FNA and pancreatic juice cytology also supports these results. It indicates that PCR-SSP has advantages in the differential diagnosis of benign and malignant pancreatic diseases compared with cytological examination, but it has little diagnostic value in ampullary carcinoma and bile duct carcinoma.

Significance of detecting k-ras gene point mutation by PCR-SSP

Pancreatic adenocarcinoma is one of the commonly encountered tumors and the incidence is increasing with age. By now there has been no satisfactory method for early diagnosis. It is still very difficult to define the character of the pancreatic mass and to differentiate between tumor-like pancreatitis and pancreatic adenocarcinoma or chronic pancreatitis and whole-pancreas adenocarcinoma. The commonly used imaging examinations (such as type B ultrasound, CT) have no qualitative diagnostic value. Determination of serum tumor markers (CA19.9, CA50, CA242, etc.) has only 60%-70% sensitivity or specificity and the false positive rate may be as high as 30%-40%. The positive rate of pancreatic juice cytology is too low (<10%) and FNA method could yield indefinite results because of the insufficient samples or atypical cellular manifestation. It is, therefore, helpful for us to use PCR-SSP technique to detect the point mutation of k-ras gene at codon 12 when we are not sure about the diagnosis of pancreas disorders. It may serve as a practical method for distinguishing pancreatic benign masses from malignant ones, and making a definitive diagnosis of pancreatic adenocarcinoma.

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Involvement of apoptosis of alveolar epithelial cells in acute pancreatitis-associated lung injury

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Subject headings pancreatitis; lung/injuries; apoptosis; protein P53; immunohistochemistry; platelet activating factor

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INTRODUCTION

Acute pancreatitis is a common and potentially fatal disease which is associated with considerable morbidity and a mortality rate of approximately 30%; its pathogenesis remains unknown^[1]. The cause of death is often due to multiple organ failure which frequently complicates severe acute pancreatitis. In particular, acute lung injury often occurs at an early stage of severe acute pancreatitis and develops into adult respiratory distress syndrome (ARDS)^[2,3].

Numerous studies have been done to clarify the mechanisms of acute lung injury in AHNP. Activated leukocytes and inflammatory mediators, such as interleukin-1 and tumor necrosis factor α -have been suggested to play a predominant role in the development of acute lung injury in AHNP^[4-6]. Further investigation have found that pretreatment of mice with antineutrophil serum results in reduction of severity of pancreatitis and complete prevention of lung injury^[7]. More recently, Hofbauer *et al*^[8-10] postulated that the pancreatitis-associated lung injury (PALI) might be dependent on the continued generation and action of platelet-activating factor (PAF). Yamaguchi *et al*^[11] have suggested that the PAF antagonist TCV-309 effectively prevented cytokine-induced neutrophil chemoattractant expression by bronchoalveolar macrophages and subsequent PALI. Furthermore, attenuation of pancreatitis and prevention of PALI could be achieved with recombinant PAF acetylhydrolase. P-selectin is a key determinant of

leukocyte recruitment, which is upregulated in the pulmonary endothelium during acute pancreatitis^[12]. However, detailed mechanisms of acute lung injury in AHNP has not been elucidated.

In the current study, sodium taurocholate-induced pancreatitis rat models was used to investigate the apoptosis of alveolar epithelial cells and expression of apoptosis-regulated gene in PALI, and the relationship between TGF β_1 and apoptosis.

MATERIAL AND METHODS

Material and chemicals

Sodium taurocholate was obtained from Sigma Chemical Co.(USA). *In situ* cell death detection kit, POD was purchased from Borehinger Mahheim Co. (Germany), proteinase K was from Merck Co.(USA), the purified goat anti-bax polyclonal antibody and mouse anti-p53 monoclonal antibody were from Santa Cruz Biotechnology Inc. (USA), the goat anti-TGF β_1 polyclonal antibody was from Promega Co.(USA), and immunochemical SP kit was from Biotech Co.(USA). Other materials and chemicals were obtained from commercial sources.

Animals

Male Wistar rats, weighing 200g-250g, were used. Animals were bred and housed in standard cages in a climate-controlled room with an ambient temperature of 22°C \pm 2°C and a 12-hour light/dark cycle. They were fed standard laboratory chow, given water *ad libitum*, and fasted overnight before each experiment.

Models for pancreatitis

Male Wistar rats were anesthetized with 2.5% pentobarbital (0.1mL/100g body weight intraperitoneally). A midline laparotomy was performed, followed by the ligation of the bile-pancreatic ducts close to the liver and duodenum. Then pancreatic duct was retrogradely injected 5% sodium taurocholate (0.1mL/100g body weight) for 1 minute, and stagnant for 4 minutes. For control, sham operation or retrograde infusion of normal saline into the pancreatic duct was performed as described above, 2, 5 and 14 hours after sodium taurocholate infusion, laparotomy was performed again and blood samples were collected aseptically from the abdominal aorta. The pancreas and right lung were rapidly removed and fixed in 10% neutral phosphate buffered formalin for histological study. Pancreatitis was confirmed by measuring amylase levels before and after the

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experiment and by histological examination.

Serum amylase activity

Serum amylase activity was measured by a chromogenic method with the phadeba amylase test.

Detection of apoptosis

Morphological examination Paraffin-embedded pancreas and lung samples were sectioned (4 μ m), stained with hematoxylin/ eosin (H & E), and examined by an experienced morphologist who was unaware of the sample identity^[13,14].

TUNEL on light microscope TdT-mediated dUTP nick-end labeling (TUNEL) was performed according to the method of Gavrieli *et al*^[15,16] with some modification. Briefly, the sections were deparaffinized by heating for 20 minutes at 60°C, rehydrated in descending concentrations of ethanol (100%, 95%, 90%, 80%) and then immersed in double distilled water (DDW). After rehydration, the sections were incubated with 20mg/L proteinase K for 15 minutes at room temperature. The slides were washed in DDW for 2 minutes 4 times and covered with 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase. The slides were rinsed with phosphate-buffered saline (PBS, pH 7.4) 5 minutes three times and incubated the slides to 50 μ L TUNEL reaction mixture for 60 minutes at 37°C. After washing in PBS, the slides were incubated with 50 μ L Converter-POD for 30 minutes at 37°C. The reaction products were visualized by immersion in diaminobenzidine solution. For positive controls, TUNEL was performed after deoxyribonuclease treatment. For negative controls, TUNEL was performed with labeling solution instead of TUNEL reaction mixture. Observations and photographs were made using an Olympus microscope. TUNEL positive nuclei were counted in fields (\times 40) magnification chosen at random, and the number of labeled nuclei per total nuclei in those fields was expressed as the apoptotic index (AI).

Immunohistochemical staining Immunohistochemical staining was performed to explore the expression of apoptosis-regulated gene bax and p53, as well as TGF β ₁. Consecutive 4 μ m paraffin-embedded tissue sections were subjected to immunostaining with streptavidin peroxidase technique. The sections were deparaffinized, rehydrated in descending concentrations of ethanol and then digested by incubation with 0.1% trypsin for 20 minutes at 37°C. Tissue sections were submerged for 15 minutes in 0.1M PBS containing 0.1% Triton X-100. Endogenous peroxidase activity was blocked by incubating the slides with 0.3% (vol/vol) H₂O₂ in methanol, followed by washing in PBS. The sections were incubated for 30 minutes

at room temperature with normal goat serum before overnight incubation at 4°C with either goat anti-bax polyclonal antibody diluted 1:50 in PBS, mouse anti-p53 monoclonal antibody diluted 1:50 in PBS or goat anti-TGF β ₁ polyclonal antibody diluted 1:100 in PBS. After washing in PBS, slides were treated with biotinylated link immunoglobulins biotinylated goat anti-mouse IgG antibody was used for bax and p53 staining. The slides were then incubated for 30 minutes with streptavidin-biotin-peroxidase complex and were visualized by a 10 minutes application of diaminobenzidine substrate. The sections were counterstained with hematoxylin to identify nuclei and observed under a light microscope. It was judged that positive staining nuclei were brown, and immunolocalized nuclei. The positive rate of bax and p53 were counted in fields (40 \times) magnification chosen at random, and the number of positive staining nuclei per total nuclei in those fields was expressed as the positive rate.

Statistical analysis

Results were expressed as the mean and standard error of the mean (SEM). The significance of changes was evaluated by using Student's *t* test when the data consisted of only two groups or by analysis of variance (ANOVA) when comparing three or more groups. A *P* value <0.05 indicated a significant difference.

RESULTS

Acute hemorrhagic necrotizing pancreatitis

AHNP was manifested by a rise in serum amylase activity and morphological evidence. In all animals a marked elevation of serum amylase levels was observed at 2, 5, and 14 hours after sodium taurocholate infusion. The morphological changes were observed after sodium taurocholate infusion and included obvious acinar cell necrosis, fat necrosis, extensive neutrophils infiltration, microthrombosis of pancreatic vessels and massive intralobular hemorrhage. In addition, 1 rat (25%) died before the end of the experiment (14 hours after sodium taurocholate infusion), whereas other animals survived the entire observation period.

Histological findings

The morphological changes observed in lung included pronounced interstitial edema associated with massive neutrophils infiltration, alveolar wall thickening and foci of vascular thrombosis in large vessels. Simultaneously, increase of pulmonary microvascular permeability was found.

Evidence of apoptosis

Morphological examination In the H&E staining, alveolar epithelial cells presented pyknotic nuclei, cell shrinkage, condensation of chromatin and formation of apoptotic bodies, etc. which were the typical morphological features of apoptosis.

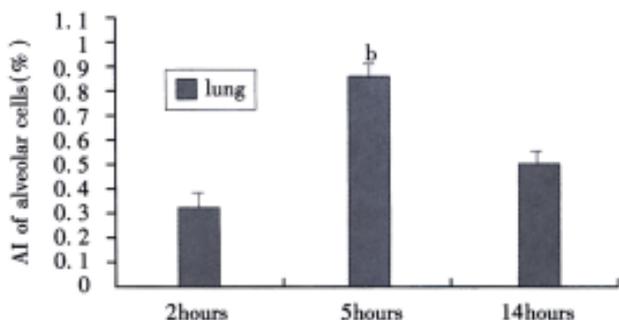


Figure 1 (PDF) AI of alveolar epithelial cells in sodium taurocholate-induced pancreatitis-associated lung injury. AHNP was induced by retrograde injections into the pancreatic ducts of 5% sodium taurocholate (0.1mL/100g body wt) in rats. Results shown are the MEAN±SEM for four or more animals in each group. Asterisks indicates ^b $P<0.01$ when the AI of alveolar epithelial cells at 5 hours after induction of AHNP compared with 2 and 14 hours after induction of AHNP.

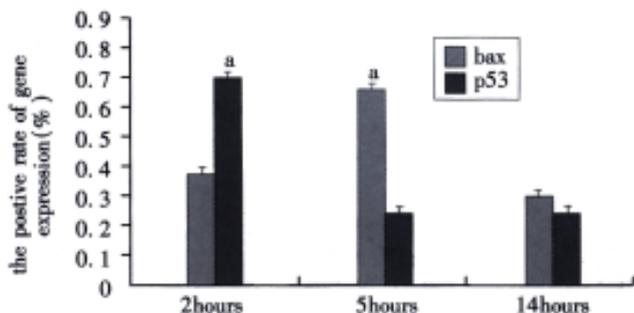


Figure 2 (PDF) The positive rate of bax and p53 protein in alveolar epithelial cells after sodium taurocholate-induced pancreatitis-associated lung injury. AHNP was induced by retrograde injections into the pancreatic ducts of 5% sodium taurocholate (0.1mL/100g body wt) in rats. Results shown were the MEAN±SEM for four or more animals in each group. Asterisks indicates ^a $P<0.05$ when the positive rate of bax protein in alveolar epithelial cells at 5 hours after induction of AHNP compared with 2 and 14 hours and the positive rate of p53 protein in alveolar epithelial cells at 2 hours after induction of AHNP compared with 5 and 14 hours.

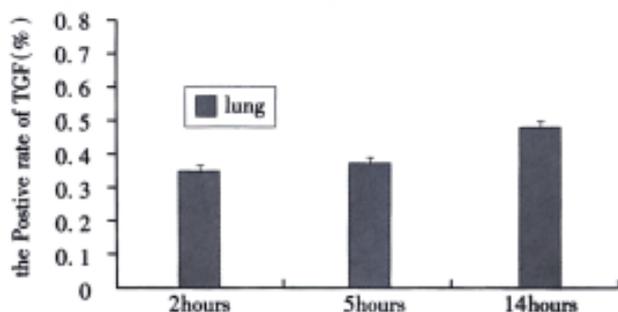


Figure 3 (PDF) The positive rate of TGFβ₁ in alveolar epithelial cells after sodium taurocholate-induced pancreatitis-associated lung injury. AHNP was induced by retrograde injections into the pancreatic ducts of 5% sodium taurocholate (0.1mL/100g body wt) in rats. Results shown were the MEAN ± SEM for four or more animals in each group. There was no significant difference among the positive rate of TGFβ₁ at 2, 5 and 14 hours after induction of AHNP.

TUNEL staining As shown in Figure 1, TUNEL selectively labeled alveolar epithelial cells nuclei. The apoptotic index of alveolar epithelial cells at 2,

5, and 14 hours after induction of AHNP were 0.33% ± 0.07%, 0.89% ± 0.06% and 0.54% ± 0.08%, respectively. Moreover, the apoptotic index of alveolar epithelial cells at 5 hours after induction of AHNP was significantly higher than those of 2 and 14 hours ($P<0.01$).

Immunohistochemical detection of bax and p53 protein

As shown in Figure 2, the positive rate of bax protein in alveolar epithelial cells at 2, 5 and 14 hours after induction of AHNP were 0.38% ± 0.11%, 0.67% ± 0.13% and 0.39% ± 0.03%, respectively. Similar to AI of alveolar epithelial cells, the positive rate of bax protein in alveolar epithelial cells at 5 hours after induction of AHNP was similarly markedly higher than those of 2 and 14 hours ($P<0.05$). In this study, the positive rate of p53 protein in alveolar epithelial cells at 2, 5, and 14 hours after induction of AHNP were 0.72% ± 0.29%, 0.28% ± 0.08% and 0.32% ± 0.04%, respectively. The positive rate of p53 protein in alveolar epithelial cells significantly decreased at 5 and 14 hours after induction of AHNP compared with that of 2 hours ($P<0.05$).

Immunohistochemical detection of TGFβ₁ Immunohistochemical staining of TGFβ-1 was scarcely detected in normal lung tissues. As shown in Figure 3, although the positive rate of TGFβ-1 in alveolar epithelial cells at 2, 5, and 14 hours after induction of AHNP were 0.35% ± 0.14%, 0.39% ± 0.15% and 0.48% ± 0.12%, respectively, no significance difference was observed among them.

DISCUSSION

Acute lung injury and adult respiratory distress syndrome (ARDS) are frequently observed during the course of severe acute pancreatitis. The pathogenesis of PALI remained unclear. Bhatia *et al*^[17] reported that neurogenic factors, such as substance P, increased release of proinflammatory mediators from the pancreas leading to increase lung injury by acting via NK1R on acinar cells. Recent studies^[18] have suggested that intercellular adhesion molecule 1 (ICAM-1) expression on pulmonary microvascular endothelial cells during pancreatitis contributed to the evolution of PALI by promoting neutrophil sequestration within the lung. Moreover, treatment with monoclonal antibodies against ICAM-1 significantly reduced the lung injury in severe acute pancreatitis^[19]. Very recently, Tsukahara *et al*^[20] noted that alveolar macrophages were activated by PLA₂, mainly by sPLA₂ type II, and produced a large amount of NO that contributed to lung injury in acute pancreatitis. Further studies had suggested that the lung injury was reduced by the administration of the PLA₂ inhibitor. Recent studies showed that the absence of T and B lymphocytes could prevent severe pulmonary injury resulting from acute pancreatitis, this indicated that

systemic lymphocyte activation modulated the systemic response, in particular, pulmonary injury caused by acute pancreatitis^[21]. Apoptotic cell death of renal tubules and hepatocytes have been reported to be involved in the mechanisms of renal failure and liver failure during severe acute pancreatitis^[22,23]. Although numerous trials had been done to clarify the mechanisms of pancreatitis-associated lung injury, there was no literature referring to involvement of apoptosis in pancreatitis-associated lung injury.

Apoptosis was initially confirmed as a specific form of cell death that served to eliminate excessive or unwanted cell types during embryogenesis and normal tissue growth^[24], but had been recently clarified to be induced in cellular injury with inflammatory disease^[25]. It was generally believed that apoptosis was a genetically regulated form of cell death, and pyknotic nuclei and formation of apoptotic bodies were the typical features of apoptosis^[26,27]. In the current work, we selected two apoptosis-regulated gene bax and p53, which were confirmed to be involved in the control of apoptosis. Proapoptotic gene bax, one of the members of apoptosis-regulated gene bcl-2 family, was a dominant inhibitor of bcl-2 and promoted cell apoptosis^[28-30]. Tumor suppressor gene p53 was a special related gene with activation of cell-cycle arrest and apoptosis^[31,32]. A very important role of normal or wild type p53 as a "guardian of the genome" was capable of inducing apoptosis upon DNA damage. Recent studies suggested that activation of NF-kappa B activity augmented p53-induced apoptosis^[32]. On the contrary, mutant p53 failed to initiate apoptosis process, so mutant p53 was thought to have effect on apoptosis inhibition. More recently, Buckley *et al*^[33] demonstrated that apoptosis via induction of p53, p21, and bax proteins was seen in alveolar epithelial cells cultured from hyperoxic rats, and apoptosis might be a protective mechanism that limits lung injury^[34].

TGF β family in mammalian cells was comprised of at least three homologous polypeptides that could regulate a variety of cellular functions, including proliferation and differentiation processes^[35,36]. Recently, some investigators reported that TGF β_1 could induce apoptosis in normal and malignant cells^[37,38]. It was demonstrated that the induction of apoptosis by TGF β_1 was due to the regeneration of reactive oxygen species in the cells, the involvement of caspase family proteases^[39-41], induction of p53 and bax^[37], or expression of TIEG (TGF β inducible early gene)^[42-44]. In this study, we sought to explore the potential relationship between TGF β_1 and apoptosis of alveolar epithelial cells in pancreatitis-associated lung injury.

We found that the apoptotic index of alveolar epithelial cells at 5 hours after induction of AHNP was significantly higher than those of 2 and 14 hours

($P < 0.01$), and the positive rate of bax protein in alveolar epithelial cells at 5 hours was markedly higher than those of 2 and 14 hours after induction of AHNP ($P < 0.05$). Interestingly, the positive rate of bax protein in alveolar epithelial cells was paralleled to the AI of alveolar epithelial cells in pancreatitis-associated lung injury. The positive rate of p53 protein in alveolar epithelial cells dramatically decreased at 5 and 14 hours after induction of AHNP compared with 2 hours ($P < 0.05$). Similarly, the positive rate of p53 protein in alveolar epithelial cells was intended to parallel to the AI of alveolar epithelial cells in PALI. Immunostaining of TGF β_1 was detected at 2, 5 and 14 hours after induction of AHNP, no significant difference was observed among them. Our data demonstrated that apoptosis of alveolar epithelial cells might be mediated by apoptosis-regulated gene bax and p53, but TGF β_1 was not implicated in apoptosis of alveolar epithelial cells.

In summary, apoptosis of alveolar epithelial cells might be involved in PALI, and the mechanisms of apoptosis probably correlates with the expression of apoptosis-regulated gene bax and p53 but is not related with the expression of TGF β_1 . Recent studies^[45] have suggested that mild pancreatitis was found to be associated with extensive apoptotic acinar cell death while severe pancreatitis was noted to involve extensive acinar cell necrosis but very little acinar cell apoptosis. More recently, some investigators^[46-48] have found that induction of apoptosis in pancreatic acinar cells attenuates the severity of experimental acute pancreatitis. Furthermore, Kaiser *et al*^[45] have demonstrated that inhibition of apoptosis by administration of cyclohexamide could enhance the severity of pancreatitis. These data have led to the concept that pharmacological induction of apoptosis in pancreatic acinar cell injury to reduce inflammatory reaction provides a new therapeutic strategy for the treatment of acute pancreatitis. Very recently, we found that the mechanisms of SSA in treating acute pancreatitis might be induction of apoptosis in pancreatic acinar cell injury to reduce inflammatory response^[49].

Future studies for clarifying the initiators, regulators, genetic control and signal transduction pathway of apoptosis of alveolar epithelial cells, would be able to reveal the pathogenesis of PALI, and further elucidate the role of apoptosis of alveolar epithelial cells in PALI.

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