PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Reviews

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

Luo DZ, Vermijlen D, Ahishali B, Triantis V, Plakoutsi G, Braet F, Vanderkerken K and **Wisse E**

Subject headings pit cells; hepatic natural killer cells; large granular lymphocytes

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 deve lopment 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 c ommon 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 immunohistoc hemistry, using mAb 3.2.3 against NKR-P1A (a specific marker of NK cells), the

Dian Zhong Luo[1,3], David Vermijlen[1], Bülent Ahishali[1], Vasilis Triantis[1], Georgia Plakoutsi[1], Filip Braet[1*], Karin Vanderkerken[2*] and Eddie Wisse[1]

[1]Laboratory for Cell Biology and Histology, [2]Department of Hematology and Immunology, Free University of Brussels (VUB), Brussels-Jette, Belgium

[3]Department of Pathology, Guangxi Medical University, Nanning, China

*Filip Braet and Karin Vanderkerken are postdoctoral research fellows of the Fund for Scientific Research Flanders.

Dian Zhong Luo, male, born on 1955-07-07 in Guilin, Guangxi, graduated from Guangxi Medical University in 1982, got Master degrees in 1987 in Guangxi Medical University and in 1994 in Free University of Brussels, Professor of Pathology of Guangxi Medical University, and now is following Ph.D. program in Medical Sciences in Free University of Brussels, Belgium, having more than 30 papers published.

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Correspondence to: Prof. Dr. Eddie Wisse, Laboratory for Cell Biology and Histology, Free University of Brussels (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium
Tel. (32)-2-477 4404, Fax. (32)-2-477 4405
Email. wisse@cyto.vub.ac.be

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number of pit cells in frozen sections of rat liver is about 13.7 per mm^2[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 cytospin 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 an d 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 an d 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 cel ls 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 N KR-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 consider ed 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 ant ibody (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 lymphocy tes is different. Most CD8$^+$ NK cells express CD8á/CD8á homodimers rather than the CD8á/CD8â heterodimers prevalent on cytotoxic T cells[33]. 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(1 0)
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 referen ces 2, 13, 16, 19, 25, and 32.

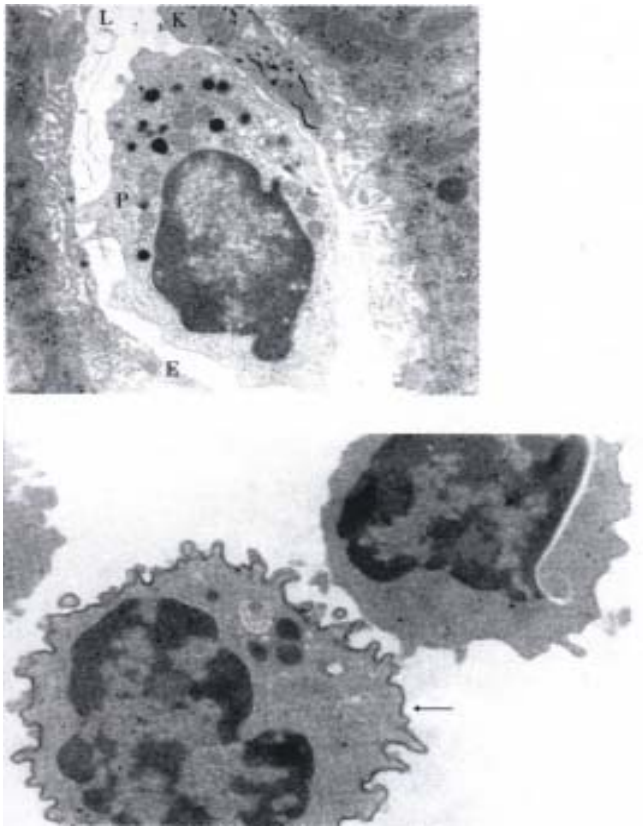

Figure 1 Transmission electron micrograph of a pit cell in a rat hepatic sinusoidal lumen (L). The pit cell shows polarity with an eccentric nucleus. The cytoplasm is abundant and contains characteristic electr on-dense granules and other organelles lying mainly on one side of the nucleus. The cell contacts 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 Immuno-transmission 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 cyt oplasm 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 centri fugation. The mononuclear cells recovered from the interface of Ficoll-Paque gr adient contained T cells, pit cells, B cells, monocytes, and a few Ec and Kc. Ad herent 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]. Howe ver, 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 heterogeneo us 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 fro m 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 asia lo-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 resist ant 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 disappear ed 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 differentiat e 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 dichloromethy lene 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 c ells 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 coincuba tion 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 spontaneo us 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 contai n 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 ^{51}Cr 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].

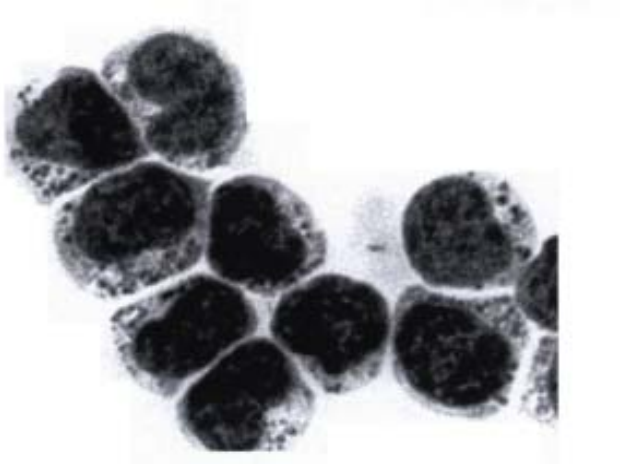

Figure 3 Light micrograph of an isolated and purified pit cell population in a May-Grünwald-Giemsa-stained cytospin. The cells contain cytoplasmic granules, which can be used to recognize and count the number of pit cells in freshly isolated liver-associated lymphocyte population. Bar = 5 μm.

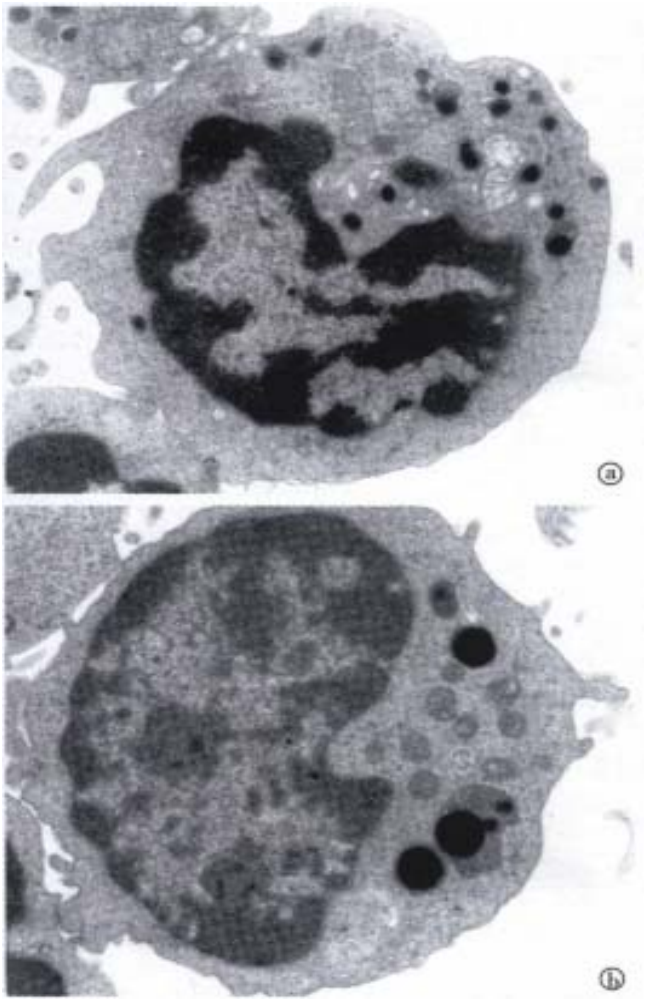

Figure 4 Transmission electron micrographs of a typical LD pit cell and a blood NK cell (B).
(A) The main morphological characteristic of LD pit cells, compared to HD cells and blood NK cells, is the presence of numerous small cytoplasmic granules.
(B) Note the few, but large granules in blood NK cell. 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]. Comp ared 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 tar get 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 CD_2[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 $Fc\gamma R^+$ 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 glycoprotei ns 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 ex tent[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 ant i-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 enc oded 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 remar kable 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 pre sent 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 sequ ence in the cytoplasmic domain and NKG2C lacks an ITIM, the CD94/ NKG2A complex acts as inhibitory receptor, whereas CD/NKG2C complex acts as a noninhi bitory ceceptor 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], mous e[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 Ca^{2+} 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 tar get 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 cont roversial results, such as activation, inhibition or no effect, depending on the NK cell population studied[27,87]. The conditions determining the outco me of the engagement of NKR-P1 in human NK cells are not known. When human NKR-P is compared with the corresponding rat and mouse proteins, it was found that all rodent NKR P1 has the C×CP motif that interacts with phosphorylated P56[1ck][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 disti nct 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 ^{51}Cr-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-medi ated 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 (CD95 L, 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 (TN F) 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 con tains 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 pi t 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/ CD 95L plays an important role in the killing of virus-infected cells and tumor ce lls 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 mediat ed 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. Gr anzymes 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 me mbrane 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 relocalizes to a nuclea r position, rapidly inducing apoptosis[102,103]. These data indicate that the cooperation of the two molecules is necessary to induce apoptosis including DNA fragmentation.

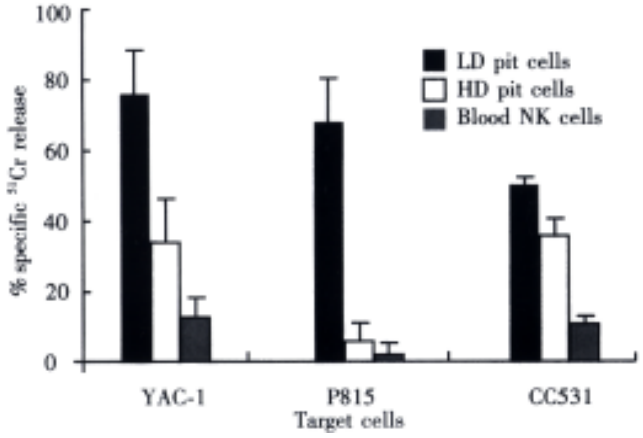

Figure 5 Comparison of cytolysis between rat blood N K, HD and LD pit cells. The ratio of freshly isolated effector cells to target cells was 20:1.The cytolysis was measured in a 4 hour ^{51}Cr-rele ase assay for YAC-1and P815 cells and a 16 hour ^{51}Cr-release assay for CC531s cells. The data show that LD pit cells are more cytotoxic against YA C-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)

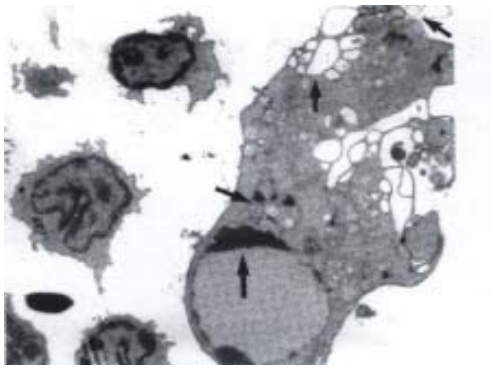

Figure 6 Transmission electron micrograph of an apoptotic CC531s cell (T) coincubated with pit cells (E) for 3 hours. The apoptotic CC531s cell (T) shows vacuolization (large arrowhead), blebbing of the cell surface (small arrowhead), chromatin condensation (thin arrow), and fragmentation of the nucleus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

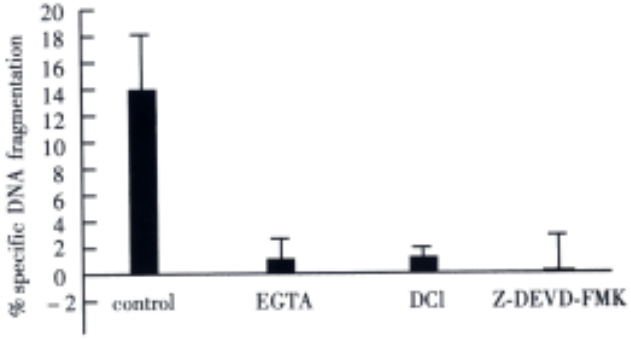

Figure 7 The involvement of the perforin / granzyme pathway in pit cell-induced CC531s apoptosis. The ratio of freshly isolated pit cells to CC531s cells was 10:1. Apoptosis was measured in a 3 hour DNA-fr agmentation assay. EGTA is a Ca^{2+} chelator that blocks granule exocytosis and the action of perforin. DCI is a granzyme inhibitor. Z-DEVD-FMK is a 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 s 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% iso-osmotic Per coll gradient centrifugation. These two populations are shown to differ morphol ogically, 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-GM 1 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 metastasing colon cancer cells. The role of pit cells in a number of liver pathologies deserves more attention.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Wei JG, Wang YC, Du F and **Yu HJ**

Subject headings hypercholesterolemia; cholithiasis; sphin cter of Oddi/ultrastructure

Abstract

AIM To study the relationship between pre-formation of galls tone 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 observatio n under electron microscope were performed.

RESULTS In groups I and II, the basal pressure in low-pressu re 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-press ure 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.

Jing Guo Wei, Yao Cheng Wang, Fan Du and Hou Jun Yu
Department of Radiology, Tangdu Hospital, the Fourth Military Medical Un iversity, Xi'an 710038, Shaanxi Province, China
Dr. Jing Guo Wei, male, born on 1944-07-12 in Shenyang City, Liaoning Province, graduated from the Fourth Military Medical University in 1968, now pr ofessor and director of department of radiology, majoring gastroenterology, coau thored two books, having 39 papers published.
Correspondence to: Dr. Jing Guo Wei, Department of Radiology, Tangdu Hospital, the Fourth Military Medical University, Xi'an 710038, Shaanxi Province, China
Tel. +86-29-3577163
Email.tdradio@fmmu.edu.cn
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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. Hyperchole sterolemia 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) a ngiographic 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 billiary 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 paraformal dehyde PBS solution at 4 ℃ for 16-18 hours, then put into 100 g/L sourose solution and preserved at low temperature according to the method of Thune A[2]. After NPDPH stain, the total number of NADPH, mean string length and spacing were measured with MPIAS 500 Multimedia Color Pathologic Imag e Pattern Analysis System (Qing Ping Imaging Corp.).

Statistical analyses

All results were expressed as $\bar{x}\pm s$. Differences were tested for sign ificance by Student's *t* test, and $P<0.05$ was considered as significan t 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 ([I]$P<0.01$, t = 2.8 *vs* control. [II]$P<0.01$, t = 3.96 *vs* control), and there was no difference between group I and II ([b]$P>0.05$, $t = 1.04$ *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 ($P>0.05$).

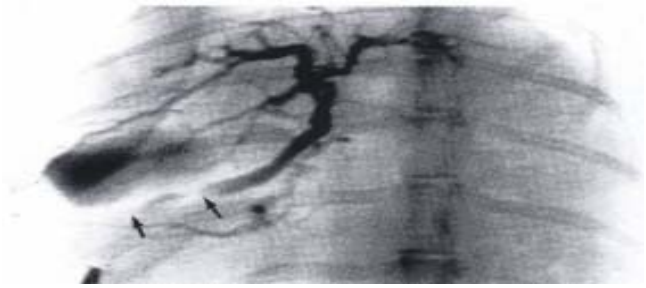

Figure 1 The cineradiographic recording of same bili ary tract of one control. The contrast clustering the proximal segment (low-pre ssure ampulla) which enlarged significantly during excretion of contrast indicat ed that part was diastoled. (↑→↑: External segment of SO)

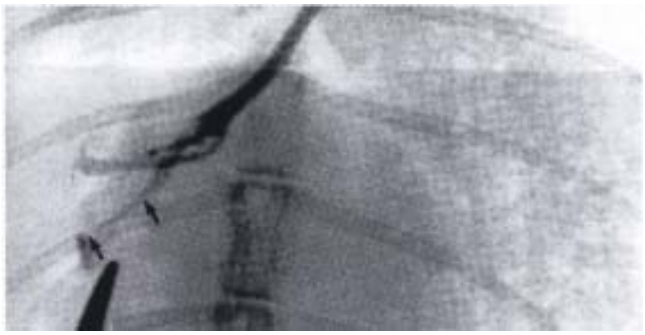

Figure 2 Biliary tract cineradiographic image of gr oup I rabbit. The caliber of proximal segment decreased, retained a fixed spasm state. (↑→↑: External segment of SO)

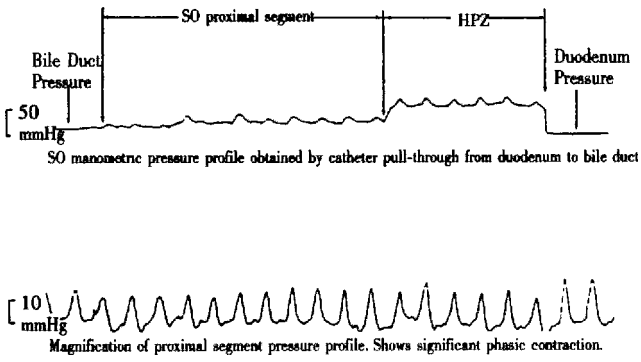

Figure 3 Pressure curve of SO segment of control rabbit.

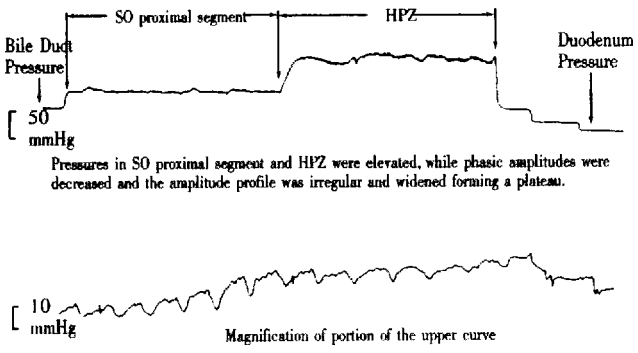

Figure 4 Pressure curve of SO segment of group I rabbit.

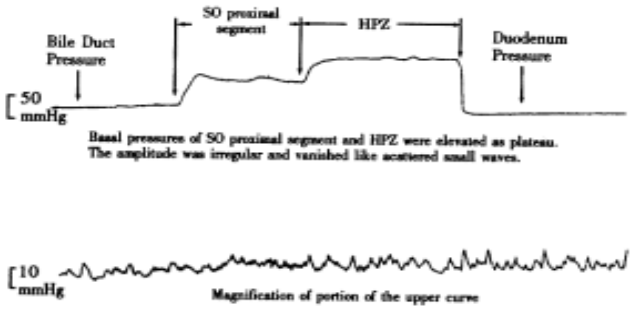

Figure 5 Pressure curve of SO segment of group II rabbit.

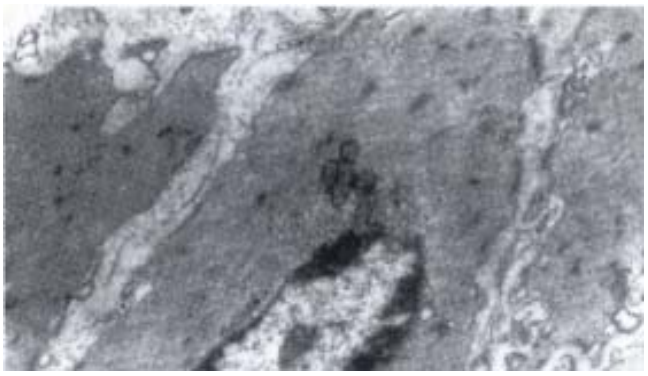

Figure 6 Electron-microscopic scanning of a control 's SO segment smooth muscle. The myofilaments were regularly arranged, kink mac ula densa clear and dense, plasmosome was normal.

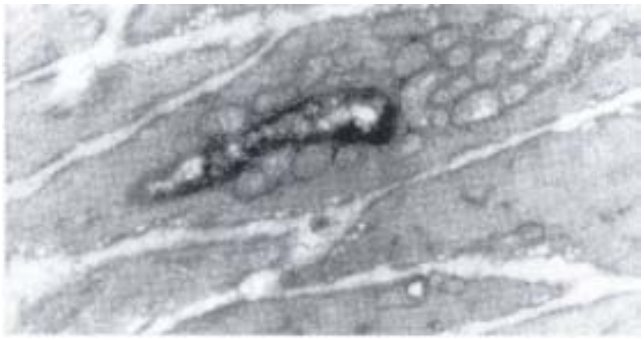

Figure 7 Electron-microscopic scanning of a group I rabbit's SO smooth muscle. Image showed the swelling of plasmosome and disap pear of intercristal space, decrease of kink macula densa and twisting of myofil aments.

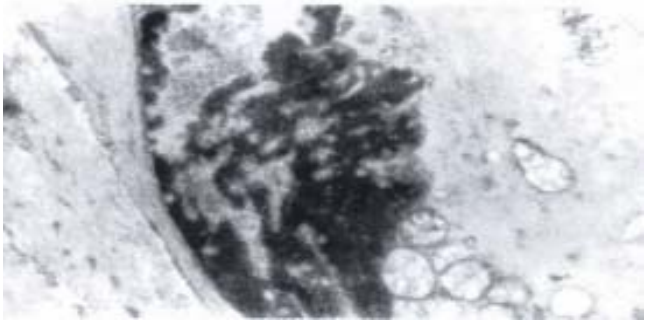

Figure 8 Electron-microscopic scanning of a group II rabbit's SO segment smooth muscle. It shows the swelling of plasmosome, irre gular arranged or fragmented intercristal space, some vacuolized, congregated at one end of nuclear, and disarragement of kink macula densa.

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 30 g/L glataraldehyde promptly and pre-fixed for 2-4 hours under 4 ℃, 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 intercristal space disappeared or vacuolized and congreg ated at the top of the nuclear of plasmosome in groups I and II, but no such ch anges 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 dyskinesis 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 phys iological anatomy consisted of a high pressure distal segment and a low-pressu re 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 construct ion 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 signif icantly decreased the phasic amplitude in the experimental animals. The cineradi ography showed that the low-pressure ampulla retained a fixed spasm state, thus, the diastolic and peristaltic functions were impaired. According to the hydrodynamic laws, Qout = -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 am pulla 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 demo nstrate 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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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Du YP, Deng CS, Lu DY, Huang MF, Guo SF and **Hou W**

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 health y 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 respectivel y than those in healthy controls (0.40 *vs* 0.20, $P = 0.003$, $P_{corret} = 0.024$) and (0.05 *vs* 0.14, $P = 0.012$, but P corret >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.

Yi Ping Du[1], Chang Sheng Deng[1], De Yin Lu[2], Mei Fang Huang[1], Shu Fang Guo[2] and Wei Hou[2]
[1]Department of Gastroenterology, the Second Affiliated Hospital, Hubei Medical University, Wuhan 430071, Hubei Province, China
[2]Department of Clinical Cancer, Institute of Virus Research of Basic Medicine, Hubei Medical University, Wuhan 430071, Hubei Province, China
Dr. Yi Ping Du, female, born on 1964-01-03 in Wuhan, Hubei Province, graduated from Hubei Medical University as a doctor in 1998, majoring peptic ulcer immunology-genetics, now working in Shenzhen Central Hospital.
Correspondence to: Dr. Yi Ping Du, Department of Digestive Medi cine, Shenzhen Central Hospital, Lian Hua Bei, Futian District, Shenzhen 518036, Guangdong Province, China
Tel. +86-755-3061340, Fax.+86-755-3062680
BB.191-7116535
Email.Szzxyy@nenpub.szptt.net.cn
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INTRODUCTION

Duodenal ulcer is a set of extensive heterogenous 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 diagnos ed endoscopically. The ulcerogenic drugs, such as aspirin, indomethacin or corti costeroids 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'-CACGGATCCGGTAGCAGC GGTAGAGTTG-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 polyenzyme and DNA dNTP were purchased from Canadian Sangon Company. Proteinase K and pBR322/*Hae*-III Mark were provided by Huamei Bioengineer Company.

Methods

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

PCR amplification A total amount of 100 uL reaction soluti on contained 1 μg DNA sample, 200 μmol/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: predenaturation at 94 ℃ for 5 min, addition of 2.5 μ Taq DNA polymerase, denaturation at 94 ℃ for 1 min, annealing at 62 ℃ for 2 min and extension at 72 ℃ for 2 min repeated 30 cycles and finally extension at 72 ℃ 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 μL) of the reaction mixture were digested with restriction endonucleases (Apal-I, Hph-I, Fok-I and Mbo-II: 5 units) at 37 ℃ for 1 h after addition of appropriate incubation buffer. When digested by Bsaj-I, the reaction mixture was incubated at 60 ℃ for 1 h.

Acrylamide gel electrophoresis Samples of the restriction enzym e-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 discriminate d with 15% polyacrylamine 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-Weinbrg theorem, gene frequencies (AF) were calculated by AF = 1-1-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].

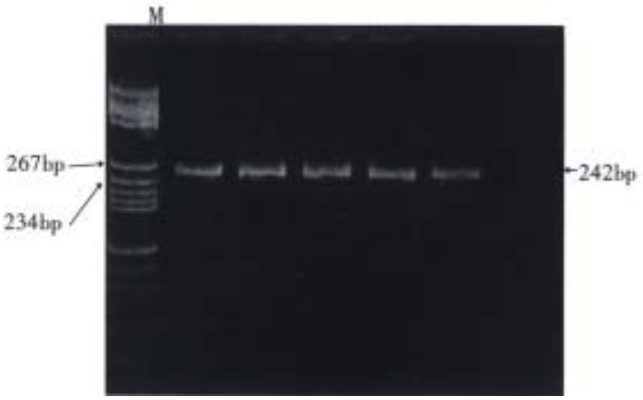

Figure 1 The size of the amplified region of the HLA-DQA1 exon 2 is 242 bp as indicated (12% PAGE). M: pBR322 / Hae-III Mark

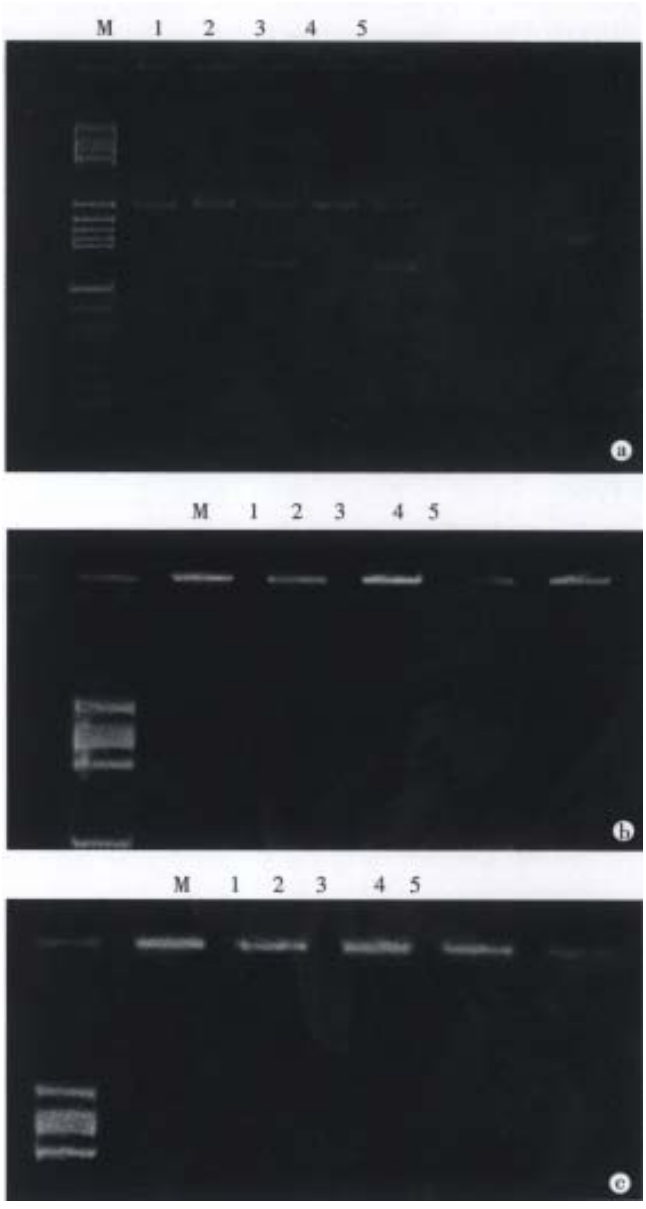

Figure 2 Cleavage patterns of polymorphic restrictio n fragments in the PCR-amplified DQA1 genes obtained with restriction enzymes (12% PAGE).
M: pBR322 / Hae-III Mark; 1: Apal-I; 2: Hph-I; 3: Bsaj-I; 4: Fok -I; 5: Mbo - II; a: 0301 / 0501; b: 0103 / 0501; c: 0102 / 0601

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 freque ncy of DQA1*0301 in patients with duodenal ulcer (0.40) was statistically si gnificant (RR = 3.20, P = 0.003, $P_{correct}$ = 0.024) compared with healthy controls (0.20). In contrast, the detect frequency and the alle le 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 pati ents 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. T he 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 in fectious 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_{corret} = 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_{corret}>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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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The relationship of lmp2 and DR3 genes with susceptibility to type I diabetes mellitus in south China Han population

Ding HL, Cheng H, Fu ZZ, Deng QL, Yan L and **Yan T**

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

Abstract

AIM To study the relationship of *lmp2* and DR3 genes with type I diabetes mellitus.

METHODS *lmp2* genotypes and DR3 were identified in 68 patien ts with type I diabetes mellitus (I-DM) and 71 healthy controls. Then, I-D M patients and controls were respectively allocated into DR3-positive and DR3- negative groups. The frequencies of *lmp2* and DR3 gene in random subjects, an d *lmp2* genotypes in DR3-matched subjects were compared between I-DM patie nts and controls. At the same time, I-DM patients were divided into 3 groups b ased 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 *lmp2* R/R in I-DM patients was lower (43% *vs* 61%, *P*<0.05) and *lmp2*-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 *lmp2*-R/R in I-DM patients was lower (47% *vs* 87%, *P*<0.05) and *lmp2*- R/H higher (47% *vs* 13%, *P*<0.05) than that in controls. In DR3-negative subjects, the frequency of *lmp2*-R/H in I-DM patients was higher than that in controls (58% *vs* 32%, *P*<0.01), but the frequency of *lmp 2*-R/R and *lmp2*-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 an younger onset age of diabetes. The *lmp2*-R/R may be the protective genotype of I-DM, and *lmp2*-R/H the susceptible genotype. These were not affected by DR3 gene. *lmp*^{2} genotypes were not related with the onset age of diabetics.

He Lin Ding, Hua Cheng, Zu Zhi Fu, Qing Li Deng, Li Yan and Tang Yan
Department of Endocrinology, Sun Yat-Sen Memorial Hospital, Sun Yat Sen University of Medical Sciences, Guangzhou 510120,China
He Lin Ding, male, born on April 18, 1963 in Feidong County, Anhui Prov ince, got master degree of medicine from Sun Yat-Sen University of Medical Scie nces in 1991. Now working as lecturer of medicine, and had 7 papers published.
Correspondence to: He Lin Ding, Department of Endocrinology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University of Medical Sciences, 107 Yanjiang West Road, Guangzhou 510120, Guangdong Province, China
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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. *lmp2* 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 *lmp2*-R or *lmp2*-H. *lmp2*-has 3 genotypes, i.e., *lmp2*-R/R, *lmp2*-R/H and *lmp2*-H/H. The r elationship between *lmp2* and I-DM is still controversial. This study aims at investigating the relationship of *lmp2* 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

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 D R3 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 preci pitation.

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 lmp2

lmp2 was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[2].

PCR amplication The PCR primers were *lmp2*-1, 5'-GCCAGCA AGAGCGGAAACAAG-3'and *lmp2*-2, 5'-GTGAACCGAGTGTTTGAGAAGC-3'. The PCR product was a DNA fragment of 252bp containing the *lmp2* polymorphism site R/H-60. PCR was performed in 50 μL of reactive volume containing 100 ng of genomic DNA, 0.8 μmol/L primer, 0.2 μmol/L dNTP, 5 μl 10×PCR buffer and 1.5 u Taq DNA polymerase. The samples were subjected to 35 thermal cycles of 50 s at 94 ℃ for denaturing, 60 s at 52 ℃ for annealing, and 60 s at 72 ℃ for extension, 7 min at 94 ℃ for denaturing before the first cycle, and 5 min at 72 ℃ for extension after the last cycle.

Hha-I digestion of PCR production The reactive volume 20 μL contained 10 μL of PCR product, and 10 u of Hha-I (Gibco). The samples were incubated in warm water bath at 37 ℃ overnight. The allele *lmp2*-R contained the *Hha*-I site, however, the *lmp2*-H did not. Therefore, the polymorphism of *lmp2* can be revealed by *Hha*-I. When Hha-I digestion was performed, the PCR products with known *lmp2* genotype were used as controls.

Statistical analysis

The χ^2 test in the 2×2 table was used to compare the frequenci es of *lmp2* 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 *lmp2* genotypes and DR3 gene were compared among groups with different onset age of diabetics by the χ^2 test in the R×C table, and if the results were significant, the Pearson's *rp* would be calculated.

RESULTS

Genotyping of lmp2 and identification of DR3 gene Figure 1 shows the DR3 gene by nested-PCR. Figure 2 shows the various genotypes of *lmp2* and the product of PCR. The identification of DR3 was made twice in 90 samples, getting 99% (89/90) precision. Genotyping of *lmp2* was performed twice in 70 samples with 100% coincidence. The primer amplifying DR3 and Hha-I digestion had excellent specificity.

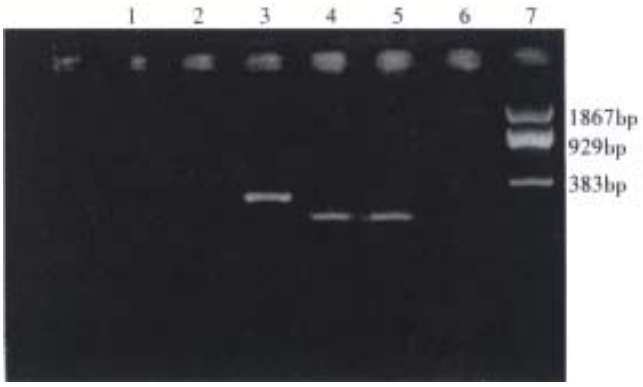

Figure 1 PCR products of DR3 and exon2 of DRB1.1. negative control of PCR; 2. exon2; 3. homozygous cell line of DR3; 4. DR3-positive sample; 5. DR3-negative sample; 6. pBR322DNA / -BstN-I Marker.

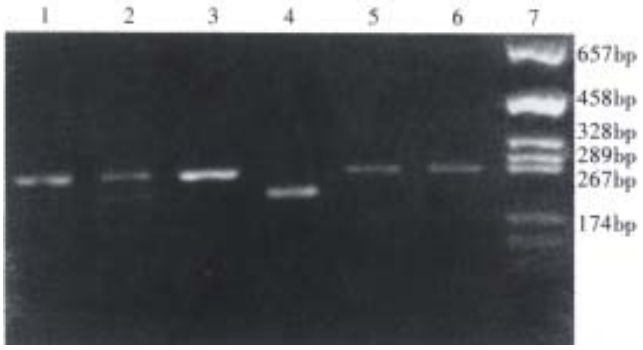

Figure 2 Genotyping of LMP2 gene by PCR-RFLP.1, 3, 5. products of PCR; 2. LMP2-R / H; 4. LMP2-R / R; 6. LMP2-H / H; 7. Pge m7Zf (+)Hae-III DNA marker.

Distribution of DR3 and lmp2 in random subjects

The frequency of *lmp2*-R/R in 1-DM patients was significantly lower, but the frequency of *lmp2*-R/H and DR3 was significantly higher than that in controls. And *lmp2*-H/H showed no significant difference between these two groups (Table 1).

Table 1 Comparison of frequencies of *lmp2* genotypes and DR3 gene between I-DM patients and controls in random subjects

	n	DR3	lmp2		
			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]x^2 = 10.44, ψ = 3.32, P<0.0 05; [b]x^2 = 4.47, ψ = 0.48, P<0.05; [c]x^2 = 8.86, ψ = 2.87, P<0.005.

Distribution of lmp2 in DR3-matched subjects

In DR3-positive subjects The frequency of *lmp2*-R/R and *lmp2*-R/H in I-DM patients was respectively lower and higher respectively than that in controls with significant difference, but *lmp2*-H/H had no significant difference between these 2 groups (Table 2).

In DR3-negative subjects The frequency of lmp2-R/H in I-DM patients was significantly higher than that in controls, while *lmp2*-R/R and lmp2-H/H had no significant differences between these two groups (Table 2).

Table 2 Frequencies of *lmp2* 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]x^2 = 7.50, ψ = 0.14, P <0.01; [b]x^2 = 4.98, ψ = 5.74, P<0.05; [c]x^2 = 6.15, ψ = 2.96, P<0.01.

Distribution of lmp2 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 *lmp2* frequencies among different groups of diabetic onset age (Table 3).

Table 3 Frequencies of *lmp2* genotypes and DR3 gene compared amon g different age groups of diabetic onset

	n	DR3		lmp2		
		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 χ^2 = 8.19, rp = 0.33, P<0.05, *lmp2* was χ^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 su ggests 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 *lmp2* and I-DM The *lmp2* encoded product is LMP protease, which is responsible for processing antigen, and may play a n important role in antigen presentation[8]. Therefore, *lmp2* may be an attractive candidate as a gene related with susceptibility to I-DM. Studies on the relation between *lmp2* and I-DM were still controversial. A recent study by Deng *et al*[9] suggested that *lmp2*-HR may be the protective genotype, and *lmp2*-R/H the susceptible genotype of I-DM, an d 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 *lmp2* genotypes had no association with I-DM, but, the samplesize was too small after divided into subgroups. The studies by Van End ert *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 *lmp2*-R/R and *lmp2*-R/H in I-DM patients were significantly lower, and higher than those in controls respectively (Table 1). Therefore, *lmp2*-R/R may be the protective genotype, and *lmp2*-R/H the susceptible genotype of I-DM. In order to make sure that the effect of *lmp2* on I-DM will be affected by DR3, we investigated the frequency of *lmp2* genotypes in DR3-matched subjects. We divided the subjects into DR3 positive and DR3 negative groups and compared the frequencies of *lmp2* 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 show ed 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 ($\leqslant$ 14 years) was significantly higher than that in adulthood ($\geqslant$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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Edited by You DY and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Xia Q, Jiang JM, Gong X, Chen GY, Li L and **Huang ZW**

Subject headings pancreatitis/ therapy; lung disease; tumor nec rosis 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 ef fect 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 meas ured, 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 majo r causes of lung injury during ANP; "Tong Xia" purgative method could allevia te the degree of lung injury mediated by TNF.

Qing Xia, Jun Ming Jiang, Xu Gong, Guang Yuan Chen, Lei Li and Zong Wen Huang
Department of TCM, The First University Hospital of West China University of Medical Sciences, Chengdu 610041, Sichuan Province, China.
Dr. Qing Xia, male, born on 1966-10-01 in Tongnan City, Sichuan Province, graduated from West China University of Medical Sciences as a postgraduate in 1996, attending physician in the Department of TCM of the university hospital, having 4 papers published.
Supported by the "8th 5-year Plan" of National Administration Bureau of Traditional Chinese Medicine and Pharmacy, No.H09301
Correspondence to: Dr. Qing Xia, Department of TCM, The First Un iversity Hospital of West China University of Medical Sciences, 37 Wainan Guoxue shiang, Chengdu 610041, China
Tel. +86-28-5422557
Email.hxydtcm@mcwcums.com
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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

in normal saline (10 mL·kg^{-1}·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 norm al 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 flu id 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 we re sectioned and preserved in 10% formalin fluid.

Tle lung wet/dry weight ratio The wet left lung was weighed and placed in the oven for 24 hours at 60 ℃, 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. The re 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 hemor rhage 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 gro up, the pulmonary edema was less severe without any patchy hemorrhage. In sham o peration group, the lung tissue was normal.

Blood amylase (Figure 1).

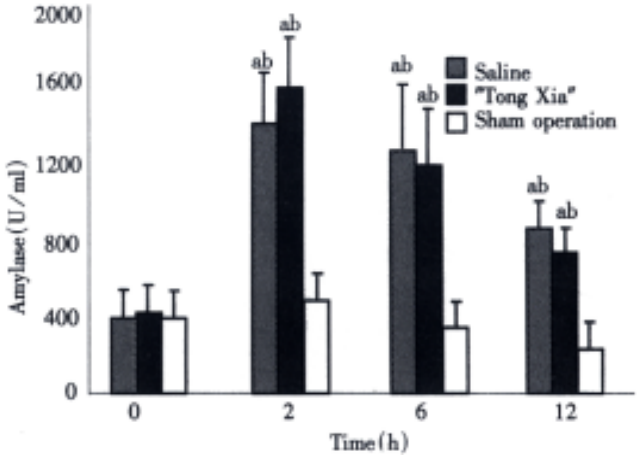

Figure 1 Serum amylase.
[a]$P<0.01$ *vs* preoperation; [b]$P<0.01$ *vs* sham operation.

Serum endotoxin(Figure 2).

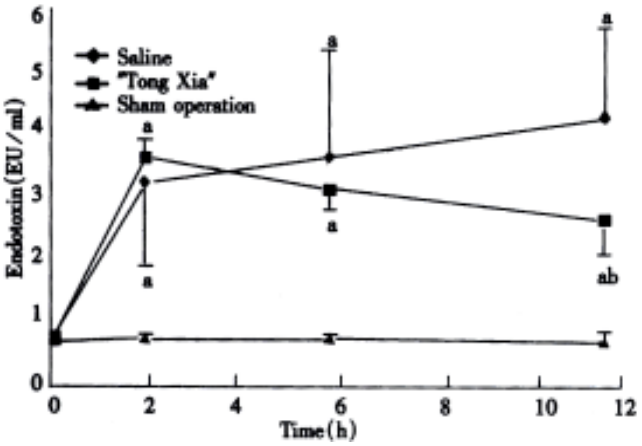

Figure 2 Serum endotoxin.
[a]*P*<0.01 *vs* sham operation; [b]*P*<0.05 *vs* saline.

Serum TNF activity(Figure 3).

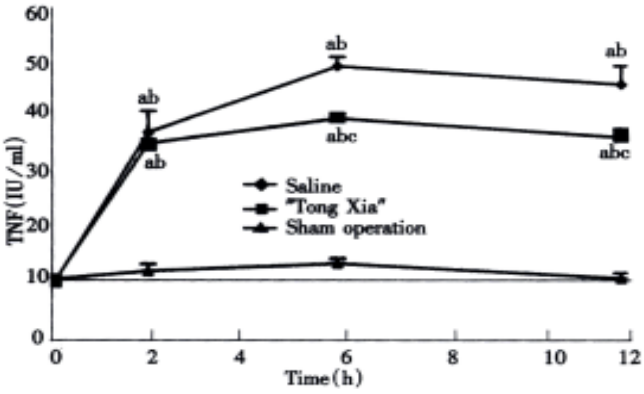

Figure 3 Serum TNF activity.
[a]*P*<0.01 *vs* preoperation; [b]*P*<0.01 *vs* sham operation; [c]*P*<0.01 *vs* saline.

Cell changes in BALF(Table 1).

Table 1 Cell changes in BALF ($\bar{x}\pm s$, $\times 10^9$/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 A_2, oxygen free radicals, cathepsin may all contribute[4]. Among them TNF plays an important role in the pr ocesses. Many authors reported that TNF activity was increased in the serum and BALF from the patients with severe acute pancreatitis and adult respiratory dist ress 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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Edited by Wu XN and Ma JY
Proofread by Miao QH

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Brief Reports

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

Hua JS, Bow H, Zheng PY and **Khay-Guan Y**

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¡æ in a humidified incubator (Forma Scientific) with 5% CO_2. 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 (BioMériux)[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℃ in 5% CO_2 atmosphere for

Jie Song Hua[1], Ho Bow[1], Peng Yuan Zheng[1] and Yeoh Khay-Guan[2]
Department of Microbiology[1] and Medicine[2], Faculty of Medicine, National University of Singapore, 5, Science Drive 2, Singapore 117597, Republic of Singapore
Dr. Jie Song Hua, male born on 1961-11-16 in Shanghai City, China, graduated from Shanghai Medical University, now a research fellow of microbiology, having 30 papers published.
This study is supported by the National University of Singapore Grant GR6431.
Correspondence to: Dr. Jie Song Hua, Department of Microbiology, National University of Singapore, 5, Science Drive 2, Singapore 117597, Republic of Singapore
Tel. +65-8743285, Fax. +65-7766872
Email. michuajs@nus.edu.sg
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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[18]. 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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Edited by Wu XN

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Schistosomiasis: progress and problems

Wu GY and **Halim MH**

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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 *Schist osoma 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 s nails, *Biomphalaria alexandra*, and Bulinus trancatus 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 (*Bulin us 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 that 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. japonicium*) have similar life cycles and develop by a succession of stages: the egg, miracidium, first stage sporocyst, second stage sporocyst, cercariae, schistosomule and adult.

George Y. Wu[1] and Mohab H. Halim[2]
[1]Department of Medicine, Division of Gastroenterology-Hepatology - University of Connecticut Health Center, Farmington, CT USA
[2]Department of Biochemistry, National Research Centre, Cairo, Dokki, Egypt
Correspondence to: George Y. Wu, M.D., Ph.D. Department of Medic ine, Division of Gastroenterology-Hepatology-University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA
Tel. (860)679-3158,Fax. (860) 679-3159
Email.wu@nso.uchc.edu
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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 ceracariae 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 penet rating 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 schistos omiasis, 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 schistoso mules 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 an d 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 month *in vitro*.

The work of Taylor *et al*[18] and Taylor[19] showed that in single-sex infestations, schistosomes migrated to the portal- mesentric 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 depen dent 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 lymp hokine 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 charact eristics of the parasite itself. After skin penetration, schistosomula 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 schistosomula 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 unres ponsive[27]. The worm tegument, which undergoes a continuous and rapid t urnover, 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 patt erns 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 bronch opulmonary manifestations attributed to the passage of schistosomules 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 alkalin e 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 com plicated 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 paranchyma, resulting eventually in liver cirrho sis 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 hepato cellular 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 cons idered to have schistosomal portal hypertension also had histological evidence of cirrhosis[39].

The portal hypertension of schistosomiasis is presinusoidal and presumably relat ed 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 prer equisite for the development of a collateral circulation, in cirrhosis the risk of bleeding cannot be directly correlated with the exact portal vein pressure, although hemo rrhage is unlikely in cases where the portal vein pressure is less than 10 mm Hg above inferior venal caval pressure[44].

Cirrhosis is defined anatomically as a diffuse process with fibrosis and nodule formation[45]. It has followed hepato-cellular 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, sperm a torrhea and hemospermia. Spontaneous recovery is rare and the condition may be complicated by the bladder ulcer, calculi, polyps, fistulae, hydroureters 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-reduc ing 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 vasculture 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 tran smission of HIV[50].

PATHOGENESIS AND PATHOLOGY

The pathological changes in schistosome infestations are caused mainly by the de position 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, neutrophiles, 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 comparis on 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 transveralis 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 schistoso miasis. 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 indivi duals[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 chemothera peutic 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 $mg \cdot kg^{-1}$) yielding 70% to 95% cure rates against all species of schistosomes infecting man. With few significant side effects and no adverse reactions o n 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 tegumentel 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 contract ion 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 toleranc e 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 ED^{50} 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 treat ment 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 does

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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Edited by You DY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Han FC, Yan XJ and **Su CZ**

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*).

Feng Chan Han, Xiao Jun Yan and Cheng Zhi Su
Chinese PLA Institute of Genetic Diagnosis, Fourth Military Medical University, Xi'an-710033, Shaanxi Province, China
Feng Chan Han, male, born on 1963-05-08 in Liquan County, Shaanxi Province and graduated from Chinese PLA Fourth Military Medical University as a postgraduate in 1998, now a lecturer, having 12 papers published.
Correspondence to: Feng Chan Han, Chinese PLA Institute of Genetic Diagnosis, Fourth Military Medical University, 17 Changle Xilu,-Xi'an 710033, Shaanxi Province, China
Tel.+86-29-3285729
Email. hanfc@263.net
Received 1999-08-10 **Accepted** 1999-10-13

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-GGGATGACTAACGAAACTATTGACC-3') and CagAp2 (5'-CAGGTCGACTTAGACTAGGG-TTCCGTTCACAC-3'). At the 5'-end of each primer, there was a restriction endo-nuclease site (*Sma*I or *Sal*I), 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 $MgCl_2$. The CagA was amplified by denaturing at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min, and extending at 72 ℃ 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 coloned the CagA gene fragment into it for sequencing.

CagA gene expression First, we digested pUC19/CagA with *Sma*I and *Sal*I 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 Eco-RI and Bam-HI, 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 ℃ overnight. We diluted (1:100) the cultured mixture of a positive clone in LB and let it grow at 30 ℃ till the late stage of logarithmic. Then we immediately raised the temperature to 42 ℃ 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 NaH_2PO_4, 11 mmol/L Na_2HPO_4 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 immonogold filtration assay (DIGFA), with staphylococci protein A as the second antibody and coloidgold 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 coloidgold and immunogold were prepared according to the published articles[10,11]. The purified FCagA was immobilized on the nitrocellulose membrane. To block n on-specific conjugate sites, the membrane was immerged in 5% bovine serum album in (BSA) for 2 h, then washed with PBS three times and laid to dry automati cally. Later, we assembled the DIGFA detecting kits. After a drop of serum was a dded 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, othe rwise, 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 DH5α with pBV220/FCagA permitted the expression of FCagA with a MW of 38 kDa when the culture temperature reached 42 ℃ (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).

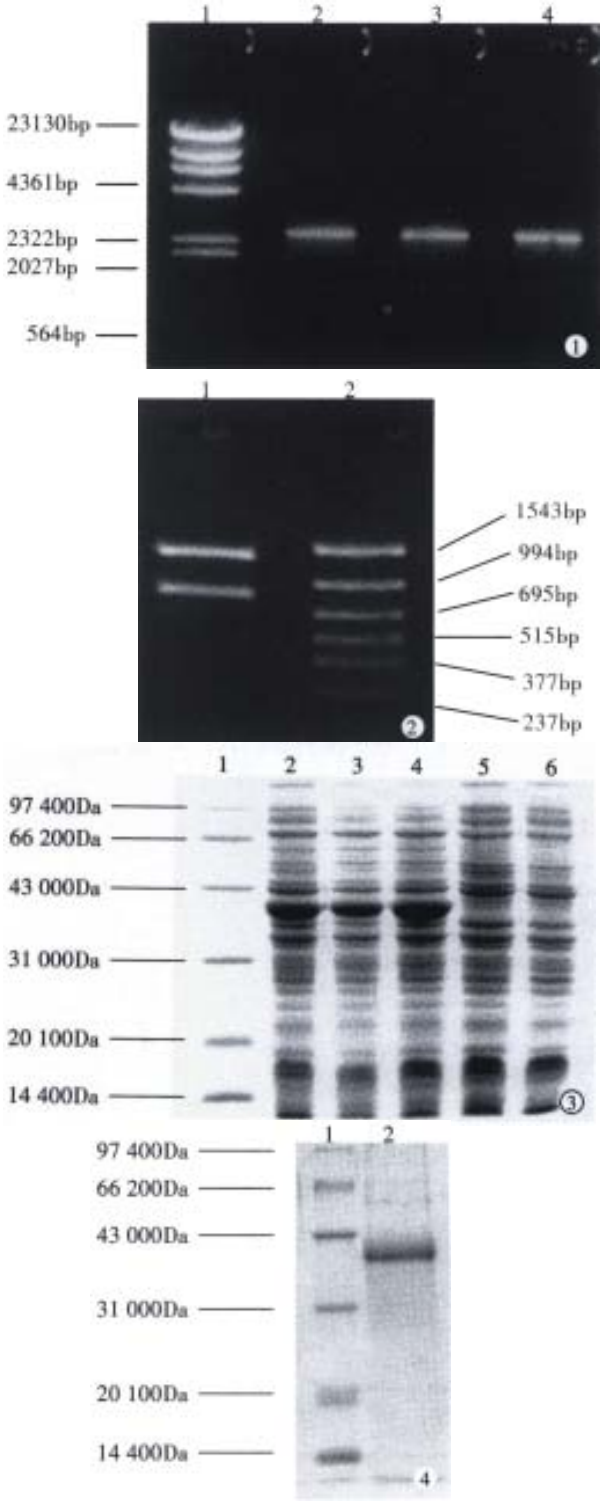

Figure 1 Analysis of the PCR product of CagA by agarose gel electrophoresis.
1: λDNA / Hind-III markers; 2: PCR product of CagA.

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-DH5α; 5:pBV220/FCagA-DH5α; 6: Heat induced pBV220/DH5α.

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 μL serum and excess amount of FCagA (10 μg) 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 ℃.

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 DH5α was transformed by pBV220/FCagA, and a 38 kDa recombinant was induced by heat when temperature reached 42 ℃. 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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Edited by Wang XL

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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The expression and clinical significance of CD44v in human gastric cancers

Chen GY and **Wang DR**

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 ℃.

Guo Yu Chen and Dao Rong Wang
Department of General Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
Dr. Guo Yu Chen, male, born on 1942-11-15, professor of Nanjing Medical University, graduated from Nanjing Medical University in 1966, specialized in gastrointestinal surgery, having more than 15 papers and 3 books published.
Supported by grant from the Science and Technology Commission of Jiangsu Province, No.98052.
Correspondence to: Guo Yu Chen M.D. The 1st affiliated hospital of Nanjing Medical University Nanjing 210029 Jiangsu Province, China.
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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 mucosas, 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 mucosas.

RT-PCR method

Primers sp1 and sp2 were taken from the cDNA sequence described by Matsumura[4]. sp1: 5'GACA-CATATTGCTTCAATGCTTCAGC3', sp2: GAT-GCCAAGATGATCAGCCATTCTGGAAT3',
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 μ TaqDNA polymerase were added, then the amplification happened in 50 μL reaction system. The conditions of PCR reaction was 94 ℃ for 50", 52 ℃ for 50" and 72 ℃ 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, ob served 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.

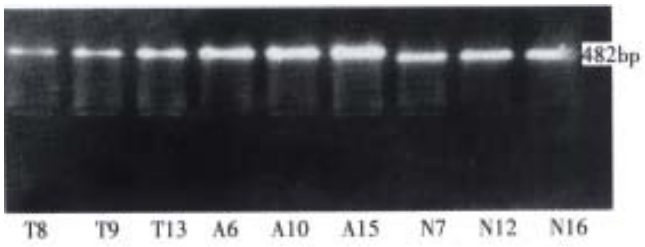

Figure 1 Electrophoretic pattern of RT-PCR product for CD44s in gastric tissues.

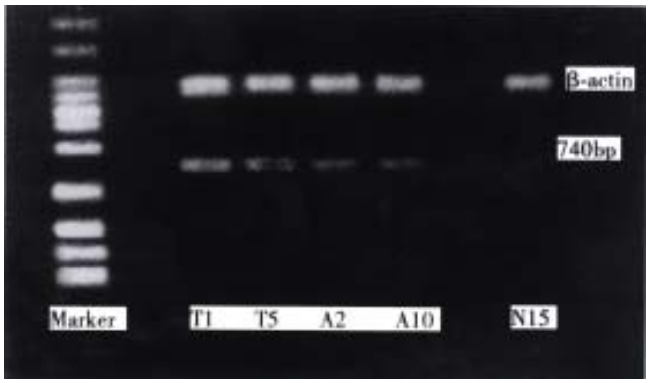

Figure 2 Electrophoretic pattern of RT-PCR product for CD44v in gastric tissues.

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

	n	CD44v mRNA		*P* value
		+	-	
Histological types				
Diffuse type	15	14	1	
Well-differentiated type	5	2	3	*P*<0.05
Lymph node metastasis				
Positive	16	15	1	
Negative	4	1	3	*P*<0.05
Tumor size				
>5 cm	13	12	1	
<5 cm	7	4	3	*P*>0.05
Clinicopathology				
Ulcerative type	13	12	1	
Infiltrative type	7	4	3	*P*>0.05
Stage				
I-II	7	4	3	
III-IV	13	12	1	*P*>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 degrated products of hyaluronic acid can motivate the growth of local vessels providing the basis for invasion and metastasis. This variated 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 munosa cells, CD44 gene only expresses CD44s protein. In metastatic cells of gastric cancer the abnormal ex pression of CD44v is strengthened more than those in primary cancer cells. There fore, 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 e asily 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 munosa 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 sig nificantly ($P<0.05$), 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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Edited by You DY and Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Zhu JS, Su Q, Zhou JG, Hu PL and **Xu JH**

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 cm^3 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.

Jian Si Zhu, Qi Su, Jian Guo Zhou, Ping Lin Hu and Jin Hua Xu
Institute of Oncology, Hengyang Medical College, Hengyang 421001, China
Dr. Jian Si Zhu, male, born in 1955 in Nanxian County, Hunan Province, Han nationality, graduated from Tongji Medical University as a postgraduate in 1988, now associate professor of pathology, majoring pathology, having 9 papers published.
Supported by the National Natural Science Foundation of China, No. 39270305
Correspondence to: Dr. Jian Si Zhu, Institute of Oncology, Hengyang Medical College, Heingyang 421001, China
Tel.+86-734-8281178, Fax.+86-734-8280907
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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 rightilium. 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, maltinucleus 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.

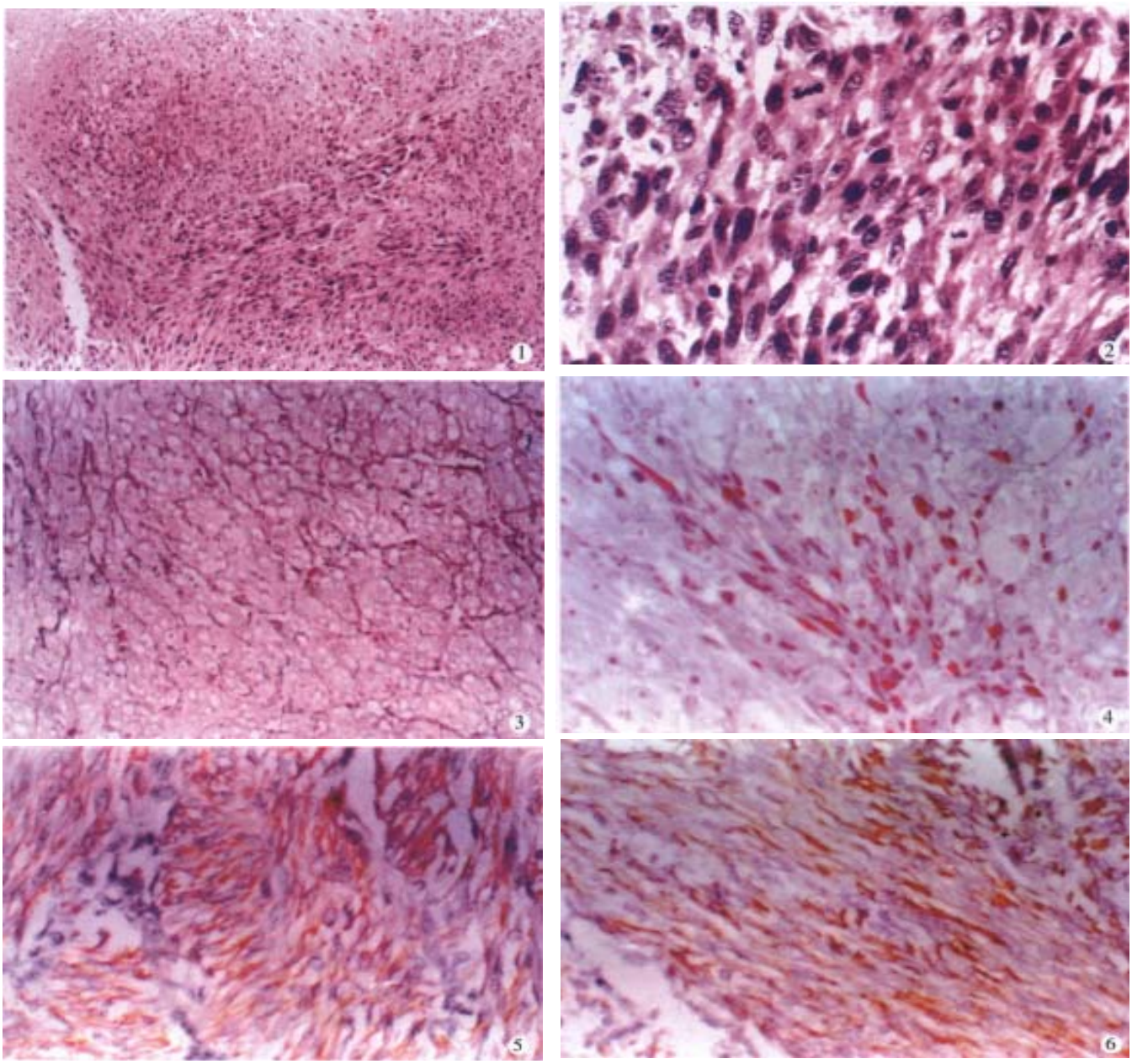

Figure 1 Interlaced arrangement of leiomyosarcoma cells stained with HE. ×20
Figure 2 Karyokinesis of leiomyosarcoma cells stained with HE. ×40
Figure 3 Reticular fibers of leiomyosarcoma cells stained with silver. ×40
Figure 4 Leiomyosarcoma cells stained red by Masson's method. ×40
Figure 5 Micrograph 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

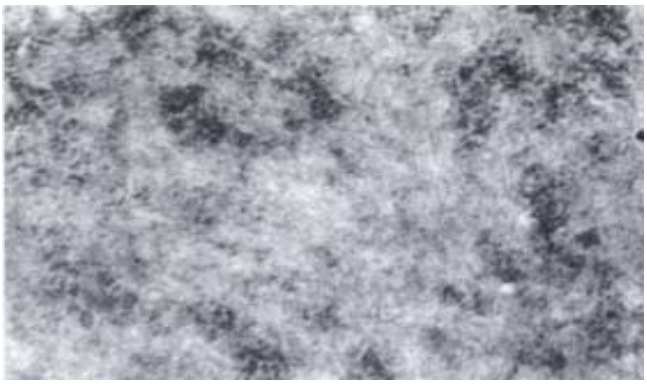

Figure 7 Electron micrograph of dense bodies and myofilaments of leiomyosarcoma. E.M.×20000

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, squmous 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 GES_1 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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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Wu ZQ, Fan J, Qiu SJ, Zhou J and **Tang ZY**

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.

Zhi Quan Wu, Jia Fan, Shuang Jian Qiu, Jian Zhou and Zhao You Tang
Liver Cancer Institute, Shanghai Medical Unviersity, Shanghai 200032, China
Prof. Zhi Quan Wu, male, born in August 1943 in Yiwu city, Zhejiang Pro vince, graduated from Shanghai First Medical College (The former of Shanghai Med ical Unviersity) in 1969. Majoring in surgical oncology of liver, having over 30 papers published.
Correspondence to: Zhi Quan Wu, M.D., Liver Cancer Institute, Shanghai Medical Unviersity, 136 Yi Xue Yuan Road, Shanghai 200032, China
Tel/Fax. +86-21-64037181
Received 1999-06-30 **Accepted** 1999-09-18

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,

1cm - 18.8 cm). The male to female ratio was 5.5:1. Among these patients, ninety two patients (87.6%) combined with posthepatitic 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±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±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.	Recurence 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Kong XB, Yang ZK, Liang LJ, Huang JF and **Lin HL**

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

INTRODUCTION

Most advanced hepatocellular garcinoma (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 investgated 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.

Xian Bing Kong[1], Zu Kui Yang[1], Li Jian Liang[2], Jie-Fu Huang[2] and Han Liang Lin[3]
[1]Department of Surgery, The First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China
[2]Division of Hepatobiliary Surgery, The First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou 510080, Guangdong Province, China
[3]Department of Pathology, Sun Yat-Sen University of Medical Sciences, Guangzhou 510089, Guangdong Province, China
Dr. Xian Bing Kong, male, born in 1961-10-17 in Pengxi County, Sichuan Province, graduated from Chongqing University of Medical Sciences as postgraduate in 1989, having 8 papers published.
Supported in part by the China Medical Board of New York, Inc., USA, Grant No.90-534
Correspondence to: Dr. Xian Bing Kong, Department of Surgery, The First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China
Tel. +86-23-68899866
Received 1999-06-23 **Accepted** 1999-09-18

Immunohistochemical stainings for P-gp

Serial sections of 4ìm 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 á of positive products in A need to be tested. Positive unit (PU) was tested and calculated with the formula in A: $PU = 100\times|G_\alpha\text{-}G_A/(1\text{-}A_{A\alpha})\times G_{max}$. 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 ex pression 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-gppositive 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_r170 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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Edited by Wu XN
Proofread by Miao QH

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Localization of HCV RNA and capsid protein in human hepatocellular carcinoma

Dai YM, Shou ZP, Ni CR, Wang NJ and **Zhang SP**

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 collect ed and processed into formalin-fixed, paraffin-embedded blocks.

Probe preparation

NS5 cDNA probe PCR product from NS_5 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 polyformaldehyte. After dehydration, prehybridization was performed and then the sections were denatured (steaming bath at 100 ℃, 10 min, then rapidly cooling down in ice bath), and hybridized again (42 ℃, overnight). After rinse, the sections were conjugated with gold-labeled strepto-biotin, stained with $AgNO_3$ 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 ℃.

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 NS_5 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.

Yi Min Dai, Zhi Ping Shou, Can Rong Ni, Neng Jin Wang and Song Ping Zhang
Department of Pathology, Second Military Medical University, Shanghai 200433, China
Yi Min Dai, professor of pathology, tutor of postgraduate, born on February 17, 1934 in Ruian City, Zejiang Province, graduated from the 2nd Military Madical University in 1961, specializing in tumor pathology and molecular pathology, having more than 40 papers published.
Project supperted by the National Natural Science Foundation of China, No.39370294.
Correspondence to: Zhi Ping Shou, Department of Pathology, 800 Xiong Ying Road, Shanghai 200433, China
Tel. +86-21-25070279
Received 1999-05-30 **Accepted** 1999-09-19

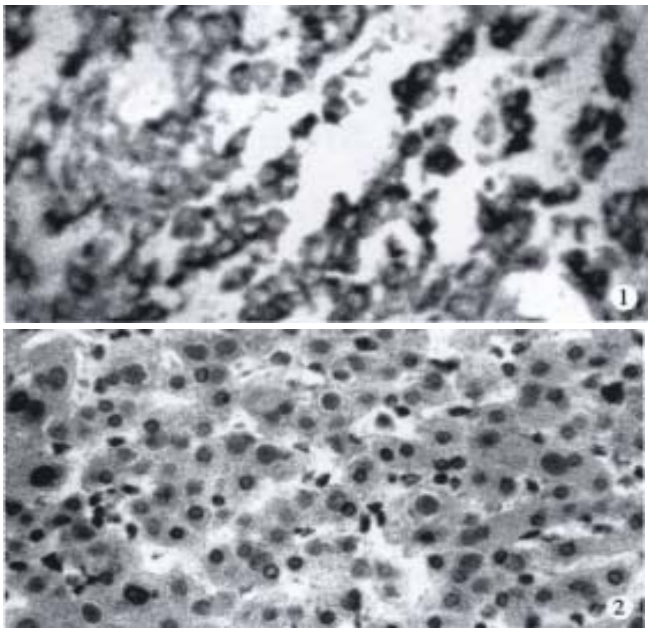

Figure 1 HCV-ISH (cytoplasmic type), biotin marker, ABC methods, HCCII,×132
Figure 2 HCAg (inclusion pattern), cancerous surrounding liver, PAP method,×200

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 infected cancers and their surrounding tissues, which was in good agreement with other authrs' reports. HCV infect ed 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 NS_5 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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Edited by Wang XL

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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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

Qin LL, Su JJ, Li Y, Yang C, Ban KC and **Yian RQ**

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, *p*21 (MAB-0143), *p*53 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 Hosp ital 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 $\mu g \cdot kg^{1} \cdot d^{1}$. LB was taken from all animals anaesthetized by ketamini. The size of the tissue s was about 0.8 cm×0.4 cm×0.4 cm, and they were fixed immediately. LB was taken repeatedly in every other 15wk after fed with AFB1.

HBsAg, HBxAg, IGF-II, *p*53 and *p*21 were detected by immunohistochemistry. Microwave citrate buffer strengthened HBxAg and *p*53. 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±96.4	7.12±7.50	0.04±0.03
B	+	-	14	71.1±72.0	1.90±2.09	0.03±0.01
C	-	+	16	72.9±70.4	2.22±2.42	0.23±0.01
D	-	-	10	19.4±19.9	0.35±0.36	0.01±0.01

Liu Liang Qin[1], Jian Jia Su[1], Yuan Li[1], Chun Yang[1], Ke Chen Ban[1] and Rue Qi Yian[2]
[1]Department of Pathology, Guangxi Cancer Institute, Nanning 530021 , China
[2]Department of Pathology, Cancer Institute, Sun Yat Sen University of Medical Sciences, Guangzhou, 510060, China
Dr. Liu Liang Qin, female, born on 1963-10-21 in Liuzhou, Guangxi, gr aduated from Guangxi Medical University with a master degree in 1996, research fellow in pathology, having 6 papers published.
Supported by the National Natural Science Foundation of China, No. 3926 0033.
Correspondence to: Liu Liang Qin, Department of Pathology, Guangxi Cancer Institute, Nanning 530021, P.R. China
Tel. +86-771-5313022 Ext.3021
Received 1999-01-03 **Accepted** 1999-03-25

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 *n*	Positive *n* (%)	Animal *n*	Positive *n* (%)	Animal *n*	Positive *n* (%)
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 wk75. 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 *n*	Positive *n*(%)	Animal *n*	Positive *n*(%)
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. Us ually 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 exper iment 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Antitumoral activity of low density lipoprotein-aclacinomycin complex in mice bearing H_{22} tumor

Bi WX, Xu SD, Zhang PH and **Kong F**

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 seques trated drug is protected from enzymatic action. ② Furthermore, LDL is not clear ed 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 rece ptor pathway. This potentially high efficient process may lead to different phar macological 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 C enter of Shandong Medical University. Murine Hepatoma 22 (H_{22}) cells were bought from Pharmaceutical Institute, Shandong Academy of Medical Sciences, and ACM from Shenzhen Wanle Pharmaceutial 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 (Na_2HPO_4 50 mmol/L, NaCl 100 mmol/L, Na_2EDTA 0.1 g/L, pH7.4) for 48 h, filtered through a me mbrane filter (Millipore, 0.22 μm pore size) and stored at 4 ℃. 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 ℃. The mixture was then subjected to extensive dialysis for 48 h aganist LDL buffer to remove free drug and thereafter filtered and stored at 4 ℃. 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, H_{22} cells (2×10^6) 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

Wen Xiang Bi[1], Song De Xu[1], Pei Hai Zhang[2] and Feng Kong[1]
[1]Department of Biochemistry, Shandong Medical University, Jinan 250012, Shandong Province, China
[2]Department of Gynaecology and Obstetrics, First Affiliated Hospital of Shandong Medical University, Jinan 250012, Shandong Province, China
Wen Xiang Bi, male, born on 1963-03-31 in Jinan City, Shandong Provin ce, graduated from Shandong Medical University in 1986 and earned master degree in Shandong Medical University in 1997, now lecturer of biochemistry majoring in lipoproteins, having 11 papers published.
Supported by the Scientific Foundation of Shandong Provincial Scientific & Technical Commission, No.971164607.
Correspondence to: Wen Xiang Bi, Department of Biochemistry, Shandong Medical University, Jinan 250012, Shandong Province, China
Tel. +86-531-2942092
Email.wwhd@jn-public.sd.cninfo.net
Received 1999-06-30 **Accepted** 1999-09-11

recorded.

On day 0, H_{22} cells (2×10^5) 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 H_{22} tumor (Table 1)

When given intravenously, the complex had a marked therapeutic effect on mice with subcutaneous H_{22} 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 agains t murine subcutaneous H_{22} 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 H_{22} tumor (Table 2)

The survival times of mice bearing intraperitoneal H_{22} 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. Administrat ion of LDL-incorporated ACM resulted in a significant increase in antitumoral a ctivity at the same dose as compared with the free ACM ($P<0.05$). Abnor mality in body weight has been noted in any lot of mice.

Table 2 Antitumoral activity of LDL-ACM complex and free ACM agains t murine intraperitoneal H_{22} 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 ℃. 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 H_{22} tumor. Results showed that entrapment of ACM into LDL particles increased the antitumoral activity as compared with the free ACM in H_{22} 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 in creased exposure of ACM to the tumor and consequently, increase the antitumoral efficacy.

LDL-drug complex might result in lessions 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-r egulate 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 t he 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 anim al 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 o n 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Zhu JY, Leng XS, Wang D and **Du RY**

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 pressue 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 μg/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. Students'*t* test was used to compare the data collected before and after the treatment.

Ji Ye Zhu, Xi Sheng Leng, Dong Wang and Ru Yu Du
Department of General Surgery, People's Hospital, Beijing Medical Unive rsity, Beijing 100044, China
Dr. Ji Ye Zhu, Male, born on 1962-07-20 in Shanghai, graduated from B eijing Medical University as a postgraduate in 1991, now associate professor of general surgery, majoring hepatobilliary surgery, having 25 papers published.
Project supported by the National Natural Science Foundation and Minist ry of Public Health of China, No.39500141
Correspondence to: Dr. Ji Ye Zhu, Department of General Surgery , People's Hospital, Beijing Medical University, Beijing 100044, China
Tel.+86-10-68314422 Ext.3500, Fax.+86-10-68318386
Received 1999-04-15 **Accepted** 1999-08-09

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 hour after somatostatin administration. The portal venous pressure decreased from 20.8±2.0mmHg (0h) to 18.2mmHg±2.0mmHg (1h) and 18.0mmHg±2.0 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

	n	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 mL/min± 757.25 mL/min to 1319.49 mL/min±622.39 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

former increased from 0.72 $cm^2 \pm 0.21$ cm^2 to 0.76cm^2±0.24cm^2, and the latter increased from 1786.22 mL/min ± 923.37 mL/m in to 1836.17mL/min±844.24mL/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 slig htly 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 slanchnic hemodynamics in cirrhotic patients with portal hypertension should be further investigated.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Radiotherapy of double primary esophageal carcinoma

Xiao ZF, Yang ZY, Zhou ZM, Yin WB and **Gu XZ**

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 ⩾ 4cm 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 irradi ation, 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 patient s 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 trea ted 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 note metastasis

Ze Fen Xiao, Zong Yi Yang, Zong Mei Zhou, Wei Bo Yin and Xian Zhi Gu
Department of Radiation Oncology, Cancer Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Zuo An Men Wai, Beijing 100021, China
Dr. Ze Fen Xiao, female, born on 1959-01-22 in Sichuan Province, graduated from West China University of Medical Sciences in 1982 and since then has been working in cancer hospital, became associate professor of radiotherapy in 1993, published several articles in international congress and journals.
Correspondence to: Dr. Ze Fen Xiao, Department of Radiation Onc ology, Cancer Hospital, Zuo An Men Wai, Beijing 100021, China
Tel. +86-10-67781331 Ext. 8286
Email.xiaozf@263.net
Received 1999-07-11 **Accepted** 1999-09-11

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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Yang YH, Fu XB, Sun TZ, Jiang LX and **Gu XM**

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 dysfun ction 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 mechan isms, i.e. the growth factors.

Both bFGF and TGFβ 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 TGFβ expression of renal origin in a gut I/R rat model, and to explore the role of bFGF and TGFβ'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 underwe nt laparotomy and dissection of the proximal SMA without occulsion 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 ℃, 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 ℃ bFGF and TGFβ 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 hybri dization, slides were washed with following solutions, 2 × SSC, 0.1 × 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 TGFβ antibody by an indirect Streptavidin / Peroxidase (SP) technique. Antib odies and immunohistochemical SP kit were products of Santa Cruz Co. and Zymed Co.. Experiments were performed following the manufacturer. Briefly, sections we re incubated with polyclonal anti-rat bFGF or TGFβ antibody for 12 hours at 4 ℃. Slides were washed with phosphate buffered saline (PBS) solution. Biotinylated

Yin Hui Yang, Xiao Bing Fu, Tong Zhu Sun, Li Xian Jiang and Xiao Man Gu
The 304th Hospital of PLA, Beijing 100037, China
Dr. Yin Hui Yang, female, born on 1966-04-28 in Hebei Province, graduated from Wuhan University in 1989 and got master degree from Academy of Military Medical Sciences in 1996, now being associate professor, having 14 papers pub lished.
Correspondence to: Dr. Yin Hui Yang, Trauma Center, the 304th Hospital, 51 Fuchen Road, Beijing 100037, China
Tel.+86-10-66867396, Fax.+86-10-68429998
Received 1999-07-11 **Accepted** 1999-09-11

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 TGFâ 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 immunohis tochemistry 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 ($\bar{x}$±s)

Group	bFGF positive staining rate (%)			TGFβ positive staining rate (%)		
	n	mRNA	Protein	*n*	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, neutrophiles, 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 TGFâ 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 regenaration 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, vascul ar endothelial growth factor (VEGF) in wound healing and tissue repair[5,6].

In our present study, bFGF and TGFâ 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 TGFβ mRNA expression increased after reperfusion, and persisted for 24 hours. These results showed endogenous bFGF and TGFβ 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 TGFâ might participate in this pr ocess. Experimental studies had also found that bFGF is a most potent angiogenic factor and TGFβ played an important role in angiogenesis. The significantly elevated expression of bFGF and TGFβ 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, neutrophiles and macrophages etc results in release of large amount of TGFβ, and high local concentration of TGFβ induces the high expression of bFGF mediating the tissue repair. Our study shows that I/R injury up-regulate endogenous bFGF and TGFβ 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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A chronic ulcerative colitis model in rats

Zheng L, Gao ZQ and **Wang SX**

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 muscosa. 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 waterad 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) phosphat e 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 carbarnate in 0.9% saline.

Instrument T25 Ultra-tukrax (German, JANKE & KUNKEL IKA-Labortechnik). 4710 series Ultrasonic homogenizer (Cole-Parmer Inst rument Co. America), GL20A Refrigerated Centrifuge (Hunan Instrument and Meter Plant China), UV-260 Spectrophometer (Shimuduzu Co. Japan), PHSJ-4 pH meter (Shanghai Leici Instrument Plant China), Libror EB-2080M Lectronic 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 carbarnat e (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 5ìm sections, stained with hematoxylin and eosin. The second segment (200mg-400mg) was immediately frozen for subsequent estimation of MPO activity[1].

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

Li Zheng[1], Zhen Qiang Gao[2] and Shu Xian Wang[3]
[1]Department of Pharmacology, National Institutes Pharmaceutical Research and Development, Beijing 102206, China
[2]Department of Pathology, Beijing Medical University, Beijing 100083, China
[3]National Administrative Center of New Drug Research, Beijing 100810, China
Li Zheng, male, born on 1965-09-01 in Hubei Province, graduated from China Pharmaceutical University, now associate professor of gastroenterology, having 14 papers published.
Supported by the National "Eighth Five-Year Plan" Program, No. 85-922-01.
Correspondence to: Li Zheng, National Institutes Pharmaceutical Research and Development, Beijing 102206, China
Tel. +86-10-69732071, Fax. +86-10-69731050
Email. nipzl@263.net or zwl@cenpok.net
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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 inf lammation.

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 50mg/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 100mg/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 a 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 E_2, thromboxane B_2, leukotriene B_4, 6-keto-prostaglandin $F_{1\alpha}$, leukotriene C_4, plet elet-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 muscosal and submuscosal 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 inflamm ation in colonic tissue[1]. MPO activity may be regarded as an index of inflammation damage[6].

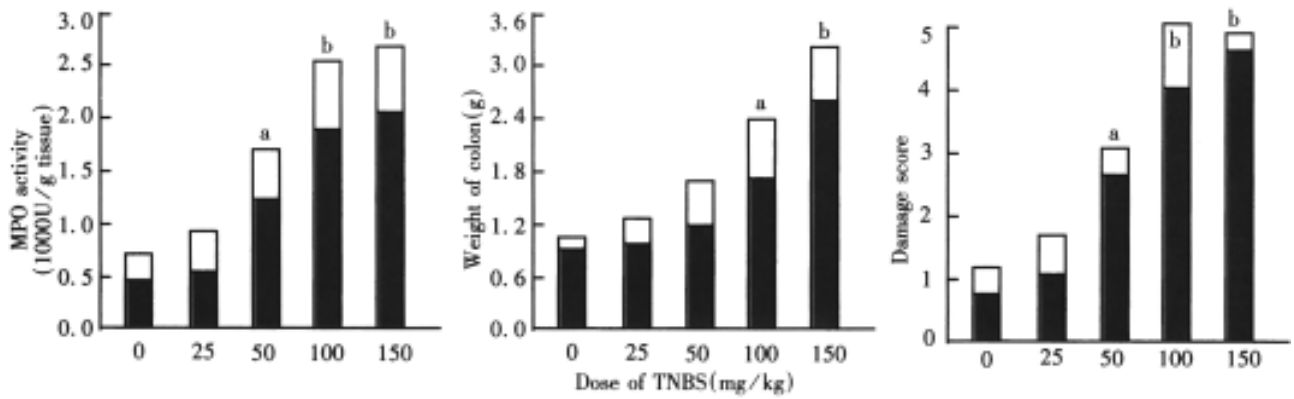

Figure 1 Rats were killed 3wk after intracolon ic administration of 25 mg/kg-150 mg/kg of TNBS. Colonic damage, colon weight and tissue MPO activity were assayed. Data of TNBS-treated rats were compared with control (30% ethanol) group by Student's *t* test, $^{a}P<0.05$, $^{b}P<0.01$.

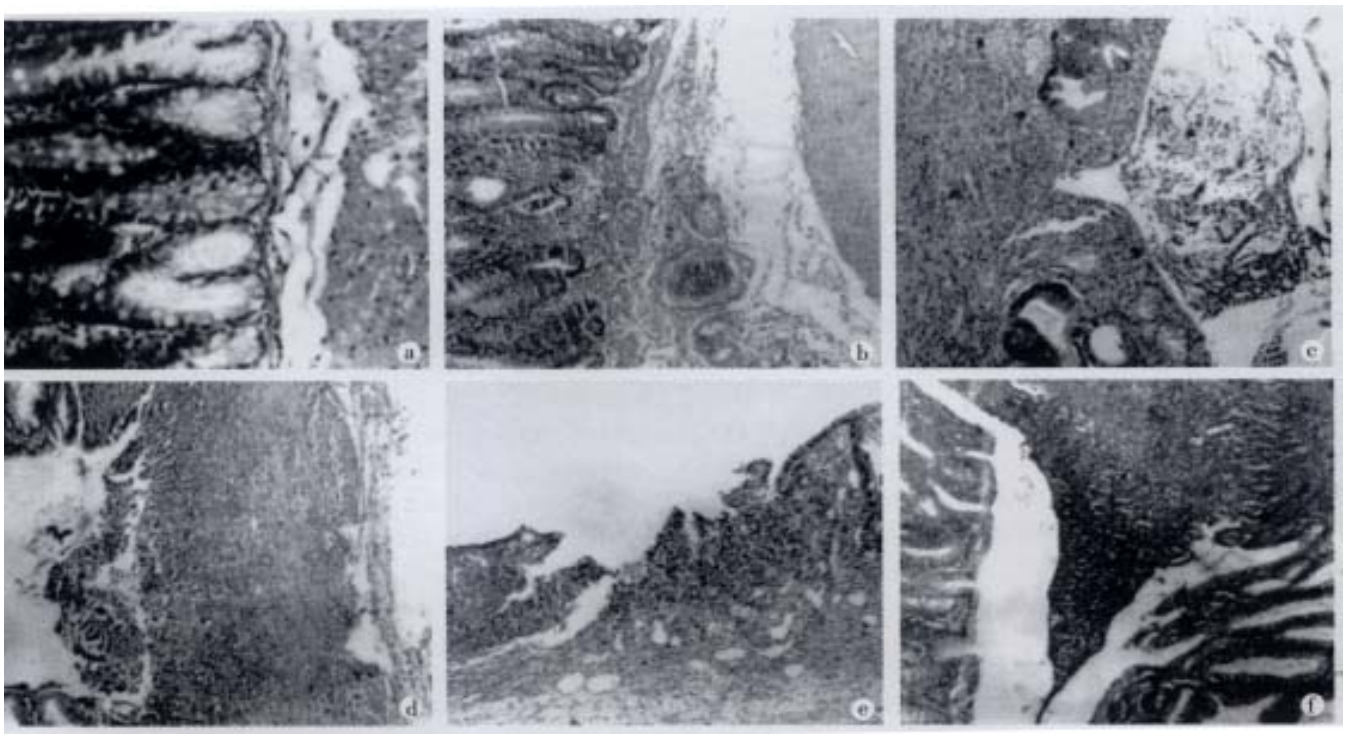

Figure 2 Histological findings in ulcerative colitis induced by different doses of TNBS ethanol.
a: TNBS 25 mg/kg, the mucosa is normal. b: TNBS 50 g/kg, small mucosal ulc ers and cryptoabscess formation. c-d: TNBS 100 mg/kg, mucosal ulcers, in flammatory exudate, proliferous granulomas and cells infiltration. e-f: TNBS 15 0 g/kg, gross ulcers and proliferous granulomas, necrosis of epithelium at mu cosal sueface and inflammatory cell infiltration (He, ×100)

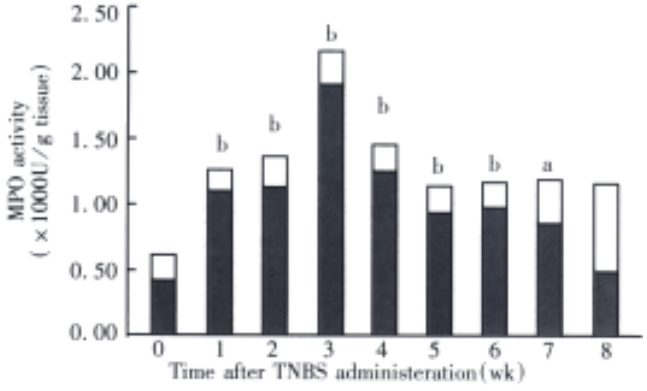

Figure 3 The effects of intracolonic administration of 100mg/kg of TNBS on MPO activity 1 wk-8 wk after administra tion. Each bar represents the $\bar{x}\pm s$ of 5 animals. All data for TNBS-tr eated rats were compared with 0 wk (control group). $^{a}P<0.05$, $^{b}P<0.01$.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Tracing method study of bacterial translocation *in vivo*

Fu WL, Xiao GX, Yue XL, Hua C and **Lei MP**

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

INTRODUCTION

Endogenesis 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℃. Restriction DNA endonucleases: *Hind*-III, *EcoR*-I (Promega Co.). Plasmid was isolated asdescribed 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 fluoresc ence-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: 10^{12}/L fluorescence-labeling E.coli were introduced into the stomach of rats by gastric tube. After 8 hours the a nimals 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 m icroorganisms 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 int estinal 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 ℃ with shaking at 50 min^{-1}-100 $^{-1}$rpm.

Plasmid isolation of the bacteria was made according to Kado's method. The product was digested 1h at 37 ℃ by restriction enzymes *EcoR*I 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.

Wei Ling Fu[1], Guang Xia Xiao[1], Xu Li Yue[1], Chuan Hua[2] and Man Ping Lei[1]
[1]Gene Center of Medical Laboratory, South-Western Hospital, the Third Military Medical University, Chongqing 400038, China
[2]Chinese PLA 252 Hospital, Baoding 071000, Heibei Province, China
Dr. Wei Ling Fu, Male, born on 1955-05-17 in Jinzhou, Liaoning Province, graduated from the Third Military Medical University in 1988, now professor of microbiology, Chief of Medical Laboratory, having 40 papers published.
Supported by the National Natural Science Foundation of China, No.39570042
Correspondence to: Dr. Wei Ling Fu, Medical Laboratory, South-Western Hospital, the Third Military Medical University, Chongqing 400038, China
Tel. +86-23-68754209, Fax. +86-23-65319609
Email.FWLDZYQS@public.cta.cq.cn
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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/* 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 apmicillin (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).

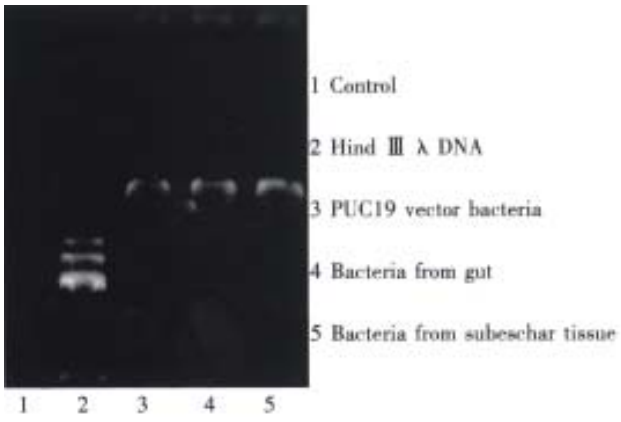

Figure 1 Plasmid fragment generated by restriction e ndonuclease-1. Control; 2. HindIII λ DNA; 3. PUC19 vector bacteria; 4. bacteria from gut; 5. bacteria from subeschar tissue.

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 report ed. 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 isoto pes labeling methods. PUC19 plasmid was constructed in the 1980s, and was used e xtensively 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 restrictione ndonuclease 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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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 carcinom a 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-s haped 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 extra-cellularly[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]. Ureasest imulates the

Yvan Vandenplas
Academisch Ziekenhuis Kinderen, Free University of Brussels, Brussels, Belgium
Professor Yvan Vandenplas, male born on 1956-02-21 in Brussels, Belgium, graduated from the Free University of Brussels in 1981, and is now Head of the Department of Pediatrics, having more than 100 papers published.
Correspondence to: Yvan Vandenplas, Academic Children's Hospit al, Free University of Brussels Laarbeeklaan 101, 1090 Brussels, Belgium
Tel. 00-32-2-477-57-80/81, Fax. 00-32-2-477-57-83
Email.pedvsy@az.vub.ac.be
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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 hydrop hobicity of the gastric mucous and mucosa. Phospholipase can also generate ulcer ogenic 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 a re 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 deve loping 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 Cag A-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* lipopolysac charide (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 pomp 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 on 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 gastro-esophageal 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 incre ased 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-alpha 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 vas ospasm 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-alpha), prostaglandins, leukotrienes, oxyradicals, C-reactive protein and fibrinogen are released in chronic infection[74]. Henoch-Sch-nlein purpura and Sj-gren’s syndrome have been correlated with the bacterium. Many patients with autoimmune thyroid diseases a re infected 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 p opulation 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 train 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[9 0]. 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., be cause 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% CO_2. 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-detect ion 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 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 cytoxic 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 a lthough *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, consider ed 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 wit h 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 heterogenecity 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 as so ciated 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* lipopolys accharide 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, H_2-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 (metron idazole or tinidazole)] for 1 week[94,160,161]. There are no specificr ecommendations 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 m^2 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 tolerate d 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 lansopr azole) 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 ddition 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 conduct ed. 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 end oscopy 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 t he 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 in hibitors (and to a lesser extent with H_2-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-lable 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 thos e 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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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Current concept of pathogenesis of severe acute pancreatitis

Wu XN

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The pathogenesis of severe acute pancreatitis is very complicated. It is a multifactorial as well as multifaceted disease. First of all, the etiologic agents in itiate 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 sequencial events are as follows.

PANCREATIC ACINAR INJURY

Pancreatic duct obstruction and bile reflux

Gallstone incarcerated in the distal common ductor 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 A_2 (PLA_2), 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]. ①It 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

Xie Ning Wu
Department of Gastroenterology, Shanghai First People's Hospital, Shanghai 200080, China
Dr. Xie Ning Wu, Professor of Medicine, B.S. M.D. Editor of ten books on hepatology and gastroenterology, having 185 papers published.
Correspondence to: Dr. Xie Ning Wu, Department of Gastroenterol ogy and Central Research Laboratory, Shanghai First People's Hospital, No.85, Wujing Road, Shanghai 200080, China
Tel. +86-21-62473001(H), Fax.+86-21-62473001
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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. ④Ethanol can change systemic and pancreatic lipid metabolism with accumulation of lipid droplets within the acinar cells, alter membrane fluidity and integrity. ⑤Ethanol 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 acut e 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 PLA^2 hydrolyzes phospholipids including cephalin, sphingo-phospholipids and lysocephalin, destroys structural phospholipid and microvasc ulature, activates platelet activating factor and increases vascular permeability and ischemia[6]. The kaillikren-kinin system produces bradykinin whic h 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, prospholipase A_2, bradykinin, complement component, histamine and some infla mmatory mediators as platelet activating factor (PAF) and prostaglandin are found.

Release of cytokines

During pancreatic necrosis and inflammation, IL-1β, 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-1 ra), IL-1 rais 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 de crease 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 PLA_2, 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 taurocho licacid 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 otherhand, 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-α 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 s 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-α and IL-1 induction, IL-8 chemotacts and a ttracts more neutrophils a cumulating 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 thrombo-fibrinolytic reaction, and produce inflamm atory mediaters from eicosanoid products. Moreover, the neutrophilic elastase is more destructive, causing multiorgan failure. Experimental studies showed that plasma neutrophilic elastase level parallelled 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 phagotyze 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 thrombo-fibrinolysis. 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 mechan isms

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 pse udocyst
2. Systemic complications	
Hypotension, hypovolemic shock	Increased vascular permeability, profuse exuda tion into peritoneal cavity Hypovolemia Hypoalbuminemia
Intestinal ileus, dehydration, hypopotassemia	Peritonitis; loss of peristaltic function; large quantity of digestive fluid sequestrated in intestinal lumen; infusion of large volume of crystaloid 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 demyelinization of grey and white matter
Transient blindness	Retinal ischemia; white cell emboli with exudation, incre ased 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 failture	Underlying ischemic heart disease; overloading of circulation by massive infusion or too rapid intravenous dripping

Pancreatic encephalopathy is primarily due to the demyelinization of the cerebral grey and white matter caused by PLA_2, 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*, *Klebsiela*, *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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Edited by Ma JY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Invited Commentary

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

Roberts LR and **LaRusso NF**

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Subject headings liver neoplasms; carcinoma, hepatocellular; tumor supressor 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.

Lewis R. Roberts and Nicholas F. LaRusso
Division of Gastroenterology and Hepatology, Department of Medicine, Mayo Medical School, Clinic and Foundation, Rochester, MN 55905, USA
Correspondence to: Dr. Lewis R. Roberts, Division of Gastroenterology and Hepatology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
Tel.(507)284-4823, Fax.(507)284-0538
Email. Roberts.Lewis@mayo.edu
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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 be en 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 bet a (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

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/ insu lin-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 chromosome1p 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 in tegration 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 know n 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 conse quence 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 sub strate 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 re pair phenotype or the clinico-pathologic features of HNPCC tumors; instead they behave in the same manner as sporadic colon cancers. Clear criteria have not be en 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 f requency 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 pat hogenesis 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 res olve 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 in itiation 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, includin g the p53 locus, does not show as clear a pattern. Fifty-eight % of 64 heterozy gous 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 can cerous 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 clinicopathologi c 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Moura RM and **Barkin JS**

Seen article on page 53

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 sceases 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.

Rossana M. Moura and Jamie S. Barkin
University of Miami, School of Medicine & Mount Sinai Medical Center Division of Gastroenterology, Miami, Florida, USA
Correspondence to: Jamie S. Barkin, M.D. Division of Gastroente rology Mount Sinai Medical Center, 4300 Alton Road, Miami Beach, FL 33140, USA
Tel. (305) 674-2240, Fax. (305) 674-3961
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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 show n 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. Cipollet ta *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 veseel 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 ac hieved 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 criticallyill 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 hemoclipping 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 hemoclipping 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 to pical ethanol injection

in a retrospective study. Endoscopic clipping was perfor med using the clipping devices Olympus HX-3L or HX-5LR and clips Olympus MD-750. Endoscopic clipping improved therapeutic outcome as determined by rebleedin g 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 hemoclipping 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 radiologi sts to identify the bleeding point during angiography when clipping fails in hem ostasis. 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Original Articles

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

Tovey FI and **Hobsley M**

Subject headings gastrectomy; nutrition; iron; vitamin B_{12}; 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 B_{12} 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 gastrect omy patients foriron, Vitamin B_{12} and Vitamin D deficiencies is emphasised.

Frank I. Tovey and Michael Hobsley
Department of Surgery, Royal Free and University College Medical School, London 67-73 Riding House Street, London W1P 7LD, UK
Frank I. Tovey OBE, ChM, FRCS (Eng) was born in England in 1921. After qualifying as a surgeon he worked in China between 1947 and 1949 and then in India from 1951 to 1967. In 1968 he was appointed as a consultant surgeon at Basingstoke in England and also as an honorary surgical research fellow at University College in London. His interests have been in leprosy reconstructive surgery and in aetiology and management of duodenal ulcer. He has travelled widely investigating the relationship between staple diets and the prevalence of duodenal ulcer in many countries. He is retired but continuing with his research projects.
Correspondence to: Frank I. Tovey, 5 Crossborough Hill, Basingst oke RG21 4AG, United Kingdom
Tel.01256-461521, Fax.01256-323696
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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 anast omosis) 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, w ho 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 Wes tern 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

discomfort or vomiting if the amount was exceeded. Early dumping consisted of weakness, fainting, sweating and palpitation 10-20 min utes 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 incl uded blood picture, serum iron and total iron binding capacity (TIBC). Iron defi ciency was defined as an iron saturation (serum iron/total iron binding capacity) below 16%.

Vitamin B_{12} deficiency Vitamin B_{12} deficiency was diagnosed when two separated bioassays repeated one month apart showed a value of less than 110 pmol/L.

Vitamin D deficiency Serum calcium, phosphate and alkaline phos phatase. 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 excl uded. A 24 h urinary calcium output below 2 mmol/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 phospha tase 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 we re used to calculate the Exton-Smith Index:

$$\frac{T2\text{-}M_2}{TL}$$

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 complaine d 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 d iarrhoea 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 signifi cant (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 B_{12} deficiency In most patients a fall in serum B_{12} 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 B_{12} deficiency, appearing in many patients during the first 10 years after operation. Vitamin B_{12} 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 B_{12} 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 durin g the first 10 years after operation (Table 1). Of those investigated, 50% had severe and 28% moderate steatorrhoea as compared with 20% and 14% respectively for the whole series.

Osteoporosis Osteoporotic changes in excess of normal ageing we re 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 satisf actory treatment has been reported for postgastrectomy osteoporosis[5].

Billroth I *versus* Billroth II gastrectomies There was no s ignificant difference in overall, moderate or severe weight loss between the two operations in women (BI6/12:BII26/45, $P = 0.1567$). In men, although there was no significant overall difference in weight loss (BI10/24:BII37/141, $P = 0.1438$), significantly more patients showed a moderate weight loss after a Billroth II gastrectomy (BI1/24: BII31/141, $P = 0.0491$), by contrast more showed severe weight loss after a Billroth I procedure (BI9/24:BII6/141, $P<0.0001$).

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 steatorrhoea the difference between the two operations was significant (BI0/37:BII32/121, $P<0.0001$).

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 se verely 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 o f iron and vitamin B_{12} deficiencies and more markedly in the occurrence of vitamin D deficiency (Table 1).

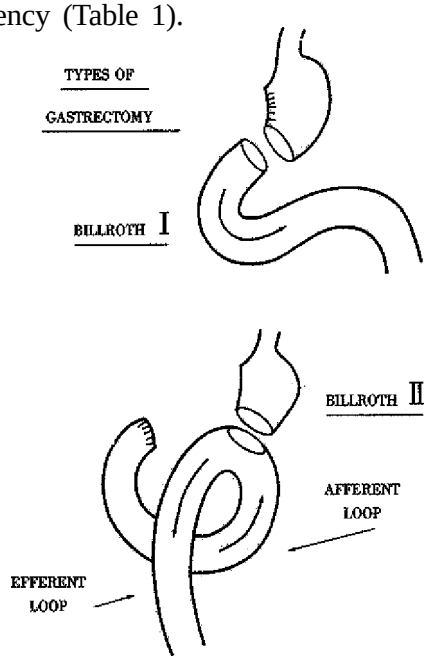

Figure 1 Types of gastrectomy.

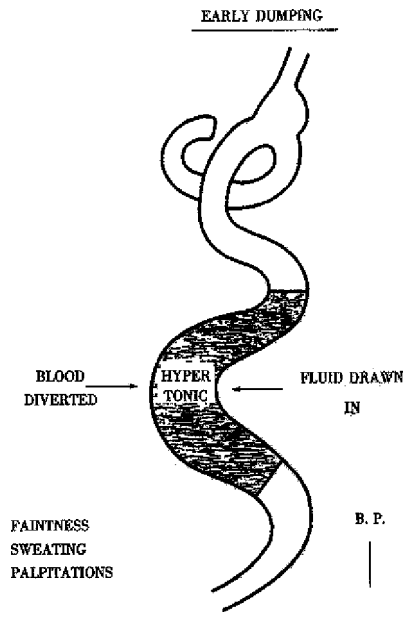

Figure 2 Early dumping.

Table 1 Prevalence of iron, B_{12} 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 disor-ders 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 intes tine, 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 B_{12} 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, i 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 B_{12} 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 B_{12} deficiency in a population after gas trectomy, reaching approximately 75% in 20-30 years. This stresses the increasing importance with passing years of regularly monitoring iron saturation and B_{12} 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 s till a large number of patients who underwent gastrectomy 20-30 years ago and a re at risk of developing nutritional deficiencies.

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Edited by Ma JY
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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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

Indaram AVK, Nandi S, Weissman S, Lam S, Bailey B, Blumstein M,Greenberg R and **Bank S**

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 fro m 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 increase d cytokine levels serve as phenotypic markers for a genetic predisposition to de veloping Crohn's disease later on, or whether they indicate early (pre-cli nical) 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.

Anant VK Indaram, Santa Nandi, Sam Weissman, Sing Lam, Beverly Bailey, Meyer Blumstein, Ronald Greenberg and Simmy Bank
Division of Gastroenterology, Long Island Jewish Medical Center, Albert Einstein College of Medicine, New York, USA
Correspondence/Reprints Anant VK Indaram, MD, Long Island Jewish Medical Center, 270-0576th Avenue, New Hyde Park, NY 11040, USA
Tel. 718-470-4692, Fax. 718-470-0827
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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 el evation[2-5]. Since there is evidence for a genetic predisposition in I BD[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 MEHODS

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 referrable 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).

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 nons pecific 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: demoaraphic 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 alon	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 = Aminosalicylic 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℃ 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 interlukins. 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±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 ± 0.56 ng/g, 1.19 ng/g ± 0.21 ng/g, 11.98 ng/g ± 2.62 ng/g, respectively, Figure 1) when compared to controls (1.86 ng/g ± 0.30 ng/g, 0.69 ng/g ± 0.01 ng/g, 5.41 ng/g ± 1.87 ng/g, respectively). No significant difference was found in the concentration of IL 1B between relatives (0.85 ng/g±0.17 ng/g) and controls (1.03 ng/g±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±0.09 ng/g and 0. 21 ng/g±0.09 ng/g, respectively) were significantly lower (P<0.05) than that of the other relatives (1.03 ng/g±0.16 ng/g and 1.47 ng/g±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.

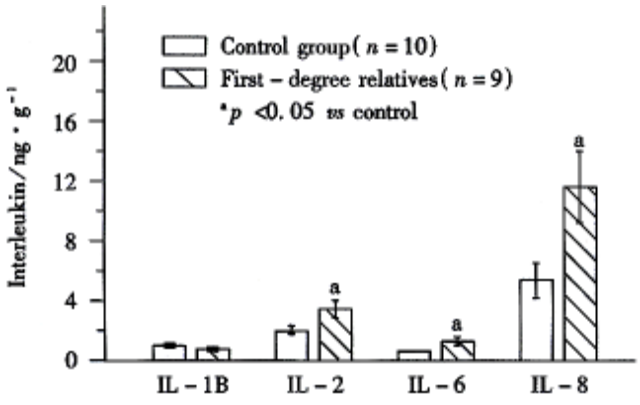

Figure 1 Interleukin levels of first-degree relatives and controls.

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 C_3 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 (PE G-400) and large sugars, including lactulose, mannitol, and rhamnose[28] , 51 Cr-labelled ethylene *diaminetetr*-aacetic 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 a ntigen) 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 gorup 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 in dex 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 questi onable 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 permea bility have been inconsistent. It may be possible that increased cytokines are an early event in the pathogenesis of the disease. Whether these increased cytoki nes lead to increased permeability and than to CD is something that needs further investigation.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Endoscopic hemoclip treatment for bleeding peptic ulcer

Lai YC, Yang SS, Wu CH and **Chen TK**

See invited commentary on page 42

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

Abstract

AIM To evaluate the efficacy of endoscopic hemoclip in the t reatment 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 e ndoscopic 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 rebl eeding 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, hemoclipping 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 av erage than did oozing bleeders (3.4 versus 2.8). We observed no obvious co mplications, no tissue injury, or impairment of ulcer healing related to hemocli pping.

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

Yung Chih Lai[1], Sien Sing Yang[1,2], Chi Hwa Wu[1] and Tzen Kwan Chen[1]
[1]Division of Gastroenterology, Cathay General Hospital, Taipei and
[2]Medical Faculty, China Medical College, Taichung, Taiwan, China
Correspondence to: Sien Sing Yang, MD, Division of Gastroentero logy, Cathay General Hospital 280 Jen-Ai Road, Section 4 Taipei 106
Tel. 886-2-2708-2121 Ext. 3120, Fax. 886-2-2707-4949
Email.yangss@tptsl.seed.net.tw
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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 pepticulcer 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, reblee ding 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 g rasping capability has been developed recently, which can make the procedure easier and save much time[20]. We therefore used this newly improved me tallic 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

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 tranversing 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 H_2-blockers and oral antacid suspensions after endoscopic examination. A nasogastric tube was inserted to observe the bl eeding condition. Blood transfusion was given if the hemoglobin level driopped 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 co ntinued 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 treatm ent. 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 considere dsignificant.

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 cla ssification 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 occur red 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 revea led 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 hemoclipping, 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 hemoclipping 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 numb er 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 ana lyze the difference of clip number used in various locations of gastric and duod enal 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. [a]Two 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).
[a]Two 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. [a]Two 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 endos cope. 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- de vice 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 an 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 a dmission, 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 hemoc lipping 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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Edited by Wu XN
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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Cloning and expression of MXR7 gene in human HCC tissue

Zhou XP, Wang HY, Yang GS, Chen ZJ, Li BA and **Wu MC**

Subject headings human HCC tissues; MXR7 gene; gene expr ession; 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 accordin g to the whole cDNA sequence of MXR7 gene. The cDNA of human HCC was taken a s the template while the cDNA of MXR7 gene was synthesized by polymerase cha in reaction (PCR). Recombinant DNA conforming to reading frame was constructed b y connecting purified PCR product of the cDNA of MXR7 gene with expression v ector pGEX-5X-1 of fusion protein. The plasmid MXR7/ pGEX-5X-1 was identi fied by sequencing. Using ^{32}P labeled MXR7 cDNA as probe, MXR7 mRNA expression was detected by Northern blot analysis in 12 different human no rmal tissues, 7 preoperatively untreated non-liver tumor tissues, 30 preoperati vely untreated HCC, the paracancerous liver tissues and 12 normal liver tissues samples.

RESULTS Restriction enzyme and sequence analysis confirmed tha t 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 si tes 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.

Xue Ping Zhou, Hong Yang Wang, Guang Shun Yang, Zheng Jun Chen, Bao An Li and Meng Chao Wu
The International Cooperation Laboratory on Signal Transduction, Easte rn Hepatobiliary Surgery Hospital, the Second Military Medical University, Shang hai 200438, China
Xue Ping Zhou, M.D., male, born on August 11, 1970, in Taihu, Anhui Province, graduated with a master degree in the Second Military Medical University in 1998, majoring hepatobiliary surgery, have 3 papers published, and now working in Department of Surgery, Shanghai Municipal Cooperation Hospital, Chinese People's Police Armed Forces.
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Correspondence to: Professor Hong Yang Wang, Eastern Hepatobi liary Surgery Hospital, No.225, Changhai Road, Shanghai 200438, China
Tel/Fax. +86-21-65566851
Email.hywangk@online.sh.cn
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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 parac ancerous 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 t issues 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.

Plasmid
Expressing vector pGEX-5X-1 (4.9 kb) of fusion protein was provided by Max-Planck Institute.

Cell
E. coli DH5α was preserved in our laboratory.

PCR primers design and synthesis
The sequences of the sense and antisense primers of *MXR7* were 5' GCGAATTCTC CCTGCGAAGCAGGATG3' with the EcoRI (GAATTC) restriction site and 5' CGCTCGAG TCAGTGCACCAGGAAGAA3' 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 DH5α 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 ^{32}P-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 ℃ for use.

Preparation of the hybridization membrane Each 50ìg 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 ℃ for 2 h and then sealed in a plastic bag for use.

Northern blot analysis Northern bloting 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 ana tomical 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 expres sion 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 ⩾400 μg/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 μg/L in 13 (43.3%) and below 30 μg/L in 10 (33.3%). The tumor size was <5 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.

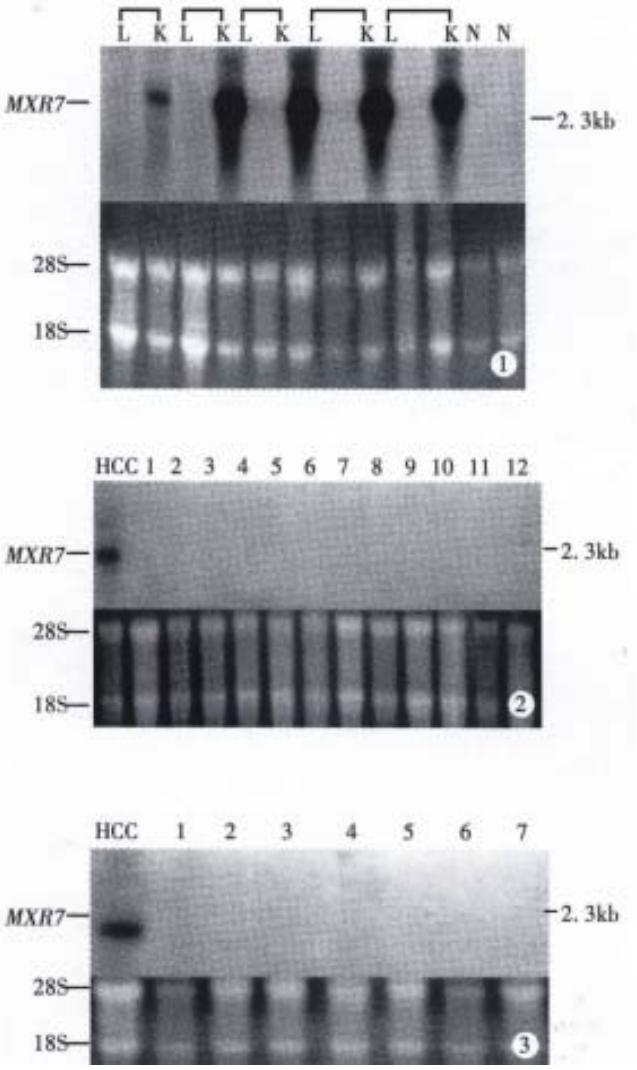

Figure 1 Northern blot analysis of *MXR7* in human HCC, paracancerous hepatic and normal liver tissues. L: paratumor tissue; K: hepatoma tissue; N: normal liver tissue. 28S and 18S rRNAs were used for evalua ting the quality and quantity of RNA loading.

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 qualit y 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 adenocarc inoma; Lane 3: gastric adenocarcinoma; Lane 4: left colon-ileal malignant mesot helioma; Lane 5: gastric adenocarcinoma; Lane 6: uterus adenomyoma; Lane 7: coli c 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*	MXR7 *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 clinicopat hological features in 30 patients with HCC

Clinical feature	*n*	MXR7 *mRNA* overexpression (%)
Serum AFP (μg/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 clinico-pathological features As shown in Table 2, *MXR7* mRNA expression did not correlate with s erum AFP elevation, tumor size, portal vein tumor thrombus, daughter nodules, HB sAg seropositivity and age. The frequency of *MXR7* mRNA expression in HCC was 70% (14 of 20 cases) with elevated serum AFP >30 μg/L, but 90% (9 of 10 cases) with serum AFP ⩽30 μg/L. In HCC <5cm, 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 rig ht 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 t he corresponding paracancerous cirrhotic tissues were 76.7% and 13.3%, respect ively. 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 h epatic 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 in completely resec ted 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 regula ted 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 overexpre ssion 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Recurrence or metastasis of HCC: predictors, early detection and experimental antiangiogenic therapy

Jiang YF, Yang ZH and **Hu JQ**

Subject headings carcinoma, hepatocellular; neoplasm metasta sis; angiogenesis; liver neoplasms

Abstract

AIM To investigate the predictors for recurrence or metastas is 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 le velless 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 an tibody 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 recur rence 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 inves tigate 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.

Yang Fu Jiang, Zhi Hua Yang and Jin Qun Hu
Cancer Institute, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China
Dr. Yang Fu Jiang, male, born on 1971-03-28 in Chongqing, graduated from West-China University of Medical Sciences in 1993, got Ph.D. at Peking Union Medical College in 1999. Now postdoctoral fellow at Albert Einstein College of Medicine, U.S.A, having 4 papers published.
Correspondence to: Dr. Zhi Hua Yang, Department of Molecular Biology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing 100021, China
Tel. +86-10-67781331 Ext. 8439, Fax. +86-10-67782259
Email.Zhyang@public.bta.net.cn
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Methods

RNA preparation The total tissue RNA was extracted with TRIzol (Life Technologies, Inc. Gaithusburg, USA), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -70 ℃.

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 μg 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 μL of PCR mixture contained 2 μL of the synthesized cDNA solution, 2.5 μL of 10× polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 μM-each of dCTP, dATP, dGTP, dTTP, 10 pmol 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 ℃ denaturation (30 s, first cycle 1 min), a 60 ℃ annealing (30 s), and a 72 ℃ 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 μL PC R products was added in 1.5% agarose gel containing 0.5 μg/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 determ ination. 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 μL 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 μL PCR products was added in 2% agarose gel for electrophoresis. If no specific band of 176 base pairs was observable, 2 μL PCR product was ream plified 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 unde rwent denaturation in 8 mol/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 a nd 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 sus ceptible 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). T he 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 expressi on 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 me tastasis or recurrence.

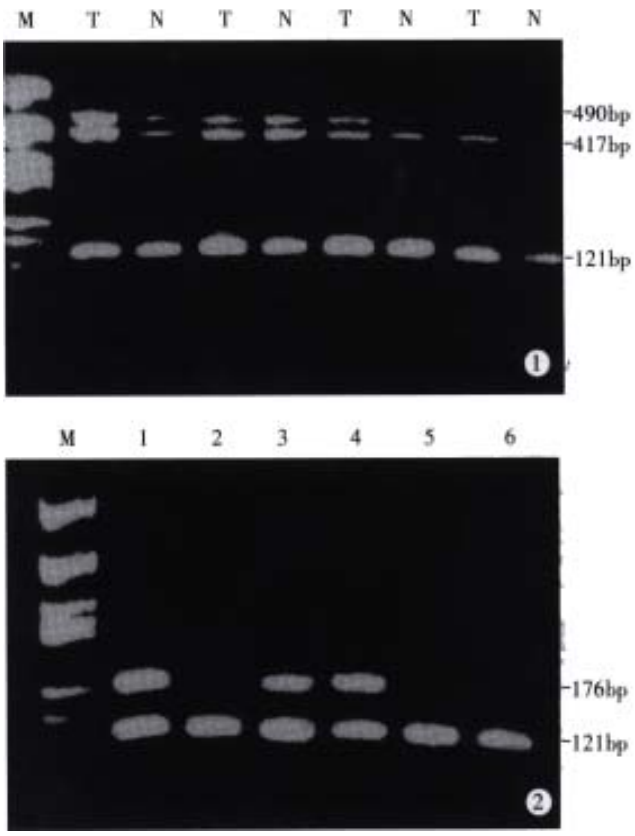

Figure 1 Electrophoresis analysis of RT-PCR product samplified from cDNA obtained from HCC and nontumorous liver tissues. M, DNA m arker (PGEM-7ZF(+) / *Hae*-III); T, tumor; N, nontumorous liver tissues. The positive bands of 417bp, 490 bp, and 12 1bp represent VEGF165, VEGF189, and β_2- MG, respectively.

Figure 2 Electrophoresis analysis of RT-PCR product s 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 implant ed 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 pos toperative recurrence or metastasis. The five year recurrence rate in liver can cers 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 degra ding 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 tumorr ecurrence 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 per ipheral blood by means of RT-PCR is a very attractive hypothesis[7]. The oretically, 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 re sult. 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 metasta sis later. Among 26 negative AFP mRNA, metastasis-free patients, 22

patients re mained 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, antiangiogen esis 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 neovasculization, 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 tre atment dramatically.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Expression of gap junction genes connexin 32, connexin 43 and their proteins in hepatocellular carcinoma and normal liver tissues

Ma XD, Sui YF and **Wang WL**

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.

Xiang Dong Ma, Yan Fang Sui and Wen Liang Wang
Department of Pathology, the Fourth Military Medical University, 17 Changle Xilu, Xi'an 710033, Shaanxi Province, China
Dr. Xiang Dong Ma, female, born on 1969-11-18 in Xi'an, Shaanxi Province, graduated from the Fourth Military Medical University as a doctor in 1999, now a lecturer, having 15 papers published.
Correspondence to: Dr. Xiang Dong Ma, Department of Obstetrics & Gynecology, Xijing Hospital, the Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
Email. maping@FMMU.edu.cn
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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 proc esses in which disorders of GJIC may play an important role[2]. This stu dy 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 verifed 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 μm thick sections.

SP immunochemistry

SP immunostaining was performed as described by SP immunochemistry 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

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 ℃ 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 ℃ for 30 min, the labeled cDNA probes were denatured in hybridization buffer at 100 ℃ for 10 min, then -20 ℃ for 3 min, added on tissues and coverslipped at 42 ℃ overnight. Sections were washed with 2×SSC, 1×SSC, 0.5×SSC and buffer I, incubated in NSS at 37 ℃ 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 hepatocar cinogenesis.

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 maligancy[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.

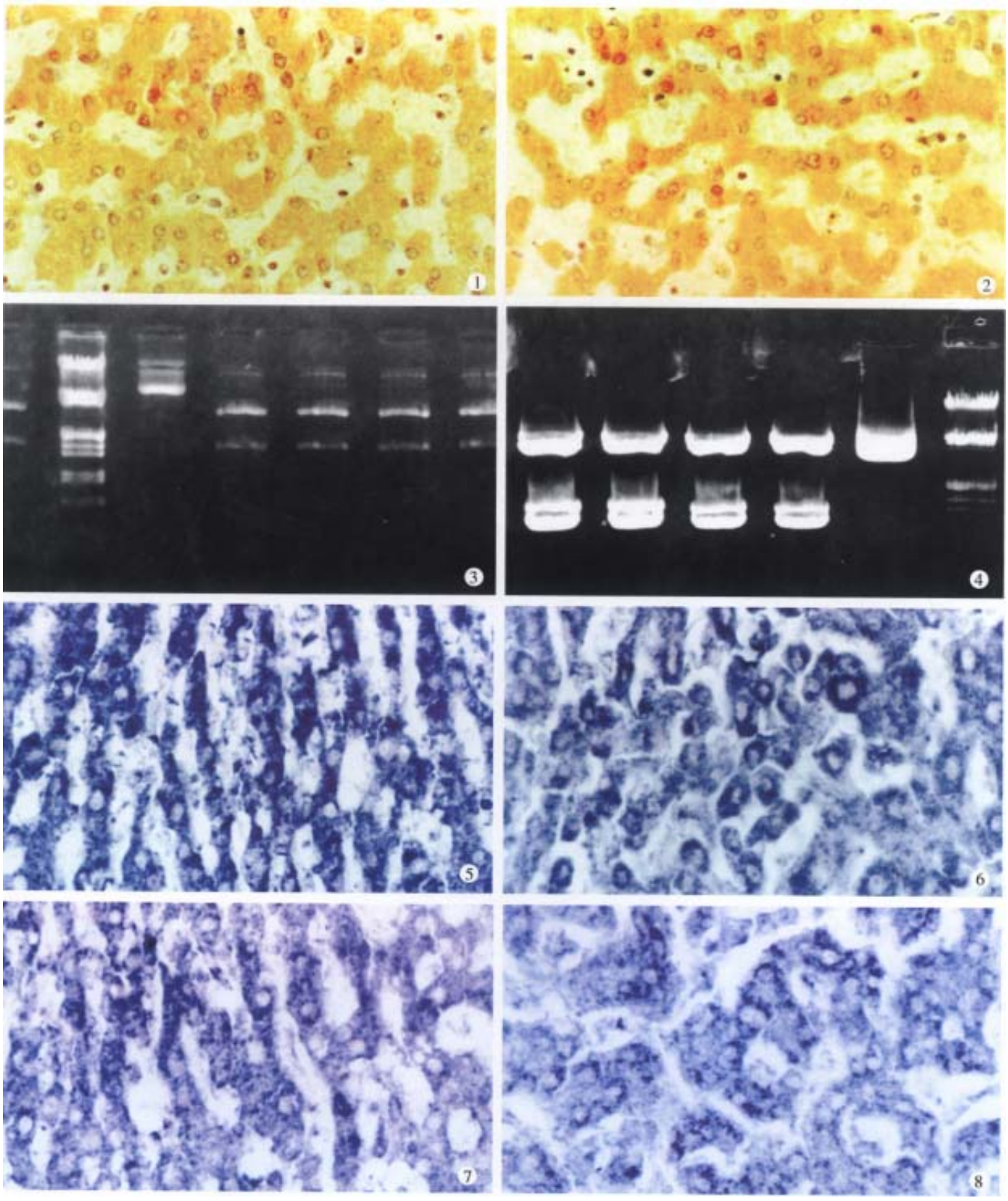

Figure 1 Cx32 protein is positive in normal liver. SP×400
Figure 2 Cx43 protein is positive in normal liver. SP×400
Figure 3 Digestion of pGEM3-cx32 by EcoR-I. A. λ DNA / Hind-III+-EcoR-I marker; B. pGEM3-cx32 plasmid; C. restric tion digestion of 1.5 kb cx32 cDNA.
Figure 4 Digestion of pSG5-cx43 by BamH-I. A . λ DNA / Hind-III+-EcoR-I marker; B. pSG5-cx-43 plasmid; C. restricti on 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-translati onal processing of Cx32 and Cx43 proteins, which may be essential for their tran sport to membrane. Post-translational phosphorylation[7] may be the imp ortant 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 no teworthy, which has direct biological significance in the process of hepatocarc inogenesis. 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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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 trans fusion 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 t he positivity for serum TTV DNA using the polymerase chain reaction method.

RESULTS Eleven and three patients, who received anti-HCV uns creened 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.

Sien Sing Yang[1,2], Chi Hwa Wu[1], Tzu Hsiu Chen[1], Yang Yang Huang[3] and Ching Shan Huang[3]

Department of Gastroenterology-1 and Laboratory Medicine-3, Cathay General Hospital, Taipei and Medical Faculty-2, China Medical College, Taichung, Taiwan, China

Professor Sien Sing Yang, male, born on 1953-11-26 in Taipei, Taiwan. Graduated from China Medical College in 1979, now associate Professor of medici ne specialized in hepatology, having 30 papers published.

Grant from Cathay Groups No. 8003.

Correspondence to: Sien Sing Yang, MD Division of Gastroenterology, Cathay General Hospital, 280 Sect. 4, Jen-Ai Rd, Taipei, Taiwan 10650, China

Tel. 886-2-2708-2121 Ext. 3121£¬Fax. 886-2-2707-4949

Email.yangss@tpts1.seed.net.tw

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INTRODUCTION

After hepatitis B screening in blood donors, the addition of antibody against he patitis 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 transfusi on[5]. In Japan, 12% of blood donors and 46% of chronic non-A-G hepat itis 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 ℃. Among them, 19 recipients were healthy hepatitis B surface antigen (HBsAg) carriers with serum alanine aminotransferase levels (ALT) <45I U/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,

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 tes ted 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 transcriptio n-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 $\geqslant$ 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 immu no-peroxidase 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 μL of serum using QIAMP Blood kit (QIAGEN Ltd., Crawley, UK) and re-suspended in 50 μL of elution buffer. For the first round of PCR, 25 μL of reaction mixture containing 2 μL of the cDNA sample, 1×PCR buffer (10 mM tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 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 am plified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. On e 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±SD, (range)] = 44±20 years (4-76 years) *vs* 45±22 years (4-75 years, $P = 0.52$), number of HBsAg carriers (9 *vs* 10, $P = 0.60$), and volume of blood transfsed [mean±SD, (range)=18.0±14.9 units (2-67 units) *vs* 18.8±12.7 units (1-70 units), $P = 0.58$] were not signif icantly 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-trans fusion 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 μmol/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, respe ctively (Figure 2). Her maximum serum ALT level was 103 IU/L, and maximum serum total bilirubin level was 8.6 μmol/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 he patitis. 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 μmol/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 max imum 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.

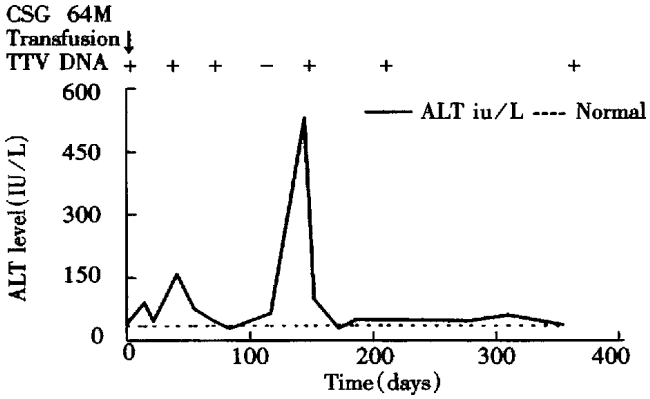

Figure 1 A 64-year-old man who received unscreened blood was positive for TTV DNA before and after transfusion.

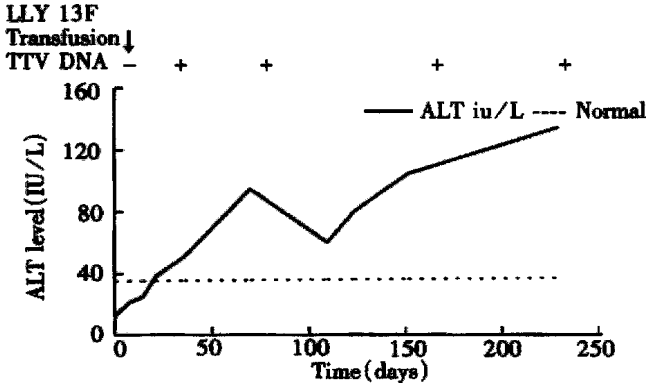

Figure 2 A 13-year-old girl who received unscreene d blood had sero-conversion of TTV DNA after transfusion.

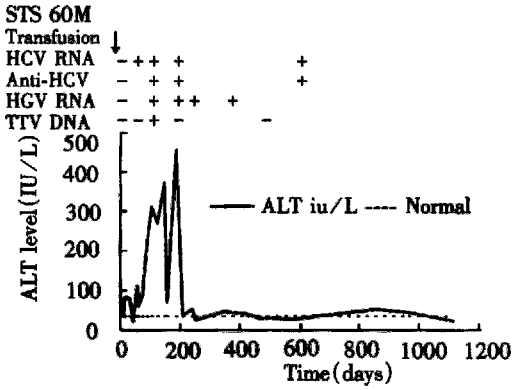

Figure 3 A 60-year-old man who received unscreened blood had sero-conversion of TTV DNA after transfusion. He was co-infected with viral hepatitis C and G.

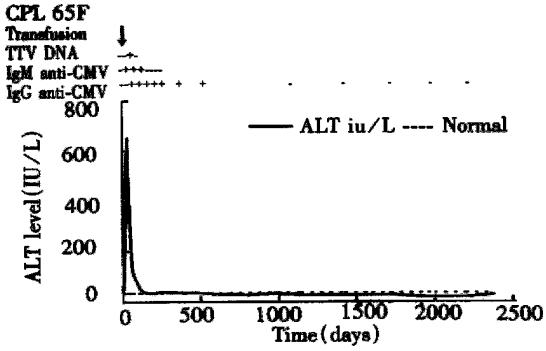

Figure 4 A 65-year-old woman who received anti -HCV screened blood had sero-conversion of TTV DNA after transfusion. She was co-infected with cytomegalovirus infection.

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 trans *fusion*[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 necessary 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 Tai wan 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-r elated 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-infect ed 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 hepatit is 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-d etectable 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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Edited by You DY

PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Stereoselective propranolol metabolism in two drug induced rat hepatic microsomes

Li X and **Zeng S**

Subject headings propranolol; enantiomers; rat hepatic micro some; phenobarbital; β-naphthoflavone

Abstract

AIM To study the influence of inducers BNF and PB on the stere oselective 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 we re assayed by HPLC.

RESULTS A RP-HPLC method was developed to determine propranol ol concentration in rat hepatic microsomes. The linearity equations for R(+)pr opranolol 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 enant iomer were 98.9%, 99.5%, 101.0% at 60 μmol/L, 120 μmol/L, 240 μmol/L respectively. At the concentration level of 120 μmol/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± 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(-)-enan tiomer 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].

Xin Li and Su Zeng
College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310031, China
Xin Li, male, born on 1966-06-27 in Zhejiang, College of Pharmacy, China Pharmaceutical University, BS in 1987; College of Pharmaceutical Sciences, Zhejiang University, MS in 1998; now associate professor, majoring in drug metabolism, having 11 papers published.
Supported by the National Natural Science Foundation of China, No. 39370 805.
Correspondence to: Prof. Su Zeng, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310031, China
Tel. +86-571-7217203
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The oxidative metabolism of propranolol is catalyzed by cytochrome p-450. Exper iments by Otton SV *et al*[8] and Ishida R *et al*[9] indicated that multiple isozymes were involved in popanolol metabolism in rat liver microsomes. Nelson *et al*[10] have observed that stereoselectivity of pro pranolol 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 micro somes 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 (hydrochlor ide), 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-hydroxymeth yl 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 ℃.

Protein concentrations of the microsomal preparations were measured by the method of Lowry *et al*[12] using arystalline 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 nico tinamide, 15 mmol/L $MgCl_2$, 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 5min pre-incubation under air at 37 ℃, reaction was started by adding 10 μL of NADPH regenerating system (10 mg NADP and 3 mg NADPH in 100 μL of 1% $NaHCO_3$). 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 μL 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 μL of the sample was applied to a reversed phase column (Shim-pack CLC-ODS 15 cm×0.6 cm id, 10 μm 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 (Vmax) and the Michaelis-Menten constant (Km) values for propranolol were determined by regression analysis of Lineweaver-Burk plots. The $\bar{x}\pm s$ of three determinations of Vmax and Km was calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of Vmax/Km. 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 μmol/L - 620 μmol/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 preciously 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 μmol/L.

Precision and accuracy The spiked drug-free microsomes at 3 concentration levels (60 μmol/L, 120 μmol/L and 240 μmol/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 (μmol/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 μmol 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 m edia 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 μmol/L for each enantiomer, BNF and PB: microsomes from the rats induced with BNF (β-naphthoflavone) or (phenobarbital) 80 mg/(kg·d), ip, 3 d, respectively. $\bar{x}$±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 μmol/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 dec reased. The ratio of R(+)/S(-) propranolol concentration in BNF group was signi ficantly 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 parame ters of propranolol enantiomers were calculated by Lineweaver-Burk method with the substrate concentrations of 20 μmol/L-600 μmol/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 metab olism *in vitro* in rat hepatic microsomes induced by β-naphthoflavone or ph enobarbital

Group	Enantiomer	Km μmol/L	V_{max} mmol/g/min	Clint L/min/g protein	R(+)Vmax: 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±0.3[fh]	111.0±1[afh]	1.14
	S(-)	39±7[d]	3.3±0.5[g]	84±5[eh]	
PB	R(+)	38±17	0.07±0.03[ef]	2.0±2[cf]	0.038
	S(-)	36±10[d]	1.94±0.07[e]	56.0±1[e]	

Clint (intrinsic clearance) is the ratio of V_{max}/Km, mean± s, n = 3. [a]P<0.05, [b]P<0.01, [c]P<0.001, compared with S(-) propranolol; [d]P<0.05, [e]P<0.01, [f]P<0.001, compared with corresponding enantiomer in control group; [g]P<0.01, [h]P<0.001, compared with corresponding enantiome r in PB group with unpaired t test.

Km of propranolol enantiomers in control group had no stereoselectivity (P>0.05), whereas Vmax and Clint had stereoselectivity of S (-)-propranolol (P<0.01). For BNF induced microsome, Km and Vmax had no stereoselectivity between R (+), S(-)-propranolol (P>0.05), and Clint had significant differenc e between the two enantiomers (P<0.05). For PB group, Km had no stereosele ctivity (P>0.05), and Vmax, Clint had stereoselectivity of S(-)-proprano lol (P<0.001).

Comparing the enzymatic parameters of R(+)-propranolol among three microsomes, Km had no statistical difference (P>0.05), whereas Vmax and Clint had stat istical differences (P<0.05, 0.01 or 0.001); compared with the control group, Vmax 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 n o statistical difference with each other; Vmax for PB group decreased about 1.5 times and that for BNF group nearly remained the same, in addition, no statisti cal 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.

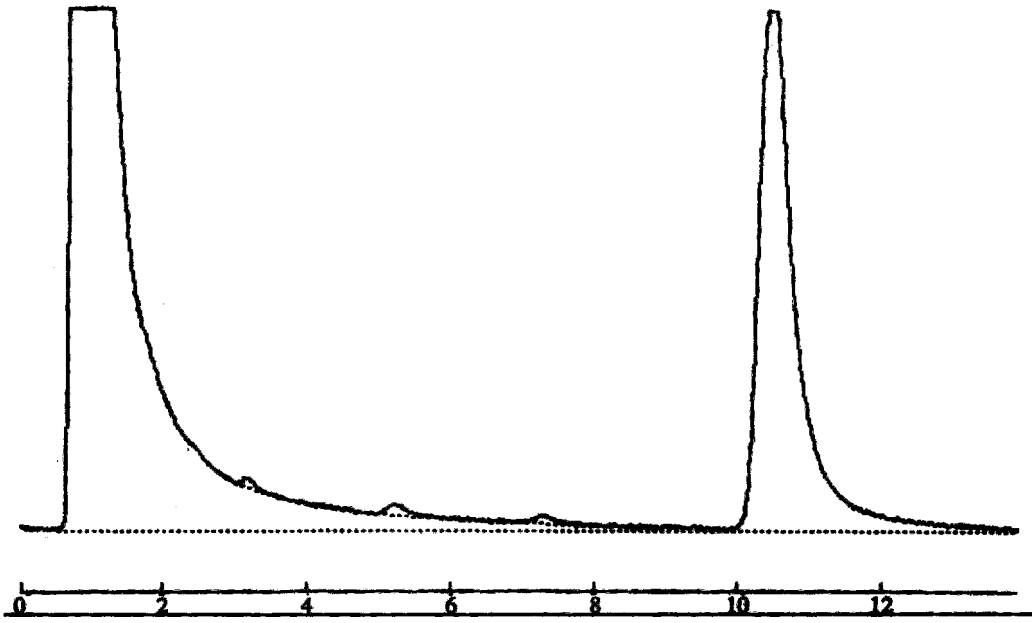

Figure 1 Chromatograms of propranolol after incubati on with rat hepatic microsome. A Shim-pack CLC-ODS column (15cm×0.6cm i.d.) was used. The mobile phase was constituted with ammonium acetate buffer (pH4.0)-methanol (50:50) with flow rate at 1.0mL / min. Propranolol was monitored at 290nm. Propranolol: ^{t}R=10.1min.

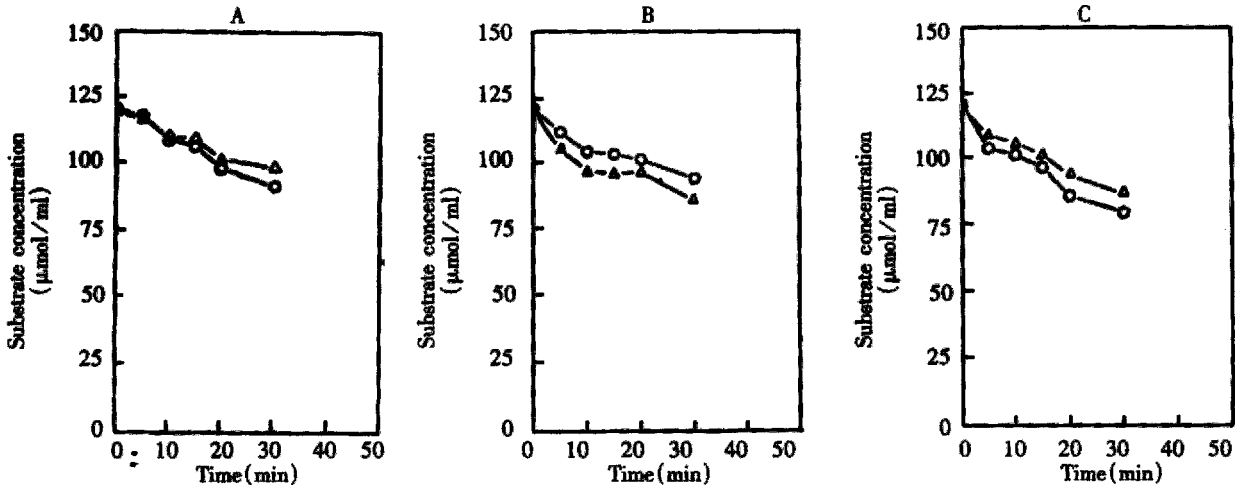

Figure 2 Concentration time curves for R(+) and S(-)-propranolol metabolism in rat hepatic microsomes.
A. Microsome of control. B. Microsome induced by BNF. C. Microsome induced by PB. △—△: R(+)-propranolol. ○—○: S(-)-propranolol.

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-hydro xylation 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(-)-enanti omer.

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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Protective effect of early enteral feeding on postburn impairment of liver function and its mechanism in rats

Zhu L, Yang ZC, Li A and **Cheng DC**

Subject headings early enteral feeding; liver; postburn impa irments

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 asparate amin otransferase (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 mucosalacidos is, reduce portal vein endotoxin level and water content of hepatic tissue, as w ell as plasma concentrations of TNF-α at all timepoints after seve re 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 conce ntrations of TP and ALB were markedly increased 24 h and 48 h posturn in EEF group compared with that of the burn without EEF group ($P<0.01$).

CONCLUSION EEF has significant beneficial effects on the impro vement 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.

Li Zhu[1], Zhong Cheng Yang[1], Ao Li[1] and De Chang Cheng[2]
[1]Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
[2]Critical Care Department, Peking Union Medical College Hospital (PUMCH), Chinese Academy of Medical science (CAMS), Beijing 100730, China
Dr. Li Zhu, male, born on 1958-02-03 in Nanyang City, Henan Province, Han nationality, graduated from Third Military Medical University as a doctor-d egree postgraduate in 1996 and now working in PUMCH as a postdoctoral research fellow, majoring in Traumatic Surgery and Critical Care Medicine, having 17 paper s published.
Project supported by the National Natural Science Foundation of China, No.39290700.
Correspondence to: Dr. Li Zhu, Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
Tel. +86-10-65233768, +86-10-65142049
Email. zhuli58@263.net
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INTRODUCTION

Acute impairement of hepatic function is one of the most common serious complica tions 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 endotxin[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±30 g, were employed in the study. They were housed in individual metabolic cages in a temperature conditioned room (22 ℃-24 ℃) with a 12 h light-dark cycle, allowed access to standard rat chow (provided by Experimental Animal Center, Third Military Medical University) and water adlibium, and acclimatized to the surro undings for 7 days prior to the experiments.

Operative procedure

All animals were weighed and anesthetized with 1%

pentobarbital sodium (30 mg/kg, ip). After laparotomy, a polyeth ylene 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 ℃ 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 ℃) 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 in to three groups: ① EEF group. Enteral feeding was initiated 1h postburn in burn ed animals via feeding tube with a total calorie of 202 $KJ \cdot Kg^{-1} \cdot 24\ h^{-1}$; 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 libium. Timepoints for different measurements and assays in all groups were made at the 3rd, 6th, 12th, 24th and 48th hpostburn, 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 heparinised blood was collected in separate tubes, spun at 3 000 g for 10 min, and the plasma frozen at -20 ℃ until analysis.

Measurements

The gastric intramucosal pH (pHi) was determined with an indirect method as previously described[11] with minor modificat ion. Briefly, animals were anesthetized and given cimetidine (15 mg) intraperit oneally 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 though 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 Pco_2 determined using the blood gas analyzer. A simultaneously obtained arterial blood sample was used for determining the $[HCO_3^-]$. pHi was then calculated as:

$$pHi=6.1+\log([HCO_3^-]/[Pco_2\times 0.03])$$

The multifunction-biochemical analyzer Beckman Synchron CX-7 was used for performing performing liver function tests. The plasma activities of alanine aminotr ansferase (ALT), asparate 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 ℃ for 5 min to inactivate the plasma inhibitor. The samples were incubated with LAL at 37 ℃ 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 ℃ for 24 h, and then weighed again. The liver tissue water contents were calculated as:

Liver tissue water contents=(wet weight-dry weight/wet weight)×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 posturn 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 % ± 0.60 %, 73.01 % ± 0.52 % and 70.18 % ± 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 ($\bar{x}$±s)

Group (samples)	Postburn hours				
	3	6	12	24	48
EEF (40)					
ALT (mmol·s^{-1}/L)	1.21±0.07^{b,d}	1.54±0.14^{b,d}	1.75±0.17^{b,d}	1.39±0.09^{b,d}	1.09±0.09^{b,d}
AST (mmol·s^{-1}/L)	8.58±0.64^{b,d}	11.47±0.81^{b,d}	14.30±1.04^{b,d}	9.75±0.80^{b,d}	7.24±0.65^{b,d}
TB (mmol/L)	16.85±2.01^{a,d}	14.97±2.36^{d}	12.90±2.01^{a,d}	10.82±1.71^{b,d}	6.59±1.61^{b}
DB (mmol/L)	7.72±1.90^{d}	4.68±1.46^{b,d}	2.42±0.78^{b}	1.72±0.36^{b}	1.74±1.09^{b}
Burn (40)					
ALT (mmol·s^{-1}/L)	2.06±0.13^{d}	2.90±0.19^{d}	3.19±0.23^{d}	2.99±0.17^{d}	2.21±0.14^{d}
AST (mmol·s^{-1}/L)	12.20±0.77^{d}	18.77±0.84^{d}	23.13±1.14^{d}	16.18±0.94^{d}	12.56±1.00^{d}
TB (mmol/L)	19.26±2.97^{d}	16.98±2.11^{d}	15.08±2.37^{d}	18.32±2.69^{d}	10.82±1.97^{d}
DB (mmol/L)	8.26±2.17^{d}	9.77±2.02^{d}	5.50±1.32^{d}	7.10±1.43^{d}	3.54±0.94^{d}
Control (40)					
ALT (mmol·s^{-1}/L)	0.61±0.09	0.57±0.07	0.63±0.08	0.58±0.07	0.64±0.10
AST (mmol·s^{-1}/L)	1.55±0.10	1.64±0.09	1.60±0.10	1.71±0.11	1.58±0.10
TB (mmol/L)	5.63±1.41	6.04±1.27	5.81±1.62	6.17±1.02	5.76±1.38
DB (mmol/L)	1.62±0.56	1.46±0.39	1.55±0.42	1.73±0.41	1.53±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 rotein and albumin levels (c/g·L^{-1}, $\bar{x}$±s)

Group (samples)	Postburn hours				
	3	6	12	24	48
EEF (40)					
Total protein	43.10±2.31^{d}	42.49±3.00^{d}	47.61±4.39^{c}	58.33±2.93^{b}	62.36±4.18^{b,d}
Albumin	19.32±1.34^{d}	19.49±1.63^{a,d}	22.76±2.19^{d}	25.70±2.40^{b}	26.77±1.25^{b}
Burn (40)					
Total protein	43.54±2.51^{d}	44.79±2.03^{d}	44.80±3.63^{d}	48.84±4.30^{d}	52.77±1.45
Albumin	19.78±2.11^{d}	20.99±1.23^{d}	21.54±1.72^{d}	21.84±1.84^{c}	22.50±0.83^{d}
Control (40)					
Total protein	53.67±2.43	57.41±1.83	52.55±2.62	55.76±3.18	53.92±2.88
Albumin	25.38±1.62	25.72±1.38	26.08±1.72	24.46±1.33	25.64±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 intram ucosal pH ($\bar{x}$±s)

Group	Samples	Postburn hours				
		3	6	12	24	48
EEF	50	7.119±0.078^{a,b}	6.943±0.089^{a,b}	7.074±0.037^{a,b}	7.285±0.098^{a}	7.257±0.077^{a,b}
Burn	50	7.017±0.037^{b}	6.826±0.049^{b}	6.802±0.080^{b}	6.949±0.082^{b}	7.074±0.041^{b}
Control	50	7.321±0.054	7.296±0.067	7.296±0.067	7.306±0.069	7.348±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 endotox 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

[a]$P<0.01$ *vs* burn group; [b]$P<0.01$ *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

[a]$P<0.01$ *vs* burn group; [b]$P<0.01$ *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 lowe ring 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 circula ting 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 no ted in animals with same amount of enteral saline therapy. In a similar rat model, Bortenschlager *et al*[20] also observed that enteral nutrient s 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 an d/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 acc ordance 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, witha 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 PO_2 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 a pplied directly to humans, the data from this study might provide valuable clues to the further improvement of prevention and treatment of post-tramatic multiple organ dysfunction syndrome. Now, EEF should not be considered merely as a sim ble 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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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

Zhu L, Yang ZC, Li A and **Cheng DC**

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 w as 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 gas tricjuice volume, total acidity and acid output were already significantly decreased ($P<0.01$), and reached the lowest point, 0.63 mL/L± 0.20 mL/L, 10.81 mmol/L±2.58 mmol/L and 2.23 mmol/ h±0.73 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, co nsistent 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.

Li Zhu[1], Zhong Cheng Yang[1], Ao Li[1] and De Chang Cheng[2]
[1]Institute of Burn Research, Southwest Hospital, Third Military Me dical University, Chongqing 400038, China
[2]Critical Care Department, Peking Union Medical College Hospital (PUMCH), Chinese Academy of Medical Sciences (CAMS), Beijing 100730, China
Dr. Li Zhu, male, born on 1958-02-03 in Nanyang City, Henan Province, graduated from Third Military Medical University as a doctor in 1996 and now working in PUMCH as a postdoctoral research fellow, major in traumatic surgery and critical care medicine, having 17 papers published.
Project supported by the National Natural Science Foundation of China, No.39290700.
Correspondence to: Dr. Li Zhu, Institute of Burn Research, South west Hospital, Third Military Medical University, Chongqing 400038, China
Tel. +86-10-65233768, 65142049
Email. zhuli58@263.net
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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 were 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 wit h 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 in hibiting agents in the prevention and treatment of burn-induced gastrointestinal complications. In recent years, however, it is increasingly and widely assumed that

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-neu tralizing 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 ℃-24 ℃) 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 libium, and acc limatized 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 ℃ 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 ℃) 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 (PBH_3, PBH_6, PBH_{12}, PBH_{24} and PBH_{48}).

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 tim epoint, 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 care fully 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 $1.5\text{-}2.0\times10^5$ 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 st arted 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]:

$$\text{GMBL (mL/min·g tissue)}=\frac{\text{Reference sample flow}\times\text{Tissue activity}}{\text{Referen ce sample activity}\times\text{Tissue weight}}$$

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 supernatent was adjusted to 7.0-7.6 with 5 mol K_2CO_3/L. Then another centrifugation was performed and the supernatent was used to assay for adenine nucleotides by using high-per formance 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 con centrations 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. Experi mental 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 co nsistent with the trend of gastric acid production changes (Tables 2, 3).

Table 1 The postburn changes of gastric acid production in rats ($\bar{x}$±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, $\bar{x}$±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 muco sa in rats ($\bar{x}$±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 cace 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 miero-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 H_2-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 presure, 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 nosocomical 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Tumor growth inhibition effect of hIL-6 on colon cancer cells transfected with the target gene by retroviral vector

Xiao B, Jing B, Zhang YL, Zhou DY and **Zhang WD**

Subject headings interleukin-6 gene; gene transfection; gene therapy; colonic neoplasms

Abstract

AIM To observe the tumor inhibitory effects by transfecting I L-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 incasing 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 exp ressing 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 t his 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 w ere 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.

Bing Xiao, Bo Jing, Ya Li Zhang, Dian Yuan Zhou and Wan Dai Zhang
Institute of Gastroenterology, Nanfang Hospital, the First Military Medical University, Guangzhou 510515, Guangdong Province, China
Bing Xiao, man, born in 1962-09-12 in Fujian of China. He received a bachelor degree from Fujian Chinese Medical College in 1984, and received Ph.D. from the First Military Medical University in 1996, now associate professor, specialized in gene therapy and gene cloning.
Correspondence to: Bing Xiao, Institute of Gastroenterology, Nanfang Hospital, The First Military Medical University, Tonghe Road, Guangzhou 510515, Guangdong Province, China
Tel. +86-20-85141544(O)
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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 exis ted 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 con firmed to have anti-tumor effect[1]. Here, we reported a colorectal car cinoma cell line transfected with IL-6 gene, which can secret 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 lab oratory, 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 supernants 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 calcu lated. 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 refe rred as hard agar layer. Then different amount of 2×10^3 HT-29/I-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 gradu ally formed, at the same time untransfected cells died completely after 1-2 wee ks 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 ca pacity 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.

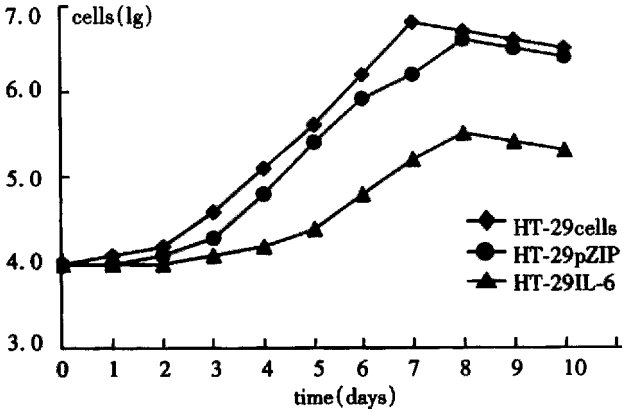

Figure 1 Growth curve of HT-29 IL-6 cells.

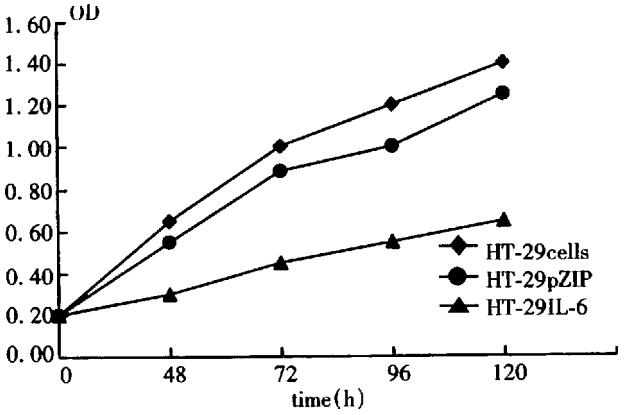

Figure 2 Cell proliferating curve by MTT.

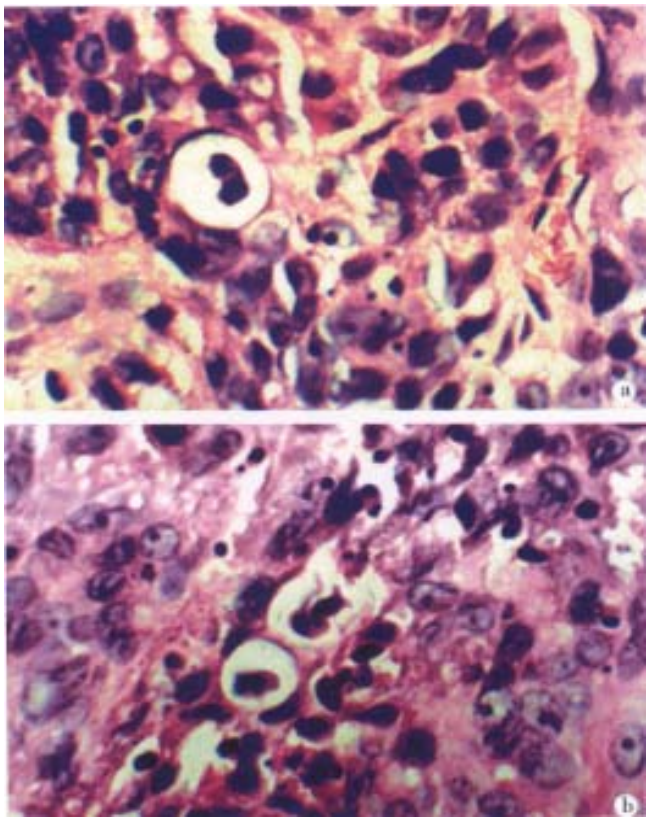

Figure 3 Histopathology of transplantation tumors of nude mice. ×400

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 in hibit 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 I L-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 d emonstrated that IL-6 not only could inhibit tumor growth directly but also pro mote 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 tranduction 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 see n any report on apoptosis induced by IL-6, even some researchers reported IL-6 could inhibit the programmed cell death in MI 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Effect of cholecystokinin and secretin on contractile activity of isolated gastric muscle strips in guinea pigs

Li W, Zheng TZ and **Qu SY**

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 t issue chamber containing 5 mL Krebs solution constantly warmed by water jacked at 37 ℃ and supplied with a mixed gas of 95% O_2 and 5% CO_2. After incubating for 1h under 1g tension, varied concentrations of CCK-8 and se cretin 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; pylous 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 mus cle (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 ampl itude 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 ind omethacin. 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.

Wei Li, Tian Zhen Zheng and Song Yi Qu
Department of Physiology, Lanzhou Medical College, Lanzhou 730000, Gansu Province, China
Wei Li, female, born on 1952-06-07 in Taiyuan, Shanxi Province, graduated from Lanzhou Medical College in 1977, now associate Professor of physiology, majoring digestive physiology, having 25 papers published.
Project Supported by the Natural Science Foundation of Gansu Province, No.ZR-94-085.
Correspondence to: Wei Li, Department of Physiology, Lanzhou Med ical College, Lanzhou 730000, Gansu Province, China.
Tel. +86-931-8289531
Received 1999-05-30 **Accepted** 1999-09-11

INTRODUCTION

CCK and secretin have important actions on gastrointestinal motility *in vivo* and *in vitro*, but few reports have covered the gastric smooth muscle motili ty 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 remoral of mucosa, each strip was suspended in tissue chamber containing 5 mL Krebs solution and constantly warmed by circulating water jacked at 37 ℃ and supplied with 95% O_2 and 5% CO_2. 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 simultane ously 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×

10^{-2} μmol/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 μL 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 = ∑ (amplitude×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/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).

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. Atropi ne (10^{-2} mmol/L) given 3 min before administration of CCK-8 (10^{-2} μmol/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}μmol/L, secretin 10^{-2}μmol/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}μmol/L, secretin 10^{-2}μmol/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 mea n 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} μmol/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

Secreatin (10^{-1} μmol/L, 10^{-2} μmol/L, 5×10^{-2} μmol/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} μmol/L) and secretin (10^{-2} μmol/L) were combined, the effect was similar to CCK-8 (10^{-2} μmol/L) alone (Tables 1, 2).

DISCUSSION

CCK-8 could increase the mean contractile amplitude of antral CM and motility i ndex 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 ampl itude of body and antral LM, but did not affect the gastric strips of the corre sponding 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 t he 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 am plitude of LM and CM of antrum by CCK-8 was eliminated in rats. There was m arked 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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PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Immunocytochemical identification and localization of APUD cells in the gut of seven stomachless teleost fishes

Pan QS, Fang ZP and **Zhao YX**

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 seattered in the intestinal mucosa of grass carp (Cyenopharyngodon idellus), black carp (Mylopharyngodon piceu s) and common carp (Cyprinus carpio) were investigated with 20 kinds of an tisera 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 amblyoce phala) 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 dis tribution density.

RESULT Gastrin (GAS), Gastric inhibitory peptide (GIP), glucagon (GLU), glucagon-like immunoreactants (GLI), bovine pancreatic polype ptide (BPP), leucine-enkephalin (ENK) and substance P (SP)-IR endocrine ce lls were found in the gut of grass carp, black carp and common carp, and somatos tatin (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, bigh ead, 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 provide d new morphological evidence for neuroendocrine and paracrine secretory function of these enteroendocrine cells.

CONCLUTION 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 e ndocrine cells scattering in the gut mucosa belong to the APUD system. Among the m, the hormones secreted by SP-, ENK-, SOM- and GLU-IR endocrine cells belon g 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, dist ribution 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.

Qian Sheng Pan[1], Zhi Ping Fang[2] and Ya Xin Zhao[2]
[1]College of Fisheries, Huazhong Agricultural University, Wuhan 43 0070, Hubei Province, China
[2]College of Animal Husbandry and Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hubei Province, China
Qian Sheng Pan, male, born on 1943-11-18 in Dushan City, Guizhou Prov ince, Han nationality, Graduated from Shanghai Fisheries University in 1968, Pro fessor of Ichthyology, major in studies on animal immunocytochemistry, having mo re than 50 papers published.
Presented at the International Conference on Gastrointestinal Hormones a nd Gastrointestinal Motility, Beijing, 26-28 October, 1993 and the 4th China-J apan Histochemistry and Cytochemistry Symposium, Chongqing, 21-24 September, 1996.
Supported by the National Natural Science Foundation of China. No. 39070 666.
Correspondence to: Prof. Zhi Ping Fang, College of Animal Husba ndry and Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, Hu bei, China
Tel. +86-27-87393766-2466, Fax. +86-27-87396057
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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-e ntero-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. amblyocephala*), 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 ℃) and sectioned (5 μm). The sections were mounted on slide, treated with gelatin and dried 12 h at 45 ℃.

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% H_2O_2-methanol for 10min at 20℃ to block auto-peroxidae; ② to incubate with normal goat serum for 30min at 20 ℃ 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 a ntisera 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 mm^2 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 end ocrine 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. amblyocephala*. 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/mm^2, 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 endoc rine 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 cros s reaction with glucagon	Dr. D Grube, Hannove
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-Hydroxytriptamine	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 Yanaigar a, Shizuoka
Synthetic porcine vasoac tive intestinal polypeptide	1:2000	R-502	Reacts against entire molecules	Dr. N Yanaihara, Shizuoka
Bombesin	1:3000	27070	Cross reaction with GRPImmunonrclear	Corp. Stillwater
Neuron specific enolase	1:1000	—	—	Dako, Copenharge
Prochymosin	1:2000	—	—	A. Andren, Uppsala
Pepsinogen	1:2000	—	—	A. Andren, Uppsala
Rabbit PAP	1:100	Z-113	—	Dako, Copenharge
Guineapig PAP	1:50	24699	—	Dako, Copenharge
PAP Kit	1:100	61-2003	—	Zymed Lab. Inc., SouthSan 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 ($\bar{x}$±S)

Fish species	Gut parts	GAS	ENK	BPP	SP	GLI	GIP	GLU	SOM
Crass carp	I	64±13	85±17	52±12	56±14	44±19	23±19	61±7	—
(*C.idellus*)	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	77±8	57±6	72±6	20±5	68±17	59±13	2±3	—
(*M. piceus*)	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	25±6	38±4	12±6	—	—	4±3	2±2	13±12
(*C. carpio*)	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=M idgut; IV=Anterior segment of hindgut; V=Rectum; —: IR endocrine cell wasn't found, PAP method was used in the table; n=5, $^aP<0.05$, $^b P<0.01$, $^cP>0.05$.

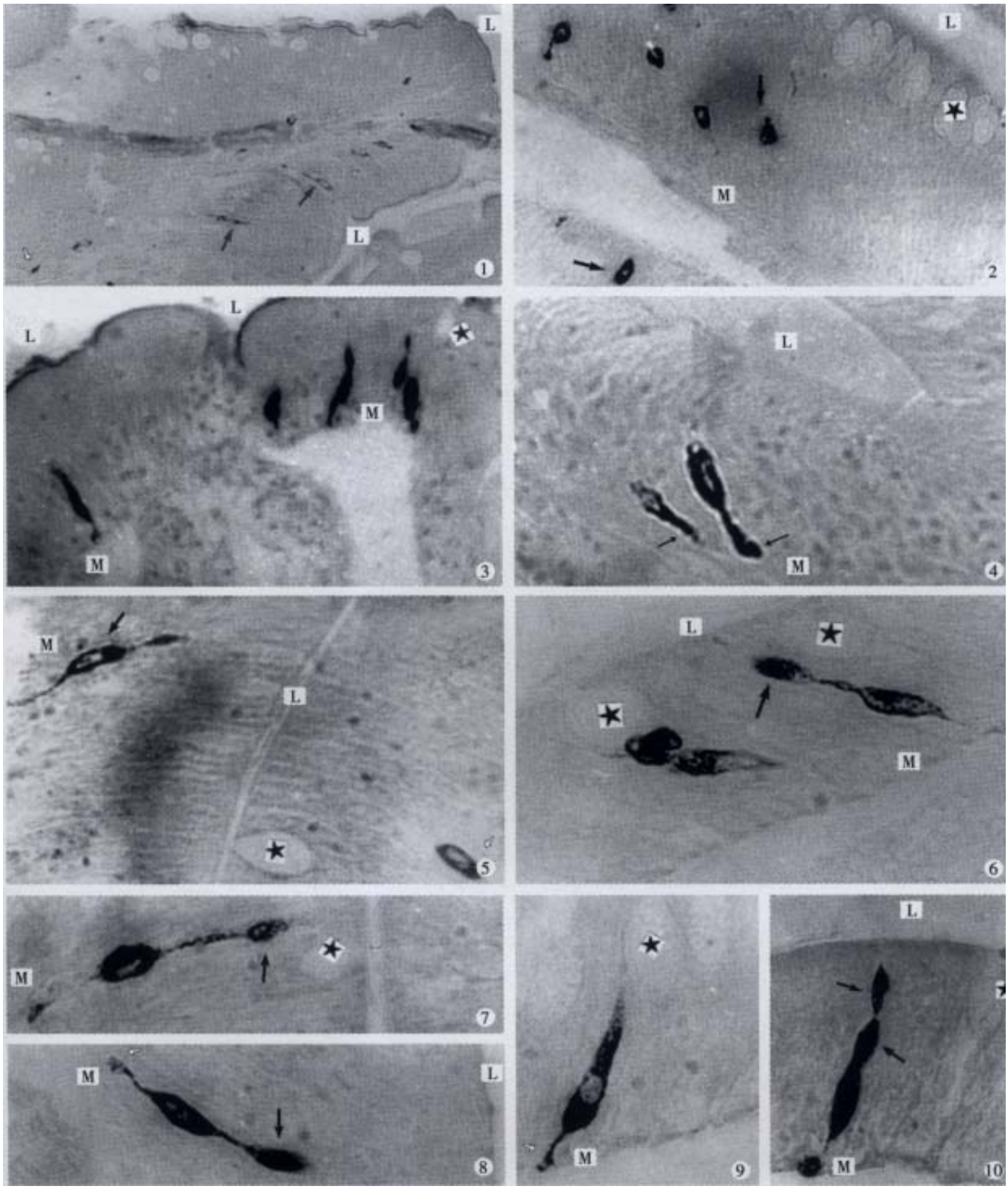

Figure 1 IR endocrine cells in epithelium (↑) and lamina propria (-), ×100
Figure 2 IR endocrine cells (↑) in bottom part of the gut f old, ×200
Figure 3 IR endocrine cells in apical part of the gut fold, ×400
Figure 4 Basal processes (↑) extended to basement membrane, ×600
Figure 5 IR endocrine cells 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 gu t of four teleost species ($\bar{x}$±s)

Fish species	Gut parts	GAS	GIP	GLU
Silver carp	I	17±5	2±2	43±10
(*H. molitrix*)	II	2±4[b]	6±3[a]	64±35[c]
	III	—	—	126±36[b]
	IV	—	—	61±26[c]
	V	—	—	30±28[c]
Bighead	I	35±10	13±3	29±16
(*A. nobilis*)	II	—	—	27±7[c]
	III	—	—	79±7[b]
	IV	—	—	60±62[c]
	V	—	—	47±46[c]
Silver crucian	I	32±17	1±2	53±18
Carp (*C. gibelio*)	II	3±3[b]	—	20±11[b]
	III	—	—	
	IV	—	—	11±13[b]
	V	—	—	—
Bluntnose black	I	31±8	4±1	32±16
Bream (*M.*	II	—	—	24±6[c]
Amblyocephala)	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, [a]P<0.05, [b] P<0.01, [c]P>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 ad jacent cells (Figures 7-10). A few of IR endocrine cells had only one cytoplasmi c 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 conchonius. 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. Conchonius*[20]. Rombout[6]considered SOM-IR end ocrine 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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