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

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Reviews

Molecular biology of liver disorders: the hepatitis C virus and molecular targets for drug development

Howard J. Worman and Feng Lin

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INTRODUCTION

Molecular biology has made a tremendous impact on the diagnosis and treatment of liver diseases[1,2]. In particular, advances in molecular biology made possible the discovery of the virus that causes hepatitis C. In this review, we use hepatitis C as an example of the impact that molecular biology has made in t he area of liver disorders. We emphasize how our growing understanding of the he patitis C virus (HCV) has lead to the identification of targets for development of new treatments.

THE HEPATITS C VIRUS (HCV)

Basic molecular virology of HCV

Investigators at Chiron Corporation were the first to discover HCV and r eported this in a landmark paper published in 1989[3]. The virus was identified by antibody screening of cDNA expression libraries made from DNA and RNA from the plasma of chimpanzees. These chimpanzees were inoculated with serum from human s with what was then called post-transfusion "non-A, non-B" hepatitis. The DNA expression library was screened with antibodies from sera of other patients with "non-A, non-B" hepatitis. This led to the isolation of clones that we re derived from portions of the viral genome and encoded fragments of viral poly peptides. Treatment with RNase and DNase showed that HCV was a positive-stranded RNA virus[3]. In an accompanying paper, the investigators who discover ed HCV and their collaborators showed that the vast majority of individuals with chronic "non-A, non-B hepatitis" had antibodies against the newly identifi ed viral polypeptides[4].

After the discovery of HCV, its entire genome was cloned and sequenced i n several laboratories[5-8]. HCV is a member of the Flavivaridae family. Once HCV infects cells, the positive, single stranded RNA genome is translated into a polyprotein of 3010 to 3033 amino acids, depending upon the strain (Figur e 1). The viral RNA is not capped and translation occurs via an internal ribosome entry site (IRES) at the 5' end of the viral RNA[9,10]. The mechanis m of translation of uncapped viral RNA therefore differs from that used by virtu ally all cellular mRNAs which are capped at their 5' ends.

Both host cell and viral proteases cleave the HCV polyprotein into severa l smaller polypeptides (Figure 1). The major structural proteins are core prote in and two envelope proteins called E1 and E2. Core protein forms the nucleocap sid of the mature virion and E1 and E2 are present in the viral envelope. A small polypeptide called P7 is also generated as a result of cleavage at the E2-NS 2 junction but its function is not clear. Four major non-structural proteins called NS2, NS3, NS4, and NS5 are generated, two of which, NS4 and NS5, are furth er processed into smaller polypeptides called NS4A, NS4B, NS5A, and NS5B. The non - structural proteins have enzymatic functions that are critical for viral replica tion in cells, such as RNA helicase (NS3), protease (NS2, NS3-NS4A complex) and RNA polymerase (NS5B) activities. NS5A has been implicated in determining sensi tivity to interferon alpha.

HCV replication and interactions with host cells

Little is known about the fundamental aspects of HCV replication, primarily beca use a robust cell culture has not been established. Although viral proteins and RNA components involved in critical steps in HCV replication are known, very li ttle is understood about the mechanistic details or the role of accessory host cell factors. Some of the basic steps in HCV replication that occur in infected cells are outlined here.

After infection of cells, HCV RNA must be translated into protein. HCV RN A translation is initiated by internal ribosome binding, not by 5'-end depend ent mechanisms[9,10]. Internal initiation is specified by an IRES ele me nt. Such elements were first discovered in the genomes of picornaviruses[11]. The IRES is believed to require the set of canonical translation initiatio n factors in order to function. In addition, IRES function is also thought to be dependent on other cell proteins. However,

Departments of Medicine and of Anatomy and Cell Biology, College of Physi cians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 100 32, USA
Introduction of Authors: Howard J. Worman is Associate Professor of Medicine and Anatomy and Cell Biology at Columbia University's College of Physicians and Surgeons and Director of the Division of Digestive and Liver Diseases of the Med ical Service at the New York-Presbyterian Hospital Columbia-Presbyterian Campus. He has over 70 scientific papers as well as publications on liver diseases for the lay audience.
Correspondence to: Dr. Howard J. Worman, Depar tment of Medicine, College of Physi cians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032, USA
Tel. 212-305-8156, Fax. 212-305-6443
Email. hjw14@columbia.edu
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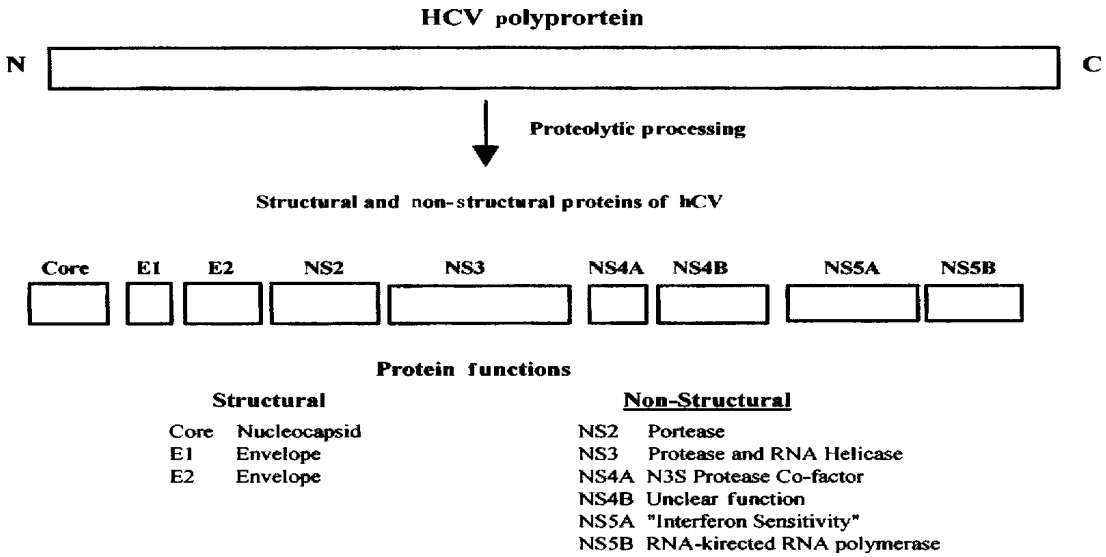

Figure 1 HCV proteins and their functions. The positive-stranded RNA of about 10,000 nuc leotides is translated into a polyprotein of approximately 3000 amino acids. The polyprotein is proteolytically cleaved into several smaller proteins. Core, E1, and E2 are structural polypeptides. Core protein is the virus nucleocapsid and E 1 and E2 are viral envelope proteins. A small polypeptide known as P7 (not shown) is also produced by additional cleavage between E2 and NS2. The major non-str uctural proteins are NS2, NS3, NS4, and NS5. NS4 is further processed into NS4A a nd NS4B and NS5 into NS5A and NS5B. NS2 and part of NS3 are proteases that proce ss the viral polyprotein. NS3 also has RNA-helicase activity. NS4A is a cofacto r for the NS3 protease and NS5B is an RNA-dependent, RNA polymerase. The functi ons of NS4B and NS5A are less well understood but NS5A is thought to play a role in determining sensitivity to interferon.

no single cell protein has been shown to be dispensable for the function of all IRESes.

HCV RNA must be unwound for efficient protein synthesis to occur. This process is catalyzed by a RNA helicase that is part of the viral NS3 protein. The three -dimensional structure of the HCV NS3 helicase domain has been determined and details about its function are emerging[12-14]. At the present time, it is not known if host cell co-factors are necessary for optimal functioning of the NS3 helicase. Cellular RNA helicases have also been shown to bind to the HCV core protein[15-17], however, it is not known if they also play a role in unwinding viral RNA.

After its synthesis, the HCV polyprotein is processed into the structural and no nstructural proteins. Proteolytic cleavages between structural polypeptides are catalyzed by signal peptidase in the endoplasmic reticulum. Two virally encode d proteases, NS2 and NS3, catalyze the other cleavages of the HCV polyprotein. The NS3 protease contains a trypsin-like fold and a zinc-binding site and is c omplexed with the viral protein NS4A[18,19].

HCV RNA must be replicated to produce more virions. The viral protein NS5B is an RNA-dependent RNA polymerase. NS5B bears some similarity and motif organizati on to poliovirus polymerase and human immunodeficiency virus 1 (HIV-1) reverse transcriptase but adopts a unique shape due to extensive interactions between th e fingers and thumb polymerase subdomains that encircle its active site[20]. The precise mechanism of action of the HCV NS5B polymerase is not known. C ellular or viral protein or RNA binding partners that function as subunits or in itiation factors may be necessary for optimal activity.

The replication rate of HCV in human hosts is estimated to be extremely high. It appears that the estimated half-life of a viral particle is 2.7 h with pr oduction and clearance of about one trillion viral particles a day[21] . This rate of virion production is approximately 1,000 times greater than that estimated for HIV-1. Factors responsible for the high rate of HCV replication ar e not entirely understood. This rapid rate of replication can explain the develo pment of mutant strains or quasispecies that occur after HCV infection. It may a lso make development of an effective vaccine difficult.

DRUG TARGETS FOR THE TREATMENT OF HCV INFECTION

"Non-specific" anti-viral agents for HCV infection

The currently available drugs for the treatment of hepatitis C are anti-viral a gents not specifically directed against HCV. The United States Food and Drug Adm inistration (FDA) has approved several preparations of recombinant interferon al pha for the treatment of chronic hepatitis C. Interferon alpha is a suboptimal t reatment in that only about 20% or less of patients who complete a one year cour se of treatment respond successfully as determined by the inability to detect HCV in serum 6 mo after the drug is stopped[22]. Numerous adverse even ts are also associated with interferon alpha, most notably flu-like symptoms,

neutropenia, thrombocytopenia, and depression. Interferon alpha must be administe red by injection 3 times a week. Newer preparations of interferon alpha-2b complexed with polyethylene glycol have been developed[23]. These so-cal led "PEG-ylated" interferon alphas are released more slowly and evenly into the bloodstream and need only be administered by injection once a week. "PEG -ylated" interferon alphas will likely be approved for the treatment of chron ic hepatitis C in the United States in the year 2000 or 2001.

The combination of interferon alpha-2b plusing oral ribavirin is approved in many countries for the treatment of chronic hepatitis C. Combination treatment for 6 mo leads to no detectable virus in serum 6 mo after stopping thera py in approximately 40% of subjects[24-26]. The major adverse event as sociated with ribavirin is hemolytic anemia, which in rare cases can be life thr eatening. VX-497 is a compound in development that inhibits inosine monophospha te dehydrogenase and may have antiviral affects similar to those of ribavirin[27]. VX-497 is being studied in combination with interferon alpha to e stablish if it is as effective as ribavirin with a similar or preferable adverse events profile.

Other cytokines have also been tested in the treatment of HCV infection. A recent report of a pilot study suggests that interleukin-10 may slow the deve lopment of liver fibrosis in subjects with chronic hepatitis C[28]. Inte rleukin-10, however, was not shown to have anti-viral activity against HCV.

Agents directed against HCV non-structural proteins

The next generation of drugs for the treatment of hepatitis C will likely be dir ected against non-structural HCV proteins with known enzymatic activities. Thre e major targets are the NS3 protease, NS3 helicase, and NS5B RNA-directed RNA polymerase. The fact that these proteins have enzymatic activities that can be mea sured *in vitro* make them amenable to high-throughput screening techniques f avored by pharmaceutical chemists. This obviates the need to grow HCV in cell cultures or in small animals, tasks that have eluded investigators.

The three-dimensional structure of the HCV NS3 protease domain has been determi ned by X-ray crystallography[18,19]. In addition, the structure of the NS3 protease domain complexed with an inhibitor has recently been established[29]. Armed with this knowledge, chemists can use rationale drug design to synthesis compounds to inhibit protease activity. Rational drug design can be c ombined with combinatorial chemistry in which a library of thousands or more str ucturally similar molecules is tested against the target. By combining rational drug design and combinatorial chemistry with high throughput screening techniques that measure enzymatic activity, N3S protease inhibitors can be identified, fu rther developed and ultimately tested in infected chimpanzees and humans.

Similar methods can be used to identify inhibitors of the N3S helicase domain an d NS5B RNA dependent RNA polymerase. The three-dimensional structures of these proteins are also known[12-14]. Although human cells have RNA helicas es, their mechanism of action is probably different than RNA helicases of viruses[30]. Animal cells do not have RNA-dependent RNA polymerases, making N S5B an attractive target for an anti-viral agent.

Agents directed against HCV RNA

HCV RNA differs from cellular mRNA. First, it has a unique ribonucleotide seque nce. Second, as outlined above, HCV RNA is uncapped and translation is initiated via an IRES. Third, the viral RNA must be efficiently packaged into the matur e virions. These features make the HCV RNA a potential target for anti-viral drugs.

Ribozymes are catalytic RNA molecules that can be designed to cleave speci fic RNA sequences. Ribozymes therefore have potential utility as drugs against RNA viruses, including HCV. Investigators at Ribozyme Pharmaceuticals have deve loped ribozymes against conserved genomic sequences in HCV[31]. These ri bozymes cut the viral RNA at specific sequences and are able to inhibit HCV RNA -directed protein synthesis and HCV RNA replication in *in vitro* systems. It is anticipated that a ribozymes against HCV will be tested in human clinical tr ials in the next couple of years.

HCV RNA is translated by internal ribosome binding mediated by an IRES[9,10]. The IRES adopts a tertiary structure that is necessary for function[32,33]. Interference with IRES structure or function is a logical approach to attacking HCV replication. Antisense olignucleotides targeted to a stem-loop s tructure within the IRES have been shown effective at inhibiting HCV gene expres sion[34,35]. Other small molecule inhibitors can potentially be designed to inhibit HCV IRES function, which can be measured using *in vitro* assays a daptable to high throughput screening methods.

HCV RNA genomes must be packaged into newly synthesized virions. This is likely mediated by specific interactions between sequences in RNA and core prote in. Synthetic oligonucleotides corresponding to sequences in the 5' region of the HCV genome have been shown to bind to core protein[36]. Agents that block HCV RNA binding to core protein could be useful as inhibitors of virion pr oduction.

Agents directed against other targets of HCV

HCV presumably gains access to hepatocytes, and possibly other cells, by binding to a plasma membrane protein receptor or receptors. HCV envelope protein s E1 and E2 have been shown to interact with plasma membranes of hepatocytes and other cells[37]. E1 and E2 may form a heteromeric complex[38], however, it is not clear if their association is necessary for binding to cell m embranes.

The receptors for HCV entry into liver cells are also not presently known. Howe ver, an interaction between HCV E2 and a plasma membrane protein CD81 has been described and characterized in some detail[39,40]. It is difficult to est ablish if this interaction mediates HCV entry

into cells, primarily because a ro bust cell culture system for HCV is not currently available. Even if CD81 or other proteins that bind to HCV E1 and E2 are not receptors that mediate viral entr y, knowledge of these interactions could lead to the development of drugs that inhibit the binding of HCV to cells. Additional experimental work that may lead to the definitive identification of HCV receptors could also lead to the developm ent of viral entry inhibitors. Structural analysis of the interactions of viral envelope proteins with cellular receptors should be of tremendous value in the d evelopment of drugs as it will be in the case of HIV-1[41,42].

The core protein of HCV is another potential target for the development of anti -viral drugs. In infected cells, HCV core protein is synthesized on the endopla smic reticulum membrane with a large domain facing the cytoplasm[43]. HCV core protein has been shown to form multimers[44] and the self-intera ction of core protein is likely important in the assembly of the virion nucleoca psid. HCV core protein expression may also influence critical processes that hav e implications for cellular pathophysiology. Core protein may play a role in tra nsformation and oncogenesis[45] or be involved in regulating apoptosis a s it has been shown to bind to the cytoplasmic domain of lymphotoxin-β receptor, a member of the tumor necrosis receptor protein family[46]. HCV core protein also binds to a cellular RNA helicase and this interaction may adversely affect host cell protein synthesis and provide the viral RNA with enhanced access to the cell's protein synthesis machinery[15-17]. Inhi bitors of core protein self-assembly or its interactions with other cellular pr oteins could therefore be useful in the treatment of hepatitis C.

CONCLUSIONS

The innovative application of standard technique in molecular biology led to the discovery of HCV. The development of treatments for HCV infection has lagged o ur understanding of the molecular biology of the virus because neither a small a nimal model of the disease nor a robust cell culture system for the virus curren tly exist. Recent advancements such as the development of "sub-genomic replic ons"[47] and an infectious viral RNA clone that can infect chimpanzees[48] may partially overcome these limitations. Nonetheless, based on our present understanding of the molecular biology of HCV, several steps in the vir allife cycle can currently be targeted for the development of anti-viral drugs .

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Edited by Lu J
proofread by Mittra S

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

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New insights into the pathogenesis of intestinal dysfunction: secretory diarrhea and cystic fibrosis

Kim E. Barrett

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INTRODUCTION

A major function of the intestinal epithelium is to control the amount of fluid entering into and being absorbed from the lumen[1]. In healthy conditio ns, net fluid movement follows an absorptive vector, although significant secret ion also takes place to subserve digestive function. Thus, the secretion of flui d, driven by the active secretion of electrolytes, is important for maintaining the fluidity of intestinal contents during various stages of digestion and there by allowing for diffusion of enzymes and nutrients. In the setting of disease, dysregulation of intestinal transport mechanisms may alter the balance between ab sorptive and secretory processes such that secretion predominates, leading to th e clinical consequence of diarrhea. However, under conditions of both health and disease, fluid secretion is driven largely by the active secretion of chloride ions. Thus, there are both basic and clinical reasons for wishing to gain a full understanding of the basis and regulation of this transport process. The goal o f my article, therefore, will be to review our understanding of intestinal chlor ide secretion and the ways in which it is regulated. Recent insights in this are a enhancing our ability to intervene in diseases where chloride secretion is over-expressed, such as infectious and inflammatory diarrheal illnesses will also be discussed. This article will also cover the implications of intestinal secretory mechanisms for a genetic disease where chloride secretion is under-expressed, namely cystic fibrosis, where significant intestinal dysfunction, including obstruction and malabsorption, may also ensue.

Professor and Vice-Chair for Research, Department of Medicine, University of California, San Diego, School of Medicine, San Diego, CA 92103, USA
Professor Kim E. Barrett received her Ph.D. degree from University College London, England, in 1982, and postdoctoral training from the National Institutes of Health, USA. She is currently Professor and Vice-Chair for Research in the Department of Medicine, University of California, San Diego, School of Medicine, and directs an NIH-funded research program that focuses on the mechanisms and regul ation of intestinal electrolyte transport. She has published over 140 original papers, book chapters and review articles, and is the current Editor-in-Chief of American Journal of Physiology-Cell Physiology.
Correspondence to: Kim E. Barrett, Ph.D. UCSD Medical Center, 8414 200 West Arbor Drive San Diego, CA 92103-8414 USA
Tel. 619-5433726. Fax. 619-5436969
Email. kbarrett@ucsd.edu
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MECHANISMS OF INTESTINAL CHLORIDE SECRETION

The secretory mechanism

The details of the ion transport pathways making up the intestinal chloride secretory mechanism have been quite well worked out at this point[1,2]. The mechanism is predominantly expressed, at least as a ssessed functionally, in epithelial cells lining the crypts of both the small in testine and colon, although there may be a small degree of secretion derived fro m villus or surface epithelial cells in addition[2]. Chloride is taken up from the bloodstream across the basolateral membrane of epithelial cells via a sodium/potassium/2 chloride cotransporter that has been cloned and designated as NKCC1[3]. This cotransporter is driven secondarily by the low intrace llular sodium concentration established by the active sodium/potassium ATPase, also localized to the basolateral membrane. This allows chloride to accumulate in the cell cytosol above its electrochemical equilibrium. When apical chloride channels are opened, this chloride is then free to flow out of the cell down this electrochemical gradient, resulting overall in net transepithelial transfer of the anion. Chloride exit occurs predominantly through a channel referred to as CF TR, which is the product of the gene that is defective in the setting of cystic fibrosis[4]. More recently, in addition, it has been recognized that the apical membrane of intestinal epithelial cells may also contain calcium-activa ted chloride channels of the CLCA family, and perhaps other channels for these a nd/or other anions[5,6]. The transport mechanism is also critically depe ndent on the presence of basolateral potassium channels which serve to recycle co-transported potassium back across the basolateral membrane, and thus prevent cell depolarization[7,8].

Positive regulation of chloride secretion

Chloride secretion is stimulate d in the intestine by a broad array of substances derived from local and more di stant endocrine cells, enteric nerves, neighbouring cell types such as immune cells and subepithelial myofibroblasts, and exogenous factors such as bacterial enterotoxins[2]. These regulatory pathways provide for minute-to-minute physiological increases in the extent of secretion (such as in response to inge stion of a meal) but also contribute to the marked upregulation of secretion that can occur in the setting of a disease[1]. Despite many hormones, neur otransmitters and other mediators that have been identified and charac terized to date as stimuli of chloride

secretion, however, appear to exert their effects t hrough two main signaling pathways[1]. The first of these is mediated by changes in intracellular cyclic nucleotides, and results in large magnitude, sustained secretory responses. At an intracellular level, the primary locus for regulation appears to be the cyclic-nucleotide mediated phosphorylation and open ing of the CFTR chloride channel, particularly via cAMP and the cAMP-dependent protein kinase, PKA[9,10]. It is increasingly recognized that at least o ne type of PKA is "scaffolded" in the proximity of CFTR via various interacting proteins, which likely contributes to the efficiency of signal transduction in response to cAMP-mobilizing agonists[11,12]. Chloride secretion can a lso be evoked by agonists that increase intracellular levels of cGMP, the best physiological example of this pathway being the stimulation by the peptide agonis t guanylin, which binds to an apical receptor also shared by the heat-stable to xin of *E.coli*[13]. cGMP-dependent secretion involves a cGMP-depend ent protein kinase and/or cross-activation of PKA, and is very similar to that induced by increases in cAMP in terms of its magnitude, kinetics and dependence on CFTR activation. Cyclic nucleotide-dependent secretion, in general, may also involve regulatory events at additional levels within the transport machinery.For example, there is evidence to suggest that sustained secretion requires the insertion of additional NKCC1 molecules into the basolateral membrane (or at least an alteration in their rate of endocytosis), and cAMP-activated potassium channels have also been postulated on functional grounds[3,8,14].

Chloride secretion can also be evoked by agonists that are capable of causing an increase in cytosolic calcium concentrations. In contrast to the cyclic-nucleo tide dependent chloride secretory responses discussed above, calcium-dependent responses are distinctive in that they are smaller and also considerably more tr ansient[2]. However, calcium-dependent secretion may nevertheless play a physiological role, particularly in the setting where only a brief, self-lim ited secretory response is called for. Moreover, synergism occurs between the se cretory effects of cAMP- and calcium-dependent agents, allowing the host, to call on markedly upregulated rates of secretory function at times of threat (such as invasion by pathogenic microorganisms). Finally, calcium-dependent secretor y responses may assume far greater significance in the setting of cystic fibrosi s, where the cAMP- dependent chloride channel, CFTR, is lost or dysfunctional[15]. At an intracellular level, at least part of the chloride secretory e ffect occurring in response to an increase in cytosolic calcium is secondary to the opening of basolateral, calcium-sensitive potassium channels. This, in turn , increases the driving force for chloride to exit across the apical membrane th rough the small proportion of CFTR channels that are hypothetically open even in resting cells[16]. However, evidence is also accumulating to suggest th at elevations in calcium, acting in concert with the calmodulin-dependent prote in kinase CaMKII, open additional apical chloride channels of the CLCA family [17-20]. This view lends additional credence by the observation that calci um-dependent agonists can cause chloride secretion in at least some epithelial cells even in the absence of any measurable CFTR function[17,21].

Negative regulation of chloride secretion

Work from our laboratory has al so indicated that certain intracellular signaling pathways may lead to the inhib ition of ongoing chloride secretion, and/or may result in its termination[2]. Such negative signals appear to be particularly pertinent in the case of c alcium-stimulated chloride secretion, with the transience of this secretory res ponse implying that endogenous factors may serve to limit its extent. We have id entified two main pathways whereby calcium-dependent chloride secretion is inhi bited. The first of these, interestingly, is activated by substances that alsos erve to initiate calcium-dependent chloride secretion. An example is the muscar inic agonist carbachol, which evokes a transient chloride secretory response tha t renders intestinal epithelial cells refractory to re-stimulation by another calcium-dependent secretagogue[22]. Both the stimulatory and inhibitory effects of carbachol are dependent on cytosolic calcium [23]. The inhib itory pathway involves the generation of a novel inositol phosphate mediator, in ositol 3,4,5,6 tetrakisphosphate [Ins (3,4,5,6) P_4], which reduces the open pr obability of calcium-activated chloride channels [22,24]. The upstream pathways leading to generation of this messenger involve tyrosine-kinase depend ent events, and the transactivation of the receptor for epidermal growth factor (EGF) and recruitment of the MAP kinase signaling cascade[25]. Inhibitors of both the EGF receptor and MAP kinase pathway potentiate and prolong secret ory responses to carbachol[25].

A second inhibitory mechanism also centers around the EGF receptor, but the mess engers and targets involved in the inhibiti on of chloride secretion are differen t[26-28]. Thus, when the EGF receptor is activated by its cognate liga nds, it heterodimerizes with another member of the ErbB receptor family, ErbB2, and thereby recruits alternative signaling events than those resulting from EGF receptor transactivation in response to carbachol[29]. This signaling d iversification, in turn, appears to activate phosphatidylinositol 3-kinase, an ovel isoform of protein kinase C (PKC-), and ultimately leads to th e inhibition of a basolateral potassium channel, thereby inhibiting the overall process of chloride secretion by preventing potassium recycling[27,28,30]. It is of interest to note that EGF, and related growth factors, inhibit chloride secreti on without themselves serving as agonists in the process. Since such growth factors and their receptors are known to be upregulated in the setting of mucosal in jury, their ability to inhibit chloride secretory responses may represent an ada ptive response that would limit diarrhea under these conditions.

ALTERATIONS IN CHLORIDE SECRETION IN THE SETTING OF DISEASE

Secretory diarrhea

An excess of chloride secretion into the intestine, a bove that which can be compensated for by the reserve capacity of intestinal abso rptive mechanisms, manifests clinically as diarrhea[1]. The classical example of this response is seen in cholera, where up to 20 liters of stool fluid can be lost per day[1]. Cholera, in common with a number of other intes tinal pathogens, elaborates enterotoxin that interact with epithelial cell signal transduction machinery to elicit profound and sustained chloride secretion due to an irreversible increase in intracellular cyclic nucleotide concentrations. Cholera toxin is internalized and induces a massive increase in intracellular cAMP, whereas the heat stable enterotoxin of *E.coli* bind to an apical recepto r that contains an integral guanylyl cyclase activity, and thereby stimulates ch loride secretion secondary to an increase in intracellular cGMP. The direct effects of enterotoxins on secretory epithelial cells are also amplified by a number of additional actions. For example, cholera toxin also activates enteric nerve endings, enterochromaffin cells and mast cells, all of which are capable of rele asing neurotransmitters and other agonists that themselves can stimulate chloride secretion either directly or indirectly[2].

Secretory diarrheal illness is also seen in the setting of infections with patho gens that are not known to elaborate enterotoxins activities. For example, Sal monella Dublin, an invasive bacterium, appears to activate a program of gene e xpression within the epithelial cell that predisposes to excessive fluid secreti on. Thus, there is upregulation of cyclooxygenase-2 and nitric oxide synthase e xpression, and in cell culture models, both of these enzymes appear to contribute to an enhanced capacity of the epithelial cell to secrete chloride in response to a range of stimuli[31]. Similarly, over-expression of cyclooxygenase-2 leads to an increased production of prostaglandins, particularly those of the E series that are known to be potent chloride secretagogues[31]. Sa lmonella invasion also evokes the synthesis of a wide range of chemokines and other cytokines by intestinal epithelial cells, and the resulting inflammatory influx that is targeted to infected cells almost certainly further amplifies secr etory responses in the intact tissue setting[32]. Furthermore, some work ers have shown that *Salmonella* infection is associated with the de novo expression of receptors for the neuropeptide galanin, a known secretory agonist[33,34]. Antibodies that interrupt galanin signaling can significantly dampen intestinal fluid secretion in a murine model of salmonellosis[33]. Thus, the host defense response of diarrhea can be generated even by pathogenic microorganisms that do not elaborate any known enterotoxins. Indeed, upregulati on of the secretory capacity of intestinal epithelial cells can even occur in the setting of infection with a non-invasive parasite also associated with diarrh eal illness, *Giardia lamblia*. These parasites adhere to, but do not penetrate, the intestinal epithelium. Co-culture of intestinal epithelial cells with G. *lamblia* trophozoites results in enhanced chloride secretory responses, and a pparent upregulation of expression of membrane transport proteins involved in th e chloride secretory mechanism such as CFTR and NKCC1 (Resta-Lenert *et al*, submitted). However, paradoxically, Giardia co-incubation appears to signif icantly reduce the ability of certain agonists to mobilize intracellular calcium. These data, in addition to being potentially significant for our understanding of infectious diarrhea, are also of interest because they underscore the concept that calcium-dependent secretagogues may exert negative as well as positive effects on secretion.

Diarrhea is also a common sequella of non-infectious, inflammatory conditions o f the intestine, such as ulcerative colitis and Crohn\s disease[1]. A substantial body of evidence now suggests that cells of the immune system that p resumably are activated in the course of such conditions can release a wide range of products capable of evoking chloride secretion. Conversely, inflammation an d tissue injury is also often accompanied by upregulation of the expression of g rowth factors, such as EGF, and their receptors, which may serve ultimately to c ounteract excessive secretion associated with inflammatory conditions of the gut[35]. Such mechanisms may underlie the efficacy of growth factors such as EGF and insulin-like growth factor that has been demonstrated in diarrheal conditions in various models of inflammation[35].

Cystic fibrosis

The disease states discussed above are characterized by e xcessive chloride secretion. However, inadequate chloride secretion, as occurs in the setting of cystic fibrosis, can be equally or perhaps even more disadvanta geous for the patient. Cystic fibrosis is characterized functionally by the abse nce of a cAMP-regulated chloride secretory pathway[15]. This is due to a series of more than 800 known mutations although about 70% of cases are relat ed to a single mutation, of the CFTR gene, △F508, that results in a channel protein that fails to traffic normally to the apical plasma membrane of secretory epithelial cells. Much of the morbidity and mortality in cystic fibrosis relates to pulmonary manifestations[36]. However, with advances in diagnosis a nd treatment, the median survival age for patients with cystic fibrosis has incr eased dramatically over the last several years. This means that disease manifest ations in organs other than the lungs are becoming more recognized, and may carry a substantial burden in terms of morbidity.

There are a number of gastrointestinal manifestations of cystic fibrosis, as wel l as complications related to dysfunction of the biliary and pancreatic systems. A large proportion of newborns with the disease are born with a form of intest inal obstruction known as meconium ileus, assumed to result from inspissated int estinal secretions that are poorly hydrated and thus difficult to clear from the lumen. In some cases, prompt surgical intervention is necessary, and evidence suggests that children presenting

with this complication will go on to have poorer nutritional status and growth, even if treated successfully, than cystic fibr osis patients without this manifestation[37,38]. Similarly, intestinal obstruction associated with cystic fibrosis may also be seen in older children and also in the increasing number of adults living with this disease[39]. The role of CFTR in promoting biliary and pancreatic ductular secretion also me ans that the majority of patients suffer from pancreatic insufficiency, up to ha lf experience biliary disorders, and approximately 5% may display Frank liver disease[39]. In addition, it has recently been postulated that unsuspected cystic fibrosis (of genotypes other than those associated with pancreatic insuf ficiency, such as △F508) may be the cause of at least some cases of idiopathic pancreatitis[40]. In each of these cases of gastrointestinal complicat ions of cystic fibrosis, the pathology suggests that any alternate chloride secr etory mechanism that are present in intestinal, pancreatic or biliary epithelial cells are insufficient to compensate for the loss of cAMP-regulated chloride secretion. In this respect, the knowledge that intestinal epithelial cells, at least, express intrinsic negative signaling pathways that limit the extent of calc ium-dependent chloride secretion may offer both an explanation for this situati on, as well as a possible therapeutic target if such negative signaling events can be countermanded.

The prevalence of cystic fibrosis in, especially, Caucasian populations, where estimates suggest that more than 3% of the population are heterozygous carriers o f the disease, raises questions about the evolutionary pressures that have drive n the retention of mutant CFTR alleles in the gene pool. Hypothetically, suchi ndividuals might have thereby gained a heterozygote advantage in resistance to enterotoxin-mediated diarrheal illnesses, such as cholera, although this may be inconsistent with the relative prevalence of cystic fibrosis in Caucasian compared with other ethnic groups. Moreover, studies in mouse models of cystic fibrosi salso fail to suggest that heterozygotes show any diminished intestinal secretory responses to cholera toxin[41,42]. However, more recent studies sugg est that tissues obtained from mice with one mutant CFTR allele do display an in creased resistance to invasion by bacteria such as *Salmonella*, although the mechanism of this effect is unknown[43]. This does provide an attractive hypothesis for cystic fibrosis prevalence, especially given that typhoid fever was common in Western Europe until relatively recent times.

CONCLUSION

Advances in our understanding of the molecular basis of intestinal chloride secr etion, as well as its regulation, may offer significant insights into disease states where this transport mechanism is either over or under-expressed in the intestine, such as secretory diarrhea and cystic fibrosis. Ultimately, it is hoped that such insights will spawn new therapies for these conditions. Secretoryd iarrheal illness, in particular, still constitutes a major burden of morbidity and mortality, particularly among children in developing countries. Moreover, in creased international travel, and the emergence of more highly pathogenic strains of certain bacteria, such as *E.coli*, will also increase the prevalence of diarrhea. Thus, improved therapies are urgently needed, in concert with the public health measures that will reduce exposure to the infectious agents that cause such disease.

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

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Alterations in gastric mucin synthesis by Helicobacter pylori

James C. Byrd[1] and Robert S. Bresalier[1,2]

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INTRODUCTION

Helicobacter pylori is recognized as a cause of chronic active gastritis, gastric and duodenal ulcers, and gastric cancer, though the mechanisms of pathogenesis for *H. pylori*-associated diseases are not yet well understood [1-4]. The ecological niche to which *H.pylori* is well-adapted is the mucous layer of the human gastric antrum, which has mucin glycoproteins as major constituents. Mucins, high-molecular weight carbohydrate-rich glycoproteins that coat the surface of the stomach and are secreted into the lumen, function to protect the stomach and could be important in *H. pylori* colonization. For further understanding the pathogenesis of *H.pylori* related diseases, it is important to consider whether *H.pylori* colonization of the surface epithelium is associated, as cause or effect, with changes in the gastric mucin synthesized by surface mucous cells.

MUCINS PRODUCED IN NORMAL STOMACH

The entire gastrointestinal tract is coated with a protective mucous layer. The main components of the viscoelastic mucous are mucin glycoproteins. Mucins are thought to protect the surface of the gastrointestinal tract from mechanical damage, from dessication, and from chemical irritants. Gastric mucins are the major components of an unstirred mucous-bicarbonate layer that protects the gastric epithelium from the high concentrations of acid in the stomach lumen and from autodigestion by pepsin. The protective functions of the gastric mucous layer imposes rigid requirements on the structure of gastric mucins (Figure 1). They must be very high in molecular weight and highly hydrated to provide the viscoelasticity necessary for protection from mechanical damage, and must also be acid-stable and have little non-glycosylated polypeptide exposed as a target for pepsin.

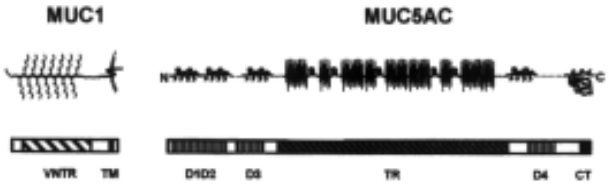

Figure 1 Models of gastric mucin structure. Lower bars represent cDNA sequences of MUC1 cell-surface mucin and MUC5AC secreted mucin, with different domains labeled. Resulting structures of the proteins with attached carbohydrate are schematically represented above.

Like mucins from a number of sources, human gastric mucins are very high in molecular weight and are heavily substituted with O-linked oligosaccharides. Human mucins are encoded by at least nine distinct mucin genes, of which three, MUC1, MUC5AC, and MUC6, are expressed at high levels in the normal stomach (Table 1).

MUC1 mucin is well characterized[5]. The protein encoded by the MUC1 gene has a large central domain (VNTR, Variable Number of Tandem Repeats) composed of a variable number (25 to 125) of tandem repeats of a 20-amino acid sequence with 25% threonine and serine and 7% proline. Unlike most other mucins, the MUC1-encoded protein has a transmembrane segment and a cytoplasmic tail that can interact with the cytoskeleton. The O-linked carbohydrates on MUC1 mucin are heterogeneous, differ between tumors and normal epithelial cells[6], and can influence the recognition of the mucin protein by different monoclonal antibodies. MUC1 mucin is produced to some extent by most epithelial cells, but has been studied most extensively in mammary, pancreatic, and colon cancer cells.

MUC5AC is expressed in the stomach and in tracheobronchial cells. Immunohistochemical studies indicate that MUC5AC apomucin is present in surface mucous cells of the gastric epithelium[7,8]. MUC5AC mucin has a small tandem repeat sequence of 8 amino acids, interspersed with cysteine-containing regions (Figure 1). The glycoprotein is very large with the bulk of the

[1]Gastrointestinal Cancer Research Laboratory, Henry Ford Health Sciences Center, Detroit, MI, USA
[2]Department of Medicine, University of Michigan School of Medicine, Ann Arbor, MI, USA
Supported by the Research Service of the Henry Ford Health Sciences Center and Research Foundation (JCB, RSB) and National Cancer Institute Grant R01 CA69480 (R-SB)
Correspondence to: Robert S. Bresalier, M.D. Division of Gastroenterology (K7), Henry Ford Health Sciences Center, 2799 West Grand Blvd., Detroit MI 48202 USA
Tel. +1-313-916-2046, Fax.+1-313-916-9487
Email. rbresal@mich.com
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molecule made up of heavily glycosylated tandem repeats of an 8-amino acid peptide sequence rich in threonine (to which O-linked oligosaccharides are attached) and proline. Interspersed irregularly within the tandem repeat region are cysteine-containing motifs. Like MUC2 intestinal mucin, both the N-terminal and the C-terminal have cysteine-rich globular domains[9] with sequence similarity to the D domains of pre-pro-von Willebrand factor and an inferred cystine-knot motif, topologically similar to epidermal growth factor (EGF). The cysteine-rich globular domains of secreted mucins may be involved in the oligomerization of mucin or in binding to collagen of basement membranes[10]. MUC5AC is expressed in the normal stomach, but not in normal colon. Aberrant expression of MUC5AC has been reported, however, in colorectal cancers and adenomas[8,11,12] and may be related to the progression of colon cancers.

MUC6 is also expressed in the normal stomach, but in mucous glands rather than surface mucous cells[7,8,13,14]. This mucin has a very large tandem repeat sequence of 169 amino acids, very high in amounts of Thr, Ser, and Pro[13]. The high content of Thr and Ser likely accounts for the large amount of carbohydrate present on this molecule. Thus, there are (at least) two completely different secreted mucins produced by the normal stomach. How these mucins differ in carbohydrate (and whether they differ in function) is speculative. Genes for both MUC5AC and MUC6 have been assigned to a region of chromosome 11 that also codes for two other secretory mucins[15], with a gene order of HRAS-MUC6-MUC2-MUC5AC-MUC5B-IGF2.

Like mucins from other sources, the carbohydrate portion of gastric mucin is heterogeneous. Several neutral oligosaccharide structures have been published [16,17], but the overall complement of oligosaccharides is not known. The results of histochemical studies suggest that the carbohydrate portion of the mucin in surface mucous cells is different from that in mucous glands. The surface mucous cells are stained by PAS, while mucous glands (cardiac gland, mucous neck cells, and pyloric gland cells) are stained by alcian blue. The "neutral" mucins in surface mucous cells are also stained by galactose oxidase/Schiff, suggesting the presence of terminal Gal or GalNAc[18]. The mucous gland cells uniquely show periodate-enhanced binding of concanavalin A, called paradoxical ConA staining. The structural basis for this is unclear, but it may detect terminal alpha-GlcNAc[18]. The mucous gel layer covering the surface epithelium has been shown to have clearly demarcated layers of two distinct mucin types [19]. On the basis of staining properties, these likely correspond to the surface-type neutral mucin (MUC5AC) reactive with galactose oxidase and to the gland-type acid mucin (MUC6), possibly sulfomucin, revealed by periodate-enhanced (paradoxical) ConA staining.

EFFECT OF *H.pylori* ON GASTRIC MUCIN IN VIVO

Though both the thickness and the hydrophobicity of the mucous gel layer is decreased in the gastric mucosa of ulcer patients[20], it has not been established whether this is associated with increased mucin degradation, decreased mucin synthesis, or a change in mucin type. It has been postulated that one important pathogenic property of *H.pylori* is its ability to weaken the mucous gel that protects the gastric epithelium[21,22], but the presence or absence of mucinase activities in *H.pylori* is controversial[23-25]. Direct analysis of mucins from *H.pylori* infected and uninfected patients show no decrease in viscosity, arguing against *H.pylori* dependent mucin degradation[26]. In spite of histochemical observations of mucous dep letion accompanying *H.pylori* infection, qualitative alterations in the type of mucin produced have only recently been studied.

In order to determine the effect of *H.pylori* infection on mucin gene expression in the gastric epithelium[7], biopsies from *H.pylori*-positive and *H. pylori* negative patients were examined by immunohistochemistry (Table 2). MUC6 was limited to mucous glands of *H.pylori*-negative patients, but 72% of *H.pylori* positive patients also expressed MUC6 on surface muco us cells. In contrast, MUC5AC mucin was seen in significantly fewer surface muco us cells of *H.pylori*-positive specimens. Overall, the percent of the surface epithelium stained by anti-MUC5 was significantly lower in *H.pylori*-po sitive specimens than in *H.pylori*-negative specimens ($P<0.01$). Carbohy drates recognized by LeX and paradoxical ConA staining were aberrantly expressed in the surface mucous cells of 16/27 and 17/23 of *H.pylori* -positive tissu es, respectively. There was a suggestive but non-significant decrease in staini ng for MUC1 mucin. Retrospective examination of clinical histories and histologi cal findings showed that the mucin alterations occur in *H.pylori* infected individuals with and without ulcers, but not in patients with non-*H.pylori* -associated gastritis or gastric ulcers (Figure 2). This indicates that the muc in alterations are not simply a secondary effect of inflammation.

For more direct examination of mucin gene expression, the presence of MUC5 AC and MUC6 message in antral biopsies were examined by in situ hybridization[7]. In antral biopsy specimens from *H. pylori*-negative patients, MUC5AC mRNA was homogeneously expressed in surface epithelial cells. MUC5AC exp ression in the surface epithelial cells of *H.pylori* -positive patients was patchy, however, and often absent from large areas of the surface epithelium. Co ncordance between the pattern of MUC5AC expression as determined by in situ hybridization and immmuno-histochemistry was 100%. MUC6 expression was limited to cells of the deep glands in *H.pylori* -negative patients, seen by both in situ hybridization and immunohisto-chemistry. In contrast, 6 of 8 *H.pylori* -positive specimens

Table 1 Human mucin genes

Gene	Locus	mRNA size	Tandem Repeats	Other structural features	Expression in normal tissues
MUC1	1q21-24	4-7 kb	20 AA	Transmembrane	Most epithelia
MUC2	11p15.5	14-16 kb	23 AA, -16 AA	D-domains, cystine knot	Colon>small intestine>respiratory tract
MUC3	7q22	16-17.5 kb	17 AA, 59 AA	Cystine knot	Small intestine>colon, gall bladder
MUC4	3q29	16.5-24 kb	16 AA		Respiratory tract, cervix>GI tract
MUC5AC	11p15.5	17-18 kb	8 AA	D-domains, cystine knot	Stomach (surface)>respiratory tract
MUC5B	11p15.5	17.5 kb	29 AA,interrupted	D-domains, cystine knot	Respiratory tract, salivary gland, cervix>GI tract
MUC6	11p15.5	16.5-18 kb	169 AA	Cystine knot	Stomach (glands), gall bladder
MUC7	4	2.4 kb	23 AA	No homology to large mucins	Salivary glands
MUC8	12q24.3	-9 kb	18 AA, 41 AA	Not Thr/Ser rich	Trachea

Table 2 Histochemical staining of mucins in gastric biopsy specimens

Antigen		*H.pylori* negative	*H.pylori* positive
MUC5AC	% Stained*[a], intensity score (surface)	(69.8 ± 3.5)%, 2.8 ± 0.1	(51.2 ± 5.7)%, 2.6 ± 0.1
MUC6	% Stained**[a], intensity score (surface)[a]	4%, 0.1 ± 0.1	72%, 1.8 ± 0.2
Le\+b	% Stained**[a], intensity score (surface)[a]	63%, 0.9 ± 0.2	96%, 1.9 ± 0.2
Paradoxical ConA	% Stained**[a], intensity score (surface)[a]	18%, 0.4 ± 0.2	79%, 2.1 ± 0.3
LeX	% Stained**[a], intensity score (surface)[a]	4%, 0.4 ± 0.4	59%, 1.0 ± 0.2
MUC1	Intensity score (surface), intensity score(glands)	2.6 ± 0.2, 1.7 ± 0.1	2.7 ± 0.1, 1.4 ± 0.2
Sialyl Tn	Intensity score (surface), intensity score (glands)[a]	1.8 ± 0.2, 1.9 ± 0.1	1.6 ± 0.1, 1.4 ± 0.1

*% of surface epithelium stained; **% of patients with surface staining; [a]$P<0.05$, *H.pylori* positive *vs H.pylori* negative

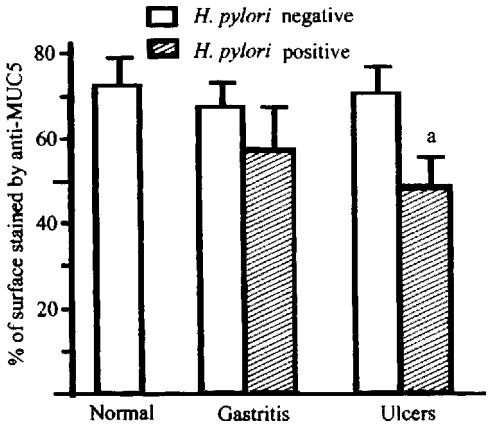

Figure 2 Expression of MUC5AC in surface epithelium of normal human stomach an d gastritis and gastric ulcer specimens. [a]$P<0.05$ *vs* corresponding *H. pylori negative* group.

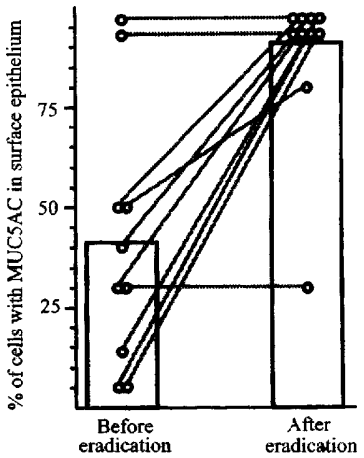

Figure 3 MUC5 expression in patients before and after eradication of H.pylor i infection. MUC5 gene expression was determined by in situ hybridization.Bars show mean percent of epithelial cells expressing MUC5. Lines show changes in MUC5AC expression in individual patients.

(0/7 *H.pylori* negative specimens) had focal MUC6 mRN A expression in surface epithelial cells. MUC5AC and MUC6 gene expression were e xamined in antral biopsies obtained from patients with *H.pylori* -associated antral gastritis (biopsy-proven) before and after documented eradication of th e bacterium. In 7 of 10 cases MUC5AC expression increased ($P = 0.004$) after *H.pylori* eradication (Figure 3). Eradication of *H.pylori* also resulted in reversal of MUC6 antigen expression toward normal patterns.

The effect of *H.pylori* on gastric mucin expression was further examined by purification and immunochemical analysis of mucins from gastric juice of *H. pylori*-positive and *H.pylori* -negative patients. For *H.pylori*-infe cted patients and uninfected patients that had been examined for immunohistochem ical staining of biopsy tissues, gastric aspirates were used as a source for muc in purification by gel filtration and CsCl density gradient centrifugation. Ther e was no significant difference in yield of mucin or carbohydrate content betwee n *H.pylori* -positive and *H.pylori* negative specimens (Figure 4). The p urified mucins were examined by ELISA for MUC6 and Le-b antigenic activity. MUC 6 activity was higher ($P = 0.026$) in mucins from the *H.pylori* -infected p atients than in mucins from uninfected patients. Mucins from the *H.pylori* - infected patients also bound monoclonal antibody to Le-b antigen to a significa ntly greater extent ($P = 0.014$) than mucins from the uninfected patients (Figu re 4). Subsequently, these purified mucins have been examined

by SDS-PAGE and Western analysis. Infection with *H.pylori was associated with an increase in* MUC6 (*detected with anti-M6P and also with anti-Le-b* and *Ulex europea aggl utinin*) and a decrease in MUC5AC (detected with anti-M5P and 45M1 antibodies and also with peanut agglutinin and *Vicia villosa agglutinin*) in these secre ted mucins.

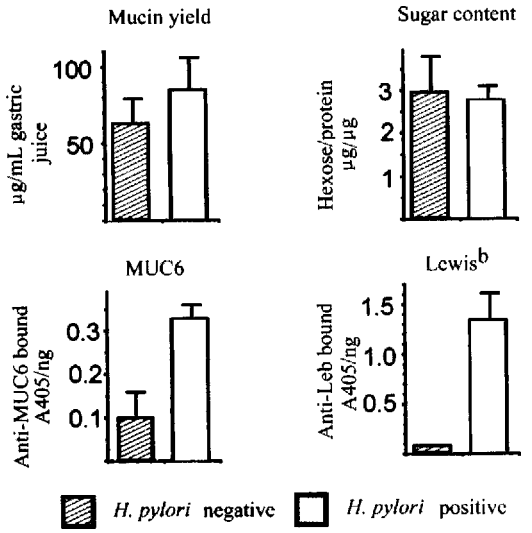

Figure 4 Purification and analysis of mucins from gastric juice. Upper left, yield of mucins purified from gastric aspirates of 5 *H. pylori* -negative and 5 *H.pylori* -positive patients. Upper right, carbohydrate content of purifi ed mucins. Lower left, binding of antibody to MUC6 peptide in ELISA. Lower right , binding of antibody to Lewis-b antigen.

These results establish that there is aberrant surface expression of gland-type gastric mucin in surface mucous cells of *H.pylori* infected patients, acc ompanied by focally decreased MUC5AC mucin. This decrease in MUC5AC mucin and ab errant expression of MUC6 might be expected to disrupt the protective surface mu cinlayer. How or whether alterations in gastric mucins would influence processes that lead to disease is an important question which requires that the specific ity and mechanisms of mucin depletion be better understood.

EFFECT OF *H.pylori* ON MUCIN SYNTHESIS IN VITRO

Analyses of tissue specimens and purified mucin glycoproteins indicate that gastric surface-type mucin expression is reversibly decreased in *H.pylori* -infected patients[7] but do not allow direct examination of mucin synthesis. Gastric cells in culture were examined to determine the effect of *H .pylori* on mucin synthesis[27]. KATO III gastric epithelial cells were incubated in the presence or absence of *H. pylori*, and the mucin produced was quantitated by labeling with[^{3}H]glucosamine and size exclusion HPLC on Superose 6 columns. The ^{3}Hlabeled high-molecular weight glycop rotein was confirmed to be mucin by CsCl density gradient centrifugation, chemical and enzymatic degradation treatments. *H.pylori* (type strain NCTC11637), under conditions that had little effect on viability, inhibited the synthesis of mucin by 82% (Figure 5). There was no inhibition of mucin synthesis by the non -gastric pathogen Campylobacter jejuni, and little inhibition by a strain (Tx30a) of *H.pylori* that is CagA-negative and non-toxigenic. Similar results were seen in five other gastric cell lines tested (Figure 5). Inhibition of m ucin synthesis was detected as early as 4h after addition of bacteria, and was partially reversible, though with a slower time-course than the onset of inhibi tion. Inhibition of mucin labeling was concentration dependent (Figure 6) and did not require the presence of intact bacteria. There was no inhibition by a solu ble extract of *H.pylori*, but the *H.pylori* pellet fraction gave inhibit ion equivalent to intact bacteria.

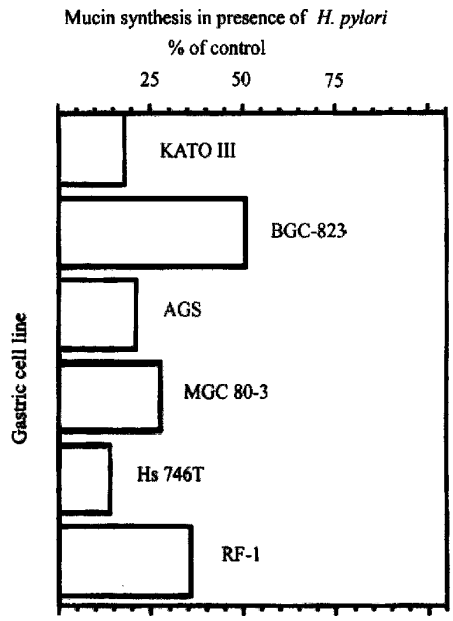

Figure 5 Effect of *H.pylori* on mucin synthesis in six gastric cell lines. Cells were labeled 22 h with [^{3} H] glucosamine in the presence or absence of 1 OD600 *H.pylori*, and labeled glycoproteins were analyzed by size-exclusion HPLC.

In a pulse-chase analysis, *H.pylori* had no effect on mucin secretion. Fur thermore, there was little or no degradation of mature mucin in the presence or absence of *H.pylori*. Further experiments, to examine the effects of *H.pylori* on mucin glycosy lation, used benzyl-GalNAc, which specifically inhibits synthesis of peripheral carbohydrate on mucin-type glycoproteins[28]. Incubation of KATO III with *H.pylori* decreased labeling of mucin to a simil ar extent in the presence or absence of benzyl-GalNAc (Figure 7), indicating th at the effect of *H.pylori* is not due to inhibition of peripheral glycosylat ion per se, but results from inhibition of synthesis of mucin core structures.

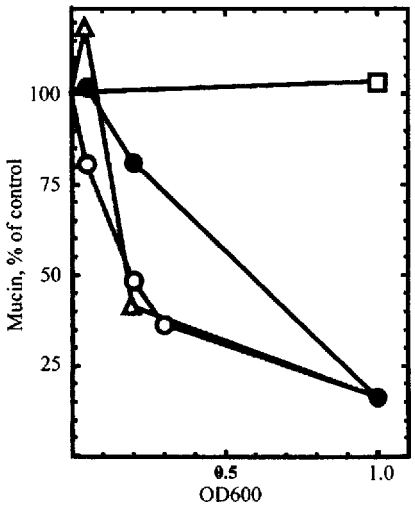

Figure 6 Inhibition of mucin synthesis by subfractions of *H.pylori*. KATO I II cells were labeled with [^{3} H]glucosamine in the presence of different concentrations of intact *H.pylori* (open circles), *H.pylori* l ysate (filled circles), the 100,000 × g pellet (open triangles), or the 100, 000 × g supernatant (open squares).

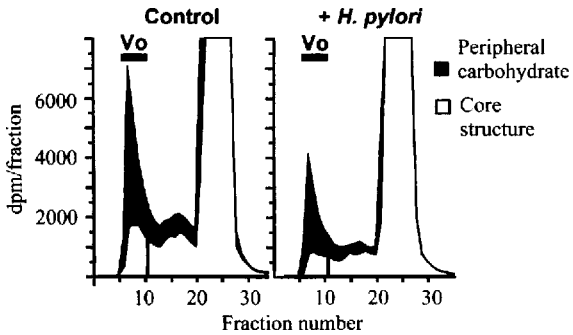

Figure 7 Effect of *H.pylori* on synthesis of peipheral and core carbohydrat e structures of mucin. KATO III cells were incubated with or without 2 mmol/L b enzy l-GalNAc, and labeled for 4 h in the presence or absence of 1 OD600 *H.pylori* . Bars show void volume (Vo) containing labeled mucin. Solid areas show labeli ng of the peripheral carbohdyrate (inhibitable by benzyl-GalNAc). Open areas sh ow residual labeling of core structures in the presence of benzyl-GalNAc.

KATO III produces MUC5AC and MUC1 mucins, and the amount of both mucin pro teins is decreased by co-incubation with *H.pylori* (Figure 8). Expression of another high molecular weight glycoprotein, CEA, and another control protein, galectin-3, was unaffected by *H.pylori*. *H.pylori* also decreased the a mount of MUC5AC protein in BGC-823 gastric cells and the amount of MUC1 protein in the BGC-823, AGS, and MGC 80-3 cell lines. The inhibition of synthesis of both MUC5AC and MUC1 protein was concentr ation dependent and associated with the insoluble fraction of *H. pylori* lysates. Kinetically, the onset of inhibiti on of MUC1 expression was more rapid than inhibition of MUC5AC expression. MUC1 inhibition was seen within 4 h while MUC5AC inhibition was slower. MUC1 recovery was also more rapid than recovery of

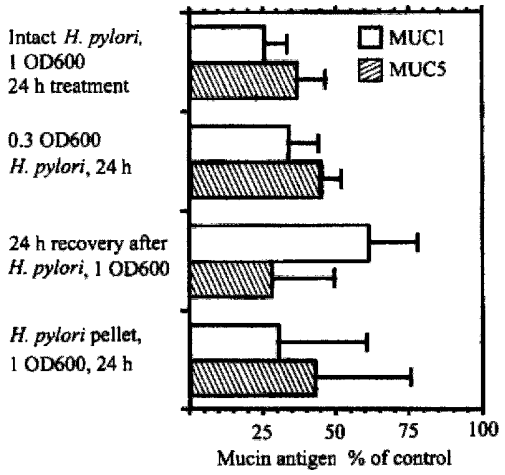

Figure 8 Effect of *H.pylori* on expression of MUC1 and MUC5AC in KATO III g astric epithelial cells in vitro. KATO III cells were incubated with or with out *H.pylori*, and cell lysates were subjected to Western analysis. Content of MUC1 antigen, detected with monoclonal antibody HMFG2, and of MUC5AC antigen, detected with monoclonal antibody CLH2, is expressed as percentage of untreated cells.

MUC5AC.

These experiments[27] demonstrate that *H.pylori* decreases the amou nt of total mucin, and MUC5AC and MUC1 proteins in gastric epithelial cells. Ind irect evidence indicates that this is due to a decrease in the synthesis of mucin protein rather than changes in glycosylation, secretion, or degradation of muc ins. These results may help to explain the mucin depletion associated with *H. pylori* infection *in vivo*[7,29].

INFLUENCE OF MUCINS ON *H.pylori* ADHESION

Most of the *H.pylori* in the stomach are present in the mucus gel layer, and appear to cause little harm to the host; adhesion of *H.pylori* to the gas tric epithelial cell surface may be required for causing disease. For example, induction of the proinflammatory chemokine interleukin-8, requires that bacteria be in contact with the epithelial cell surface[30,31]. Mucin glycoprot eins produced by the gastric epithelial surface could influence the process of *H.pylori* adhesion in two ways: First, secreted glycoproteins could bind to bacterial adhesins and help to keep the bacteria in the mucous gel layer, preventing their approach to the epithelium. Although *H.pylori* has several differ ent adhesins which could be involved in binding to mucins[32-35] and human gastric mucin has been shown to inhibit bacterial binding to other cell types, e .g., erythrocytes and HEp-2 cells[36,37], it is not known whether secr eted gastric mucin can inhibit the adhesion of *H.pylori* to gastric epithelial cells. Second, cell-surface mucin glycoproteins could shield the epithelial cell surface from exposure to contact-dependent virulence factors, preventing adhesion-dependent synthesis of pro-

inflammatory chemokines. In MUC1-expressing cells, the highly glycosylated tandem repeat domain extending out from the cell surface can interfere with cell-cell interactions, for example, integrin-med iated aggregation[38]. Since adhesion of *H.pylori* to gastric epithe lial cells requires their close proximity to the cell surface where they can int eract with integrins or other cell-surface receptors, cell-surface mucins migh t be expected to block adhesion of *H.pylori* to gastric epithelial cells.

Since previous results indicated that gastric surface-type mucins are decr eased by *H.pylori* both in vivo[7]and in vitro[27] , we sought to determine the influence of mucin on adhesion of *H.pylori* to cultured gastric epithelial cells. For measurement of the adhesion of *H.pylori* to gastric epithelial cells, an assay was established using biotinylated *H .pylori*, with bacteria attached to the BGC-823 gastric epithelial cells quan titated with avidin-biotin-peroxidase complex and ABTS as chromogen. The bindi ng of bacteria was characterized with regard to time dependence, temperature dep endence, and bacterial strain dependence. Optimal conditions for adhesion were f ound to be 30 min incubation at 37℃. Under conditions where the CagA/cytotoxin positive type strain of *H.pylori*, NCTC 16137, bound well to BGC-823 cells, there was little binding of the CagA-negative, cytotoxin-negative strain of *H. pylori*, Tx30a or of the non-human pathogen Helicobacter mustelae. As further validation, the standard binding assay was compared to colony counts for detection of viable *H.pylori* bound to BGC-823 cells (Figure 9). Binding of biotinylated bacteria was equivalent to binding of viable bacteria.

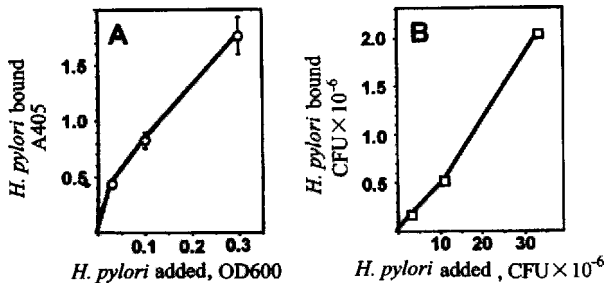

Figure 9 Assay of adhesion of *H.pylori* to gastric epithelial cells. Biotin -labeled *H.pylori* were incubated with BGC-823 cells for 30 min at 37℃, and attached bacteria were quantitated using the avidin-biotin-complex assay (A, left panel) or by colony counts (B, right panel).

BGC-823 cells are well differentiated, attach well to tissue culture plast ic, and are susceptible to *H.pylori* -dependent inhibition of mucin synthesis [27,39]. In comparison to other gastric epithelial cell lines, there w as more binding of *H.pylori* to BGC-823 cells (which produces MUC5AC mucin and has relatively low levels of MUC1 cell-surface mucin) than to two cell line s (AGS and MGC-803) which do not produce MUC5AC mucin but produce high levels o f MUC1 mucin[27,40]. This inverse correlation between MUC1 expression and *H.pylori* adhesion in gastric epithelial cell lines suggests that MUC1 mucin could interfere with *H.pylori* adhesion.

In order to test the role of mucin in *H.pylori* adhesion, we sought to inh ibit mucin synthesis in BGC-823 gastric epithelial cells and examine the effect on binding of bacteria. It was initially confirmed that treatment of BGC-823 cells with the mucin-specific glycosylation inhibitor benzyl-GalNAc inhibits to tal mucin synthesis (measured by labeling with [^{3} H] glucosamine and size-exclusion chromatography) by approximately 80% (Figure 10). Treatment of 823 cells with benzyl-GalNAc significantly increased the adhesion of *H.p ylori* (treated/control = 1.43 ± 0.14, n = 6). These results indicate that inhib ition of mucin glycosylation is associated with an increase (rather than a decre ase) in *H.pylori* adhesion, suggesting that mucins protect against (rather t han facilitate) binding of the bacterium to the gastric epithelial surface.

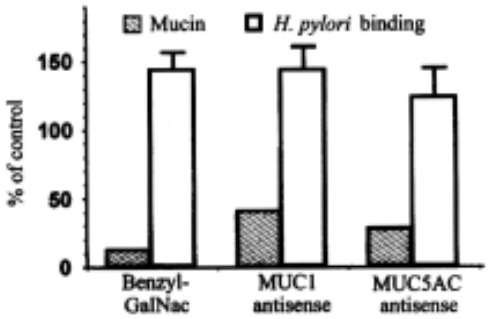

Figure 10 Effect of inhibitors of mucin synthesis on *H.pylori* adhesion. BG C-823 cells were treated with 2 mmol/L benzyl-GalNAc, with 5 μmol/L MUC1 ant isense (5'-GGG-CTG-GGG-GGG-CGG-TGG-3'), or with 5 μmol/L MUC5AC antisense (5'-AGA-GGT- TGT-GCT-GGT-TGT-3'). Solid bars show, as percent of control, amo unts of total mucin (left), MUC1 mucin (middle), and MUC5AC mucin (right). Open bars show binding of biotinylated *H.pylori*, expressed as percent of control .

In order to specifically decrease the synthesis of MUC1 and MUC5AC phospor othiolate antisense oligodeoxynucleotides were designed and targeted against th e VNTR regions (M1TR) of MUC1 and MUC5AC genes. By Western analysis (Figure 10) , MUC1 antisense treatment decreased MUC1 protein (treated/control = 0.39 ± 0.15, n = 6), and MUC5AC antisense treatment decreased MUC5AC protein (treated/contr ol = 0.27 ± 0.13, n = 3). MUC1 antisense oligodeoxynucleotide treatment of BG C-8 23 cells significantly increased the adhesion of biotinylated *H.pylori*, (tr eated/ control =1.43 ± 0.18, n = 4). MUC5AC oligodeoxynucleotide treatment ha d no significant effect on adhesion (treated/control = 1.23 ± 0.22, n = 5), but it sh ould be noted that the culture medium, which would contain most of the secreted (MUC5AC) mucins, was removed before the

adhesion assay was performed.

These results indicate that cell-surface mucin glycoprotein decreases adhe sion of *H.pylori* to gastric epithelial cells. Since inhibition of mucin syn thesis in vitro is associated with an increase in *H. pylori* adhesion, *H.pylori*-dependent mucin depletion *in vivo* would be expected to facilitate further binding of the bacterium to the gastric epithelial surface.

CONCLUSION AND WORKING HYPOTHESIS

Our working hypothesis (Figure 11) is that *H. pylori* alters the synthesis of gastric mucin in surface mucous cells and that the resultant alteration in the surface mucous gel layer facilitates adhesion of *H.pylori* to the epithelial cell surface, which could lead to increased inflammation. Based on *in vitro* results, *H.pylori* adhesion decreases mucin synthesis, and decreased mucin synthesis increases *H.pylori* adhesion. If asimilar cycle applies *in vivo*, the pathogenetic effects of *H.pylori* infection could be mechanistically tied to the mucin depletion observed histologically.

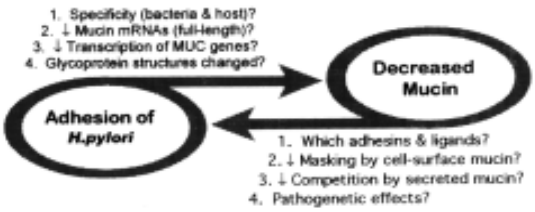

Figure 11 Working hypothesis: interaction of *H.pylori* and gastric mucins.

The diseases caused by *H.pylori*, gastroduodenal ulcers and gastric cancer, take years to develop, but only short-term effects can be studied *in vitro*. *H. pylori* infection causes little overt damage to the gastric epithelium in most of the host population. *H.pylori* may, therefore, cause ulcers thro ugh a subtle disturbance in the interaction between the bacteria and the host, r ather than acute damage to one critical component of the host cell. Non-physiol ogical conditions are necessary for observable effects in cultured cell systems. Caution must therefore be exercised in extrapolating these data to the in viv o interactions between *H.pylori* adhesion and mucin expression. Neverthele ss, if, as we hypothesize, *H.pylori* adhesion to gastric epithelial cells de creases mucin synthesis, and decreased mucin synthesis further increases *H.py lori* adhesion, even small effects on both processes could eventually lead to d isease.

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Edited by Lu J
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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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Current medical therapy of inflammatory bowel disease

Kiron M. Das and Sherif A. Farag

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INTRODUCTION

The 1990's have brought a significant promise and the hope for a better and brighter future in the new millennium for patients with inflammatory bowel disease (IBD). A better understanding of the pathophysiology of IBD symp toms has led to newer treatment modalities and streamlining of therapy for specif ic subsets of patients.

ULCERATIVE COLITIS

The treatment for ulcerative colitis (UC) is aimed at modulating the inflammator y response. The drugs which are found to be effective are sulfasalazine (Azulfi dine, Salazopyrin) and its 5ASA derivatives, glucocortico-steroids, immunomodul ators/immunosuppressants, and other new potential drugs (Table 1).

Table 1 Medical therapy in IBD

Sulfasalazine and 5-amino salicylates	Azulfidine
	Olsalazine, Asacol, Pentasa, Balsalazide
Corticosteroids	Hydrocortisone, ACTH, Prednisone, Budesonide
Immunosuppressive/Immunomodulators	Immuran/6MP, Cyclosporin A, Methotrexate,
	Anti-TNFα Antibody, (Remicade, CD P571)
	FK506, IL-10, IL-11
	IL-1 Receptor Antagonist, Anti-CD4 Antibody
Antibiotics	Metronidazole, Ciprofloxacin,Clarithromycin,
	Trimethoprim-sulfamethoxazole
New potential drugs	Nicotine
	Heparin
	ISIS 2302 (ICAM-1 inhibitor)
	Hydroxychloroquine, Leukotrienes inhibitors,
	Short chain fatty acids
	Antioxidants & free radical scavengers
	Probiotics

Sulfasalazine and 5-ASA compounds

Initially developed in the 1940s for the treatment of rheumatoid arthritis, sali cyl-azo-sulfapyridine, or sulfasalazine was quickly recognized as being effec a cious in the treatment of colitis. Consisting of a molecule of 5-aminosalicylic acid (5-ASA) joined by an azo bond to a molecule of sulfapyridine (Figure 1), sulfasalazine has been a mainstay in the treatment of UC for more than 50 years[1].

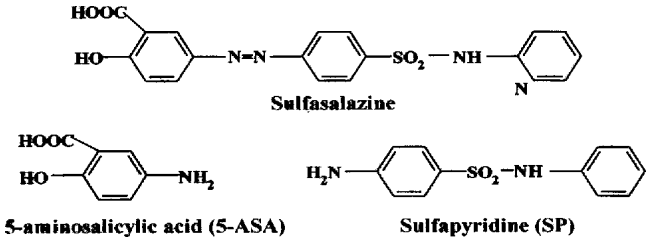

Figure 1 Structure of salicyl-azo-sulfapyridine or sulfasalazine (azulfidine , salazopyrin).

The active moiety in sulfasalazine is 5-ASA, with the sulfapyridine acting as a carrier to prevent absorption of 5-ASA in the small bowel. In the distal ileum and colon, bacteia that possess azo reductase split the molecule, releasing free 5-ASA and sulfapyridine (Figures 1,2). Almost all colonic bacteria have azore ductase enzyme. The sulfapyridine is readily absorbed from the colon, acetylate d in the liver, and conjugated with glucuronic acid, and excreted in the urine[1]. The 5-ASA is only minimally absorbed, with the majority being excreted in the feces unchanged (Figure 2). 5-ASA's mechanism of action is by direct contact with colonic mucosa to suppress various pro-inflammatory pathways including both cyclooxygen ase and lipoxygenase derived products such as prostaglandins and leukotrienes from arachidonic acid and from suppression of superoxide dismutase and possibly by other mechanisms.

Sulfasalazine has been well studied in UC and has proven efficacy in inducing remisson in patients with mild-to-moderate disease, as well as in maintaining r emission. Its use has not been studied in a controlled manner for severe UC, but it is commonly used as an adjunct to approximately 80% of patients with mild-to-moderate disease, compared with 30% to 35% of those receiving placebo. Studies examining maintenance of remission have shown that sulfasalazine's effect is d ose-dependent, with relapse rates of 33% with 1 g/day, 14% with 2 g/day, and 9 % with 4 g/day[2].

The toxic effects of sulfapyridine are the limiting factor in using sulfasalazin e. Common adverse reactions include headache, nausea, anorexia, and dyspepsia. These

Department of Medicine, Division of Gastroenterology and Hepatology, and Crohn's and Colitis Center of New Jersey, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ 08903, USA
Correspondence to: Kiron M.Das, M.D.,Ph.D. Chief, Div. of Gastroenterology & Hepatology, UMDNJ-Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, New Brunswick, NJ 0 8903, USA
Tal. 732-235-7784, Fax. 732-235-7792
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symptoms relate to plasma levels of sulfapyridine and usually occur at dosa ges greater than 3 g/day [3]. Because of sulfasalazine's substantial to xicity and the limitation of dosing due to side effects, efforts were ma de to develop 5-ASA products with other delivery systems to prevent proximal sm all-bowel absorption. Three such oral preparations are now available in the Un i ted States: two products containing mesalamine (Asacol, Pentasa), olsalazine sod ium (Dipentum) and balsalazide. Each uses a different mechanism to deliver the 5-ASA moiety to the sites of inflammation such as distal small bowel and colon, bypassing the absorption by jejunum.

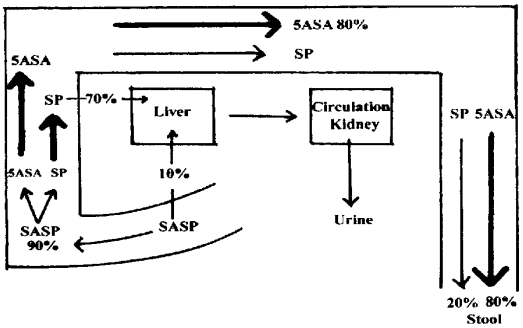

Figure 2 Metabolism and pharmacokinetics of salicylazo-sulfapyridine. Adopted from the Ph.D. thesis entitled, "Salicylazosul fapyridine Metabolism in Clinical Practice" submitted by Das KM to the University of Edinburgh, U.K., J anuary 1973.

Asacol(mesalamine) contains 5-ASA coated with an acrylic-based resin.Theres in releases 5-ASA in a pH-dependent manner at pH 6 or above. This causes releas e of 5-ASA in the distal small bowel and colon, making this drug ideal for the treatment of UC. Another mesalamine product, Pentasa, is 5-ASA encapsulated in ethylcellulose microgranules. This time-release formulation allows for release of 5-ASA throughout the small and large in testines. Thus, this drug appears to be superior for small bowel Crohn's disea se (CD) in addition to its use in UC. While these products are effective and safe, there have been rare reports of granulom atous hepatitis, interstitial neph ritis and recurrent thrombocytopenia on rechallenge with mesalamine/ mesal azine[4,5,6].

Olsalazine sodium consists of two molecules of 5 ASA linked by a diazo bond. B a lsalazide consists of 5-ASA linked with 4-amino-benzoyl-β-alan ine (an iner t compound) by a diazo bond. The presence of the azo bond in both of these comp ounds, similar to sulfasalazine's, prevents small-bowel absorption and allows for delivery of the drug mainly in the colon, where bacterial azo reductase libe rates the 5-ASA. These drugs would therefore be useful in the same circumstances as sulfasalazine. However, the 5-ASA products may cause diarrhea due to decreased water absorpti on in the small bowel, and this side effect occurs most frequently with olsalazine. Since the diseases these drugs are used to treat are diarrheal illnesses, their use may be limited by this untoward effect. In a recent study [7] bal salazide was found to be more effective and better tolerated than mesalamine as treatment for UC.

In the treatment of distal colitis, enema preparation of 5-ASA (Rowasa enema) is efficacious. In the initial study to identify the active moiety in sulfasalazi ne, patients with distal UC were treated with sulfasalazine, 5-ASA, or sulfapyr idine enemas[8]. Three quarters of the patients in the sulfasalazine and 5-ASA groups showed improvement, while only about one third of patients in the sulfapyridine grop improved. These data supported the hypothesis that 5-ASA was the active therapeutic moiety, and subsequent studies confirmed the efficacy of 5-ASA enemas in distal colitis[9]. 5-ASA is also available in suppo sitory form (Rowasa) that is beneficial for the treatment of proctitis.

Distal ulcerative colitis

About two thirds of the patients with proctitis and proctosigmoiditis respond to hydrocortisone enema (Cortenema) or 5-ASA enemas (Rowasa). However, in the pat ients with proctitis and/or proctosigmoiditis that do not respond to this conven tional treatment of hydrocortisone enema of 5-ASA enemas, several maneuvers are helpful. A clinical trial that compared the efficacy of nightly 4 g 5-ASA retention enemas with continued administration of 100-mg hydrocortisone enemas in distal UC after failure of a 3-week trial of the latter, with or without oral sulfasal azine, demonstrated that a significantly greater number of refractory patients responded to 5-ASA enemas than to continuation of standard therapy[10].

Several studies show that mesalamine enemas 4 g/day along with oral mesalamine 2 .4-4 g/day are useful in inducing and maintaining remission. The data also suggest that a higher dosage (such as 4 g/day orally) of all but one 5-ASA agent offers an important therapeutic advantage in some of the patients who are apparen tly "refractory" to conventional dosage. The exception to this is olsalazine, which, because of dimer-induced intestinal secretion, delivers more fluid to the colon and may cause diarrhea. Other data suggest that daily or every-other- day administration of 5-ASA enemas is sufficient to maintain remission, but that this effect is lost if administration is reduced to every third day.

A recent 6-week multi-center, randomized, double-blind comparative study of oral mesalamine versus rectal mesalamine versus combination therapy in the treat ment of mild-to-moderate distal UC evaluated the differences among these regimens. A total of 60 patients were enrolled in the study; patient demographics, in cluding UC history, did not differ significantly among treatment groups[11]. This study suggests that a combination of oral and rectal mesalamine in patients with mild-to-moderate UC produces earlier relief of rectal bleeding tha n either therapy alone and more complete relief of rectal bleeding than oral the rapy alone. 5-ASA by the oral route and intermittently by topical route was found to be more effective than oral

therapy alone in maintaining remission[12].

Glucocorticoids

Like sulfasalazine, glucocorticoids and adrenocorticotropic hormone(ACTH) have b een used in the treatment of UC for more than 40 years. The first controlled trial was done in 1955 by Truelove and Witts [13], and this, and other subs equent studies, clearly established the efficacy of glucocorticoids. Several stu dies have established that for mild-to-moderate disease, a daily dose of 40 mg of prednisolone is optimal, while for severe disease the optimal dosage is 60 mg of prednisolone or equivalent in divided doses. Even in severe disease, intravenous glucocorticoids have been shown to induce remission in up to 80% of patients. While ACTH is effe ctive, studies have shown that it has no significant benefit over glucocorticoids, and it is now rarely used due to its expense and the fact that it must be used intravenously. For distal colitis, local therapy with glucocorticoid enemas is effective. However, unlike sulfasalazine and 5-ASA compounds, glucocorticoids have not been found to have any benefit as long-term maintenance therapy in UC.

Because of the numerous systemic side effects of glucocorticoid therapy, attempts have been made to develop poorly absorbed, topically active steroids and gluco corticoids with high topical activity and high rate of metabolism in the liver. The most promising of these agents appears to be budesonide. It is readily absor bed from the gut and rapidly degraded to metabolites with low glucocorticoid sys temic activity during the first passage through the liver. It is not yet available in the United States, but several recent studies with oral administration suggest its efficacy in treating UC[14], and ileal Crohn's disease[15,16], as well as distal UC in the form of enemas[17,18].

In a randomized trial of Budesonide 8 mg/day vs prednisone 40 mg/day for 8 wk in patients with CD, there was equivalent remission by CDAI<150 in both groups. However, twice as many responders in the Budesonide groups responded to treatme nt with no side effects as compared to the prednisone group[19].

Immunosuppressive/immunomodulatory agents

The first immunosuppressive agents used in the treatment of IBD were 6-mercapt o purine (6-MP) and its *S*-imidazole precursor, azathioprine. Azathioprine was de veloped with the intent of allowing delayed release of 6-MP, which is the active metabolite. In clinical practice, azathioprine and 6-MP have similar efficacy and toxicity, and their use is usually based on personal preference and experience.

Several controlled and uncontrolled trials have been conducted to evaluate the efficacy of 6-MP (1-2 mg/kg body weight per day) and azathioprine which is metabolized in the liver and releases 6-MP. The overall results show a response rate of about 70%, with a significant steroid-sparing effect in most patients. In patients who stop taking these agents after remission is induced, the relapse rate is about two thirds[20,21].

The main impedance to use these agents by many gastroenterologists is the concer n of toxicity. While many patients receiving either agent may develop a decreased white blood cell (WBC) count, few develop marked leukopenia. Because of the potential for bone marrow suppression, blood counts must be checked frequently (e.g. initially bi-weekly and then once a month), but at the dosages used problems are rare. In one of the largest series reviewing the toxicity of 6-MP in infla mmatory bowel disease, the authors found marked bone marrow suppression in 2% of patients (WBC<2500/mm\+3), infections in 7.4%, and severe infections in 1.8%. Off note, however, many patients were receiving concomitant corticosteroids, an d it is unclear what role this may have played in the infections. Pancreatitis c an be seen in 3.3% of patients[22]. This is believed to be an allergic r eaction and usually occurs within 1 month of onset of therapy and abates on with drawal of the agent. Pancreatitis precludes further use of these drugs, while leukopenia may be supported with low-dose corticosteroids or by using a smaller dosage of 6-MP. Rarely, patients may develop a cholestatic hepatitis-like picture even after long term use. Therefore, periodic check-ups (about every 6 months) for liver function tests are necessary. Abnormal results are reversible after cessation of therapy.

A uheoretical risk with the use of purine analogues is teratogenicity and the de velopment of malignancy, particularly lymphoma. Although there have been occasional reports of malignancy in patients receiving 6-MP or azathioprine, little data support a causative effect in patients with IBD[23]. In reviews looking at teratogenicity, no difference in premature births or congenital anomalies was found in patients taking 6-MP compar ed with the general population[24].

Cyclosporine is an immunomodulatory agent that has shown some promise in refractory IBD. It acts by inhibiting T-lymphocyte function, does not have the myelos uppressive effects of the purine analogues, and produces an effect quickly, usually within 1 wk. The first report of cyclosporine use in IBD was in 1984, and multiple open trials for use in UC have been conducted since then.

Several recent trials have investigated the efficacy of cyclosporine. These stud ies, although with small patient numbers, indicate that cyclosporine, at least temporarily, and induced remission in up to 80% of patients with severe UC unresp onsive to corticosteroid therapy[25]. However, the majority of these patients do not sustain clinical remission with oral cyclosporine and eventually required colectomy before one year[26]. Recently, a study showed lower relapse rate with azathioprine maintenance therapy after remission was induced wit h intravenous cyclosporine[27].

Antibiotics

Antibiotics are not generally useful as a primary therapy for

UC. Although antib iotics do have a clear role in treating complications of UC, such as abscesses, they have never been shown to be efficacious as a direct treatment for the unde rlying disease[28].

A recent double blind, placebo controlled trial found that ciprofloxacin treatm ent was slightly better than placebo at 3 months but approached placebo without significant difference in response at 6 months in UC patients[29].

Nicotine

While UC is more common among the previous smokers, the efficacy of nicotine as a therapeutic agent in UC remains controversial[30,31]. Given the addictive property of nicotine and significant adverse effects on cardiovascular system and the lack of clear benefit in UC, nicotine cannot be recommended at this time[32].

Nutrition

Since UC is a condition of bowel inflammation, several investig ators have studied bowel rest as therapy. Total parenteral nutrition (TPN) allows for bowel rest, eliminates dietary macromolecules and, thus, reduces mucosal immune response, and helps to correct the malnutrition associated with UC. Several randomized controlled trials have looked at the benefit of TPN in active UC. They found that TPN offered no benefit over the control group with regard to UC[33]. Patients with distal colitis/proctitis should be on a high roughage diet [34] and those with anemia refractory to iron and vitamin supplements may benefit from treatment with oral iron and recombinant erythropoietin[35].

Other agents

Heparin was found to paradoxically induce remission in 9 of 10 patients with UC refractory to standard therapy [36]. It may act as an anti-thrombotic a gent or may be directly anti-inflammatory. Further multicenter studies are curr ently in progress to examine the efficacy of heparin in patients with active UC.

Deficiency of short-chain fatty acid (SCFA) is associated with diversion colitis, and studies with SCFA enemas in patients with distal UC showed good response in an initial study[37]although a subsequent study could not reproduce these data[38]. Further studies are warranted to explore this simplified treatment.

CROHN'S DISEASE

The management of CD is similar in many respects to the management of UC since the two diseases share many common features. However, there are differences.

Sulfasalazine and 5-ASA products

Being a transmural disease, the medical management of the inflammatory process in CD is often more difficult than it is in UC. Pharmacologically active compounds that exert their effect from the luminal side of the intestine as a local anti - inflammatory agent have limited response because of the lack of transmural ava ilability of the drug. Oral sulfasalazine or 5-ASA derivatives are commonly employed initially for the col onic manifestations of the disease. A multicenter study that evaluated the role of Pentasa in active CD demonstrated that a dose of 4 g/ day was more effective in inducing remission than placebo[39].

Glucocorticoids

Corticosteroids are often used on a short-term basis to manage acute exacerbati ons of the disease. However, intra-abdominal and perineal sepsis, common compli cating factors, must be ruled out before corticosteroids are administered. Oral budesonide (9 mg daily) treatment has been found to be useful to induce remissi on with less steroid-related side effects[40]. The efficacy was lower than prednisone[41]. Recently, budesonide (9 mg daily) was also found to be superior in inducing remission than mesalamine (2 g twice daily) over 16 wk of therapy in active ileocolonic CD[42]. However, on a long term bas is, (over 12 months), the use of budesonide (6 mg/ day) did not sustain clinical remission[43]. Further studies are currently in progress to ascertain th e dosage, duration and clinical response.

Immunosuppressive /immunomodulatory agents

In a double-blind placebo controlled study, 6-mercaptopurine (6-MP) was found to induce remission or significant clinical improvement in about two-thirds of patients with symptomatic CD[44]. If symptoms cannot be controlled with corticosteroids or if it is not possible to taper steroid dosage while maintai ning symptom control, then immunomodulatory agents such as azathioprine or 6-MP may be used in conjunction with reduced doses of steroids to induce and m aintain remission. 6-MP may be used in conjunction with reduced doses of steroids to induce and maintain remission. 6-MP has also been demonstrated to be effective in closing fistulas and reducing steroid requirements in patients with CD. Unfor tunately, fistulas frequently recur upon cessation of immunosuppressive treatment[44]. IV loading of 6-MP has not been shown to decrease the time to re spond in patients requiring ongoing steroid therapy for Crohn's disease. However, Casson has a report of a rare response of IV 6-MP in patients with fulminant colitis who failed steroid management and refused to have surgery[45,46]. Cyclosporine and methotrexate have been found to be benefi cial in subgroup of patients with chronically active CD[47,48]. Tacrolim us (FK506) has a mechanism similar to cyclosporine and preliminary results have shown some benefits to proximal small bowel or fistulizing CD[49]. In a recent small study, 7 of 11 patients with steroid refractory Crohn's disease and UC, tacrolimus with azathioprine and mesalamine achieved rapid remission and allowed for tapering of steroids[50]. However, the dat a related to the use of these drugs is very limited, and at this time, these drugs may be considered in a subgroup of patients who are refractory to more conven tional treatment.

Nutrition

Low residue diets have been found to be beneficial in patients with CD[51]. In patients with proximal CD there was a significantly increased incidence of lactose malabsorption[52]. A defined formula diet can induce remission, probably through a decrease in immunostimulation by luminal contents[53]. This treatment is controversial because remission lasts only as long as the diet is continued. Because fishoil has anti-inflammatory actions, studies have shown a reduction in relapse rates in CD, but its use is limited by the unp leasant taste and smell[54].

Antibiotics

Antibiotics (metronidazole, ciprofloxacin, and clarithromycin and trimethoprimsu lfamethoxazole) can be useful intreatment of CD[55,56]. Antibiotics are also used to treat complications such as intra-abdominal sepsis and perineal fistulas. Although long-term use of antibiotics for treatment of CD has been advo cated by some physicians, there is no convincing support for this strategy.

Maintenance of remission in CD

One of the most vexing issues in the treatment of CD is maintaining remission. The recent data come from trials that compare maintenance regimens following surgical resection. Both mesalamine and metronidazole have been useful in prolonging the time from surgery to symptomatic relapse[57,58,59].

A meta-analysis of sulfasalazine and 5-ASA for maintenance therapy of CD demon strated that 5-ASA was more likely to maintain remission than sulfasalazine and that it demonstrated greater efficacy for patients with ileal desease than for those with combined ileocolonic disease; ileocolonic disease responded better than colonic disease[60].

Although oral mesalamines have demonstrated some benefit in maintaining remission, there are many unresolved issues, e.g. which mesalamine preparation is prefer able, in which clinical group such as phlegmonous type, stricture type or perfor ating type of CD, what dose should be used, and whether drugs are equally effect ive in maintaining remissions induced initially by medical and/or surgical interventions. A recent study suggested that 6-MP is also effective in preventing post-operative recurrence of CD and may be more effective than 5-ASA preparation s[61].

Newer agents

Tumor necrosis factor (TNF-α) is a proinflammatory cytokine present in excess in the mucosa of patients with active CD. A chimeric mouse/human an ti-TNF-α monoclonal antibady (cA2, infliximab, Remicade; Centocor, Malvern, PA)was developed and its efficacy was assessed in patients with severe CD[62]. There was no apparent dose relation between 5 mg per kg to 20 mg per kg body weight given as intravenous infusion as a single dose over a 2 h period. After a single dose of infusion 5 mg/kg clinical response was achieved in 65% of the patients. About half of these patients in the treatment group (compared with 4% in the placebo group) went into remission by wk 4. However, by wk 12, the number of patients maintaining their clinical response after the single dose infusion had decreased to 41% (compared to 12% in the placebo group). Repeated doses in initial responders appear to maintain remission at least in the short term study reported so far. The patients with no response to the first infusion of cA2 were less likely to have a response to a second infusion. Thus, this group of patients may clinically differ from the responder group. Preliminary results from another anti-TNF antibody (CD P571) also showed promising results[63]. The adverse effects of cA2 therapy were mostly transient and not serious and they included headache, nausea, upper respiratory tract infection, fatigue, myalgia, rhinitis, pain, pruritus and dyspnea[62]. Of the 29 patients who received two cA2 infusions, two had a reaction with chest pain, dysp nea and nausea necessitating discontinuation of the infussion. At least 6 % of t he patients developed anti-cA2 antibody by 12 wk. This percentage may be hig her because cA2 was still detectable in serum samples in two-thirds of the pati ents and the presence of cA2 may have interferred with the anti-cA2 assay. As use of infliximab has increased, reports of hypersensitivity have been published. In our experi ence, this can be avoided by premedication with benadryl and glucocorticoids[64]. It is unknown at this time whether the presence of anti-cA2 will in fluence the efficacy of the therapy, particularly after subsequent infusions. Remi cade has recently been approved by the FDA for use in moderate and severe Crohn's disease, with or without fistula since closure of fistula has also been reported with this therapy. In another review, infliximab was effective in achi eving fistula closure 68% *vs* 26% placebo[64].

Interleukin-10 (IL-10) is a cytokine with both anti-inflammatory as well as immunosuppressive properties. In a recent study in patients with steroid-refractory active CD, daily administration of IL-10 for one week resulted in 50% of p atients achieving complete remission within 3 wk (compared to 23% in the placebo group)[65].

IL-11 has been known to have a mucosal protective effect. Besides its anti-inf lammatory effects, it also has a trophic effect on intestinal villi. Sands *et al* are currently investigating dosing strategies for possible use of this cyto kine in the treatment of active Crohn's disease[66,67].

Intercellular adhesion molecule-1 (ICAM-1)is an inducible transmembrane glycop rotein involved in the activation of leukocytes and is upregulated in inflamed m ucosa in CD. ISIS 2302 is a 20-base phosphorothioate oligodeoxynucleotide which selectively inhibits cytokine-induced ICAM-1 expression. In patients with steroid-resistant active CD, it was found to achieve remission in 7 of 15 patients immediately after treatment and 5 of 7 patients were still in remission at the end of six months[68]. This modality of treatment opens upyet anoth er novel

approach of administering antisense therapy for IBD.

It is recognized that NSAIDs may be harmful in patients with inflammatory bowel disease. Since the arrival of COX_2 inhibitors on the market, only one study has attempted to evaluate their safety in IBD. The results do not point to any ad vantage or safety of COX_2 drugs over NSAIDs[69].

Probiotics

There has been recent investigation into the bacterial makeup and possible assoc iation of intestinal bacteria with inflammation. Investigators have focused on use of nonpathogenic *E.coli* and other probiotic preparations in maintaining remis sion of UC. In one study by Rembacken *et al* at 12 months there was a non-st atistically significant difference between two groups, one taking mesalamine, the other taking nonpathogenic *E.coli*[70,71].

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Edited by Lu J
proofread by Mittra S

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

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Pathogenesis and managenent of pain in chronic pancreatitis

C.S.Pitchumoni

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INTRODUCTION

Of the three cardinal manifestations of chronic pancreatitis-pain, diabetes mel litus and steatorrhea, it is pain that brings the patient to the physician and is the most difficult to manage. The intractabale pain that is quite debilitating disrupts lifestyle and leads to functional incapacity, drug and alcohol dependency, and a drug-see king behavior that occasionally might push the desperate patient to suicidal ten d ency. Painless CP is an exception that has been observed in nearly 5% to 10% of patients with all forms of chronic pancreatitis. Lack of pain is also a feature of the late onset idiopathic CP.

CHARACTERISTICS OF PANCREATIC PAIN

The pain of pancreatitis is steady and agonizing, is felt in the epigastrium, so metimes in the left upper quadrant with radiation to the back between T12 and L2 or to the left shoulder. Typically pain is postprandial in nature suggesting a gastric lesion. Colicky pain is unusual in CP. Associated nausea and persistent vomiting that does not relieve the pain are in contrast to the pain of gastritis or pyloric obstruction. The pain may be accompanied by flushing and shortness of breath. The severity of pain varies greatly in defferent patients and indeed in the same individual in different episodes, for no obvious reason. The onset of the persistent abdominal pain 12 to 48 h after a drinking bout or "on the afternoon after the night before" is said to be characteristic of alcoholic pancreatitis, but exacerbation of pain may occur even during abstinence from alco hol with no identifiable cause. At times of pain patients bend forward to the so-called "pancreatic position" or lie in the knee-chest position on their ri ght or left side. The duration of pain-free intervals is unpredictable and may last from weeks to many months, making it difficult to assess the value of dif ferent modalities of pain therapy. As the disease advances, the postprandial rel ationship is lost and pain becomes steady and unrelenting[1].

Recently Ammann *et al* reported characteristics of pancreatic pain in 207 pat ients with alcoholic chronic pancreatitis[2]. They identified two patter ns of pain Type A and B based on natural history. In Type A pain in nearly 44% o f patients, who never, who never needed surgery for pain relief the duration of pain was short usually less than 10 days. In contrast Type B pain occurred in 56% of patients who had episodes of constant pain defined as prolonged periods of daily persistent pain, occurring 2 or more days per week for at least 2 months. All these patients underwent surgery. The two types of pain noted by Ammann in S witzerland has not been observed in other series[3]. What Ammann has not ed in his Zurich patients based on the natural history of pain in chronic pancre +atitis as "burning out of the pancreas" and pain relief after 10 or more years is also not seen in many other countries[4-6]. Although it is not clear why geographical differences occur in the natural history of chronic pancreatit is, one observation is that the different studies quoted include patients with a lcoholic and non-alcoholic pancreatitis while Ammann's group is exclusively of alcoholic pancreatitis.

PATHOGENESIS OF PAIN IN CP

Pain in CP is multifactorial in pathogenesis[1]. The current thoughts in clude (a) increased intraductal pressure, (b) increased pancreatic tissue pressure (interstitial-hypertension), (c) pancreatitis-associated neuritis, (d) pa ncre atic ischemia, and (e) ongoing pancreatic injury. Complications of CP, such as pseudocyst, common bile duct obstruction, and associated gastroduodenal diseases (e.g., peptic ulcer), may contribute to the pain. Pancreatic cancer may mimic CP and may be a complication in some patients with CP.

Intraductal hypertension

Increased intraductal pressure is the most important cause of pain. Intraoperative ductal pressure measurements, direct measurements through endoscopic approach , and studies on surgical and autopsy specimens of pancreas have substantiated the concept.

The normal intraductal pressure, depending on the

Professor of Medicine/Community and Preventive Medicine, New York Medical College
Director of Medicine and Chief of Gastroenterology, Our Lady of Mercy Medical Center, Bronx, New York 10466
Correspondence to: Prof C.S.Pitchumoni, Director of Medicine and Chief of Gastroenterology, Our Lady of Mercy Medical Center, Bronx, New York 10466.
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methodology used in the unstim hulated pancreas, is about 7 mmHg, and it is markedly increased in CP. DuVal in 1 958 measured the pancreatic duct pressures in nine patients with CP using a smal l-caliber Foley catheter inserted into the distal pancreatic duct at pancreatic ojejunostomy and in four patients with cutaneous pancreatic fistulae who served as controls [1]. In those with CP the intraductal pressure was invariably higher. Hi stopathological studies reveal that strictures of the ducts and ductules, obstru ction by calculi in major ducts or its branches are the essential features of CP and could all cause pre-stenotic intraductal hypertension.

Interstitial hypertension

The pathogenesis of interstitial or tissue hypertension is the same as intraduc tal hypertension[7]. Even minimal obstruction to the ductules can lead to tissue fluid hypertension. Indeed, interstitial hypertension can theoretically occur much before intraductal hypertension and ERCP changes of ductal morphology are detectable. In the so-called minimal change chronic pancreatitis, the severe pain cannot be explained with the above[8].

Pancreatic pain maybe ischemic in nature analogous to the pain in compartment sy ndromes. High interstitial pressure could greatly increase vascular resistance and reduce pancreatic blood flow. Karanjia *et al* have shown in their cat mode l of CP that the blood flow to the pancreas was 40% lower than in normal pan creas, whereas secretory stimulation of the pancreas further decreased the blood flow[9]. Decompression of the obstructed pancreatic duct reversed all these changes. Increase in ductal or interstitial pressure has been questioned in a few studies and no correlation has been found between ductal morphology and pain[10]. In those with normal ERCP findings, one cannot rule out the poss ibility of interstitial hypertension.

Ongoing pancreatic injury

Kloppel hypothesizes that the dynamics of the disease rather than the end result are possible factors for pain [11]. During progressive scarring the pancreatic nerves and ducts become irregularly entrapped in fibrotic tissue. Recurrent tissue necrosis causes pain in early stages, whereas the persistent pain in a dvanced CP is a result of incomplete obstruction of the ducts.

The role of pancreatic stellate cells in this regard is notable. Pancreatic stel late cells are perivascular and derived from Vitamin A-containing cells. These cells because of their contractile potential and perivascular location could cause microvascular ischemia and pain[12].

Inflammatory mass

Nearly 30% of patients with painful chronic pancreatitis have enlargement of the pancreatic head caused by inflammation[13]. The inflammatory mass cause d pain by involving pancreatic nerves and producing obstruction of bile duct, pancreatic duct or duodenum[14,15]. Operations have been devised to remove inflammatory masses[16, 17]. The studies of Ammann *et al* did not find these inflammatory masses in the head of the pancreas[2].

Neuronal changes

Keith *et al* (1985) studied pancreatic tissue obtained from 50 patients who underwent pancreatic resection or decompression[18]. They found perineur al accumulation of inflammatory cells, predominantly eosinophils correlating with severity of pain and alcoholism-scores, but not with ductal morphology, indic ating a role of these inflammatory cells in the causation of pain.

It is well known that eosinophils are toxic to nerve tissue. The mean diameter of nerves in the pancreas of CP patients is significantly greater than in controls[19]. The perineural sheath is altered such that it no longer provides a barrier be tween the surrounding connective tissue and the internal neural components. The absence of the normal barrier provided by the perineurium exposes the nerves to activated eczymes, plasma components, and bioactive materials released from infl a mmatory cells. Increased mean diameters of nerves argue against an old thought that pain is caused by strangulation of nerves by fibrosis. The weakness of the n euronal theory of pain lies in its failure to explain the relief of pain with the cessation of pancreatic function when it shows the same histologic changes in nerves, as in painful pancreatitis. It also does not explain the relief of pain with surgical procedures that reduce intraductal pressure but do not alter the neuronal changes.

NATURAL HISTORY OF PAIN IN CP

Pain in CP as the disease progresses to pancreatic exocrine and endocrine insuff iciency is unpredictable in an individual case. Levra *et al* (1970) reporting on a longitudinal study of 113 patients followed for 4 years, observed that the pain decreased in 42%, was stable in 32%, and worsened in 26%[20]. The often quoted study of Amman *et al* (1984) from Switzerland concluded that 85% of patients obtained lasting relief from pain at a median of 4.5 years from the onset of disease accompanied by a marked increase in pancreatic dysfunction and calcifications suggesting a relation between pain and the onset of pancreatic insufficiency.

Although there seems to be no consensus with regard to the perce ntage of patients who get spontaneous relief, some observers have confirmed a fi nal pain-free stage in the natural history of CP in 30% to 50% of patients. How ever, in an individual case no one can predict how long it will take to reach a pain-free stage of CP or whether that individual will ever get a “burned out pancreas”. Surgical management cannot be delayed hoping that spontaneous relief would ever occur.

The study of Ammann *et al* quoted earlier is relevant here[2]. Nearly 44% of patients had only intermittent pain

and had a favorable course without invasive therapy. In evaluating various treatment modalities the group of patients who underwent the therapy has to be clarified. An endoscopic therapy of clearing stones from the pancreatic duct by a group claimed that the best predictor of pain-free int erval after therapy was infrequent attacks before therapy[22]. One might criticize studies with similar results as expected response even without the therapy.

MANAGEMENT OF PAIN IN CP

Management of pain in CP is to be approached by a team of physicians that includes a gastroenterologist, surgeon, radiologist, and a psychiatrist. It is difficu lt to assess the severity of pain in patients with CP since many of them are add icted to alcohol and/or narcotics. Equally important is to recognize that in pat ients with alcoholic pancreatitis it is not uncommon to see malingering or a drug-seeking behavior in the form of exaggerated complaints of pain. The available modes of therapy in the management of pain are tabulated. Bef ore the initiation of therapy, treatable complications such as pseudocysts, bile duct abstructions, and peptic ulcer disease should be ruled out.

Table 1 Management of pain in chronic pancreatitis

Medical (conservative)	Abstinence from alcohol
	Analgesics (nonopioid and opoid NSAIDs)
	Tricyclics
	Pancreatic enzyme supplements
	Parenteral nntrition
	Octreotide
Endoscopic therapy	Sphincterotomy, stone extraction, extracorporeal shock wave
	Lithotripsy (ESWL) of pancreatic calculi
	Septotomy+
	Stent placement
Neurolytic therapy	Drainage procedures
	Surgical therapy
	Resections
	Denervation procedures
Experimental	Dissolution of calculi with oral medications
	Direct chemical dissolution of calculi
	Duct injection to cause complete obstruction (prolamin, acrylate, latex)

Abstinence from alcohol

The help of a psychiatrist or an alcoholics anonymous group is often necessary. The rate of pain relief is usually higher in abstinent patients, and deteriorati on of pancreatic function is slower. The importance of alcohol abstention thus cannot be overemphasized. Considerable time should be spent in discussing with the patient and making him or her understand the inevitable progression of disease if alcohol use is continued.

It is difficult to interpret the results of studies showing no correlation betwe en abstinence from alcohol and abdominal pain. It is likely that the clinical stage of the disease alters the effect of alcohol or pain. In the early stages, with well-preserved pancreatic function, secretagogoues such as alcohol may exagg erate pain, whereas in advanced stages they may have very little stimulatory effect on the fibrotic pancreas[21].

Diet

The goal is to provide rest to the pancreas or avoid excessive stimulation while maintaining adequate nutrition. The conventional diet is one that is low in fat and small in quantity. In some patients hospitalization, total cessation of oral intake of food, and short-term use of partial or total parenteral nutrition may be needed for urgent management of severe pain. The diet is also determined by the fact whether there is associated diabetes and/or steatorrhea. Supplement ation with oral antioxidants is reported to reduce the intensity and frequency of pain.

Antioxidant supplementation

The basis of antioxidant therapy was the hypothesis that CP is a disease caused by unopposed free radical (FR) injury[23,24]. Banks and colleagues in their attempt to prevent generations of FR used allopurinol 300 mg/d. There was no significant reduction in pain.Despite a definite proof for FR injury in relation to CP or clinical trials conf irming the use of antioxidants, there is widespread enthusiasm for the therapy. Antioxidant therapy is inexpensive and harmless. For a disease with no better substitute for treatment the simplicity of antioxidant therapy is attractive.

Cigareete smoking is noted to be an associated factor in the pathogenesis of CP, it is prudent to advise against smoking[25,26]. Most alcoholics who develop CP are heavy cigarette smokers.

Analgesics

The first step is to try nonopioid analgesics, such as acetaminophen, salicylate s and nonsteroidal anti-inflammatory drugs (NSAIDs) with or without antidepress ants. However, most patients need opiod analgesics for symptomatic relief, and the initial doses should be low and administered less frequently. Small doses of codeine derivatives with acetaminophen are the usual drugs of choice. The use o f an H_2 receptor antagonist or a proton pump inhibitor to suppress gastric acid production, reduce destruction of orally administered pancreatic enzymes and theoretically reduce pancreatic stimulation is advocated. As an adjunct to the therapy, a small do se of an antidepressant may be helpful. If a potent narcotic is needed to offer pain relief, surgery should be considered as early as possible before the developme nt of narcotic addiction.

Pancreatic enzyme therapy

The basis for oral enzyme therapy for pancreatic pain is as follows. Secretory status of exocrine tissues and obstruction to outflow determine intraductal pressure. Deficiency of proteases (trypsin, chymotrypsin) within the duodenal lum en causes release of cholecystokinin from the mucosa,

leading to enhanced stimulation of the exocrine tissues [27-29]. Experimental evidence indicates that the presence of intraluminal proteases in the proximal small intestine inhibits the release of cholecystokinin and thereby stimulation of the exocrine parenchyma.

To effect feedback inhibition of pancreatic secretion, it is important to admini ster large doses of commercially available pancreatic enzymes. One should choose the type of preparation that increases the intraduodenal proteases[28]. The ideal patient for enzyme therapy is a female with idiopauhic pancreatitis with normal fecal fat excretion. Individuals with large dilated ducts tend not to respond to enzyme therapy. Enzyme therapy is simple, easy to initiate, but some what expensive. However the cost of enzyme therapy cannot be considered high when the alternative is major pancreatic surgery. The selection of an enzyme prepar ation is to be based on the protease concentration within the preparation, the s tability of the enzyme in withstanding gastric acidity, and the timely release of the enzyme from capsules in the proximal intestine. The inconvenience of taking l arge doses of enzyme preparations is justified if the preparations relieve painor reduce the need for analgesics. Hence, it is worth trying enzyme therapy in all patients with painful CP for at least 6 to 8 weeks.

If there is no pain relief with a nonenteric coated preparation, addition of an H_2 receptor antagonist or sodium bicarbonate (650 mg) before and after each do se of pancreatic enzymes to protect them from gastric acid is a consideration.

The side effects of enzyme therapy are few. Pancreatic extracts from insoluble comple xes with folic acid, as a result folic acid deficiency can develop. Hyperuricemi a and hyperuricosuria are described in cystic fibrosis patients treated with large doses of pancreatic extracts. Allergic reactions to the porcine proteins in extr acts may occur. There is a valid criticism that studies supporting response to enzyme therapy are few and have used only a small number of patients.

The vast majority of patients with CP are those with longstanding history of alc oholism, more often men, with large duct disease. Even the protagonists of enzyme therapy do not consider the above group as ideal patients to obtain relief with oral pancreatic enzymes. It appears that at this time the majority of gastroen terologists are not quite enthusiastic about enzyme therapy for pancreatic pain[30]. The role of enzyme therapy is limited despite its wide populari ty which is so lely based on its simplicity and the fact that it appears to be a non-invasive form of therapy compared to endoscopic therapy or surgery.

Somatostatin and octreotide

Somatostatin is a naturally occurring hormone that inhibits pancreatic secretio n. Octreotide is a synthetic long-acting analogue of somatostatin that inhibits cholecystokinin release and both basal and oral stimulated pancreatic secretion . The duration of action of octreotide is longer than that of somatostatin, but it increases the contractability of the Sphincter of Oddi while somatostatin dec reases it. One multi-center randomized study of 91 patients with CP showed promising results [31]. At a dose of 200 mg TID octreotide offered good pain relief especially in those with constant pain. Another recent study by Mal fertheiner *et al* however did not show any benefit[32].

Endoscopic therapy in CP

Endoscopic therapies are based on the premise that the most important, mechanism of pain in CP is impairent of outflow of pancreatic secretion by strictures or calculi in the main pancreatic duct[33-36]. The attractive feat ure of endoscopic drainage procedures is that it offers an alternative to surgic al forms of drainage. The optimistic belief that these methods may achieve the same results as surgical drainage but without its operative morbidity (20% to 40%) or mortality (2% to 5%) rates is attractive.

The forms of endoscopic procedures currently available include sphincterotomy, internal drainage of pancreatic cysts, extraction of stones from the pancreatic duct, guidewire-catheter dilation of strictures, and placement of pancreatic stents. Endoscopic intervention can be considered in the following situations: (1) biliary strictures, (2) pain or recurrent pancreatitis associated with a dominant s tricture at the proximal end (head), (3) recurrent pancreatitis with pancreas divisum, (4) pancreatic cysts, (5) pancreatic stones, and (6) Sphincter of Oddi dys function.

Lithotripsy

Although it is controversial whether stones or calculi directly cause pain it is logical to assume that removal of stones would relieve intraductal hypertensi on. Although endoscopically it is possible to remove one or two stones in the head end of the pancreas, it is only after fragmentation by shock wave or laser li thotripsy that the more proximal innumerable stones can be drained. Some studies have noted excellent pain relief[35-38]. In a series of 123 patients with pancreatic calculi ESWL was helpful in 122. After ESWL the pancreatic duct could be completely cleared with endoscopic papillotomy in 595 of patients [37]. Adamack and group recently (1999) studied 43 patients who were success fully treated with ESWL [38]. The only feature associated with treatment success was the presence of a single stone rather than multiple stones. Successfully treated patients achieved some degee of reduction in pain. The factors favoring stone removal and clinical response include three or less stones, absence o f multiple strictures, stones confined to the head of the pancreas, stone diameter of <10 mm and absence of impacted stones[35]. Lithotripsy with stone removal is not yet a well accepted form of therapy for pain relief in chronic pancreatitis. Lack

of expertise, a skepticism generated by lack of adequate well controlled trials and the theoretical opinion that removal of stones is only cosmetic to the total histology of the pancreas have dampened the enthusiasm for this form of therapy in the U.S. among academicians.

Sphincterotomy

The best indication for endoscopic sphincterotomy is when the patient has a soli tary ductal stone in the head of the pancreas without any evidence of proximals tricture. Relief of pain can be predicted if a postsphincterotomy pancreatogram demonstrates immediate reduction in size of the main pancreatic duct.

Stenting

Another procedure used in the treatment of pain is endoscopic stenting of the ma in pancreatic duct after sphincterotomy. The stents have side holes at approxima tely 1-cm intervals that permit better drainage of pancreatic juice from side b ranches. Plastic stents have been noted to be inferior to self-expandable metallic stents (Wallstent), which provide better dilation of the stricture and do not clog as often. Cremer's group noted that in addition to pain relief marked im provement in nutritional status of patients occurred as a result of better delivery of bile and pancreatic secretions in the duodenum helping digestion[37]. Excellent improvement in pain has been reported. Prolonged symtomatic impr ovement even after the stent has been removed is observed. This unexpected result may be explained by the fact that before or during stenting, many small calculi are removed, eliminating small ductular obstructions.

The risk of pancreatic stenting is beginging to be appreciated[33,39,40,41]. A number of complications are reported after stent placement:(1)clogging o f the endoprosthesis is frequent, and it must be changed every 4 to 6 months; (2) migration of the prosthesis may be serious problem (3) the stent can be passed spontaneously without any adverse effect; (4) migration, clogging, and occlusion of the stent may lead to pancreatic abscess formation or infect a pseudocyst th at is in communication with the main pancreatic duct; (5)duodenal erosion may be caused by improper positioning of the stent; (6) pancreatitis may be induced by stent placement but is usually mild and self-limiting; and (7) ductal changes mimicing CP may develop even after stent placement.

Celiac ganglion blocking

It is reasonable to expect that pharmacologic denervation of the celiac plexus w ill result in pain relief. Celiac plexus blocking is technically difficult; the pain relief is transient and sometimes lasts only a few days. It is also associa ted with complications such as hypotension, epidural or intraperitoneal hematomas, and sexual dysfunction. Neurolytic block is thus considered a last resort the rapy.[41] Recent reports on celiac ganlion blocking using endoscopic ultr asound guidance offer much hope[42,43].

A variant of celiac block is intrapleural analgesia achieved by instilling local anesthetic into the pleural space[44]. Dramatic pain relief lasting for 4 months has been reported. Diffusion within the interpleural space seems to block several intercostal nerves. The scientific explanation is that pancreatic pa in is transmitted along afferent fibers from the pancreas to the splachnic nerve s and also by the lower intercostal nerves, which innervate the peritoneum.

Acupuncture and transcutaneous nerve stimulation (TENS)

A pilot study showed that 3 patients with chronic pain responded well to both el ectroacupuncture and TENS [45]. In 23 patients with chronic pancreatitis with daily pain for 3 minths the efficacy of TENS was studied. In two prospectiv studies with cross over design active acupuncture was compared with sham acupun cture and TENS of the segemental points of the pancreas with sham treatment.Neit her acupuncture nor TENS brought about pain relief that could substitute for or supplement medical treatment (Ballegaard, et al. 1985). This small study con ducted in an institution not specializing in acupuncture should not discourage experts in the field from trying it. In the absence of an effective therapy for pain in CP, there is a great need to look at alternate medicine for possible answers. The expertise of the Chinese is greatly needed here.

Surgery for pain in CP

Surgery is a consideration when pain is severe enough to interfere with day-to-day life and when it cannot be managed safely with medical treatment alone. Re cent review articles well describe the surgical aspects of CP[46,47] Eva luation for surgery must be individualized and the following should take into co nsideration: frequent hospitalizations, disruption of employment and social life, nutritional status, depression of other psychiatric manifestations, and drug d ependence. Preoperative assessment should include abdominal computed tomography, ERCP, and if appropriate, psychiatric evaluation.

The operative candidates can be divided into two broad groups: those with dilated pancreatic ducts (who more likely benefit from ductal drainage) and those normal size ducts (who may need pancreatic resection or a denervation procedure).

Drainage procedures Many authors recommend longitudinal pancreaticojejunostomy or lateral pancreaticojejunostomy (modified Puestow precedure) when the pancreatic ducts are large enough for anastomosis[46]. Long-term pain relief is achieved in more

than two thirds of patients with CP and a dilated (more than 7 mm diameter) pa ncreatic duct. Pain relief is immediate in 80% to 90% of patients, although there is an unpredictable recurrence rate in some of them. Some 60% to 75% of patien ts are pain free for 5 to 6 years. Recurrence of pain after an initially success ful longitudinal pancreaticojejunostomy suggests stricture formation and may indicate the need for reoperation.

The operative mortality rate averages about 4%, and diabetes, does not result fr om the procedure. However, many patients may go on to become insulin-requiring diabetics because of continual destruction of the pancreas as a result of CP.

Pancreatic resection Resection is the procedure of choice in a pancreas whose ducts are not dilated, when a previous drainage procedure has failed, or if pathologic changes predomin antly involve a particular area of the gland. Resection offers good pain relief that tends to be more permanent than that after pancreaticojejunostomy. Resectio ns generally involve one of the following procedures: (1) pancreaticoduodenecto my, (2) local resection of the head of the pancreas, and (3) subtotal or distal pancreatectomy. However, exocrine and endocrine insufficiency is a major drawback with resections.

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Edited by Zhu QR
proofread by Mittra S

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

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Therapeutic methods for diarrhea in children

S.K. Bhattacharya

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DEFINITION

Acute diarrhoea is defined as passage of loose or watery stools at least three t imes in a 24 h period. When loose stools contain blood, it is called bloody diarrhoea (dysentery). It is the consistency of the stools which is most important rather than the frequency. Breast-fed babies often pass "pasty" stools fr equently which is not diarrhoea. The mother can often tell accurately whether child has diarrhoea or not.

MAGNITUDE OF THE PROBLEM

Acute diarrhoea is an important cause of mortality and morbidity particularly in young children in the developing countries. Of the 11.6 million deaths among ch ildren less than five years old in all developing countries (1995) due to infect ious diseases, 19% deaths are attributed to diarrhoea[1]. In 1993, an es timated 3. 2 million children below five years of age died from diarrhoea alone; 80% of these deaths occurred in the first two years of life[2].

AETIOLOGY

Acute diarrhoea is usually caused by different infectious agents. The microbial agents causing diarrhoea may be classified as bacterial (*Vibrio cholerae* O1 and O139, Vibrio *parahaemolyticus,* enterotoxigenic *E.coli, Shigella, Salmonella, Campylobacter jejuni, Aeromonous etc*.), viral (Rotavirus, Norwalkv i rus etc.), parasites (*E. histolytica, Giardia lamblia, Cryptosporidium* etc.). The various agents produce diarrhoea either by production of toxin(s) or by inv asion of the gut mucosa. Those organisms which produce diarrhoea by production of toxin(s) produce watery diarrhoea. *Vibrio cholerae* and enterotoxigenic *E .coli* are the prototype organisms producing watery (also called secretory) dia rrhoea. Those organisms which invade the gut mucosa usually produce bloody diarr hoea (dysentery). *Shigella* and *E.histolytica* are the prototype organisms producing bloody diarrhoea (also called invasive diarrhoea).

In watery diarrhoea usually there is loss of lots of fluid and electrolytes from the body which results in dehydration which is a conspicuous clinical feature in watery diarrhoea. In contrast, in invasive diarrhoea not much fluid and electr olytes are lost in the stool. Therefore, dehydration is not a major feature. It is most practical to base treatment of acute diarrhoea on the clinical type of illness (watery or bloody). Laboratory studies are usually not needed.

DEHYDRATION

Management of acute watery diarrhoea includes replacement of fluid and electroly tes losses, proper feeding and use of appropriate antibiotic in selected cases. It has been mentioned that dehydration occurs due to loss of fluid and electroly tes from the body. Dehydration is now clinically assessed as diarrhoea with "no signs of dehydration", diarrhoea with "some dehydration" and diarrhoea wit h "severe dehydration". Table 1 describes how to determine the degree of dehy dration clinically. The signs typical of children with no signs of dehydration are shown in column A, signs of some dehydration in column B and those with sever e dehydration in column C. The sign in bold print with asterisks (*) are the mos t valuable signs for assessing dehydration and are called "key signs". If two or more of the signs in column C are present including at least one key sign the child has severe dehydration. If this is not the case, but two or more signs from column B (and C) are present, including at least one key sign, the child has some dehydration. If this also is not the case, the child is classified as havi ng no signs of dehydration.

PREVENTION OF DEHYDRATION (PLAN A)

Diarrhoea with no signs of dehydration may be managed safely and effectively at home with the administration of extra fluid, proper feeding and watching for dan ger signs. The mother may be educated to give her child extra fluid in the form of coconut water, salt and sugar solution, rice water with salt, mild tea (these fluids are called "home available fluid"-HAF) or oral dehydration salt solution (ORS). At any stage if the child becomes either very thirsty, passes many watery stools, vomits repeatedly, or has fever, or blood in stool, the mother s hould be alerted to take the child to a doctor for further management.

Director, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, S cheme XM, Beliaghata, Calcutta 700010, INDIA
Correspondence to: Dr S.K. Bhattacharya, Director, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, S cheme XM, Beliaghata, Calcutta 700010, INDIA
Tel. 350-1176/350-8493/321-6566 (Res), Fax. 350-5066/353-2524
Email.niced@cal2.vsnl.net.in
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Table 1 Assessment of diarrhoea patients for dehydration

1. Look at:	Condition[a]	Well, alert	Restless, irritable	*Lethargic or unconscious; floppy*
	Eyes[b]	Normal	Sunken	Very sunken and dry
	Tears	Present	Absent	Absent
	Mouth and tongue[c]	Moist	Dry	Very dry
	Thirst	Drinks normally, not thristy	*Thirsty, drinks eagerly*	*Drinks poorly, or not able to drink*
2. Feel:	Skin pinch[d]	Goes back quickly	*Goes back slowly	**Goes back very slowly*
3. Decide:		The patient has no signs of dehydration	If the patient has two or more signs, including at least one *sign*, there is some dehydration severe dehydration	If the patient has two or more signs, including at least one *sign*, there is
4. Treat:		Use Treatment Plan A	Weigh the patient, if possible, and use Treatment Plan B	Weigh the patient and use Treatment Plan C urgently

[a] Being lethargic and sleepy are not the same. A lethargic child is not simply asleep: the child's mental state is dull and the child cannot be fully awa kened; the child may appear to be drifting into unconsciousness.
[b] In some infants and children the eyes normally appear somewhat sunken. I t is helpful to ask the mother if the child's eyes are normal or more sunken th an usual.
[c] It is necessary to look inside the child's mouth. The mouth may be dry in a child who habitually breaths through the mouth. The mouth may be wet in a dehydrated child owing to recent vomiting or drinking.
[d] The skin pinch is less useful in infants or children with marasmus or kw ashiorkor, or obese children.

TREATMENT OF DEHYDRATION (PLAN B & C)

Studies have shown that 90% of cases of watery diarrhoea with some dehydration can be safely and effectively managed with ORS solution alone[3]. WHO/ UNI CEF recommended ORS contains sodium chloride 3.5 g, 85 mm potassium chloride 1.5 g, sodium bicarbonate 2.5 g, or trisodium citrate, dihydrate 2.9 g and glucose 20 g dissolved in 1 L of water. For the treatment of some dehydration ORS should be administered (50-100 mL per kg). Table 2 gives the guidelines for treating c hildren and adults with some dehydration. The mother should be taught to prepare and give ORS solution. The solution should be given to infants and young childr en using a clean spoon or cup. Use of a feeding bottle is strongly discouraged. If vomiting occurs (usually during the first hour of treatment) the mother should wait for 5-10 minutes and then start giving ORS solution again but more slow ly. The disadvantages of WHO/UNICEF ORS are that it does not reduce the stool vo lume or duration of diarrhoea and thus are sometimes not acceptable to the mothe rs. Several clinical trials have shown that an ORS solution containing cooked rice powder in place of glucose substantially reduces the rate of stool loss due to acute diarrhoea. Rice based ORS solution significantly reduces the rate of stool output during the first 24 hours of treatment by 36% in adults with cholera and by 32% in children with cholera. In contrast, the rate of stool loss in infants and children with acute non-cholera diarrhoea treated with rice ORS solution was only recuded by 18%[4]. A small but significant proportion of dehyd rated patients might benefit from using a low osmolarity solution in which glucose concentration has been slightly reduced[5]. However, the real benefit of using sucha solution as well as their exact composition remains to be determ ined.

The preferred treatment for children with severe dehydration is rapid intravenous rehydration. Such treatment should preferably be carried out by admitting the patient to the hospital. Guidelines for intravenous rehydration are given in Tab le 3. The preferred solution is Ringer's Lactate.

FEEDING DURING DIARRHOEA

During diarrhoea the child should be fed properly. Previously it was thought tha t during diarrhoea, nutrients are not absorbed adequately and hence the bowel sh ould be given a rest. Recent studies indicate that during and after diarrhoea mo st of the nutrients are sufficiently absorbed. In fact, proper feeding during diarrhoea has been shown to be beneficial and prevents malnutrition. Breast feeding should be continued throughout the duration of diarrhoea. Easily digestable, energy-rich, high potassium containing, non-fibrous food should be given to the child. During convalescence at least one extra feed daily for several weeks is recommended. Locally available and culturally acceptable foods are preferred.

ROLE OF DRUGS

All cases of bloody diarrhoea where dehydration is present should be managed wit h rehydration therapy as detailed for the acute watery diarrhoea. In addition, a ll the cases of bloody diarrhoea in children below 5 years of age should be trea ted with an appropriate antibiotic assuming that the child is suffering from shi gellosis. Studies have shown that antibiotic therapy definitely hastens recovery [6]. The drug of choice for shigellosis is nalidixic acid[7]. Ot her drugs (ampicillin[8] or cotrimoxazole[9]) may be used depend ing upon the drug resistance pattern of the circulating shigella strains in the area. It has been shown that norfloxacin[10] or ciprofloxacin[11] are also highly effective in the treatment of shigellosis. These drugs are co ntraindicated for young children because of the potential cartilage toxicity rep orted in experimental animals[12]. However, more and more information is coming that these drugs may turn out to be safe even in children for such short term use[13]. Amebiasis rarely occurs in children below 5 years of age and therefore random use of antiamoebic drugs for childhood diarrhoea is

Table 2 Guidelines for treating children and adults with some dehydration

	Approximate amount of ORS solution to give in the first 4 hours					
Age[a]	Less than 4 months	4-11 months	12-23 months	2-4 years	5-14 years	15 years or older
Weight:	Less than 5 kg	5-7.9 kg	8-10.9 kg	11-15.9 kg	16-29.9 kg	30 kg or more
In mL	200-400	400-600	600-800	800-1200	1200-2200	2200-4000
In local measure						

[a] Use the patient's age only when you do not know the weight. The ap proximate amount of ORS required (in mL) can also be calculated by multiplying the patient's weight in kg by 75.

- If the patient wants more ORS than shown, give more.
- Encourage the mother to continue breastfeeding her child.
- For infants under 6 months who are not breast fed, also give 100-200 mL clean water during this period.

NOTE: During the initial stages of therapy, while still dehydrated, adults can consume up to 750 mL per hour, if necessary, and children up to 20 mL per kg body weight per hour.

Table 3 Guidelines for intravenous treatment of children and adults with sever e dehydration

- Start IV fluids immediately. If the patient can drink, give ORS by mouth until the drip is set up. Give 100 mL / kg Ringer's Lactate Solution[a] divided as follows:

Age	Fisst give 30 mL / kg in:	Then give 70 mL / kg in:
Infants (under 12 months)	1 hour[b]	5 hours
Older	30 minutes[b]	2.5 hours

- Reassess the patient every hour. If hydration is not improving, give the IV dr ip more rapidly.
- After six hours (infants) or three hours (older patients), evaluate the patien t using the assessment chart. Then choose the appropriate Treatment Plan (A,B or C) to continue treatment.

[a] If Ringer's Lactate Solution is not available, normal saline may be used.
[b] Repeat once if radial pulse is still very weak or not detectable.

not recommended. However, if antibiotic therapy fails and *E.histolytica* trophozoites are seen by microscopic examination of stool, metronidozole or tini dazole may be used in the recommended doses.

Routine use of antibiotic(s) in acute watery diarrhoea is not recommended and is actually harmful. The only indication for use of antibiotics is for the treatme nt of suspected severe cholera cases as an adjunct to rehydration therapy. Chole ra should be suspected when a child of more than two years of age suffers from acute watery diarrhoea with severe dehydration in an endemic area. The drugs of c hoice for the treatment of cholera are tetracycline and doxycycline. Norfloxacin [14]and ciprofloxacin[15] have been shown to be also highly eff ective. Antibiotic therapy shortens the volume and duration of diarrhoea thereby reducing the fluid requirement, and duration of hospitalisation and excretion o f *Vibrio cholerae* in stool. Other drugs, as for example, antiemetics, antich olinergics, antidiarrhoeals like opium, charcoal, kaoline and pectin, steroid an d cariotonics are not required for the treatment of diarrhoea. In fact some of t hem are not only useless but may also be harmful. Antiemetics produce sedation a nd therefore, interfere with oral rehydration therapy and may produce or aggrava te hypotension thereby interfering with the renal circulation. Anti-cholinerg ics may produce paralytic ileals.

Recently, supplementation with micronutrients especially zinc as an adjunct to r ehydration therapy for the treatment of acute diarrhoea has been suggested[16].

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Edited by Zhou XH
proofread by Mittra S

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

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Original Articles

A study on relationship of nitric oxide, oxidation, peroxidation, lipoperoxidation with chronic cholecystitis

Jun Fu Zhou[1], Dong Cai[1], You Gen Zhu[2], Jin Lu Yang[2], Cheng Hong Peng[1] and Yang Hai Yu[1]

Subject headings nitric oxide; oxidation; peroxidation; lipoperoxidation; chronic cholecystitis

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Abstract

AIM **To study relationship of injury induced by nitric oxide, oxidation, peroxidation, lipoperoxidation with chronic cholecystitis.**

METHODS **The values of plasma nitric oxide (P-NO), plasma vitamin C (P-V C), plasma vitamin E (P-VE), plasma β-carotene (P-β-CAR), plasma lipoperoxides (P-LPO), erythrocyte superoxide dismutase (E-SOD), erythrocyte catalase (E-CAT), erythrocyte glutathione peroxidase (E-GSH-Px) activities and erythrocyte lipoperoxides (E-LPO) level in 77 patients with chro nic cholecystitis and 80 healthy control subjects were determined, differences of the above average values between t he patient group and the control group and differences of the average values bet ween preoperative and postoperative patients were analyzed and compared, linear regression and correlation of the disease course with the above determination values as well as the stepwise regression and correlation of the course with th e values were analyzed.**

RESULTS **Compared with the control group, the average values of P -NO, P-LPO, E-LPO were significantly increased ($P < 0.01$), and of P-VC, P-VE, P-β-CAR, E-SOD, E-CAT and E-GSH-Px decreased ($P < 0.01$) in the patient group. The analysis of the lin ear regression and correlation s howed that with prolonging of the course, the values of P-NO, P-LPO and E-LPO in the patients were gradually ascended and the values of P-VC, P-VE, P-β-CAR, E-SOD, E-CAT and E-GSH-Px descended ($P < 0.01$). The analysis of the stepwise regression and correlation indicated that the correlation of th e course with P-NO, P-VE and P-β-CAR values was the closest. Compared with the preoperative patients, the average values of P-NO, P-LPO and E-LPO were significantly decre ased ($P < 0.01$) and the average values of P-VC, E-SOD, E-CAT and E-GSH-Px in postoperative pa tients increased ($P < 0.01$) in postoperative patients. But there was no signif icant difference in the average values of P-VE, P-β-CAR preope rative and postoperative patients.**

CONCLUSION **Chronic cholecystitis could induce the increase of nitric oxide, oxidation, peroxidation and lipoperoxidation.**

INTRODUCTION

Chronic cholecystitis is a frequently encountered disease of the digestive syste m. Some studies point out that in blood of patients with acute cholecystitis the levels of inducible nitric oxide (*i* NO) and lipoperoxides are markedly incre ased, while the level of vitamin C, the activities of superoxide dismutase and g lutathione enzyme are significantly decreased[1-5]. However, up to now , there has been no reports on the above in patients with chronic cholecystit is. In order to observe the metabolic state of nitric oxide and other free radi cals in patients with chronic cholecystitis, and the degree of injury induced by oxidation, peroxidation, lipoperoxidation due to the chronicity of cholecystiti s, we determined nitric oxide (P-NO), vitamin C (P-VC), vitamin E (P-VE), β-carotene (P-β-CAR) and lipoperoxides (P-LPO) levels in the pla sma as well as superoxide dismutase (E-SOD), catalase (E-CAT), glutathione peroxidase (E- GSH-Px) activity and lipoperoxides (E-LPO) level in the erythrocytes in 77 patients with chronic cholecystitis and 80 healthy controls.

[1]The Second Affiliated Hospital of Medical College of Zhejiang University, Hangzhou 310009, Zhejiang, China
[2]The People's Hospital of Jinhua City, Jinhua 321000, Zhejiang, China
Prof. Jun Fu Zhou, M.D, male, born on 1945-03-07 in Hangzhou, Zhejiang Province, graduated from Zhejiang Medical University, having 150 papers published and having won 10 Advanced Prizes in Science and Technology from Zhejiang Province People's Government and PLA
The item of science and technology research plans of Zhejiang Province (No 1999-2-121)
Correspondence to: Prof. Jun-Fu Zhou, The Second Affiliated Hospital of Medical College of Zhejiang University, 68 Jiefang Road, Hangzhou 310009, Zhejiang, China
Tel. +86-571-778-3768 or 605-2515, Fax. +86-571-721-3864
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We also an alyzed and compared differences of the above average determination values betwee n the patient and the control group, and between preoperative and postoperative patients. Additionally, we analyzed the relationship between the course of the d isease and the above values in the patients by the linear regression and correla tion as well as the stepwise regression and correlation.

SUBJECTS AND METHODS

Subjects

Patients Seventy-seven patients suffering form chronic cholecystitis with gallsto nes who were confirmed diagnostically through abdominoscopy and biopsy in the People's Hospital of Jinhua City were randomly sampled. Their ages ranged fro m 31 to 69 years (52.4 a ± 10.3 a), and their courses of disease were from 2 to 20 years (5.2 a ± 5.6 a). Of them, 32 were male and 45 were female. No patients had abnormality in the routine examination of blood, urine, feces, ECG and X -rays, and medical history about heart, brain, lung, liver, kidney, diabetes, a utoimmune disease, peripheral vascular disease, cataract, tumor, and so on. The gallbladders of all the patients with chronic cholecystitis were removed by abdo minoscopy.

Control Eighty healthy adults confirmed through the comprehensive he alth examination by the 2nd Affiliated Hospital of Zhejiang University were randomly sampled, their ages were from 31 to 70 years (52.7 a ± 9.6 a), and 40 were male and 40 were female. The healthy adults were all normal in the routine examination of blood, urine, feces, ECG and X-rays, with no medic al history regarding heart, brain, lung, liver, kidney, cholecystic disease, diabetes, autoimmune disease, peripheral vascular disease, cataract, tumor, etc .

All the patients and the healthy adults had neither exposure to kind of radiation, nor contacted any kind of pesticide and poison. Within a month prior to the study they had not taken any antioxidants such as vitamin C, vitamin E, g inkgo leaf agents, tea-polyphenol etc. There was neither any significant di ffer ence ($P > 0.05$) between the average age of the patient and the control group a s determined by *t* test, and nor any significant difference ($P > 0.05$) betw een the gender proportion of the patient and the control group as determined by χ^2 test.

Methods

Blood samples Fasting venous blood samples were collected in the morning for all the subjects with heparin sodium as an ant icoagulant. The separated plasma and erythrocytes were stored immediately at 4℃[6].

Plasma NO (P-NO) level Colloidal aluminium hydroxide without nitrite was used to absorb yellow pigments and to cause protein sedimentation in the plasma. The nitrite in the supernatant, which contained sodium acetate (0.20 mol/L) an d sulphanilic acid (3.30 mmol/L), reacted with β-Naphthyla mine and formed a colored product, which was detected spectrophotometrically, using sodium nitrit e (2.50 μmol/L) as the standard and at a wavelength of 520 nm. The P-NO concentration was expressed in nmol/L[6,7].

Plasma LPO (P-LPO) level Trichloroacetic acid (TCA) solution (20.0 g%, w /v) was used to cause protein sedimentation in the plasma. The protein sediment reacted with thiobarbituric acid (TBA) solution (0.67 g%, w/v) and produced red colored compounds following incubation in a water bath at 100℃. This was detect ed spectrophotometrically at 532 nm, using tetraethoxypropane (TEP, 5.0 μmol/L) as the standard. The P-LPO concentration was expressed as μmol/L[6,8].

Plasma VC (P-VC) level TCA (5.0 g%, w/v) was used to cause protein sedim entation in the plasma, and ferric trichloride was added to the supernatant. Vit amin C in the supernatant reduced Fe^{3+} in ferric trichloride to Fe^{2+} . Fe^{2+}, on reacting with ferrocene, produced a colored product which was detected spectrophotometrically at 563 nm, using vitamin C as the standa rd. The P-VC concentration was expressed as μmol/L[6,9] .

Plasma VE (P-VE) level Absolute ethyl alcohol was used to cause protein sedimentation in the plasma and to extract vitamin E. Vitamin E in the supernata nt reduced Fe^{3+} in ferric trichloride to Fe^{2+}. Fe^{2+} reacted wi th ferrocene to form a colored product that was detected spectrophotometrically at 563 nm, using vitamin E as the standard. The P-VE concentration was expressed as μmol/L[6,10].

Plasma β-CAR(P-β-CAR)level A mixture of a bsolute ethyl alcohol and petroleum ether was used to cause protein sedimentati on in the plasma and to extract β-carotene. The petroleum ether extract containing β-carotene was an alyzed colorimetrically, using β-carotene as the standard at a wav elength se tting of 440 nm. The P-β-CAR concentration was expressed as μmol/L[6,11].

Erythrocyte LPO (E-LPO) level A mixture of absolute ethyl alcohol and t richlorom ethane (5 : 3) was used to precipitate hemoglobin (Hb) from a hemolytic solution (HS) of RBC without WBC and platelets. Hb level was determined in the HS. LPO in the extracted solution reacted with TBA-glacial acetic acid solution (1.0 g%, w/v) in a water bath at 100℃ and produced red colored compounds. These were det ected using TEP (5.0 μmol/L) as the standard at 532 nm. The E-LPO concentration was expressed as nmol/g Hb[6,12].

Erythrocyte SOD (E-SOD) activity A mixture of absolute ethyl alcohol and trichloromethane (5 : 3) was used to precipitate Hb from the HS of RBC without WBC and platelets. Hb level was determined in the HS. Pyrogallol (6.0 mmol/L) au to-oxidized in Tris-HCl buffer (50

mmol/L, pH 8.20), SOD was added to the buff er to inhibit its auto-oxidation and SOD activity was calculated according to t he auto-oxidation rate of pyrogallol and the rate of SOD-inhibited pyrogallol auto-oxidation. The WL of 420 nm was used and the E-SOD activity was indicated as U/g Hb[6,13].

Erythrocyte CAT (E-CAT) activity H_2O_2 (0.20 mol/L) was added to phos phate buffer (10 mmol/L, pH 7.0) containing HS of RBC without WBC and platelets.The Hb level was determined in the HS. After a reaction time of 60 s, a s olution of potassium dichromate (0.169 mol/L) and glacial acetic acid (1 : 3) was added to the reacting mixture to stop the reaction, and the reacting mixture was heated for 10 min at 100℃. Colorimetry was done at 570 nm. The E-CAT activity was indicated as K/g Hb[6,14].

Erythrocyte GSH-Px (E-GSH-Px) activity A mixture of absolute ethyl alc ohol and trichloromethane (5 : 3) was used to precipitate Hb from the HS of RBC without WBC and platelets. Hb level was determined in the HS. GSH-Px in the ext ract catalyzed the reaction of glutathione and 5, 5'Dithiobis-*p*-nitr obenzoic acid (DTNB) and produced yellow colored compounds which were detected at 422 nm, using glutathione (1.0 mmol/L) as the standard. The E-GSH-Px activity was expressed as U/g Hb[6,15].

Major analytical reagents such as Vitamin C, Vitamin E, β-Carotene , Superoxi de dismutase, Catalase, β-Naphthylamine, 1,2,3-Trihydroxybenzene (pyrogallol), 1, 1,3,3-Tetraethoxypropane, 2-Thiobarbituric acid were all purchased from SIGMA CHEMICAL COMPANY, USA; and the other analytical-grade reagents were all procured form China. The main analytical instruments were 721-spectrophotometer and UV-754-spectrophotometer.

Statistic analysis

All data were analyzed with SPSS/8.0 and Statistica/6.0 statistic software u sing Compaq Pentium III/600 computer. Statistical testing methods inc luded unpaired and paired *t* test and chi square test (χ^2 test), linear reg ression and correlation analysis, stepwise regression and correlation analysis, and confidence interval (CI) of 95%. The level of significance of hypothesis testing was $P < 0.05$ a nd the power of test (power) > 0.75.

Results

Comparison between the above mentioned determinations in the patien t and the control group The average values determined for P-NO, P-LPO and E-LPO in the patient group were significantly increased ($P < 0.01$) with respect to the control, whereas the average values of P-VC, P-VE, P-β-CAR, E-SOD , E-CAT and E-GSH- Px in the patient group were significantly decreased ($P < 0.01$) (Table 1).

Comparison between the above mentioned determinations in the preop erative an d the postoperative patients The average values of P-NO, P-LPO and E-LPO in the postoperative patients were significantly decreased ($P < 0.01$), whereas the average determination values of P-VC, E-SOD, E- CAT and E-GSH-Px in the postoperative patients were significantly increased ($P < 0.01$) , but there was no significant difference between the average values of P-VE, P-β-CAR in the pre- and postoperative patients (Table 2).

Linear regression and correlation analysis between the course of disease and the above mentioned values determined in the patients In pace with gradua l prolongi ng of the course of disease in the patients, the values of P-NO, P-LPO, E-LPO in the patients were gradually increased ($P < 0.01$), the values of P-VC, P-VE, P-β-CAR, E-SOD, E-CAT, E-G SH-Px were gradually decreased ($P < 0.01$) (Table 3).

Table 1 Comparison of various determinations between patient group and control group (CI 95%, $\bar{x} \pm s$)

Group	*n*	P-NO nmol/L	P-VC μmol/L	P-VE μmol/L	P-β-CAR μmol/L	E-SOD U/g Hb	E-CAT K/g Hb	E-GSH-Px U/g Hb	P-LPO μmol/L	E-LPO nmol/g Hb
Patient	77	514 ± 142	44.3 ± 10.9	19.1 ± 4.6	1.35 ± 0.38	1813 ± 249	233 ± 57	22.7 ± 4.8	13.6 ± 1.9	38.2 ± 7.2
		482-546	41.8-46.8	18.0-20.1	1.26-1.44	1757-1869	220-246	21.6-23.8	13.2-14.0	36.6-39.8
Control	80	365 ± 157	55.2 ± 12.8	25.4 ± 5.3	1.72 ± 0.45	2057 ± 212	309 ± 61	27.2 ± 5.5	11.3 ± 1.7	29.4 ± 6.7
		330-400	52.4-58.0	24.2-26.6	1.62-1.82	2010-2104	295-323	26.0-28.4	10.9-11.7	27.9-30.9
t		6.2290	5.7344	7.9415	5.5559	6.6197	8.0588	5.4535	8.0001	7.9316
P		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 2 Comparison of various determinations between preoperative and postoperative patients (CI 95% $\bar{x} \pm s$)

Group	*n*	P-NO nmol/L	P-VC μmol/L	P-VE μmol/L	P-β-CAR μmol/L	E-SOD U/g Hb	E-CAT K/g Hb	E-GSH-Px U/g Hb	P-LPO μmol/L	E-LPO nmol/g Hb
Postoperative	77	514 ± 142	44.3 ± 10.9	19.1 ± 4.6	1.35 ± 0.38	1813 ± 249	233 ± 57	22.7 ± 4.8	13.6 ± 1.9	38.2 ± 7.2
		482-546	41.8-46.8	18.0-20.1	1.26-1.44	1757-1869	220-246	21.6-23.8	13.2-14.0	36.6-39.8
Postoperative	77	436 ± 139	48.5 ± 11.3	19.3 ± 5.1	1.34 ± 0.36	1915 ± 242	274 ± 59	25.1 ± 5.3	12.4 ± 1.8	34.9 ± 6.9
		404-468	45.9-51.1	18.1-20.4	1.26-1.42	1860-1970	261-287	23.9-26.3	12.0-12.8	33.3-36.5
*t**		8.9773	7.5376	0.8739	0.5732	7.9814	10.8058	9.5453	9.2384	10.3493
P		<0.01	<0.01	>0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*Paired *t* test

Table 3 Linear regression and correlation analysis between the course of disease and the values determined in the patients

Correlative item	*n*	Regression equation	*r*	t_r	*P*
Course with P-NO	77	Y = 368.6527+18.8688X	0.7162	8.8874	<0.01
Course with P-LPO	77	Y = 11.8836+0.2212X	0.6958	8.3888	<0.01
Course with E-LPO	77	Y = 31.5301+0.8807X	0.6369	7.1553	<0.01
Course with P-VC	77	Y = 53.7124-1.1505X	0.6495	7.3982	<0.01
Course with P-VE	77	Y = 23.3849-0.4532X	0.5572	5.8114	<0.01
Course with P-β-CAR	77	Y = 1.7887-0.0486X	0.7428	9.6093	<0.01
Course with E-SOD	77	Y = 2075.87-29.3446X	0.6239	6.9146	<0.01
Course with E-CAT	77	Y = 282.0238-6.7284X	0.7227	9.0545	<0.01
Course with E-GSH-Px	77	Y = 28.6710-0.7006X	0.7233	9.0702	<0.01

Stepwise regression and correlation analysis for the course of disease and the a bove-mentioned values determined in the patients Supposing the course of disease in the patients to be y, the determination valu es of P-NO, P-VC, P-VE, P-β-CAR, E-SOD, E-CAT, E-GSH-Px, P- LPO and E-LPO in the patients to be x_1, x_2, x_3, x_4, x_5, x_6, x_7, x_8 and x_9 respectively, after stepwise regression and corre lation, the stepwise regression equation was $y = -0.2706 + 0.0203x_1 + 0.6844x_3 - 11.3731x_4$, $r = 0.7902$, $F = 40.4440$, $P < 0.01$. The e quation suggested that the correlation of the course of disease was the closest with the values determined for P-NO, P-VE and P-β-CAR.

DISCUSSION

The metabolic status of nitric oxide and functional status be tween oxidation and antioxidation systems in human body are in close relationship with health[6-10,12-73]. If the metabolism of nitric oxide is abnorm al and the dynamic balance between oxidation and antioxidation is disturbed, free radicals (FRs) concentration will unusua lly increase and a series of FRs chain reactions will pathologically aggravate i n human body. This status can speed senility of human cell s, and induce many diseases[6-10,12-73]. Vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) are the most imp ortant antioxidants in human body, and they play an important role in scavenging superoxide anions ($O^{\cdot}_2$), hydxoy l radic al (• OH), hydroperoxyl radical (HO • $_2$), lipid FRs, lip ox yl FRs, alkyl FRs, alkoxyl FRs, singlet oxygen ($^1O_2$), hydrogen perox ide (H_2O_2) and others, thereby protecting biological membranes a gainst oxidation, peroxidation and lipoperoxidation[6,9,10,44-54, 56-69] injury. And they can promote synthesis and stabilization of immunoglobulin in human body and obstruct formation of carcinogens such as nitrosamine [6,9,1 0,44-54,56-69]. Superoxide dismutase (SOD), catalase (CAT) and glu tathione peroxidase (GSH-Px) are the most important specific antioxidases in hu man body, SOD is able to clean $O^{\cdot}_2$, obstruct and preven t the pathological aggravation of a series of FRs chain reactions induced by O • $_2$, CAT enables toxic active mass H_2O_2 to degrad e into non-toxic O_2 and H_2O, GSH-Px may decompose toxic active mass LPO[6,13-15,29-39,41,42,46-54,56-69]. LPO and its metabolic products such as mal ondialdehyde (MDA), conjugated diene (CD) and others are important po i sonous residual products that enable biological membranes to be injured by lipop eroxidation. Marked increase in LPO level in h uman body can strongly attack DNA, proteins, enzymes, biological membranes and so on, which leads to the lipoperoxidation injury of the biological membranes, etc [5-8,39-43,48-53,62-71]. Nitric oxide (NO) is a neurotransmitter and en dothelium-derived factor that reduces tone of vascular smooth muscle, and disor der of NO metabolism can induce many diseases[6,7,16-28,46-49,55,62-66].

In this study the results that the average values of P-NO, P-LPO and E-LPO in the patient group were significantly higher than those in the control group ($P < 0.01$), and that the average values of P-VC, P-VE, P-β-C AR, E-SOD, E-CAT and E-GSH-Px in the patient group were significantly lower than those of the cont rol group ($P < 0.01$) showed that there was a severe disorder o f the NO metabolism and imbalance between oxidation and antioxidation, and there was the pathological aggravation of the oxidation, peroxidation, lipoperoxidat ion reactions in the bodies of the patients with chronic cholecystitis and galls tones. The causes probably were as follows. The cytokines, particularly interleukin -I (IL-1), which were released out by inflammatory cells such as phagocytes namely lymphocytes, neutrophilic granulocytes, macro-phagocytes in the cholec ystic inflammatory reaction, can activate inducible nitric oxide synthase (iNOS). The iNOS enables NO to be produced excessively in the body of p atien ts, thereby resulting in a significant increase in the P-NO value in the patients[6,7,16-28,46-49, 55,62-66]. Excessive NO was diffused into near by tissues and cells, thus furthe r leading to injury of the tissues and cells[6,7,16-28,46-49,55,62-66]. The excess NO can combine with iron ions in heme group, with activated guany late cyclase and lipoperoxidation reaction. The excess NO also inactivated antioxidases such as SOD, CAT, GSH-Px by means of the reaction of NO and hydrosul-fide group (-SH) in the enzymes, which furthe r resulted in marked decrease in SOD, CAT, GSH -Px activi ties and further injured cells and biologic membranes. Excessive NO in the body was able to be speedily oxidated into nitrogen dioxide (NO_2). Both NO and NO_2 thems elves are extremely active FRs, NO_2 was still able to react with the organ ic molecules in cystic bile, and activate the neutrocytes and phagocytes in the cho lecystic focus, thereby releasing out a vast amount of O • $_2$, • OH, HO • $_2$, and H_2O_2 etc. Meanwhile, the phagocytes such as polymorphonuclear leukocyte were speedily activated, and a large number of O • $_2$, • OH, HO • $_2$ etc were released out, and continuously got into the blood stream i n the patients, thereby inducing the pathological aggravation of a series of FRs c hain reactions[6,7,16-28,46-49,55,62-66].

It must be stressed that excessive NO was capable of reacting speedily with O • $_2$, thereby forming another kind of free radical, i.e. sup erox ide nitroso free radical ($ONOO^-$) which possessed still more strong oxidative

properties. $ONOO^-$ can further attack and injure the various cells in the body, and deact ivate the antioxidases such as SOD, CAT and GSH-Px. Excess NO and NO_2 in hu man body injured DNA by way of th e deamination of the base and the chain scission[6,7,46-49,55,62-66]. So, on one hand the P-NO level in the body of patients was significantly increased, and on the other hand the body had no choice but to put to goo d us e a great quantity of antioxidants and antioxidases in the body so as to catch and clear these excess $O\bullet_2$, $\bullet OH$, $HO_2\bullet$ and others [6-10,12-73], which resulted in significant decrease of the levels of P-VC, P-VE, P-β-CAR and the act ivities of E-SOD, E-CAT, E-GSH-Px in the patients. Besides the gallbladder calculi such as bilirubin, cholesterol and other organic substances themselves also produce a large number of FRs[2-5,29,34,35,70-78].

NO_2 is a very active catalyst, and NO_2 can aggravate lipoperoxidation of the polyunsaturated fatty acids (PUFAs) through hydrogen-extractive process. The excess NO, NO_2, $O\bullet_2$, $\bullet OH$, $HO\bullet_2$ also can attack upon directly PUFAs, aggra vate significantly the lipoperoxidation, thereby resulting i n a large number of PUFAs which get lipoperoxidated, and subsequently form LPO. With the addition of the significant reduction in th e synthesis or regeneration of GSH-Px decomposing LPO, and the marked lose of the GSH Px activity, it goes without saying that finally th is status resulted in significant increase of P-LPO and E-LPO levels[6,7,46-49, 5 1,52,55,62-66].

In general most anti-oxidative vitamins such as VC, VE, β-CAR, etc, must b e acquired from dietary sources because they cannot be synthesized in the body. It is generally recognized that the chronic chole cystitis patients have poor appetite because their diets are controlled, and digestion of VC, specially digestion of fat-soluble vitamin s such as VE and β-CAR, markedly reduced. And the anti-oxidative vitamin-poor diets cannot provide sufficient free radical scav engers to keep the balance between oxidation and antioxidation. For this reason, the values of P-VC, P-VE, P-β-CAR in the bodies of the patients were further significantly decreased[6,9,10,47-54,56 -68].

In this study the average values of P-NO, P-LPO and E-LPO in the pos toperative patients were significantly decreased, the average values of P-VC, E-SOD, E-CAT, E-GSH-Px were significantly increased, but there was no significant difference in the average values of P-VE and P-β-CAR between the preoperative and postoperative patients. The findings showed that the series of FRs chain reactions in the body of patients were marked lysis, and the dynamic balance between oxidation and antioxidation, obtained resumption to a very marked degree because of the elimination of inflammatory focus after operati on. However, before the compensation of common bile was established the absorpti on of fats and lipids was still limited, thus the absorption of fat-solubl e VE and β-CAR obviously reduced[6,9,10,47-54,56-68]. Therefore, the normal levels of P-VE, P-β-CAR in the patients were diffi cult to be resumed shortly after operation.

In this study there was the linear correlation between the course of disease and the above determined values, specially the stepwise correlation with the course with P-NO, P-VE, P-β-CAR values was the closest. This st atus suggested t hat in chronic cholecystitis, a large amount of NO p roduced by iNOS induced and activated by the long-time infection and stimulatio n of the calculi in gallbladder provoked the pathological aggravation of a serie s of FRs chain reactions[2-5,16-29,34,35,46-54,56-78]. As a result, the patients with chronic cholecystitis over a long time were in the state of serious imbalance between oxidation and antioxidation as well as injuries induced by oxidation, peroxida tion and lipoperoxidation[2-5,29,34,35,70-78]. The findingss also sho wed that the metabolic status of nitric oxide and the changes in vitamin E and β-carotene levels in the body played an important part in chronic cholecystitis. Therefore, the above values, particularly the dynamic determination of P-NO, P-VE and P-β-CAR values, to a g reat degree, contribute to wards monitoring the condition and course in patients with chronic cholecystitis.

We think that in treating preoperative and postoperative patients with chronic cholecystitis with suitable dosage of antioxidants such as vitamin C, vitamin E, β-carotene, ginkgo leaf agents, tea-polyphenol daily to the patients may alleviate the injuries induced by oxida tion, peroxidation and lipoperoxidation.

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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 comparison between intravenous and peritoneal route on liver targeted uptake and expression of plasmid delivered by Glyco-poly-*L*-lysine

Chang Qing Yang, Ji Yao Wang, Guo Ting Fang, Jian Jun Liu and Jin Sheng Guo

Subject headings intravenous injection; intraperitoneal injection; glyco-poly-L-lysine; lwcr targeted uptake

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Abstract

AIM To compare the effects of intravenous route and peritoneal route on liver targeted uptake and expression of plasmid delivered by galactose-t erminal glyco-poly-L-lysine (G-PLL).
METHODS The plasmid pTM/MMP-1 which could be expressed in eukaryotic cells was bound to G-PLL, and was then transferred into Wistar rats by intra venous and intraperitoneal injection. The expression and distribution of the plasmid were observed at different time periods by *in situ* hybridization and im munohistochemistry.
RESULTS The plasmid could be expressed significantly within 24 h a fter being transferred *in vivo* by both intravenous and intraperitoneal ro utes. One week later the expression began to decrease, and could still be observ ed three weeks later. Although both the intravenous and intraperitoneal route co uld target-specifically deliver the plasmid to the liver, the effect of the fo rmer was better as compared to that of the latter.
CONCLUSION Intravenous route is better for liver targeted uptake and expression of G-PLL-bound plasmids than the peritoneal route.

INTRODUCTION

The efficient transference and the high expression of exogenous genes in specifi c cells or tissues are critical steps for both *in vitro* and *in vivo* gene therapy[1-12]. Gene transference mediated by receptors is carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, and then the fore ign gene can be delivered into the cells by phagocytosis[13,14]. There also exist some specific receptors on the surface of hepatocytes such as asialog lycoprotein receptors (ASGP-R)[15-19], which facilitate the researche rs to deliver exogenous genes into hepatocytes specifically using the ligand-re ceptor interaction. Galactose-terminal glyco-poly-L-lysine (G-PLL) cont ains the saccharide group of galactosan that can be specifically ligated to the asia loglycoprotein receptor (ASGP-R) on the surface of hepatocytes. At the same time, the cationic poly-L-lysine can bind to nucleotides with high affinity, so it can serve as a good carrier to deliver exogenous DNA to liver specifically and steadily[20-24].

Both peripheral veins and abdominal cavity can be used as the delivery route to target drug or nucleotide to liver[25-28], but the comparison of their effects on the targeted liver uptake has seldom been reported.

Using rats as the experimental animals, we compared *in vivo* the difference in distribution and expression of the plasmid given through intravenous or intrap eritoneal route.

MATERIALS AND METHODS

Preparation of the carriers

The original plasmid of rat interstitial collagenase was kindly provided by Prof. John J Jeffrey[29], and we reconstructed it with the plasmid of pT argetT (TM) (Promega Co., Madison, MI, USA), which could be expressed in eucaryo tic cells. We also inserted a segment of nucleotides (GAC TAC AAG GAC GAC GAT GA T AAG) before the terminator codon (TAA) of the rat interstitial collagenase. Th e 'Flag Domain' peptide (DYKDDDDK) encoded by the segment of nucleotide above, which was usually called 'Tag', could be fused in the rat interstitial colla genase[30] and could be specifically recognized by an M2 monoclonal anti body (Kodak, New Haven, CT, USA). This recombinant plasmid was named pTM/MMP-1. The plasmid of pTM/MMP-1 was extracted and purified using QIAGEN-Tip 500 kit (QIAGEN Inc., Valencia, CA, USA) according to the manufactures instructions. The plasmid was mixed with different amounts of galactose-terminal glyco-poly- L-lysine (kindly provided by Dr. Shou-Ming Wen of Air-Force General

Division of Gastroenterology, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Chang-Qing Yang, male, 35 years old, got his M.D. in 1990 and Ph.D. in 1998 from Xiangya Hospital of Hunan Medical University, now is working as a postdoctoral fellow in Zhongshan Hospital of Shanghai Medical University, majoring in liver targeted uptake and hepatic fibrosis.
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Correspondence to: Ji-Yao Wang, Division of Gastroenterology, Z hongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Tel. +86-21-6404-1990 ext 2420, Fax. +86-21-6483-3680
Email. xhk@shmu.edu.cn
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Hospi tal o f PLA, China. The mean molecular weight of this GPLL was 8500 and the ratio of glactose topoly-L-lysine was 15:28). The optimal proportion of the plasmid pTM/MMP-1 binding to galactose-terminal glyco-poly-L-lysine was determin ed by electrophoresis in 1% agarose gel.

Animal experiments

Eighteen male Wistar rats, with body weight of 130-150 g, were randomly divided into three groups of six rats each. Poly-L-lysine intravenous (PI) group: 50 μg plasmid pTM/MMP-1 bound to galactose-terminal glyco-poly-L-lysine wa s administered through cauda vein; poly-L-lysine intra-peritoneal (PP) g roup: each rat was given the same amount of pTM/MMP-1 bound to galactose-termin al glyco-poly-L-lysine intraperitoneally; normal group: control animals. Tw enty-four hours, 48 h, 72 h, 1 wk, 2 wk, 3 wk after the administrat ion of the plasmid, one rat from each group was randomly selected and anaestheti zed with 2% pentobarbital sodium intraperitoneally. Then 1 mL blood was obtained by cardiac puncture for the assay of alanine transaminase (ALT), aspartic trans aminase (AST), and creatinine (Cr) to observe the functions of important organs. After perfusion of the whole body with 20 mL phosphate-buffered saline and 40 mL 4% paraformaldehyde through ventricular injection, the tissues of liver, spl ee n, lungs, and kidneys were collected and fixed in 4% paraformaldehyde, encapsula t ed in paraffin and cut into sections 4-μm thick. This procedure above was approved by the Laboratory Animal Committee of Shanghai Medical University .

Immunohistochemistry

Immunohistochemistry was performed according to the literature[31,32]. The first antibody used was M2 monoclonal antibody which was specific for flag -domain tag (Kodak, New Haven, CT, USA) and the second antibody used was Horse anti-mouse IgG, labeled with biotin (Vecter, Burlingame, CA, USA). After the treatment with avidin and biotin (ABC kit, Vecter, Burlingame, CA, USA), color dev elopment was followed using dimethylaminoazobenzene (DAB) and counterstained with hematoxylin. Five fields were observed under high power from every immunostained section and the positive signals were counted.

In situ hybridization

The procedure of *in situ* hybridization was also described previously[2 5,26]. To state briefly, the oligonucleotide probe (5'-TGG TGT GAC TAC AAG GAC GAC GAT GAT AAG-3') was synthesized in Cell Biology Institute of Chinese Academy of Sciences (Shanghai), which could hybridize with the (mRNA) of the fl ag-domain tag in the plasmid pTM/MMP-1, and the 5' end of the probe was labeled with biotin. After the hybridization of the target mRNA with the probe, the rest of the procedure was similar to that followed in immunohistochemistry excluding the step of hematoxylin counterstaining.

Other biochemical assays

ALT, AST, and Cr were assayed using the 7170A Automatic Analyzer (HITACHI, Ja pan) to observe the changes in the important organs' function.

Data analysis

All of the data were analyzed by the software SPSS 7. 0 for windows (one-way ANOVA).

RESULTS

Ratio of G-PLL to plasmid

According to the electrophoresis results, we found that 0.3 μg of g alacto se-terminal glyco-poly-L-lysine could throughly bind to 1 μg of the plasmid , which meant that about 72 molecules of G-PLL could carry one molecule of the plasmid pTM/MMP-1 (Figure 1).

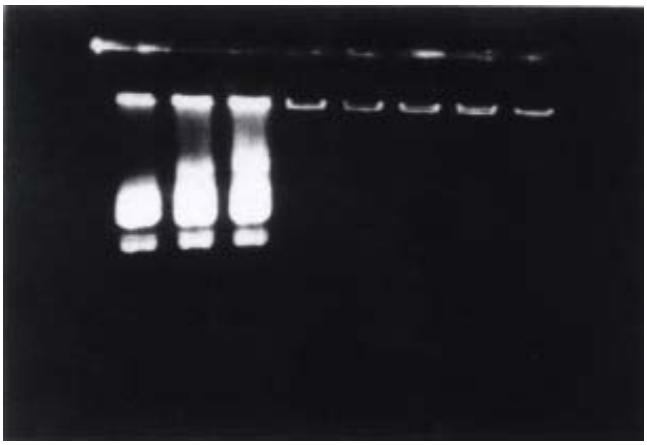

Figure 1 Determination of optimal proportion of G-PLL bound to plasmid by 1% agar ose electrophoresis. Lane 1-8 are respectively 0.05 μg, 0.1 μg, 0.2 μg, 0.3 μg, 0.4 μg, 0.5 μg, 1.0 μg, and 1.5 μg G-PLL mixed with 1 μg pTM/MMP- 1 p lasmid. pTM/MMP-1 1 μg plasmid could only be bound completely by more than 0. 4 μg G-PLL.

The changes in the functioning of important organs

Compared with the normal group, there was no obvious elevation of the ALT, AST, and Cr levels in the PI and PP groups.

The distribution and expression of the plasmid in liver, spleen, lung and kidney

The results of the immunohistochemistry and *in situ* hybridization showed tha t the plasmid binding to G-PLL could be expressed *in vivo*, regardless of th e introducing route and the results of immunohistochemistry were more sensitive a nd stable. In addition, the protein product of the plasmid could be secreted ext racellularly (Figures 2 and 3), similar to the expression of interstiti al collag enase in the physiological state[30,33]. We found that both intravenous route and intraperitoneal route could make liver as the major distribution organ of the plasmid bound to G-PLL.

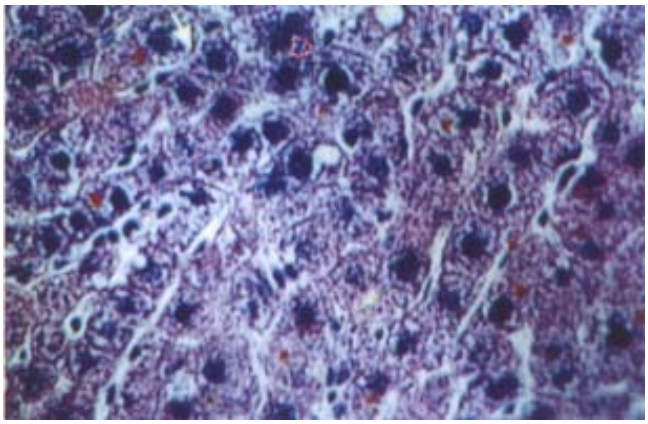

Figure 2 Immunostaining of flag-domain tag in the liver all hours after admin istration of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) via cauda vein. × 200

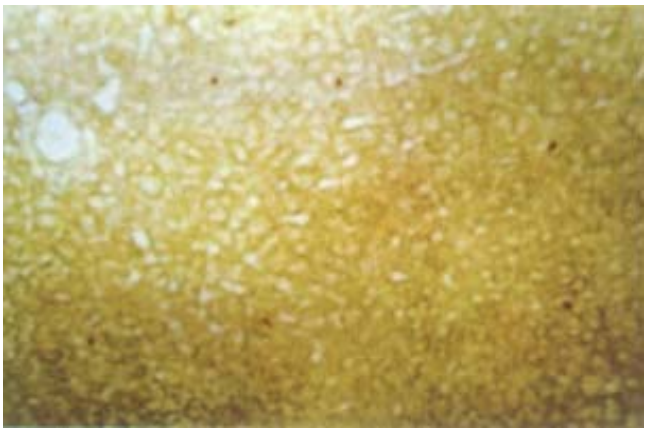

Figure 3 *In situ* hybridization with biotin labeled oligonucleotide probe in th e liver 3 wk after the administration of the plasmid bound to G-PLL (galacto se-terminal glyco-poly-L-lysine) via abdominal cavity. × 100

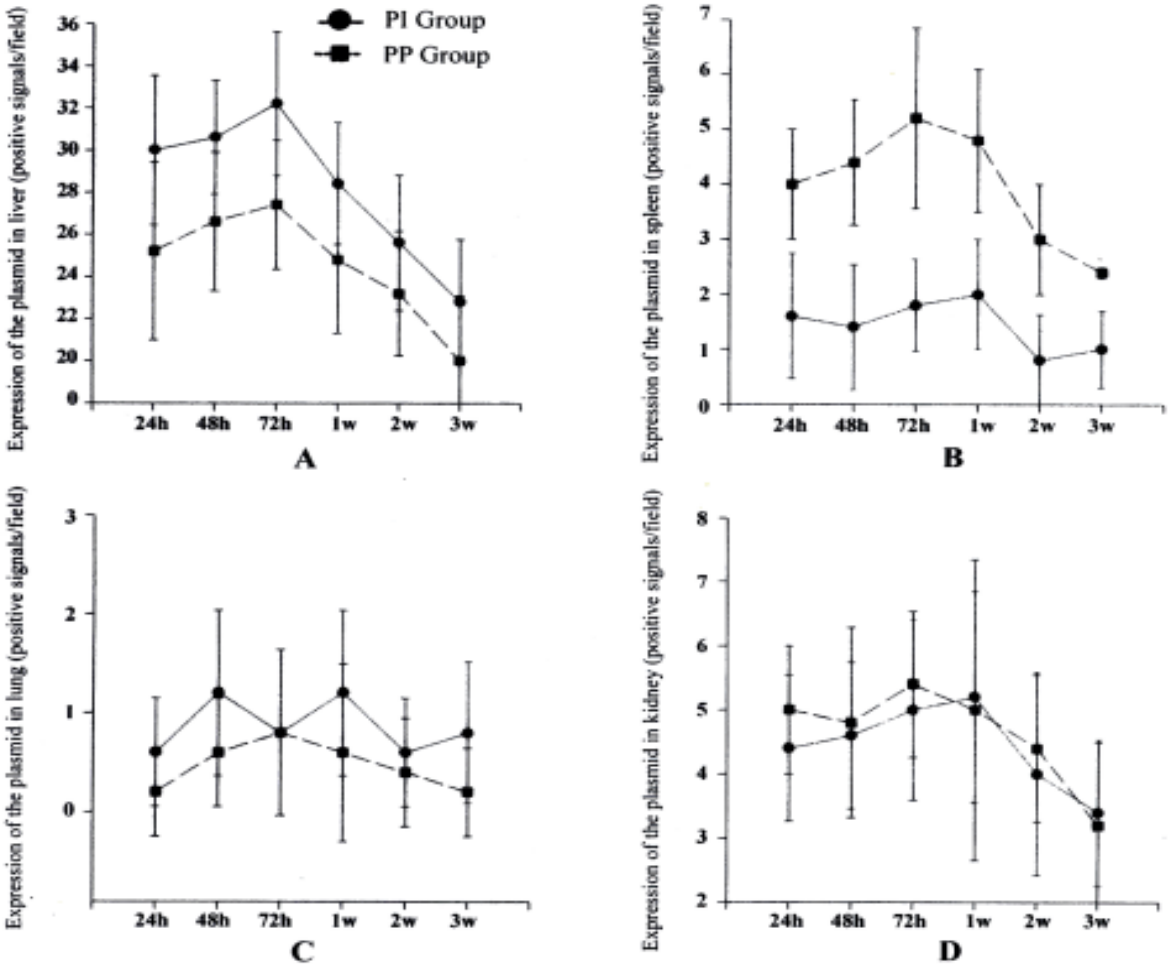

Figure 4 The distribution and expression of the plasmid bound to G-PLL (galactosetermina l glyco-poly-L-lysine) in different tissues and at different time period and administered intraperitoneally or intravenously. A: liver, B: spleen, C: lung, D: kidney. PI: plasmid bound to G-PLL introduced intravenously, PP: plasmid bound to G-PLL introduced intraperitoneally.

The obvious expression of the plasmid could be observed 24 h after the administration and began to decrease one week later, although it could still be observed weakly even two or three weeks later. Among the two groups, we

observed that the expression and distribution of the plasmid in the liver of the PI group was significantly higher than that of the PP group. Besides the liver, the plasmid in PI group could also be expressed in lung at a lower level, and almost could not be expressed in spleen and kidney. As for the PP group, most of the distribution and expression was located in the liver and a relatively higher level could be seen in the spleen and kidney, whereas low expression could be observed in the lungs (Figure 4).

DISCUSSION

Regarding the gene therapy of hepatic diseases, efficient delivery of the exogenous genes to the liver and its high expression there could increase its local accumulation and minimize the side-effects on other tissues and organs as well[34-36].

For the gene therapy of hepatic diseases in the animal experiments, exogenous ge nes were usually delivered to the liver through the portal vein, bile duct injec tion, or even by direct liver injection[37-41]. From the viewpoint of clinical application these methods have limitations concerning its invasive trauma and possible risk. If the gene transference to liver could be accomplished by a peripheral vein or abdominal cavity, the limitations could t hen be avoided or decreased and it would also be easily accepted by the patients . So we observed the efficacy of liver targeted gene delivery using intravenous and intraperitoneal routes.

Receptor mediated gene transfer was carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, such that the foreign gene could be delivered into the cells by phagocytosis[24,42]. It has been reported that the ratio of the targeted uptake by the liver delivered by G-PLL could reach up to 70%-90% *in vivo*[43,44]. In our experiments we found that in additio n to its main location in the liver, the plasmid binding to G-PLL could be also expressed in kidneys, spleen, and lungs. It might be due to the existence of AS GP-R in the other extrahepatic tissues[45,46].

Recently Zhang *et al*[42]found that intravenous injection was an us eful method for delivery to liver by a target carrier. We found that the peripheral vein route was better than abdominal cavity in the targeted delivery of the plasmid bound to G-PLL. We conjectured that although the majority of the DNA/ ca rrier complex would reach the liver through portal vein after the absorption by the abundant capillary bed of peritoneum, quite a lot of the complex would possibly be absorbed or degraded by other organs in the abdominal cavity, thus leadin g to the decreased distribution and expression of the plasmid in the liver. In the same way, it might be one of the reasons that its distribution in the spleen reached to a relatively high level. In this experiment, we also observed whether G-PLL binding to the plasmid would induce some toxicity to the body. We did not find any detrimental effect on the functioning of important organs such as liver, heart, and kidney, and this further indicated that G-PLL could be used safely *in vivo* as the delivery carriers for drug or nucleotides.

In conclusion, we found that the plasmid bound to GPLL could be delivered to the liver efficiently and safely, and the intravenous transference was better than peritoneal transference for the targeted uptake by the liver when G-PLL was used as a carrier. But whether G-PLL can be used to deliver drugs or nucleotides for the treatment of liver diseases in human beings by intravenous route deserves further investigations.

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Edited by Zhu QR
proofread by Mittra S

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

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Overexpression of p27^{KIP1} induced cell cycle arrest in G_1 phase and subsequent apoptosis in HCC-9204 cell line

Jiang Li[1], Xin Ke Yang[2], Xin Xin Yu[2], Meng Liang Ge[3], Wen Liang Wang[1], Jie Zhang[4] and Yun De Hou[2]

Subject headings p27^{KIP1}; apoptosis; cell cycle; inducible expression system; carcinoma , hepatocellular; liver neoplasms

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Abstract

AIM **We have previously reported that inducible over-expression of Bak may prolong cell cycle in G_1 phase and lead to apoptosis in HCC-9204 cells. This study is to investigate whether p27^{KIP1} plays an important role in this process.**

METHODS **In order to elucidate the exact function of p27^{KIP1} in this process, a zinc inducible p27^{KIP1} stable transfectant and transie nt p27^{KIP1} -GFP fusion transfectant were constructed. The effects of inducible p27^{KIP1} on cell growth, cell cycle arrest and apoptosis were examined in the mock, control pMD vector, and pMD-KIP1 transfected HCC-9204 cells.**

RESULTS **This p27^{KIP1}-GFP transfectant may transiently express the fusion gene. The cell growth was reduced by 35% at 48 h of p27^{KIP1} induction with zinc treatment as determined by trypan blue exclusion assay. T hese differences remained the same after 72 h of p27^{KIP1} expression. p27^{KIP1} caused cell cycle arrest after 24 h of induction, with 40% inc rease in G_1 population. Prolonged p27^{KIP1} expression in this cell line induced apoptotic cell death reflected by TUNEL assay. Fourty-eighth and 72 h of p27^{KIP1} expression showed a characteristic DNA ladder on agarose gelelec trophoresis.**

CONCLUSION **Bak may induce cell cycle arrest in G_1 phase through upregul ating expression of p27^{KIP1} and subsequently lead to apoptosis in HCC -9204 cells. The p27^{KIP1}-GFP fusion protein can be transiently expr essed in HCC-9204 cells. The inducible p27^{KIP1} expressing cell line provides a model to assess p27^{KIP1} function.**

[1]Department of Pathology, Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China
[2]State Key Laboratory for Molecular Virology and Genetic Engineering, Beijing 100052, China
[3]Department of Dermatology, Beijing Hospital, Beijing 100016, China
[4]Institute of Radiation Medicine, Beijing 100085, China
Jiang Li, male, born on 1972-5-28 in Chongqing, Sichuan Province, graduated from Fourth Military Medical University, Ph.D candidate, having 8 papers published.
Correspondence to: Jiang Li, Department of Pathology, Fourth Mil itary Medical University, Xi'an 710033, Shaanxi Province, China
Tel. +86-29-3374039, Fax. +86-29-3287001
Email. lijiang@fmmu.edu.cn
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INTRODUCTION

Cell cycle regulatory proteins play a critical role in both normal cell growth and tumorigenesis. The cyclin dependent kinases (CDKs) are the major regulators o f cell cycle progression, and thus are important candidates for therapeutic tumo r suppression[1]. The CDKs, which are responsible for the phosphorylati on of the retinoblastoma protein (pRb) and pRb related proteins, are in turn reg ulated by changes in cyclin levels, phoshphorylation and the presence of cyclin kinase inhibitors (CKIs)[2-3]. Two classes of CKIs are present within mammalian cells. One class, the INK4 (inhibitors of CDK4) family, is a group of ankyrin repeat protein whose members p16^{INK4A}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} are specific inhibitors of cyclin D1/CDK4 or CDK6 complexes[4-6]. The second class of CKIs, the p21 family, c onsists of p21$^{CIP/WAF1}$, p27^{KIP1} and p57^{KIP2} which are general inhibitors of the G_1/S CDKs[7-9]. Homology between the f amily members is limited to a conserved amino-terminal 60-residue domain respo nsible for kinase binding and inhibition[10].

p27^{KIP1} regulates cell cycle progression by interacting with,and the reby inhibiting,various cyclin-CDK complexes.Physiologically, p27$^{K IP1}$ is believed to act primarily to regulate progression of cells from late G_1 into S through its interaction with cyclin E-CDK2 complexes[11]. p2 7^{KIP1} has been implicated as a mediator of growth arrest due to TGF-β, cAMP, and other extracellular factors[12]. Moreover, elevated expression of p27^{KIP1} leads to G_1 arrest in many cell types and pro motes neuronal differentiation in mouse neuroblastoma cells, while inhibition of p27^{KIP1} expression through use of antisense technology prevents G_1 arrest and/or suppresses entry of fibroblasts into a state of quiescence in resp onse to mitogen depletion[13-14]. The phenotype of mice null for the p27^{KIP1} gene includes increased body size, female sterility and a high incidence

of spontaneous pituitary tumors[15].

We have previously shown that overexpression of Bak might prolong cell cycle in G_1 phase though this new property of Bak had never been reported before. We provided a hypothesis that Bak's translocated expression from the cytoplasm to the nuclei of HCC-9204 cells may trigger the expression of certain cell cycle regulators such as cyclins, CDKs, or CDK inhibitors through DNA-protein or prote in-protein interaction. Since $p27^{KIP1}$ is one of major cyclin-CDK reg ulators in G_1 phase, we currently executed an investigation of whether overexpression of Bak in HCC-9204 cells upregulates the expression of $p27^{KIP1}$ and whether $p27^{KIP1}$ itself may induce cell cycle arrest in G_1 pha se and even apoptosis in HCC-9204 cells.

MATERIALS AND METHODS

Cell line and cell culture

HCC-9204 cell line derived from a human hepatocellular carcinoma and establishe d in our laboratory was cultured in Eagle's medium containing phenol red, with 50 mL/L fetal bovine serum (FBS) (HyClone Laboratories, USA) in a humidified at mosphere of 950 mL/L air, 50 mL/L CO_2 at 37℃[16]. All media were s upplemented with 2 mmol/L-L-glutamine, 100 mg/L penicillin and 100 kU/L str epto mycin. Culture medium and supplements were obtained from GIBCO BRL. Of the HCC- 9204 cell line, p53 was mutated. Medium was changed every 3 days. Cells were removed from culture flasks for passage by washing once with Hank's balanced salt solution, followed by a 5 min incubation with 0.5 mmol/L EDTA and 0.5g/L trypsin at pH 7.4, RT.

RT-PCR analysis

Total RNA was extracted from HCC-9204 cells using an RNA extraction reagent, TR IZOL (Life Technologies, USA), according to standard acid-guanidium-phenol-ch loroform method[17]. About five μg of total RNA were revers e tra nscribed at 42¡æ for 60 min in a total 20 μL reaction volume using a first-Strand cDNA synthesis kit (Boehringer Mannheim, Germany). cDNA was inc ubated at 95℃ for 5 min to inactivate the reverse transcriptase, and was serve d as template DNA for 32 rounds of amplification using the GeneAmp PCR System 2400 (Perkin-Elmer Applied Biosystems, CA, USA). PCR was performed in a standard 50 μL reaction mixture consisting of 10 mmol/L Tris-HCl, 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride (pH 8.3), 0.2 mmol/L dNTPs, 5 0 pmol of each sense and antisense primer and 2.5 U of Taq DNA polymerase (M BI, Canada). Amplification was performed for 30 s at 94℃, 45 s at 58℃ and 45 s at 72℃ after heat-start for 5 min. Finally, an additional extension step wa s carried out for 7 min. As negative control, the DNA template was omitted in th e reaction. The amplification products were separated on 12 g/L agarose gels and visualized by ethidium bromide staining. PCR primers for $p27^{KIP1}$ were as follows: forward primer, 5'-ggggtaccatgtcaaacgtgcgagt-3'; reverse primer , 5'-gcgtcgacacgtttgacgtcttc-3': according to the $p27^{KIP1}$ gene structure in Genebank. PCR product of 597 bp was obtained. Kpn-I and Sal-I sites were designed in the 5'and 3'end of cDNA encoding $p27^{KIP1}$ and the terminal code for $p27^{KIP1}$ was mutated so as to construct a fu sion protein.

DNA sequencing

The PCR product was purified through Wizard Plus Minipreps DNA Purification System (Promega, USA). The pGEM-T Easy Vector Systems (Promega, USA) was applie d for the cloning of the PCR product. Then the recombinant plasmid DNA was trans formed into *E.coli* and was sequenced by dideoxy-mediated chain-termination method, using ABI PRISM-TM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA). This kit was developed specifically for the preparation of samples for sequence analysis on the ABI PRISM 377 DNA Sequencer. Cycle sequ encing was performed on the GeneAmp PCR systems 2400 (Perkin Elmer, USA) according to the instructions.

Plasmid construction

Plasmids were constructed using standard molecular biology techniques[18]. In order to construct a hybrid DNA encoding a chimerical $p27^{KIP1}$- GFP fusion protein, pGEM-T Easy KIP1 was digested with Kpn-I and Sal-Ito yield a 0.6 kb fragment. The resulting fragment then ligated to Kpn-I and Sal-I digested pGREEN LANTERN-1 (Clontech, USA) containing the humanized T hr^{65} GFP cDNA under the control of CMV enhancer/promoter and the SV40 poly adenylation signal. Correct reading framework of the fusion gene was confirmed b y DNA sequencing. Human $p27^{KIP1}$ (pBluescript SK-p27) was a gift f rom Dr. Polyak[19-20]. PCR primers for $p27^{KIP1}$ were as foll ows: forward primer, 5'-ggggtaccatgtcaaacgtgcgagt-3'; reverse primer, T7 primer. PCR product of 1.0 kb was obtained and subsequently ligated into pGEM-T E asy Vector System. To construct pMD-KIP1, pGEM-T Easy KIP1 was digested with EcoR-I and Xho-I to yield a 1.0 kb fragment. T he resulting fragment then ligated to EcoR-I and Xho-I digested pMD-neo. Plasmids for transfection were purified using a kit (Quiagen Inc., USA).

Transient and stable transfection

pKIP1-GFP and pMD-KIP1 were used for transient and stable transfections respec tively. Clonfectin (Clontech, USA) was used to transfect plasmids into HCC-9204 cells. According to the instructions, Clonfectin transfection reagent was an effective liposome transfection reagent for many mammalian cell types with high tr ansfection efficiencies and required only 1-4 hour incubation for optimal resu lts, especially effective transfections in serum-containing media. Green fluore scence was detected 4, 24 and 48 h after transfection. For stable transfection, the transfected HCC-9204 cells were grown for 2 weeks in a

medium containing 0. 5 g/L G418 (Life Technologies, Inc., USA), after which the G418 concentration wa s reduced to 0.2 g/L. Stable cell line of HCC-9204 tranfected with pMD-KIP1 vector was treated by continuous exposure to 100 μmol/L of $ZnSO_4$.

Cell viability

Cells were seeded at 10^5 per dish. HCC-9204 cells viability was determined by Nikon Eclipse TE200 inverted microscopic examination of cells stained by 1 g/L trypan blue (trypan blue exclusion), counting cells on a hemocytometer at the ti me point indicated[21].

Apoptosis analyzed by TUNEL assay

Cells were harvested for TUNEL staining. The proportion of cells showing DNA fra gmentation was measured by incorporation of fluorescein isothiocyanate (FITC)-1 2-dUTP into DNA by using terminal deoxynucleotidyltransferase (TdT)[22]. Briefly, a kit from Boehringer Mannheim (In Situ Cell Death Detection Kit, F ITC) was used. After a 30 min (RT) incubation with 30 g/L BSA, 200 mL/L normal bovine serum in PBS, pH 7.4, slides were covered with the TUNEL mix (calf thy mus TdT, FITC-12-dUTP and cobalt chloride in 1 × reaction buffer) for 1.2 h at 37 ℃. The morphologic features were visualized by fluorescence microscopy. Routine HE staining was also conducted. Negative control was performed by omitting TdT. We used paraffin-embedded sections of HCC as a positive control [23].

DNA laddering

Laddering of DNA in extracts from untransfected and transfected cells was carried out. DNA was extracted from 5×10^6 cells 24 h post-induction. DNA fragmenta tion was assayed as described previously[24]. Briefly, 24 h following ad dition of 100 μmol/L $ZnSO_4$, 5×10^6 cells were lysed in buffer c ontaining 5 mmol/L Tris (pH 8.0), 20 mmol/L EDTA and 5 g/L Triton X-100 on ice for 30 min. High molecular weight DNA was removed by centrifugation at 14 000 r/min for 15 min at 4℃, and the supernatant was sequentially extracted with phenol: chlorofo rm, and chloroform. The low molecular weight DNA was recovered by ethanol precip itation, resuspended in 30 μL of TE buffer, and treated with RNase A for 3 h at 37℃ prior to electrophoresis on 12 g/L agarose gels.

Western blot analysis of transgene expression

Monolayers were rinsed with PBS and lysed with SDS-PAGE loading buffer (50 mmol /L Tris HCl pH 6.8, 100 mmol/L dithiothreitol, 20 g/L SDS). Mock and pMD-neo tr ansfected cells served as a control. Samples were analyzed by SDS-PAGE and tran sferred into Hybond-C super membranes (Amersham, UK). The membranes were blocke d with 50 mL/L skim milk, 1 g/L Tween-20 and then probed with primary antibody overnight according to manufacturer's instructions, washed in PBS, 2 g/L Tween-20 and then incubated with the appropriate horseradish-peroxidase (HRP) conju gated secondary antibody. After washing, the membranes were developed by DAB det ection reagents according to manufacturer's guide (Dako Co. USA). $p27^{K\ IP1}$, rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) was detected f ollowed by HRP-conjugated anti-rabbit IgG (Fc) was used. The level of β-actin was used as a control for equal loading of protein.

Immunohistochemistry analysis

HCC-9204 cells were harvested at different times of induction with $ZnSO_4$. Cel l preparations were fixed with 700 mL/L ethanol for two hours, washed in PBS, an d incubated with $p27^{KIP1}$ antibody diluted 1 × 100 in PBS containing 10 mL/L bovine serum albumin (BSA, Sigma, USA). Immunostaining was performed using LSAB1 kit (Dako, Peroxidase, USA), according to the instructions of the manufac turer.

Fluorescence microscopy and laser confocal microscope

Nikon Eclipse TE200 inverted fluorescence microscope was used to observe and pho tograph. Images were collected on a laser confocal microscope, model MRC-1024 (BioRad, UK) with a Plan-Ne-ofluar 40 × 1.3 NA Apochromat objective (Zeiss, Ger many). The 488 nm line of a krypton/argon laser was used for fluorescence excita tion of FITC. Images were processed using Adobe Photoshop 5.0 software (Adobe Sy stems Inc., USA)

RESULTS

RT-PCR for $p27^{KIP1}$ in Bak transfected HCC 9204 cells

Bak transfected HCC-9204 cells were collected 0, 24 and 48 h post-induction as the previous study mentioned and were used to extract total RNA by TRIZOL reagent. The findings as shown in Figure 1 suggested that overexpression of Bak const antly induced expression of CDK inhibitor, $p27^{KIP1}$, at mRNA level in H CC-9204 cell line.

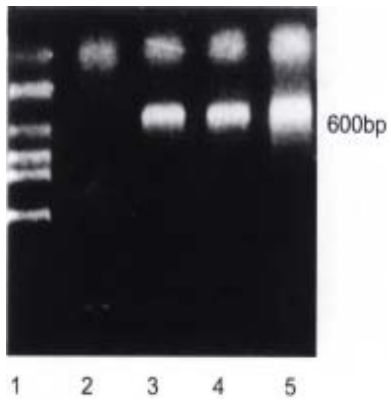

Figure 1 RT-PCR results of human $p27^{KIP1}$ RNA in Bak transfected HCC- 9204 cell line. Predicted PCR product size is about 600 bp. lane 1: Molecular we ight Marker (Takara, DL 2000); lane 2: Bak (0 h); lane 3: Bak (24 h); lane 4: Bak (48 h)

Sequencing of p27^{KIP1} and p27^{KIP1}-GFP fusion genes

The purified p27^{KIP1}- PCR product was cloned into pGEM T-Easy vector a nd sequenced. Comparison of the sequences with published data in Genebank indica ted only one base pair of disparity while the amino acid residue encoded was the same as what the Genebank indicated (Figure 2). pGEM-T Easy KIP1 was digested with Kpn-I and Sal-I to yield a 0.6 kb fragment. The resulting fragment then ligated to Kpn-I and Sal-I digested pGREE N LANTERN-under the control of CMV enhancer/promoter and the SV40 polyadenylation signal. Correct reading framework of the fusion gene was confirmed by restriction enzyme analysis or DN A sequencing.

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gtcaaacgtgcgagtgtctaacgggagccctagcctggagcggatggacgccaggcag  60
ggagcaccccaagccctcggcctgcaggaacctcttcggcccggtggaccacgaagag  120
aacccgggacttggagaagcactgcagagacatggaagaggcgagccagcgcaagtgg  180
tttcgattttcagaatcacaaacccctagagggcaagtacgagtggcaagaggtggag  240
gggcagcttgcccgagttctactacagacccccgcggcccccccaaaggtgcctgcaag  300
gccggcgcaggagagccaggatgtcagcgggagccgcccggcggcgcctttaattggg  360
tccggctaactctgaggacacgcatttggtggacccaaagactgatccgtcggacagc  420
gacggggttagcggagcaatgcgcaggaataaggaagcgacctgcaaccgacgattct  440
tactcaaaacaaaagagccaacagaacagaagaaaatgtttgagacggttccccaaat  540
cggttctgtggagcagacgcccaagaagcctggcctcagaagacgtcaaacgt       595
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Figure 2 The nucleotide sequences of p27^{KIP1}. Only one nucleotide is changed from c→t at site No 524, while the sequences of amino acids are the same

Transient expression of p27^{KIP1}-GFP fusion proteins in HCC-9204 cell line

reen fluorescence was visible 8 h after transient transfection with pKIP1-GFP fusion constructs. The p27^{KIP1}-GFP fusion proteins localized primarily to cell nuclei, with some GFP presented in the cytoplasm (Figure 3). However, 24 h post transfection, the green fluorescence disappeared. Some cells exhibited typical active cell death including condensed and rounded cells, cell detachment under Nikon Eclipse TE200 inverted microscope as shown in Figure 4.

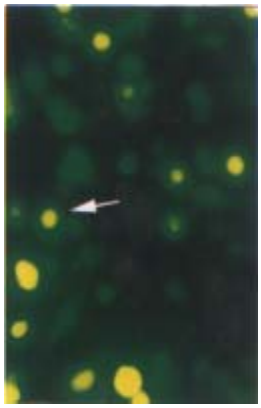

Figure 3 Cell slides under Nikon Eclipse TE200 fluorescence microscope using excitation light at either 488 nm to detect GFP (emission filter 522 nm). Distribu tion of GFP p27^{KIP1} fusion protein expressed in living HCC-9204 cel ls 8 after 100 μmol / L $ZnSO_4$ treatment, indicated by an arrow. × 200

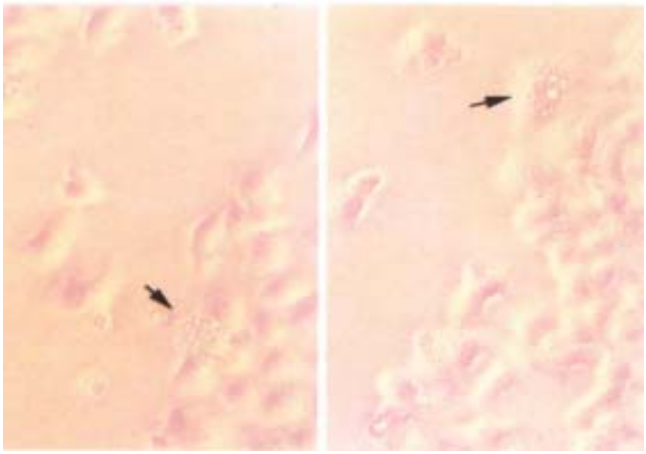

Figure 4 Transient transfected HCC-9204 cells exhibiting typical active cell death such as cell blebbing, condensed and rounded cells as seen under, Nikon Ecli pse TE200 phase contrast microscope indicated by an arrow. × 200

pMD vectors direct the inducible expression of p27^{KIP1}

Inducible pMD-KIP1 vectors were constructed to express human cyclin kinase inhi bitors, p27^{KIP1}, as shown in Materials and Methods. Transgene expressi on was under the control of the metallothionein-II (MT-II) promoter and the BGH polyadenylation signal. Previously it was found that M T-II promoter gave higher expression levels in HCC-9204 cells as c ompared with those obtained with the HCMV promoter. To study the effects of pMD -vector driven expression of p27^{KIP1}, HCC-9204 cells were left untre ated (mock), or were transfected with control pMD-vector or pMD-KIP1, at 4, 24 and 48 h, respectively after addition of 100 μmol/L $ZnSO_4$. West ern blot analysis showed a strong signal of Mr 27 000 protein appeared in H CC-9204 24 h post induction. However no band was detected in normal HCC-9204 cells as shown in Figure 5. Immunohistochemistry of p27^{KIP1} as shown in Figure 6 was consistent with Western blot analysis. Nuclear staining signals were observed in p27^{KIP1} stable transfectant 24 h post addition of $ZnSO_4$.

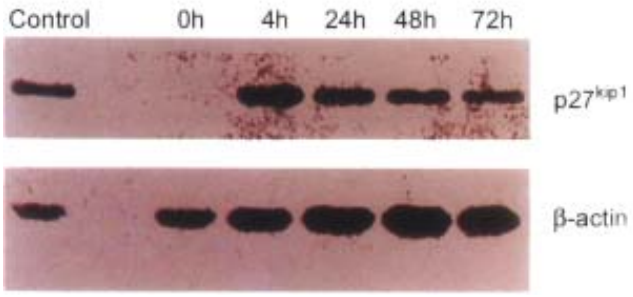

Figure 5 The results of Western blots of lysates from transfected HCC-9204 cell s, probed with antibodies against human p27^{KIP1}. Excellent p27^{KIP1} expression was observed in HCC-9204 cells even 4 h after induction with zinc.

Figure 6 $p27^{KIP1}$ detected by DAKO LABC kit in transfected HCC-9204 ce lls after 24 h of addition of $ZnSO_4$. $p27^{KIP1}$ dark-brown granules are present in the nuclei of $p27^{KIP1}$ transfected HCC-9204 cells, as in dicated by an arrow. × 200

Inhibition of tumor cell growth by exogenous $p27^{KIP1}$

$p27^{KIP1}$ gave a very pronounced inhibition of growth. HCC-9204 cells viability was determined by microscopic examination of cells stained by 1 g/L try pan blue (trypan blue exclusion) (Figure 7). Time course effect of $p27^{KIP1}$ on HCC-9204 cells growth was observed under Nikon Eclipse TE200 inverted mi croscopic (Figure 8). Within one day post-induction, a block in cell growth was observed in HCC-9204 cells with a few cells staining positive with Trypan blue, indicating that the exogenous $p27^{KIP1}$ was having a cytostatic effect on growth. More dramatic effects were observed 24 h after addition of zinc. Moc k and control pMD vector transfected cells grew until confluent (day 3) and then entered a growth arrest following which significant numbers of trypan blue-sta ining cells were observed, indicative of cell death, accounting for the decline in the cell number, which was especially apparent in confluent HCC-9204 cells. By 3 days post-induction, cells staining positive with trypan blue-staining cells were observed in the cultures transfected with $p27^{KIP1}$ even though the monolayers were not confluent, and this cell death accounted for the over- all reduction in cell number that was observed during the remainder of the exper iment. Cell death was greatest in HCC9204 cells transfected with pMD-KIP1.

From Figure 7, we could obviously figure out that the cell growth was reduced by 35% after 48 h of $p27^{KIP1}$ induction with zinc treatment as determined by trypan blue exclusion assay. These differences remained the same at 72 h , and even at 96 h post addition of zinc.

pMD vector expression of $p27^{KIP1}$ alters cell cycle distribution profiles

To see if the $p27^{KIP1}$ had an ability to induce G_1 arrest, cell cycle analysis was performed on transfected HCC-9204 cells. At 24 h post-induction, HC C-9204 cells expressing exogenous $p27^{KIP1}$ showed an accumulation of cells in G_1 compared to mock and control pMD transfected cells, consistent with $p27^{KIP1}$ inhibiting tumor cell growth. (Figure 9)

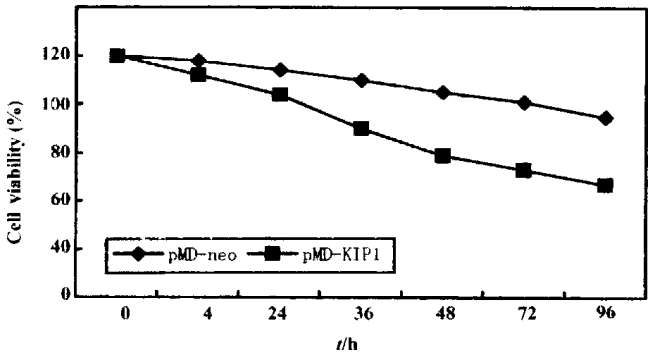

Figure 7 Time course of growth of cells transfected with pMD-KIP1 expressing $p27^{KIP1}$. HCC-9204 cells were induced by 100 μmol/L $ZnSO_4$. HCC-9204 cells viability was determined by microscopic examination of cells stained by 1 g/L trypan blue, counting cells on a hemocytometer at the time point indicated. Each treatment was triplicate.

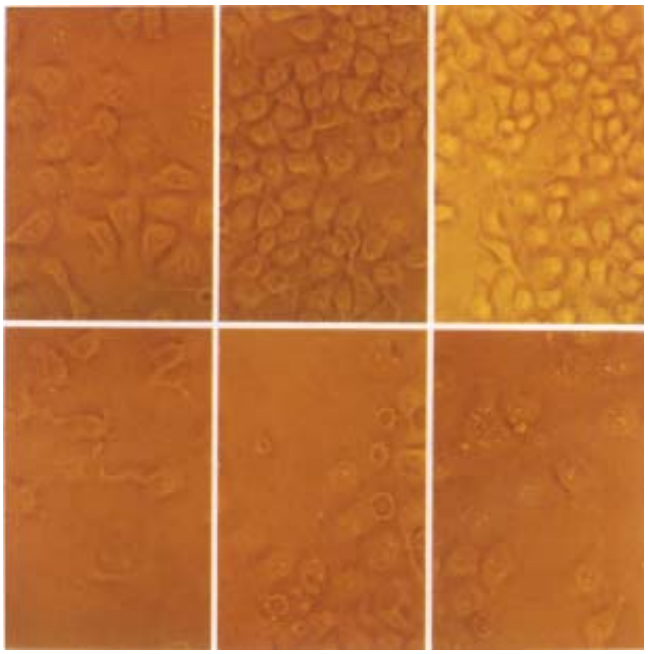

Figure 8 Time course effect of $p27^{KIP1}$-on HCC-9204 cells growth. Cells were seeded at 10\+5 per dish. HCC-9204 cells were induced by 100 μmol/L $ZnSO_4$ at the time indicated. Cells morphology was observed under Nikon Eclipse TE 200 phase-contrast microscopic at 0, 24 and 48 h post addition of zinc. "Uppe r-row" represents pMD-neo transfected cells; "Lower-row" represents pMD- Bak transfected cells. × 200

FACS analysis showed that overexpression of $p27^{KIP1}$ resulted in an i ncrease of G_1 population in HCC-9204 cells from 35.21% to 76.31%. Together, these data indicated that overexpression of $p27^{KIP1}$ alone is sufficie nt to suppress the proliferation of human tumor cells. We are currently investig ating the role of $p27^{KIP1}$ in mediating cellular responses to a variety of external signals and the mechanism of inhibition.

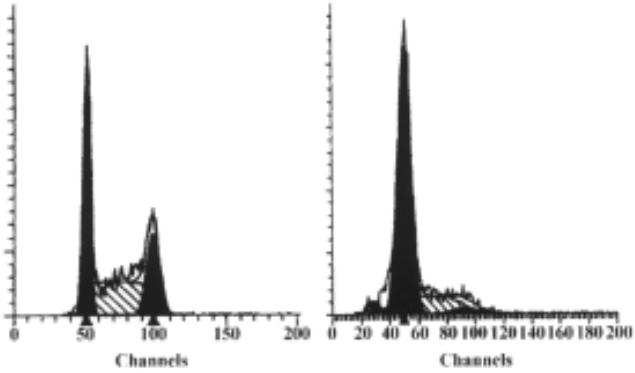

Figure 9 Result of flow cytometric analysis. Cells were stably transfectd with p27^{KIP1} and induced by 100 μmol/L $ZnSO_4$. The percentage of each phase for each group (before or 24 h after addition of zinc) is indicated in each panel.

Induction of apoptosis in cells transfected with vectors expressing p27^{KIP1}

Since by day 3, a significant proportion of HCC-9204 cells transfected with the vectors expressing p27^{KIP1} stained positive with trypan blue, indicat ive of cell death, we attempted to ascertain whether p27^{KIP1} was induc ing apoptosis. To confirm that over-expression of p27^{KIP1} induced cel l death was apoptotic, a variety of apoptosis associated detection assays were a pplied. In stable transfectants after addition of 100 μmol/L $ZnSO_4$, cell growth was significantly inhibited according to FACS analysis as shown i n Figure 9. TUNEL analysis (Figure 10) and DNA laddering (Figure 11) confirmed that part of cells underwent apoptosis 48 h or 72 h after addition of 100 μmol/L $ZnSO_4$.

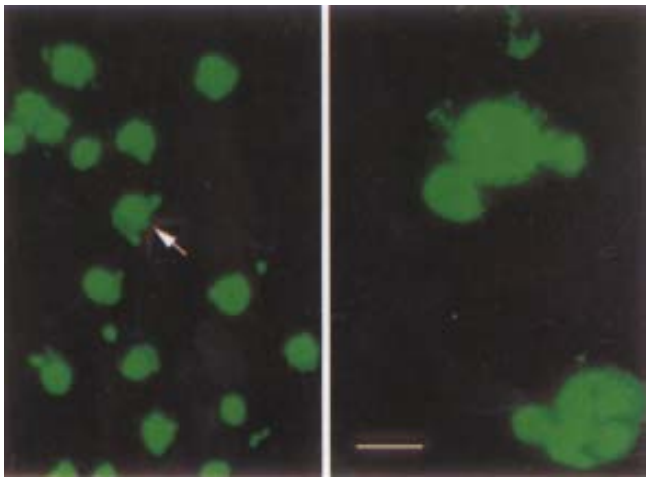

Figure 10 TUNEL assay demonstrating obvious apoptotic changes in HCC-9204/p 27^{KIP1} 24 h after addition of zinc under laser confocal microscope as in dicated by an arrow. (Bar represents 10 μm)

Taken together, p27^{KIP1} overexpression induced rapid morphological changes in HCC-9204 cells. p27^{KIP1} overexpression inhibited prolifera tion of HCC-9204 cells and subsequently induced apoptosis.

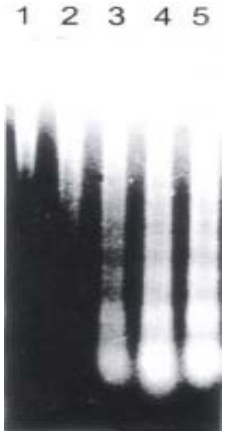

Figure 11 Analysis of DNA ladder formation at 0 (lane 1), 4 h (lane2), 24 h (lane 3), 48 h (lane 4), 72 h (lane 5) post addition of zinc in HCC-9204 cell line. Eac h lane was loaded with 10 μg DNA.

DISCUSSION

Since its introduction into cell biological research, the green fluorescent protein (GFP) of the jellyfish-Aequorea victoria has become a versatile tool for the analysis of protein function and dynamics at the cellular level[25] . GFP, which consists of 238 amino acids, has been used as a tag for localizatio n of a broad range of proteins in a wide variety of eukaryotic cells[26]. A mutant of GFP, S65T with an excitation peak of 489 nm and an emission peak o f 511 nm, emits four to six times more fluorescence energy compared with wild-type GFP. GFP may be fused generically to a target protein, and the fluorophore of GFP forms spontaneously in the presence of oxygen, thus rendering it an ideal probe for *in vivo* applications. The *in vivo* expression of GFP obviates t he fixation and permeablization of cells for immunofluorescence or the microinj ection of labeled proteins[27].

GFP fusion proteins constitute a major advance in the study of the dynamics of intracellular processes in living cells[28-32]. A major concern in the application of GFP as a fluorescent tag relates to whether the distribution of GFP fluorescence is identical to that of the protein to which it is fused. We have constructed GFP p27^{KIP1} fusion gene and transfected into HCC-920 4 cells. The results indicated that the fusion protein was still express in the nuclei of HCC-9204 cells.

Previous studies indicated that cell survival following transfection with pGRE EN LANTERN-1 was similar to transfection with pCMV • SPORT-βgal. No trypan blue-stained cells were seen to fluoresce green, and the fluorescence intensity of green fluorescent cells treated with trypan blue remained the same when judged by microscopic observation indicating that fluorescent cells were viable cells[33]. A comparative study has been performed to determine wheth er apoptotic cells still can express GFP. A

GFP positive clone was chosen and co unterstained with PI. The cells which had a property of apoptosis were stained with PI and no longer expressed GFP. We used PI counter-staining to show that the cells appearing as red fluorescence were not viable and only viable cells had a potential to express GFP. The fluorescence green vanished as soon as the cells become unviable (Figure 12). Therefore, transient expression of KIP1 - GFP fusion protein was disrupted when these cells underwent apoptosis.

The cell cycle progression is controlled by a family of cyclins and their spec ific catalytic partners, cyclin-dependent kinases (CDKs). D-type cyclins g over n the G_1/S transition in association with their proper physiological partners, CDK4 or CDK6, and CDK2, respectively[34-35]. For cells to complete the G_1/S transition, the activities of both D-type cyclins and cyclin E are essential. CDK inhibitors stop the cell cycle progression by negatively and stoichi ometrically regulating cyclin/CDK activities and are the targets of various extr acellular growth-modifiers. These inhibitors have similar N-terminal regio ns which are responsible for the blockade of CDK kinases. They can inhibit any G_1 cyclin/CDK activity. The CDK inhibitors also served as the common mediators of various biological processes including DNA-damages, cell differentiation, cell to cell contact inhibition and cell senescence[36]. Recent studies indicate that oncogene signals converge to cyclin/CDK and CDK inhibitors[37].We have reported that overexpression of Bak may prolong cell cycle in G_1 phase in HCC-9204 cells. We postulated that translocated expression of Bak in this process transferred signals to the cell cycle regulators in the nulcei, and thus induced cell cycle arrest in G_1 phase.

$p27^{KIP1}$, as a member of CDKIs, is constitutively expressed in many ce lls, and the level of this protein is elevated in G_0 phase and declines as cells in culture enter the cell cycle[38-40]. $p27^{KIP1}$ may play an important role in governing the growth factor restriction point by ensuring that CDK activity is suppressed during G_0 and early G_1 phase[41]. Int erestingly, in many types of tumors including breast, colon, prostate and ovaria n carcinomas, the expression of $p27^{KIP1}$ gene was down-regulated[42-43]. From the sequencing analysis, we could figure out that in some types of tumor, $p27^{KIP1}$ gene was null or mutated, while in other types it was still intact. HCC-9204 cells, as a target cell line, has intact $p27^{KIP1}$ which was confirmed by fluorescence *in situ* hybridization (FISH) as shown in Figure 13 and DNA sequencing. Researchers are attempting to discover the mech anism of controlling the expression of $p27^{KIP1}$ and cyclin-CDK activat ion in these malignant cells.

The correct timing of cyclin-CDK activation is regulated at several levels, in cluding the control of cyclin-encoding gene expression, cyclin stability and cellular localization, as well as CDK subunit phosphorylation and dephosphorylatio n[44-46]. Another level of regulation is provided by the CDKIs which consist of two families. The potential role of $p27_{KIP1}$ in control of the hepatocyte cell cycle is suggested by the implication of mitogens and anti-mit ogens known to control $p27^{KIP1}$ levels in the regulation of liver cell proliferation[47]. The discovery of $p27^{KIP1}$ has facilitated the elucidation of these mechanisms. In addition, in all organs including the liver, p27/, but not p21/mice show an increased cell density withou t any obvious alteration in liver cells differentiation or structure[48] .

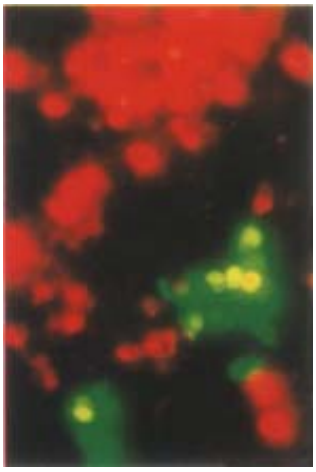

Figure 12 Comparative study of whether apoptotic cells can still express GFP. A GFP positive clone was chosen and counterstained with PI. Apoptotic cells were stained with PI and no longer expressed GFP. HCC-9204 cells were observed under Nikon Eclipse TE200 fluorescent phase-contrast microscopic. × 200

Figure 13 In situ fluorescence hybridization with FITC labelled $p27^{KIP1}$ probe. Two signals were observed in the chromosome under laser confocal micro scopy. (Bar represents 5 μm)

Most studies examining the function of $p27^{KIP1}$ had been performed in tissue culture systems using transformed cell lines or embryonic cells. Previous studies suggested that $p27^{KIP1}$ overexpression causes cell cycle arrest in G_1 phase and/or apoptotic death of mammalian cells and supported the potential utility of gene therapeutic approaches aimed at elevating $p27^{KIP1}$ expression for

treatment of human cancers and inhibition of tumorigenicity[49]. Recent evidences demonstrated that high levels of $p27^{KIP1}$ protein, induced by adenovirus vector, led to growth arrest as well as enhancement of apoptosis in several cell lines from different species and tissues of various origins[50]. However, as far as we know, no study has been conducted previously to examine the expression of $p27^{KIP1}$ in human hepatocelluar carcinoma tissue in relation to apoptosis. We obtained $p27^{KIP1}$ gene fragment in Bak transfected HCC-9204 cells and constructed a transient or stable transfectant. The purpose of the study was to examine the potential role of $p27^{KIP1}$ during Bak overexpression in HCC-9204 cells. The results suggest that overexpression of Bak may induce expression of $p27^{KIP1}$ at least in mRNA level. $p27^{KIP1}$ may exert its effect on G_1 phase elongatio n induced by Bak and $p27^{KIP1}$ itself may play an important role in the regulation of cell cycle during this process.To our knowledge, this is the firs t time that a significant correlation between $p27^{KIP1}$ expression and cell cycle arrest and apoptosis has been demonstrated in HCC-9204 cell line.

However, the mechanism of tumor growth suppression appears to include not only $p27^{KIP1}$ but also various other proteins, such as p53 etc. As weknow, p53 is mutated in HCC-9204 cell line and is often null in other cell lines[51-53]. Therefore, how these cell types undergo cell death is of co ncern since the p53-dependent apoptosis pathway has been abolished. p53 serves as a major G_1 checkpoint regulator of the induction of p21-CIP1 but not of $p27^{KIP1}$ and $p57^{KIP2}$, while it induces apoptosi s[54]. The p53-mediated CDK inhibitor $p21^{CIP1}$ does not le ad to significant apoptosis, though both $p21^{CIP1}$ and $p27^{KIP1}$ induce G_1 arrest through their potential abilities to inhibit CDK activity[55-56]. These findings suggest that the inhibition of CDK activity may not be sufficient to induce apoptotic cell death. A possible explanation of these differences between $p27^{KIP1}$ and p53-mediated $p21^{CIP1aa}$ functions is that $p27^{KIP1}$ may have other functions in addition to its notable function as a CDK inhibitor. It can also be speculated that $p27^{KIP1}$, which has the potential to induce growth arrest and apoptosis, may play a more important role in tumor growth suppression than p53-mediated $p21^{CIP1}$.

ACKNOWLEDGEMENTS Special thanks are extended to Mr. Dong Bo for technical assistance with f luorescence-activated cell sorter analysis; Ms. Zhang Fan for technical assista nce with laser confocal microscope; Dr. Polyak SS for pBluescript SK-p27; Dr. Zhang Jie for pMD-neo plasmid and technical support; Ms. An Jianhua for photo processing; Dr. Zheng Jian-Yong for other cooperation.

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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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Bile acid formation in primary human hepatocytes

Curt Einarsson[1], Ewa Ellis[1], Anna Abrahamsson[1], Bo-Göran Ericzon[2], Ingemar Björkhem[2] and Magnus Axelson[4]

Subject headings bile acid formation; cell culture; cholesterol metabolism; cyclosporin; human hepatocytes

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Abstract

AIM To evaluate a culture system for bile acid formation in primary human hepatocytes in comparison with HepG2 cells.

METHODS Hepatocytes were isolated from normal human liver tissue and were cultured in serum-free William's E medium. The medium was collected and re newed every 24 h. Bile acids and their precursors in media were finally analysed by gas chromatography-mass spectrometry.

RESULTS Cholic acid (CA) and chenodeoxycholic acid (CDCA) conjugated with glycine or taurine accounted for 70% and 25% of total steroids. A third of CDC A was also conjugated with sulphuric acid. Dexamethasone and thyroid hormone alo ne or in combination did not significantly effect bile acid formation. The addit ion of cyclosporin A (10 μmol/L) inhibited the synthesis of CA and CDCA by about 13% and 30%, respectively.

CONCLUSION Isolated human hepatocytes in primary culture behave as in the intact liver by converting cholesterol to conjugated CA and CDCA. This is in contrast to cultured HepG2 cells, which release large amounts of bile acid precursors and unconjugated bile acids into the medium.

[1]Division of Gastroenterology and Hepatology, Department of Medicine
[2]Department of Transplantation Surgery
[3]Department of Clinical Chemistry-Karolinska Institutet at Huddinge Unive rsity Hospital, Stockholm, Sweden
[4]Department of Clinical Chemistry-Karolinska Institutet at Karolinska Hospital, Stockholm. Sweden
Dr. Curt Einarsson graduated from the Karolinska Institutet in 1969, now Professor of Gastroenterology. Main research interests are bile acid formation and its regulation, biliary lipid secretion and gallstone formation. Dr Einarsson has published 290 papers.
Supported by grants from the Swedish Medical Research Council (03X-4793 and 03X-7890)
Correspondence to: Dr. Curt Einarsson, Division of Gastroenterology and Hepatology, Department of Medicine, Karolinska Institutet at Huddinge University Hospital, SE-141 86 Stock holm, Sweden
Tel. +46-8-58580000, Fax. +46-8-58582335
Email. Curt.Einarsson@medhs.ki.se
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INTRODUCTION

The degradation of cholesterol to bile acids is a major way for elimination of cholesterol from the body. Disturbances of bile acid formation may lead to clinic al disorders in humans like atherosclerosis, cholesterol gallstone formation and liver diseases. Primary bile acids-cholic acid (CA) and chenodeoxycholic acid (CDCA) are formed from cholesterol in human liver and excreted via the bile as conjugates to the intestine. In the intestine, the bile acids are partly deco njugated and 7α-dehydroxylated by microbial enzymes to the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA). The bile acids, with the exception of lithocholic acid, are efficiently absorbed from the intestine and return to the liver via the portal vein. In the liver (70-90)% of the bile acids a re extracted and resecreted to the bile. Thus, the main bile acids in human bile are CA, CDCA, and DCA. Small amounts of LCA and ursodeoxycholic acid (UDCA), a metabolite of CDCA are also found.

Two major pathways of bile acid synthesis from cholesterol have been described[1-5]. The main pathway, also called the neutral pathway, is initiated by a 7α-hydroxylation, catalyzed by the rate-limiting enzyme cholest erol 7α-hydroxylase. This hydroxylation is followed by further tra nsformations of the steroid nucleus and oxidative cleavage of the side chain. An alternative pathway, also called the acid pathway, is initiated by a 27-hydrox ylation of cholesterol. The quantitative importance of the latter pathway is not yet clear. It may be of importance for the formation of particularly CDCA and a s a "back-up" pathway in conditions in which the neutral pathway is represse d for some reasons.

Bile acid formation in humans has been studied by different in vivo and *in vitro* techniques. Also cultures of human hepatoblastoma cells (HepG2) have been employed. However, these cells have been found to be defective in CA formation and conjugation by some authors[6-9]. The cells have also been found to prod uce large amounts of bile acid precursors.

More recently, techniques have been developed for preparation of primary cultures of normal human hepatocytes. These offer now possibilities to study bile acid synthesis in normal hepatocytes under various experimental conditions.

MATERIALS AND METHODS

Preparation and culture of human hepatocytes

Primary hepatocytes were prepared from normal donor liver tissue. The project wa s approved by the Ethics Committee at Huddinge University Hospital and by Human Ethics

Committees of the Western Sidney Area Health Service, Australia. Liver se ctions weighing 100-200 g and with only one cut-surface were used. Hepatocyte s were isolated using a 2-step perfusion technique utilizing EGTA and collagena se type IX-S as recently described[10]. They were seeded on 60-mm dis hes at a density of 3.5 × 10^6 cells per dish. Dishes were precoated with 0.2 mL of matrigel, a laminine-rich extracellular matrix prepared from Engelbreth -Holm-Swarm mouse sarcomas[11]. Hepatocyte viability was >85% as det ermined by trypan blue exclusion. The cells were then incubated in serum-free W illiam's E medium (3 mL/dish), supplemented with glutamine (292 mg/L), Na_2SeO_3 (173 μg/L), insulin (2 mIU/mL), penicillin G s odium, streptom ycin sulphate and gentamycin. In some experiments, triiodothyronine (T3) (0.1 μmol/L) and/or dexamethasone (0.1 μmol/L) were adde d. In some other experiments, on d 4, cyclosporin A (CsA), an inhibitor of the sterol 27-hydroxylase[12,13], was added to the medium (0-10 μmol/L). The medium was collected and renewed every 24 h and frozen at -20 ℃ for later analysis of bile acids. At the end of the incubation, the hepatocytes were harvested and stored at 20℃ until extraction.

Analytical procedures

For determination of the primary bile acids CA and CDCA formed, deuterium-label ed bile acids were added to the collected culture medium. After alkaline hydroly sis and extraction, the bile acids were analysed by gas chromatography-mass spe ctrometry as described recently[10]. For more detailed analysis of neutral and acidic steroids-unconjugated and conjugated with glycine or taurine a nd/or sulphuric acid-group separation was performed by anion exchange chromat ography after addition of isotopically labeled steroids. Deconjugation was perfo rmed by an enzymatic method. Sulphate groups were cleaved by solvolysis in acidi fied tetrahydrofuran. The identification of steroids was performed by gas chroma tography-mass spectrometry. Further details are given in a recent paper[14].

RESULTS AND DISCUSSION

Formation of bile acids

CA and CDCA were the two dominating steroids formed, accounting for as much as 7 0% and 25% of total, respectively (Figure 1). CA and CDCA are the two bile acids normally synthesized in human liver but the ratio between the production rates of the two bile acids in the hepatocytes was higher (about 3:1) than that of nor mal subjects (about 2:1). However, the ratio is similar to that obtained in pati ents with complete biliary drainage[15]. Small amounts of two other bil e acids, 3β-hydroxy-5-cholenoic acid and 3β, 7β-dihydroxy-5β-cholanoic acid were also detected (about 1% of total of each).

Bile acid formation was lowest on d 1 and 2 and then increased several-fold on d 4 to 6, whereafter it declined slightly (Figure 2). The reason for this is not clear. However, it has previously been observed that the activity and mRNA l evels of cytochrome P450 enzymes involved in drug metabolism show a similar prof ile in human hepatocytes e.g., a sharp recovery followed by a steady decline (Li ddle C *et al*, unpublised observations).

About 3% of the total amount of the isolated steroids consisted of bile acid pre cursors. Among those, 7α-hydroxy-cholesterol and 7α-hydroxy-4-cholesten-3-one are established intermediates in the neutral pat hway and 27-hydroxycholesterol is the first intermediate in the acidic pathway. Since 7α-hydroxycholesterol can also be formed by autooxidation o f cholesterol, 7α-hydroxy-4-cholesten-3-one is a better marker for the neutral pathway. The daily release of 7α-hydroxy-4-chol esten-3-one increases in parallel to those of CA and CDCA, whereas the release of 27-hydroxycholesterol decreases. Since the hepatocytes are incubated in as erum-free medium devoid of lipoprotein cholesterol, the decrease in amounts of 27-hydroxycholesterol is consistent with the hypothesis that cholesterol utili zed for the acidic pathway is mainly derived from plasma lipoproteins[2,7].

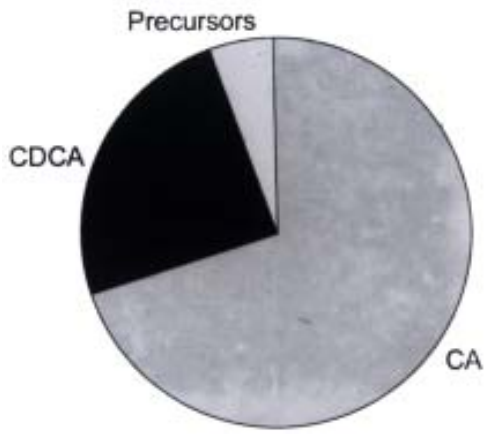

Figure 1 Distribution of cholic acid, chenodeoxycholic acid and precursors formed in prim ary cultures of human hepatocytes.

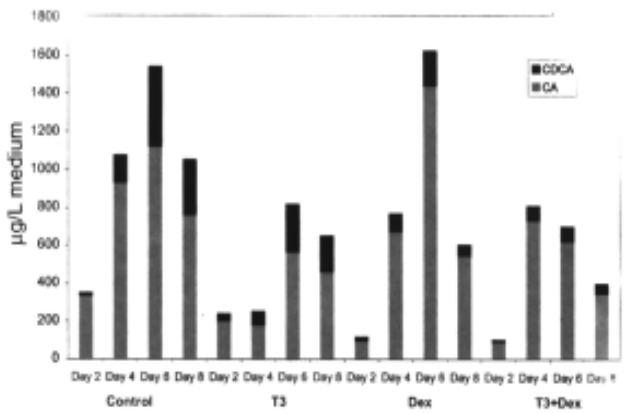

Figure 2 Formation of cholic acid and chenodeoxycholic acid in primary cultur es of human hepatocytes during d 2, 4, 6 and 8. Effects of adding T3 and dexa methasone (DEX) alone and in combination.

As can be seen in Figure 3, HepG2 cells release large amounts of bile acid p recu rsors into the incubation medium. It cannot be excluded that the bile acid synth etic pathways also may be abnormal in cultured HepG2 cells.

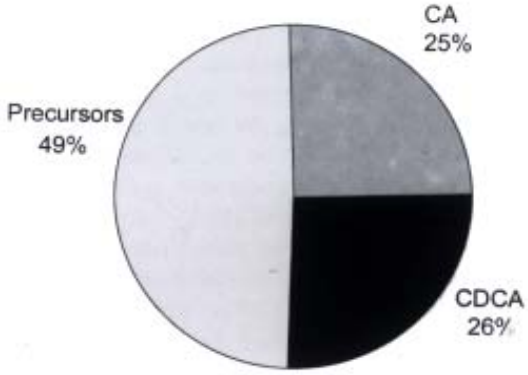

Figure 3 Distribution of cholic acid, chenodeoxycholic acid and precursors fo rmed in cultures of HepG2 cells (data from ref. 7).

Conjugation

CA and CDCA were conjugated with glycine or taurine to more than 99.5% (Figure 4). About one-third of CDCA was also conjugated with sulphuric acid (Figure 5). 3β-Hydroxy-5-cholenoic acid and 3β, 7α-dihydroxy-5β-cholanoic acid were also sulphated. 27-Hydroxych olesterol and other oxysterols were partly sulphated.

In contrast to primary human hepatocytes, Hep G2 cells release large amounts of unconjugated bile acids into the medium (Figure 6).

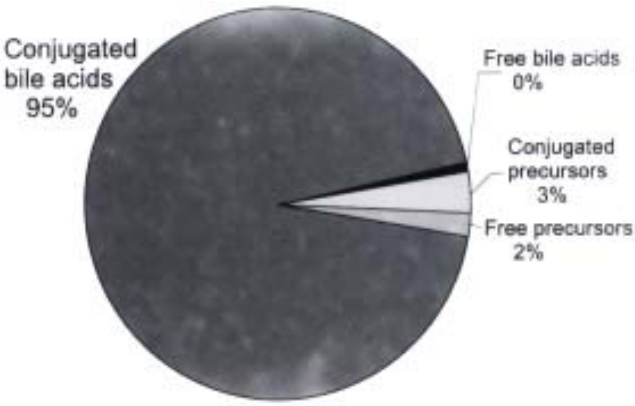

Figure 4 Conjugation of bile acids and precursors formed in primary cultures of human hepatocytes.

Effect of cyclosporin A

Cyclosporin A (CsA) is a potent inhibitor of the sterol 27-hydroxylase activity, especially towards nonpolar substrates like cholesterol[12,13]. When added to the culture medium, 1 μmol/L and 5 μmol/L CsA had l ittle or no effect on the formation of bile acids, but 10 μmol/L Cs A de creased the formation of CA by about 13% and CDCA by about 30% (Figure 7). No ac cumulation of bile acids occurred in the hepatocytes. However, a significant accumulation of 7α-

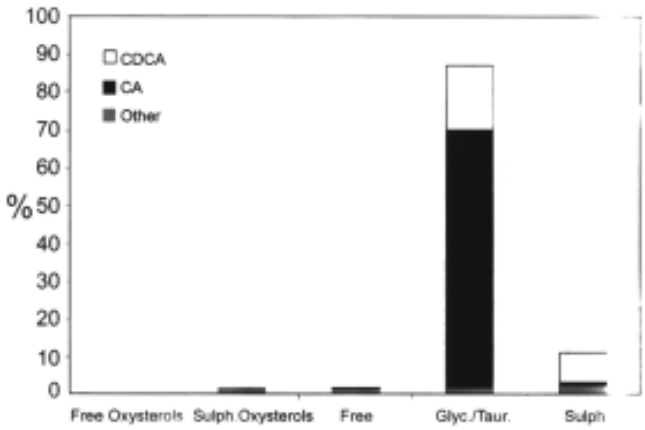

Figure 5 Distribution of free and conjugated bile acids and potential interme diates isolated from medium on the fifth day of incubating primary human hepatoc ytes. CDCA = chenodeoxycholic acid; CA = cholic acid.

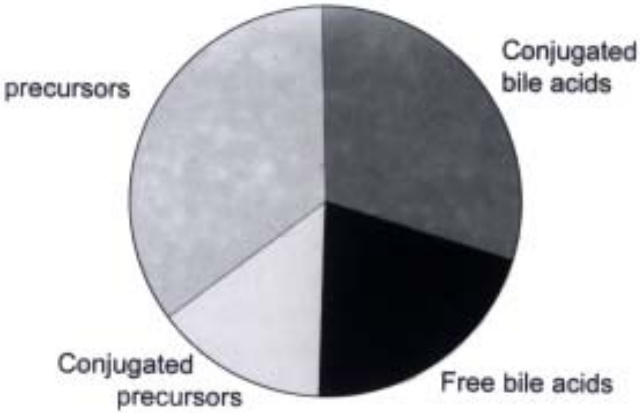

Figure 6 Conjugation of bile acids and precursors formed in cultures of HepG2 cells (data from ref. 7).

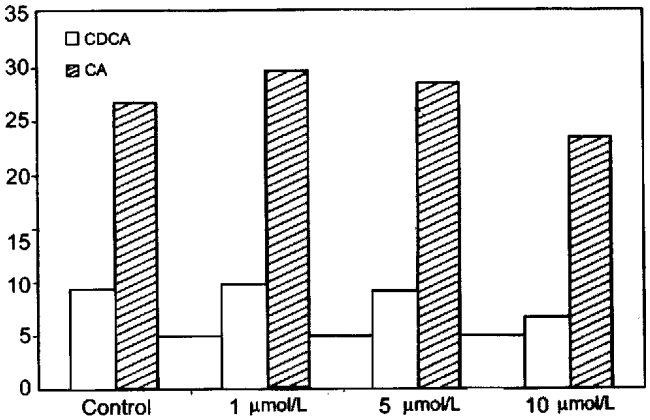

Figure 7 Effect of cyclosporin A on bile acid formation in primary human hepa tocytes.

hydroxy-4-cholestene-3-one and 7α, 12α-dihydroxy-4-cholestene-3-one, an intermediate in CA synthesis, was found indicating that not only the acidic pathway but also the ne utral pathway of bile acid formation was affected by CyA. In agreement with thes e results, it has previously been reported that patients with cerebrotendinous xanthomatosis, who lack the sterol 27-hydroxylase[16,17],

accumulate 7α, 12α- dihydroxy-4-cholestene-3-one more than other intermediates in the liver[18]. However, the decreased formation of tot al bile acids (CA and CDCA) during CsA treatment may mainly be due to an inhibit ory effect of CsA on the hepatic synthesis of cholesterol[12].

Influence of hormones

Previous studies have shown that the combined addition of T3 and dexamethasone s timulates bile acid formation, cholesterol 7α-hydroxylase activity and mRNA levels in rat hepatocytes, whereas the addition of T3 or dexamethas one alone has little or no stimulatory effect [10,19]. The addition of T 3 and dexamethasone alone or in combination to human hepatocytes did not increas e bile acid synthesis (Figure 2). If anything, bile acid formation tended to dec rease. The different effects of T3 and dexamethasone in rat and human hepatocyte s agree well with recent studies of the hormonal regulation of the cholesterol 7α-hydroxylase gene promoter[20,21]. These studies showed that the rat cholesterol 7α-hydroxylase gene promoter is stimulated by dexamethasone whereas thyroid hormone has no significant effect. The promot er of the human cholesterol 7α-hydroxylase gene, on the other hand , is suppressed by both dexamethasone and thyroid hormone.

In conclusion, isolated human hepatocytes in culture behave as in the intact liver by almost quantitatively converting cholesterol to conjugated CA and CDCA. In contrast, cultured HepG2 cells release large amounts of bile acid precursors an d unconjugated bile acids into the medium. Human hepatocytes in culture represent a useful model for further studies on the synthesis and regulation of bile acids.

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Edited by Lu J
proofread by Mittra S

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

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Glyco-poly-L-lysine is better than liposomal delivery of exogenous genes to rat of liver

Chang Qing Yang, Ji Yao Wang, Bo Ming He, Jian Jun Liu and Jin Sheng Guo

Subject headings liposomes; glyco-poly-L-lysine; targeted liver uptake; exogenous gene

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Abstract

AIM To compare the effects of liposomes and glyco-poly-L-lysine on liver tar geted uptake and expression of plasmid in rat liver.

METHODS After binding with lipofectamine or galactose-terminal glyco-poly-L-lysine, the plasmid could be expressed in eukaryotic cells when injected into Wistar rats by intravenous route. At different time intervals after the injection, the distribution and expression of the plasmid in liver of rats were observed and compared using *in situ* hybridization and immunohistochemistry.

RESULTS The expression of the plasmid binding to liposomes or G-PLL could be markedly observed 24 h later, and began to decrease one week later, but it still could be observed up to three weeks. Both liposomes and G-PLL could deliver the plasmid to the liver effectively, but the effect of the latter was better than the former concerning the distribution and expression of the plasmid targeted uptake in the liver.

CONCLUSION G-PLL is better than liposome as the targeted carrier for delivering exogenous genes to the liver.

INTRODUCTION

Liver is one of the important metabolizing organs, and is closely related to many kinds of diseases, such as hereditary disease, infectious disease, metabolic disease, tumors and so on. Since the development of chemical drugs for the trea tment of these diseases is comparatively slow, gene therapy has opened up a prospective way for them[1-5].

The efficient delivery and the high expression of exogenous genes in specific cells or tissues are critical steps for gene therapy both *in vitro* and *in vivo*[6-15]. As for the gene therapy of hepatic diseases, efficient delivery of the exogenous genes to the liver and its high expression could increase its local accumulation while minimize the side-effects on other tissues and organs as well.

Both liposome and glyco-poly-L-lysine (G-PLL) are often used as carriers to deliver exogenous genes to the liver in gene therapy experiment[16-22]. Most of the liposomes are cationic when used as the carriers of gene transfe rence. Both *in vitro* and *in vivo* studies have showed that liposomes could be applied to transfer exogenous genes to hepatocytes [23,24], but its specificity varies in different studies[24-26]. Poly-L-lysine is a kind of polycation which can be bound to DNA by interacting with the oppositeele ctric charge on DNA. After binding with other specific ligands through covalent linkage, the resulting ligand-poly-L-lysine-DNA complex can be formed[27] and can thus be used to deliver foreign DNA to specific cells or tissues. Similarly, after being saccharified by galactose, glyco-poly-L-lysine (G-PLL) formed is then capable of delivering exogenous genes to the liver specifically [28-30].

Using rats as the experimental animal, we compared the *in vivo* potency of l iposomes and glyco-poly-L-lysine on delivering the plasmid, which could be expressed in eucaryotic cells.

MATERIALS AND METHODS

Preparation of the carriers

The original plasmid of rat interstitial collagenase was kindly provided by Prof. John J Jeffrey[31], and we reconstructed it with the plasmid of pTa rgetT (TM) (Promega Co., Madison, MI, USA), which could be expressed in eucaryot ic cells. We also inserted a segment of nucleotides (GAC TAC AAG GAC GAC GAT GAT AAG) before the terminator codon (TAA) of the rat interstitial collagenase. The 'Flag Domain' peptide (DYKDDDDK) encoded by the segment of nucleotide above, which was usually called 'Tag', could be fused in the rat interstitial collag enase[32-33] and could be specifically recognized by a M2 monoclonal an tibody (Kodak, New Haven, CT, USA). This recombinant plasmid was named

Division of Gastroenterology, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Chang-Qing Yang, male, 35 years old, got his M.D. in 1990 and Ph.D. in 1998 from Xiangya hospital of Hunan Medical University, now is working as a postdoctora l fellow in Zhongshan Hospital of Shanghai Medical University, majoring in liver targeted uptake and hepatic fibrosis.
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Correspondence to: Ji-Yao Wang, Division of Gastroenterology, Z hongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Tel. +86-21-6404-1990 ext 2420, Fax. +86-21-6483-3680
Email. xhk@shmu.edu.cn
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pTM/MMP- 1. The plasmid of pTM/MMP-1 was extracted and purified using QIAGEN-Tip 500 kit (QIAGEN lnc., Valencia, CA, USA) according to manufacturers of the instructions. The plasmid was mixed with different amounts of lipofectamine (GIBCO, Grand I sland, NK, USA) or galactose-terminal glyco-poly-L-lysine (kindly provided by Dr. Shou-Ming Wen of Air-force General Hospital of PLA, China. The mean mol ecu lar weight of this kind of G-PLL was 8500 and the ratio of glactose to poly-L-lysine was 15: 28). The optimal proportion of the plasmid pTM/MMP-1 to lipo fec tamine or galactose-terminal glyco-poly-L-lysine was determined by elect rophoresis in 1% agarose gel.

Animal experiments

Eighteen male Wistar rats, with body weight 130-150 g, were randomly divided into three groups. Lipofectamine intravenious (LI) group: 50 μg of plasmid pTM/ MMP-1 encapsulated by lipofectamine was given through cauda vein; Poly-L-lysi ne intravenous (PI) group: 50 μ plasmid pTM/MMP-1 binding to gala ctose-termi nal glyco-poly-L-lysine was administred through cauda vein; Normal group: control animal. Twenty-four hours, 48 h, 72 h, 1 wk, 2 wk, 3 wk af ter the administration of the plasmid, one rat from each group was randomly sele cted and anaesthetized with 2% pentobarbital sodium intraperitoneally. Then 1 mL blood from each rat was obtained by cardiac puncture for the assay of alanine t ransaminase (ALT), aspartic transaminase (AST), and creatinine (Cr) to follow the functions of important organs. After perfusion of the whole body with 20 mL phosphate-buffered saline and 40 mL precold 4% paraformaldehyde through ventricula rinjection, the tissues of liver, spleen, lungs and kidneys were collected and fixed in 4% paraformaldehyde, encapsulated in paraffin and cut into sections of 4 μm thick. The animal experiments above were approved by the La boratory Animal Committee of Shanghai Medical University.

Immunohistochemistry

Immunohistochemistry was performed according to the literature[34,35]. The first antibody used was M2 monoclonal antibody which was specific for flag - domain tag (Kodak, New Haven, CT, USA) and the second antibody used was Horse anti-mouse IgG, labeled with biotin(Vecter, Burlingame, CA, USA). After the tre atment with avidin and biotin (ABC kit, Vecter, Burlingame, CA, USA), color deve lopment was followed using dimethylaminoazobenzene (DAB) and counterstained with hematoxylin. Five fields were observed under high power from every immunostaine d section and the positive signals were counted.

In situ hybridization

The procedure of *in situ* hybridization was also described previously[3 4,35]. To state briefly, the oligonucleotide probe (5'-TGG TGT GAC TAC AAG GAC GAC GAT GAT AAG-3') was synthesized in Cell Biology Institute of Chinese Academy of Sciences (Shanghai), which could hybridize with the (mRNA) of the flag-domain tag in the plasmid pTM/MMP-1, and the 5' of the probe was labeled with biotin. After the hybridization of the target mRNA with the probe, the rest of the procedure was similar to that followed in immonohistochemistry excluding the step of hematoxylin counterstaining.

Other biochemical assays

ALT, AST, and Cr were assayed using the 7170A Automatic Analyzer (HITACHI, Jap an) to observe the changes in the important organs' function.

Data analysis

The data was analysed using the software SPSS 7.0 for windows (One-way ANOVA).

RESULTS

Ratio of liposomes and G-PLL to plasmid

According to the electrophoresis results, we found that 5 μl of li pofectamin e could encapsulate 1 μg of plasmid pTM/MMP-1 completely; While 0. 3 μg of ga lactose-terminal glyco-poly-L-lysine throughly could bind to 1 μg of the pl asmid, which meant that about 72 molecules of G-PLL could carry one molecule of the plasmid pTM/MMP-1 (Figures 1 and 2).

Figure 1 Determination of the optimal proportion of liposome bound to plasmid by 1% agarose electrophoresis. Lane 1-8 are respectively 1-8 μL lipofectamine mixed with 1 μg pTM / MMP-1 plasmid. Plasmid 1 μg could only be encapsulated complet ely by more than 5 μL lipofectamine.

The changes in the functioning of important organs

Compared with the normal group, there was no obvious elevation of the ALT, AS T, and Cr levels in the LI, PI groups.

The distribution and expression of the plasmid in liver, spleen, lung and kidney

The results of the immunohistochemistry and *in situ* hybridization showed that the plasmid binding to liposomes or G-PLL could be expressed *in vivo* , and the results of immunohistochemistry were more sensitive and stable. In addition, the protein product of the plasmid could be secreted extracellularly (Figures 3 and 4), similar to the expression of interstitial collagenase in the phy siological state[36]. We found that both liposomes and G-PLL could deli ver the plasmid to the liver very efficiently, making liver as its major distrib ution organ. The obvious expression of the plasmid could be observed 24 h af ter the administration and began to decrease one week later, although it could s till be observed weakly even two or three weeks later (Figure 5). Among the thre e groups, we also observed that the expression and distribution of the plasmid in the liver was most in the PI group, followed by the LI group. Besides the liver , the exogenous gene could also be expressed highly in lungs, and expressed in ki dney in a relatively lower level in the LI group; while for the PI group, a rela tively lower level of the expression could be seen in the kidney, the spleen, and the lung.

DISCUSSION

Both liposomes and G-PLL can be used as the targeted carriers to deliver drugs or nucleotides to liver[17-21,24-29], but the comparison of their effects on the liver targeted uptake have not been reported extensively.

Gene transfer mediated by receptors is carried out by high affinity linkage between the ligands (binding to the foreign gene) and specific receptors on the surface of different kinds of cells, and then the foreign gene can be delivered into the cells by phagocytosis[37-40]. There also exist some specific receptors on the surface of hepatocytes such as asialoglycoprotein receptor (ASG P-R)[41,42], which facilitate the delivery of exogenous genes into hepa tocytes specifically using the ligand-receptor interaction. Galactose-terminal glyco-poly-L-lysine contains the saccharide group of galactosan that can be specifically ligated to the ASGP-R on the surface of hepatocytes. At the same time, the cationic poly-L-lysine can bind to nucleotides with high affinity , thus forming a good carrier to deliver exogenous DNA to liver specifically and ste adily[28-30]. It has been reported in the literature, that the ratio of the liver targeted delivery could reach up to 70%-90% *in vivo*[27,43]. In our experiments we found that in addition to its main locati on in the liver, the plasmid binding to G-PLL could be expressed in kidney, spl een, and lung also. It may be due to the existence of ASGP-R in other extrahepa tic tissues[44,45].

Liposomes are a kind of annular closed vesicles made from double layers of l ipid molecules[46]. Having no toxicity and no immunogenicity[47]. Gene transfer mediated by liposomes is achieved by the fusion between liposomes and the membrane of the cells. Liposomes encapsulating the foreign DNA can be integrated with the membrane of

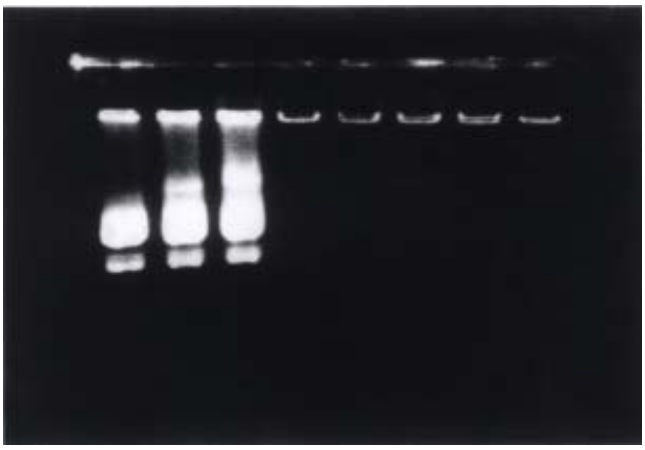

Figure 2 Determination of the optimal proportion of G-PLL bound to plasmid by 1% agarose electrophoresis. Lane 1-8 are respectively 0.05 μg, 0.1 μg, 0.2 μg, 0.3 μg , 0.4 μg, 0.5 μg, 1.0 μg, and 1.5 μg G-PLL mixed with 1 μg pTM / MMP-1 plasmid. Plasmid 1 μg could only been bound completely by more th an 0.4 μg G-PLL.

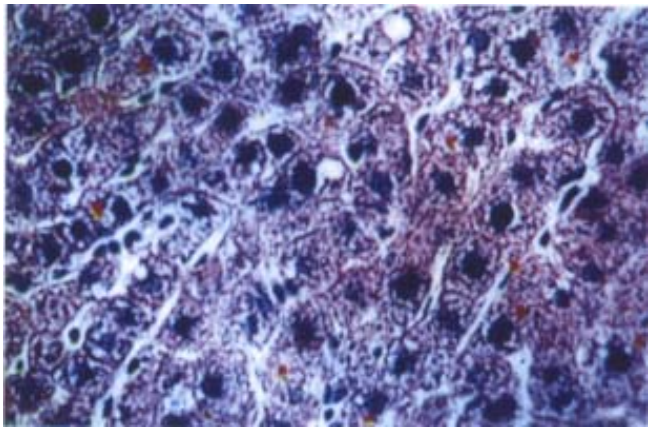

Figure 3 Immunostaining of flag-domain tag in the liver of rat 24 h after the admini stration of the plasmid bound to G-PLL (galactose-terminal glyco-poly-L-lysine) via cauda vein. × 200

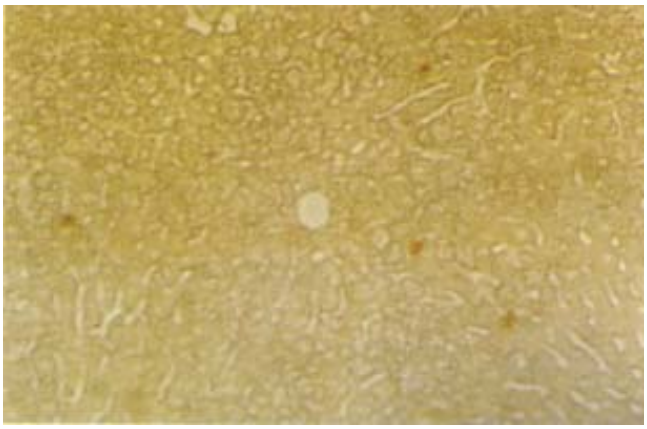

Figure 4 *In situ* hybridization with biotin labeled oligonucleotide probe in the li ver 3 wk after the administration of the plasmid encapsulated by liposome (lipofectamine) via cauda vein. × 100

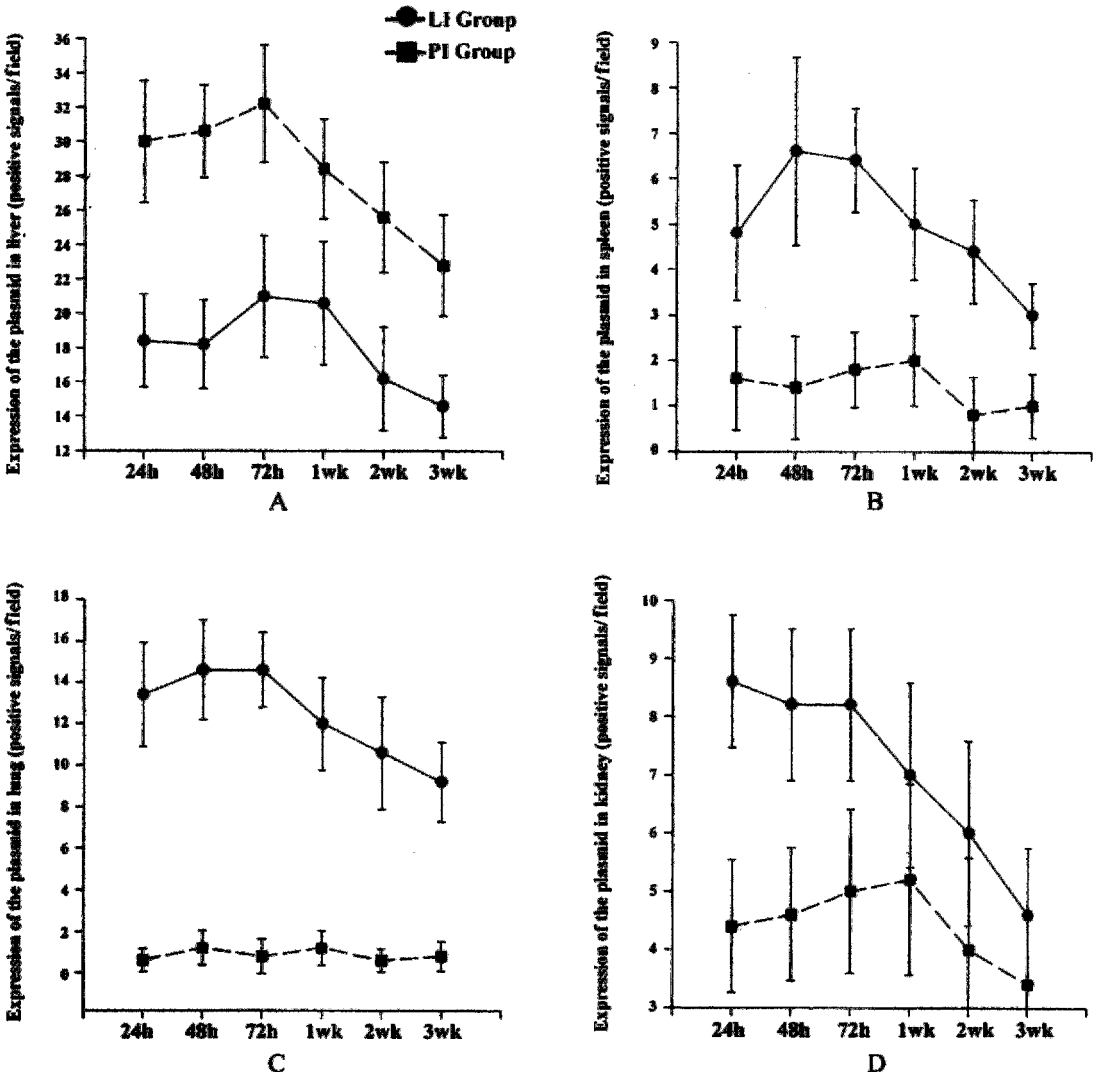

Figure 5 The distribution and expression of the plasmid bound to liposomes or G-PLL (gal actose-terminal glyco-poly-L-lysine) in different tissues and at differe nt time points. A: liver, B: spleen, C: lung, D: kidney. LI: plasmid encapsulate d by liposomes given through cauda vein, PI: plasmid bound to G-PLL introduced intravenously.

the cells, and thus the foreign gene can be deli vered inside the cells by phagocytosis[24,48-50]. Because it contains lecithin and lactosylceramide that can bind to ASGP-R on the surface of hepato cytes specifically, liposome could also be used as targeted delivery carrier to the liver[51,52]. Our experiment al results demonstrated that the plasmid encapsulated by liposomes could also be expressed in lung and spleen to a certain extent despite its major expression in the liver, maybe due to the macrophage system which existed in the lung, spleen, and other tissues. This phenomenon demonstrates further that the efficacy of liver targeted delivery by liposomes i s inferior to that of G-PLL.

In this study, we also observed whether liposomes and G-PLL, both binding to the plasmid, would induce some toxicity to the body or not. We did not find any detrimental effects on the functioning of important organs like liver, heart, and kidney and this indicated further that liposomes and G-PLL could be used *in vivo* safely as delivery carriers for drug or nucleotides.

In conclusion, we found that the plasmid binding to liposomes or G-PLL could be delivered to the liver efficiently and G-PLL was better than liposomes regar ding the distribution and expression of the plasmid in the liver. Both liposomes and G-PLL can be used as carriers to deliver drugs or nucleotides to rat liver, but whether they can be used in human beings in the future deserves further in vestigation.

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Edited by Zhu QR
proofread by Mittra S

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

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Induction of apoptosis by arsenic trioxide and hydroxy camptothecin in gastriccancer cells in vitro

Shui Ping Tu[1], Jie Zhong[1], JI Hong Tan[1], Xiao Hua Jiang[1], Min Min Qiao[1], Yu Xin Wu[1] and Shi Hu Jiang[1]

Subject headings gastric cancer; apoptosis; arsenic trioxide; hydroxycamptothecin

Tu SP, Zhong J, Tan JH, Jiang XH, Qiao MM, Wu YX, Jiang SH. Induction of apoptosis by arsenic trioxide and hydroxy camptothecin in gastric cancer cells *in vitro*. ***World J Gastroentero***, 2000;6(4):532-539

Abstract

AIM **To study the effects of arsenic trioxide and HCPT on dif ferent degrees of differentiated gastric cancer cells (SGC-7901, MKN-45, MKN-28) with respect to both cytotoxicity and induction of apoptosis *in vitro*.**

METHODS **The cytotoxicity of As_2O_3 and HCPT on gastric cancer cells was determined by MTT assay. Morphologic changes of apoptosis of gastric cancer cells were observed by light microscopy and transmission electron microscopy. Apoptosis and cell cycle changes of gastric cancer cells induced by HCPT and As_2O_3 were investi gated by TUNEL method and flow cytometry.**

RESULTS **As_2O_3 and HCPT had remarkable cytotoxic effects on different degrees of diff e rentiated gastric cancer cells. The IC^{50} of As_2O_3 on well differentiat ed gastric cancer cell MKN-28, moderately differentiated gastric cancer cell SG C-7901, and poorly differentiated gastric cancer cell MKN-28 were 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respectively. The IC^{50} of HCPT on MKN-28, SGC-7901, and MKN-45 were 9.35 mg/L, 10.21 mg/L, and 12.63 mg/L respectiv ely after 48 h treatment. After 12 h of exposure to both drugs, gastric cancer cells exhibited morphologic features of apoptosis, including cell shrinkage, nuclear condensation, and formation of apoptotic bodies. A typical subdiploid pe ak before G_0/G_1 phase was observed by flow cytometry. The apoptotic rates of SGC-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively after 48 h exposure to 10 μmol/L As_2O_3. The apoptotic rates of SGC-7901, MKN-4 5, and MKN-28 were 21.88%, 12.35%, and 30.26%, respectively after 48 h expo sure to 10 mg/L HCPT. The apoptotic indice were 7%-15% as assessed by TUNEL method. The effect of As_2O_3 on SGC-7901 showed remarkable cell cycle specificity, which induced cell death in G_1 phase, and blocked G_2/M phase. HCPT also showed a remarkable cell cycle specificity, by inducing cell death and apoptosis in G_1 phase and arrest of proliferation at Sphase.**

CONCLUSION **As_2O_3 and HCPT exhibit significant cytotoxicity on gastric cancer cells by induction of apoptosis. As_2O_3 and HCPT might have a promising prospect in the treatment of gastric cancer, which needs to be further studied.**

INTRODUCTION

Gastric cancer is one of the most common malignant tumors in China. Evidences ha ve demonstrated that stomach cancer is a disease caused not only by excessive cellular proliferation and poor differentiation, but also by decrease in apoptosis of the gastric cells[1]. Though the disease in its early stage can be treated by surgical resection, in advanced stage its response to conventional che motherapy or radiotherapy is usually not satisfactory. Therefore, we think, that indu ction of apoptosis of gastric cancer cells might be a new means in the treatment of gastric cancer. Arsenic trioxide (As_2O_3) and hydroxycamptothecin (HCPT) have long been used in China. The former was first reported by investigators in Harbin and Shanghai to be an effective drug in the treatment of patients with a cute promyelocytic leukemia (APL)[2,3]. It induces apoptosis of the leuk emic cells at a concentration achieved in the plasma of treated patients (0.5×10^{-6} mol/L-1×10^{-6} mol/L), as demonstrated by studies on all -trans-retinoic acid (ATRA)-susceptible or resistant APL cell lines, on primary APL cell culture ,and on patients' blood samples

[1]Department of Gastroenterology, Ruijin Hospital,Shanghai Second Medical University, Shanghai 200025, China
Dr. Shui Ping Tu, male, born on 1966-06-29 in Nanfeng, Jiangxi Province, grad uated from Suzhou Medical College with bachelor degree, now physician-in-charge,and PhD, majoring in gastrointestinal tumor research, having 8 papers published
Supported by the Natural Science Foundation of Committee of Science and Technology of Shanghai Municipality (No964119035)
Correspondence to: Shui Ping Tu, Department of Gastroenterology, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China
Tel. 0086-21-64370045 Ext, 661144
Email. Tushuiping@sohu.com
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obtained during treatment with arsenic[4]. It is not known whether As_2O_3 is effective in the treatment of solid tumors such as gastric cancer or not.

HCPT is a unique antitumor drug that has been extracted and synthesized by Chine se scientists from *Camptotheca accuminata* which is a native plant in China[5]. HCPT can act directly on topoisomerase I inhibiting its activity. HCP T possesses stronger cytotoxicity to tumor cells with less side effects, as com pared to camptothecin[6]. More recent studies have shown that camptothec in have strong apoptosis induction effects in human leukemia cell lines[7]. However, there is yet no report about HCPT-induced apoptosis in stomach cells. Therefore, we studied the effects of HCPT and As_2O_3 on induction of apoptosis in gastric cancer cells.

MATERIALS AND METHODS

Cell culture and chemicals

Human moderately differentiated gastric adenocarcinoma cell line SGC-7901 was o btained from Shanghai Sixth People's Hospital, human poorly differentiated sto mach adenocarcinoma cells line MKN-45 and well differentiated stomach adenocarc inoma cell line MKN-28 were kindly provided by Japanese Cancer Research Resourc es Bank Corp (Tokyo, Japan). SGC-7901, MKN-45 and MKN-28 were maintained in a humidified, 5% CO_2 atmosphere and cultured in RPMI 1640 (GIBCO) supplemented with 10% FCS, 2 μmol/L L-glutamine, 100 units/mL penicillin, and streptomycin. HCPT 1 g/L and 0.1% As_2O_3 preparation for iv administr ation were kindly provided by Hubei Huangshi Second Pharmaceutical Company,and Shanghai Institute o f Hematology, respectively.

MTT method and cytotoxicity

One hundred μL cancer cells in exponential growth at 1×10^4/mL w ere added into flat-bottomed 96-well plates (NUNC) 24 h prior to drug treatment. Cells w ere treated with 1 mg/L-100 mg/L HCPT, (0.5-10) μmol/L As_2O_3 an d with no drugs (control) in triplicate for 24 h and 48 h, respectively. After washing th e medium was replaced by 100 μL RPMI 1640 (GIBCO) medium containing 1 g/L3(4,5dimethylthiazol)-2,5diphenyltetra-zolium(MTT, MERCH). After 4 h , plat es were centrifuged at 800 × g for 5 min, the MTT medium was removed, and th e blue dye was dissolved in 200 μL of warm dimethylsulfoxide (DMSO). Absorban ce was measured at 570 nm. The cytotoxicity rates were measured by the formula:

$$\text{The cytotoxicity vate} = (1-\frac{OD_{570}\ \text{test}}{OD_{570}\ \text{control}} \times 100\%$$

The IC_{50} was evaluated from the cytotoxicity curve.

Apoptosis cell morphology

One hundred μL cancer cells at concentration of 5×10^6/mL were in cubated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation with drugs for 12 h, 24 h, 48 h, and 72 h, glass slides with cancer cell growth were fixed with 4% p olyformalin, and stained with hematoxylin-eosin. Cell morphology was examined under light microscopy.

Transmission electron microscopy

Culture cells were fixed in 2% glutaraldehyde in 0.1 mol/L, pH 7.4 PBS at 4 ℃ and postfixed with 1% osmium tetroxide for 2 h, then the cells were embedded wi th Ep pon 812 and ultrathin sections were cut. Cells were observed under transmission electron microscope (H-500, Japan).

TUNEL assay

Apoptosis was assessed by dUTP labeling of DNA nicks with terminal deoxynucleoti dyl transferase (TUNEL). One hundred μL cancer cells at concentr ation of 5×10^6/mL were inoculated into 6-well plates with previously placed glass slides. After 24 h, the medium was replaced with the drug containing medium. After incubation w i th drugs for 24 h and 48 h, glass slides with cancer cells growth were fixed wit h 4% polyformalin. The TUNEL assay was performed according to the instructions in the *In Situ* Cell Death Detection Kit (Boehringer-Mannheim, Germany). Briefly, after washing twice with pH 7.4 PBS, 50 μL TUNEL reaction soluti on was add ed to the well, then incubated at 37℃ for 2 h. After substrate reaction, s tained cells were examined under light microscopy. Apoptotic cells were scored a nd expressed as the number of positively stained cells per 500 cells (n = 4-5) .

Flow cytometry

Apoptotic cells were also detected by flow cytometry which was performed as des cribed previously. About 1×10^6 cells were treated with drugs for 24 h and 48 h. After trypsin digestion, the cells were collected by centrifugation, then fix ed in 70% ethanol /phosphate buffered saline for at least 12 h at 4℃. After 100 μL (1 g/L) RNase treatment, cells were stained with 50 mg/L propidium iodide. Cells were examined by flow cytometry using a FACScan (Becton-Dickinson , USA). The results were analyzed with Lysis II software (Becton-Dickinson).

RESULTS

As_2O_3 and HCPT cytotoxicity

HCPT and As_2O_3 had strong cytotoxic effect on gastric cancer cells (Figure 1). The IC_{50} of As_2O_3 on MKN-28, SGC-7901, and MKN-45 was 8.91 μmol/L, 10.57 μmol/L, and 11.65 μmol/L, respecti vely.The IC_{50} of HCPT on MKN-28, SGC-7901, MKN-45 was 9.35 mg/L, 1 0.21 mg/L, and 12.63 mg/L, respectively.

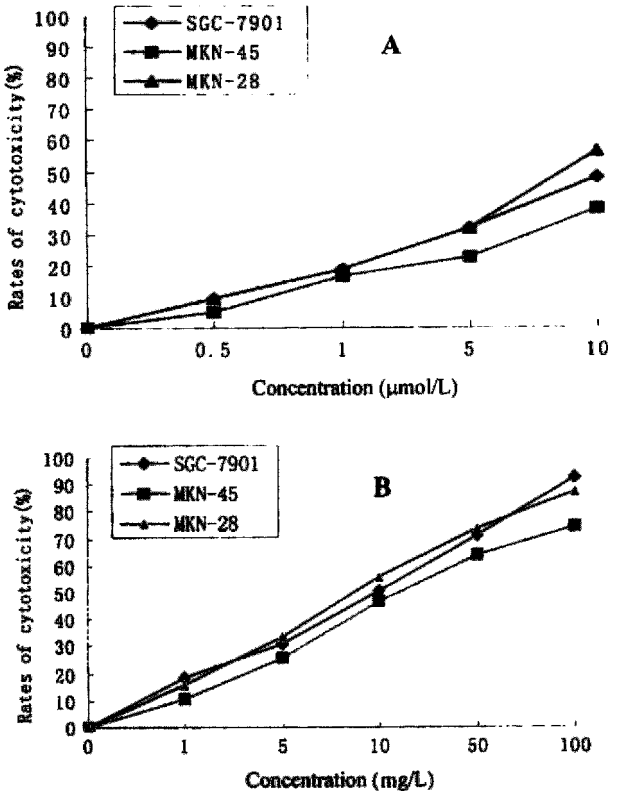

Figure 1 Cytotoxicity of As_2O_3 (A), and HCPT (B), on gastric cancer cells.

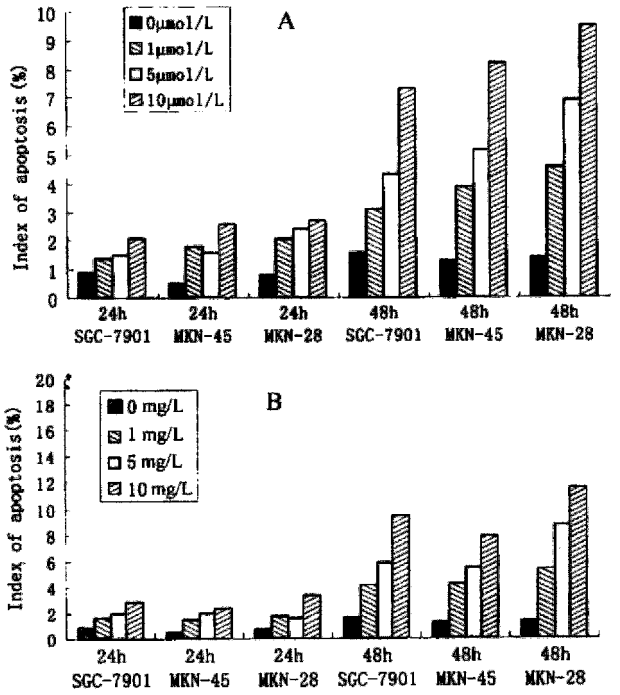

Figure 3 The index of apoptosis of gastric cancer cells induce d by As_2O_3 (A) and by HCPT (B).

Induction of apopotosis by As_2O_3 and HCPT

After 12 h of exposure to As_2O_3 and HCPT, gastric cancer cells MKN-28 a nd SGC-7901, began to show morphologic features of apoptosis. The apoptotic cells increased on prolongation of exposure time to drugs. The ultrastructural feat ures of apoptosis was observed in MKN-45 and SGC-7901 by transmission electron microscopy, including cell shrinkage, cytoplasmic blebs, condensation of chromatin, nuclear condensation, fragmentation of nucleus, and formation of apoptotic bodies, and so on. The results are presented in Figure 2.

As_2O_3 and HCPT induced time- and dose-dependent apoptosis in three strains of gastric cancer cells. The indice of apoptosis were measured by TUNEL. The results are presented in Figure 3A and 3B.

To further study the inducing effect of As_2O_3 and HCPT on gastric cancer cells, we analysed the DNA fragment reflecting the endonuclease activity during apo ptosis. (Figure 4). Examination of histogramic related nuclear DNA contents on FACS showed a distinct region below G_1 phase, which is the typical profile of apop totic cells in which DNA stainability is reduced due to degradation and subseque nt leakage of DNA from cells. After 48 h exposure to 10 mg/L HCPT, the apoptot ic rates of SGC-7901, MKN-45, and MkN-28 were 21.88%, 12.35%, and 30.26%, res pe ctively. After 48 h of exposure to 10 μmol/L As_2O_3, the apoptotic rates of SG C-7901, MKN-45, and MKN-28 were 13.84%, 22.52%, and 9.68%, respectively (F igure 4).

Effect of As_2O_3 and HCPT on cell cycle of SGC-7901 cells

The effect of As_2O_3 and HCPT on SGC-7901 cells show remarkable cell cycle specificity. There was no significant change in cell cycle after 10 μmol/L A s_2O_3 treatment for 24 h. The fraction of G_0/G_1 was decreased from 54.2% to 17.7%, while the fraction of G_2/M was significantly increased from 20.2% t o 63.4% with 10 ¦Imol/L As_2O_3 treatment in SGC-7901 cells for 48 h. The re sults showed that As_2O_3 induced gastric cancer cell death was in G_1 phase , blocked at G_2/M phase. The fraction of G_0/G_1 phase was decreased from 54 .2% to 37.6%, while the fraction of S phase was increased from 25.6% to 38.6% with 10 mg/L HCPT treatment in SGC-7901 cells for 48 h. The results showed that HCPT-induced apoptosis was at G_1 phase, and arrested at S phase.

DISCUSSION

Since the first observation of the relationship between arsenic and skin canc er in 1820s, arsenic compounds have been generally recognized as potent environ mental carcinogens, more likely as a co-mutagen and co-carcinogen for human sk in and lungs[8], although no animal model had been established[9]. Biochemically, it is documented that arsenic can inactivate some important enzymes by binding their sulfhydryl groups. Arsenic can also interfere with the p hosphorylation-dephosphorylation process by replacing the phosphorylation reaction. It has

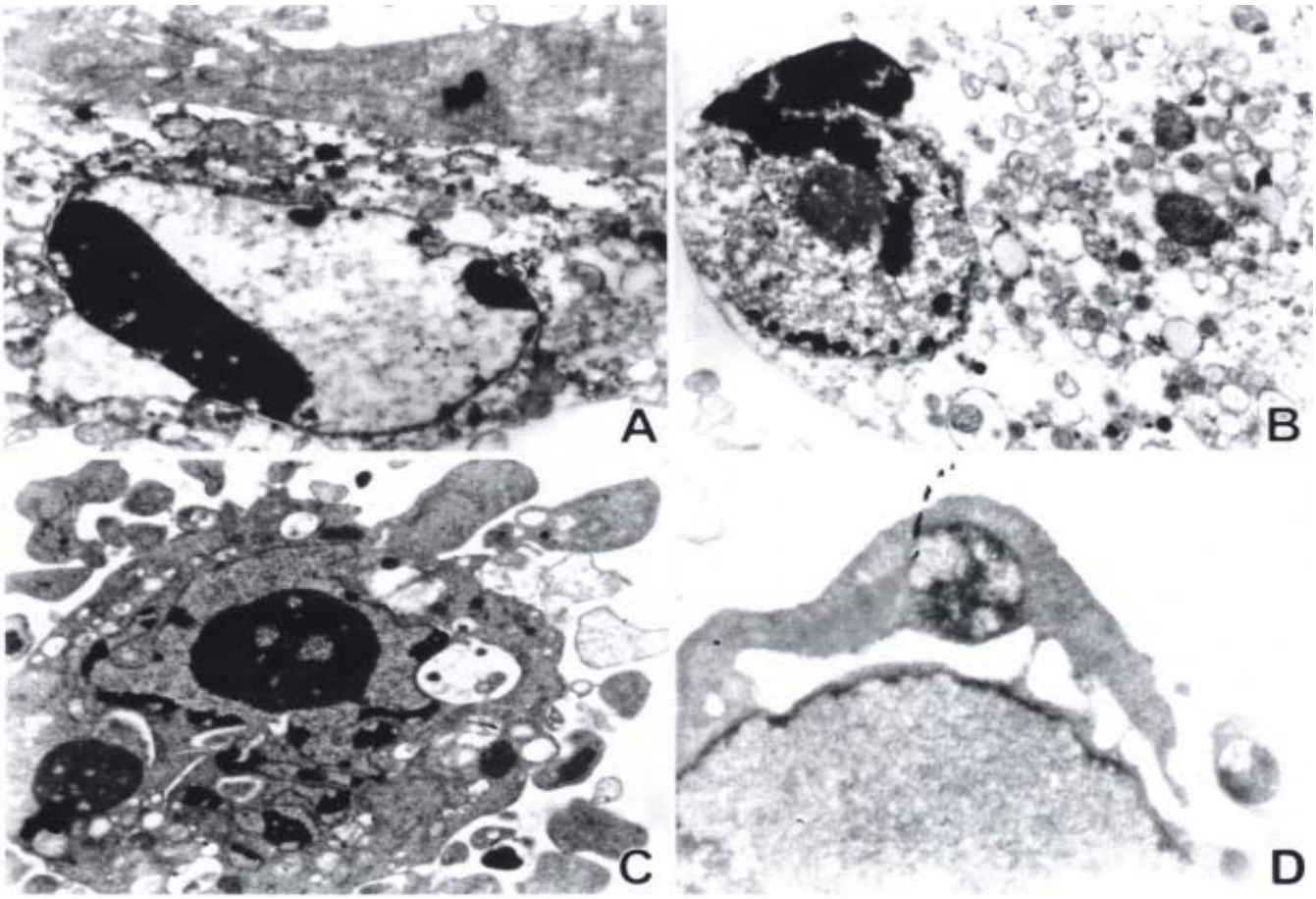

Figure 2 Electron microscopic observation of ultrastructural changes in gastric cancer ce lls treated with 10 μmol / L As_2O_3 for 48 h. The early change of apoptosis, nuclear chromatin condensation, looks like a new moon subjacent to the nuclear membranes (A). The intermediate stage of apoptosis presents cytoplasmic blebs, nu clear condensation, overflow of nuclear chromatin, and like-sprout (B). The late stage of apoptosis is characterized by splitting of nuclear membrane, nuclear chr omatin condensation, overflow of some parts of nuclear chromatin, and fragmentation of nucleus (C), and formation of apoptotic bodies (C) (D).

also been shown that arsenic can induce chromosome aberrations, sister-chromatin exchanges, DNA-protein cross links and protein-associated DNA-strand breaks in mammalian cells[10,11]. However, low concentration of some arsenic compounds also had some benefits to human physiologically,such as stimulation of human hematopoiesis. The use of arsenic compounds as drugs has a long history in Chinese traditional medicine. For example, it was recorded that ar senic had therapeutic effects on some human diseases such as psoriasis, syphilis, and rheumatosis. Recently, it has been shown that two arsenic compounds As_2O_3[2] and arsenic disulfide[12], which were used in some tradit ional Chinese prescriptions, are very effective in APL treatment. For instance, a report from the northeastern region of China showed that As_2O_3 (10 g/L v ia intravenous infusion for 28 to 60 days) induced clinical complete remission (CR) in 65.6% of APL patients. More interestingly, 28.2% (9/32) of patients survived more than 10 years. A more recent clinical trial with As_2O_3 trea tment also demonstrated that CR was achieved in 15 of 16 APL patients who relapsed after ATRA-induced and chemotherapy-maintained CR[4].

It has been suggested that As_2O_3 might induce apoptosis selectively in APL cells[13]. Furthermore, at pharmacological concentrations, it has no eff ect on the growth and survival of the leukemia myeloid cells U937 and HL60[3]. However, preliminary reports suggest that the apoptotic effect of As_2O_3 is not specific for APL cells but can be observed in various lines of either myeloid or lymphoid origin[14-18]and in blast cells from patients wi th non-M3 acute myeloid leukemia [19]. Another arsenic-containing comp ound, the melaminyl-phenyl-arsenoxide melarsoprol, which is used in the treatm ent of human African trypanosomiasis, has a broad efficacy against leukemia cells of both lymphoid and myeloid lineage[20]. Both As_2O_3 and melarsoprol also markedly induce apoptosis in plasma cell lines and in plasma cells from multiple myeloma[21]. Recent reports have demonstrated that As_2O_3 can induce apoptosis in various cell lines of solid tumor. As_2O_3 inhibited the growth and survival of solid tumor cell

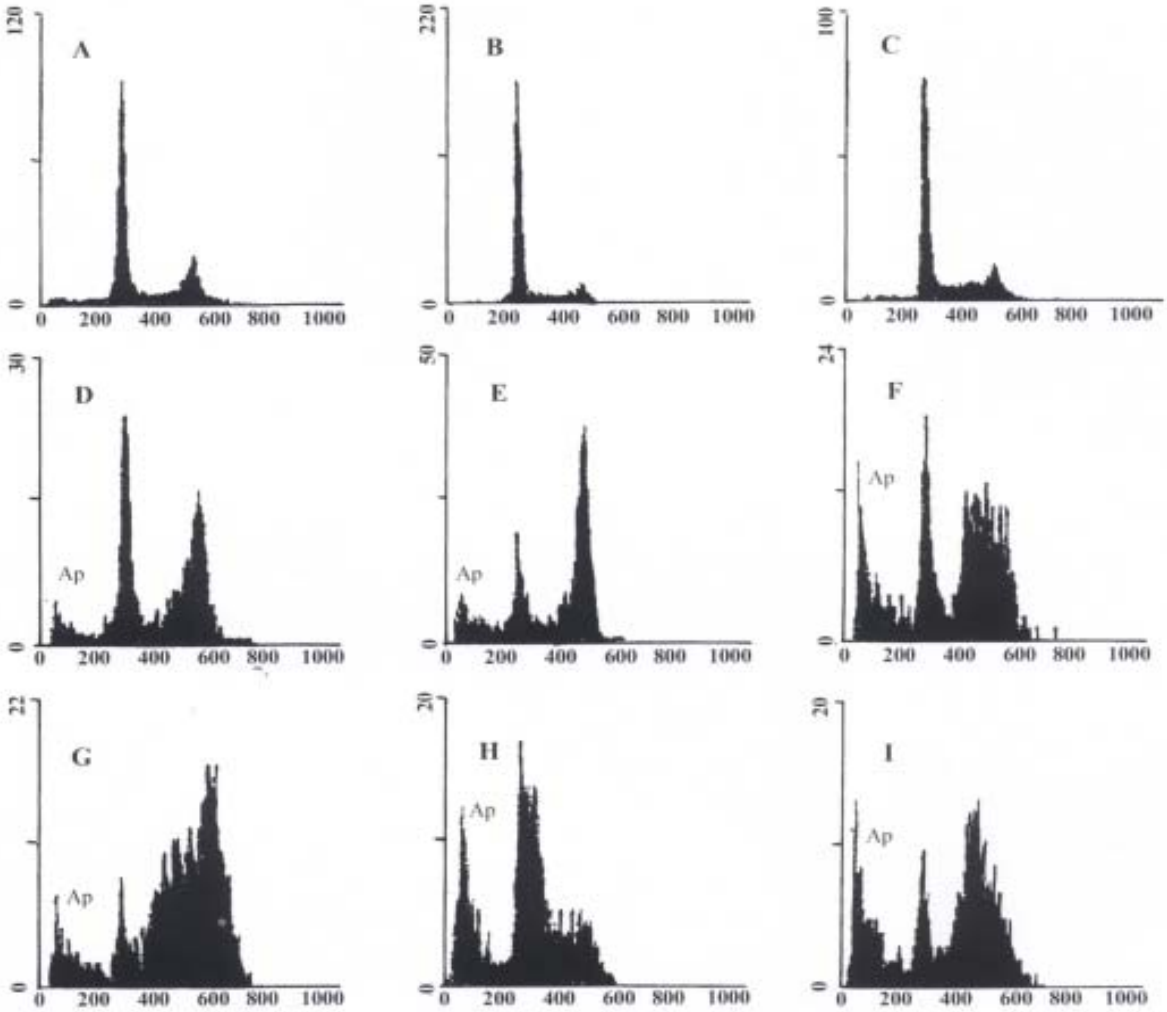

Figure 4 Nuclear DNA contents measured by flow cytometry in As_2O_3 and HCPT-induced apoptosis in gastric cancer cells at 48 h. Ap represents apoptotic cells. A: Untreated MKN-45 cells; B: Untreated SGC-7901 cells; C: Untreate d MKN-28 cells; D: MKN-45 cells treated with 10 μmol/L As_2O_3; E: SGC-79 01 cells treated with 10 μmol/L As_2O_3; F: MKN-28 cells treated with 10 μmol/L As_2O_3; G: MKN-45 cells treated with 10 mg/L HCPT; H: SGC-7901 c ells treated with 10 mg/L HCPT; I: MKN-28 cells treated with 10 mg/L HCPT.

lines in gastric[22], esop hageal[23,24], lung[24], cervix [25], liver[26] tumor and neuroblastoma[27] by triggering programmed cell death. These re sults suggest that As_2O_3 may be an effective drug in treatment of solid tum ors. As_2O_3 has been intensively investigated for the treatment of cancer.

There is growing evidence indicating that apoptosis plays a crucial role in both carcinogenesis and developement of stomach tumors[1,28-30]. Induct ion of apoptosis in gastric cancer cells might be a new means in the treatment of gastric cancer. It was reported that both radiation and chemotherapy (5-fluor ouracil, cisplatinum, mitomycin and trichosanthin) can induce apoptosis in gast ric cancer[31-33]. Our previous study showed that As_2O_3 had an effect on inhibiting proliferation and inducing apoptosis in gastric cancer cell SG C-7901[22]. In this study, we found that As_2O_3 exhibited a dose- and time-dependent cytotoxicity on gastric cancer cells at different degrees of differentiation. The IC_{50} of As_2O_3 on MKN-28, SGC-7901, and MKN-45 were 8.9 μmol/L, 10.5 μmol/L, and 11.6 μmol/L, respectively. Toxicity of As_2O_3 was not significantly different among gastric cancer cells at different de grees of differentiation. As_2O_3-treated gastric cancer cells presented char acteristic morphological changes of apoptotic cells. Results showed DNA degrada tion into oligonucleosomal fragments and these changes occurred in a time-and dose-dependent manner. The apoptotic index was 7%-15% as assessed by TUNEL. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. Af ter 48 h of exposure to As_2O_3, the apoptotic rates of SGC-7901, MKN-45 , and MKN-28 were 13.8%, 22.5%, and 9.68%,respectively. We concluded th at one of the main effects of As_2O_3 on gastric cancer cells at the concentrations u sed is to induce cell death by apoptosis.

It was recently shown[45] that the cell cycle time was prolonged in As_2O_3 treated malignant lymphocytes

Namalwa and Raji cells, and that no substan tial increase in cell cycle time was found in Jurkat cells treated with As_2O_3, as compared with untreated cells. This result suggests that As_2O_3 can inhibit proliferation of some malignant lymphocyte cell lines by prolonging the cell cycle instead of arresting cells in a specific phase. In the present study, we demonstrated that the effect of As_2O_3 on SGC-7901 showed remarkable cell cycle specificity in inducing cell death at G_1 phase, and blocking prolifer ation at G_2/M phase. This result is consistent with the report of Deng *et al*[46], in which As_2O_3 induced apoptosis in HeLa cells by arresting G_2/M phase of the cell cycle.

Camptothecin and its analogues are agents with a unique spectrum of antitumor activity mediated by a selective inhibition of eukaryotic DNA topoisomerase I (Topo I). The cytotoxicity of these compounds is pr edominantly exerted during S phase, and is associated with an inhibition of DNA replication. This inhibition is generally thought to be mediated by stablization of the CPT-Topo I-DNA cleavable complex[34]. Recent stud ies have shown that camptothecin and its analogues can strongly induce apoptosis in human leukemic cells[35-36], prostate[36-37], colon[38]and breast[39-40]cancer cells as well as glioma cells[41].

HCPT has been used in the treatment of gastrointestinal tumor[42,43], but its mechanism of anticancer action is still not completely understood. Recently, there were reports that HCPT can induce apoptosis of cancer cells of colon[47], pancreas[48], and bladder[49]. Our previous study showed HCPT can induce apoptosis in gastric cancer cell SGC-701 [44]. In this study, we found that HCPT had a strong dose- and time-dependent cytotoxicity to gastric cancer cells at different degrees of differentiation. The IC_{50} of HCPT on MKN-28, SGC-7901, and MKN-45 was 9.35 mg/L, 10.21 mg/L, and 12.62 mg/L, respectively. There was no significant difference in toxicity of HCPT on gastric cancer cells at different degrees of differentiatio n. Gastric cancer cells treated with HCPT presented characteristic morphological changes of apoptosis. The effects of inducing apoptosis in gastric cancer cells could be co rrelated with time and dosage of HCPT treatment. A typical subdiploid peak before G_0/G_1 phase was observed by flow cytometry. After 48 h of exposure to 10 mg/L HCPT, the apoptotic rates of SGC-7901, MKN-45, and MKN -28 were 13.8%, 22.5%, and 9.68%, respectively. In this study, the fraction of G_0/G_1 p hase was decreased from 54. 2% to 37.6%, while the fraction of S phase was in creased from 25.6% to 38.6% with 10 mg/L HCPT treatment of SGC-7901 c ells for 48 h. The result suggested that HCPT also showed a remarkable cell cycle specificit y, induced cell death and apoptosis at G_1 phase, and arrested proliferation at Sphase.

The molecular mechanism of As_2O_3 on APL cells showed that the inhibition of cell proliferation was due to a direct induction of apoptosis through downreg ulation of bcl-2 expression and modulation of PML/RARα/PML pro tein *in vitro* studies[3]. Furthermore, the activation of caspases was also involved in As_2O_3-induced apoptosis in APL cells [50]. Akao *et al* also reported that As_2O_3 induced apoptosis through the down-regulation of Bcl-2 protein and activation of caspases in B-cell leukemia cell lines[51]. It was also reported that As_2O_3 induced apoptosis in neuroblastoma cell line s through the activation of caspase-3[27]. Litvak *et al* reported that inhibition of gastric cancer by camptothecin involves apoptosis and multiple cellular pathways, and induction of apoptosis of gastric cancer cell was mediated by up-regulation of p53, p21Waf1/Cip1, and p27Kip1 and the down-regulation of Bcl-2 and Bcl-XL[52]. Camptothecin induced apoptosis thr ough activation of caspase in U-937 cells[53]. Our results showed that HCPT and As_2O_3 exerted significant cytotoxicity on gastric cancer cells and induction of apoptosis *in vitro*. The molecular mechanism of apoptotic effect of HCPT and As_2O_3 on gastric cancer cells the remains to be further investi gated, and the effect of apoptosis must be confirmed by *in vivo* studies. However , the results of the present study suggest that HCPT and As_2O_3 might be cand idate drugs to be used in the treatment of gastric cancer, thus needing further stud ies.

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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 AT1 receptor antagonist, losartan, on rat hepatic fibrosis induced by CCl4

Hong Shan Wei, Ding Guo Li, Han Ming Lu, Yu Tao Zhan, Zhi Rong Wang, Xin Huang,Jing Zhang, Ji Lin Cheng and Qin Fang Xu

Subject headings liver cirrhosis/drug therapy; renin-angiotensin system; angiotensin II type 1 receptor antagonist; losartan

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Abstract

AIM **To investigate effect o f losartan, an AT1 receptor antagonist, on hepatic fibrosis induced by CCl_4; and to determine whether or not AT1 receptors are expressed on hepatic stellate cells.**

METHODS AND RESULTS **Fifty male Sprague-Dawley rats, weighing (180 ± 20) g, were randomized into five groups (control group, model group, and three los artan treated groups), in which all rats were given the subcutaneous injection o f 40% CCl_4 (every 3 days for 6 weeks) except for rats of control group. Rats of losartan-treated groups were treated with losartan (20mg/kg, 10mg/kg, 5mg/ kg, daily gavage). After 6 weeks liver tissue and serum samples of all rats were examined. Serum hyaluronic acid (HA), procollagen type III (PC III) were detected by radioimmunoassays. van Giesion collagen staining was used to evaluate the extracellular matrix of rats with liver fibrosis. The expression of AT1 receptors, transforming growth factor-beta (TGF-β), and alpha-smooth muscle actin (α-SMA) in liver tissue were determined by immunohistochemical techniques. Compared with model group, serum ALT and AST of losartan-treated groups were significantly reduced (t = 4. 20, P <0.01 and t = 4.57, P<0.01). Serum HA and PC III als o had significant differences (t = 3.53, P<0.01 and t = 2.20, P<0.05). The degree of fibrosis was improved by losartan and correlated with the expressions of AT1 receptors, TGF-β, and α-SMA in liver tissue.**

CONCLUSION **AT1 receptor antagonist, losartan, could limit the p rogression of the hepatic fibrosis induced by CCl_4. The mechanism may be relat ed to the decrease in the expression of AT1 receptors and TGF-β, a meliorating the injury of hepatocytes; activation of local renin-angiotensin system mi ght relate to hepatic fibrosis; and during progression of fibrosis, activated hepatic stellate cells might express AT1 receptors.**

Department of Gastroenterology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Dr Hong Shan Wei, male, born on 1965-12-06 in Shandong Province, graduated from Jining Medical Collage and earned doctoral degree from Shanghai Second Medical University, majoring in hepatic fibrosis, having 15 papers published.
Correspondence to: Dr Hong-Shan Wei, Department of Gastroenterology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Tel. +86-21-65790000, Ext. 5319
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INTRODUCTION

Hepatic fibrosis, which may ultimately lead to cirrhosis, is associated with most chronic liver diseases, and is characterized by the net accumulation of extrac ellular matrix (ECM), including collagen, glycoproteins, and proteoglycans [1,2]. Many reports have suggested that hepatic stellate cells (HSCs) are the major producers of ECM in liver injury, and play a prominent role in liver fibro sis[3-7]. Tissue repair after acute liver damage involves "activation" of "quiescent" HSCs to myofibroblast-like cells[8-12]. Transform ing growth factor-beta (TGF-β) is a pleiotropic cytokine that has been assigned a key role in epithelial repair, and HSCs were shown to its main source[13-16]. In cultured HSCs, TGF-β-mediated up-regul ation of collagen and other ECM components mRNA was time and dose-dependent[17,18]. In the past years, significant progress has been made in our understanding of this pathologic mechanism, however, few effective drugs can slow the progression of the fibrosis[19,20].

Over the past decade, preventing the formation of angiotensin II by angiotensin-converting enzyme (ACE) inhibitors has revolutionized the therapy of hypertension and especially of congestive heart failure[21]. Recen tly, a number of studies demonstr ated that ACE inhibitors also effectively limited the progression of cardiac, renal and pulmonary interstitial fibrosis[22 - 26]. Recent work has shown that angiotensin II type 1 (AT1) recep tor antagonist, losartan, can also ameliorate the renal and cardiac fibrosis[27,28]. The prevailing hypothesis for the main mechanism was suppressing the expression of TGF-β in kidney and heart, rather than its dynamic effects[29-32].

Based on this and other information, we hypothesized that the AT1 receptor antagonist, losartan, could also limit the progression of hepatic fibrosis. To explore our speculation, the present study was designed to investigate the effect of losartan on rat's hepatic fibrosis induced by CCl_4, and determine whether or not there was expression of AT1 receptor on hepatic s tellate cells.

MATERIALS AND METHODS

Animals and reagents

Fifty male Sprague-Dawley rats, weighing 180 ± 20 g, were purchased from Animal Center of Shanghai Medical University (Shanghai, China). Losartan was obtained f rom MSD Co. (England). Polyclonal rabbit antibody to rat TGF-β was purchased from Boster Biotech Co. (Wuhan, China). Monoclonal antibody of alpha -sm ooth muscle actin (α-SMA) was purchased from Maixin Biotech Co. (Fuzhou, China). Hyaluronic acid (HA) and procollagen type III (PC III) radioimmunoassays kits were purchased from Navy Shanghai Medical In stitute (Shanghai, China).

Serum function tests

Fifty rats were randomized into five groups (control group, model group and thre e losartan-treated groups) in which all rats were given subcutaneous inject ion of 40% CCl_4 (0.3 mL/100 g, every 3 days for 6 weeks) except for rats o f co ntrol group (only given injection of same dose of olive oil). In an initial exp eriment, rats of losartan-treated groups were treated with losartan (20 mg/kg, 10 mg/kg, 5 mg/kg by daily gavage). After six weeks, all rats were sacrificed. Serum was collected and stored at -20℃ for analysis of aspartate transaminase (AST) and alanine transaminase (ALT) activity by standard enzymatic methods. The serum levels of PC III and HA was determined by radioimmunoassays.

Immunohistochemical detections and histological data

The liver sections were fixed in a 10% solution of formaldehyde in 0.1 mol/L pho sphate-buffered saline (pH 7.4), and embedded in paraffin. Five-micrometer sli des were prepared. van Giesion collagen staining was used to evaluate the ECM o f rats. According to van Giesion collagen staining, the degree of fibrosis was divided into five grades (0-5). Specimens were scored blindly by the histologist and were also ranked blindly for severity of fibrosis. The expression of AT1 receptor (anti-rat rabbit polyclonal antibody was the product of Santa Cruz Biote ch Co), TGF-β and α-SMA were detected by immunohisto chemical techniques.

Statistics

Data are presented as $\bar{x} \pm s_{\bar{x}}$. Comparision between two groups was made using Student's *t* test. Difference of fibrosis between model and losartan-treated groups was compared using Ridit analysis. The test was con sidered significant at $P < 0.05$.

RESULTS

Serum function tests

Compared with control group, ALT and AST increased significantly in fibrotic rats in the model group, but only marginally in losartan treated rats. ALT and AST activities were significantly lower in 20 mg losartan-treated group than in model group of CCl_4 rats. The effects were associated with doses of losartan (Tab le 1).

Table 1 Serum function tests

	n	LT (U/L)	AST (U/L)
Control	10	99.50 ± 18.78	244.50 ± 46.52
Model	7	1863.29 ± 893.68[a]	2680.00 ± 1039.12[a]
Losartan (20 mg/kg)	7	432.14 ± 112.26[d]	824.57 ± 265.41[d]
Losartan (10 mg/kg)	7	535.25 ± 200.78[d]	77.50 ± 270.32[d]
Losartan (5 mg/kg)	6	771.71 ± 237.18[c]	1643.00 ± 810.36

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* model; [d]$P < 0.01$ *vs* model

Serum components of ECM

As expected, serum levels of HA and PCIII increased in rats of model group. Serum HA levels were approximately three times higher in rats of model g roup than rats of control group. There was a tendency towards a decrease in HA a nd PC III levels in losartan treated group (Table 2).

Table 2 Serum component of ECM

	n	HA (ng/L)	PC III (μg/L)
Control	10	331.42 ± 42.31	19.06 ± 4.43
Model	7	911.66 ± 345.49[a]	31.82 ± 6.90[a]
Losartan (20 mg/kg)	7	425.05 ± 115.80[d]	22.78 ± 8.38[c]
Losartan (10 mg/kg)	7	556.11 ± 195.22[c]	24.49 ± 2.73[c]
Losartan (5 mg/kg)	6	734.03 ± 318.93[c]	24.19 ± 6.76

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* model; [d]$P < 0.01$ *vs* model

Histological data

Piecemeal and lobular necrosis was obvious in the CCl_4 model compared to rats in control group. The lobular necrosis was significantly decreased by losartan i n three treated groups. There was an increase in the area of fibrosis in model r ats compared with rats in control group. There was a significant decrease in the losartan treated rats (Table 3, Figure 1).

Table 3 Degree of fibrosis

	n	0	I	II	III	IV	*U* -value
Control	10	10	0	0	0	0	
Model	7	0	0	1	2	4	
Losartan (20 mg/kg)	7	0	2	3	1	1	2.05[a]
Losartan (10 mg/kg)	8	0	2	4	1	1	2.31[a]
Losartan (5 mg/kg)	7	0	1	3	2	1	1.49

[a]$P < 0.05$ *vs* model

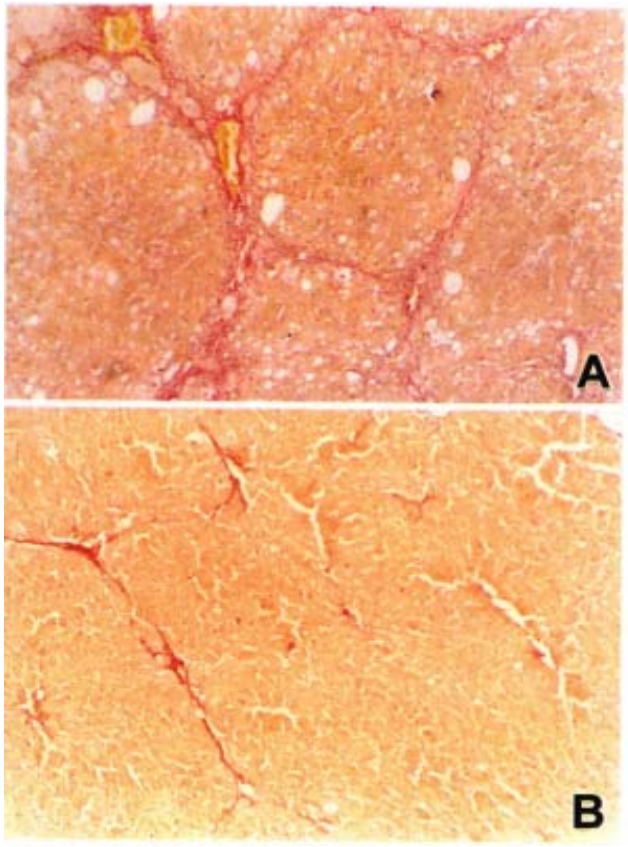

Figure 1 Collagen expression in liver section of model rat (van Gieson staining, × 200) (A), and of losarta n treated group (van Giesion staining, × 200). Significantly less fibrosis can be noted compared to that in the model group (B).

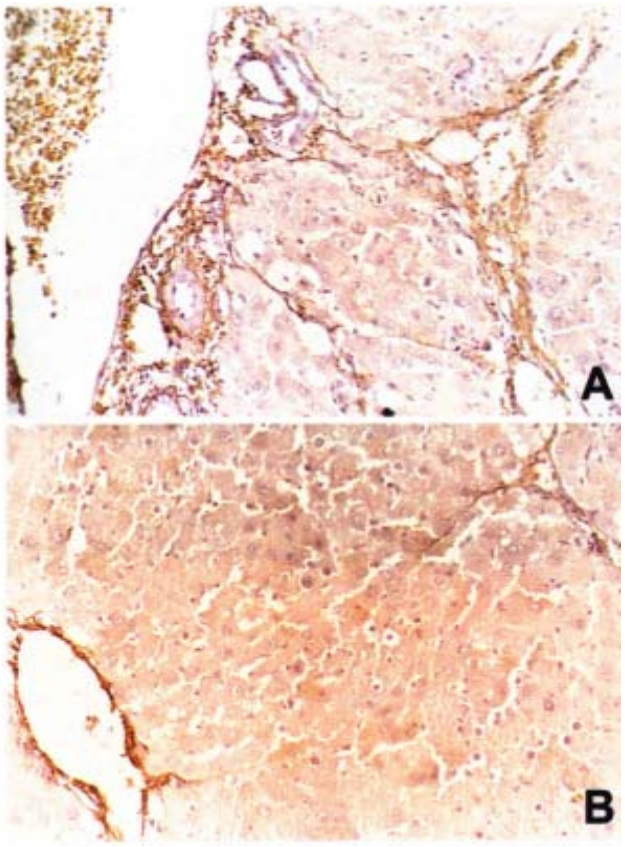

Figure 2 AT1 receptor expression in liver section of model rat (DAB staining, × 200). AT1 receptor is seen mainly scattered in fibrotic areas and vascular wal l (A), and of los artan treated group (DAB staining, × 200). Note significantly less AT1 expression than that seen in the model group (B).

Expression of AT1 receptors, TGF-β, and α-SMA

Compared with normal liver tissue, in which AT1 receptors mainly locate in vasculature, in the fibrotic liver tissue, the expression of AT1 receptors significan tly enhanced, and scattered in fibrotic areas. The express ion of AT1 receptors was markedly reduced by losartan (Figure 2). The expressio n of α-SMA was a marker of HSC activation. Immnohistochemical dete ction demonstrated that vascular smooth muscle cells and pericytes were positive for α-SMA in control livers, whereas HSCs strongly positive of α-SMA were observed in rats of model group, and they were scattered along the sinusoidal walls. Many α-SMA-positive HSCs were detecte d in the area of centrilobular and periportal fibrotic bands in rats of model group. Compared with model group, liver of rats treated with losartan, showed mark edly reduced numbers of α-SMApositive HSC. At same time, its serum levels of PC III and LN were also significantly decreased ($P < 0.05$).

Similar to the α-SMA, TGF-β was also strongly expre ssed in areas of periportal fibrotic bands in rats of model group. In contrast, livers of rats treated with losartan showed significantly reduced numbers of TGF -β-positive cells (Figure 3).

DISCUSSION

Our study firstly demonstrated that AT1 receptor antagonist, losartan, could slow the progression of hepatic fibrosis induced by CCl_4. Activated HSC might express AT1 receptors in fibrotic liver tissue.

Over the last decade, many lines of evidence have demonstrated that local RAS ac tivation was the major mechanism of cardiac and renal interstitial fibrosis[33-37]. *In vivo* studies have shown that ACE inhibitors and AT1 receptor s antagonists can limit the progression of cardiac, renal, and pulmonary fibrosi s[23,38-41], and the mechanism is independent of their dynamic effects. This is based on several *in vitro* and *in vivo* findings. First, all cardi ac and renal fibroblasts express AT1 receptors; secondly, Ang II ind uces mitogenic response, increases protein synthesis, production of collagen, and TGF-β in fibroblasts in a dose-dependent manner[29,42,43]. These results support the notion that Ang II can both directly act on fibroblasts and/or enhance the expression of TGF-β.

In the present study, the three different doses (20 mg/kg, 10 mg/kg, 5 mg/kg) of losartan were given to the rats. As the results shown, losartan could limit the progression of the hepatic fibrosis in a dose-dependent

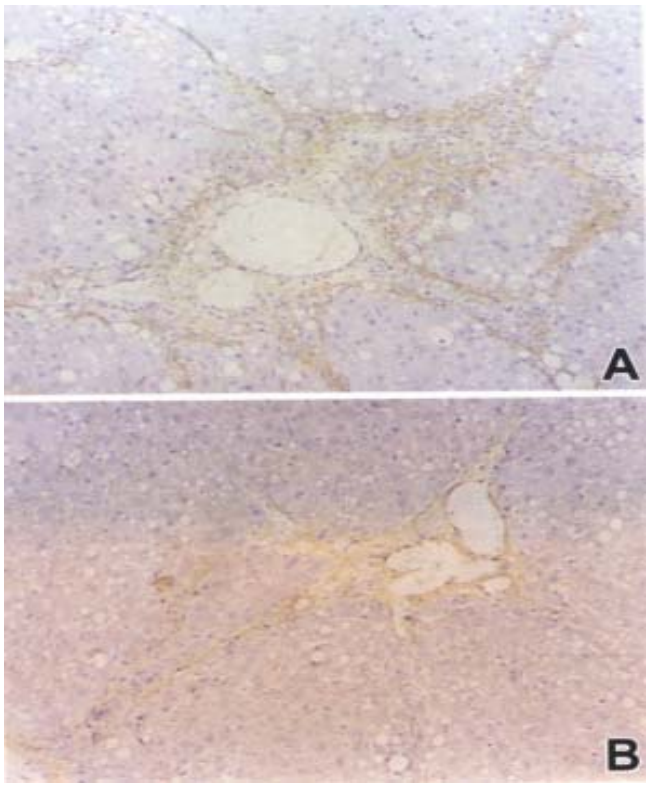

Figure 3 TGF-β expression in liver section of model rat (DAB staining, × 200). TGF-β expression is seen scattered in the fibrotic areas and vascular wall (A), and of losart an treated group (DAB staining, × 200). Note significantly less TGF-β expression than that seen in the mod el group (B).

manner. At the dose not influencing systolic blood pressure (10 mg/kg) in the normotensive rats[44], losartan could attenuate the fibrosis, but even at a very low dose (5 mg/kg) , had a weak but significant effect. In agreement with this study, Boffa *et a l*[45] also reported that losartan completely prevented collagen I gene activation and attenuated the degree of fibrosis without influencing the systolic pressure in the kidneys of transgenic mice. This suggested that Ang II might act as an important regulator in the progression of hepatic fibrosis induced by CCl_4.

Using the immunohistochemical methods, we observed the expression of AT1 recepto rs in liver tissue. As the results shown, this is the first report that confirms that the liver tissue expresses AT1 receptors. Compared with normal rats, wher e AT1 receptors are mainly located in the vasculature, the expression of AT1 rec eptors were significantly enhanced in rats of model group, which were mainly loc ated in fibrotic area, and correlated with the degree of fibrosis. The results s ugge sted that the expression of AT1 receptors might relate to hepatic fibrogenesis. Furthermore, immunostaining indicated that the distribution of AT1 receptor corr ela ted with the expression of TGF-β and α-SMA. As has b een recently reported, in a model of chronic cyclosporine (CsA), TGF-β played a role in CsA-induced tubulointerstitial fibrosis and arteriolopath y by stimulating ECM protein synthesis and inhibiting ECM degradation. Losartan resulted in decreasing expression of TGF-β and synthesis of ECM[46]. Zhang *et al*[28] have also reported that losartan is effe ctive in reducing the increasing expression of AT1 receptors and ECM protein in infarcted heart tissue. Based on the above data, we postulated that expression o f AT1 receptor might be related to the activation and ECM synthesis of HSCs.

Another interesting finding in our study was that losartan could ameliorate the hepatocyte injury, as reflected by the release of liver enzymes. The explanatio n for this observation is not known, but it might relate to protection of the h epatocyte from free radical-mediated damage. Recently, Anthuber *et al*[47] demonstrated that non-thiol-containing ACE inhibitor, enalapril, could attenuate the hepatocyte injury induced by ischemia/reperfusion. The prevaili ng mechanism of action was considered to relate with modulation of the angiotens in, bradykinin, and prostacyclin metabolism. Whether losartan has some as-yet-unknown, specific, protective property, remains to be determined in future studies.

That transforming growth factor β (TGF-β) is a key m olecule responsible for tissue fibrosis, provides a basis for targeting TGF-β as an antifibrotic agent [48-50]. Recently, Sun *et al*[31]found that the early induction of TGF-β1 via the angiot ensin II type 1 receptor played a major role in the development of cardiac fibrosis in infarcted heart. Shihab *et al*[46] described that losartan reduced TGF-β overproduction in a dose-dependent manner, slowing the rate of renal interstitial fibrosis[22].

Overproduction of TGF-β and activation of HSC are key processes in the progression of hepatic fibrosis. Our results demonstrated that losartan could reduce the expression of TGF-β and α-SMA in liver tissue and suppress fibrosis procession. It is suggested that RAS also parti cipates in the progression of hepatic fibrosis induced by CCl_4 in rats.

In conclusion, our results demonstrated that (1) AT1 receptor antagonist, losar tan, could limit the progression of the hepatic fibrosis induced by CCl_4. Th e mechanism may be related to the decrease in the expression of AT1 receptors an d TGF-β, ameliorating the injury of hepatocyte; (2) activation of local renin-angiotensin system might relate to hepatic fibrogenesis; (3) in the progression of fibrosis, activated hepatic stellate cells might express AT1 receptor.

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Edited by Zhu QR
proofread by Mittra S

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

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Participation of CD45, NKR-P1A and ANK61 antigen in rat hepatic NK cell (pit cell)mediated target cell cytotoxicity

Dian Zhong Luo1,4, David Vermijlen1, B¨1lent Ahishali1, Vasilis Triantis1, Karin Vanderkerken2 Peter J.K. Kuppen3 and Eddie Wisse1

Subject headings hepatic NK cells; pit cells; cytolysis; apoptosis; perforin/granzyme pathway

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Abstract

AIM **Several triggering receptors have been described to be involved in natural killer (NK) cell-mediated target cytotoxicity. In these studies, NK cells derive d from blood or spleen were used. Pit cells are liver-specific NK cells that possess a higher level of natural cytotoxicity and a different morphology when com pared to blood NK cells. The aim of this study was to characterize the role of t he NK-triggering molecules NKR-P1A, ANK61 antigen, and CD45 in pit cell-media ted killing of target cells.**

METHODS **^{51}Cr-release and DNA fragmentation were used to quanti fy target cell lysis and apoptosis, respectively.**

RESULTS **Flow cytometric analysis showed that pit cells expressed CD45, NK R-P1A, and ANK61 antigen. Treatment of pit cells with monoclonal antibody (mAb) to CD45 (ANK74) not only inhibited CC531s or YAC-1 target lysis but also apopto sis induced by pit cells. The mAbs to NKR-P1A (3.2.3) and ANK61 antigen (ANK61) had no effect on pit cell-mediated CC531s or YAC-1 target cytolysis or apoptosis, while they did increase the Fcγ receptor positive (Fcγ R$^+$) P815 cytolysis and apoptosis. This enhanced cytotoxicity could be inhibited by 3,4-dichloroi socoumarin, an inhibitor of granzymes.**

CONCLUSION **These results indicate that CD45 participates in pit cell-med iated CC531s and YAC-1 target cytolysis and apoptosis. NKR-P1A and ANK61 antig en on pit cells function as activation structures against FcγR$^+$ P 815 cells, which was mediated by the perforin/granzyme pathway.**

INTRODUCTION

Natural killer (NK) cells can kill certain tumor cells or virus-infected cells without prior sensitization and thus play an important role in host anti-tumor defense[1]. Cytotoxic activity of NK cells is achieved by two distinct mechanisms, i.e. necrosis (cytolysis) and apoptosis, and is believed to be regula ted by triggering and inhibiting receptors on NK cells[1-3]. Several candidate receptors involved in triggering a cytotoxic reaction, such as NKR-P1A (CD161A) and an NK-activation structure recognized by monoclonal antibody (mAb) ANK61 (ANK61 antigen), have been described on rat NK cells [2,4,5].

NKR-P1A is a lectin-like surface molecule expressed on all rat NK cells[4]. The ligands for NKR-P1A are oligosaccharides. Interactions of these oligo saccharides on target cells with NKR-P1A on NK cells are crucial for target cell recognition and for NK cytotoxicity[6]. Monoclonal antibody to NKR -P1A (3. 2.3) has be en shown to stimulate NK cell phosphoinositide turnover, calcium mobilization, degranulation and NK cytotoxicity against certain Fcγ receptor posit ive (FcγR$^+$) tumor target cells[4,7]. These findings demonstrate that NKR-P1A is an activating molecule on rat NK cells that recognizes target cells and induces cytotoxicity.

Recently, a rat NK-activation structure (ANK61 antigen) recognized by mAb ANK61 has been described [5]. Cross-linking of the ANK61 antigen and FcγR$^+$ tumor targets with mAb ANK61 resulted in enhanced killing of the targets by NK cells[5].

CD45, which is expressed by most leukocytes, including NK cells, has been shown to be involved in NK

[1]Laboratory for Cell Biology and Histology.
[2]Department of Hematology and Immunology, Free University of Brussels (VUB), Brussels-Jette, Belgium; K Vanderkerken is a postdoctoral researcher of the Fund for Scientific Research Flanders.
[3]Department of Surgery and Pathology, Leiden University Medical Center, Leiden, The Netherlands
[4]Department of Pathology, Guangxi Medical University, Nanning, China LUO Dian-Zhong, graduated from Guangxi Medical University in 1982, got Master d egrees in 1987 in Guangxi Medical University and in 1994 in Free University of B russels, 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. 32-2-4774404, Fax. 32-2-4774405
Email. wisse@cyto.vub.ac.be
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cell-mediated target cell cytotoxicity[8-10]. It is reported that mAbs against CD45 inhibit NK cellmediated target cell killin g[8,9] and CD45-negative NK cells fail to lyse tumor targets[10]. The cytoplasmic domain of CD45 has tyrosine phosphatase activity, which is believed to play an important role in the regulation of NK activity[8,11] .

Pit cells are liver-specific NK cells[12]. Compared to blood NK cells, rat pit cells possess a higher level of cytolytic activity against a variety of tumor target cells, including NK resistant P815 cells, which is comparable to the cytotoxicity level of lymphokine-activated killer (LAK) cells[13]. These data indicate that pit cells have different characteristics from blood NK cells. Although much is known about the NK receptors that participate in NK cell a ctivation and target cell killing, the surface receptors involved in pit cell-mediated target cell killing have not yet been clarified. Moreover, involvement o f the above mentioned antigens in NK cell-mediated target cell apoptosis has re mained elusive, since ^{51}Cr-release assay employed in the previous stu dies[4,5,10] indicates only cytolysis, rather than apoptosis. In the pre sent work, we used mAbs against CD45, NKR-P1A, and ANK61 antigen to evaluate their roles in pit cell-mediated target cell lysis and apoptosis.

MATERIALS AND METHODS

Isolation and purification of pit cells

Male Wistar rats (Proefdierencentrum, Leuven, Belgium) weighing (250-280) g were used at an age between 8 to 12 wk. Hepatic NK cells (pit cells) were iso lated according to protocols described before[14]. The purity of the rec overed pit cells was at least 90%, as evaluated by morphology on May-Giemsa-st ained cytospin preparations and by flow cytometric analysis using mAb 3.2.3. The viability of the recovered cells was more than 95%, as determined by Trypan blue exclusion. The procedures used in this study were approved by the local ethical committee (license No LA1230212).

Tumor cell line

CC531s, a dimethylhydrazine-induced colon carcinoma of Wag/Rij (inbred strain o f Wistar) rats[15], P815, a murine mastocytoma, and YAC-1, a mouse T cell lymphoma, were maintained in culture medium RPMI-1640 (Gibco, Life Technolog ies, Gent, Belgium), supplemented with 10% fetal calf serum (Eurobiochem, Bierges, Belgium), benzylpenicillin (100 kU/L), streptomycin (100 mg/L) , and glutamine (0.2 mmol/L) (Gibco, Life Technologies, Gent, Belgium).

Reagents and antibodies

The following mouse anti-rat mAbs were used: 3.2.3 (anti-NKR-P1A, IgG_{2b} or IgG_1)[4],ANK74 (anti-CD45, IgG_1)[8],and ANK61 (anti- r at NK activation structure, IgG_1)[5] were developed in Department of S urgery and Pathology, Leiden University Medical Center, the Netherlands. 3,4-d ichloroisocoumarin (DCI), a granzyme inhibitor[16]was purchased from Sigma (Bornem, Belgium).

Flow cytometry

The expression of surface antigens on the cells was measured by one or two-colo r flow cytometric analyses as described previously[17]. Briefly, a quant ity of 0.5 × 10^6 cells per sample was incubated (30 min, 4℃) with the prim ary antibodies, 3.2.3, ANK74, and ANK61. Cells were then washed thrice w i th cold phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.02% sodium azide. Subsequently, cells were incubated with fluorescein-conjuga ted anti-mouse IgG_1 and biotin-conjugated anti-mouse IgG_{2b} (Gilbertsv ille, PA). After incubation and washing, cells were incubated with streptavidin-phycoerythrin (Gilbertsville, PA). Then, cells were washed and fixed with 2% p araformaldehyde in PBS and analyzed (FACstar, Becton Dickinson, Mountain View, CA). Isotype-matched irrelevant antibodies were used as a control.

^{51}Cr-release assay

Cytolysis was measured after 4 h incubation of P815 and YAC-1 cells, and 18 h for CC531s cells. ^{51}Cr-release assay was performed using round -bottomed 96-well microplates as described previously[18]. Briefly, 1 × 10^6 target cells/0.5 mL were labeled with 9.25 MBq Na $^{51}CrO_4$ (A mersham Belgium, Gent, Belgium) for 80 min at 37℃. Before the coincubation, freshly isolated pit cells were preincubated with either saturating amounts of the mAbs (final concentration for each mAb was 10 mg/L), 3.2.3, A NK61, ANK74, or irrelevant mouse IgG_1 for 15 minutes at room temperature. The mAbs were al so present during the assay and the treatments with these mAbs did not affect th e viability of the cells. The target cells were seeded at a concentration of 1 × 10^4 cells/ well. Suspensions of effector cells were then added to the wells at effector to target (E:T) ratios of 10:1 in a final volume of 200 μL . All assay s were done in triplicate. For the experiments using DCI, pit cells were preincu bated with 50 μmol/L DCI for 30 min at 37℃. After washing twice with medium, the cells were coincubated with the target cells as mentioned above. After a coincubation at 37℃ for 4 or 18 h, 100 μL of supernatant per well was aspi rated and counted in a gamma counter to determine experimental release. Results were expressed as percentage of specific lysis according to the formula:

$$\% \text{ specific cytolysis} = \frac{\text{Experimental release-Spontaneous release}}{\text{Maximal re lease-Spontaneous release}} \times 100\%$$

Experimental release represents cpm (counts per minute) release from target cells in the presence of effector cell and mAbs. Spontaneous release was obtained fr om wells containing labeled target cells and medium only. Maximal release of ^{51}Cr-labeled target cells was determined

after adding 20 μL 10% sodium dodecyl sulfate (SDS) detergent to the labeled target cells.

Quantitative DNA fragmentation assay

DNA fragmentation in the target cells was determined as described previously[19]. Briefly, to label the DNA of the target cells, 5×10^5 cells in 250 μL medium were incubated with 0.37 MBq [methyl-^{3}H] thymidi ne ([^{3}H]-TdR) (Amersham, Buckinghamshire, U.K) for 3 h at 37℃. Bef ore the coincubation, freshly isolated pit cells were preincubated with the mAbs as mentioned above. The pit cells (10^5 cells in 100 μL) and the target cells (10^4 cells in 100 μL) (E:T = 10:1) were placed in trip licate in 1.5 mL microcentrifuge tubes. The total volume per microcentrifuge tub e was 200 μL. For the experiments using DCI, pit cells were preincu bated with 50 μmol/L DCI for 30 min at 37℃. After washing twice with medium, the cells were coincubated with the target cells as mentioned above. After 3 h coincubation at 37℃ and centrifugation at 300 × g for 10 min, the incubation medium was removed from the tubes. Sub sequently, the pelleted cells were lysed with 0.5 mL of cold lysis buffer (4℃) (5 mmol/L Tris, 2 mmol/L EDTA, 1% Triton X-100, pH 7.4) for 30 min at 4℃. Then, the lysates were ultracentrifuged (10,000 × g for 15 min at 4℃) to se parate fra gmented DNA from intact DNA. Radioactivity (cpm) in the incubation medium, in th e 10,000 × g supernatant and in the 10,000 × g pellet was determined in a beta count er (Beckman, Fullerton, CA, USA). The percentage fragmented DNA was calculated u sing the following formula:

$$\%\ \text{specific DNA fragmentation} = \frac{cpm_{fr,exp} - cpm_{fr,spont}}{cpm_{total} - cpm_{fr,spont}} \times 100\%$$

in which: cpm_{fr} = the radioactivity in the incubation medium plus the cpm i n the 10,000 × g supernatant; cpm_{total} = cpm_{fr}+radioac tivity in the 1 0,000 × g pellet; exp = experimental (CC531s cells with pit cells); spont = spont aneous (CC531s cells and medium only).

Hoechst 33342 (HO 342)/propidium Iodide (PI) staining

The method of HO 342 and PI staining has been described previously[19]. In short, target cells, at a concentration of 1×10^4 cells per well in a 96m ultiwell plate, were coincubated with pit cells (E:T = 10:1). After 3 h coinc ubation, the cells were stained with HO 342, which stains DNA blue, and PI, which only penetrates necrotic cells through the damaged cell membrane and stains DN A red. Cells were viewed under a Leica DM IRB/E inverted fluorescence microscope (Leica, Heidelberg, Germany) with ultraviolet excitation at 340 to 380 nm. The apoptotic target cells were determined by their characteristic apoptotic morphol ogical changes of the nucleus, i.e. condensation of chromatin and nuclear fragmentation. Target cells alone acted as spontaneous apoptosis control.

Statistical analysis

Results were given as the mean with the corresponding standard deviation. Statis tical analysis was performed by Students't test or one-way ANOVA with pos t-hoc multiple comparison analysis made by Duncan test, using SPSS statistical package (SPSS Inc., Chicago, IL, USA). Statistical significance between two groups was considered at the level of $P < 0.05$.

RESULTS

Expression of surface antigens on pit cells

Flow cytometric analysis was used to determine the expression of surface antigens on pit cells and target cells. In two-color flow cytometric analysis, mAb 3.2 .3 was used to stain pit cells[20]. Pit cells were shown to express CD45 , which was recognized by mAb ANK74, and an NK activation structure, which was recognized by mAb ANK61 (Figure 1). CC531s, P815, and YAC-1 cells did not express these NK-associated antigens (data not shown).

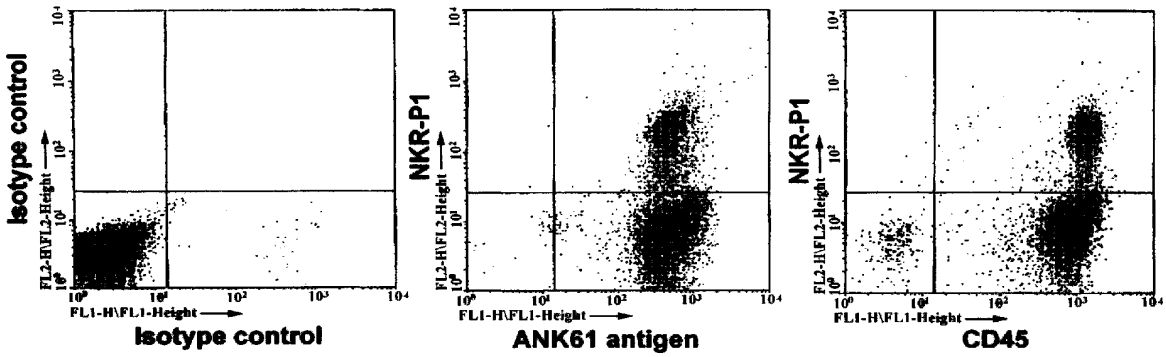

Figure 1 Expression of CD45 and ANK61 antigen on hepatic NK cells (pit cells). T he cells were washed out of the liver by sinusoidal lavage. After Ficoll-Paque gradient centrifugation and nylon-wool adherence to remove erythrocytes, granul ocytes, monocytes and B lymphocytes, the cells were stained with anti-NKR-P1A mAb (3.2.3) (phycoerythrin) and anti-ANK61 antigen (ANK61), anti-CD45 mAb (ANK 74) (fluorescein) and analyzed by two-color flow cytometry. CD45 and ANK61 anti gen were expressed on the x-axis (fluorescein), NKR-P1A on the y-axis (phycoe rythrin).

Effect of CD45, NKR-P1A, and ANK61 antigen on pit cell-mediated target cell lysis

To investigate the function of these surface antigens in pit cell-mediated cyto lysis of targets, the mAbs against these antigens were used in ^{51}Cr-r elease assays. In the presence of anti-CD45 mAb ANK74, the lysis of CC531s and YAC-1 cells by pit cells was significantly inhibited ($P < 0.01$, $P < 0.05$) (Figure 2A, 2B). The presence of mAbs 3.2.3 and ANK61 had no effect on pit cell- mediated lysis against-CC531s and YAC-1 targets (Figure 2A, 2B). These data in dicate that CD45 is involved in pit cellmediated cytolysis against CC531s and YAC-1 targets.

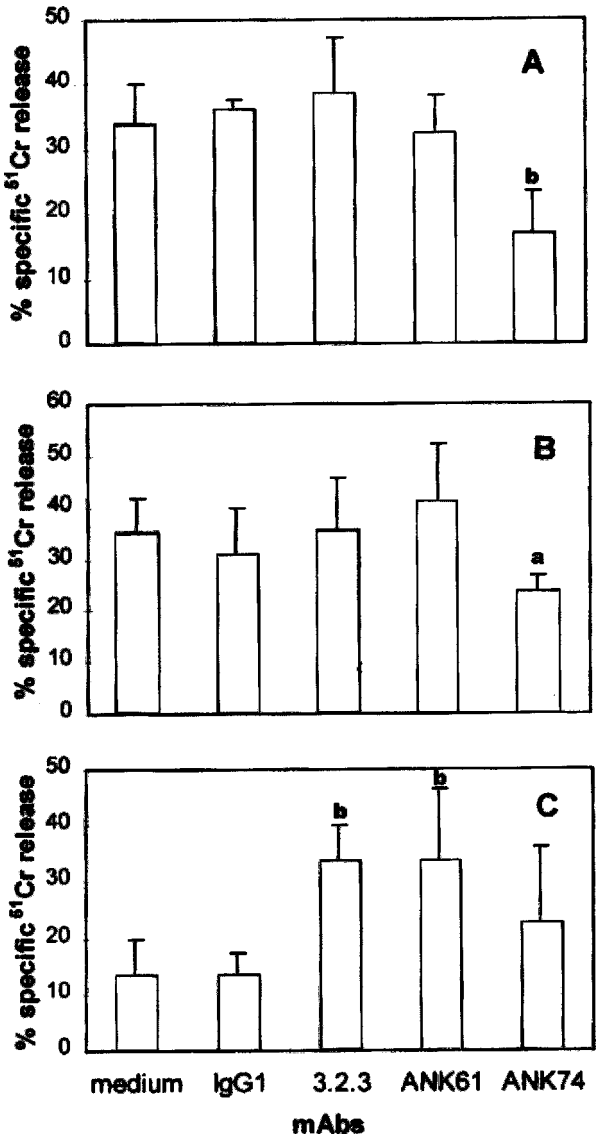

Figure 2 Effect of the mAbs on pit cell-mediated CC531s (A), YAC-1 (B) and P81 5 (C) target cell cytolysis.^{51}Cr-labeled target cells were incubated at an E:T ratio of 10:1 with freshly isolated pit cells in the absence or prese nce of the mAbs. (A), Cytolysis of CC531s cells was measured in an 18h ^{51}Cr-release assay. (B), Cytolysis of YAC-1 cells was measured in a 4-h ^{51}Cr-release assay. (C), Cytolysis of P815 cells was measured in a 4-h ^{51}Cr-release assay. Values are mean ± SD from four differen t experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the control .

It is reported that mAbs 3.2.3 or ANK61 can induce cytolysis in Fcγ R^{+} target cells by NK cells by redirected antibody- dependent cellular cytotoxicity (rADCC)[4,5]. In order to investigate whether these mAbs play a similar role in pit cells, cytolytic activity of pit cells was measured by a 4-h ^{51} Cr-release assay using the Fc γR^{+} P815 cell line[21], a murine mast ocytoma, as target cells. As shown in Figure 2C, the mAbs 3.2.3 and ANK61 signif icantly increased the cytolysis of P815 cells by pit cells ($P < 0.01$). Ant i-CD45 mAb ANK74 did not significantly increase the cytolysis (Figure 2C).

Effect of CD45, NKR-P1A, and ANK61 antigen on pit cell-mediated target apoptosis

When CC531s[19], P815 (Vermijlen D, unpublished data) or YAC-1 targets (Figure 3) were coincubated with pit cells, these target cells showed typical mo rphological characteristics of apoptosis like nuclear fragmentation. To address the effect of the surface antigens in pit cell-mediated apoptosis of these targets, a quantitative DNA fragmentation assay was used. The results showed that the anti-CD45 mAb ANK74 significantly inhibited pit cell-mediated DNA fragmentat ion of CC531s and YAC-1 cells ($P < 0.05$, $P < 0.01$) (Figure 4A, 4B). The pre sence of mAbs 3.2.3, ANK61 had no effect on DNA fragmentation of CC531s and YAC -1 cells by pit cells (Figure 4A, 4B), but increased the DNA fragmentation of P 815 cells induced by pit cells ($P < 0.01$) (Figure 4C). The anti-CD45 mAb ANK7 4 did not significantly affect the DNA fragmentation of P815 cells by pit cells (Figure 4C).

Inhibition of anti-NKR-P1A and ANK61 antigen mAb-enhanced P815 cell cytotoxicity by DCI

To address whether mAbs 3.2.3 and ANK61 enhanced FcγR^{+}P815 cell k illing by pit cells via the perforin/ granzyme pathway, we used DCI, an inhibitor of granzymes in intact cells, to investigate the role of granzymes in the process. When pit cells were preincubated with 50 μmol/L DCI, the mAb-enhanced P81 5 cytolysis and apoptosis by pit cells were completely inhibited ($P < 0.05$, $P < 0.01$) (Figure 5A, 5B). These results suggest that mAbs 3.2.3 or ANK61 induce cytotoxicity in FcγR^{+} P815 cells by pit cells via the perforin/granzyme pat hway.

DISCUSSION

Pit cells are liver-specific NK cells and, together with Kupffer cells, constit ute the natural cellular defense against invading cancer cells in the liver[12]. It has been shown that pit cells are four to eight times more cytotoxi c against YAC-1 and CC531s cells than blood NK cells and are able to kill NK-r esistant, LAK-sensitive P815 cells[13]. However, the surface receptors involved in pit cell-mediated cytotoxicity against tumor targets have remained elusive. Present data show that the mAbs against the NK-triggering molecules NK R-P1A and ANK61 antigen increased not only cytolysis but also apoptosis of FcγR^{+} P815 cells, but had no effect on Fc γ R YAC-1 and

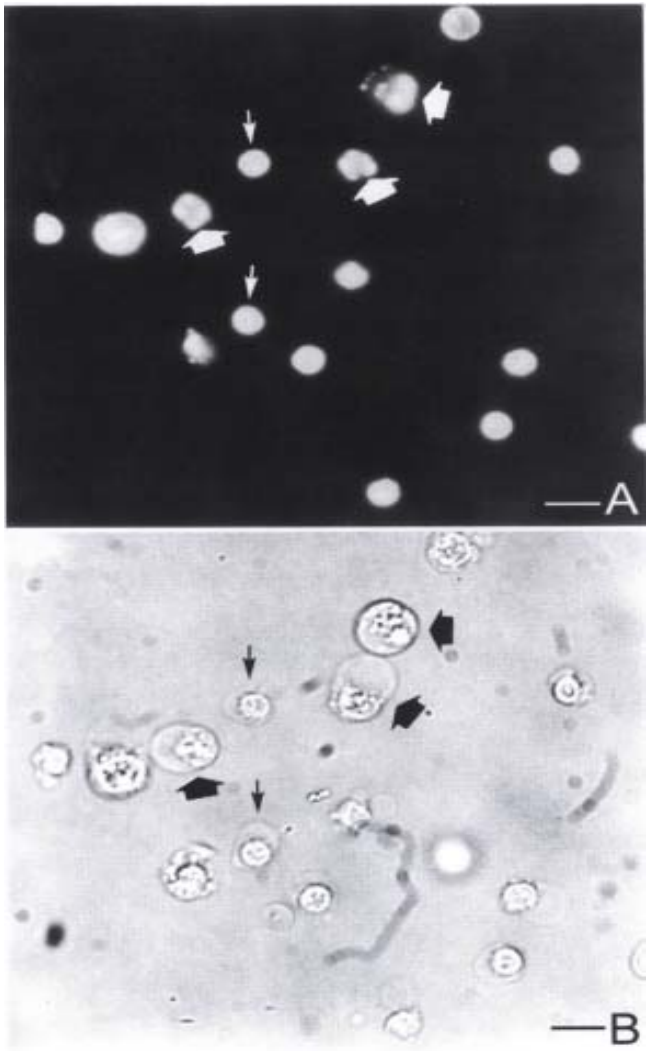

Figure 3 Fluorescence and light micrographs of YAC-1 cells coincubated with pit cells at an E:T ratio of 10:1 for 3 h. (A) Fluorescence micrograph showing the apoptotic YAC-1 cells with fragmented nuclei (thick arrows) and pit cells (thin arrows). Light micrograph shows the same field as (A). Bar = 5 μm.

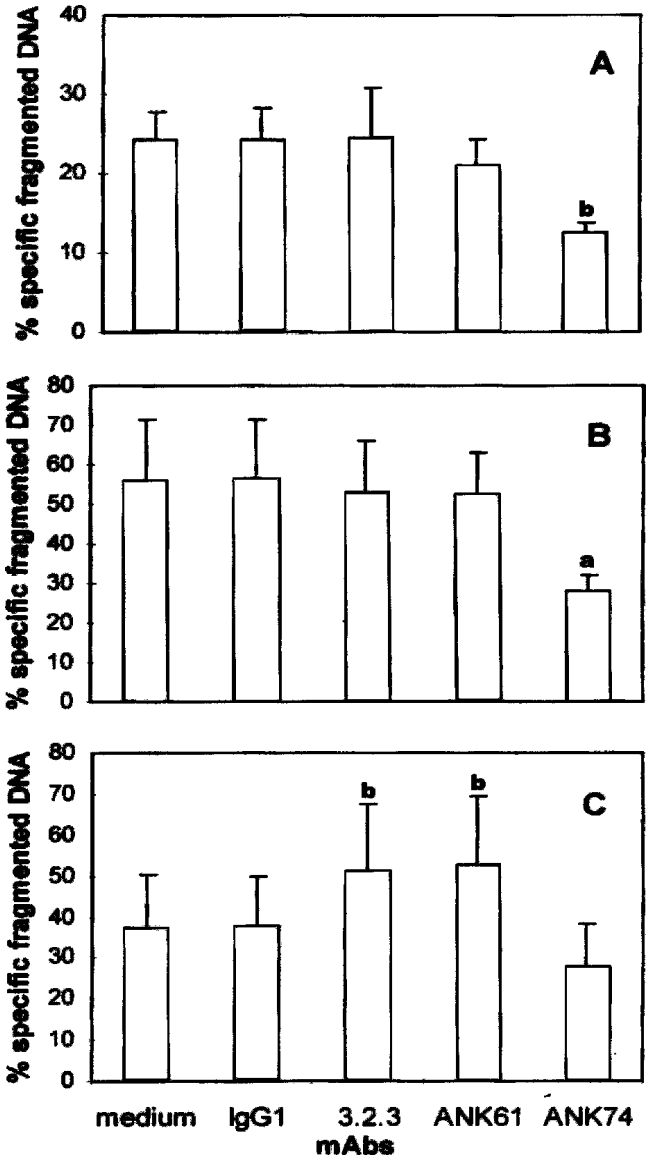

Figure 4 Effect of the mAbs on pit cell-induced CC531s (A), YAC-1 (B) and P8 15 (C) target cell apoptosis. [^{3}H]-TdR labeled target cells were incubated at an E:T ratio of 10:1 with freshly isolated pit cells for 3 h in the absence or presence of the mAbs. Values are mean ± SD from four different ex periments.$^a P < 0.05$, $^b P < 0.01$ *vs* the control.

CC531s cytotoxicity ind uced by pit cells. These results indicate that NKR-P1A and ANK61 antigen on pit cells function as triggering molecules against FcγR$^+$ target cells, but not against FcγR- targets. It has been demonstrated that mA bs 3.2.3 and ANK61 enhance cytolytic activity of NK and LAK cells only against FcγR$^+$ tumo r target cells by rADCC[4,5,7]. Other evidence to support this observati on was that, in our experiments, cross-linking of NKR-P1A on pit cells with mAb 3.2.3 did not induce granule exocytosis from pit cells, as observed in May-Giemsa-stained cytospin preparations (data not shown). This is in contradiction with a report describing that mAb 3.2.3 can induce degranulation and release of granzymes fro m NK and IL-2 activated NK cells[22]. Furthermore, after incubation of pit cells with mAb 3.2.3, the medium and the pit cells were separated by centrifugation. The medium did not kill CC531s and YAC-1 cells, whereas the pit cells'cytotoxicity against these targets was comparable to freshly isolated pit cells (data not shown). Moreover, it has also been shown that NKR-P1A negative NK clones are able to lyse the same type of target cells as NKR-P1A positive NK clones [23] and expression of NKR-P1A is not an absolute requirement for NK activity[24]. These data suggest that NKR-P1A is not the sole activation molecule on NK cells. Actually, a diversity of activation molecules on NK cells has been described[2,3,5,25]. NK cells might use these differe nt activation molecules to lyse different target cells.

It is believed that mAbs 3.2.3 or ANK61-enhanced cytotoxicity in FcγR$^+$ target cells by NK cells is a rADCC reaction[4,5]. The present data show further that these

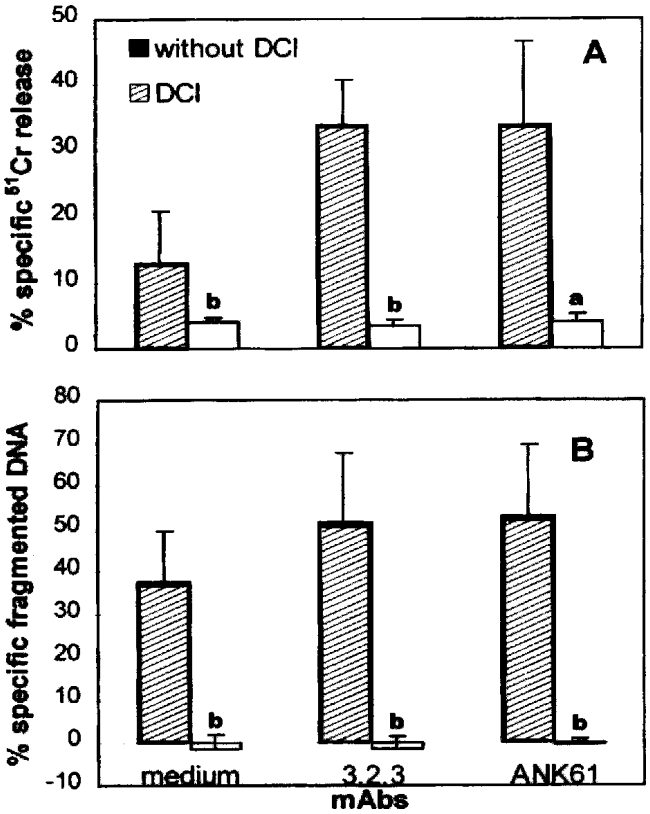

Figure 5 Effect of DCI on mAbs 3.2.3 and ANK61-enhanced FcγR+ P815 cell cytolysis (A) and apoptosis (B) by pit cells. Pit cells were preincubated with 50 μ mol/L DCI for 30 min at 37℃. After washing twice, the cells were coincubated with P815 cells as mentioned in materials and methods. Values are mean ± SD from three different experiments. $^aP < 0.05$, $^bP < 0.01$ *vs* the corresponding control.

mAb-enhanced c ytolysis and apoptosis of P815 cells by pit cells are mediated by the perforin/g ranzyme pathway because this enhanced cytotoxicity can be completely inhibited by the granzyme inhibitor DCI.

Although the function of CD45 is well known in NK cells[8-11], its rol e in pit cells has not yet been clarified. We showed here that CD45 was involved in the pit cell induced cytolysis and apoptosis of CC531s and YAC-1 cells but not of P815 cells. The reason that CD45 on pit cells had different effects on pi t cell-mediated cytotoxicity against different targets is not clear. It has bee n reported that CD45 is not essential for FcγR-mediated function in NK cells, since NK cells in CD45-/-mice have normal cytotoxic activities, comparable to normal mice (CD45+/+)[26]. Another possible explanation is that CD4 5 plays a regulatory role in signal transduction through surface receptors on NK cells, such as NKR-P1A, LFA-1, and killer cell inhibitory receptors (KIR) in NK cell-mediated target killing[8,10,11]. A diversity of receptors is found to be involved in NK cell activation[2,3]. It could be that CD45 plays a different regulating role in different NK activation receptors. On the other ha nd, the regulatory role of CD45 in the inhibitory signal pathway via KIR is dependent on the expression level of MHC class I on target cells[8]. The ant i-CD45 mAb inhibits only the lysis of target cells expressing low level of MHC class I, such as CC531s[8] and YAC-1[27], whereas the lysis of target cells expressing high level of MHC class I, such as P815 [27], is not inhibited[8].

In summary, CD45 on pit cells participated in pit cell-mediated CC531s and YAC-1 but not P815 cytolysis and apoptosis. NKR-P1A and ANK61 antigen on pit cell s were involved in pit cell-mediated FcγR+ P815 cell cytolysis a nd apoptos is, but not in FcγR- CC531s and YAC-1 cell cytotoxicities. The mAbs 3.2.3 an d ANK61-induced cytotoxicities of FcγR+ P815 cells by pit cells were mediated by the perforin/granzyme pathway. These findings provide evidence that the same m olecules on pit cells and blood or spleen-derived NK cells regulate target cell lysis and apoptosis. The difference in cytotoxic capacity between these cell ty pes therefore may be on a quantitative rather than a qualitative level.

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Edited by Zhu QR
proofread by Mittra S

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

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Brief Reports

Study on the risk factors of lymphatic metastasis and the indications of less in vasive operations in early gastric cancer

BoJian Jiang[1], Rong Xun Sun,Hao Lin , You Fu Gao

Subject headings stomach neoplasms/diagnosis; neoplasm metastasis; precancerous conditions; risk factors; surgery/operative; lymphatic metastasis; lymph node excision

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INTRODUCTION

The principle of surgical treatment for gastric cancer is the radical resectioni ng although the suitable resecting range for different cases of gastric cancer is still being argued upon[1-9]. However, the diagnostic accuracy of early gastric cancer (EGC) without lymphatic metastasis has obviously improved with an improvement in the diagnostic technique and due to the accumulation of kn owledge on the biological profiles of EGC[10-17]. The D_2 lymph node excision was used as a regular operation to treat the EGC previously. But the conc ept for the EGC without lymphatic metastasis has gradually changed and the less invasive resections has been applied in some cases[18-20]. This study aimed at investigating the risk factors of lymphatic metastasis in EGC in order to find out the proofs for the suitable indications for less invasive operations such as endoscopic mucosal resectioning (EMR), laparoscopic and laparotomic res ectioning.

MATERIALS AND METHODS

From 1978 to 1999, 520 cases of EGC with operative and pathological analyses were studied. Among them, 287 were male and 233 female, with an average age of 62 years (32-81). The samples from the primary tumor and the metastatic lymph nodes were collected and examined as previous reports distinguishing the t umor was limited in mucosal layer (m Ca) or submucosal layer(sm Ca) of stomach[10] were scanty. According to the gross differences of the following groups were constituted of II_c+III, II_c, II_b, II_a and I.

Sixty-six cases diagnosed by the ultrasound endoscopical examination received t he less invasive operative technique. This resection retained the partial omentu m and resected the 1st station and No 7 of lymph nodes. In this operation, the dissection was made and the omentum was cut off in (3-4) cm places away from or along the blood vessels in greater and less curvature of the stomach. And the No 7 lymph nodes were resected and blocked or picked out. Hepatic branch of vagus had better to be protected possibly. Mouth margin and anal margin for gastr ectomy was over 2 cm away from the tumor site. All of 259 cases received radical resectioning of N_2 lymph nodes or a more radical resection.

The rate of lymphatic metastasis and the morbidity of intestinal obstruction after operation and the expression of long-term survival were statistically analyzed by Check test or by log-rank test respectively. *P* value less than 0.05 was considered statistically significant.

RESULTS

In this study, the risk factors regarding the occurrence of lymphatic metastasis were investigated. The long-term survival analysis and the complications relat ing to the less invasive operation were studied at the same time.

Gross type

Lymphatic metastasis happened the most easily in II_c+III subgroups of both m Ca and sm Ca, and secondly in II_c subgroups. In II_c+III subgroups, the lymphatic metastasis could be identified in 2 cases of m Ca and 20 cases of sm Ca, and one n_2 lymphatic metastasis was observed in m Ca and 10 in sm Ca (Table 1). No n_2 lymphatic metastasis could be found in other subgroups of m Ca. However, this n_2 lymphatic metastasis could be observed in sm Ca in all groups except II_b.

Invading depth

Forty-four cases of n_1 lymphatic metastasis and 14 cases of n_2 lymphatic metastasis (58 cases of lymphatic metastasis in

[1]Department of General Surgery, Shanghai Jing-An Central Hospital, Shanghai 200040, China
Bo Jian Jiang, male, Born on 1958-02-08 in Shanghai, graduated from Shanghai Second Medical University for M.D. in 1983 and Asahikawa Medical College for Ph.D. in 1996, now an Associate Professor/Vice Chairmen, Dept. of General Surgey, J ing-An Central Hospital, Shanghai, majoring in gastrointestinal oncology, hepatocyte transplantation, and having 30 papers published.
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Correspondence to: Bo-Jian Jiang, MD., Dept of General Sur gery, Shanghai Jing-An Central Hospital, No 259, Xi-Kang Road, Shanghai 200040, China
Tel. +86-21-62474530, Ext. 6009
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total, 11.2%) could be demonstrated in all cases of EGC. Four (1.5%) of 275 cases suffering from the m Ca had lymphatic metastasis, of which one case shared n_2 lymphatic metastasis (Table 2). Lymphatic metastasis was identified in 54 (22.0%) out of 24 5 cases in sm Ca group, of which 13 cases were of n_2 lymphatic metastasis. Thi s result showed that lymphatic metastasis was more frequent in sm Ca than in m C a ($P < 0.05$).

Table 1 Lymphatic metastasis relating to gross types (%)

Gross types	m Ca (n = 275)	sm Ca (n = 245)
II_c+III	2.5 (2/85)[*,b]	25.3 (20/79)
II_c	1.7 (2/120)[b]	20.2 (17/84)
II_b	0 (0/3)	0 (0/4)
II_a+II_c	0 (0/29)	23.8 (10/42)
II_a	0 (0/34)	25.0 (2/8)
I	0 (0/4)	17.9 (5/28)

*Number of positive lymph nodes/total numbers of cases. [b]$P < 0.01$, *vs* each subgroup of II_b, II_a+II_c, IIa orItypes .

Table 2 Lymphatic metastasis relate(LMR) of m and sm Ca (%)

Invading depth	n_1 LMR	n_2 LMR	Total LMR
m Ca (n = 275)	1.1 (3)[*]	0.4 (1)	1.5 (4)[b]
sm Ca (n = 245)	16.7 (41)	5.3 (13)	22.0 (54)
Total cases (n = 520)	8.5 (44)	2.7 (14)	11.2 (58)

[*]Numker of cases. [b]$P < 0.01$, *vs* sm Ca.

Tumor size with or without ulcer

With tumor size less than 20 mm in m Ca, there was no lymphatic metastasis in 122 of 275 cases regardless whether accomp anied with ulcer or not. In 153 cases with tumor larger than 20 mm, there were 4 cases of lymphatic metastasis in m Ca ($P < 0.01$, Table 3). Among 164 cases with m Ca and without ulcer, the lymphatic metastasis cou ld be observed only in one with 25 mm of tumor. On the other hand, 3 of 111 case s of lymphatic metastasis could be found in ulcer group, in which all tumors were larger than 30 mm. These findings indicated that lymphatic metastasis was more significantly in cases with tumor size larger than 20 mm subgroups, especially those with ulcer ($P < 0.05$).

Table 3 Lymphatic metastasis related to tumor size with or without ulcer (%)

Ulcer diameter (cm)	m Ca (n = 275)		sm Ca (n = 245)	
	With ulcer (n = 111)	Without ulcer (n = 164)	With ulcer (n = 110)	Without ulcer (n = 135)
≤2	0 (0/43)	0 (0/79)	16.7 (3/18)	3.1 (1/32)
~3	0 (0/21)	2.4 (1/42)	20.8 (5/24)	21.1 (8/38)
~4	5.3 (1/19)	0 (0/15)	20.0 (4/20)	24.0 (6/25)
~5	6.7 (1/15)	0 (0/14)	18.8 (3/16)	18.2 (4/22)
≥6	1 (1/13)	0 (0/14)	43.8 (14/32)	33.3 (6/18)
Total No	2.7 (3/111)[a]	0.6 (1/164)	26.4 (29/110)[a]	18.5 (25/135)

[a]$P < 0.05$, *vs* ulcer subgroup in each group.

In sm Ca, 4 out of 50 (8.0%) cases of lymphatic metastasis could be identified in cases with tumor size less than 20 mm with or without ulcer, which showed that the radicalres ection with standard lymph node excision could be carried out even in small tu mor sized sm Ca.

In 135 cases of sm Ca without ulcer, 25 (8.5%) cases were demonstrated to have ly mphatic metastasis, out of which 12 (11.3%) cases belonged to n_2 lymphatic meta stasis. But 29 out of 110 (26.4%) cases of sm Ca with lymphatic metastasis were prov en to occur in the ulcer subgroup, in which 7 (6.4%, $P < 0.05$) cases had n_2 lymphatic metastasis. Therefore, the n_2 lymphatic metastasis happened more sig nificantly in sm Ca than in m Ca.

Pathological classification

One hundred and forty-three out of 275 cases (52.0%) with m Ca had well-differentiatied adenocarcinoma in which there was n o lymphatic metastasis. Two out of 58 cases (3.4%) and 2 out of 57 cases (3.5%) sharing lymphatic metastasis were respectively identified in the poorly-differe ntiatied group and the non-differentiatied group of EGC. Fifty-four out of 245 (22.0%) cases with lymphatic metastasis were observed in sm Ca, out of which th ere were 21 (8.6%), 11 (4.5%), 15 (6.1%), and 7 (2.9%) cases respectively in eac h group of well-differentiatied, poorly-differentiatied, non-differentiatied and ringed cells cancer. Difference in pathological classification could not si g nificantly result in the increase of lymphatic metastasis, but n_2 lymphatic me tastasis was more easily recognized in worst differentiatied groups.

Comparison of D_2 and D_{1+7}

resections In a study regarding the le ss invasive D_{1+7} resection, this resection and D_2 or more radical lymph node excision were separately carried out in 66 cases and 259 cases during 1988 -1999. All cases resulted in the curable resection and the longest follow-up was of 10 years. In D_{1+7} group, only one case was histopathologically demons trated for the occurrence of lymphatic metastasis. Twelve cases diagnosed as m C a preoperatively were sm Ca and others were m Ca. In investigating the postopera tive complications, 1 out of 66 cases (1.5%) with D_{1+7} resection and 39 out of 259 cases (15.1%) with D_2 resection suffered from the intestinal obstruct ion ($P < 0.01$). But no mortality occurred in D_{1+7} resection group and 2 cases (0.8%) died within one month after D_2 operation.

The investigation of long-term survival showed that the survival rates of 1 and 5 years in D_{1+7} and D_2 operation groups were respectively 98.3% vs 97.6% and 90.2% vs 92.1%, in which no significant differences could be obse rved.

DISCUSSION

As is well known, the degree and the range of lymphatic metastasis is dependant on the biological features of the primary tumor[10,12-14]. This study showed that one of the main risk factors for the lymphatic metastasis was the tumor invading depth infiltrating through the mucosal layer and

the tumor size bei ng larger than 20 mm. In sm Ca with tumor size larger than 20 mm, the morbidity of n_2 lymphatic metastasis happened with an increase in the tumor size. Theref ore, correct determination of the invading depth and the tumor size are key poin ts for the less invasive surgeries in EGC. The indications for endoscopical muco sal resection (EMR)[20-21], laparoscopic resection[22-31] and less invasive resection by laparotomy[20,32-33] should be limited in t he cases of EGC without lymphatic metastasis. Even for less invasive resection b y laparotomy, it should be indicated only for n_1 lymphatic metastasis without ulcer, I, II_a and II_b gross types as II_c types with turnor size less than 2 0 mm although such indications have not been accepted ubiquitously[10,20,32 -33]. The less invasive surgery by laparotomy has become one of the main choi ces in surgical treatment of EGC currently. The range of less invasive operation includes the partial gastrectomy and regional lymph node excision as well as th e retains of the omentum, vagus, pylorus and so on[10,14,27]. The less i nvasive operations for m Ca or sm Ca have been proven to share the good results of long-term survival[14,33], and at the same time, less invasive surg ery is required.

Development of less invasive operations by laparotomy have made both MER and lap aroscopic resection applicable in the treatment of EGC[18-32]. The rate of complete resection for primary tumors and metastatic lymph nodes by EMR or l aparoscpic resection was between 45%-85%. This resection rate was lower becaus e most tumors were larger than 10 mm and some were located in the cardia, lesser curvature of gastric body as well as latter wall of stomach [9,11,14]. M u cosal resectioning by laparoscopic surgery including lesion lefting resection[31] and gastric mucosal resection[20,23] comes in between EMR and less invasive laparotomy. Advantages of this laparoscopic surgery are to get more satisfactory resectioning of the entire layer of the gastric wall at the lesion site and the regional lymph nodes of n_1 around the tumor in comparison to EMR[20, 29-31]. If the tumor infiltrated to the sm Ca or the vessels in the gastric wall, the radical resectioning at the 2nd station of lymph nodes sho uld be carried out as the first choice[1,9,32,34].

Judgements for the invading depth of EGC before operation are mainly based on the gastric fiber examination with the ultrasound. On the application of the ultr a sound endoscopy, especially at 20 MHz, correct diagnostic has been observed in over 80% cases and identification of m Ca or sm Ca was easier[15-17,34]. At the same time, n_2 l ymph atic metastasis happened more easily in sm Ca. Therefore, D_2 lymph node excisi on should be applied in sm Ca if the diagnosis is doubtful before or during op e ration. The investigations on the metastasis and the regional invasion of gastric cancer has the support of not only clinicopathological studies but the genetic and molecula r biological studies also[35-42]. At present, a very careful choice should be m ade for the less invasive operations, especially for EMR and laparoscopic surgery, as making a correct judgement for the invading depth of tumor in stomach before operation[16-17,34]is very difficult.

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Edited by Zhou XH
proofread by Mittra S

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

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Sutureless end-to-end bowel anastomosis in rabbit using low-power CO_2 laser

Zhong Rong Li, Yong Long Chi and Run Cong Ke

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INTRODUCTION

The use of laser energy to weld biological tissues and produce sutureless anasto mosis has its advantages over conventional silk- sutured anastomosis since it was reported in small vessels[1] and fallopian tubes[2], in the la te 1970s. Since then, more investigators have welded a larger variety of tissues[3-13] and have expanded its application to welding trials of ent ertomies of rabbit and rat small intestine[14-17]. Sauer *et al*[18] reported results from Nd: YAG laser in reconstruction of end-to-end wel ding in rabbit small intestine. Recently, controlled temperature during YAG and argon laser-assisted welding of entertomies of rabbit and rat was implemented to eliminate exponential increases in the rate of denaturation associated with r apidly increasing temperature[19,20]. Yet there was no report of sutureless end-to-end bowel anastomosis using low-power CO_2 laser. This is a repor t of a circumferential end-to-end laser welding bowel anastomosis in rabbit by using 3 different CO_2 laser powers to explore the feasibility of CO_2 laser welding of a circumferential intestinal tissue and to determine the optimal la ser-welding parameter. Then the appropriate CO_2 laser power was chosen to weld bowels in rabbit and its long-term healing effect was evaluated.

MATERIALS AND METHODS

Animals and equipment

Twenty-eight Japanese white rabbits weighing between 1.8 kg and 2.5 kg (6 for a cut e and 22 for chronic phase of experiment) regardless of sex were supplied by Experimental Animal Center of Wenzhou Medical College. A hand-held Model JZ-5, po wer 500 mW-5 W adjustable CO_2 laser made in Shanghai Optical Machinery Institute, which was modified to provide a 100 mW-1000 mW potentimeter, was used. The laser spot diameter was 0. 4 mm. The laser power output was calibrated by a Model SD2490 CO_2 laser power meter produced by Subei Electronic Equipment. The pres sure was measured by a PT-6B pressure transducer and PTM-6B physiological pre ssure meter, jointly produced by Shanghai Fudan University and Zhejiang Ouhai El ectronic Equipment Factory, with normal saline intraluminal infusion at an average rate. The flow was monitored by a computed infusion pump (DYB-1) made in Jia ngsu Haimeng Electronic Equipment Factory.

Acute phase

A rabbit was fasted for 24 h prior to surgery and sodium pentobarbitol (30 m g/kg, im) was used for anesthesia. Using a 5-cm upper abdominal incision, the ileum was identified. At 10 cm proximal to the ileocecal junction, intestinal circ umference was measured. Ileum was cut apart and then reconstructed. Each subsequent test was moved approximately 10 cm and a bowel anastomosis was welded by using 3 different CO_2 laser powers of 250, 500 and 1000 mW respectively. At two circumferential cut-edges of ileum, 3 silk stay sutures were placed to hold tissue together and allow for accuratere-approximation and inversion. Before and a fter each welding, the laser power output was calibrated and modified. Tissue blanching and constriction, and fusing of both egdes of the anastomosis were marks of the completion of a tissue weld. The circumferential welding was completed in sequence.

One min after each laser welding, a 10-cm segment of bowel including the anas tomosis was isolated between occlusive bowel ligature, and the bursting pressure of anastomosis was tested by the intraluminal infusion of normal saline at a rate of 16 mL/min. The pressure was recorded until the anastomosis burst which was visible both as leakage of infusate from the anastomosis and as a sharp drop of pressure. Each rabbit was welded 3 to 5 times and all subjects were sacrificed after the experiment.

Chronic phase

A rabbit was fasted for 24 h prior to surgery and sodium pentobarbital (30 mg/kg, im) was used for anesthesia. Using upper midline abdominal incision, ileum was circumferentially cut apart for a laser-welded anastomosis and a conventio nal one-layer silk sutured anastomosis. It was randomly determined which anasto mosis was 10 cm or

Department of Pediatric Surgery, Yu-Ying Children's Hospital, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China
Dr. Zhong-Rong Li, surgeon-in-chief and associated professor, male, born on 1963-05-21 in Wenzhou City, Zhejiang Province, graduated from Shanghai Second Medical University as a postgraduate in 1989. Major in pediatric general surgery.
Supported by the Youth Scientific Research Foundation of Zhejiang Provincial Department of Public Health.
Correspondence to: Dr. Zhong-Rong Li, Department of Pediatric Surgery, Yu-Ying Children's Hospital, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China
Tel. +86-577-881-6176, Fax. +86-577-883-2693
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35 cm proximal to ileocecal junction. Three silk stay sutures were placed to hold the cut intestinal edges in welded anastomosis, and 16-20 stiches of interrupted one-layer 0 silk suture were used in sutured anastomosis.

The laser power output 500 mW was used in the laser welding. Tissue blanching and constriction, and fusing of both egdes of the anastomosis by naked-eye were marks of the completion of a tissue weld. The time used for laser welded anastomosis was about 5-7 min while sutured anastomosis needed 10-15 min.

Gentamycine (4 mg, im) was administered 12 h pre and post-operatively. Al l rabbits were sacrificed at d 3, wk 1, 3 and 5 and pathological study was done macroscopically and microscopically.

RESULTS

Acute phase

The intestinal circumference was 26-30 (28.2 ± 1.3) mm. The laser was delivered within 1 mm of both intestinal cut-edges. Based on this calculation, the laser delivery area of each anastomosis was (26-30) × 2 mm. The delivery time for 250 mW group, 500 mW group and 1000 mW group was 92 s-153 s (average 123 s), 59 s-84 s (average 70 s) and 25 s-45 s (average 40 s) respectively. According to the above pa ra meters, the laser power density and energy density of each anastomosis could be calculated. The number of bowel anastomosis using 3 different CO_2 laser powers and the bursting pressure are shown in Table 1.

Table 1 The bursting pressure of the welded and sutured anastomoses

Groups	Number of ananstomosis	Bursting pressure ($x \pm s$) kPa (mmHg)
Silk-sutured	5	6.1 ± 1.9 (46 ± 14)
250 mW-welded	7	1.1 ± 0.4 (8 ± 3)
500 mw-welded	6	2.7 ± 0.7 (20 ± 5)[a]
1000 mW-welded	6	1.7 ± 0.8 (13 ± 6)

[a]$P<0.05$ *vs* other three groups

Chronic phase

In chronic phase experiment, except that one animal died at 1 day and another at 3 days after operation from unknown causes without anastomotic disruption by au topsy, the remaining 20 rabbits,which were dissected at a different postoperative times, were divided into four groups, each consisting of 5 rabbits.

At d 3 after operation, both modes of anastomoses healed well without leaks. Loose fibrous adhesion, stiffness, and edema were found in the adjacent bowel including the anastomosis and mesentery. Suture was seen in the sutured anastomosis while the laser welded anastomosis was smooth.Microscopically, both anastomoses showed edema, acute inflammatory cell infiltration, consisting of mainly neutrophils, lymphocytes, and macrophages, but comparatively less in the laser-welded a nastomosis. At 1 wk after operation, edema was markedly reduced but the cut-edge in the sutured anastomosis was still swollen. Adhesion was observed surroun ding both anastomoses, less fibrous adhesion was seen in all laser-welded anast omose except in one that had a small walled-off leak surrounded by fibrous form ation without pus or purulent fluid in the abdominal cavity. Some inflammatory cells infiltration, mainly lymphacytes and plasmacytes, was found in both groups. Broad gap of mucous due to excessive inversion was seen in the sutured anastomos is while the gap was much narrower in the laser-welded anastomosis.

At 3 wk after operation, anastomotic tissues were morphologically normal and soft. Linear scar along the cut-edge of sutured anastomosis was seen while lase r anastomosis showed less scar formation. Fibrous adhesion was remarkably decrea sed in the sutured anastomosis than before. No adhesion in 2 cases and very little adhesion in 3 cases was found in the laser-welded group. Microscopically, in flammatory cell infiltration and fibrous proliferation still existed in both ana stomoses. In the sutured anastomosis, there was more and thicker fibrous tissue than in the laser-welded anastomosis, especially at the sutured site. At 5 wk after operation,white linear seams were seen around the sutured anastomoses. Slightly sunken scar was found on one anastomosis and some fibrous adhesion in an other. At both cut-eges of anastomosis dotted line of scars resulted from the silk suture were vaguely observed, but no residual black suture remained. Two la ser-welded anastomoses were hardly distinguishable and the calibre of the other three anastomoses was identical to that of the normal bowel, though thread-like scarring was found at the cut-edges.

DISCUSSION

Laser which is used to weld living tissue differs from high energy laser for other medical purposes in that the former requires specific low power energy. Which type of laser is to be chosen depends on the structure of the target tissue and welding requirement as well. Sauer *et al*[16] used CO_2 laser to weld a 0.5 cm longitudinal incision in rabbit bowel and acquired the bursting power of 5.4 kPa. In this experiment low-power CO_2 laser was used to weld circum ferential bowel anastomosis,which attempted to make laser-welding more applicable to clinical practice. The busting pressure of 500 mW group was 2.7 kPa. The bu rsting pressure was lower probably because the welded anastomoses in this group were circumferential instead of a small longitudinal incision and therefore greater tension was expected. Besides, the distance between each stay suture to be welded was as long as 1.0 cm, much longer than Sauer's 0.5 cm[16]. This study shows that the 500 mW group produced remarkably higher bursting pressure than the other two groups. Group 250 mW cannot produce a desired welding effect due to much too low power and prolonged delivery while charring tissue occurs in the 1000 mW group so that the tensile strength of the welded anastomosis is affected. Furthermore, Cilesiz *et al*[19,21] believed that temperature feedback control improved the

quality and stability of laser- assisted enterotomy closures in surviving animals, temperature (90-95) ℃ was considered optimal.

Though no information is available about the maximum bursting pressure of bowel of the conscious rabbit, Abbott *et al* reported that the basic pressure of the distal bowel was 0.83-1.03 kPa, and Fink reported that the pressure of normal ileum was no more than 0. 92 kPa with the peak pressure less than 2.03 kPa in the hum an body. Therefore we infer that the bursting pressure produced in the anastomoses of the 500 mW group is adequate to avoid the anastomotic disruption caused by peak tensile strength.

The healing process of the welded bowel reveals that fibrous tissue or intestina l adhesion to various degree form around both kinds of anastomoses early after o peration. Later, the laser-welded anastomosis is hardly distinguishable from the normal intestine, while suture remnant and white linear scar are found in the sutured anastomoses. Microscopically, compared with the sutured anastomosis, the laser-welded anastomosis has less inflammatory cells early after operation and less fibrous proliferation, less scar formation, and no suture granulation later postoperatively. We therefore conclude that the laser-welding in intestinal an astomosis, as a new type of sutureless surgical technique, has its potential cli nical application value with the advantages including sutureless anastomosis, no foreign material, avoidance of needle trauma and suture remnant, minimal inflam matory response with reduction in stricture and infection. It is especially suit able for mini-bowel anastomosis in congenital intestinal atresia.

It is universally accepted that laser thermal effect is involved in the mechanism of laser-welding of biological tissue. It is also shown that adequate tissue seals occur in laser-welded wounds only when the tissue edges are directly oppo sed,whereas any blood in the interface selectively absorbs the laser energy and forms a fibrin seal which is too weak to tolerate increased intraluminal pressure[14,22]. According to Jain[1] in his study of YAG laser welding of the microvessels, it is the physical coherence of fibrous collagen that weld ed successfully the vascular walls, meanwhile laser does not result in degen era tion, coagulation, and necrosis. In contrast, it is held by most scholars that, based on the observation that coagulation and necrosis exists at the welded site , strong weld is not the result of physical coherence of collagen, but rather th e result of chemical bonding caused by laser thermal effect which can lead to collagenic degeneration and tissue necrosis[23-26]. In our welding experim ents, ideal bonds formed when the opposed cut edges of the bowel were directly and strongly held together and tissue blanching was seen during laser welding. Microscopically, slight tissue degeneration and necrosis could also be seen near the laser-welded anastomosis. However, recent studies show that solid protein used as solder can improve vascular welding effect[27,28]. Meanw hile, it is reported that collagen synthesis is stimulated during the healing process after laser welding[29,30], therefore, further study on mechanism of laser-welding is needed.

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Edited by Zhu QR
proofread by Mittra S

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

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Can the rat donor liver tolerate prolonged warm ischemia ?

Ji Qi Yan, Hong Wei Li, Wei Yao Cai, Ming Jun Zhang and Wei Ping Yang

Subject headings liver transplantation; cadaver; tissue donors; organ procurement; tissue survival; non-heart-beating donors; warm ischemia

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INTRODUCTION

The last two decades of the twentieth century have witnessed increasingly successful rates of liver transplantation. The number of liver transplantations has increased steadily while the number of organ donors has remained relatively constant. Thus a great disparity has developed between the demand and supply of donor organs and remains a major limiting factor for further expansion of liver transplantation. Although many procedures, such as split liver[1], living-related transplantation[2], and xenotransplanta tion[3], have been attempted clinically to overcome the shortage, it is hoped that livers harvested from non-heart-beating donors (NHBDs) would alleviate the problem of organ shortage, which again becomes the focus of attention[4-9]. However, sensitivity of the liver to warm ischemia remains a major worry for use of the NHBDs. The aim of this animal study was to assess if murine liver could tolera te prolonged period of warm ischemia and to determine the optimum timing of inte rvention in the cadaver donor in order to preserve liver viability.

MATERIALS AND METHODS

Animals

Male Sprague-Dawleyrats, weighing 180 to 220 g, were obtained from Sh anghai BK Lab Animals Co. Ltd. Donor and recipient rats were matched for size. R ats were housed in the standard animal room without fast before the surgery and with free activity and standard diet after surgery.

Department of Surgery, Ruijin Hospital Affiliated to Shanghai Second Medic al University, Shanghai 200025, China
Dr. Ji Qi Yan, male, born in December 1971 in Shanghai, graduated from Shanghai Second Medical University with a Ph.D degree in 2000, majoring in portal hypert ension and liver transplantation, having 10 papers published.
Supported by Shanghai Science and Technology Development Fund, №964119027.
Correspondence to: Prof. Hong Wei Li, Department of Surgery, Ruijin Hospital Affiliated to Shanghai Second Medical University, Shanghai 200025, China
Tel. 0086-21-6437-0045, Fax 0086-21-6433-3548
Email. digsurg@online.sh.cn
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Experimental design

Orthotopic liver transplantation was carried out in 5 groups with non-heart-beating time in the donor ranging from zero to 15, 30, 45 and 60 min respectively (Group 1-5). The period of non-heart-beating was counte d from the donors cardiac arrest to the beginning of the cold perfusion of the liver. Each group consisted of 13 rats; 5 rats were sacrificed one hour after surgery for sample collection and graft biopsies; the other 8 rats were used to observe one-week survival rate; and rats that survived for more than 7 d after transplantation were considered as survivors. Except for 1 or 2 survivors that were randomly selected to observe the possibility of long-term survival, the others were also sacrificed 7 d after surgery.

Animal surgery

Both donor and recipient surgery was performed at room temperature of 21℃ to 22℃ under ether anesthesia. No immunosuppressive agents were given to either donor or recipient. Five min after the intravenous administration of 500 IU o f hep arin, donor cardiac arrest was induced by opening the chest and cross clamping t he base of the heart with hemostats. Donor surgery began 15 min prior to the end of the designated warm ischemic time in each group. All livers were perfused with 10 mL of 4℃ Ringer's lactate solution through the abdominal aorta, and stored in the same cold solution for an average cold ischemia of 60 min. The don or liver was implanted to the recipient according to the cuff technique of Kamad a and Calne [10] with an average anhepatic phase of 16 min. No heparin was administrated to the recipientrat.

Sample collection and analysis

The blood samples were obtained one hour and seven days after hepatic implantation for measurement of serum ALT and AST, meanwhile liver tissues were also taken for histologic examination. Furthermore, one hour after reperfusion serum TNF level was detected by the bioassay using L-929 cell lines as described elsewhere. The graft tissues were fixed in 10% formaldeh yde, and sections were stained with hematoxylin and eosin.

Statistics

Values are expressed as the $\bar{x} \pm s$. Statistical significance was c ompared with group 1 and tested by unpaired Student's *t* test. The survival rates in each group were compared with group 1 using Fisher's exact test. *P* values less than 0.05 were considered significant.

RESULTS

Survival

No recipient death was directly related to operative echnical failur e in this study. The one-week survival rate and the long-term survival period of rats that underwent liver transplantation are listed in Table 1. With a stepw ise increase of the non-heart-beating period from zero to 15, 30, 45, and 60 min in donors, the survival rates in recipients were 100, 75, 62.5, 25, and 0 percent respectively. Moreover, rats that received grafts having suffered 15, 30, or 45 min warm ischemia before implantation showed the possibility to su rvive more than 60 d. The longest time for using NHBDs in our study was 45 min. A total of 19 recipient rats died within 7 d, among which 15 deaths were caused by primary graft nonfunction, with massive ascites and patchy liver ne crosis at autopsy. Two rats died of biliary complications and 2 died of pulmon ary infection. The survivor in Group 3 also died of pulmonary infection 18 d after surgery.

Table 1 Survival status

Group	Warm ischemia / min	One-week survival rate	Long-term survival / day
1	0	100% (8 / 8)	>60, >60
2	15	75% (6 / 8)	>60, >60
3	30	62.5% (5 / 8)	18, >60
4	45	25% (2 / 8)[a]	>60
5	60	0% (0 / 8)[a]	

[a]$P<0.01$ *vs* Group 1

Hepatic function

The mean serum ALT and AST concentration one hour after reperf usion was significantly higher in Groups 2, 3, 4, 5 compared with Group 1, and the concentration of enzymes dropped markedly after 7 d. The postoperative increase in enzyme concentration appeared to be proportional to warm ischemia time.

Table 2 ALT and AST values after transplantation

Group	One hour after reperfusion		7 d after operation	
	ALT(u / L)	AST(u / L)	ALT(u / L)	AST(u / L)
1	362 ± 49	623 ± 75	38 ± 9	115 ± 19
2	546 ± 68[a]	873 ± 95[a]	64 ± 11[a,b]	345 ± 66[a,b]
3	1096 ± 195[a]	1435 ± 238[a]	124 ± 35[a,b]	454 ± 120[a,b]
4	1505 ± 326[a]	2001 ± 376[a]	184	732
5	2357 ± 534[a]	2886 ± 590[a]		

[a]$P<0.01$ *vs* Group 1; [b]$P<0.01$ *vs* 1 h after RPF

Serum TNF

The mean serum TNF levels one hour after reperfusion in Group 1 to 5 were (1.84 ± 0.21) U/mL, (2.62 ± 0.29) U/mL, (4.0 ± 0.4) U/mL, (4.5 ± 0.4) U/mL and (7.4 ± 0.6) U /mL respectively. The degree of serum TNF elevation was associated with the severity of warm ischemia injury to donor liver.

Histological examination

One hour after reperfusion, the livers whether or not subjected to warm ischemia all showed basically well-preserved hepatic architecture without lymphocyte in filtration in the portal area. In Group 5, mild diffused hepatocyte vacuolizatio n was identified, accompanied by dilatation of the sinusoids. One week after transplantation, the livers of the different groups manifested lymphocyte infiltrat ion in the portal area and congestion in the sinusoids. Neutrophil infiltration in the portal area was also identified in Groups 3 and 4. Furthermore, mild lobu lar necrosis surrounding hepatic vein was found in Group 3; moderate lobular nec rosis and ballooning degeneration were found in Group 4.

DISCUSSION

Ever since the guidelines for defining brain death have been established, brain dead donors with beating hearts have been the commonest source of transplant organs[8]. However, the concept of utilizing organ allograft from non-heart-beating donors for transplantation is not new. During the early stage of tran splantation, NHBDs were the only source of unpaired allograft, i. e. hearts and livers. With refinements of surgical techniques, improvements in immunosuppressi on and development of effective organ preservation, the increasing success of and widening indication for liver transplantation has exacerbated the shortage of donor organs, and resulted in an increasing number of potential allograft recipi ents dying on waiting list. To increase the number of livers available for liver transplantation, grafts procurement from NHBDs has again become the focus of at tention. Furthermore, recent estimates indicate that an increase of 20% to 25% i n organ donors could be realized if NHBDs were used routinely[12].

During the past two decades, the belief in the extremely high sensitivity of hepatic parenchyma cells damage from warm ischemic injury has been challenged. In both clinical and experimental studies, it has been suggested that the live r can tolerate warm ischemia up to 60 min[13-18]. Warm ischemia of graft is considered a risk factor for postoperative graft dysfunction after liver transplantation. However, in liver transplantation from NHBDs, the effect of warm ischemic damage is subsequently compounded by preservation (cold ischemia) a nd reperfusion injuries. The tolerance of the liver to warm ischemia in such situations is still controversial.

Generally liver allografts can be preserved at tow temperatures safely for up to 24 h in University of Wisconsin solution. In transplantation setting, howe ver, there are a few studies about the limit of the period of warm ischemia before transplantation. In rodents, Soejima *et al*[19]reported that none of their control rats exposed to 30 min warm ischemia before transplant ation survived more than 2 d, and also the similar result was seen in Sumimot o's study[20]. However, Ikeda[21]reported that all

the rats suffering 30 min warm ischemia before transplantation survived more than 3 wk . Moreover, in 1995 Xu and Jones reported 50 per cent of one-week survival rate after 100 min of non-heart-beating time[22], and also Takada re ported his successful pig liver transplantation from NHBDs exposed to 60 min warm ischemia[23]. Our data were compatible with Ikeda's[21] and Richter's[24]. The present study unexpectedly found that rat livers harvested from NHBDs after 30 min of cardiac arrest could be successfully gr afted to the recipients with a 62.5% one-week survival rate and hav e the possibility to survive more than 60 d. No statistical significance of one-week survival rates was found between Group 1 and 3. In Group 4, even the don or liver suffered 45 min of warm ischemia, two of eight (25%) rats s urvived more than a week, also with a chance of long-term survival. The results confirmed that the liver is less sensitive to warm ischemia than formerly belie ved.

Hepatic ischemia-reperfusion injury is characterized by sinusoidal perfusion failure, accumulation and adherence of leukocyte in sinusoids, loss of the end othelial integrity with concomitant extravasation of blood cells, and, finally, breakdown of the liver microcirculation[25]. Under inflammatory conditi ons, there are several factors that would favor neutrophil trapping. First swell ing of Kupffer cells and endothelial cells occurs during exposure to inflammatory mediators. Second, an imbalance of vasoconstrictor (e.g. endothelin-1) and vasodilator (e. g. nitric oxide) formation can further narrow the sinusoidal diameter[26,27]. Moreover, inflammatory mediators such as activated complemen t factors reduce the deformability of neutrophil. The combination of these factors appears to be responsible for the microcirculation failure.

It is widely accepted that warm ischemia and cold ischemia represent two differe nt and distinct types of injury to the liver. While cold ischemia mainly causes injury to the cells of the sinusoidal lining, hepatocytes are the most vulnerable to warm ischemia[21,28,29]. The postoperative elevations in the serum liver enzyme concentration observed in the present study may reflect the extent of hepatocyte disruption, which was more severe in proportion to the duration of warm ischemia. TNF procuded by Kupffer cells plays an important role in the pathogenesis of early graft failure[30-33]. In this study, we demonstrated an elevation of serum TNF levels in association with increasing prolonged wa rm ischemia time. The degree of histological damage and serum ALT and AST value changes correlated positively with the TNF levels.

In conclusion, rat liver can tolerate a prolonged warm ischemia time up to 45 min for transplantation with a chance of recipient survival. Based on our experience, warm ischemia is not the only factor limiting the success of liver transplantation from NHBDs[34]. More attention should be paid to other factors such as the mechanical injury during the harvest of cadaver donor liver and to the cold preserve time and anhepatic phase. Actually, clinical experience with the use of liver allograft from NHBDs has been reported[4,5,35]. The patients, who had irreversible neurologic damage but did not meet standard brain death criteria, were transported to the operating room where ventilation w as discontinued, and rapid cold perfusion was performed after declaration of death. In such controlled circumstances, the warm ischemia time was less than 30 min in most cases. Some of the procured grafts were actually transplanted, exh ibiting satisfactory initial function. However, in the patients who suffered a sudden cardiac arrest, in whom cardiopulmonary resuscitation was necessary before organ procurement (referred to as uncontrolled NHBDs), there was a high inciden ce of primary graft nonfunction. In this regard, the present study is a simulati on of well-controlled NHBDs, and further intensive experimental studies are required to approach this goal, because human NHBDs have much more complicated clin ical conditions than healthy rat donor. In clinical liver transplantation, unsta ble hemodynamic conditions before cardiac arrest may produce a warm ischemia injury. New strategies to attenuate ischemic liver injuries will be required to est ablish the safety and efficacy of liver transplantation from human NHBDs.

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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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Serum vascular endothelial growth factor is a potential biomarker of metastatic recurrence after curative resection of hepatocellular carcinoma

Qi Niu, Zhao You Tang, Zeng Chen Ma, Lun Xiu Qin and Lian Hai Zhang

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China. To date, surgery is still the best solution to it. However, metastatic recurren ces after curative hepatic resections are very common. Tang *et al* have repor ted that recurrence rate within 5 years of curative hepatic resection is 61.5%[1]. As curative hepatic resection has a high tendency for metastatic recu rrence, therapeutic interventions such as transarterial embolization and antiang i ogenesis have been tried to further improve prognosis of HCC patients. Therefore, establishing a dependable, sensitive, easy, and economical method to predict me tastatic recurrence following curative hepatic resection is of clinical urgency.

Neovascularization has been shown to be essential for the growth and metastasi s of solid tumors. Vascular endothelial growth factor (VEGF), a dimeric hepari n-binding glycoprotein with a molecular weight of about M_r45000, is one of the most important angiogenic factors. In addition to increasing permeability of blood vessels, VEGF has potent mitogenic effect on vascular endothelial cells[2-9]. Serum VEGF levels have previously been shown to be raised in patients with various tumors, including brain, renal, melanoma, breast, gastrointest inal, and liver malignancies particularly in metastatic diseases[10-15].Because VEGF plays an essential role in tumor angiogenesis and hence the meta stasis and recurrence of HCC, its elevation in serum may be a candid at ebiomarker of metastatic recurrence. Consequently, we set out to study whether preoperative serum VEGF could be used as a biomarker of metastatic recurrence following curative hepatic resection in HCC. Since 84.6% of HCC patients have acc ompanied cirrhosis to some extent we also examined serum concentrations of VEGF in cirrhotic patients and normal healthy controls[16]. In addition, we studied the relationship between serum VEGF concentrations and immuno histological expressions of two known metastatic recurrence parameters-*p*53 and PCNA in tumor tissues.

MATERIALS AND METHODS

Subjects

The current study registered 12 normal healthy controls, 12 patients with cirr hosis, 8 patients with benign liver tumors including hemangioma and focal nodular hyperplasia, and 85 HCC patients who received curative resection. The healthy controls were selected randomly from people coming to our hospital for a medical checkup and found to be healthy. Cirrhotic patients were diagnosed clinically. HCC and benign liver tumor were diagnosed histologically. All HCC patients had u nderlying cirrhosis to some degree as confirmed by operations. Thrombi, intra- and extra-hepatic dissemination, were confirmed by operation, and/or ultrasonog raphy, and computed tomography. According to generally recognized standards, we set HCC patients with thrombi, intra- and extra-hepatic dissemina tion, and tumor size larger than 5 cm as high-tendency metastatic recurrence (HTMR) group, and less than 5 cm as low-tendency metastatic recurrence (LTMR) group. Hepatic resection with no signs of tumor lesion within the liver, and no metastatic lesion outside the liver after operation as well as no tumor thrombi in major bran ches of portal, hepatic vein, and intrahepatic biliary ducts before operation wa s considered as curative hepatic resection.

Blood samples were taken from all subjects. The serum was separated after 20-30 min of coagulation at room temperature and was stored at -80℃ until the assay. Repeated thawing and freezing of samples was avoided.

Liver Cancer Institute & Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Dr. Qi Niu was born on April 8,1970 in Guoyang, Anhui Province. In 1994 he graduated from Bengbu Medical College as a Bachelor of Medicine and in 1997 as a Mast er of Science. From 1997 to 2000 he studied as a Ph. D. and M.D. student in Shan ghai Medical University majoring in metastasis and recurrence after curative hepatic resection in HCC and published 4 papers.
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Correspondence to: Dr. Qi Niu, Liver Cancer Institute & Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, China
Tel. 0086-21-64037181, Fax. 0086-21-64037181
Email. qiniu99@yahoo.com
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VEGF assay

The VEGF determinations were performed in duplicate following the manufacturer's instructions using the R&D Systems Quantikine enzyme-linked immunosorbent assay (ELISA) kit. The VEGF concentration in a sample was determined by computer software-generated interpolation (Microsoft Origin software) from the standard curve. The internal VEGF standards ranged from 0 to 2000 ng/L, and the intens ity of chromogen was measured at a wavelength of 450 nm with a reference wavelength of 595 nm using the dual wavelength mode on the BIO-RAD 450 Microplate Reader. Standard curve was generated and plotted using a log-log linear regression.

Immunohistochemistry

For the immunohistochemical demonstration of p53 and PCNA protein, formalin fixed, paraffin embedded sections were deparaffinized in xylene and alcohol and placed for 15 min in alcohol-H_2O_2 for blocking endogenous peroxidase. The samples were processed in a microwave oven, placed in a thermoresistant plas tic box with 10 mmol/L pH 6.0 citrate buffer. Tissue sections were treated in th e oven twice for 5 min while the buffer was boiling. Tissue sections were left a t room temperature in the buffer solution for 20 min without drying. Section s were treated with bovine serum albumin to prevent background staining and incu bated for 1 h with a primary nondiluted ready-to-use murine anti-p53 a ntibody (Dako, Carpentaria, CA) or murine anti-PCNA antibody (Dako, Carpentaria , CA) diluted at 1:500 at room temperature in a humidified chamber. Slides were rinsed with phosphate buffered saline for 3 min and incubated first with the biotinylated linked goat anti-mouse antibody for 30 min and then with the l abeling reagent, peroxidase conjugated streptavidine, for 30 min. After the slides were rinsed, the peroxidase label was demonstrated using 3-amino-9-eth ylcarbazol (AEC) for 15 min, and counterstained with Mayer hematoxylin. AEC produced a red product which was soluble in alcohol and was used with an aqueous mounting media. Positive and negative controls were included in each experiment. Specifically, for the latter the primary antibody was substituted with nonspec ific mouse IgG. p53 or PCNA immunopositivity was recorded when more than 15 carcinoma cell nuclei were stained in one or more fields[10].

Statistics

Analyses were performed using SAS (Version 6.12; SAS Institute, Inc., Cary, NC). Student's *t* test and Oneway ANOVA were used to determine the differences between the means of different groups. Results were expressed as mean ± SD. Th e level of significance was $P<0.05$.

RESULTS

The VEGF concentrations in the normal controls and groups of cirrhotic, benign liver tumor, and HCC patients were 158.46 ± 41.84 ng/L, 90.00 ± 22.42 ng/L, 156 .34 ± 41.32 ng/L, 164.42 ± 76.07 ng/L, respectively (Table 1). Cirrhotic patients had the lowest levels of VEGF in the four groups. Compared with the cirrhotic group, HCC group had a significantly higher level of VEGF in the serum ($P<0.01$). Yet, no significant differences could be found between serum levels of VEG F in HCC and benign liver tumor or normal healthy control group ($P>0.05$) (Fi gure 1). Since large HCC has a high tendency to recur after hepatic resection, we next divided HCC patients into small HCC and large HCC group. The VEGF concent rations of large HCC group were a little higher than those of small HCC patients (173.52 ± 52.34 ng/L *vs* 154.46 ± 37.23 ng/L, $P > 0.05$). However, this diffe rence was not significant. In patients with thrombi, VEGF levels were significan tly higher than those in patients without (182.46 ± 35. 61 ng/L *vs* 157.62 ± 53. 42 ng/L, $P < 0.05$). On dividing the HCC patients into HTMR and LTMR groups, HT MR patients were observed to have significantly higher VEGF concentrations in th e serum than LTMR patients (185.33 ± 92.88 ng/L *vs* 144.75 ± 51.37 ng/L, $P < 0.05$) (Figure 2). Anotable case observed was that of a female patient having a tumor growth of just 1.8 cm diameter with no thrombi but with the highest levels of VEGF (819.37 ng/L), she was the first to metastasize (within three months). As p53 is reported to play a role in regulating the production of VEGF, we f urther divided HCC patients into *p*53 positive and *p*53 negative groups. We found that serum VEGF levels in p53 positive patients were significantly h igher than those in p53 negative patients (176.56 ± 53.29 ng/ L *vs* 149.26 ± 41.29 ng/L, $P < 0.05$). Despite PCNA being a commonly used clinical indicator of metastatic recurrence after curative hepatic resection in HCC, we did not fi nd any significant difference in VEGF levels between PCNA positive and PCNA nega tive groups (176.56 ± 53.29 ng/L *vs* 165.26 ± 54.29 ng/L, $P > 0.05$).

Table 1 Serum VEGF levels in different HCC groups which received curative hepatic resect ion, benign liver tumor group, and normal control group ($\bar{x} \pm s$)

Group	Case (*n*)	VEGF concentrations (ng/L)
Normal control	12	158.46 ± 41.84
Liver cirrhosis	12	90.00 ± 22.42
Benign	8	156.34 ± 41.34
HCC	85	164.42 ± 76.07[a,b]
Small HCC	34	154.46 ± 37.23
Large HCC	51	173.52 ± 52.34[c]
Without thrombi	71	157.62 ± 53.42
Thrombi	14	182.46 ± 35.61[d]
p53 negative	34	149.26 ± 41.29
p53 positive	38	176.56 ± 53.29[e]
PCNA negative	36	165.26 ± 54.29
PCNA positive	31	176.56 ± 53.29[f]

[a]$P<0.01$, *vs* liver cirrhosis; [b]$P > 0.05$, *vs* benign; [c]$P < 0.05$, *vs* small HCC; [d]$P < 0.05$, without thrombi; [e]$P < 0.05$, *vs* p53 negative; [f]$P > 0.05$, *vs* PCNA negative.

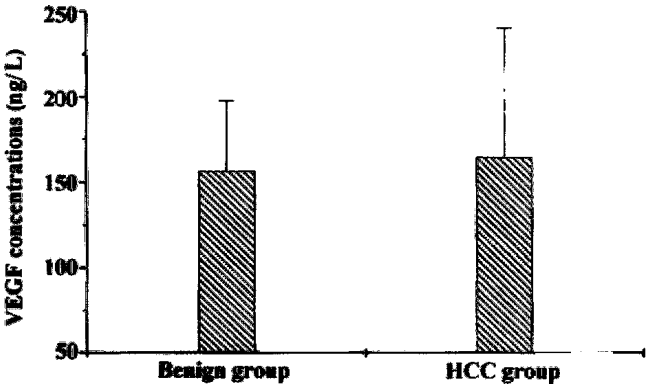

Figure 1 Serum VEGF levels in benign liver tumor and HCC (hepa tocellular carcinoma) groups.

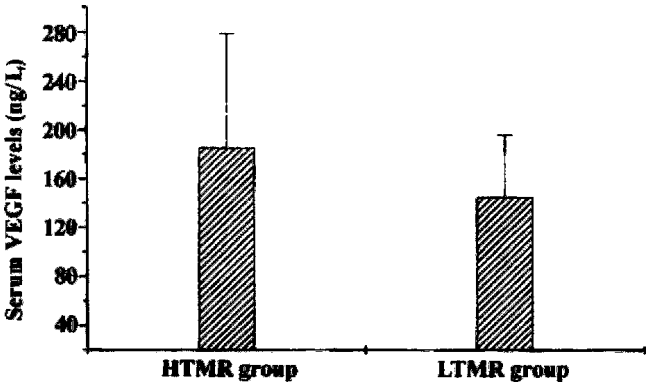

Figure 2 Serum VEGF levels in HTMR and LTMR groups. HTMR: high-tendency metastatic recur rence group; LTMR: low tendency metastatic recurrence group.

DISCUSSION

VEGF is produced by a wide variety of tumor cells, helping the growth and diss emination of the solid tumor by making it more vascular. In HCC, it acts in a paracrine fashion and plays an essential role in tumor angiogenesis[2-5, 17].

The prognostic value of VEGF has been shown in breast and gastric cancer based on VEGF expression in tumor tissue detected by immunohistochemistry, with VEGF concentrations being high in highly vascular rich breast tumors. The VEGF positi vity in gastric cancer correlates with vessel involvement, lymph node metastasis as well as liver metastasis and is associated with an overall poor prognosis[18-23]. Because local tumor invasion and metastatic spread are angiogenes is-dependent, it is hypothesized that metastatic recurrence after curative hep atic resection in HCC may be associated with up-regulation of angiogenic factors. Our present study showed that HTMR patients had significantly higher levels o f VEGF than LTMR patients. This indicates that VEGF is a potential biomarker of metastatic recurrence in HCC patients after curative hepatic resection. The sens itive elevation of VEGF in one female patient further strengthens the hypothesis that raised VEGF levels may predict metastatic recurrence in HCC. The range of serum VEGF levels among healthy controls was from undetectable to 481.02 ng/L. The relevance of normal levels of VEGF is not clear at present and further studies are required to clarify it. As about 84.6% of Chinese HCC patients have some degrees of cirrhosis, it would be proper to compare VEGF levels between cirrhos is group and HCC group, rather than normal healthy controls and HCC patients[16]. Compared with cirrhotic patients, HCC patients had significantly hig her levels of VEGF in their serum. It indicates that VEGF could play an importan t role in transforming liver cirrhosis into HCC. Unexpectedly, the mean serum le vels of VEGF in HCC and benign liver tumor patients were observed to be very clo se. This suggests that VEGF can not be used as a marker to distinguish benign li ver tumor from malignant one (HCC). The main reason for this phenomenon may be t hat all of the benign liver patients in our study have noncirrhotic liver. Since tumor thrombi is a putative indicator of early metastatic recurrence following curative hepatic resection and poor prognosis[24,25], we detected VEGF levels in HCC patients with thrombi and found that consistent high levels of VEG F reflected a high tendency towards metastatic recurrence in the thrombi group. Meanwhile, we found that there was no significant difference between small HCC group and large HCC group regarding VEGF concentrations although mean serum VEGF levels in big HCC patients were higher than those in small HCC patients. Despite the fact that tumor size is a commonly used prognostic indicator of HCC, our re sult does not sufficiently reflect its role as an important parameter of metastatic recurrence in HCC[26,27]. Here the point to contemplate is that the ability of metastatic recurrence of HCC cannot be predicted by a single parameter alone, such as presence of tumor thrombi, intrahepatic dissemination or tumor size, rather all of them combined together can give a more precise indication.

*p*53 and PCNA have been reported to be indicators of HCC metastatic recurr ence[28-31]. Meanwhile, p53 also plays an important role in regula ting the production of VEGF. Wild-type *p*53 down-regulates whereas mutated *p*53 up-regulates VEGF expression according to some studies[32-35] . In the present study, *p*53 positive patients had significantly higher levels of VEGF than *p*53 negative counterparts. confirming previous reports[2 5]. However, the difference in VEGF levels between PCNA positive and PCNA neg ative groups was not significant. The role of PCNA in the regulation of VEGF is currently being investigated in our lab.

In conclusion, this study demonstrates that serum VEGF is a potential biomarke r of metastatic recurrence in HCC patients following curative hepatic resection. However, it can not distinguish HCC from benign liver tumor. *p*53 positive patients have a significantly higher VEGF level in the serum than their counterp arts. Further follow-up studies are needed to delineate the ability of VEGF in predicting metastatic recurrence after curative hepatic resection in HCC.

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Edited by Zhu QR
proofread by Mittra S

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

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Comparative ultrastructural study of endoplasmic reticulum in colorectal carcinoma cell lines with different degrees of differentiation

Shu Feng[1] and Jin Dan Song[2]

Subject headings colorectal neoplasms; endoplasmic reticulum; ultrastructure; microscopy, electron; microscopy, electron, scanning; cell diff erentiation

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INTRODUCTION

The endoplasmic reticulum (ER) consists of a complex system of tubules, lamellae , and flattened vesicles, and has a variety of morphologies in different cells. It is believed to play a central role in the biosynthesis of cholesterol, phosph olipids, steroids, prostaglandins, membrane and secretory proteins[1]. C ancer cells have different functions and ultrastructure from their original cel ls[2-4]. The studies on ER membrane system of cancer cells are of great significance in understanding their malignant behavior. In the present work, the ultrastructural characteristics of ER in human colorectal carcinoma cell lines with different differentiation degrees were investigated.

MATERIALS AND METHODS

Materials

Well differentiated human colorectal carcinoma cell line CCL229 and poorly d ifferentiated cell line CCL227 were generous gifts from Dana-Farber Cancer Inst itute of Harvard Medical School, USA.

Methods

Cell culture The human colorectal carcinoma cell lines CCL229 and CCL227 were grown in Dulbecco's modified Eagle's medium (DMEM, G1BCO, BRL) suppleme nted with 10% calf serum and 2 kU/L gentamycin. Cell cultures were incubated a t 37℃ in a humidified atmosphere of 95% air and 5% carbon dioxide.

Transmission electron microscopy Exponential cells cultured in flasks were collected, washed thrice in phosphate buffer saline (PBS), and then fixed in 2.5% glutaraldehyde in a buffer containing 0.1 mol/L sucrose and 0.1 mol/L sodium cacodylate (cacodylate buffer, pH7. 4) for 2 h. After being washed in cacod ycate buffer, the cells were post-fixed in 1% osmium tetroxide for 30 min, dehydrated through graded alcohol and acetone, and embedded in Epon 812. Ultrathin sections were cut with glass knives on LKB 2088 ultratome, stained with uranyl ac etate and lead citrate and examined under a Hitachi H600 transmission electr on microscope.

Scanning electron microscopy Cells grown on coverslips were rinsed thrice in a buffer containing 60 mmol/L sodium citric acid, 25 mmol/L KCl, and 35 mmo l /L $MgCl_2$ (sodium citric acid buffer, pH 7.4), then fixed in 125 mmol/L potassi u m permanganate in sodium citric acid buffer for 7 min. Cells were rinsed in sodium citric acid buffer, dehydrated through graded alcohol, replaced with iso-amy l acetate, dried at CO_2 critical point in a Hitachi HCB-2 critical point drie r, gilded on a BIKO TB-3 ion film-plating machine and then examined under a Hi tachi S-450 scanning electron microscope.

RESULTS

Well differentiated colorectal carcinoma CCL229 cells appeared round or polygona l with many microvilli and pseudopodia and a large elliptic nucleus containing 1 -2 dense nucleolus. The cytoplasm contained some mitochondria, Golgi apparatus, lysosomes, polyribosomes as well as abundant ER. The ER consisted of a complex system of tubules, lamellae, and flattened vesicles distri buted throughout the cytosol. There was a great amount of vesicle-like and flattened cisternal ER in pseudopodia (Figures 1, 2).

More apparent heteromorphism was observed in poorly differentiated colorectal ca rcinoma CCL227 cell. There were less microvilli and pseudopodia on the cell surf ace. The ratio of nuclei to plasma was higher than that found in CCL229 cell. An abundance of free polyribosomes and less

[1]Microbial Engineering Department, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110015, Liaoning Province, China
[2]Key Laboratory of Cell Biology, Ministry of Public Health of China, China Medical University, Shenyang 110001, Liaoning Province, China
Dr. Shu Feng, female, born on 1965-02-09, graduated from China Medical University as a postgraduate and got a doctorate degree in 1996, and is now working as a professor at the Institute of Applied Ecology, Chinese Academy of Sciences, and has having 32 papers published
Supported by the National Natural Science Foundation of China, № 3904005, and "Hundred-Person plan" of Chinese Academy of Sciences, (№ 10989902)
Correspondence to: Dr. Shu Feng, Microbiol Engineering Department, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110015, Liaoning Province, China
Tel. +0086-24-23899941
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of mitochondria, Golgi apparatus and l ysosomes were found in the cytoplasm, and its ER, which mainly consisted of vesi cles and short tubules was much less than that in well differentiated CCL229 cell (Figures 3, 4).

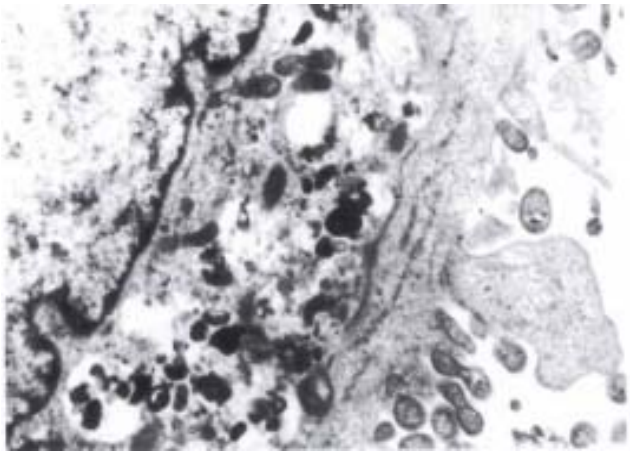

Figure 1 Ultrastructure of well differentiated colorectal carcinoma cell line CCL229 under TEM. × 800

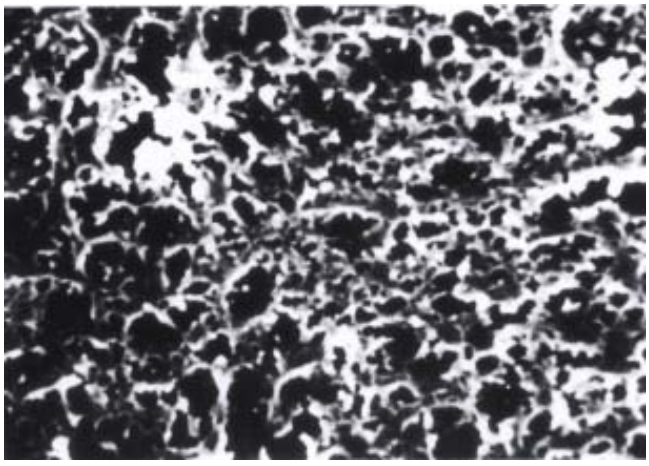

Figure 2 Ultrastructure of well differentiated colorectal carcinoma cell line C CL229 under SEM. × 7500

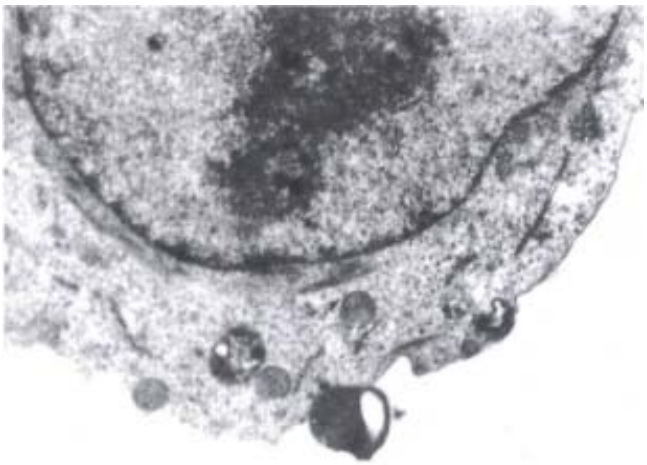

Figure 3 Ultrastructure of poorly differentiated colorectal carcinoma cell line CCL227 under TEM. × 500

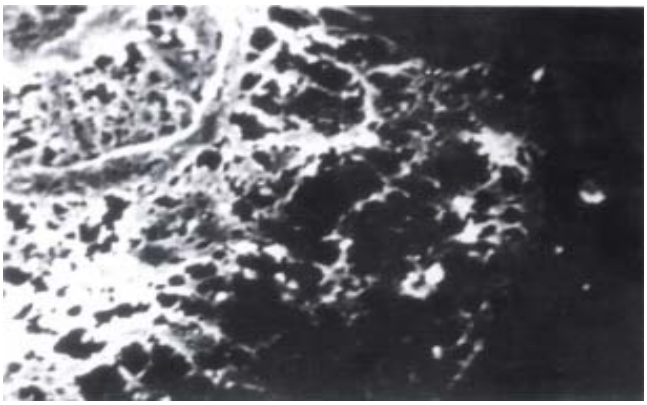

Figure 4 Ultrastructure of poorly differentiated colorectal carcinoma cell line CCL227 under SEM. × 5500

DISCUSSION

Overlap with other compartments and limits of regular light and electron microsc opy make it difficult to study the ultrastructure and cellular distribution of ER membrane system. Terasaki *et al*[5] reported a rapid and simple technique for localizing the structure of whole-mount ER both in living and gluta raldyde-fixed cells by fluorescent microscopy which can also be detected by pha se-contrast microscopy and scanning electron microscopy in potassium permangana te-fixed cells. With this technique, the ultrastructure and distribution of ER in many normal and tumor cells have been studied[6-11].

The ER is a highly specialized structure which performs many distinct functions. Hence a well developed ER may be looked upon as an expression of cell different iation and functional activity. It is abundantly clear that immature or undiffer entiated cells such as stem cells, embryonic cells, and cells in culture have, as a rule, a poor complement of ER as compared with their normal mature function ing counterparts, and this concept also applies to tumor cells. In general, there is a meaningful correlation between ultrastructural signs of anaplasia and mal ignancy. In this study, using regular transmission microscopy and whole-mount E R scanning electron microscopy of potassium permanganate fixation, the ultrastru cture and distribution of ER in well and poorly differentiated colorectal carcin oma cell lines CCL229 and CCL227 was investigated comparatively. The results sho wed that well differentiated cell line CCL229 had abundant endoplasmic reticulum , which consisted of a complex system of tubules, lamellae, and flattened vesicl es distributed throughout the cytoplasm. A great amount of vesicle-like and fla ttened cisternal endoplasmic reticulum were found in pseudopodia. Poorly differe ntiated cell line CCL227 had relatively less ER. This result is coincident with the above-mentioned concept.

In poorly differentiated CCL227 cell, the ER is not abundant, but free polyribos omes are very rich in the cytoplasm. This presumably reflects the active synthes is of endogenous proteins needed for cell growth and division.

Well differentiat ed cell CCL229 is a highly invasive colorectal carcinoma cell line, its high inv asiveness correlates to its cell's ultrastructure[12,13]. Pseudopodia of tumor cells play an important role in the invasion process[14], tumor cells are supposed to secrete a great amount of proteolytic enzymes to cleave the basement membrane[15-18]. In this work, cell CCL229 was found to have many microvilli and pseudopodia with abundant ER which presumably reflect its a ctive synthesis of secretory proteins. This may be the ultrastructural basis of its high invasiveness.

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Edited by Zhu QR
proofread by Mittra S

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

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Study on environmental etiology of high incidence areas of liver cancer in China

Nian Feng Lin[1], Jie Tang[1] and Hoteyi S. Mohamed Ismael[2]

Subject headings liver neoplasms; etiology; epidemiology; water pollution; environmental carcinogens; nitrosamines; china; geology

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INTRODUCTION

China is a country with a high incidence of liver cancer in some areas[1]. Liver cancer has a wide distribution and threatens human health seriously. A rough estimation shows that out of a population of 1.2×10^8 in liver cancer areas patients are more than 1.0×10^5. The environment of the liver cancer area is very complicated and has different characteristics in different regions. But the epidemic regularity of liver cancer is obvious, and the environment in the cancer areas has also distinct characteristics that contribute to the study on the environmental etiology of liver cancer[2-5].

THE EPIDEMIOLOGICAL CHARACTERISTICS OF LIVER CANCER

The liver cancer discussed here has epidemiological characteristics like endemic , comparison and trend. The general investigation of liver cancer all over the country of China or local area shows that the high rate of liver cancer has been found in some provinces, counties, towns, and islands with the back-ground of the low death rate ($10/10^5$)[1,6]. The main areas of high rate of liver cancer chiefly concentrate in the coastal areas such as Jiangsu, Guangxi, Fujian, Zhejiang, Guangdong Provinces, and Shanghai City[7,8] (see Figure 1). Qi dong in Jiangsu Province and Fusui in Guangxi Province are two counties of the highest death rate of liver cancer in China and the death rates are $47.76/10^5$ and $46.87/10^5$[1,9]. As far as macroscopic environment is concerned, the risk area often has focused distribution and has a strict and steady dividing l ine between light and non-liver cancer areas. The risk area has peculiar charac teristics regarding landmark, geology, hydrogeology, and geochemistry[9,10]. It is an important way to study the cause of high incidence of liver cancer from the angle of environmental etiology.

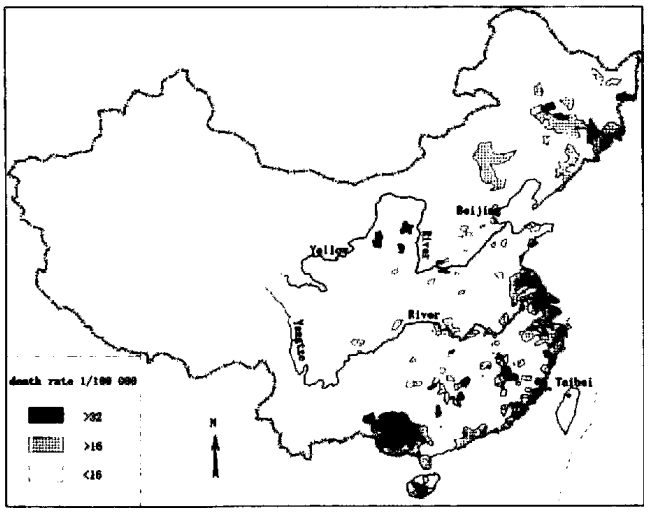

Figure 1 Sketch map of liver cancer in China.

The area of high incidence of liver cancer is adjacent to that of the low i ncidence area and the death rate has great disparity. For example, the death rate in Fus ui is $46.87/10^5$ while that in Niming County is $7.47/10^5$, and the death rate in Qidong is $47.76/10^5$ while that of Rudong County adjacent to Qidong is $20.6\ 1/10^5$. The death rate in Tongxiang Town in Qidong County is $50.67/10^5$ while that in adjacent Xining Town is $17.28/10^5$. All these show that the carcinogens are distinctly different in the two adjacent environments[9].

The three epidemic trends of liver cancer may be summarized as follows: the change in epidemic curve from low to high, parallel development or from high to low. The trend reflects the change in the intensity of carcinogenic factor with time, and the epidemic curve of many high incidence areas of liver cancer belongs to the first type. The curve rose in the 1970s, while it gently went down from the end of the 1980s to the beginning of the 1990s. We think that organic pollution of the drinking water may be the reason for the rising of the curve, while the downward trend of the curve may be the result of changed water.

[1]College of Environment and Construction Engineering, Changchun University of Science and Technology, Changchun 130026, China
[2]Baiqiuen Medical Science University, Changchun 130021, China
Prof. Nian-Feng Lin, male, born on 1932-12-31 in Wanhan City, Hubei Province, graduated from Baijing College of Geology in 1956, majoring in eco-environmental geology, having 52 papers published.
Project supported by National Natural Science Foundation of China, № 48970193
Correspondence to: Prof Nian-Feng Lin, College of Environment and Construction Engineering, Changchun University of Science and Technology, Changchun 130026, China
Tel. +86-431-850-2261
Email. Tang@cust.jl.cn
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THE ECO-ENVIRONMENTAL GEOLOGICAL TYPES IN LIVER CANCER AREAS

The cancer of digestive system in China has wide distribution and threatens huma n health seriously and liver cancer is the most serious one of all[1,11]. Distinct environmental characteristics were discovered in the high incidence a reas of liver cancer according to a survey and analysis on a large scale. It ref lects comprehensively the climate, hydrology, landmark, geology, geochemistry, drinking water, the degree of pollution, economic condition, the food custom and so on. However, the organic and reduction environment and the drinking water polluted by organisms are the most obvious and common characteristics. The high inc idence areas are classified into five geo-environmental types[10,12-14].

(1) The denuded mountain environmental type: it is mainly distributed at the jun ction of three provinces of Hebei, Henan, and Shanxi in south Taihang Mountain. Esophagus, liver and stomach cancer are the common cancers. The climate is dry and the rainfall is about 500 mm-600 mm. The slopes are bare and are made up of carbonate and clastic rock. The ground has sparse plantation and the surface water and groundwater are very scarce. The drinking water condition is poor, and only a few residents drink stream and spring water, while most have to drink pool a nd pond water polluted with organic waste.

(2) The Karst mountain environmental type: it is mainly distributed in Fusui and Longan County in Guangxi Province, the main cancers are liver and stomach cancer. The rainfall is sufficient about 1100 mm-1300 mm. The carbonate rock layer distributes extensively in the cancer areas belonging to Karst geographical are a. Underground rivers are developed while surface water is very scarce. People h ere mainly drink the water from dirty ponds or wells besides the ponds.

(3) The river network plain environmental type: it is mainly distributed in Jian hu and Huai'an in the north of Jiangsu Province, Yangzhong and Taixing in the middle and lower reaches of Yangzi River. Main cancers are of the esophagus and liver. The area belongs to the alluvial and lake plains of the lower reaches of Yangzi River, and most of the cancer areas are in the lake-marsh depression whi ch is rich in humus sludge, peat, and biological deposition and CH_4 is easily found escaping from the earth's surface. The surface water and groundwater is rich but the runflow is stagnated.

(4) The delta type: it is distributed in Qidong, Haimen, Rudong, Chongming Island in Yangzi River Delta and Nanhui County belonging to Shanghai City, Shunde, Jiangmen, and Foshan in the Zhujiang delta. Liver and esophagus cancer are the main cancers. The climate is humid with plenty rainfall. Most of the areas are sea and land mutual depositions that are mainly made up of sand, humus, peat, a nd biological deposition. The surface water and groundwater flow is sluggish and is severely polluted by organisms.

(5) Coastal plain and island type: stomach, liver, and esophagus cancer are the m ain diseases. The cancer areas of coastal plain type are mainly distributed in Changle and Putian in Fujian Province. Most of them are sea and land mutual depos itions of coastal plains that are made up of clay, soil, and biological depositi on. The groundwater and surface water are rich, but severely polluted by organis ms. Dongshan Island in Fujian and Nan'ao Island in Guangdong Province belong to the island type which is a rocky hill and is covered by thin Quaternary deposit ion, and fresh water is scarce.

The classification of eco-environmental geology has contributed towards a deep study and contrast between the macroscopic and microscopic characteristics in disease areas, and searching for questionable carcinogenic factors of environment, and giving comprehensive protective measures.

DRINKING WATER TYPES AND LIVER CANCER

Comparing the high incidence areas of liver cancer in China, we discovered that there exists a common faction in cancer incidence in these areas although the natural environment is different. It is that the groundwater or surface water is rich in humuns, the water flow is stagnant and is in an organic and reduction envi r onment consisting of pools, ponds, cellars, trenches, channels, and the ground water in the swampland. The people in non-cancerous areas mainly drink groundwa ter or surface water which is not polluted by organisms. The water flow is smooth an d is in an oxidation environment such as springs, rivers, and shallow or deep well water[9]. Taking Fusui and Qidong as examples, we have constructed a figure that reflects the relation between the type of drinking water source and the death rate due to liver cancer (Figure 2). The figure illustrates that the death rate due to drinking the pond and pool water is the highest and due to deep well water is lowest.

RELATIONSHIP BETWEEN DRINKING WATER AND LIVER CANCER

After affirming a close relationship between drinking water type and liver cancer, we tested the inorganic and organic compositions of all kinds of drinking water. We did not find any peculiar contents regarding common and trace elements, but some cancer areas were low in minerals and Se in water [13-16]. Howe ver the organisms in water were different and the death rate due to liver cancer was found to have a very close relationship with drinking water type and the de gree of water pollution[17-18] (Table 1).

From Table 1, it can be observed that water pollution indicator such as nitrate, nitrite, COD, and humic acid have an obvious relation with the drinking water type and death rate from liver cancer increases with a rise in the

above indicators. In order to study the relationship between death rate and water quality, 29 water samples that corresponded to death rate due to liver cancer were chosen. The death rate and COD, DO, BOD, NO^-_2, and humic acid were used to mak e a correlation analysis to study the relationship between the death rate and th e five factors. The regression equation is y = a+bx, y stands for the death rate due to liver cancer, xstands for the five factors. The results of the analysis are listed in Table 2.

Table 1 The organic pollution indicators of drinking water and death rate from cancer

The drinking water type	Qidong in Jiangsu					Fusui in Guangxi		
	Pond	Channel	River	Shallow well	Deep well	Pond	River	Deep well
Death rate	61.53	58.17	41.99	18.80	0.0	115.05	37.49	0.0
Number of samples	20	18	22	21	16	7	18	25
COD	4.66	3.85	3.55	3.20	1.28	48.27	0.75	0.197
Humic acid	0.43	0.36	0.28	0.18	0.08	0.58	0.18	0.042
NO_3^-	14.52	18.20	21.46	67.91	1.53	0.74	0.75	0.480
NO_2^-	0.54	0.48	0.38	0.28	0.04	0.061	0.008	0.0096

The death rate is $1/10^5$ and the unit of NO_3^-, NO_2^- is ppm.

Table 2 The analysis between various drinking water factors and death rate due to liver cancer

Content PPM	Regression a	Coefficient b	Interrelation coefficient r
Humic acid	29.4985	78.6384	0.5981^b
NO_2^-	53.1249	75.9767	0.4232^a
COD	43.8098	0.4280	0.4881^b
BOD	45.0576	0.6231	0.4872^b
DO	191.8340	-19.0087	-0.7285^b

$^aP < 0.05$, $^bP < 0.01$, *vs* control.

The results shows that humic, COD and BOD have a very remarkable positive re lati on with the death rate ($P < 0.01$), NO^-_2 has a significant positiv e correlation. While DO has a very marked negative correlation ($P < 0.01$). That is to say, the lower the DO is in the water, the higher is the death rate. Pond water is severely polluted by organisms and is in a strong reduction environmen t lacking of DO. We postulate that the five factors given above can be used as i mportant signs to judge the water quality and the death rate due to liver cancer .

THE RELATION BETWEEN NITROSAMINE AND LIVER CANCER

It has been affirmed that nitrosamine is carcinogenic. Nitrosamine contents were observed in water from high incidence areas of liver cancer in the 1970s and 1980s in China, but the test method used was not very sensitive for semi-quan tit y[18].

We collected 24 water samples from cancer-prone villages and non -cancer-prone villages in Fushui County to quantify the contents of nitrosamin e compound in water. Nitrosamine was not found in sixteen water samples taken fr om river, stream, and deep well water except in 8 samples of pond water. Dimethy l nitrosamine was the main compound in the pond water samples, only three pond wa ter samples contained diethyl nitrosamine. The death rate seems to have positive relation with the contents of nitrosamine (Table 3).

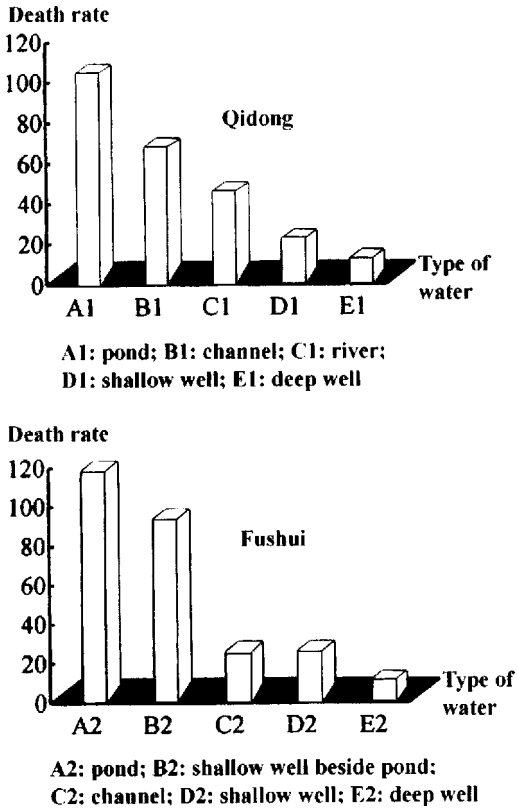

Figure 2 Relation between the types of drinking water source and the death rates from liv er cancer.

Table 3 The content of nitrosamine in pond water and the death rate due to liver cancer in Fusui

Location of samples	Death rate of liver cancer $1/10^5$	Dimethyl nitrosa mine ppb	Diethyl nitrosamine ppb
Sihe	74.96	3.8	
Jiuchen	35.42	3.2	
Yiliu	31.68	3.6	
Mumin	80.80	2.4	2.2
Qutun	112.59	5.6	
Zhongyuan	148.49	4.8	2.4
Lailu	95.36	4.2	
Jiuta	18.38	0.8	0.4

MUTATION TEST OF DRINKING WATER

Professor De Long Su[1] holds that the high incidence of liver cancer h as relation with water polluted by micro organisms. He carried out an Ames test on the drinking water in Qidong disease area and got a positive result. Li Sheng Zhang[9] carried out a micronucleus test using Zilu Grass from America on pond water in Fusui disease area and extracted liquid from the pond mud. His res ults show

that high pollution degree corresponds to high micronucleus rate of pl ant cell and high death rate due to liver cancer. He also found that the micronu clei in clean deep well water in Karst area and corresponding water are very low .

Following some studies[19-24], we decided to carry out an HHPL muta tion test on pond water, shallow well water, and deep well water. Distilled water was used as a blank contrast.

The first test was the cell micronuclei test and the results showed that HHPL micronuclei rate found in the water that has been poisoned had a dose-effect relationship with the density of the samples and the higher the density of samples content was, the higher the micronuclei rate was. The micronuclei rate had a marked difference (pond water > shallow well water > deep well water ⩾ blank). On evaluation by *t* test, the micronuclei rates of poisoned pond water and shallow well water were significantly different ($P < 0.001$).

The second test used was the mutation test of cell chromosome malformation test. The results showed that the mutation rate of chromosome aberration of HHPL has dose-effect with the density of each polluted water sample. Rates of chromo som e aberration caused by polluted water are statistically significant and similar t o micronuclei test as evaluated by t test, $P < 0.001$ (Table 4). This can be an important test for studying environmental etiology of liver cancer.

Table 4 Result of cell mutation test and polluted water samples

Water source type	Cell micronuclei rate %	Cell chromosomal aberration ra te %
Pond water 1	63.34	6.42
Pond water 2	59.66	4.76
Shallow well water 1	48.32	3.76
Shallow well water 2	42.66	3.21
Deep well water	3.30	0.75
Blank contrast	2.43	0.17

STUDY ON THE WATER QUALITY BEFORE AND AFTER THE CHANGE IN WATER SOURCE

It has been accepted that liver cancer has close relations with the polluted dri nking water. The changed water project has been made in many cancer prone areas since 1980's, especially in Fusui County, the changed water project has been implemented on a large scale, and the present water source is from the limestone layer. The depth of well is about 100 m-200 m. Most cancer prone villages have used this supply of water with beneficial results. We chosed nine villages supplied with changed water and observed the change in the water quality and human hea lth before and after. Before the water was changed, the pond water was severely polluted, and the color of water was brown and grey green, the water smelled ter rible, and residents had many unhealthy symptoms such as pale cheeks, fatigue, distension of abdomen, menoxenia and liver cancer. After the water was changed, t he deep well water was clear and sweet, the symptoms noted above gradually disap peared, residents felt sound and the liver cancer death rate lowered. The result s of water quality before and after the water was changed and mutation test are listed in Table 5.

Table 5 Contrast in water quality before and after the change in water

Test item (ppm)	NO_2^-	Humic acid	COD	DO	NH_4
1	0.0224	0.53	44.24	6.10	0.34
2	0.0106	0.05	0.34	8.21	0.07
Test item (ppb)	**Se**	**Fe**	**Mn**	**Zn**	**nitrosamine**
1	0.56	1110.88	198.33	9.29	4.20
2	0.39	164.60	10.14	50.47	0

Test item	Plant cell micronuclei rate / %	HHPL micronuclei rate / %	HHPL chromosome aberration rate / %
1	10.34	59.61	4.76
2	5.96	3.30	0.75

Note: 1. pond water, 2. deep well water.

From Table 5 it can be observed that the water quality of deep well is better th an that of pond water. Thus using a change in water source and additional trea t ments,an improvement in human health and prevention in the occurrence of liver cancer[25-32]may be observed.

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Edited by Zhu QR
proofread by Mittra S

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

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Influence of splanchnic vascular infusion on the content of endotoxins in plasma and the translocation of intestinal bacteria in rats with acute hemorrhage necrosis pancreatitis

Ren Yi Qin, Sheng Quan Zou, Zai De Wu and Fa Zu Qiu

Subject headings acute hemorrhage necrosis pancreatitis; microcirculation/splanchnic organ; endo toxins/plasma; intestinal bacterial trans-location

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INTRODUCTION

The main reason for the death of the patient with acute hemorrhage necrosis panc reatitis (AHNP) is pancreatic infection and multi-organ failure caused by endot oxemia and intestinal bacterial translocation [1-7]. However, the patho genesis of endotoxemia and intestinal bacterial translocation remains a question [8-10]; moreover, no effective method of prevention and cure for it has been found till now[11-15]. In the present study, we infused low dose dopamine and low molecular weight dextran through the catheters to abdominal aort a and portal vein, and observed its influence on the endotoxin concentration in plasma and the rate of translocation of intestinal bacteria in AHNP rats.

MATERIALS AND METHODS

Animals

A total of 48 Sprague-Dawley rats weighing 295-320 g were divi ded into 4 groups (with 12 rats in each group): Group A (healthy rats), Group B (AHNP rats), Group C (femoral artery and femoral vein infused rats), and Group D (abdominal aorta and portal vein infused rats).

Department of Surgery, Tongji Hospital, Tongji Medical University, Wuhan 430030, Hubei Province, China
Dr. QIN Ren-Yi, male, born in 1963-10-04, in Zhun Yi City, Gui Zhou Province. G raduated from Gui Yang Medical College in 1986. Receiving the degree of Medical Doctor (Zhejiang Medical University) in 1995, he is now an Associate Professor a nd is doing Postdoctoral research since 1997, and has 21 paper published.
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Correspondence to: Dr. Ren-Yi Qin, Department of Surgery, Tong ji Hospital, Tongji Medical University, Wuhan 430030, Hubei Province, China
Tel. 0086-27-8366-2389, Fax. 0086-27-8364-6605
Email. ryqin@tjh.tjmu.edu.cn
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Experimental methods

Bacterial labeling Following the way previously described by Wells, we di rectly labeled $O_{55}B_5$ Escherichia coli (*E. coli*) with flourescei n isothiocyanate to prepare the solution of 5×10^6 cfu/L tracer.

Induction of AHNP model and blood vessels catheter insertion and infusion Firstly, all rats were deprived of food 24 h before laparotomy, and were given a gavage of fluorescein-labeled *E.Coli* (0.7 mL/100 g) 12 h later, then they w ere kept for 12 h before being anesthetized ip with 2% pentobarbital sodium (0.15 mL/100 g). Secondly, after the abdominal hair was removed and t he abdominal cavity was opened through a midline laparotomy, the pancreas was exposed and 5% taurodexycholic acid sodium solution (0.15 mL/100 g; Sigma) was sl owly injected into the pancreatic duct with retrograde pressure. Five min la ter, hemorrhage, necrosis, swelling and exudation appeared in the pancreas. Thir dly, 2 h after the AHNP models were completed, the animals of group B were infused continuously with saline through the catheters which were connected to the fe moral artery and femoral vein and portal vein (the catheter was inserted from th e ileocolic vein to the main trunk of portal vein), the animals of group C were infused continuously and alternately with low dose dopamine ($5 \mu g \cdot kg^{-1} \cdot min^{-1}$) and low molecular weight dextran (1.5 mL through catheters inserted into femoral arte ry and femoral vein, while being infused continuously with saline through the portal vein. The animals of Group D were infused continuously and alternately wi th low dose dopamine and low molecular weight dextran through the portal vein and abdominal aorta (catheters were inserted from the femoral artery and above the junction between the abdominal aorta and abdominal cavity artery), while being infused continuously with saline through the femoral vein. The total amount of infusion (6 mL/100 g) was the same in each group. Lastly, after 4 h of sustained infusion, the abdominal cavity was found to be hemorrhagic on beingre-open ed, and it was more evident in both Group B and Group C than in Group D. The pan creas showed pathological changes such as hemorrhage, necrosis, swelling and exu dation, while Group A (control group) had no pathological changes.

Indicators and methods

Plasma endotoxin One mL vein blood was put into the heparin-containing tes t tube under aseptic and non-pyrogenic conditions, and after being centrifuged 500 rpm at for 10 min, the plasma was absorbed and preserved in a refr igerator at -20℃. The content of plasma endotoxin was investigated by quantitative azo stromatic coloration limulus test microassay (kit from Institute of Medi cine in Shanghai).

Investigation of mesentric lymph mode (MLN) labeled-bacteria MLN of the ileocecum were excised, weighed, triturated and diluted into 10% tissue plasma a nd was observed under fluorescein microscope for the existence of labeled-bacteria. The bacterial translocation rate was also calculated.

The microcirculation of pancreas and mesentery The diameter of pancreatic and mesenteric small vein at the ileocecum end was directly measured under the microscope and recorded on the video-camera.

Pathological changes in the intestinal mucosa: Observed under light and transmission electron microscopy.

RESULTS

Changes in content of plasma endotoxin and rate of translocation of labeled-bac teria in MLN (Table 1)

Table 1 Changes in plasma endotoxin concentration ($\bar{x} \pm S_{\bar{x}}$, EU/mL) and the rate of translocation of labeled-bacteria in MLN

Group	Plasma endotoxin (2 h)	Plasma endotoxin (6 h)	Rate of translocation of labeled-bacteria (%)
A	0.023 ± 0.004	0.033 ± 0.006	0
B	0.028 ± 0.002	0.340 ± 0.038	91
C	0.025 ± 0.007	0.270 ± 0.048	83
D	0.027 ± 0.001	0.103 ± 0.018	33

There was a significant statistical difference between group D and either group B or C ($P < 0.05$), regarding the content of plasma endotoxin and translocation rate of MLN labeled-bacteria. However, there is no statistical difference betwe en group B and group C ($P > 0.05$), while a significant difference exists when group a is compared with either group B, C, or D ($P < 0.05$).

Microcirculatory changes in pancreas and mesentery (Table 2)

Table 2 Change in the mesenteric small vein diameter after 2 h and 4 h AHNP

Group	2 h MVD	6 h MVD
A	0.66 ± 0.04	0.63 ± 0.04
B	0.69 ± 0.05	1.03 ± 0.05
C	0.72 ± 0.03	1.09 ± 0.05
D	0.77 ± 0.07	0.68 ± 0.05

Between 2 h and 4 h after AHNP, in Group B and Group C, the diameter of panc reatic and mesenteric small vein increased significantly ($P < 0.05$), and the velocit y of blood was observed to be retarded or even blocked, while there was no siguificant increase in the diameters of pancreatic and mesenteric small veins in group D ($P > 0.05$) and no st atistical difference regarding the velocit y of blood stream between group D and group A ($P > 0.05$).

Pathological changes in intestinal mucosa

Optical microscopic observation It was seen that a large-number of mucos al chorionic epithelium were exfoliated, the upper parts of villus intestina were in significant edema, the central chylectasia was expanded, the blood vessels congested, the proprietary membrane was in moderate edema and the inflammatory cells infiltrated in group B and group C. While the damage of mucosa in group D w as alleviated as compared to group B or group C, it was seen that only the villus became shorter, the proprietary membrane was in edema and the inflammatory cel ls infiltrated.

Electronmicroscopic observation Rarefaction and exfoliation of the epithe lium microvilli of intestinal mucosa, exudation of matrix vacuolar degeneration of mitochondria, swelling of endoplasmic reticulum, and break-down of epitheliu m bridges were observed in groups B and C; and in group D only slight dera n gement of intestinal mucosa epithelium and slight swelling of mitochondria and endoplasmic reticulum were seen.

DISCUSSION

An extensive amount of experimental and clinical work reveals that the disorder of pancreatic microcirculation, the production of many inflammatory mediators an d cytokines and the translocation of intestinal bacteria are all thought to play a critical role in the pathogenesis of acute hemorrhage necrosis pancreatitis [15-26]; furthermore, the disorder of splanchnic organic microcirculation, especially the disorder of pancreas microcirculation in AHNP is closely connected with the production of many inflammatory mediators and cytokines[26-37]. Dopamine has been seen to possess complicate pharmacological function s[38-40], in above 10 μg • kg^{-1} • min^{-1}, alpha adr energic receptors are additional ly activated, causing splanchnic vascular contraction. At a dose range of 1-4 μg • kg^{-1} • min^{-1}, the effect is predominantly on dopaminergic receptors, leading to splan chnic dilatation. At the 4-10 μg • kg^{-1} • min^{-1}, beta a drenergic receptors are increasi ngly stimulated, which stops the increase in microvascular permeability caused by histamine and bradykinin. It also activates dopaminergic receptors leading to an increase of blood flow in splanchnic organs. Low molecular weight dextran can lower blood viscosity and hemaglutination leading to a halt in microvascular th rombogenesis in portal vein system[41-42]. Therefore, low dose dopamine and low molecular weight dextran can be used to improve splanchnic

microcirculat ion. With the aid of catheters inserted into the aorta and portal vein, we i nfused low dose dopamine and low molecular weight dextran continuously and alter natively, which enhances the drug concentration in pancreas, liver and intestin al tract. The results have revealed that by this method the microcirculation was improved, and the content of endotoxin and the rate of intestinal bacterial t ranslocation were decreased with increasing blood supply to the pancr eas and intestinal tract of AHNP rats. This effect can be related to the followi ng factors: Firstly, low dose dopamine and low molecular weight dextran can dire ctly improve the ischemic status of intestinal tract and inhibit the damage of t he barrier function of intestinal mucosa. It can also inhibit the pancreatic hem orrhage, necrosis and decrease the production of inflammatory mediators by impro ving microcirculation of pancreas, liver, and intestinal tract. On the other hand they can lighten the damage of inflammatory mediators and endotoxin on intesti nal tract by enhancing the ability of the liver in clearing from inflammatory me dia tors and endotoxin. Hence, the method can alleviate the injury of intestinal mucosa and protect its barrier function, and inhibit endotoxemia and translocation of the intestinal bacteria, and so indicate that the disorder of microcircul a tion of pancreas and intestinal tract and liver are critically important to endo toxemia and the bacterial translocation from the intestine. The study also shows that the infusion through catheter to femoral vein and artery has no obvious in fluence on the content of endotoxin in plasma and the bacterial translocation in AHNP rats, the reason of which may be related to the low concentration of drugs in pancreas, liver and intestinal tract.

To achieve clearance of inflammatory mediators in patients with AHNP, in addition to drainage and removing the necrotic tissues by op e ration, we can also infuse low dose dopamine and low molecular weight dextran in to the abdominal cavity after operation through catheter inserted either from t he right gastroduodenal vein to the portal vein or from the femoral artery, which can improve the microcirculation disorder of pancreas, liver and intestinal t ract. Moreover, we can infuse enzyme inhibitors and other anti-inflammatory med iators through the catheter to the portal vein, so as to eliminate inflammatory mediators before they reach the liver.

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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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Regulatory effects of electro-acupuncture at Zusanli on ir-SP content in rat pituitary gland and peripheral blood and their immunity

Wei Gao[1], Yu Xin Huang[1], Hong Chen[2], Da Yong Song[3] and Qin Li Wang[1]

Subject headings electro-acupuncture; pituitary; point ST36; substance P (SP); erythrocytes; fl ow cytometry; rats; immunity; immunosuppression

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INTRODUCTION

It has been reported in many studies that electro-acupuncture (EA) can positive ly regulate erythrocytic immunity and T-lymphocytic subgroups[1-8]. Nevertheless, its mechanism remains to be explored. In the present study, a multi -group, multi-stepped and multi-indexed observation was conducted on the effe cts of EA on erythyrocytic immunity and T-lymphocytic subgroups. A simultaneous assay of the changes in immunoreactive substance-P (ir-SP) content in the pit uitary gland and peripheral blood was also carried out. The objective of the study was to investigate the regulatory effects of the immune system and their poss ible mechanism in the treatment of relevant diseases with EA.

MATERIALS AND METHODS

Animals and groups

Forty healthy Shanghai SD white rats weighing 150 g-180 g, aged 12 wk-14 wk, p rovided by the Center for Laboratory Animals, 4th Military Medical University, Xi'an, were divided into 5 groups with 8 rats in each group: the normal control group, the Zusanli group, the immunosuppressive model group, the non-acu point group, and the Zusanli + immunosuppressive group.

[1]Department of Gastroenterology
[2]Medical Affair Office, Tangdu Hospital, 4th Military Medical University, Xi'an 710038, Shanxi Province, China
[3]Department of Gastroenterology, Chinese PLA General Hospital of Guangzhou Command Area, Guangzhou 510210, Guangdong Province, China
Dr. Wei Gao, female, graduated from Xi'an Medical University, postgraduated in 1996, Presently resident doctor, she has studied gut hormones and motility for years and published 3 papers.
Supported by the National Natural Science Foundation of China, № 39970888.
Correspondence to: Dr. Yu-Xin Huang, Department of Gastroentero logy, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, China
Tel. +86-29-3577721
Email. chentx007@sohu.com
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Instruments and agents

G6805-A EA Instrument was purchased from Shanghai Medical Instrument Factory, Shanghai, China. Radioimmunoassay kit was provided by Beijing Haikerui Biotech Co . Ltd., Beijing, China. Epics-profile II Flow Cytometer was product of Coulter, USA. Anti-rat CD4 FITC/CD8PE was provided by SEROTEC, Britain.

Methods

Rat model of immuno-suppression Rat model of immuno-suppression was est ablished by injecting 100 mg/kg of newly- prepared cytoxan (CY) solution into the rats'abdominal cavity on the 1st and the 4th day. Same amounts of normal saline was injected into the abdominal cavity of the rats of the other 4 groups.

Electro-acupuncture All the rats were raised in a quiet place. The acupo int chosen for EA was *Zusanli* (He-Sea Point, St 36)[9]; the non-a cupoint for EA was 0.5 cm off Zusanli. EA parameters were intermittent waves at 2 Hz and 3 V. The visual param eter was the slight shivering of the hind lim bs of the rats. After they were fixed on a wooden board, the rats of the Zusanli-group were electro-acupunctured at *Zusanli* continually for 30 min at a fixed time for 7 d on a daily basis. The immunosuppressive group and the *Zusanli* + immunosuppressive group were also established according to the above method. EA treatment for the *Zusanli* + immunosuppressive group was the same as that for the *Zusanli* group. Rats of the control and the immunosuppre ssive group were tied by the same method for 30 min at a fixed time on a daily basis but did not undertake EA stimulation.

Sampling and assay Thirty mg/kg of 1% pentobarbital sodium was injected into the abdominal cavity of all the rats on the 7th day of EA. Blood samples were taken by decapitation after anesthesia with 15 U/ml of heparin as, an anticoagulant. Ir-SP content in the pituitary gland and peripheral blood was assayed by radioimmunoassay (RIA) accord ing to the manufacturer's instructions. By micro-whole-blood direct immunofl uorescence staining and flow cytometry, T-lymphocytic subgroups were assayed to reflect the cellular immunity. The rats'RBC-C_3bRR and RBC-ICR were assayed by immune adherence rosette[10].

Statistical analysis SPSS 8.0 (a statistical analysis system) was applied for the data analysis. The methods included variance analysis and linear correlation.

RESULTS

In the Zusanli group, the values of CD4+, RBC-C_3bRR and RBC-ICR an d the conte nt of SP in pituitary gland and peripheral blood were all markedly highe r than that of the control ($P < 0.01$). The values of CD8+ did not change significantly ($P > 0.05$), and the rate of CD4+ positively correlated with RBC-C_3bRR ($r = 0.719$, $P < 0.05$), and so did the content of SP. Th e values of CD4+ and RBC-C_3bRR and the content of SP in rats'pituitary gla nd and peripheral blood of the immunosuppressive group were significantly lower than that of the control ($P < 0.01$, $P < 0.05$). The values of CD8+ did not change significantly ($P < 0.05$). In contrast, after EA at *Zusanli*, there was significant improvement in the indexes of the immuno-suppressive group ($P < 0.01$) whereas there was no statistical difference between the non-point and the control group (Tables 1 and 2).

Table 1 Changes in the values of T-lymphocytic subgroups and erythrocytic immu nity ($n = 8$, $\bar{x} \pm s$)

Group	CD4+	CD8+	RBC-C-3bRR	RBC-ICR
Control	43.1 ± 3.1	23.6 ± 2.6	8.6 ± 2.1	6.8 ± 2.3
Non-point	44.7 ± 4.6	21.0 ± 4.3	11.1 ± 1.2	8.9 ± 2.9
Immuno-suppressed	34.5 ± 2.5[a]	26.2 ± 8.4	5.2 ± 1.2[b]	9.8 ± 4.4
Zusanli	65.6 ± 8.4[a]	29.5 ± 8.2	15.9 ± 3.0[a]	12.1 ± 1.2[a]
Zusanli +Immuno suppressive	48.8 ± 6.0[c]	22.6 ± 7.0	16.4 ± 4.1[c]	15.0 ± 5.2[c]

[a]$P < 0.01$, [b]$P < 0.05$ *vs* control group; [c]$P < 0.01$ *vs* immuno-suppressed group. EA: electro-acupuncture

Table 2 The contents of substance P in pituitary gland and peripheral blood in rats ($n = 8$, $\bar{x} \pm s$)

Group	Pituitary gland	Peripheral blood
Control	569 ± 8	20 ± 7
Non-point	547 ± 22[b]	20 ± 8[b]
Immuno-suppressed	273 ± 106[a]	12 ± 4
Zusanli	592 ± 142[ab]	92 ± 22[a]
Zusanli +immuno-suppressive	554 ± 32[b]	30 ± 9[b]

[a]$P < 0.01$ *vs* control group; [b]$P < 0.01$ *vs* immuno-suppressed group. EA: electro-acupuncture

DISCUSSION

Regulatory effects of EA at Zusanli on the neuroendocrine system

Zhao *et al*[11] discovered the cubic structure of the microangium of Zusanli and Lin *et al*[12] reported that there might be a nerve network in the colloid of the spine and brain stem that corresponds with the stomach channel of Foot Yangming, St, runs through the spine, and reaches the nucleus of spinal tract of trigeminal nerve. These studies have proved the regulatory effect of EA at *Zusanli* on neuroendocrines. With the development of studies on the mechanism of acupuncture, the relation between acupuncture and endocrine hormone has become a focus of interest for many researchers. Many studies have indicated that acupuncture can regulate thyroid hormones, sex hormones, adrenocortical hormones, insulin, brain-gut peptide, etc[13]. Substance P, an important cerebral and brain-gut peptide is widespread in the central nervous system and gastrointestinal tract. It exists not only in the endocrine and paracrine cells, playing the roles of hormones and the local transmitter, but also in the endogenous and exogenous neurons, playing the role of a neural transmitter. The present study has proved that EA at *Zusanli* can significantly increase the synthesis of substance P in the pituitary gland, and its content in the peripheral blood, thus bringing into play its unique physiological and pathophysiological regulatory functions.

The general regulatory effects of Zusanli EA and the theory of neuroendocrine-immune network

It has been reported that many hormones can modify the body's immunity by their intervention at macrophages, T-lymphocytes and B-lymphocytes[14-20] receptor sites whereas immune cells can also secrete various neuropeptides and hormones as immune transmitters. What is more, some neuroendocrine cells can secrete cellular factors. These cells can influence both the neuroendocrine system and the immune system. The three systems of nerve, endocrine and immunity depend mainly on the peptidergic factors they generate and the receptors of the latter to communicate with each other. Therefore, immune cells can also be regarded as a kind of receptors in the body that receive stimulation from antigens, secrete immune transmitter, transmit information to the central nervous system before it regulates, as a feedback, the body's immunity by means of transmitting nerves and hormones. This is the so-called "theory of neuroendocrine-immune regulatory network". The regulatory effect of EA at *Zusanli* on the nervous, endocrine and immune systems cannot be separated from its internal association with the neuroendocrine-immune regulatory network. In addition to its direct regulation of the above systems, acupuncture can also indirectly act upon the systems through their internal association with each other. This is probably the theo retical basis of Traditional Chinese Medicine's general regulatory principle. It is also a point of collision between Traditional Chinese Medicine and modern medicine, namely the holistic medical mode[21].

Role of SP in the mechanism of acupuncture's effects on the immunoregulation

SP is an important biologically active substance of information channel transmission[22]. Modern immunological research has proved that cells of the immune system can release SP and contain SP conjugative sites of

high affinity[23]. SP can affect the immune system in the following ways. It can 1) stimulate and proliferate lymphocytes *in vivo* and *in vitro*; 2) improve the proliferative reaction of the spleen, mesenteric lymph nodes and intestinal aggregated lymphatic follicles[24]; 3) at milli-Molar level, it can induce interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factors (TNF) from monocytes[25]; 4) increase lipopolysacchraide (LPS)-induced secreti on of IL-10 from monocytes in human umbilical cord blood and reverse the inhibitory effect of INF-¦Ã on LPS-induced secretion of IL-10[26]; 5) stimulate the synthesis of PGE2, which in turn stimulates the generation of cellular factors[27]. The present study indicates that after electro-acupuncture at Zusanli, ir-SP in the rats'pituitary gland and peripheral blood increased significantly in comparison with that of the control and were positively correlated with the change in CD4+. This attests that electro-acupuncture can regulate cellular immunity by exciting organism synthesis and releasing SP and affect the neuroendocrine-immunoregulatory network through the indirect ef fect of SP on the change of various cellular factors. However, the change in SP after electro-acupuncture at Zusanli had nothing to do with the immune indexes of RBC, which indicates that electro-acupuncture probably does not depend on SP to influence RBC immune indexes.

The effects of EA at Zusanli on the mucosal immunity of gastrointestinal tract

Modern immunological studies have confirmed the significant regulatory effects of gastrointestinal hormone on the intestinal mucosal immunocytes[28-30]. The intestinal mucosal immuno-tissues are controlled by peptidergic nerve fibres, which were located adjacent to lymphatic systems in the epithelial layer, lamina propria and immunocytes such as macrophages, mastocytes, etc. can directly regulate the intestinal immune system. In the intestineassociated lymphatic tissues, the existence of T-lymphocytes and SP receptors specific to B cells in dense SP teleneurons and aggregated lymphatic follicles indicates that SP might be acting as a nutritious factor for the intestinal immunocytes. It is by stimulating its organic synthesis and release that EA at *Zusanli* regulates the intestinal immunity, thus adjusting gastrointestinal diseases and functions. Furthermore, EA at *Zusanli* can also positively regulate the general immune system by the exchange between GALT and the general lymphatic tissues. According to Traditional Chinese Medicine, pathogenesis boils down to three factors, i.e., spiritual, exopathic and constitutional factors. The three factors constitute the gist of the theory of neuroendocrine-immunoregulation network.

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Edited by Lu J
proofread by Mittra S

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

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The serological prevalence and risk factor analysis of hepatitis G virus infection in Hubei Province of China

De Ying Tian[1], Dao Feng Yang[1], Ning Shao Xia[2], Zheng Gang Zhang[1], Hong Bo Lei[1] and Yuan Cheng Huang[1]

Subject headings hepatitis G virus; enzyme linked immunoabsorbent assay; risk factors; polymeras e chain reaction; prevalence; transcription, genetic

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INTRODUCTION

Hepatitis G virus (HGV),also known as GB virus C, is a recently cloned virus which may be associated with human non A-E hepatitis[1,2]. It is parenterally transmitted and usually coinfected or superinfected with hepatitis B or hepat itis C virus[3-5]. Some investigations have been reported on the serop revalence and molecular prevalence of HGV infection in different areas and diffe rent population[6-15]. Current infection of HGV is diagnosed by detectio n of HGV RNA, and past infection with HGV is detectable by testing anti-HGV env elope protein (E2)[16-17]. To investigate the prevalence of HGV in Hubei Province, a central area of the People's Republic of China, ELISA and RT - PCR were employed to detect serum anti-HGV and HGV RNA in 1516 patients who were divided into 16 groups.

MATERIALS AND METHODS

Samples

One thousand five hundred and sixteen serum samples were obtained from 16 groups in Hubei Province, including 299 drug users (19.72%), 98 prostitutes (6. 46%), 504 blood donors (33.25%), 61 hemodialysis patients (4.02%), 351 cases with viral hepatitis (23.15%), 41 with primary hepatocellular carcinoma (PHC, 2.70%), 51 with hemopathy (3.36%), 53 renal transplant recipients (3.49%) and 3 liver trans plant recipients (0.20%). 55 freshmen, nurses and doctors (3.63%) were employed as healthy controls.

Reagents and detecting methods

Serum HBV markers, HAV IgM, HEV IgM, and anti-HCV were detected by enzyme linked immunoabsorbent assay (ELISA). Anti-HGV was also detected by ELISA and the tes t kits were procured from Wantai Biological Preparation Co Ltd, Beijing. HGV RNA was assayed by reverse transcript polymerase chain reaction (RT-PCR) with the primers of 5'-UTR, and the kits were purchased from the Center of Hepatitis R ea gents, Beijing. Both anti-HGV and HGV RNA were simultaneously measured in the drug users, prostitutes, and healthy subjects. For other groups, HGV RNA was dete cted only when their anti-HGV was positive due to insufficient outlay.

Data statistics

All data were analyzed by means of the Chi-square test.

RESULTS

HGV infections in drug users and prostitutes

The positive rates of anti-HGV and HGV-RNA in drug users were 9.06% (34/375) and 27.20% (102/375); those in prostitutes were 38.77% (38/98) and 20.41% (20/98). The analysis of risk factors of HGV infections in drug users and prostitutes is listed in Tables 1 and 2.

Table 1 Positive rates of anti-HGV and HGV RNA in drug users and prostitutes

	n	Anti-HGV	HGV RNA
Drug users*			
po	211	6.63 (14)	27.96 (49)
iv	164	12.20 (20)	32.92 (54)
Prostitutes			
Non-addict	22	9.09 (2)	13.36 (3)
Addict	76	14.43 (36)	22.36 (17)
Healthy group	55	1.82 (1)	0 (0)

*Seventy-six prostitutes who were addicted to drugs were added to this g roup.

The positive rate of HGV RNA in intravenous drug users (IVDU) was higher than in oral users ($\chi^2 = 4.36$, $P < 0.05$). The positive rates of anti-HG V in the p rostitutes who were addicted to drugs were higher than those who were not ($\chi^2 = 12.19$, $P < 0.01$).

[1]Department of Infectious Diseases, Tongji Hospital, Tongji Medical University, Wuhan 430030, Hubei Province, China
[2]Unit of National Genetic Engineering, Xiamen University, Xiamen 361005, Fujian Province, China
Professor De-ying Tian, graduated from Tongji Medical University in 1975, and is now Director of the Department of Infectious Diseases, having 40 papers published.
Supported by a grant from the National 863 Plans, № 102-07-02-07
Correspondence to: Tian de-ying, Department of Infectious Diseases, Tongji Hospital, Tongji Medical University, Wuhan, 430030, Hubei Province, China.
Tel. +86-027-83663326, Fax. +86-027-83662688
Email. tianyawu@sina.com
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Table 2 Risk factors of HGV infections in drug users

	Sex		Addiction duration		HBV infection	
	Male	Female	< 2 years	≥2 years	HBsAg (+)	HBsAg (-)
Anti-HGV	8.65	9.23	6.48	12.58	13.33	9.37
HGV RNA	3.46	32.84	9.72	52.56	16.67	22.78

The statistical difference of anti-HGV positive rate was tested between users a ddicted for more than 2 years and users for less than 2 years (χ^2 = 4.29, P < 0.05). The positive rate of HGV-RNA in female users was higher th an that in mal e users(χ^2 = 11.55, P < 0. 01), that in the patients who were addicted for more t han 2 years was also higher than those addicted for less than 2 years (χ^2 = 79.06, P < 0.01).

HGV infections in blood donors

Seven percent (29/417) of blood donors were found to be anti-HGV positive and 27.5% anti-HGV positive donors were detected to be HGV RNA positive.

HGV infections in the cases of PHC, hemodialysis, liver or kidney transplanta tion (Table 3)

Table 3 HGV infections in the cases of PHC, hemodialysis, liver or kidney tran splantation

	n	Anti-HGV	HGV RNA in anti-HGV (+) cases
Hemodialysis	61	13.11 (8/61)	37.50 (3/8)
PHC	41	4.80 (2/41)	100.00 (2/2)
Liver transplantation	3	33.30 (1/3)	100.00 (1/1)
Kidney transplantation	53	1.82 (1/53)	0
Total	158	7.5 (12/158)	50.00 (6/12)

HGV infections in the patients with hemopathy

Anti-HGV was detected in 6 (11.7%) out of 51 cases with hemopathy, among them, 4 cases with acute or chronic leukemia, one with aplasia anaemia and one with leukopenia. No HGV RNA was assayed in six anti-HGV positive cases.

HGV infections in the patients with viral hepatitis (Table 4)

Table 4 Positive rates of anti-HGV and HGV RNA in patients with viral hepatitis

	n	Anti-HGV	HGV RNA in anti-HGV (+) cases
Hepatitis A	35	14.29 (5/35)	20.00 (1/5)
Hepatitis B	214	17.29 (37/214)	35.14 (13/37)
Hepatitis C	62	14.52 (9/62)	55.56 (5/9)
Hepatitis E	25	4.00 (1/25)	0
Hepatitis NA-E	15	26.67 (4/15)	50.00 (2/4)
Total	351	15.05 (56/351)	37.50 (21/56)

The positive rates of anti-HGV and HGV RNA did not correlate with the sex, age, duration, and severity of diseases. The mean levels of ALT and T-Bil in patients with hepatitis B whose anti-HGV was positive were higher than those whose anti-HGV was negative.

DISCUSSION

HGV is a new pathogenic agent which was discovered in 1995, its genome structur e resembles other flaviviruses containing a positive, single plus-strand RNA[1,2]. It has been demonstrated that the distribution of HGV is global [1,2,6-14]. The transmission route of HGV is similar to that of HBV and HC V[1,3,4,11,12]. The investigation on HGV infection rates in Hubei Province of China showed that the infection rates of HGV in cases with viral hepatitis , hemodialysis, hemopathy, transplantation, intravenous drug users (IVDU) and prostitutes were 15.05%, 13. 11%, 16.7%, 9.1% and 14.43%, higher than that in h ealt hy controls (1.82 %). The result indicate that HGV infection is common in China .

The infection rate of HGV in drug users varies widely on different documents with a range from 23.8% to 77.4%[7,18-20]. However, there is no contr ov ersy regarding the fact that drug addiction is a high risk factor for HGV infect ion. We found that the positive rate of HGV RNA in IVDU was higher than that in oral drug users (12.82% vs 2.97%, P < 0.05). This suggests that the un sterilized injections contribute to HGV transmission among IVDU.

It is known that sexual contact is a common route for horizontal transmission of HBV, HCV and HDV. Recently, sexual transmission of HGV has been demonstrated[18,21-25]. Our survey found that most of the prostitutes were also addicted to drugs, this may be one of reasons that they had a high HGV infection rate . HGV infection was found in 15.05% of patients with viral hepatitis. This is much higher among the blood donors in the same geographic area. However, there may be bias in this statistical inference, since blood donors who are found to be positive for HBsAg or for anti-HCV through screening programs are told not to donate blood. Thus, the low prevalence of HGV infection may represent a selection bias. This viewpoint was demonstrated by Handajani *et al* [14].

No agreement has been reached on the pathogenicity of HGV. Most of the recent in vestigations show that HGV infection in patients with hepatitis B and hepatitis C is not associated with any changes in indices of liver diseases, including se rum ALT level, Knodell score or histology activity index (HAI)[25-28], and the consequences of hepatitis[27,29]. However, some reports inclu ding this paper found that HGV infection was associated with liver damage, even fulminant hepatitis [31,32]. So further prospective studies are needed to demonstrate its relative significance in causing hepatitis and other diseases.

High infection rates of HGV were also discovered in patients with hemodialysis, PHC, and transplantation. This result may be associated with the more transfusions received by these cases than healthy persons.

A conclusion which can thus be drawn from this study

is that HGV infection is co mmon in this area, and the drug users, prostitutes, transplant recipients, blood donors, patients with hemopathy, hemodialysis, and liver diseases are high risk groups for HGV infection.

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Edited by Lu J
proofread by Mittra S

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

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High-level expression of human calmodulin in *E.coli* and its effects on cell proliferation

Xiao Jun Li, Jian Guo Wu, Jun Ling Si, Da Wen Guo and Jian Ping Xu

Subject headings calmodulin; gene expression; biological activity; *Escherichia coli*; cell proliferation; trifluoperazine; polymerase chain reaction; monoclonal antibodies

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INTRODUCTION

Calmodulin (CaM), widely distributed in almost all eukaryotic cells,is a major intracellular calcium receptor responsible for mediating the Ca^{2+} signal t o a multitude of different enzyme systems and is thought to play a vital role in the regulation of cell proliferative cycle[1,2]. Recently, many studies showed that CaM is also present in extracellular fluid such as cell culture media and normal body fluid and has been reported to stimulate proliferation in a range of normal and neoplastic cells, apparently acting as an autocrine growth factor[3-11]. In 1988, Crocker *et al* reported for the first time that addition of extracellular pure pig brain CaM could promote DNA synthesis and cell proliferation in K_{562} human leukaemic lymphocytes[7]. After th at, more and more research was done on extracellular CaM and evidences demonstra ted that extracellular CaM could also stimulate cell proliferation in normal human umbilical vein endothelial cells[5], keratinocytes[4], suspen sion-cultured cells of Angelica Dahurica, etc[6].CaM is a monome ric protein of 148 amino acids that contains four homologous Ca^{2+} binding domains. CaM has been highly conserved throughout the evolution. Only 1 out of 148 amino acids of human CaM is different from that of fish CaM. Complementary DNAs encoding rat, eel, chicken, human, and trypanosome CaM have been cloned.

In this paper, we describe the expression and purification of recombinant human CaM (rhCaM) and the role of extracellular rhCaM in SP2/0 mouse myeloma cell proliferation.

Department of Immunology, Center of Laboratory Medical Science, Nanjing General Hospital of Nanjing Command, PLA, Nanjing 210002, Jiangsu Province, China
Xiao Jun Li, M.D., female, graduated from Second Military Medical University in 1988, now an associate professor of Immunology, engaged in the research of laboratory diagnosis and pathogenesis of autoimmune diseases, having more than 30 papers published.
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Correspondence to: Xiao Jun Li, Department of Immunology, Center of Laboratory Medical Science, Nanjing General Hospital of Nanjing Command, PL A, 305 Zhongshan East Road, Nanjing 210002, Jiangsu Province, China
Tel. 0086-25-4826808-58177, Fax. 0086-25-4803061
Email. li.xj@china.com
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MATERIALS AND METHODS

Construction of expression plasmid

PCR amplification was used for the insertion of the coding sequence of human CaM III (hCaM III) between BamHI and EcoRI sites of the expre ssion vector pBV220 (constructed by Dr. Zhang[12,13]). The primers for P CR based on the reported sequence of hCaM III were synthesized by DNA synthesizer (Sangon). Upper primer:5'-CGGAATTCATATGGCTGACCAGCTGAC -3', containing EcoRI restriction site. Down primers: 5'-CGGGATCCTTACTTTGCAGTCATCA TC-3', containing BamHI restriction site. The vector pUC/hCaM III (a generous gift from Dr.Strehler EE[14]) was used as template. PCR amplif ications were performed on a thermocycler (PE 9600). The PCR conditions used were 95℃ for 1 min, 58℃ for 1 min, 72℃ for 1 min, for 30 cycles.The product was a single band of about 400 bases detected by 1.2% agarose gel electrophoresis. After purifying the PCR product by High Pure PCR Product Purification Kit (Boehr inger Mannheim) and isolating the vector pBV220 by the Wizard Plus Minipreps DNA Purification Kit (Promega), the amplified gene fragments of hCaM III digested with EcoRI and BamHI were ligated into the pBV220 vector that had been previously digested with the same enzymes. The recombinant construct (pBV220/hCaM III) was then used to transform *E.coli* DH5α (a ge nerous gift from Beijing Institute of Basic Medical Sciences) competent cells by the $CaCl_2$ method.

Expression and purification of rhCaM

A single colony from *E.coli* DH5α cell harboring the pBV220/hCaM III construct was used to inoculate a 5-mL LB culture. After overnight growth a t 30℃, the 5 mL culture was used to inoculate a 1-L culture in LB medium and grown at 30℃ for 4-6 h until the absorbance reached 0.5 at 600 nm. The bact erial culture was then induced at 42℃ for additional 5 h. Stable rhCaM-express ing recombinant *E.coli* was harvested by centrifugation at 5000 r/min for 5 m in. The pellets were washed twice with PBS, resuspended in 0.05 mol/L Tris-HCl buffer (pH7.5, containing 0.25 mmol/L PMSF, 2 mmol/L EGTA and 2 mmol/L β-merca ptoethanol) and disrupted by sonication (Cyclone/Tempest IQ_2) at 100 W for 30 min with 10 s of pulse and 10 s of pulse off time. Supernatant fractions were o btained by centrifugation at 12 000 r/min for 30 min. $CaCl_2$ solution 0.1 mol/L was added to the supernatant to

adjust to 5 mmol/L $CaCl_2$ final concentration. The supernatant was applied to a Phenyl-sepharose CL-4B affinity column (Pharm acia) equilibrated with buffer I (0.05 mol/L Tris-HCl buffer, pH7.5, 0.1 mmol/L $CaCl_2$), then the column was washed with buffer I followed by another wash with buffer I containing 0.5 mol/L NaCl. CaM then was eluted from the column with b uffer I containing 1 mmol/L EGTA. The solution containing purified rhCaM was dialysed against ddH_2O for 48 h. The protein concentration was determined by Brad ford assay. RhCaM was filtered through micropore filter membrane (0.22 μm) to remove bacteria and stored at -20℃.

SDS-PAGE and immunoblot assay

Expression and purified protein were analyzed by 15% SDS-PAGE. Gels were stained with Coomassie brilliant blue G. For immunoblot analysis, protein were separated by 15% SDS-PAGE and electroblotted on to nitrocellulose (NC) membrane (Bio R ad). After blotting, nonspecific protein binding sites were blocked with 3 g/L B SA in 50 mmol/L Tris-HCl, pH 8, and 150 mmol/L NaCl, 0.5% Tween 20. The NC memb rane were incubated with a 1:200 dilution of anti-CaM McAb (Sigma). After wash ing, the membrane were incubated for 1 h with HRP conjugated goat anti-mouse immunoglobulin antibody (1:200, Boehringer Mannheim). Color development was obtai ned by adding DAB substrate.

Amino acid composition

The amino acid composition of purified expression product was analyzed using an automatic amino acid analyzer, Hitachi 835-5Q.

DNA sequence analysis

The DNA sequence was determined with ABI PRISM™ 377 DNA sequenser.

NAD kinase assay

The activity of CaM-dependent NAD kinase(NADK) was detected as described by Har mon[15].

Cell culture

SP2/0 cells were cultured in RPMI 1640 medium (GIBCO), supplemented with 20% neon atal calf serum (NCS) under 37℃, 5% CO_2 conditions.

Measurement of cell proliferative rate

Cell proliferative rate was determined by MTT colorimetric assay[16,17] . SP2/0 cells were collected at the logarithmic growth phase by centrifugation. The cells were washed twice and seeded in 96-well plate and cultured for 48 h with rhCaM and CaM- antagonist trifluoperazine (TFP). MTT 10 μL (Sigma, 0.5 mg M TT in 1 mL PBS) was added to each well of 96-well plate and cultured for another 4 h. After the formazan was dissolved with 100 μL DMSO, absorbance value of each well at 490 nm (A_{490nm}) was read on a BioRad Model 550 microplate reader.

Proliferative rate = $A_{490\,nm}$ value of experimental group/$A_{490\,nm}$ value of control group × 100%. Improved proliferative rate = Proliferative rate-100%.

RESULTS

Expression of rhCaM in E.coli

The results of both restriction enzyme digestion of pBV220/hCaM III a nd PCR identification in which pBV220/hCaM III was used as template s howed a specific single band with the same molecular weight as reported hCaM gen e on agarose gel (Figure 1). DNA sequence analysis also indicated that the recom binant vector had been constructed successively. After heat induction, the total extract of the *E.coli* DH5α cell harboring pBV220/hCaM III was a nalyzed by 15% SDS-PAGE under reducing conditions. As shown in Figure 2, a unique protein band with an apparent molecular weight (17 000) was similar to that o f standard human brain CaM. This protein accounted for over 20% of the total cell ular protein. The study on solubility of expression protein indicated that CaM protein was expressed predominantly in the soluble form. Western blot analysis sh owed that anti-CaM McAb specifically bound to the 17 000 band of expression product. The expression product purified by phenyl-sepharose CL-4B affinity chrom a tography was shown as a single band on argarose gel by SDS-PAGE. The protein concentration was determined by Bradford method and approximately 3-4 mg of the purified protein were obtained from 1 L of bacterial culture.

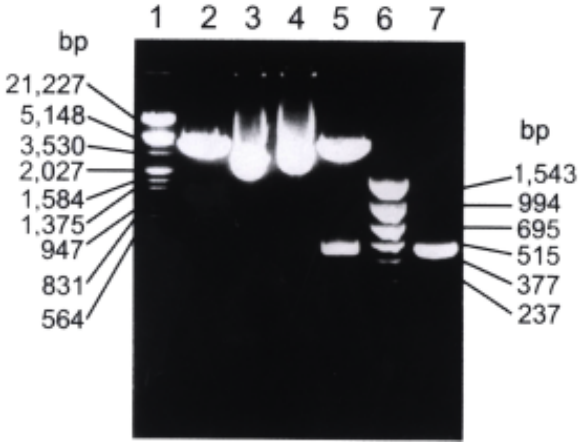

Figure 1 Restriction enzyme digestion and PCR analysis of recombinant plasmid pBV220/hCaM III.
1. λDNA/Hind III+EcoR I marker
2. pBV220 digested with EcoR I and BamH I
3. pBV220
4. pBV220/hCaM III
5. pBV220/hCaM III digested with EcoR I and BamH I
6. pBR322/Hinf I marker
7. PCR product of hCaM III

Amino acid composition analysis

The amino acid compositions of purified rhCaM were identical to those of the pre viously reported bovine brain

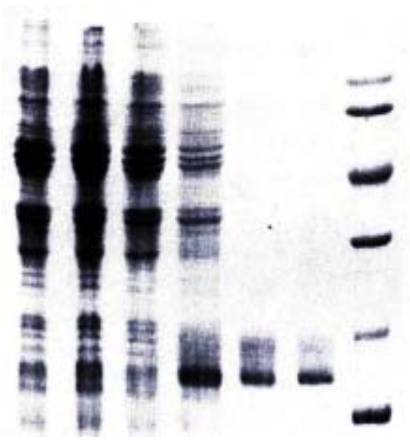

Figure 2 SDS-PAGE analysis of hCaM expression and purification.
1. Uninduced DH5α / pBV220
2. Induced DH5α / pBV220
3. Uninduced DH5α / pBV220-hCaM III
4. Induced DH5α / pBV220-hCaM III
5. Purified rhCaM by Phenyl-sepharose CL-4B column
6. Standard Human brain CaM (Sigma)
7. Protein molecular weight marker

CaM. The acidic amino acid (such as Asp and Glu) composition was about 30% or more.

NAD kinase assay

The results of NAD kinase (NADK) assay showed that purified rhCaM was able to activate CaM-dependent NADK activity to the same extent as the standard human brain CaM (Sigma, Figure 3).

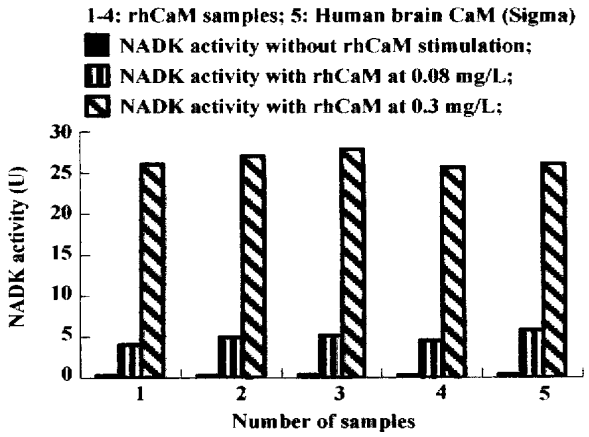

Figure 3 Activation of NADK by rhCaM.

Effect of extracellular rhCaM on cell proliferation

Cells were diluted with RPMI 1640 (containing 0. 5% NCS) to 1-5 × 10^5/mL and se eded in 96-well plates. rhCaM was added to the final concentration of 0.1-10 μg/mL and each concentration was triplicated. Cells were incubated with different concentrations of rhCaM under 37℃, 5% CO_2 conditions for 48 h prior to MTT assay. The effect of extracellular rhCaM on cell proliferation was investigated (Figure 4). Evidence indicated that rhCaM within a certain concentration (0.1- 7.5 mg/L) could stimulate cell proliferation. The stimulatory effect declined when the rhCaM concentration was higher than 10 mg/L. Effect of a ddition of pu re rhCaM was also dependent in some degree upon the cell density of the initial culture. For a certain CaM concentration, the lower the cell density of the init ial culture, the higher the promoting effect. Moreover, the effect of exogenous rhCaM was also influenced by the amount of NCS added to the culture medium. When NCS in medium accounted for 0.5%-1%, a significant stimulatory effect could be observed. However, when NCS in medium was increased to 2%-10%, no stimulatory effect was observed for rhCaM (data not supplied).

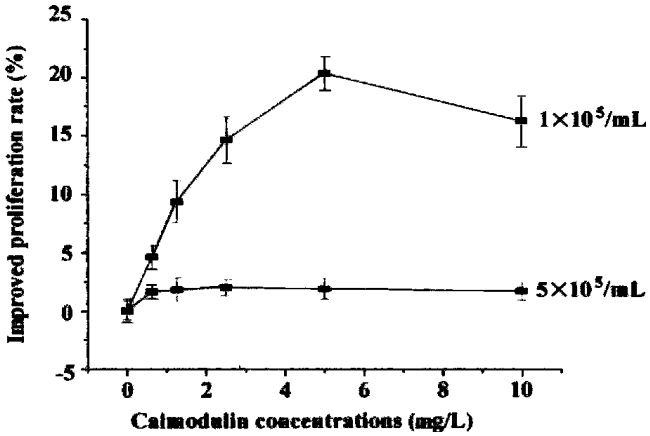

Figure 4 The effect of extracellular rhCaM on cultured SP2/0 cells.

Inhibitory effect of TFP on cell proliferation

Cells were seeded in 96-well plates as above. CaM-antagonist TFP (Sigma) was added to the final concentration of 0.025-250 μmol/L. Each concentra tion was do ne in triplicate. Cells were incubated with TFP under 37℃, 5% CO_2 conditions for 48 h and determined by MTT assay. Results indicated that addition of various concentrations of TFP to cell culture medium could significantly inhibit cell proliferation rate and the inhibitory effect strengthened with increase in the co ncentrations of TFP (Figure 5).

Effect of extracellular rhCaM on TFP-inhibited cells

TFP was added to the SP2/0 cell (1 × 10^5/mL) culture medium until the final co ncentrations were 25 μmol/L. Cells were cultured in 5% CO_2 incuba ted under 37 ℃ for 6h, washed twice with RPMI 1640, then seeded into 96-well plate at the density of 1 × 10^5/mL. Various concentrations of rhCaM were added and cells were cultured for 48 h. Results demonstrated that addition of rhCaM could alleviat e the inhibitory effect of TFP. rhCaM 5 mg/L could offset the inh ibition almost to normal (Figure 6).

DISCUSSION

CaM, a heat-stable, acidic and multifunctional calcium-binding protein, exists in almost all eukaryotic cells. On the gene level, a multigene family of th ree maximally

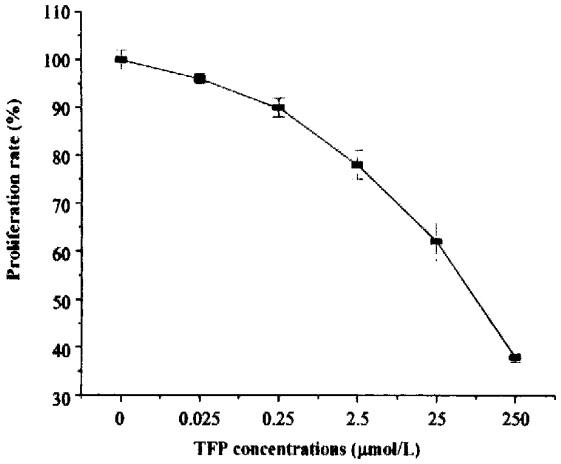

Figure 5 The inhibitory effect of CaM-antagonist TFP on cell proliferation.

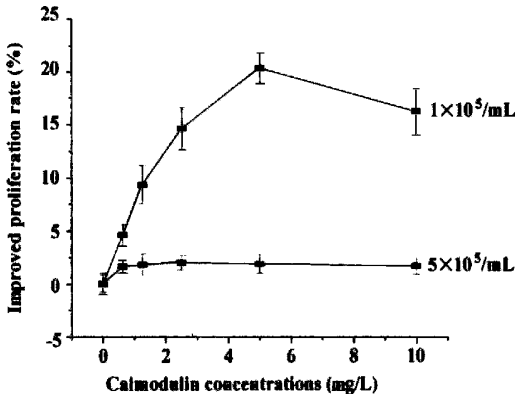

Figure 6 The effect of extracellular rhCaM on TFP inhibited cells.

divergent members is responsible-at least in mammals-for the gene ration of the single CaM protein with the same amino acid sequence. Three human CaM cDNAs called hCaM I, hCaM II, and hCaM III have been cloned[14,18-23]. In this paper we described the constructi on of expression plasmid for human CaM III gene and expression of hCa M protein in *E. coli*. About 3-4 mg of purified rhCaM was obtained from 1 L of *E.coli* culture. This level of production is higher than that achieved by the classic purification method. If the genetic engeneering techniques are used for a large scale expression, the product can be much higher.

It is well known that intracellular CaM plays an important role in the regulatio n of cell proliferation. The levels of intracellular CaM and CaM mRNA vary during cell cycle and accelerate cell proliferation. Moreover, CaM antagonists TFP, W_7, W_{13} and anti-CaM antibodies can block the effect of CaM and arrest cell cycle at G_1/S boundary[24-27]. In the last few years, extracellular CaM was detected and evidence accumulated that extracellular CaM could also affect cell proliferation.

In our study, effects of purified rhCaM and CaM antagonist TFP on SP2/0 prolife ration rate were examined. The results revealed that certain concentrations of extracellular rhCaM (0.1-7.5 mg/L) could stimulate cell proliferation in a dose-dependent manner and the stimulatory effect was dependent upon the cell density of the initial culture. The lower the cell density, the higher the stimulatory effect.The results are consistent with previous literature[4-7]. TFP inhibited cell proliferation and the inhibition could be alleviated by addition of extracellular rhCaM, which further supports that extracellular CaM could acce lerate cell proliferation. CaM was also detected in many extracellular body flui ds such as breast milk, saliva and serum. Serum, which is necessary for the succ essful culture of many cell types, contains high levels of CaM which could amoun t to 0.9-8 mg/L[28]. This might explain why the effect of CaM was inf luenced by NCS added to culture media. While CaM could facilitate cell prolifera tion, compared with NCS, the effect of CaM was less important.

So far, how extracellular calmodulin achieves its effects is yet unclear. Evidence indicates that the mechanisms may be as follow: (1) CaM can interact with so me cell proliferation-related factors such as epidermal growth factor (EGF), transforming growth factor (TGF) and platelet derived growth factor (PDGF). It is possible that extracellular CaM exerts its effects by strengthening these factors' binding to their membrane receptors. It was reported that EGF receptor conta ned a CaM-binding domain[29] and the binding of EGF to cells could be inhibited by CaM antagonist[30]. Mac Neil *et al* showed that there wa s a significant positive correlation between CaM level and EGF concentrations in normal body fluids, indicating that CaM together with EGF, TGF, PDGF etc may be members of a functionally co-ordinated group of mitogens[28]. (2) Ca M may directly interact with CaM-binding proteins (CaMBPs) on cell membrane. CaMBPs were found to exist on cell wall of wheat coleoptiles [31]. Extrac ellular CaMBPs were also detected in the suspension-cultured cells of Angelica Dahurica and carrot[32]. CaMBP is a kind of glycoprotein which can bind with CaM and may function as a bridge between CaM and intracellular metabolic processes.

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Edited by Zhou XH
proofread by Mittra S

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

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Lactosamination of liposomes and hepatotropic targeting research

Yong Peng Chen, Lian Zhang, Qiao Sheng Lu, Xiao Rong Feng and Kang Xian Luo

Subject headings liposomes; asialoglyco-protein; liver; interferon-alpha; antigens, viral; drug carriers; drug therapy; rats

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INTRODUCTION

Site-specific delivery of therapeutic drugs to their target cells is a major s cientific challenge for the pharmaceutical sciences. It offers a number of advantages over conventional drug administration. With drug targeting, high local conc entrations of the drug can be achieved, thus circumventing many unwanted side ef fects. Various carriers have been suggested for the delivery of drugs, including liposomes[1-5] and (neo) glycoproteins[6-8]. The asialoglycopr otein receptor (ASGP-R) has frequently been utilized for targeting drugs to the parenchymal liver cell[6-12]. Liposomes have several advantageous cha racteristics as drug carrier, and particularly, ligand-tacked liposomes achieve a highly effective targeting[13]. Hara *et al* reported that asialofetuin (AF)-tacked liposomes distributed to rat hepatocytes selectively *in vivo*[14], and ASGP-R mediated the uptake of AF-liposomes encapsulating IFN-γ by isolated rat hepatocytes *in vitro* [15]. Lacto saminated huma n serum albumin (L-HSA) is a neoglycoprotein taking number of galactose residue as terminal sugar[6].

In this paper, we studied the preparation and rat hepatocyte uptake of the conjugate of L-HSA and liposomes, and the inhibitory effect of L-HSA-liposomes containing IFN-α on replication of hepatitis B virus (HBV) on 2.2.15 cells line.

MATERIALS AND METHODS

Materials

Sodium cyanoborohydride was purchased from Aldrich, Chemical Co., Milwaukee, WI, USA, α-lactose, phosphatidylcholine (PC), cholesterol (Chol), and N-succini midyl-S-acetylthioacetate (SATA) were obtained from Sigma, St. Louis, MO, U SA. Maleimido-4-(p-phenylbutyryl) phosphatidylethanolamine) (MPB-PE) was pu rch ased from Avanti Polar lipids, Birmingham, AL, USA. Dulbecco's modified eagle medium, penicillin and streptomycin were obtained from GIBCO, Grand Island, NY, USA. MTT was from Boehringer Mannheim, Germany. Fetal calf serum was a product of Hyclone, USA. Other reagents were of analytical grade.

Methods

Lactosamination of human serum albumin Lactosamination was performed according to Schwartz & Gray[16]; human se rum albumin (HSA) 500 mg was allowed to react at 37℃ with 1.0 g α-lactose and 1.0 g NaBH3CN in 20 ml 0.05 mol/L potassium phosphate buffer solution (PB S), pH 8.0. The reaction was continued for 144 h. And the mixture was dialyse d against double distilled water. The lactose content of lactosaminated human serum albumin (L-HSA) was determined by the phenol-sulphuric acid method of Dubois *et al*[17].

Liposomes preparation PC, Chol, and MPB-PE were mixed in a molar ratio of 23:16:1 and dissolved in ether, dried by a rotary evaporator, dissolved in HN-buffer (10 mmol/L HEPES, 135 mmol/L NaCl, pH 6.7) and lyophilized. Phospholipid phosphorus of liposome preparation was measured by phosphate assay after perchloric acid destruction. Conventional liposomes were prepared with PC and Chol in a molar ratio of 23:16.

Coupling of L-HSA to MPB-PE containing liposomes (MPB-liposomes) L-HSA was coupled to MPB-liposomes by a sulfhydryl-maleimide coupling techni que according to Derksen *et al*[18]. Using SATA as a heterobifunctional reagent, free sulfhydryl groups were introduced in L-HSA[19]. After separation of SATA from the protein by Sephadex G-25 gel permeation chromatog raphy, the acetylthioacetate-L-HSA was deacetylated by a freshly prepared solution of 0.5 mol/L hydroxylamine-HCl, 0.5 mol/L HEPES, 25 mmol/L EDTA, pH 7.0. After deacetylation, the thioacetyl-L-HSA was allowed to react with the MPB-PE containing liposomes for 4 hours at room temperature, in a ratio of 3 mg of prote in per mg liposomes. N-ethylmaleimide was added to cap un-reacted sulfhydryl groups. Liposomes were separated from unconjugated protein by Sepharose 4B

Department of Infectious Diseases, Nanfang Hospital, The First Military Medical University, Guangzhou 510515, Guangdong Province, China
Dr. Yong Peng Chen, male, graduated with a bachelor degree in 1993, and with a masters degree in 1999 from the First Military Medical University, majoring in infectious diseases, and published 5 papers
Supported by Science Fundation of Science and Technology Committee of Guangdong Province, № 97031
Correspondence to: Prof Lian Zhang, Department of Infectious Diseases, Nanfang Hospital, Tonghe tow n in Baiyun borough, Guangzhou 510515, Guangdong Province, China
Tel. +86-20-8514-1941, Fax. +86-20-8771-4940
Email. lianzh@fimmu.edu.cn
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infiltrat i on. The L-HSA- liposomes conjugates were characterized by determining protein a nd phospholipid phosphorus content, lyophilized, and stored at 4℃.

Tissue distribution of L-HSA-liposomes L-HSA-liposomes were labled with ^{131}I by Iodogen's method. Male Sprague-Dawley rats weighing 200-250 g were anaesthetized by intraperitoneal injection of 20-25 mg sodium pentobarbital. Radiolabled liposomes were injected via the penile vein and the abdomen was opened. At the end of the experiment, liver lobules and other tissues were removed and weighed. Radi oactivity was determined by γ-counter. And the results were registered as cpm per gram tissue.

Inhibitory effect of L-HSA-liposomes encapsulating interferon-α on HBV replication To assess the effects of L-HSA- liposomes en c apsulating interferon-α on HBV replication, 2.2.15 cells were plated at a dens ity of 2×10^5 per 17 mm culture dish and pre-incubated in Dulbecco's modified eagle medium containing 10% fetal calf serum for 24 h. After being washe d with phosphate -buffered saline, they were cultured at 37℃ for 12 d, with fresh medium changed every 3 d supplemented with L-HSA-liposomes encapsulating interferon-α (LL-IFN), conventional liposomes encapsulating interferon-α (CL-IFN), and free interferon-α (IFN) at an appropriate concentr ation. The culture medium was collected every 3 days and the cells were used in subsequent experiments. The viability of cells was examined spectrophotometrically by the MTT methods, and the cytotoxicity of the compounds was also monitored by the MTT as say.

RESULTS

Synthesis and characterization of L-HSA-liposomes

Having reacted with lactose, the HSA and lactose mixture was dialysed against water for 3 days. There was no lactose in the final dialysis solution, suggesting the L-HSA had been purified completely. Each HSA molecule was modified with about 17 molecules of lactose. When L-HSA was coupled to MPB-liposomes, the amount of L-HSA that could be coupled to 1 μmol MPB-liposomes was 538.7 μg. A number of about 107 000 L-HSA molecules per liposome particle were calculated, assuming that the molecular weight of L-HSA was 7 600 and the average diameter of MPB-liposomes was 400 nm. The liposomes conjugate was stored at 4℃ for at least 8 weeks and filtered by Sepharose 4B. The infiltration figure showed as a single absorbing peak, suggesting that the liposomal conjugate was stable at 4℃.

Tissue distribution of L-HSA-liposomes

Forty minutes after i.v. injection of ^{131}I labled L-HSA-liposomes, r adioactivity in liver was higher than the other organs ($P <= 7.84 \times 10^{-6}$) (Table 1). Spleen uptake of L-HSA-liposomes was less than half of that of liver, and the uptake by kidney, heart, and lung was 1/4 to 1/9.

When L-HSA was pre-injected, the liver uptake decreased significantly, showing no statistic difference with spleen ($P = 0.38$) (Table 2). However, ^{131}I labled L-HSA-liposomes uptake was also observed in the other organs.

Table 1 Tissue distribution of ^{131}I-labeled L-HSA-liposomes in rat

Rat No.	Tissue distribution *in vivo* (cpm per gram tissue)				
	Liver	Spleen[a]	Kidney[b]	Heart[c]	Lung[d]
1	31777.2	15394.2	7912.1	4427.5	9568.5
2	30476.1	15464.1	5424.5	3180.8	6044.1
3	30013.1	14041.4	7932.3	3777.5	5886.1
4	36099.3	10817.5	6372.3	3722.7	8717.3
5	36479.8	17882.6	7228.6	3885.5	7695.8
Average	32969.1	14719.9	6974.1	3798.8	7582.4

^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min after injection as described in *Mate rials and Methods*.
Compared with liver: [a]$P = 7.84 \times 10^{-6}$; [b]$P = 1.06 \times 10^{-7}$; [c]$P = 2.98 \times 10^{-8}$; [d]$P = 2.1 \times 10^{-7}$

Table 2 Tissue distribution of ^{131}I labeled L-HSA-liposomes in rat, after L-HSA pre-injection

Rat No.	Tissue distribution *in vivo* (cpm per gram tissue)				
	Liver	Spleen[a]	Kidney[b]	Heart[c]	Lung[d]
1	34682.0	35187.6	5535.7	2474.2	6368.0
2	20587.4	19791.1	4541.8	2277.2	8507.7
3	24288.8	27297.1	4564.4	2089.5	7280.7
4	33733.1	17637.5	5560.3	2698.3	12561.1
5	25600.9	18994.5	6999.2	3227.0	11087.1
Average	27778.4	23781.6	7699.1	2553.2	8679.4

Fifteen min after L-HAS pre-injection, ^{131}I labeled L-HSA-liposomes were injected into rats. Radioactivity in different tissues was determined 40 min af ter injection as described in Materials and methods.
Compared with liver: [a]$P = 0.38$; [b]$P = 4.35 \times 10^{-5}$; [c]$P = 1.66 \times 10^{-5}$; [d]$P = 2.52 \times 10^{-4}$

Table 3 Inhibitory effect on HBeAg expression in 2.2.15 cells of di fferent types of interferons

Treatment	Inhibitory effect on HBeAg expression (%)			
	3 days	6 days	9 days	12 days
Liposomes	0	1.8	0	0
L-HSA-Liposomes	0	0	0	0
IFN 0.08 MU[a]	0	4.7	12.7	2.3
CL-IFN 0.08 MU[b]	0	0	22.8	5.8
LL-IFN 0.08 MU	44.8	60.7	43.5	45.5
LL-IFN 0.04 MU	6.8	47.4	28.6	12.1
LL-IFN 0.02 MU	0	24.6	23.8	4.6
LL-IFN 0.01 MU	0	4.8	15.4	5.2

IFN: interferon; CL-IFN: interferon entraped in conventional liposomes; LL-IFN : interferon entraped in L-HSA-liposomes.
Compared with LL-IFN 0.08 MU: [a]$P = 0.003$, [b]$P = 0.015$

Anti-HBV activity of L-HSA-liposomes encapsulating interferon-α

Table 3 shows the antiviral activity of L-HSA-liposomes encapsulating interferon-α tested against HBV *in vitro*. The liposomes and L-HSA-l iposomes did not show any anti-

HBV effect. However, when entraped in L-HSA-liposomes, int erferon was 3 to 5 times more effective against HBV replication in 2.2.15 cells than CL-IFN and IFN (*P* value was 0.015 and 0.003, respectively). To achieve similar antiviral effects, the dose of LL-IFN was only 1/4, 1/8 of CL-IFN and IFN, respectively. The treatments did not appear to be cytotoxic to 2.2.15 cell s at the concentrations used in the anti-HBV assay.

DISCUSSION

Worldwide, HBV infection is the main cause of chronic liver disease. HBV carrie r s are at risk for chronic hepatitis, cirrhosis, and hepatocyte carcinoma. Previo us studies have indicated the correlation of HBVX gene to hepatocyte carcinoma[20]. An effective treatment for HBV infection is therefore urgently needed. Interferon-α, nucleotide analogy, Chinese medicinal herbs, and other i mmunological approaches have shown promising results in a subset of patients treated for prolonged periods[21-24]. But overall response rates have been unsatisfactory. Selective drug delivery to the parenchymal liver cell by drug conjugation to a carrier would improve curative effect, reduce therapeutic dosage and side effects of drugs. In this report we describe the preparation of a liposomal drug carrier system, which could target the therapeutic drug to liver via the asialoglycoprotein receptor. Lactose was coupled to human serum albumin by reductive lactosamination in the presence of sodium cyanoborohydride. Then L- HSA was covalently coupled with liposomes using the heterobifuntional reagent N-Succinimidyl-S-acetylthioacetate. This is a well established method for coupli ng proteins to liposomes, based on the reaction of thiolated proteins and liposo mal maleimido-4-(*p*-phenylbutyryl) phosphatidylethanolamine[10]. T hio lation of L-HSA, in which about 17 of the free ε-amino groups of lysine were derivatized with lactose, still allowed the introduction of several sulfhydryl molecules in this protein. This indicates that SATA is suitable for coupling deri vatized protein to liposomes.

Mammalian liver contains a unique asialoglycoprotein receptor responsible for the rapid serum clearance and lysosomal catabolism of desialylated glycoproteins bearing terminal, non-reducing galactose residues. Previous studies demonst rated that, for bovine serum albumin, at least 13 lactosyl groups were needed for high affinity recognition by the ASGP-R[25]. In our study, about 107 000 molecules L-HSA perliposome were coupled, and each HSA molecule was modifi ed with 17 lactose molecules, hence the liposomal conjugate had a high affinity to ASGP-R. The *in vivo* study indicated that the liver uptake of liposomal c onjugate was higher than other organs, which was about 2 times higher than that of spleen, and 4 to 9 times higher than kidney, heart, and lung. L-HSA pre-injection almost prevented the selective liver delivery of liposomal conjugate, suggesting that the galactose-specific nature of hepatic uptake. Modified with 17 lactose molecules, L-HSA was capable of inhibiting the hepatic uptake of liposomal conjugate, owing to the high affinity for the hepatic galactose-recognizin greceptor. Another study also showed that liposomes modified with several galatose residues had a similar liver targeting[26].

2.2.15 cell is a stable expression system of transfetced HBV DNA, which could produce HBV particle, and is often taken as a cell model for screening anti-hepatitis B virus drugs[27,28]. Interferon had the inhibitory effect on HBV replication in this system[29]. Wu *et al*[30] proved that this system contained ASGP-R. *In vitro*, interferon-α entraped in liposomal conjugate had a higher activity against HBV free than interferon and than interf eron entrapped in conventional liposomes. To achieve similar antivirus effects, the doses of LL-IFN capable of inhibiting virus growth were 4 to 8 times less than t hat of CL-IFN and IFN.

In conclusion, the results obtained in this study indicated that the conjugate of lactosaminated human serum albumin and liposomes achieved good liver targeting , and allowed the development of a potent liver-targeting drug carrier system.

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Edited by Zhu QR
proofread by Mittra S

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

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Intestinal acariasis in Anhui Province

Chao Pin Li and Jian Wang

Subject headings mite infestation; intestinal diseases, parasitic; epidemiology; data collecti on; tick control; leukocytes count

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INTRODUCTION

The mites found in stored food and house comprise a large group of subclass Acar i, belonging to the suborder Acardida of the order Acariformes. They can be found in dust and vacuum samples from floors, furniture, mattresses, Chinese herb al medicine, dry fruit, grain, flour, sugar, and bedding. These mites are nidico lous and feed on organic debris, including sloughed human skin, fungi, spilled food, pollen, etc. These mites are particularly prevalent in Chinese herbal medi cine, dry fruit, grain, flour, sugar, beds, though carpeted floors near beds or couches may also have large numbers. The most common species are Acarus siro, Ty rophagus putrescentiae, Dermatophagoides farinae, D. pteronyssinus, Glycyphagus domesticus, G. Ornatus, Carpoglyphus lactis and Tarsonemus granarius, etc. The viability of mites in storage is quite strong and they can invade and parasitize the intestines of humans[1-15]. They can cause pulmonary acariasis[16-25], urinary acariasis [26-33] and so on. The dejecta of mites is a quite strong allergen and can cause different allergic diseases [34-44]. Intestinal acariasis can be caused by some mites related to the way of diet intake and invading against intestinal mucosa, intestinal muscle [45-58]. The first report of intestinal acariasis caused by these mites was made by Hinman *et al* (1934)[45]. From then on, all kinds of studies on the disease have been reported gradually. In order to make an epidemiological survey of intestinal acariasis the investigation of the disease was taken in so me areas of Anhui Province from 1989 to 1996.

School of Life Sciences, University of Science and Technology of China, Hefei 230027, Anhui Province, China
Dr. Chao-Pin Li, male, graduated from Suzhou Medical College, with Master Degree in 1983. Professor, director of Parasitology, member of International Acarology Commission, council member of Chinese Parasiology Commission, having more than 100 papers and 8 books published. Now working in School of Medicine, Huainan Institute of Technology.
Supported by the grants from Science Foundation of the Ministry of Coal Industry of China
Correspondence to: Chaopin Li, School of Medicine, Huainan Insti tute of Technology, Huainan, 232001, Anhui Province. China
Tel. +86-554-66-6870, Fax. +86-554-666-2469
Email. hyjlcp@mail.ahbbptt.net.cn
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MATERIALS AND METHODS

Materials

The site of investigation was in the Chinese traditional medicine storehouses, factories and rice storehouses, rice mills, primary schools and so on, the subjects of investigation were sellers of the traditional Chinese medicine, workers of granary and processing, primary school pupils in grades 1-6 and 152 patients with diarrhea who sought medical advice and workers in certain processing factories.

Methods

Case history Included the history of present illnesses, the history of past illnesses, the patients' occupation.

Feces examination First, the stool of the subject was collected in dispos able feces boxes. Secondly, mites were separated by saturated saline flotation methods and identified with the help of microscope.

Blood examination Differential leukocyte counting was made in patients who had positive results of feces mite examination.

RESULTS

The rate of detection of mites in stool and clinical symptoms

The total number of samples of feces examination were 3416 (male 1879, female 15 37), the positive number of mites in stool were 225 (male 128, female 97) and the detectable rate of mite was 6.59% (male 6.81%, female 6.31%). Among them, the samples of feces examined in the groups of age 5-14, 15-24, and >24 were 1829 (5. 74%), 508 (3.54%), 927 (6.36%) respectively. There were 43 positive samples in 152 patients with diarrhea who were suspected to suffer from intestinal acariasis, the detectable rate was 28.29%. Of the 225 mite positive cases observed after stool examination, the detectable number of mite cases together with other intestinal parasites was 131, the number of cases with only mite symptoms was 94.

Results of analysis of digestive canal symptoms in 30 cases positive of mites al one in stool showed that the number of patients with diarrhea, abdominal pain, abdomen discomfort, mucous stool, pus and blood stool, burning sensation of anus were 26 (86. 67%), 20 (66.67%), 19 (63.33%), 15 (50.00%), 13 (43.33%), an d 14 (46.67%) respectively. Besides, some of the patients were accompanied with the symp toms of hypodynamia, magersucht, lassitude, asthma, vomiting, anorexia, low fever, dysphoria, general

malaise, etc.

Mites seperated from stool

The detectable rates of adult mites, larval mites, both adult and larval nutes, both adult mites and eggs, adult and larval mites and eggs, both larvel mites and eggs, and both hypopus and eggs were 44.00% (99), 10. 22% (23), 34.67% (78), 2.67% (6), 4.89% (11), 2. 22% (5), and 1.33% (3) respectively. The population identification analysis showed that there were Acarus siro, Tyrophagus putrescentiae, *Dermatophagoides farinae*, *D. pteronyssinus*, *Glycyphagus domesticus*, *G. Ornatus*, *Carpoglyphus lactis* and *Tarsonemus granariu s* in stool. Furthermore, the hypopus of mites could be found. The statistics of this investigation showed that the concentration of mites with a count of 1-2/cm^3, 2 -4/cm^3, >5/cm^3 was 11, 55, 159 respectively.

Blood examination

Among the 30 patients, differential leukocyte count in most cases was (5.55-10. 4) × 10^9/L with the exception of 4 cases [(11.0-12.9) × 10^9/L]. The eosinophilic granulocyte count was high [(0.32-0.78) × 10^9/L]. The average value of constituent ratio of eosinophilic granulocyte was 0.09 (0.04-0.11) and was higher than that of normal control ($P < 0.01$).

The rate of detection in different occupations

The field investigation showed that different rate of invasion existed in different occupations. The detectable rates of mites in stool were 6.71% (20/298), 6.5 9% (89/ 1350), 6.14% (98/1596), 4.17% (5/120) and 25.00% (13/ 52) in herb stuff processors and sellers, food storage and processor workers, primary school pupils, machinery workers, and some others.

Anamnesis

Among the 30 patients with obvious symptoms, the total misdiagnosis rate was 63. 33%. The patients who had been misdiagnosed for chronic colitis, allergic enteritis, intestinal neurosis, amebiasis, hookworm disease, ascariasis, chronic appen dicitis, pelvic inflammation, metroendometritis, intestinal adhesion, and chronic diarrhea were 2, 5, 3, 1, 1, 1, 2, 1, 1, 1, and 1 respectively.

Mites seperated from work environment

Different breeding mites were separated from the samples of work environment and food. Collecting the samples of mill floor dust (30 shares), stores of traditio nal Chinese medicine, and traditional Chinese herbs (146 species) of wolfberry fruit, ophiopogon root, liquorice, boat-fruited sterculia seed, safflower, and so on. The results showed that the number of breeding mites per gram were 91-1862, 21-186, 0-483, 10-348, 51-712, and 311-1192, in mill floor dust, traditional Chinese medicine stores, traditional Chinese herbs such as candied fruit, dry fruit, brown sugar, and expired cake. Twenty-six species of mites could be identified out of them (most of them were unidentifiable), and belonged to nine families of Acaridae, *Carpoglyphidae*, *Glycyphagidae*, *Pyroglyphidae*, *Histiostomidae*, *Chorto glyphidae*, *Lardoglyphidae*, *Tarsonemoidae* and *Cheyletidae*. The results of this study showed that the mites separated from work environment were identical to those f rom stored food.

DISCUSSION

The main pathogenic mites have been verified as Acarus and *Tarsonemidae* by Zuo Zuoxue (1951). However, most of the mites causing intestinal acariasis that have been confirmed by Li Yousong (1980), Zhou Hongfu (1986), Li Chaopin (1987)[56] *et al*, belong to 6 families Astigmate order like, *Acaridae*, *Car poglyphidae*, *Glycyphagidae*, *Pyroglyphidae*, and *Tarsonemidae*. The results of our investigation are in bas ic agreement with the views that had been previously reported. With regard to t he path of infection of intestinal acariasis, the general idea was that the infe ction of the disease was caused by the mites coming from stored and swallowed food[50-58]. Our study has verified that mites separated from stool were identical to those from storage, and belonged to common species of mites in our l iving environment and stored food. With the help of dust sampler, eight sampling sites were set up in a traditional Chinese medicine plant. Thirteen mites (adult 9, larval 4) were separated from the 640 L volume of air in the work environment of the plant. Thus another path of infection may be through respiration and swal lowing of the mites in the air. The results of our study also showed that intestinal acariasis was associated with the hobby of drinking of tea, including ophio pogon root, liquorice, boat-fruited sterculia seed; and eating dry fruit, inclu ding da teplum persimmon, preserved fruit, candied fruit, dried persimmon, and even dried flesh of fish. Previous reports [45-58] and the conclusions of this s tudy verify that the disease is associated with breeding of mites in the diet, work environment, and stored food; but unassociated with age or sex.

The vitality of some mites causing intestinal acariasis is quite strong. The stu dy report of Kodama *et al*[48] had confirmed that the mites could l ive in 10% formalin for 12 days and then oviposite after two weeks, they could live in stool for 83 days and then oviposit and hatch to larva. In our study, tw enty mites were soaked in distilled water at the temperature of 20-25℃, and an other twenty in running water at room temperature for 7 weeks. The results showed that the mites collected from the bottom of the test tubes were all alive. After the stool with *Acarus siro* had been stored in a sealed plastic bag for 7 weeks, active mites could still be found. Kampmeier's report verified that not all mites could be killed by gastric acid or digestive juices[49]. Mites could be found alive by differential leukocyte count.

After mites came into the intestinal tract, mechanical stimulation and injuries of tissues were caused by the

gnathosoma, chelicera, feet, and so on[59] . The dejecta, products of metabolism, and cleaved body parts of mites could cau se different allergic reactions of the digestive system, such as abdominal pain, diarrhea, burning sensation of anus, and so on. The reports in past have shown that the dejecta, metabolites, cleaved body parts were all quite strong alle rgens and could result in high levels of eosinophilic granulocytes, total IgE, special IgE for mites in serum. The clinical manifestation of this was allergic urticaria, allergic rhinitis, allergic asthma *et al*[34-44]. The results of our studies showed that the levels of eosinophilic granulocytes in patients with in testinal acariasis were high too. Its possible mechanism could be similar to tha t of the allergic reaction caused by other mites. The intestinal acariasis may n ot only cause injury of the intestinal mucosa and invade the intestinal muscle but also stimulate the immune system by the dejecta, metabolites, and cleaved bo dy of the mite itself. The disease may be easily misdiagnosed for allergic enter itis, chronic colitis, intestinal neurosis, amebiasis, pelvic inflammation, or schistosomiasis japonica[60]. As a result, proper treatment might be thus delayed.

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Edited by Zhou XH
proofread by Mittra S

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

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Study of T-lymphocyte subsets, nitric oxide, hexosamine and *Helicobacter pylori* infection in patients with chronic gastric diseases

Hui Zhang1, Shu Lin Jiang1 and Xi Xian Yao1

Subject headings stomach disease; T-lymphocyte; nitric oxide; *Helicobacter pylori*; hexosamine; Helicobacter infections; gastric mucosa

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INTRODUCTION

Chronic gastritis (CG) and peptic ulcer (PU) are frequently-occurring diseases. It is now well recognized that Helicobacter pylori (Hp) is a major fact or that leads to CG and PU[1-8]. In order to study the relationship among T lymphocyte subsets, NO, Hexosamine and *Hp* infection in patients with chronic gastric diseases, the levels of blood T lymphocyte subsets, plasma NO and hexosamine in gastric mucosa were measured respectively in 30 patients with CG and 32 patie nts of PU+CG.

MATERIALS AND METHODS

Clinical materials

Thirty-two patients with PU and CG (23 males and 9 females, aged 20 to 68 years , mean age 43.2)and 30 patients with CG (18 males and 12 females, aged 24 to 66 years, mean age 44. 3) were enrolled in the study. Twenty healthy people with comparable ages acted as control. All subjects were excluded of hepatic and other diseases.

Methods

Vein blood 2 mL from each fasted patient was obtained for blood T lymphocyte su b sets and NO determinations. Four gastric biopsies were taken from gastric antrum 3 cm from pylorus (2 from curvatura ventriculi minor and the other 2 from ante -wall) for *Hp*, pathology and hexosamine determinations. T-lymphocyte subsets were determined by FCM methods (FACS 420, by Becton-Dickinson Company, U.S.A.). Laser light source was 2 Wargon ionic laser, wavelength 488 nm. The emerging gr een fluorescence by FITC was used for fluorimetry by 520 nm long filter disc. The data were processed by an *HP*-300 computer programme. *Hp* infection was dia gnos ed if any two of the following methods were positive: (1) rapid urase test (test kit procured from San Qiang Company); (2) gastric mucosa smear with gram's stain. The Hp density gradients were according to Monshoy staging grades: 0, free; I, a few; II, obvious (in all fields of microscopy); III, plenty or piled; (3) Warthin-Starry stain of gastric mucosa.

Pathology

HE stain was used for observing mucosal inflammation. The severity of gastritis was graded as: mild: infiltrated inflammatory cells only at gastric pit or inte stinal villas; moderate: infiltrated cells in gland lamina propria; and heavy: infiltrated inflammatory cells in muscular layer of mucosa. Determination of hexo samine: two gastric biopsies were ground into 1 mL liquid, centrifuged it, and supernatant was kept at -20℃ for measuring. Protein content in supernatant was m easured by taking 20 μL sample and 500 μL Coomassie brilliant blue G250 reagent at the wavelength of 600 nm by automatic analysis appa ratus. 0.5 mL of the supernatant and 0.5 mL enriched hydrochloric acid were mixe d together at high pressure (147 KPa) for 30 min, put into 0.5 mL NaOH (8.3 mo l/L) and acetyl acetone solution, boiled for 15 min and cooled, and then analy zed. The results were expressed as mg/mL. Hexosamine was calculated as following: hexosamine = supernatant hexosamine (mg/mL)/supernatant protein (g/L). NO measureme n t: (1) pre-measurement: Took 200 μL plasma and the same amount of a cetonitrile, shook them, and centrifuged for 10 min (5000 r/min), repeating once again as above, taking 10 μL of supernatant overflow for measurement. Baseline 810 high effect liquid phase apparatus, made by Waters Company, U.S .A. and 486 outer purple detector were used. Conditions of chromatostrip includ ed: m obile phase 2.5 mmol/L LiOH water solution +50 g/L acetonitrile, velocity of flow 1.2 mL/min, column temperature 40℃, detection wavelength 214 nm, analysis cl oumn IC-Pak Anion, 4.6 mm × 5 cm (Waters Company, U.S.A.), detection time 10 min .

Department of Gastroenterology, Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei Province, China
Hui Zhang, female, graduated from Hebei Medical Univercity as a Bachelor in 1986 and as a Master in 1998, associate chief doctor, majoring in chronic gatric diseases, having over 10 articles and 7 science books published
Correspondence to: Dr. Hui Zhang. Department of Gastroenterology, Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei Province, China
Tel. 0086-3117046901-6413
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RESULTS

T-lymphocyte subsets in CG patients

Total lymphocyte (CD_3^+), helper lymphocyte (CD_4^+), suppressor lymphocyte (CD_8^+), CD_4^+/CD_8^+ in CG patients were significantly lower than thos e of normal control ($P < 0.05$-0.01, Table 1). Eighteen out of 30 CG patients had *Hp* infection (60%). CD_3^+, CD_4^+ in Hp^+ group were significantly decreased ($P < 0.01$, < 0.05), but CD_4^+/CD_8^+ was not significantly changed (Table 2). In addition, the study of the relationship between different pathological changes and T lymphocyte subsets indicated that: CD_3^+, CD_4^+, CD_4^+/CD_8^+ in severe CG were significantly decreased than those in mild to moderate CG (P<0.01, <0.05, <0.05 respectively, Table 3).

Table 1 T-lymphocyte subsets in CG patients ($\bar{x} \pm s$, %)

Lymphocyte	CG (n = 30)	Normal control (n = 20)
CD_3^+	60.7 ± 2.4[b]	68.3 ± 3.9
CD_4^+	35.8 ± 2.5[b]	43.0 ± 3.8
CD_8^+	25.2 ± 2.4[a]	26.4 ± 1.7
CD_4^+/CD_8^+	1.43 ± 0.15[a]	1.64 ± 0.18

[a]$P < 0.05$, [b]$P < 0.01$, *vs* normal group

Table 2 T-lymphocyte subsets in CG patients with *Hp* infection ($\bar{x} \pm s$, %)

lymphocyte	*Hp* (+) (n = 18)	*Hp* (-) (n = 12)
CD_3^+	59.7 ± 2.5[b]	62.2 ± 0.9
CD_4^+	34.8 ± 2.6[a]	36.9 ± 2.0
CD_8^+	25.3 ± 2.5	25.0 ± 2.3
CD_4^+/CD_8^+	1.42 ± 0.17	1.45 ± 0.12

[a]$P < 0.05$, [b]$P < 0.01$, *vs Hp* (-)

Table 3 Pathological stages and T-lymphocyte subsets in CG ($\bar{x} \pm s$,%)

Lymphocyte	Mild-moderate (n = 13)	Severe (n = 17)
CD_3^+	62.3 ± 1.7	59.5 ± 2.1[b]
CD_4^+	37.4 ± 1.8	35.4 ± 2.7[a]
CD_8^+	25.5 ± 2.1	24.9 ± 2.6
CD_4^+/CD_8^+	1.50 ± 0.13	1.40 ± 0.13[a]

[a]$P < 0.05$, [b]$P < 0.01$, *vs* mild-moderate

Hexosamine levels

The hexosamine levels in patients with severe lesions (38.0 mg/g ± 3.8 mg/g) were significantly lower than those with mild and moderate CG (47.0 mg/g ± 7.6 mg/g, P<0.01). The hexosamine levels in Hp^+ group were significantly lower than those in Hp^- group ($P <$ 0.05, Table 4). In addition, 24 out of 32 PU+CG patie nts (75.0%) had *Hp* infection. The hexosamine levels in Hp^+ group w ere significantly lower than those in Hp^- group (P<0.01, Table 4).

Table 4 Changes in hexosamine levels in CG, PU+CG patients with *Hp* infectio n ($\bar{x} \pm s$, mg/g)

	CG		PU + CG	
	Hp (+) n = 18	*Hp* (-) n = 12	*Hp* (+) n = 24	*Hp* (-) n = 8
Hexosamine	40 ± 6	45 ± 7	39 ± 8	51 ± 7

[a]$P < 0.05$, [b]$P < 0.01$, *vs Hp* (-)

Plasma NO levels

In CG, PU+CG patients, the levels of plasma NO (2514 μg/L ± 364 μg/L, 2824 μg/L ± 673 μg/L) were significantly higher than those of normal control (2228 μg/L ± 214 μg/L, $P < 0.05$, $P < 0.01$), and the level of plasma NO in PU+CG patients (2824 μg/L ± 673 μg/L) was higher than those of CG patients (2514 μg/L ± 364 μg/L, $P < 0.05$). The levels o f NO in CG, PU+CG *Helicobacter pylori* positive group were significantly hi gher t han in Hp^- group ($P < 0.01$, $P < 0.05$, Table 5). The study of the relat ions hip between different pathological changes and NO levels indicates that the levels of NO in both groups with severe lesion were significantly higher than those in mild to moderate lesions ($P < 0.05$, Table 5).

Table 5 Changes in plasma NO, pathological stages and *Hp* infection in CG an d PU+CG ($\bar{x} \pm s$, μg/L)

	CG	PU+CG
Hp (+)	2671 ± 258	3071 ± 398
Hp (-)	2282 ± 387	2579 ± 668
Mild-moderate	2328 ± 413	2403 ± 284
Severe	2656 ± 251	2880 ± 802

[a]$P < 0.05$, [b]$P < 0.01$, *vs Hp* (-); [c]$P < 0.05$, *vs* severe.

DISCUSSION

As we all know, CG is closely related to *Hp* infection [9-12], but so far any report regarding the relationship between CG and T lymphocyte has not bee n observed. Our results showed that CD_3^+, CD_4^+, CD_8^+, CD_4^+/CD_8^+ T cells in CG patients were observed to be significantly lower than in normal control. This indicated that the functioning of cellular immunity was impaired in CG patients. In order to explore the relationship between T lymphocyte subse ts and *Hp* infection, this experiment divided CG patients into Hp^+ grou p and Hp^- group. The results showed that CD_3^+, CD_4^+ T cells in Hp^+ group wer e significantly decreased showing that *Hp* infection is related to the decrease in the function of cellular immunity. In addition, the majority of the severe cases of CG (71%) had *Hp* infection, and the levels of CD_3^+, CD_4^+, CD_8^+, CD_4^+/CD_8^+T cells were significantly decreased than in mild to moderate CG patients. This implied that the impaired function of cellular immunity in CG patients especially in severe cases, may possibly be related to *Hp* infection.

Gastric mucoitin-bicarbonate barrier is one of main constituents of gastric mu cosal barrier[13,14]. Gastric mucoitin contains polymer glucoproteins, electrolytes, peptides, lipides, etc. The present study showed that: the orientat ion of *Hp* is related to pathogenic factors, such as lipopolysaccharide, urea enzyme[15-18], vacuole toxin[19-21] and adhesiveness. Hexosamine[22] reflects the glucoprotein. So, the purpose of this study was to evaluate the lesion of gastric mucosal barrier by measuring the levels of hexosamine in gastric mucosa. The results showed that in CG, and PU+CG patients, the levels of hexosamine in Hp^+ group were significantly decreased than in Hp^- group. The more severely the gastric mucosa was impaired, the higher the *Hp* infection rate was (moderate 60.7%, severe 74.2%). *Hp* infection leads to the decrease i n hex osamine levels in gastric mucosa especially in severe cases. It is thus indicate d that *Hp* is one of main pathogenic factors for endogastritis or severe gast ritis.

NO is a multifunctional regulatory substance of the body[23-30]. It has many biological activities. It participates in many physiological functions and pathogenesis in digestive tract. Endogenous NO is an important transmitter maintaining the blood flow of gastric mucosa, and has a role in inhibiting platelet aggregation in gastric mucosa microcirculation. The results in this study showed that the levels of plasma NO in CG and, PU+CG patients were increased to varying extents as compared to normal control, and the levels of plasma NO in PU+CG patients were significantly increased than those in CG patients. In addition, th e plas ma NO levels in Hp^+ group in CG and PU+CG patients were significantly increased as compared to Hp^- group. According to the pathological stages, the more the gastric mucosa was impaired by *Hp* infection, the higher the levels of NO were. It is suggested by our results that *Hp* infection in gastric mucosa may induce an increase in NO production by inflammatory cells[31]. It might be possibly an immune response of our body to resist *Hp* infection, but the exact pat hogenesis needs to be further confirmed.

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Edited by Zhou XH
proofread by Mittra S

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

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Helicobacter pylori vacA genotypes and *cagA* status and their relationship to associated diseases

Peng Hou,Zhen Xing Tu,Guo Ming Xu, Yan Fang Gong,Xu Hui Ji and Zhao Shen Li

Subject headings *Helicobacter pylori*; gastritis; peptic ulcer; stomach neoplasms; genotype; p olymerase chain reaction

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INTRODUCTION

Helicobacter pylori (*H.pylori*) is a major causative bacterium of chronic gas tri tis, peptic ulcer and mucosa-associated lymphoid tissue lymphoma in humans, and associated with an increased risk of gastric cancer[1-8]. An important virulant factor of *H.pylori* is the vacuolating cytotoxin (VacA) encoded by vacA that induces cytoplasmic vacuolation in target cells both *in vitro* and *in vivo*[9-11]. VacA is produced as a 140 kDa precursor which contains an N-terminal signal peptide and an approximately 33 kDa C-terminal outer membrance exporter. The precursor is cleaved at both N-terminal and C-te rminal and secreted into the extracellular milieu as a 95 kDa mature protein. The mature protein futher undergoes specific cleavage to yield 37 kDa and 58 kDa subunits[12-14]. Although *vacA* is present in all *H.pylori* strains, only about 50% to 60% of strains can induce vacuolation of epithelial cells as assessed by the HeLa cell assay. *vacA* shows considerable genetic variation in *H.pylori* isolated from all over the world and contains at least two variable regions. The s region exists as s1 or s2 allelic types. Among type s1 strains, subtypes s1a and s1b have been identified. The m region occurs as m1 or m2 allelic types. Specific *vacA* genotype of H. pylori strains are associated with the production of the cytotoxin *in vitro*, epithelial damage *in vivo* , and clinical consequences[15-27]. The other virulant factor is the cytotoxin-associated protein (CagA) encoded by the cytotoxin-associated gene (cagA). The *cagA* gene is present in about 60% to 70% of strains and all of these strains express the *cagA*. The presence of *cagA* is also associated with the production of the cytotoxin *in vitro*, and clinical outcome[24-30]. The aim of this study was (i) to identify *vacA* genotypes and *ca gA* status of *H.pylori* isolated from Chinese patients; (ii) to evaluation t he relatioship beween *vacA* genotypes, *cagA* status and related gastroentero logical disorders.

MATERIALS AND METHODS

Patients

Seventy-four clinical isolates of *H.pylori* were obtained f r om patients which underwent gastroduodenoscopy in Changhai Hospital, Shanghai, China. *H.pylori* strain CCUG17874 and G50 obtained from IRIS, Italy were also used in this study. No patient had received nonsteroidal anti-inflammatory drugs or antacids. Of these, 31 patients (mean age 47 years) had peptic ulcer including 21 duodenal ulcers, 5 gastric ulcers and 5 complete ulcers; 39 patients (mean age 41 years) had gastritis including 18 supper gastritis, 6 atrophic gastritis and 15 erosive gastritis; and 4 patients (mean age 56 years) had gastric adenocarci noma.

Isolation and culture of H.pylori

Two gastric biopsies were obtained res pectively from gastric antrum and corpus of each patient by using endoscopy. Each specimen was placed in a transport medium and sent to the laboratory within 3 h. The specimens were then incubated onto Campylobacter selective agar (MERCK) c ontaining 10% sheep blood for 3 d at 37℃ under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2). All *H.pylori* strains were positive for urease, oxid ase, catalase and were identified by Gram and Giems staining under light microscopy. Colonies directly harvested from the plates were used for RNA extraction.

Preparation of total RNA and RT-PCR amplification

The extraction of tot al RNA was performed using Promega's SV total RNA isolation system according to manufacturer's instructions. The DNA sequences of the primer oligonucleotides u sed for RT-PCR and the size of the corresponding PCR products are listed in Table 1. RT-PCR reactions were performed as follows: denaturation at 95℃ for 2 min, followed by 30 cycles consisting of 95℃ for 40 s, 55℃ for 1 min, and 67℃ for 2 min, and final extension at 67℃ for 7 min. The RT-PCR products were elect rophoretically separated on a 1.5% agarose gel and stained with ethidium bromide.

Department of Gastroenterology, Changhai Hospital, Shanghai 200433, China
Dr. Peng Hou, male, graduated from Fourth Mi litary Medical University in 1986, now graduate student for doctor degree in Second Military Medical University, having 12 papers published
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Correspondence to: Professor Zhen-Xing Tu, Department of Gastro enterology, Changhai Hospital, Shanghai 200433, China
Tel. 0086-21-25070556, Fax. 0086-21-35030384
Email. Xiaohua@smmu.edu.cn
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Statistical methods

Analysis of data was performed using the χ^2 test. Probability levels (P) of <0.05 were considered statistically significant.

Table 1 Oligonucleotide primers used in this work

Region amplified	Primer sequence	Size of PCR product
cagA	5'ATAATGCTAAATTAGACAACTTGAGCG 3'	297 bp
	5'TTAGAATAATCAACAAACATCACGCCA 3	
vacA		
s1a	5'ATGGAAATACAACAAACACAC 3'	190 bp
	5'GTCAGCATCACACCGCAAC 3	
s1b	5'ATGGAAATACAACAAACACAC 3'	187 bp
	5'AGCGCCATACCGCAAGAG 3'	
s2	5'ATGGAAATACAACAAACACAC 3'	286 bp
	5'CTGCTTGAATGCGCCAAAC 3'	
m1	5'GGTCAAAATGCGGTCATGG 3'	290 bp
	5'CCATTGGTACCTGTAGAAAC 3'	
m2	5'GGAGCCCCAGGAAACATTG 3'	352 bp
	5'CATAACTAGCGCCTTGCAC 3'	

RESULTS

vacA genotypes of H.pylori strains

Typing of the *vacA* gene of 74 clinical isolates and strain CCUG17874 and G50 was performed based on signal and middle sequences by RT-PCR as described in mate rials and methods. All of the 74 clinical isolates and strain CCUG17874 were s1a type whose fragment was 259 bp products as predicted by RT-PCR-amplification. Strain G50 was s1b type whose fragment was 286 bp products predicted by RT-P CR-amplification. No clinical isolates was of type s1b. Neither strain CCUG1787 4 and G50 nor clinical isolates was of type s2. Seven isolates (9.5%) and strai n C CUG17874 were of m1 type whose fragment was 290 bp products. Sixty-seven isolates (90.5%) and strain G50 were of m2 type whose fragment was 352 bp products. O verall, 7 strains (9.5%) were type s1a/m1, 67 isolates (90.5%) were type s1a/m2. An s1a/m2 type strain was predominantly found in clinical isolates from Chinese patients living in Shanghai.

cagA positivity of H.pylori strains

The *cagA* gene was determined in 74 isolates by RT-PCR. Sixty-nine Chinese *H.pylori* strains (93.2%) were *cagA* positive. When the *vacA* genotype wa s compared with *cagA* status, all (100%) of 7 s1a/m1 type strains and 62 (92. 5%) of 67 s1a/m2 typr strains were *cagA* positive.

Relationship between vacA genotype, cagA status and related gastroenterological disorders

Infection with type s1a/m1 strain was found in 4 (12.9%) of 31 patients with peptic ulcers compared with 3 (7.7%) of 39 patients with gastritis. Infection with s1a/m2 strain was found in 27 (87.1%) of 31 patients with peptic ulcer compared with 36 (92.3%) of 39 patients with gastritis. There was no statistical signifi cance between *vacA* genotype and the clinical outcome. ($P > 0.05$). *cagA* positivity was found in 29 (93.5%) of 31 patients with peptic ulcer compared with 37 (94.9%) of 39 patients with gastritis. There was also no relationship between *cagA* status and related gastroenterological diseases ($P > 0.05$).

DISCUSSION

Based upon the methodology for typing *vacA* gene developed by Atherton *et a l*[19], we analysed and typed *vacA* genotype of clinical isolates fr om Chinese patients living in Shanghai by using RT-PCR. The product amplified by RT-PCR was obtained in all clinical isolates, CCUG17874 and G50 strains. The results suggested that *vacA* transcription occurred in each of *H.pyroli* s t rains tested and was in agreement with previous reports[19]. According to typing of *vacA* alleles, these Chinese patients infected prevalently with *H.pylori* contained the type s1a/m2 of *vacA* alleles. The finding reported here was different from that in United States, Germany, and Japan[15-26] . The reason why the s1a/m2 strains have accumulated in Shanghai, China is unclear. It is known that genomic rearrangement may be associated with the uptake of D NA by natural transformation. Chinese have the same habits and customs, and little intermarriages with other nations. The high incidence of the s1a/m2 allele in Chinese population may indicate that it was first acquired in this population and the chance for genomic diversity may be little. Alternatively, the s1a/m2 allele may have a selective advantage in the Chinese population due to human polymor phism and ethnic background.

Recently, van Doorn *et al* found a novel subtype of *vacA* signal region fr om s1a subtype, designated as s1c. Type s1c allele was obtained exclusively in isolates from East Asia[32]. We have done *vacA* gene sequencing from 5 clinical isolates (in press). The s region was considered as type s1a. Compared with s1c sequence, two of the five strains were of type s1c. These results ind icated that s1c subtype could be identified as subtype s1a. Whether s1c strains are phenotypically different from s1a strains remains to be determined.

The findings here showed that all strains but five possessed the *cagA* gene and suggested that *cagA* positive strains were predominantly found in Shanghai also. The relationship between type s1a *vacA* allele and *cagA* positive has been described[31]. All (100%) of Chinese (Shanghai) *H.pylori* strains were of type s1a *vacA* allele and 93.2% of strains were *cagA* positive and were highly prevalent in China.

Subtype analysis of *vacA* alleles and *cagA* positivity demonstrated that the *vacA* subtype and *cagA* status was not independently associated with the clinical outcome of *H.pylori* infection. The factors affecting the clinical consequences need further studies.

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Edited by Zhou XH
proofread by Mittra S

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

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Case Reports

Hepatic angiomyolipoma-misdiagnosis as hepatocellular carcinoma: A report of 14 cases

Ding Rong Zhong and Xiao Long Ji

Subject headings angiomyolipoma; liver neoplasms/ diagnosis; immunohistochemistry; microscopy, electron; carcinoma, hepatocellular/pathology; case-control studies

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INTRODUCTION

Angiomyolipoma (AML) is a rare benign mesenchymal tumor of the liver, composed o f a varying heterogeneous mixture of three tissue components: blood vessels, smooth muscle, and adipose cells. It has recently been proposed that the perivascula r epithelial cell (PEC) is the common progenitor[1,2]. Since its first d escription by Ishak in 1976 [3], there have been more than 100 cases repo rted in the English literature[4-6]. With the advance of radiological techniques, many more tumors are being diagnosed by the means. But radiological findings of AML may only be suggestive of the lesion; its definitive diagnosis r equires histological confirmation[9-19]. Some authors regard renal and hepatic AMLs, pulmonary and soft tissue lymphangiomyomatosis[2], pulmon ary and pancreatic clear cell "sugar" tumor, and cardiac rhabdomyoma as closely related groups of tumors, based on their morphologic overlap and common immuno reactivity for HMB-45[1]. They show different microscopic appearances, however, according to their organ of origin. The goals of this study were to hig hlight more subtle morphology and to gain possible insights into the differential diagnosis that could provide important information about this disease.

MATERIALS AND METHODS

Fourteen cases of AML were identified in the pathology files at the Department of Path ology, Chinese Military General Hospital, four of which were consulted cases. All the cases were independently reviewed by two pathologists, and the most import ant diagnostic criterion was the presence of HMB-45-positive cells. The clinical data and follow-up information were obtained in each case (Table 1). All the tumor tissues had been fixed in neutral buffered formalin and were routinelyem bedded in paraffin. Hematoxylin and eosin-stained sections were examined. Accor ding to the morphological aspect, one block was selected for each of these cases . Immunohistochemical study was performed on representative blocks by using an a vidin-biotin peroxidase complex technique. Selective cases were also examined w ith antibodies to CD68, CD31, CD34, factor VIII-related antigen.

RESULTS

Clinical findings

The clinical findings in all 14 patients are summarized in Table 1.There was am arked female predominance (women: men = 9:5) and the average age of patients at diagnosis was 40.07 years (range 30-63 years). Only four cases (case 4,7,10,13) had symptoms of space-occupying lesions while others were incidentally discovered on imaging studies (Figure 1). Two cases had two masses each (the other m ass was diagnosed as a hemangioma by a pathologist) and one of them had calcifi ed nodes (confirmed by a pathologist). None of 14 cases had tuberous sclerosis syndrome, angiomyolipoma of kidney, lymphangiomyoma or clear cell tumor of lung or other parts. For patients with complete follow-up data,all were well with no tumor recurrence and metastasis after excision.

Gross findings

Thirteen cases were solid and one was cystic (the content of cyst was a brown liquid). All tumors were well circumscribed, but not encapsulated (Figure 2). T he average size of all thirteen cases was 9.4 cm (range 2.5 cm-26 cm). The surfaces of tumor sections were yellowish but not uniform and some parts of tumors were fish-like.

Histological features

The most common pattern was that of solid sheets of myoid cells intermixed with areas of adipose cells and vessles,most of which were thin-walled (Figure 3) and few of which were thick-walled but had to be carefully observed. In 12 out of -14- cases there were clusters of hematopoietic

Department of Pathology, General Hospital of PLA, Beijing 100853, China
Ding Rong Zhong, male, graduated from West China University of Medical Sciences in 1994, presently working in the Department of Pathology, General Hospital of PLA.
Correspondence to: Xiao Long Ji, Department of Pathology, Gener al Hospital of PLA, Beijing 100853, China
Tel. +86-10-6822-8362
Email. xlji@public.bta.net.cn
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Table 1 The clinical findings in all 14 patients

NO.	Sex	Age(years)	Tumor size (CM)	Location (LOBE)	Primary diagnosis	Follow-up
1	F	38	4.5	R	Angiosarcoma	Well 44 mo after excision
2	F	33	2.5	R	Hepatic carcinoma	Well 35 mo after excision
3	F	33	6.0	R	Hepatic carcinoma	Well 33 mo after excision
4	F	49	26.0	R,L	Hemangioma	Well 153 mo after excision
5	F	48	3.5	R	Hepatic carcinoma	Well 31 mo after excision
6	F	38	12.5	L	Hepatic carcinoma	Well 12 mo after excision
7	F	35	14.5	L	Hepatic carcinoma	Well 9 mo after excision
8	M	63	17.0	R	Cystic adenoma or cystic adenocarcinoma	Well 9 mo after excision
9	M	37	3.5	R	Hepatic carcinoma	Well 16 mo after excision
10	M	34	10.0	R	Hepatic carcinoma	Well 11 mo after excision
11	M	30	7.5	L	Hepatic carcinoma	Well 16 mo after excision
12	F	43	6	L	Hepatic carcinoma	Well 3 mo after excision
13	M	37	12	L	Hepatic carcinoma	Well 2 mo after excision
14	F	43	6	L	Angiosarcoma	Well 1 mo after excision

Table 2 Immunohistochemical findings in 12 cases

Serial number	HMB45	Actin	S-100	EMA	AFP	Ki-67%
1	++	++	local+	-	-	<1
2	++	++	Local+	-	-	<1
3	++	++	Local+	-	-	<1
4	++	+	Local+	-	-	<1
5	++	+	Local+	-	-	<1
6	++	+	Local+	-	-	<1
7	++	+	Local+	-	-	<1
8	+	+	Local+	-	-	<1
11	++	+	Local+	-	-	<1
12	++	++	Local+	-	-	<1
13	++	++	Local+	-	-	<1
14	++	++	+	-	-	<1

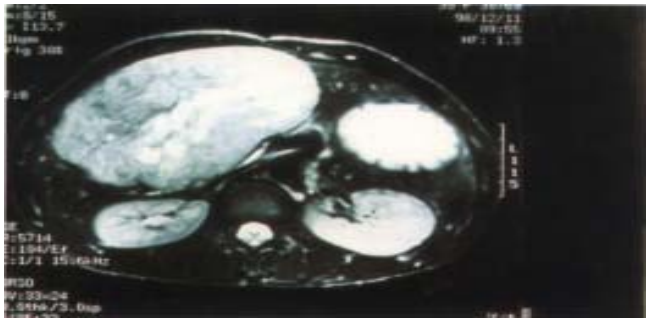

Figure 1 MRI image of case 7.

Figure 2 Macroscopic appearance of AML with yellowish fatty areas (case 7).

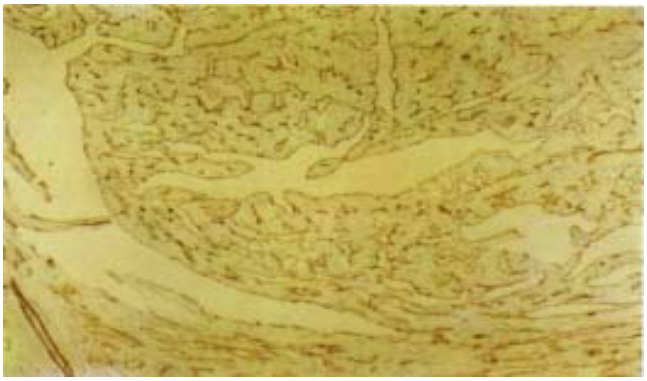

Figure 3 Thin-walled vessles and trabecular tumor cells of AML. IH: CD31 × 100

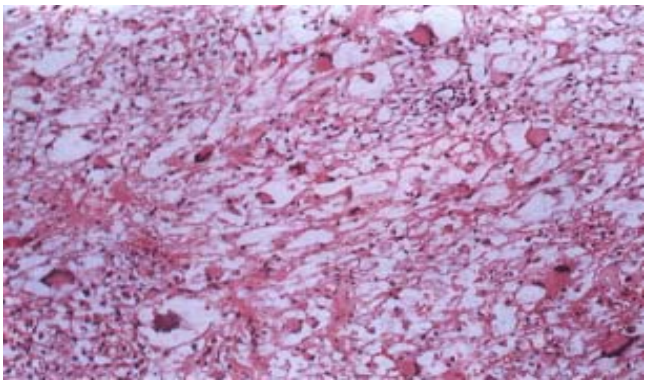

Figure 4 Multinucleus cells in a large number in AML of case 9. HE × 100

cells and in 1 of 14 cases there were lots of multinu clear cells (Figure 4). The myoid cells usually predominated and their morphology varied from epithelioid to intermediate (ovoid or short s pindle) spindle. The epithelioid cells in our 12 cases were main cell patter, cytoplasm of which varied from clear to vacuolated and eosinophilic granular, nuclei of which were partly normalchromatic with moderate pleomorphism, with delicate chromatin,and a single distinct eosinophilic nucleolus. Furthermore, there were some large bizarre cells in 10 cases out of 14 cases but without nucleus mitoses

(Figure 5). Long spindle cells with elongated eosinophilic cytoplasm could be seen but very rarely lipoblast-like cells were observed in sheets in local areas. Only two cases had multiple local necrotic areas but their total size was smaller than 5 percent of the tumor (Figure 6).

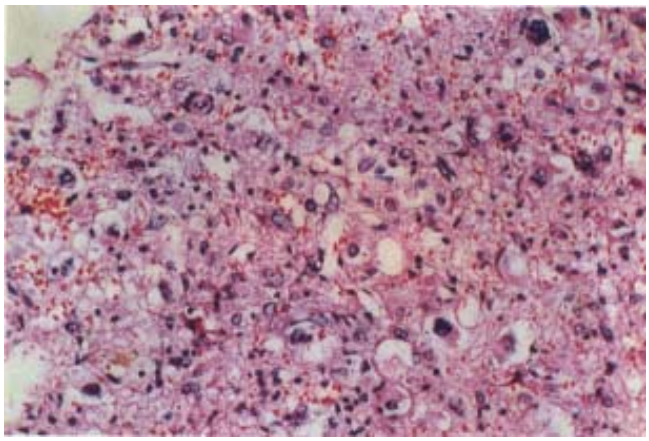

Figure 5 Pleomorphistic and large bizarre cells. HE × 200

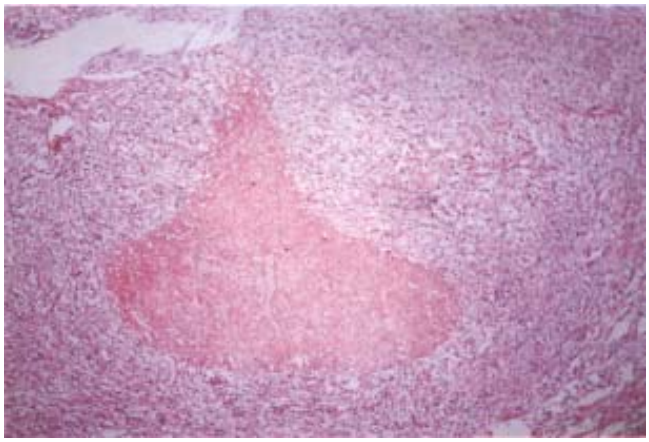

Figure 6 Local necrotic area of case 9. HE × 50

Immunohistochemical findings (Summarized in Table 2) The tumor cells were positive for HMB-45, but negative for EMA and AFP in all cases.Local tumor cells were positive for Actin and S-100. HMB-45 staining was intense, granular, and concentrated in the perinuclear pink cytoplasm. Although the epithelioid cell s were most consistently stained, the spindle cells were also weakly positive. A ctin staining in spindle tumor cells was more intense than in other cells. The l ipoblast-like cells were positive for both HMB-45 and S-100 protein. Lots of multinucleus cells presenting in 1 of 12 cases were positive for HMB45, Actin, S-100 and CD68. Furthermore, positive rate for Ki-67 of tumor cells in all 12 cases was no more than 1 percent.

Ultrastructural finding Six cases were examined under H-7000 electron microscopy. Neoplastic cells gene rally were polygonal and closely arranged with minimum intercellular material. In the cytoplasm, glycogen and round-to-oval mitochondria were common features, together with a characteristic finding of lots of electron-dense, membrane-bound granules (these granules were 60-100 nm in diameter with some filament-like structures in them which could be seen under high magnification. These were de emed to be either premelanosomes or atypical lysosomal bodies) (Figure 7).

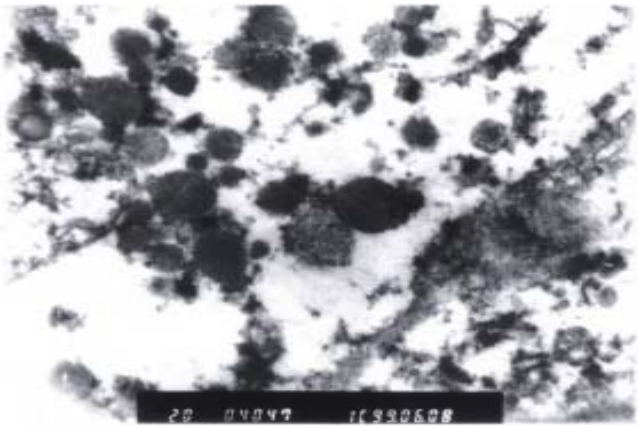

Figure 7 Ultrastructure of neoplastic tissue: glycogen, electron-dense granules. EM × 20 000

DISCUSSION

Angiomyolipoma, which occurs frequently in kidney but rarely in other sites[7,8], previously considered as a hamartomatous growth rather than a true neoplasm, is a rare benign mixed mesenchymal clonal neoplasm of the liver. Since the first case described by Ishak[3], AML has been diagnosed with increas ing frequency with advances in MRI, CT, CD-SO and angiography. Despite the claim that the imaging features are highly characteristic[9-19], the preoperative diagnoses are erroneous in more than half of the cases in the present s eries. None of our 14 cases were correctly diagnosed before operating, furthermore, 5 cases were misdiagnosed as hepatic cell carcinoma or sarcoma by pathologist even after operating.

The diagnosis of AML is usually made on recognition of three or four components of the tumor, namely blood vessels, smooth muscle, mature fat, and hematopoietic tissue[4-6,20]. These elements, however, are variable in proportion and distribution. The adipose cells have no characteristic features in themselves to distinguish between various lipomatous tumors. Extramedullary hematopoiesis is not an integral part of AML and is seen in many primary benign and malignant hepatic tumors. Its occurrence is probably more closely related to the hepatic s inusoidal endothelium which plays an important part in hematopoiesis in the feta l liver. This also explains that hematopoietic cells are found only in hepatic but not renal AMLs. Blood vessels are present in all kinds of tumors which easily escape attention. The more distinctive features in AML are their tortuosity, thick walls (but rare in AML of live) and perivascular muscle proliferation. It th us appears that the myoid component is the only specific and diagnostic

component in AML and it can exist in epithelioid, spindle, and intermediate forms. The e ssential component in this tumor appears to be PECs[1-6]. It has been speculated that the distinctive epithelioid cells are primitive mesenchymal cells that have an ability to differentiate towards both myoid and fat cells. Spindle myoid cells and lipocytes probably represent the mature derivatives of the epi thelioid cells. Immunohistochemistry is particularly useful for diagnosing, as m any authors have found. HMB-45 has been shown to be a promising marker for renal and hepatic AMLs and can even be applied to minute samples such as fine-needle aspirates[21,22].

Because of the rarity and the pleomorphism of histological features of hepatic A MLs, histological diagnosis may be difficult, especially with needle biopsy (one of our cases was diagnosed by this means). Many features in AML can mislead the unwary pathologist to a diagnosis of HCC: polygonal cells in trabecular arrange ment, peliosis, nuclear pleomorphism, prominent eosinophilic nucleoli, deficient reticulin framework, presence of glycogen, eosinophilic globules, and tumor nec rosis. Four of our cases were misdiagnosed by pathologists because of causes mentioned above. In AML with spindle cells and pleomorphic features, sarcoma is the most common incorrect diagnosis. For this reason, one case in our series was midiagnosed as angiosarcoma. Lipomatous AML has to be differentiated from true lipoma and focal fatty change. Evidently, a careful histologic survey of the entire specimen usually reveals the presence of the classic mixed pattern of the tumor. Immunohistochemical result (positive for HMB45 and Actin but negative fo r AFP or EMA), ultrastructural stucture and the low proliferation of tumor cells can further confirm the diagnosis[25-35]. The histogenesis of AML is unclear, but immunohistochemical and ultrastructural studies provide important insights into its cell differentiation[4-6,20,2 3,24]. The concept of "perivascular epithelioid cells" as the unified feat ure has been proposed[1]. Presumably, the PECs are an aberrant type of mesenchymal cells proliferating to form tumorous lesions, possibly due to chromos omal aberrations in the tuberous sclerosis (TS) complex gene. Because they are capable of dual differentiation, the argument for a hamartomatous proliferation i s unsound. Recent molecular studies have shown tumor clonality and 5q deletions with a common region of deletion spanning 5q33 to 5q34, indicating a clonal neop lastic process[36-40].

Preoperative identification of AML is desirable because of differences in clinic al course and treatment between this disease and other hepatic neoplasms[41]. Although some imaging features may suggest hepatic AML, in such cases, his tological analysis must be performed. No matter whether or not the liver biopsy contains fat component, the diagnosis can be established based on myoid component positive for HMB45.

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Edited by Zhou XH
proofread by Mittra S

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

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Editorials

progress of gastric cancer etiology: N-nitrosamides 1999s

Da Jun Deng

Subject headings stomach neoplasms/etiology; nitroso compounds; nitrosamides/nitrosourea; epidemiology; chromatography; liquid; microorganisms

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INTRODUCTION

Stomach carcinoma is still the leading cause of cancer death in China and the second one in the world. Its possible causes include: A) chemical factors such as intragastric formation of *N*-nitroso compounds (NOC) and high salt intake; B) biological factors such as infection of *Helicobacter pylori* and biotoxins intake; and C) nutritional factors such as deficiency of vitamin C, selenium, a nd other antioxidants. Nitrogenous precursors of NOC, e.g. alkylamines, alkylur eas, alkylguanidines, and alkylamides, occur widely in nature and potential nitrosating agents, e.g., nitrite (NO_2-) and NO_x (the gaseou s oxides of nitrogen) are similarly widespread. Relationship between exposure to NOC and causes of human cancer was investigated extensively ten years ago. Resu lts indicated that the exposures of NOC might contribute to the occurrences of m alignancy in the upper digestive tracts including stomachs. It was also observed that both high salt intake and deficiency of some micronutrients enhanced NOC- induced carcinogenicity. Recent studies show that infection of *H. pylori* can lead to atrophic gastritis and achlorhydria, and promote endogenous formation of NOC indirectly[1]. Much attention has been paid to stomach cancer and NOC regarding the characterization of natural *N*-nitrosamides in human envir onment in the 1990s.

N-nitrosamides, one kind of direct-acting NOC, can be synthesized endogenously in stomach lumens and damaged DNA of gastric mucosal epithelium *in situ*. Most of epidemiological investigations showed that the occurrence of stomach cancers was correlated positively with exposure levels of nitrosating agents and ni trogenous precursors of NOC[2]. Laboratory synthesized *N*-nitrosamides are strong animal stomach carcinogens. Intragastric *N*-nitrosamide formation may play an important role in the etiology of gastric carcinomas[3]. However, most *N*-nitrosamides are chemically reactive, thermal, photo, and alkali-labile compounds. It is very difficult to detect *N*-nitrosamid es in human environments chemically. Little was known about the existence of n atu ral *N*-nitrosamides before the 1980s because of lack of a convenient sensi tive method to detect them precisely [4]. Several progresses have been ma de on the study of *N*-nitrosamides in the past ten years, including setup of detection meth ods for trace *N*-nitrosamides in the early 1990s[5,6] resulting in recent discovery of natural *N*-nitrosoureas in human environments[7-9].

This article will present and discuss results of studies on stomach cancer and *N*-nitrosamides in the past decade.

DEVELOPMENT OF SENSITIVE AND SELECTIVE METHODS TO DETECT *N*-NITROSAMIDES CHE MICALLY

Many *N*-nitrosamines can be analyzed sensitively (detection limit, less than 1 ng/injection) by a standard commercial Thermal Energy Analyzer Detector (TEA), relying on their thermal cleavage of the *N-N* bond to produce a nitrog en oxide (NO) radical. However, *N*-nitrosamides and related compounds, unlike *N*-nitrosamines, typically rearrange on pyrolysis to yield molecular nitro gen (N_2) instead of nitrogen oxide. Because of the possible etiological role o f *N*-nitrosamides in human gastric carcinogenesis, it is necessary to setup a sensitive and selective method to detect *N*-nitrosamides in human environm ents.

A liquid chromatography (HPLC) with postcolumn photolysis device was assembled first at Shuker and Tannembaum's laboratory, Massachusetts Institute of Technol ogy in 1983 (Figure 1A)[10]. In the device *N*-nitrosamides were cle aved photolytically by ultraviolet (UV) irradiation to produce nitrite ion in aqueous solution, which was determined colorimetrically with Griess reagent in a postcolumn reactor. However this method is not *N*-

Laboratory of Cancer Etiology, Beijing Medical University School of Oncology & Beijing Institute for Cancer Research, 100034, China
Professor Da Jun Deng is Director of Laboratory of Cancer Etiology, Beijing Medical University School of Oncology. He is also Vice Chairperson of Cancer Aetiology Committee, Chinese Anti-Cancer Association. His work on N-nitrosamides and causes of stomach cancer has been supported by grants from national scientific foundations/or ganizations such as 7.5 and 8.5 Key Investigations of Science and Technology, 211 Project, etc.
Correspondence to: Dr. Da Jun Deng, Laboratory of Cancer Etiology, Beijing Medical University School of Oncology & Beijing Institute for Cancer Research, Western Destrict, Beijing, 100034, China
Tel. +10-6616-2978, Fax. +10-6617-5832
Email. dengdajun@sina.com
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nitrosamide-specific. It is of low sensitivity (detection limit, ng/injection): 20 for *N*-me thyl-*N*-nitrosourea (NMU) and 8 for *N*-methyl-N'-nitro-*N*- nit rosoguanidine (MNNG). The polarity of *N*-nitrosamides ranges widely from str ongly polar to non-polar. Non-polar *N*-nitrosamides do not unde rgo photohydrolysis because of their nondissolubility in aqueous so lution. Though the device could not be used to detect trace amounts of *N*-ni trosamides in nature, it has been often used to further develop sensitive met hods (Figure 1B and 1C)[5,11]. Fine *et al*[12] at New Englan d Institute for Life Sciences have modified the pyrolysis chamber in a standard TEA such that *N*-nitrosamides release nitric oxide on pyrolysis (sensitivities for standards, less than 1 ng/injection) in 1987. However, details o f the instrument were not provided and further development was needed.

An HPLC-photolytic interface-TEA method was reported to precisely detect *N*-nitrosamides and other non-volatile NOC at Hotchkiss's laboratory, Institute of Food Science, Cornell University in 1988 (Figure 1B)[5]. A chromat ographic effluent containing separated NOC is introduced into a glass coil with a purge stream of He and irradiated with UV in a photolysis device. Nitric oxide, cleaved by photolysis, is separated rapidly from the solvent through a series of cold traps and carried by the He into the reaction chamber in a standard TEA. The maximum sensitivity of the approach was approximately 8 ng for NMU and 16 ng for MNNG, and less than 1 ng for *N*-nitrosoamino acids and *N*-nitrosam ines. The selectivity for *N*-nitrosamides was not mentioned.

A selective and sensitive HPLC-Photolysis/ Pyrolysis-TEA method (Figure 1C) was set up for *N*-nitrosamides at Beijing Institute for Cancer Research, Beijing Medical University School of Oncology (Chen *et al*; Li and Deng)[6,11]. In the photolysis device, *N*-nitrosamides are first cleaved photolytic ally by UV irradiation to produce nitriteion in aqueous chromatographic effluen t. Then nitrite ion in the effluent is introduced into a pyrolysis tube (made of Al_2O_3) at 550℃ with carrying gas stream of He or N_2, and releases nitric oxide on pyrolysis. Nitric oxide is separated rapidly from the solvent-spray t hrough a series of cold traps, and is lead into the reaction chamber in TEA by t he carrying gas. Detection limits (ng/injection) are: 5.2 for NMU, 8.5 for MNNG, about 1 for *N*-nitrosamines, and 16 for *N*-nitrosoproline. In addition, when UV lamp of the photolysis device is turned off, the responses decreased up to 90%-100% for *N*-nitrosamides, but only 0%-45% for other kinds of NOC. I t indicates that the method is a selective one, which can be used to differentiate *N*-nitrosamides preliminarily from other kinds of NOC by comparing the difference in response UV when the lamp is on or off. This special feature can be useful for chemists to select the right chromatogra phic components for further identification of trace *N*-nitrosamides in human environments.

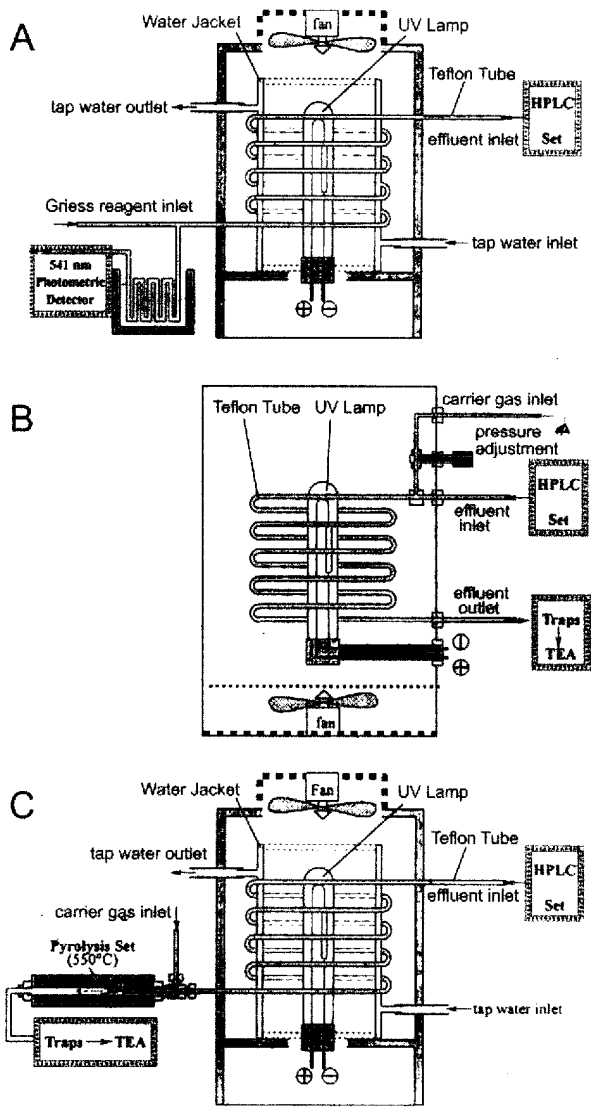

Figure 1 Diagrams of HPLC-Photolysis Device-UV Detector (A), HPLC-Photolytic Interface-TEA (B), and HPLC-Photolysis/ Pyrolysis-TEA (C).

Zhang *et al*[13] developed a method to detect the total amount of *N*-nitrosamides in biological samples with the Photolysis/Pyrolysis-TEA detector. It can be used only to roughly evaluate exposure levels of total *N*-nitr osamides, because calculation of the concentration of total *N*-nitrosamides is based on the difference inresponse when the UV lamp is on or off. Sampl e purification by extraction with organic reagents is required in the method. Th erefore, *N*-nitrosamides with strong polarity in the samples will be lost du ring the process of extraction. Only medially polar ones can be detected by the method.

IDENTIFICATION OF *N*-NITROSAMIDES IN NATURE

There is an indispensable evidence to show existence of *N*-nitrosamides in human environments in order to prove etiological role of *N*-nitrosamides in gastric carcinogenesis. However, little is known about the detailed chemical structures of the natural *N*-nitrosamides except stre-

ptozotocin and therape utic *N*-nitrosoureas[14]. Establishment of above the sensitive methods to determine *N*-nitrosamides make it possible to detect the trace amounts of *N*-nitrosamides in nature.

Caffeine is a normal component of coffee. Kumar *et al*[15] rep orted fo rmation of caffeidine and caffeidine acid from pure caffeine treated under condi tions similar to preparation of salted tea practised in Kashmir, which could result in two *N*-nitrosamides, dinitrosocaffeidine and N, N'dimethyl-*N*-nitrosourea by nitrosation. However, there is no report to show that th ese *N*-nitrosamides are detectable in the nitrosated-salted tea. Mende *et al*[16] characterized the nitrosamide precursor pyrrolidin(2)one in food and tobacco. A volatile *N*-nitrosamide, *N*-nitroso pyrrolidin (2) one, was detectable in the nitrosated precursor by gas chromatography-TEA me thod. They also mentioned the existence of trace amounts of the *N*-nitrosami de in natural Indian nasal snuff. But no detailed supporting materials were p rovided.

Fish sauce is a liquid product of small marine fish and sodium chloride (7:3). T he main species of fishes used are Sardinella aurita (Val.) and Decapt erus maruadsi (T & S). The fishes are completely liquidized after being fermented for 1-2 years. The product is consumed daily (about 30 mL/capita) by r esidents as a traditional seasoning in the Chinese southeast coast, the highest risk area for stomach cancer in China (the male standard mortality of stomach ca ncer in Changle County, 134.44/10^5 in 1986-1988). Epidemiological studies sho wed that the intake of fish sauce is a high risk factor for gastric carcinogenes is for the local residents[17]. It was reported that extract of fish sauce samples is markedly and directly mutagenic toward S. typhimurium TA100 induced high sister chromatid exchanges and micronucleus in Chinese hamster V79 cells after nitrosation with sodium nitrite under the simulated gastric conditions. But the extract of non-nitrosated samples had no such effect. The nitrosated fish sauce also induced SOS in *E. coli* PQ37 and alkylation of calf thymus D NA. The potency of nitrosated fish sauce to induce unscheduled DNA synthesis in human normal gastric mucosal cells was increased about fivefold compared with fish sauce. When the extract of nitrosated fish sauce was given to newborn rats by gavage, dysplasia and adenocarcinoma were induced in the glandular stomach in t he 4th and 16th experimental week, respectively[13,18,19]. Because of the high exposure level of nitrosating agent nitrite for the local residents [20], dietary fish sauce may contribute to the causes of the high mortality due to stomach cancer in the areas. It is necessary to identify che mical carcinogens in the nitrosated fish sauce.

Chen *et al*[21]reported that 100% (n = 21) of fish sauce samples contained some kinds of volatile *N*-nitrosamines by HPLC -TEA and gas chromat ography-TEA. Concentrations of total NOC in 49 fish sauce samples ranged from 0 .2 to 16 μmol/L and rose by up to 4800- and 100-fold after being nitrosated at pH 2 and pH 7, respectively[19]. Deng *et al*[7]characterized a chromatographic component of thermal-unstable *N*-nitroso compounds in nitrosated fish sauce by above HPLC-Photolysis/Pyrolysis-TEA method. A strong chromatographic peak with the same retention times as that for authentic NMU, was obtained under two different liquid chromatographic conditions after the sample was nitrosated by 5 mmol/L of sodium nitrite (final concentration) at 37℃ and pH 2.0 for 1 h. Like NMU, the chemical could not be detected by the method when UV lamp in the photolysis device was turned off. In a confirmation study, the chemical structure of the component was compared with authentic NMU by HPLC-elec tric spray ionization-mass spectrometer and HPLC-UV diode array detector. The chemical showed the same mass spectrum (m/zvalues 64, 102, 145) and spectrum of ultraviolet-absorbency (λ_{max} = 230 nm) as those of NMU[8]. These results indicated that the component was NMU. This was the first study reporting that there is *N*-nitrosamide, NMU, in nitrosated food. In addition, NMU could also be detected in the nitrosated human gastric juice sample spiked with fish sauce[7]. The formation of NMU in the sample was pH- and nitrite-dependent (Table 1). These results provide direct evidences that NMU formation could occur in human gastric juice samples spiked by fish sauce during nitrosation under simulated gastric conditions *in vitro*. Another *N*-nitrosamide, one of *N*-nitrosodipeptides, was separated recently and confirmed in our laboratory.

Table 1 Comparison of formation of NMU at various doses of $NaNO_2$ in gastric lumen of pig model *in vivo* and control experiments *in vitro*[9,22]

Amount of $NaNO_2$ (μmol)	Formation of NMU			
	Concentration (μmol/L)		Total amount (μmole)	
	in vivo	*in vivo*	*in vivo*	*in vivo*
3480	25.40	29.20	4.27	1.75
870	7.97	6.48	1.91	0.38
220	ND	2.77	ND	0.17

ND, below detection limit; the value was the average of two independent experime nts.

Furthermore, experimental mini-pig model and human volunteers were used to study the possibility of intragastric formation of NMU *in vivo*[9,22]. Fish sauce sample (20-30 mL) and nitrite were administrated into gastric lumen of experimental pigs by perfusion via pig stomach cannula, or taken orally by hum an volunteers. Gastric juice samples were taken out 30 min later. Concentration of NMU in condensed extracts of these samples was analyzed with HPLC-Photolysis / Pyrolysis-TEA. Results showed that NMU was formed in gastric lumens of both models *in vivo* and also that the formation of NMU was nitrite and pH-dependent (Tables 1 and 2).

Zhang *et al*[20] reported that concentration of nitrite in

fastin g g astric juice samples in China was up to 100 μmol/L. Pignatelli *et al*[23] reported that the level of nitrite in fasting gastric juice in Columbia w as up to 472 μmol/L. It was reported that NMU was still detectable in the conde nsed extract of 100 mL of mixture of pooled fasting human gastric juice samples and fish sauce sample (9 v:1 v) after treatment of 500 μmo l/L of nitrite *in vitro*, which is within the range reported in human gastric contents as veported by Deng *et al*[9]. These results suggest that low micromolar amo unts of *N*-nitrosoureas can be formed in the normal stomach when nitrite is consumed in amounts to which humans are commonly exposed.

Table 2 Status of gastric juice samples from four human volunteers and formatio n of NMU 30 min after taking 40 mL of diluted fish sauce and 500 μmol of nitri te[9]

Sample's origin	Total volume of sample (mL)	pH of sample	Total amount of NMU detected in gastric lumen (nmol)
Male A	24	5.0	4
Male B	110	3.0	85
Female A	50	2.0	100
Female B	50	2.0	22

Fish sauce is rich in nitrosable amines, i.e. dipeptides, free amino acids, crea tine, creatinine, and putrescine[24]. It was reported that 16-31 mg/kg of methylurea could be detected in dried, salted bonito fish after nitrosation and denitrosation, though no methylurea could be detected in the fish directly[25]. Further studies showed that methylurea was synthesized through 5-oxocreatinine 5-oxime and 1-methyl-5-oxohydrantoin 5-oxime duri ng nitrosation of creatinine[26,27]. NMU in the nitrosated fish sauce might be synthesized from creatinine.

POPULATION STUDY ON TOTAL *N*-NITROSAMIDES IN STOMACH

Previous knowledge on status of human exposure to *N*-nitrosamides is deduced indirectly from data obtained in studies to exposure of NOC precursors. The situation has been changed since the setup of chemical methods to detect total *N*-nitrosamides in biological specimens[13]. To elucidate the correlat ion between exposure level of *N*-nitrosamides and causes of gastric carcinom as, a pilot cases-control study and a population study were reported by Zhang *et al* in 1991 and Deng *et al* in 1997[13,28]. Total amount of natural *N*-nitrosamides was detected with the Photolysis/Pyrolysis-TEA method in fasting gastric juice samples from subjects in high and low risk areas for stomach cancer (Table 3, 4).

Gastric carcinogenesis is a multistage process. Its precancerous lesions include chronic atrophic gastritis, intestinal metaplasia, and dysplasia. In the case-control study, high levels of total *N*-nitrosamides were detected in the gastric juice samples from patients with chronic gastritis in Putian area, a county along the Chinese southeast coast (Table 3)[13]. The positive rates and mean concentrations

Table 3 Relationship between presence of total *N*-nitrosamides in gastric juice samples from subjects with pathological changes in gastric mucosa

Gastric mucosal status	Positive rate of total *N*-nitrosamides in gastric juice samples (%)**		
	High risk areas		Low risk area
	Putian[13]	Linqu[28]	Cangshan[28]
*N and CSG	2/12 (16.7)	1/4 (25.0)	4/23 (17.4)
CAG	10/14 (71.4)[b]	38/76 (50.0)[c]	18/56 (32.1)
CAG and IM	12/13 (92.3)	17/56 (28.8)[e]	0/4 (0.0)
CAG and DYS	12/13 (92.3)	18/44 (40.9)	5/10 (50.0)

*N, normal mucosa; CSG, chronic superficial gastritis; CAG, chronic at rophic gastritis; IM, intestinal metaplasia; DYS, dysplasia; **sample was classified as *N*-nitrosamides-positive when more than 184 nmol/L (de tection limit) of the chemicals was detected; [b]Significantly different fr om N and CSG with $P < 0.01$; [c]Significantly different from low risk a rea with $P < 0.05$; [e]Significantly different from CAG with $P < 0.05$.

Table 4 Presence of total *N*-nitrosamides (NAD) in gastric juice samples (GJ) from low and high risk areas for stomach cancer

	High risk area (Linqu)	Low risk area (Chongshan)	*P*-value
No. of GJ samples	176	99	
Proportion, pH≤3	48.0%	84.0%	<0.01
pH≥5	45.3%	13.5%	<0.01
Positive rate, all	40.9%	30.3%	= 0.03*
GJ, pH≤3	46.2%	27.4%	<0.01
GJ, pH≥5	43.3%	53.9%	
Conc. (μmol/L)	0.91	0.73	

*after age-adjustment.

in the three groups of patients were positively correlated with the severity of pathological changes in the gastric mucosa. In the populat ion study, the exposure status of total *N*-nitrosamides in stomach of subjects aged 35-68 years from high risk area for stomach cancer was further compared with that in low risk area under the same geographical and socioeconomic conditions in Shangdong Province, China (Table 3)[28]. Similar relationship between presence of *N*-nitrosamides and pathological changes of gastric mu cosa was obtained both in Linqu and Cangshan areas. However, the percentage of *N*-nitrosamides-positive samples was decreased in subjects with chronic atrophic gastritis when intestinal metaplasia was developed. The mechanism of decre ase in concentration of NOC is not clear. *N*-Nitrosamides are alkali-labi le compounds. It is a common step to adjust pH of sample-extractant mixture t o 5.0 with 10% sodium hydroxide in order to accelerate stratification of aqueous phase and organic extractant during extraction of NOC. During extraction of NMU in sample (aqueous solution), it was observed that addition of even one-drop (about 50 μL) of sodium hydroxide solution would destroy all NMU in the sample (Deng *et al*, unpublished data). Gastric mucosa with intestinal metaplasia secretes alkali-mucus. It is necessary to study whether the alkali-mucus catalyzes decomposition (activation) of *N*-nitrosamides and contributes to the dec rease of amount of total *N*-

nitrosamides in fasting gastric juice from patie nts with intestinal metaplasia of gastric mucosa. In addition, development of in testinal metaplasia of gastric mucosa is not suitable for colonization of *H. p ylori* in the alkali-mucus closely adjacent to the surface of gastric epithelium and finally eradicates them from there. It is interesting to study the relati onship between disappearance of *H. pylori* and the decrease in *N*-nitro samides amounts in gastric juice.

In the population study, more *N*-nitrosamides-positive samples in the high risk area (Linqu) were observed than in the low risk area (Chongshan). The diffe rence was significant after age-adjustment (Table 3)[27].

Chemical formation of *N*-nitrosamides and other NOC occur mainly under acidi c conditions. It had observed that 84% of samples was pH$\leqslant$3 in Chongshan, whe reas only 48% in Linqu (Table 4, P <0.001). That *N*-nitrosamides-positiv e rate is higher in samples with pH$\geqslant$5 than with pH$\leqslant$3 in the low risk area indi cates that there are some factors which could catalyze formation of *N*-nit rosamides in the achlorhydric stomach. Colonizations of microorganisms are commo n in the achlorhydric stomach. Some species of bacteria in stomach contain nitra te-reductase and could lead to high concentration of nitrite in gastric juice. It is well known that microorganisms catalyze formation of *N*-nitrosamines. Pan *et al*[29] reported that synthesis of *N*-nitrosamides , NMU, could also be accelerated by *Pseudomonas aeruginosa* at pH of 6-7, simulating achlorhydric stomach conditions. He *et al*[30] reported further that 6 out of 46 strains of bacteria, isolated from patients with gastrit is in Linqu County, promoted formation of NMU at pH 6.0. Biological formation of *N*-nitrosamides mediated by microorganisms in the achlorhydric conditions m ight account for the high concentration of total *N*-nitrosamides in gastric juice sample with pH$\geqslant$5.

High *N*-nitrosamides-positive rate was observed both in gastric juice sampl es with pH$\geqslant$5 and pH$\leqslant$3 from subjects in Linqu, the high risk area. The result s uggests that there is a high chemical formation of *N*-nitrosamides in the acidic stomach. It is supported by the observation that *N*-nitrosamides-posit ive rate in the samples with pH$\leqslant$3 from Linqu is higher than that from Chongshan , the low risk area (Table 4).

Above results indicate that human intragastric exposure to *N*-nitrosamides is positively correlated to risk of stomach cancer. *N*-Nitrosamides may be synthesized chemically in the acidic stomach and biologically in the ach lorhydric stomach.

PROSPECTS

Establishment of sensitive methods to detect trace *N*-nitrosamides enabled identification of this sort of NOC in human environments greatly. More natural *N*-nitrosamides need to be discovered in order to understand aetiological role of *N*-nitrosamides in gastric carcinogenesis in populations. Rediscovery of *H. pylori* is an important event in the history of oncology of stomach. It c auses atrophic gastritis, gastric ulcer, and correlates with the occurrence of m ucosa-associated lymphoid tissue lymphoma closely. It might also contribute to the causes of gastric carcinoma. *H. pylori* mediated gastritis induces high levels of nitrogen oxide (NO) in gastric mucosa. Tissue nitrogen oxide could damage DNA directly if it penetrates into cytoplasma during S-phase and further into nucleus. When Fe^{2+} exists in the tissue simultaneously it also nitrosates nitrogenous precursors to form NOC. It is noted that *H. pylori* infection leads to an increase in concentration of nitrite in the stomach lumen, decrease in secretion of ascorbic acid from gas tric mucosa, and might promote formation of *N*-nitrosamides in achlorhydric stomachs. However, relationship between occurrence of *N*-nitrosamides and *H. pylori* is not investigated extensively. Such investigation may bring to light the mechanism of carcinogenesis caused by *H. pylori*.

Because of the instability of *N*-nitrosamides, it is almost impossible for the chemicals to exist in diet. Most of the human exposure might originate from intragastric formation. That provides a good target to prevent stomach cancer by inhibition of nitrosation and elimination of nitrite in stomach. Garlic and related components are ideal candidates for such interventional study. They inhibit proliferation of microorganisms, combine nitrite, block nitrosation, and destroy formed NOC. An interventional study with garlicoil and other chemicals has been undertaken in Linqu area[31].

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