ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2013 May 21; 19(19): 2841-2978

WJG World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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2010-2013

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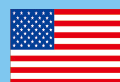

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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1352 experts in gastroenterology and hepatology from 64 countries.

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INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16951 (7/74); Current Articles: 677 (1/74); and Eigenfactor® Score: 0.06035 (5/74).

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza, 315-321 Lockhart Road,
Wanchai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
May 21, 2013

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SPECIAL STATEMENT
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INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

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http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2841

World J Gastroenterol 2013 May 21; 19(19): 2841-2846
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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EDITORIAL

Biliary complications following liver transplantation

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Received: February 17, 2013 Revised: March 23, 2013
Accepted: April 10, 2013
Published online: May 21, 2013

Abstract

Biliary tract complications are the most common complications after liver transplantation. These complications are encountered more commonly as a result of increased number of liver transplantations and the prolonged survival of transplant patients. Biliary complications remain a major source of morbidity in liver transplant patients, with an incidence of 5%-32%. Post liver transplantation biliary complications include strictures (anastomotic and non-anastomotic), leaks, stones, sphincter of Oddi dysfunction, and recurrence of primary biliary disease such as primary sclerosing cholangitis and primary biliary cirrhosis. The risk of occurrence of a specific biliary complication is related to the type of biliary reconstruction performed at the time of liver transplantation. In this article we seek to review the major biliary complications and their relation to the type of biliary reconstruction performed at the time of liver tranplantation.

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Key words: Liver transplantation; Complication; Biliary; Stricture; Leak; Endoscopic retrograde cholangiopancreatography

Core tip: Biliary complications continue to be a major cause of morbidity in liver transplant recipients. In this article, we review the etiology, as well as the main types of biliary complications according to the technique of biliary reconstruction and liver transplant procedure performed. Their management is also discussed with endoscopic techniques emerging as the preferred treatment option, obviating the need for surgery in majority of patients.

Kochhar G, Parungao JM, Hanouneh IA, Parsi MA. Biliary complications following liver transplantation. *World J Gastroenterol* 2013; 19(19): 2841-2846 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2841.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2841

INTRODUCTION

Since the first experiences with liver transplantation in the 1960s, this procedure has become a standard treatment for end stage liver disease. Limited primarily by the donor liver supply, the number of orthotopic liver transplants (OLT) has continued to increase. In the United States alone, according to the American Liver Foundation, 6500 liver transplantations were performed in 2005. Although, because of constant improvements in surgical techniques, the rate of biliary complications following liver transplantation has been decreasing; they remain a major source of morbidity and mortality[1,2]. Post liver transplantation biliary complications include strictures, leaks, stones or debris, and sphincter of Oddi dysfunction (SOD). T-tube biliary reconstruction, Roux-en-Y anastomosis, ischemia, reperfusion injury, hepatic artery thrombosis (HAT), cytomegalovirus infection, and primary sclerosing cholangitis are some of the risk factors that have been implicated in biliary complications.

TYPES OF BILIARY RECONSTRUCTION

The choice of biliary anastomosis is a major determinant of the risk of biliary complications after OLT[3,4]. The two most common forms of biliary reconstruction are choledochocholedochostomy (CC, duct-to-duct anastomosis) and choledochojejunostomy (CJ, connection of the bile duct to a portion of jejunum). The choice of biliary reconstruction is determined by multiple factors, including the underlying liver pathology, the size of donor and recipient bile ducts, prior transplant or previous biliary surgery, and the preference of the performing surgeon. There are no clear-cut guidelines on the optimal type of biliary reconstruction, and considerable variability exists between surgeons.

CC is the most common biliary reconstruction procedure performed during OLT. This type of reconstruction is usually preferred because the procedure is technically easier, it preserves the function of Sphincter of Oddi, and it also allows easy endoscopic access to the biliary system after the surgery[5]. Furthermore, the preservation of the sphincter of Oddi, theoretically decreases the risk of ascending cholangitis as it serves as a barrier against the reflux of enteric contents into the biliary tree. CC can be performed either with or without a T-tube. Routine use of a T-tube allows direct measurement of bile output and color in the early post-operative period, maintains easy access for radiological evaluation of the biliary system and allows rapid decompression of the biliary tree if needed. It also may reduce the risk of anastomotic stricture formation. On the other hand, the use of T-tubes has been associated with bile leak and cholangitis at the time of their removal. A recent retrospective study of 180 patients demonstrated an increased rate of overall complications (33% *vs* 15.5%) and an increased rate of cholangitis (10% *vs* 2.2%) in patients with a T-tube compared to those without. In that study patients without a T-tube had an increased survival rate compared to the T-tube population (80.1% *vs* 72.8%), an observation that was attributed to higher complication rates among those with a T-tube[5]. This observation is supported by a recent meta-analysis consisting of 1027 patients, in which those without a T tube had a decreased incidence of cholangitis and peritonitis with overall decreased rate of biliary complications. Interestingly, this meta-analysis did not show any significant differences between the T-tube and non T-tube groups in terms of other complications such as anastomotic bile leaks, fistulas, choledochojejunostomy revisions, stenting of the bile duct, hepatic artery thromboses, retransplantation, and mortality due to biliary complications[6].

CJ is another type of biliary reconstruction usually recommended in patients with pre-existing biliary disease such as primary sclerosing cholangitis, or prior biliary surgery, and also when there is a size mismatch between donor and recipient ducts. Compared to CC, CJ takes longer to perform and adversely affects the ability to perform an endoscopic evaluation of the biliary system after the liver transplantation. Potential complications of CJ include intestinal perforation, stricture, leakage, and bleeding at jejuno-jejunostomy site.

DIAGNOSIS OF BILIARY COMPLICATIONS

The presentation of biliary complications varies considerably. Some complications such as bile leaks may occur immediately in the post-operative period, while others may take weeks to develop. The clinical presentation can vary from asymptomatic patient with moderate liver enzyme elevations to a septic patient with fever and hypotension due to ascending cholangitis. Whenever a biliary complication is suspected, work-up usually begins with laboratory evaluation and an abdominal doppler ultrasound. Abdominal ultrasounds are relatively inexpensive, and are easy to perform. An abdominal ultrasound allows for the evaluation of the biliary tree and the corresponding hepatic vasculature. The positive predictive value of abdominal ultrasound is very high, especially in the presence of dilated bile ducts. In the absence of dilated bile ducts, the sensitivity of the ultrasound for detecting biliary obstruction ranges from 38%-68%[7]. In the event that the ultrasound does not reveal evidence of bile duct dilatation despite clinical suspicion, the next step can be magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP), depending on their availability. MRCP has excellent sensitivity (93%-100%) in detecting biliary strictures; and can also offer a road map for the endoscopist in planning the necessary intervention[8]. Another advantage of MRCP is that it does not carry the invasive risk involved with ERCP or other interventions such as percutaneous transhepatic cholangiography (PTC). The upside of ERCP and PTC, on the other hand, is that they both offer a potential therapeutic advantage over MRCP. It should be noted, however, that ERCP is associated with a high failure rate in patients with Roux-en-Y reconstruction, except when double balloon enteroscopy is available to assess the biliary tree. PTC is usually reserved in cases where ERCP cannot be performed.

Bile leaks after OLT

Bile leaks, along with strictures, account for the majority of complications post-OLT. Bile leaks occur in 2%-25% of cases after liver transplantation and can be classified in two categories: early bile leaks, which present within 4 wk of OLT; and late bile leaks, which present beyond this time[9-12].

Etiology: Early bile leaks usually occur at the anastomotic site or at the T-tube insertion site. They can be caused by ischemia, relative downstream obstruction, sphincter of Oddi hypertension, or as a result of T-tube removal[13]. The majority of bile leaks after OLT are associated with either planned or inadvertent T-tube removal, and the leak often occurs at the T-tube insertion site. One factor that has been shown to predict development of bile leak after T-tube removal is the presence of mucosal duct irregularities on cholangiography.

Presentation: The presentation varies with extent of the leak. Bile leak should be suspected in any patient who develops abdominal pain, fever or any sign of peritonitis after liver transplant, especially after T-tube removal. Bile leaks not related to T-tube removal typically present within the first 30 d after OLT. Some patients, especially those on corticosteroids, may be asymptomatic, with no signs of pain or fever. In such cases, any unexplained elevations in serum bilirubin, fluctuation in cyclosporine levels, or bilious ascites should raise suspicion for a bile leak.

Management: Once the clinical suspicion of a bile leak following T-tube removal is raised, initial management usually involves pain control with analgesics, intravenous fluids, and supportive care. Biliary leaks due to ischemia are difficult to treat since the cause is usually not corrected by endoscopic or radiologic intervention. Leaks due to other causes usually respond to non-operative diversion of biliary flow, such as unclamping of the T tube, endoscopic sphincterotomy with or without endoscopic stenting at the time of ERCP, or placement of a PTC. ERCP with stenting of the bile duct, sphincterotomy, nasobiliary drainage, or a combination of these techniques has been shown to have high rates of success[9,14]. Most studies report resolution of symptoms in 85%-100% of the cases[15]. Although one study reported better results with nasobiliary drainage; most centers use an internal biliary stent to overcome the difference in pressure in the bile duct and the gut. The stent usually remains in place for several weeks. PTC is commonly used in cases where ERCP cannot be performed, or in patients with Roux-en-Y reconstruction where the biliary orifice cannot be reached with a regular sideview endoscope. In rare cases, surgical intervention may become necessary[9].

Biliary strictures

Post liver transplantation biliary strictures are usually classified as anastomotic or non-anastomotic. The incidence of biliary stricture ranges from 5%-15% after deceased donor liver transplantation and 28%-32% after living donor liver transplantation[7]. Strictures are commonly seen as late complications, occurring approximately 5-8 mo after transplantation.

Anastomotic strictures

Anastomotic strictures (AS) at the site of biliary anastomosis are frequent after OLT and can occur in both CC and CJ type of reconstruction. AS are more common after CJ than CC due to the direct bilioenteric connection[1,12].

Etiology: The pathogenesis of AS is believed to be from inadequate mucosa-to-mucosa anastomosis, surgical technique, local tissue ischemia, and the fibrotic nature of the healing process[16]. Early bile leak is also considered to be a risk factor for developing AS[9]. In those with a T-tube, strictures at the CC anastomosis are often not typically evident until after removal of the T-tube. A slight and transient narrowing of the biliary lumen occurs frequently in biliary anastomosis shortly after the OLT due to post-operative edema. It is uncertain how many of these cases progress to clinically significant strictures.

Presentation: Biliary stricture should be suspected in any OLT patient who presents with jaundice, fever, abdominal pain, or even in patients with asymptomatic biochemical cholestasis. Dilatation of the bile ducts proximal to the biliary anastomosis may be observed on imaging studies in some patients but is not a pre-requisite for diagnosis. Histologic findings may be suggestive of biliary obstruction, such as pericholangitis or bile duct proliferation.

Management: When a clinically significant AS is found, treatment is warranted. In recent years, ERCP has seen an increase in popularity in the management of AS. Although results differ markedly, studies have demonstrated good response after endoscopic therapy in over 75% of the patients[17,18]. Endoscopic treatment is thus regarded as the treatment of choice for AS, especially in the CC group of patients. Stenting of the stricture during ERCP is performed with or without balloon dilatation of the stricture. The initial stent is then exchanged for a larger stent or multiple stents every 3 mo for an average of 1 year to dilate the stricture and prevent clogging and stone formation. In patients with CJ reconstruction, the initial treatment usually involves stenting by percutaneous approach. Some centers have reported that early strictures respond better to therapy than late strictures. If a stricture does not respond to endoscopic or percutaneous therapy, surgery may be indicated. Previous endoscopic or percutaneous treatment has not shown to influence the success rate of surgery in treating such complications.

Non anastomotic strictures

Non anastomotic strictures (NAS), also known as ischemic type strictures, are well known and have been described since the beginning of liver transplantation. They are frequently hilar in location, but can also be diffusely intrahepatic. NAS tend to be longer and multiple on presentation. NAS incidence ranges from 5%-15% with mean time to presentation of 3.3-5.9 mo post-OLT[19,20].

Etiology: A few theories have been proposed for the development of NAS. The blood supply to the supraduodenal bile duct is predominantly from vessels which are resected during OLT. The remaining blood supply to the donor bile duct then comes from the hepatic artery and its branches, which are tenuous and highly susceptible to ischemic injury. In patients with NAS, up to 50% have demonstrable HAT[21]. Prolonged cold ischemia time has also been shown to be responsible for the development of NAS. Besides ischemia, an immunological cause has also been proposed. This is mainly due to the observa-

tion of an increased incidence of NAS in cases with ABO-incompatible grafts, in patients with autoimmune hepatitis or primary sclerosing cholangitis, in patients suffering from chronic ductopenic rejection, and those with a CC chemokine receptor 5delta32 polymorphism. In many cases, NAS are probably multifactorial in origin with injury resulting from one or more of the above mechanisms[7,22].

Management: The presentation of NAS is similar to that of AS. NAS are more difficult to manage than AS, as treatment in each case has to be individualized. It is therefore difficult to make generalized recommendations for management of NAS. In cases with early HAT, aggressive management with either revascularization or early re-transplantation has been recommended. In NAS not associated with HAT, endoscopic or percutaneous therapy are often attempted first. Repeated dilatation with stenting seems to be the most accepted treatment form[14]. Treatment success depends upon stricture severity, number, and location. Extra-hepatic strictures generally respond better to therapy. Different studies report variable treatment success rates, ranging from 50%-70%[10,23]. If radiological and endoscopic therapies fail, surgery may become necessary. Success rates are higher if surgery is done within 2 years of OLT and if the liver biopsy does not show any significant fibrosis. Retransplantation may also be considered in patients with treatment failure, or in the presence of secondary biliary cirrhosis, recurrent cholangitis, or progressive cholestasis.

SOD

Another common occurrence after OLT is a mild increase in the size of donor and recipient common bile ducts. In certain cases, significant dilatation of both recipient and donor bile duct in association with biochemical abnormalities occurs in the absence of cholangiographic evidence of obstruction. In these cases, SOD is suspected. The incidence of SOD is reported to be up to 7%[11,24].

Etiology: The pathogenesis of SOD is attributed to denervation of the sphincter during OLT. This leads to an increase in basal pressure, thus causing increased pressures in the choledochal duct[24]. Very few studies have directly assessed the pressures in the sphincter of Oddi post-OLT. Two types of SOD have been proposed on the basis of pathogenic mechanisms: stenosis and dyskinesia[16,25,26]. Any process that leads to chronic inflammation and fibrosis, can lead to sphincter stenosis. Dyskinesia, on the other hand, is usually seen as a result of functional disturbance of the sphincter.

Management: There have been virtually no clinical trials that demonstrate the best treatment option for SOD. In recent years, endoscopic therapy with sphincterotomy with or without stenting has been the most acceptable treatment option for SOD.

Biliary stones, sludge, and casts

On ERCP, stones, sludge and casts are usually seen as a defect in the contrast column and described as “filling defects”. Intrinsic bile duct obstruction, in the form of biliary stones and sludge, can virtually occur at any time following the OLT. Sludge is described as a thick collection of mucous, calcium bicarbonate and cholesterol cystals, which, when left untreated, can go on to form biliary stones. Sludge and casts usually occur within the first year of transplant, while stones tend to occur later on.

Etiology: Theoretically, anything that increases the viscosity of bile or reducing flow can predispose to the formation of sludge, and stones. Bile duct mucosal damage due to obstruction, ischemia, or infection is thought to play a role in the development of casts. Of patients presenting with biliary stones and sludge, most will have an underlying stricture. In addition, medications such as cyclosporine may play a role in bile lithogenicity by inhibiting bile secretion and promoting functional biliary stasis. Bile in transplant patients has been shown to be supersaturated with cholesterol, which is aggravated by T-tube drainage and depletion of the bile acid pool.

Presentation: Patients commonly present with abdominal pain, cholestatic liver tests, and uncommonly with cholangitis.

Management: A study demonstrated that cholangiography is the only reliable imaging method for sludge; ultrasonography and CT scans are of limited value. If sludge alone is present, then it would be reasonable to first attempt medical treatment with ursodeoxycholic acid. Endoscopic therapy with sphincterotomy, lithotripsy and stone extraction are successful in treating majority of filling defects, especially biliary stones[23].

Biloma

Bile rupture and spilling of bile within the liver and abdominal cavity may result in the formation of a biloma. Small bilomas, especially ones that communicate with the biliary tree, may resolve on their own. Although bilomas can generally be treated with antibiotics and percutaneous drainage, some may require placement of a biliary stent in the extrahepatic bile duct[9]. Surgical drainage of a biloma is viewed as a last resort option.

Hemobilia

One of the rare complications seen after OLT is hemobilia. It is usually associated with percutaneous liver biopsy or PTC. The commonly described triad of right upper quadrant pain, jaundice, and gastrointestinal bleed is seen only in a minority of patients. Treatment of hemobilia requires both hemostasis and treatment of any associated biliary obstruction by clots. The bleeding stops spontaneously with supportive therapy and correction of coagulopathy in some cases. Embolization of the

bleeding vessel by interventional radiology is required if bleeding is persistent or severe[27]. Removal of clots from the biliary tree for relief of obstruction is usually done by ERCP.

Ductopenia

Ductopenia (also referred to as bile duct paucity and vanishing bile duct syndrome) is a descriptive term for small intrahepatic bile duct loss from any cause. In post liver transplantation patients, acute and chronic rejection and ischemia are the most common culprits. The diagnosis is established by liver biopsy in the appropriate clinical setting. Treatment depends mainly on the underlying etiology of ductopenia.

CONCLUSION

Biliary complications following liver transplantation are relatively common and continue to be a challenging aspect in the management of such patients. The development of these complications is heavily influenced by the type of anastomosis during surgery. The majority of biliary complications after liver transplantation are now a days being managed endoscopically rather than surgically. ERCP, in particular, has proven to be relatively safe and effective in the management of these complications.

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P- Reviewers Ayoub WS, Bogdan D **S- Editor** Gou SX
L- Editor A **E- Editor** Xiong L

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Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2847

World J Gastroenterol 2013 May 21; 19(19): 2847-2863
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Chemokines, chemokine receptors and the gastrointestinal system

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Received: September 12, 2012 **Revised:** March 22, 2013
Accepted: April 27, 2013
Published online: May 21, 2013

Abstract

The biological properties of tumor cells are known to be regulated by a multitude of cytokines and growth factors, which include epidermal growth factor receptor agonists and members of the transforming growth factor β family. Furthermore, the recent explosion of research in the field of chemokine function as mediators of tumor progression has led to the possibility that these small, immunomodulatory proteins also play key roles in carcinogenesis and may, therefore, be potential targets for novel therapeutic approaches. In this review, we will summarize recently reported findings in chemokine biology with a focus on the gastrointestinal tract.

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Key words: Chemokine; Receptor; Signal transduction; Tumor progression; Targeted therapeutics; Digestive system; Cancer

Core tip: The chemokine network makes an attractive target for therapeutic intervention in many tumor types, including those of the gastrointestinal tract. However, we need to define more selective and specific targets, to minimize systemic side effects during treatment.

Miyazaki H, Takabe K, Yeudall WA. Chemokines, chemokine receptors and the gastrointestinal system. *World J Gastroenterol* 2013; 19(19): 2847-2863 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2847.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2847

INTRODUCTION

Cancer development and progression in the gastrointestinal tract

Cancer is a disease in which normal cells acquire genetic and epigenetic abnormalities[1,2], leading to disorientation of conventional processes for the maintenance of normal cell physiology. These aberrant genetic and epigenetic modifications to the normal cell induce abnormal cell motility, proliferation, and survival, eventually enabling these cells to invade into adjacent tissues and even to migrate to regional or distant organs where they may grow continuously as metastatic lesions[3]. For many cancer patients, metastasis is generally the major cause of disease-related death[4,5]. Therefore, it is indispensable to elucidate the basic molecular mechanisms of tumor development to identify effective therapeutic targets, which can possibly reduce the side-effects of treatment, and define useful molecular markers for early detection and prediction of disease course. Especially, the newly emerged concept of personalized medicine may require in-depth assessment of potential therapeutic molecular target(s) in each individual case through analysis of sig-

naling pathways and subsequent validation of treatment efficacy[6].

Numerous reports have identified molecules that play key roles in development of gastrointestinal cancer. Amongst these, which include growth factors and their receptors[7-9], signaling pathway components[10,11], transcription factors[12,13], matrix remodeling enzymes[14,15] and cytokines[16-18], a milestone finding by Müller *et al*[19] made chemokines one of the most intensively studied molecular targets to understand the mechanisms of organ-specific metastasis. The chemotactic cytokines or chemokines contribute to the tumor microenvironment by establishing a chemokine gradient, which is important for the process of chemoattraction and subsequent cell motility and infiltration for metastasis[20,21]. In this review, we will summarize the current status of chemokine-related studies in digestive tract cancer.

Recent progress has made it clear that the non-tumor cells, such as stromal fibroblasts and inflammatory cells present in the microenvironment, also play critical roles in establishing the metastatic phenotype of tumor cells[22,23]. Indeed, a role for inflammation in cancer progression is well-recognized, with many different cancer types having an inflammatory component[24]. Tumor cell infiltration is aided by the presence of tumor-associated macrophages (TAMs), dendritic cells and lymphocytes[25-28]. While TAMs are able to kill tumor cells, they also play an important role in enhancing tumor development by secreting matrix metalloproteinases (MMPs)[29,30], growth factors [interleukins (ILs), vascular endothelial growth factors (VEGFs)] and pro-angiogenic factors that support tumor cell growth, neovascularization, and tumor cell invasion through the stromal tissues[31].

Chemokines and signal transduction

Chemokines and chemokine receptors: In humans, the chemokine superfamily is comprised of more than 50 small secreted proteins. These molecules function as immune modulators, chemoattractants and as activators of lymphocytes. They are sub-divided into four major groups based on the relative position of conserved cysteine residues near to the N-terminus: CXC-, CC-, C-, and CX$_3$C-chemokines[32-34]. The CXC-chemokines can be further subdivided according to the presence or absence of a three amino acid motif immediately N-terminal to the first cysteine. Therefore, a glutamic acid-leucine-arginine (ELR) sequence defines the ELR$^+$ CXC-chemokines, which generally function as activators and chemoattractants for neutrophils. In contrast, lymphocytes constitute the target cell type for most ELR$^-$ CXC chemokines. The CC-chemokines play both chemoattractant and immunomodulatory roles. CCL5 attracts monocytes, eosinophils and memory T-cells, and is one of the human immunodeficiency virus (HIV)-suppressive factors secreted by CD8$^+$ T-cells[35]. Similarly, CCL3 and the closely related CCL4 are also capable of inhibiting HIV infection of target cells, in addition to their pro-inflammatory and chemoattractant functions[35]. Furthermore, CC-chemokines are also reported to be involved in lymphocyte recirculation and homing to secondary organs (CCL21), as well as T-cell trafficking within the thymus (CCL19)[36-38].

While the major biological functions ascribed to chemokines regulate leukocyte trafficking and recruitment to inflammatory foci, chemokine receptors are also expressed on non-immune cells, and act as key modulators of the biological functions of other cell types. For example, CCL3 is a negative regulator of keratinocyte growth[39], while the CXC-chemokine ligand (CXCL) 5[40], CXCL8[41,42] and other ELR$^+$ chemokines stimulate endothelial cell migration during angiogenesis[43].

To date, 19 chemokine receptors are known to be expressed in mammals[44]. Chemokines activate these specific G-protein-coupled receptors (GPCRs) on the surface of target cells. Chemokine receptors consist of an extracellular ligand binding domain, seven transmembrane spans, and an intracellular carboxyl terminus. The transmembrane domain consists of three intracellular domains and three hydrophobic extracellular domains. Variation of the amino-terminus sequence of these receptors defines the specificity for recognition of chemokine ligands, the binding of which results in phosphorylation of Ser/Thr residues in the intracellular domain and induction of f to the N-terminus of the receptor, specific signal transduction will be induced by the release of these heterotrimeric G-proteins (guanine nucleotide-binding proteins -G/G/ G) that are bound to the intracellular carboxyl terminus. Each of these heterotrimeric G-proteins consists of several different subtypes[45]. Chemokine receptors are subdivided into four types in relation to the class of ligand to which they bind: CXC-chemokine receptors (CXCR), which bind CXC-chemokines; CC-receptors (CCR), for CC-chemokines; and XC-receptors and CX$_3$C-receptors (CX$_3$CR) for XC- and CX$_3$C-chemokines, respectively. Among these receptors and ligands, there is promiscuous binding between several receptors and multiple chemokines (Table 1)[38].

Mechanisms of signal transduction: Binding of chemokines to their innate receptors can activate a number of intracellular signaling pathways that lead to cell proliferation and migration. Downstream mediators identified to date include the small GTPase Ras; extracellular signal-regulated kinases (ERK)[46,47]; phosphatidyl inositide-3-OH kinase (PI-3K)[47-49], and the other small GTPases Rho, Rac and Cdc42[50,51]; and some of these are active in pathways that regulate the actin cytoskeleton. For example, it was shown that migration of Jurkat cells in response to CXCL12 through CXCR4 is mediated by both Rac- and Rho-dependent mechanisms. While Rac is activated by G_i and $G_{\beta\gamma}$ subunits, activation of RhoA occurs *via* $G_{\alpha 13}$ and leads to phosphorylation of myosin light chains[52]. Furthermore, CXCL8 (IL-8) signaling through CXCR1 has been reported to mediate migration of leukocytes in a β1-integrin-dependent manner which requires downstream activation of p38 and c-Jun N-terminal kinases[53]. The quality and the quantity of chemokine signaling is not controlled only by the expression volume of chemokine ligand/receptor

but, also, by proteolytic processing of chemokine ligands. In this regard, MMPs are major modulators of chemokine signaling. For example, MMP9 can activate CXCL8 by processing its amino terminus, but it also abolishes the function of CXCL1 by cleaving it. Likewise, chemotactic activity of CCL7 is decreased by MMP2, and CXCL12 is decreased by multiple MMPs. However, numerous other proteases have also been documented to act upon chemokines and modulate their activity[54]. Furthermore, recent observations suggest more complexity in signal transduction mechanisms that are induced by heterodimerization of GPCRs[55]. As indicated above, chemokine and chemokine receptor interactions are complex and are connected to multiple combinations of effector proteins and divergent intracellular signaling pathways (Figure 1).

Effects of chemokines on tumor cell proliferation

Many growth factors and cytokines act to control cell proliferation, either in a positive or a negative manner[56]. For example, epidermal growth factor (EGF) and related family members activate the EGF receptor (EGFR) and initiate divergent biochemical cascades that result in transcription of genes involved in cell cycle progression and other processes necessary for growth. In contrast, transforming growth factor-beta negatively regulates epithelial cell growth by inhibiting cell cycle transit. Signal transduction pathways regulated by these and other growth factors frequently become altered during tumorigenesis, resulting in deregulated cell growth. It has now become clear that deregulated function of multiple chemokines also contributes to enhanced tumor cell proliferation.

The ELR$^+$ CXC-chemokines play important roles in melanoma cell growth[57,58]. CXCL1 has also been implicated in non-melanoma skin cancers, including tumors of neural origin and squamous cell carcinomas. Zhou *et al*[59] demonstrated that CXCL1 is highly expressed in anaplastic astrocytomas *in vivo*, and reported that this enhanced cell growth, motility, adhesion to extracellular matrix, and invasion *in vitro*, as well as enhanced aggressiveness *in vivo*. Constitutive expression of CXCL1 in squamous cell carcinomas results in formation of an autocrine growth loop through the CXCR2 receptor, while overexpression of CXCL1, CXCL2 and CXCR2 in esophageal cancer also enhances proliferation[60]. Furthermore, it was found that CXCL2 activates signal transduction through an ERK dependent pathway[61].

Transcriptional upregulation mediated through nuclear factor κB (NFκB)-dependent pathways has been reported to be largely responsible for the enhanced levels of CXCL1 in tumor cells. The tumor promoter okadaic acid, which inhibits protein phosphatases and results in hyperphosphorylation of proteins at serine and threonine residues, activates transcription through two response elements in the CXCL1 promoter, utilizing three distinct NFκB subunits (p65, p52 and c-Rel)[62]. Upregulation of NFκB by the upstream NFκB-inducing kinase is also an important mechanism by which CXCL1 is upregulated[63]. NFκB-dependent induction of CXCL1 is further regulated by poly (ADP-ribose) polymerase-1 (PARP-1). Inactive PARP-1 binds to the CXCL1 promoter and blocks transcription by excluding NFκB. However, activation of PARP-1 causes its promoter binding ability to be lost leading to NFκB upregulation of CXCL1. In melanoma cells, PARP-1 is highly expressed and active[64], and may be a major contributor to melanoma cell proliferation *via* chemokine-dependent mechanisms, together with constitutively expressed NFκB.

Chemokines in tumor cell migration, invasion and homing

In terms of cancer metastasis, chemokine-dependent mechanisms for targeting to specific secondary sites is now widely recognized after studies showed upregulation of CXCR4 and CCR7 in breast cancer cells and that activation of these receptors could induce actin polymerization, migration and invasion[19]. Importantly, ligands for these receptors were shown to be expressed in organs that represent the primary sites for breast cancer metastasis, strongly suggesting that ligand-receptor "homing" functions *in vivo* to target tumor cells to sites of secondary growth. Organ-specific metastasis has been reported for different tumor types, including breast[65], ovary[46] and epidermoid carcinomas[60]. Further, more than twenty tumor types have been documented to overexpress CXCR4[66]. Upregulation of CXCR4 expression in tumor cells through the action of VEGF also appears to be an important mechanism to further enhance invasiveness[67].

CXCR4/CXCL12 overexpression is associated with metastasis to lung, liver, lymph nodes and bone marrow. Rearrangement of the actin cytoskeleton and alteration in cell polarity are fundamental processes required for cell motility, regulated at least in some cases by CXCL12-CXCR4 pathways[68]. CXCL12-CXCR4 signaling may also contribute to tumor progression by upregulating protease expression. In prostate cancer cells, various MMPs were shown to be modulated by CXCL12[69]. However, these effects were not consistent for all cell lines examined, suggesting that cell-specific factors may influence the response to CXCL12. In glioma cells, CXCL12 induced expression of MMP15 but not gelatinases. RNA interference studies proved that glioma cell invasiveness *in vitro*, and tumor aggressiveness *in vivo*, is due to the upregulation of MMP15 by CXCL12[70]. Chemokine-receptor interactions in skin, as a frequent metastatic site of malignant melanoma, may be another example: CCL27 is highly expressed in skin and the CCR10 receptor for this ligand is frequently upregulated in melanoma cells[71,72].

Furthermore, mounting evidence points to cancer-associated stromal fibroblasts playing important roles in modulating tumor cell behaviour[73,74]. As p53 mutation or loss has been shown to occur in stromal cells[75,76], this may upregulate CXCL12 and enhance proliferation and motility[77].

Chemokines and tumor cell survival

The majority of metastatic tumor cells fail to colonize secondary lesions successfully, most likely due to induction of programmed cell death[78]. For metastasized lesions to grow, enhancement of growth factors as well as

positive molecular interactions with surrounding cells in the lymph node or other organs are indispensable. For example, EGFR signaling can activate survival pathways regulated by protein kinase B (AKT)[79]. However, despite the presence of available growth factors, tumor growth at the secondary lesion may still be uncertain: it may also require other factors and/or pathways in order for cells to survive and proliferate. Studies have also shown that resistance to cell death induced by loss of attachment to the extracellular matrix (anoikis) is an important element at least for certain tumors[80]. Interestingly, some chemokine-receptor combinations, such as CXCL5-CXCR2[48] and CCL19/21-CCR7[81], can activate PI-3K and AKT, key regulators of cell survival. Thus, interaction of chemokines with their receptors could play roles in multiple and complex biological processes that are important for successful survival in metastasized locations. For example, CCL2 is well known for regulating cell migration and is a key mediator of breast cancer cell migration[82]. Also using breast cancer cell lines, Fang *et al*[83] demonstrated enhanced cell migration and survival along with increased phosphorylation of Smad3 and mitogen-activated protein kinases (MAPKs) in response to CCL2 and, moreover, they found that levels of the innate receptor CCR2 were elevated in breast cancers, accompanied with CCL2 expression. These investigators also suggested that MAPK and Smad3 signaling function as an independent/alternative mechanism for cell survival. Furthermore, they showed that CCL2-induced Smad3 signaling through MAPKs regulates expression and activity of Rho GTPase, thereby facilitating breast cancer cell motility and survival. Therefore, beyond well-described canonical chemokine ligand/receptor signaling, new molecules may need to be considered as critical players in chemokine signaling in cancer. The CXCL12-CXCR4 signaling pathway has been shown to be important for the survival of leukemic B cells in chronic lymphocytic leukemia (CLL) through activation of AKT and ERK1/2[84,85]. Moreover, O'Hayre *et al*[86] recently identified additional molecular targets and novel phosphoproteins as possible mechanisms for cell survival in CLL. Amongst these is programmed cell death factor 4, found in all CLL cells examined, and also heat shock protein 27, which mediates anti-apoptotic signaling and has previously been linked to chemotherapeutic resistance, which was detected in a subpopulation of CCL patients. If the roles of these cell-survival-related proteins are supported by further future studies, it may be worth considering identifying these and other phosphoproteins whose functions are modified by certain chemokines in specific pathological conditions, such as cancer and/or inflammation in gastrointestinal disease.

Chemokines and angiogenesis

Development of microvessels is an another critical event that enables oxygen delivery and nurtures tumor cell survival at both primary and secondary sites[87]. Recently, intensive studies in normal angiogenic development using mouse model systems have revealed the importance of the angiogenic chemokine CXCL12 for the organized development of vessel branching along with neural development (https://intramural.nhlbi.nih.gov/labs/ldbsn/pages/publications.aspx). Furthermore, Komatsu *et al*[88] reported the importance of the small-G protein R-Ras for the development of abnormal collateral capillary systems which may play critical roles in the survival of localized tumors by supporting nutrient and oxygen delivery[89]. Nonetheless, it remains unclear how the tumor cells, which express chemokine receptors, respond to chemokines released from these pathological vessels, which are inherently "leaky". Several different chemokines are known to be pro-angiogenic, notably CXCL5 and CXCL8. Koch *et al*[90] clearly demonstrated that CXCL8 could induce neovascularization in a rabbit corneal pocket assay. Furthermore, they also showed that the angiogenic activity present in conditioned media derived from macrophages or monocytes from rheumatoid synovial tissues was dependent upon CXCL8. Indeed, macrophages have been reported to induce malignant progression in a breast cancer model by initiating the angiogenic switch[91]. Additionally, CXCL8-CXCR2 signaling facilitates migration and proliferation of endothelial cells[92], and the AKT pathway is important for GPCR-dependent angiogenesis[93] following CXCR1 and CXCR2 activation[49,81]. Also, the human herpesvirus-8, an etiological agent of the highly vascular Kaposi's sarcoma, induces expression of CXCL8[94], providing further evidence for chemokine involvement in tumorigenesis[95].

Notably, in contrast to the ELR$^+$ chemokines discussed above, most ELR$^-$ chemokines have anti-angiogenic or angiostatic activity[96]. Among these, CXCL9, CXCL10 and CXCL11 are inducible by other cytokines, including members of the IL family and interferons. The angiostatic response of these cytokine-inducible chemokines is mediated through the CXCR3 receptor, found on the surface of endothelial cells[97]. Specifically, an alternatively spliced variant of the receptor - CXCR3B - has been shown to mediate this activity[98]. In addition to inhibiting endothelial cell migration, these chemokines also block proliferation. A further critical review focused on the development of tumor angiogenesis is available[99].

CHEMOKINES IN DIGESTIVE SYSTEMS/ DEVELOPMENT AND PROGRESSION

Compared to normal cells, many cancer cells overexpress chemokine and chemokine receptors. Ligation of overexpressed chemokine receptors on tumor cells and the specific chemokines released from target organs seem to be critical regulators of metastasis[19,100]. As outlined above, chemokines and their receptors play various important roles in the regulation of invasion, metastasis and dissemination of cancer cells. Here we review chemokine/chemokine receptor interactions, specifically in digestive organs.

Oral cavity

In the head and neck region, which includes oral cavity,

Table 1 Ligand specificity of chemokine receptors implicated in gastrointestinal disease

CC-chemokines		CXC-chemokines		CX$_3$C-chemokine	
Ligands	Receptors	Ligands	Receptors	Ligands	Receptors
CCL2	CCR2	CXCL1	CXCR2/CXCR1	CX$_3$CL1	CX$_3$CR1
CCL3	CCR1/CCR5	CXCL2	CXCR2		
CCL4	CCR5	CXCL4L1	CXCR3		
CCL5	CCR1/CCR3/CCR5	CXCL5	CXCR2		
CCL7	CCR1/CCR2/CCR3	CXCL7	CXCR2		
CCL8	CCR3/CCR5	CXCL8	CXCR1		
CCL13	CCR2/CCR3	CXCL9	CXCR3		
CCL19	CCR7	CXCL10	CXCR3		
CCL20	CCR6	CXCL11	CXCR3		
CCL21	CCR7	CXCL12	CXCR4/R7		
CCL25	CCR9	CXCL14	Unknown		
CCL27	CCR10	CXCL17	Unknown		

CXCL: CXC chemokine ligand; CXCR: CXC-chemokine receptor; CCR: CC-receptor; CX$_3$CR: CX$_3$C-receptor.

pharynx, larynx, nasal cavity and paranasal sinuses, the head and neck squamous cell carcinoma (HNSCC) accounts for more than 90% of malignant neoplasms[101]. Despite intensive efforts, survival rates have shown limited improvement over the decades. When primary tumor location is taken into account, the outcome can be even worse, with advanced hypopharyngeal tumors having a 4% five-year survival[102]. Metastasis of HNSCC is generally *via* the lymphatic system to loco-regional sites. Recently, several different chemokines were shown to be highly expressed in HNSCC derived cell lines and patient tumor samples. For example, in a series of 94 HNSCCs, CXCL1 was found to be overexpressed in around 40% of lesions[103]. Measurement of microvessel density (MVD) in HNSCCs revealed a correlation between CXCL1 expression and angiogenic activity, as well as with nodal metastasis and infiltration of leukocytes. CXCL8 has long been recognized to participate in autocrine (and possibly paracrine) regulation of HNSCC proliferation[60]. Recent studies using global gene expression profiling of primary and synchronous metastatic HNSCC further support previous reports of CXCL8 upregulation[104]. Along with this observation, Chen *et al*[105] reported that hydrogen sulfide produced by *Porphyromonas gingivalis* bacteria in the oral cavity induced expression of CXCL8 in gingival and oral epithelial cells. Potentially, this may provide a link between tumor development and the induction of inflammation in periodontal disease, which is associated with persistent bacterial infection, and similar results to this study have also been reported[106].

In addition to CXCL8, another ELR$^+$ angiogenic chemokine, CXCL5, is highly expressed in some HNSCCs. Data suggest that CXCL5 enhances tumor development by stimulating proliferation, cell motility and invasion[107] as knockdown of CXCL5 by siRNA completely inhibited tumorigenicity in a mouse xenograft model.

Furthermore, Delilbasi *et al*[108] reported upregulated CXCR4 expression in squamous carcinomas of the tongue by an immunohistochemical method. Of interest, their experiment exhibited no difference in expression between primary tumors of early and more advanced stage, although invading cells and those which had metastasized to lymph nodes exhibited higher CXCR4 expression, suggesting *in vivo* selection for this phenotype with malignant progression. Clatot *et al*[109] also studied the possible correlation of CXCL12/CXCR4 expression and tumor recurrence and survival in HNSCC patients. They found no meaningful correlation between CXCR4 expression and either recurrence or survival, but a significant difference in CXCL12 expression. Further prospective studies are required to clarify this.

The loss of CCR6 expression in metastatic lesions with concomitant elevation of CCR7 in some HNSCCs was also documented[110]. CCL19 and CCL21, ligands for CCR7, induced migration of metastatic cells *in vitro*, whereas primary tumor cells responded to the CCR6 ligand, CCL20. Together, these data suggest that CCR7 upregulation might play a role in targeting tumor cells to sites of secondary growth *in vivo*, by facilitating entry into the lymphatic system and migration to regional lymph nodes. CCR7 signal transduction was also shown to activate cellular invasion and pro-survival pathways by PI-3K and PLCγ-dependent, but EGFR-independent, mechanisms[111].

Esophagus

In the esophagus, several cytokines and chemokines are reported as possible mediators of gastroesophageal reflux, esophagitis, pre-cancerous change (typically Barrett's esophagus) and adenocarcinoma[112]. Amongst these, CXCL8 is reported as a molecular marker indicative of response to therapeutic procedures. For example, Oh *et al*[113] compared the expression level of CXCL8 between pre- and post-operation of Nissen fundoplication in reflux esophagitis. They found that CXCL8 expression was significantly reduced postoperatively, as measured by quantitative real-time polymerase chain reaction (qRT-PCR). These authors also reported that CXCL8 expression was higher in patients with reflux compared to those without reflux. Furthermore, they found that patients with the highest CXCL8 expression were those with Barrett's dysplasia and adenocarcinoma. Chemokines and several other cytokines, such as ILs, CXCL8, and VEGFs were found to be upregulated in cancer-related cachexia, although the underlying mechanism is not understood[114]. Moreover, chemokine expression in tumors is complicated. Verbeke *et al*[115] screened 51 patients operated on for colon adenocarcinoma, esophageal adenocarcinoma, or esophageal squamous cell carcinoma (SCC) by immunohistochemical staining to examine the expression of CXCL4L1, CXCL8, CXCL10, CXCL12, and VEGF. According to their study, the angiostatic chemokine CXCL4L1 was strongly expressed in colorectal cancer, while there was weaker expression in esophageal cancer. CXCL12 staining was almost negative in esophageal SCC, while stronger staining was observed in adenocarcinoma of the esophagus and colon. VEGF was moderately-to-strong-

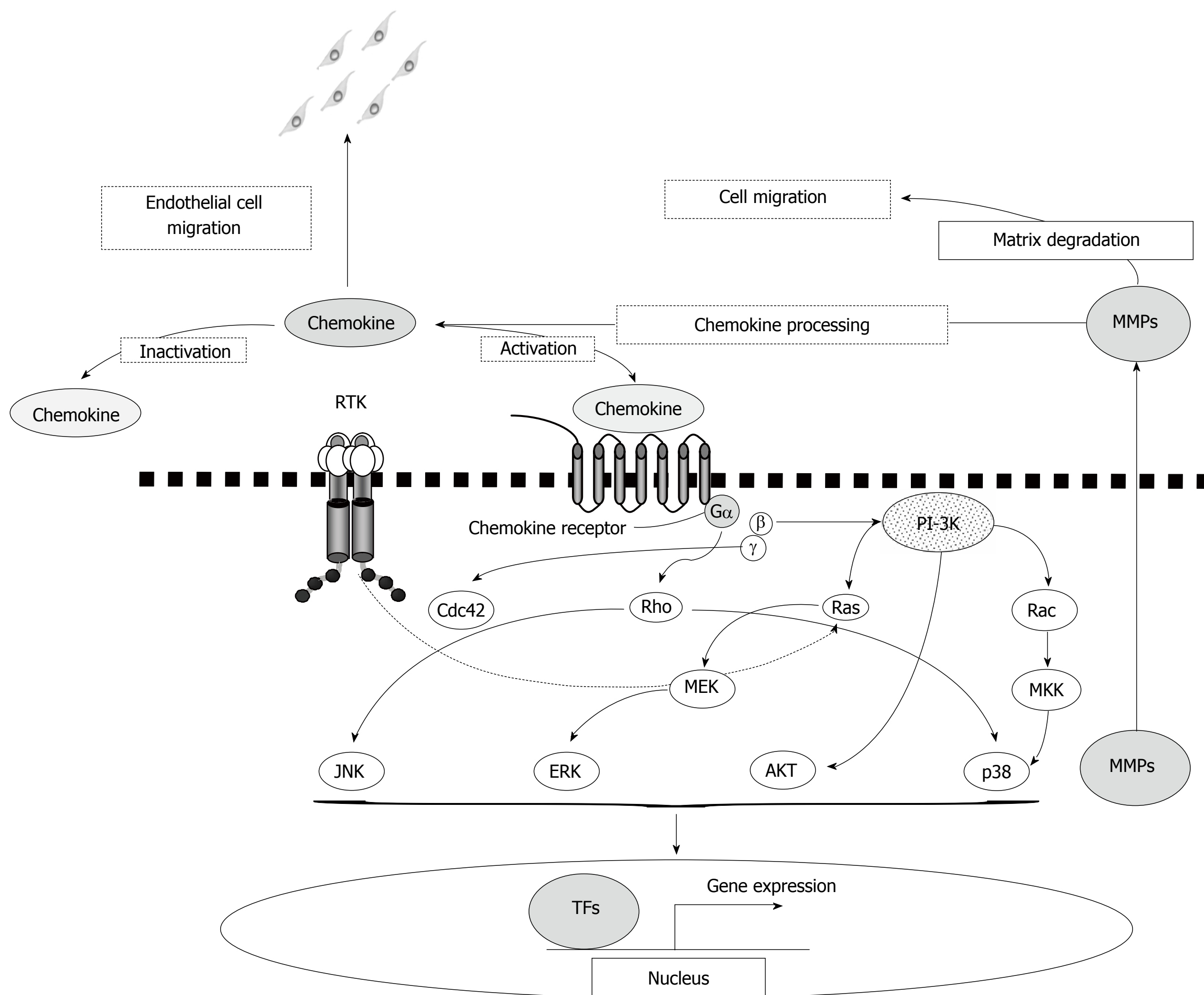

Figure 1 Chemokine-induced signal transduction pathways. Schematic representation of signaling pathways activated by binding of chemokine ligands to their seven transmembrane G-protein coupled receptors. MMPs: Matrix metalloproteinases; TFs: Transcription factors; RTK: Receptor tyrosine kinase; ERK: Extracellular signal-regulated kinase; MEK: Mitogen-activated protein/extracellular signal-regulated kinase kinase; PI-3K: Phosphatidyl inositide-3-OH kinase; MKK: Mitogen-activated protein kinase kinase; JNK: c-Jun N-terminal kinase; AKT: Protein kinase B.

ly positive in all 3 types of cancer, but relatively weaker in esophageal adenocarcinoma. Interestingly, not only was the expression of the angiogenic chemokines CXCL8 and CXCL12 heterogeneous in the samples, but so was the expression of the angiostatic chemokines CXCL10 and CXCL4L1. Based on these results, the roles of chemokines are likely complex in these tumors[115]. Recently, *in vitro* and *in vivo* studies using AMD3100, a pharmacological CXCR4 inhibitor, and the human HER2 inhibitor, trastuzumab, have indicated CXCR4 to be a positive regulator of human epidermal growth factor receptor 2 (HER2) expression in esophageal cancer. In this study, the authors also suggested a possible complex relationship between HER2 and CXCR4 in tumor development and metastasis[116]. These observations indicate further the involvement of multiple chemokines in molecular events that underpin different stages of cancer development.

Stomach

In gastric cancer treatment, detection at an early stage of tumor development is still the most effective curable approach. Amongst different types of gastric cancer, peritoneal dissemination is one of the most incurable conditions and no radical and effective treatment is available to date. Hashimoto *et al*[117] showed that the CXCL12/CXCR4 axis is important in peritoneal dissemination of gastric cancer cells. They found that CXCL12 ligation to CXCR4 on the cancer cell surface strongly and rapidly activates AKT-mammalian target of rapamycin signaling and co-activates production of other metastatic mediators, such as MMPs[117,118]. Graziosi *et al*[119] also suggested possible involvement of the p38 MAPK pathway in the development of peritoneal dissemination. Using an *in vivo* mouse system and administering p38 MAPK inhibitors ML3403 or SB203580, they observed decreased tumorigenesis. Microarray studies using these cancer cells identified several downregulated genes, such as CXCR4, fms-related tyrosine kinase 4, non-receptor spleen tyrosine kinase and collagen α2(IV). Interestingly, p38 MAPK inhibitors induced significant downregulation of multidrug resistance-1 gene expression, a well-

defined marker of resistance to chemotherapy, which possibly induced susceptibility to the cisplatin treatment in this model[119]. In a separate study, Zhi *et al*[120] examined the expression of CXCL12 in normal gastric tissue, gastric cancer cell lines, 35 primary gastric carcinomas and corresponding normal gastric tissues. They found that CXCL12 was downregulated in gastric cancer cell lines and patient samples of primary gastric carcinomas compared to normal samples. They provided evidence to show that CXCL12 expression was inversely associated with lymph node metastasis and histological grade. They concluded the possible cause of this downregulation of CXCL12 expression to be hypermethylation of the CXCL12 promoter, as treatment of highly metastatic cancer cell lines with a demethylating agent impaired its invasive phenotype[120]. One possible explanation for this reduction in CXCL12 might be to permit CXCR4-expressing gastric cancer cells to sense a CXCL12 gradient from target lymph nodes or other organs, which would otherwise be masked by CXCL12 expressed by the cancer cells. Together, these reports suggest that the peritoneal dissemination may be induced by the dissemination of CXCR4-expressing gastric cancer cells directed into the peritoneal area, which expresses high levels of CXCL12. Therefore, blocking molecular targets such as AKT and/or CXCR4 could be possible treatment strategies to prevent the activation of signaling events induced by CXCR4 ligation mediated by CXCL12 on the cancer cell surface. Moreover, preventing peritoneal dissemination itself might be possible by developing specific inhibitors that prevent binding between CXCR4 and its ligand. In this regard, Manu *et al*[121] investigated plumbagin, which is a CXCR4 expression inhibitor, and it was widely effective in downregulating CXCR4 expression in cancer cell lines irrespective of the tissue of origin. The suggested mechanism of CXCR4 downregulation by plumbagin is through inhibition of NFκB. However, regulation of CXCR4 expression in cancer cells may not be so straightforward as initially expected. Bao *et al*[122] found that HER2, which is frequently overexpressed in gastric cancer, interacts with CD44 and induces CXCR4 expression by blocking expression of microRNA-139. Additionally, EGF signaling may play an important synergistic role in concert with the CXL12/CXCR4 axis in gastric cancer metastasis, as Yasumoto *et al*[123] found that the EGFR ligands amphiregulin and heparin-binding EGF (HB-EGF)-like growth factor, as well as CXCL12, are highly expressed in malignant ascites. Their work showed that HB-EGF and CXCL12 together enhanced tumor necrosis factor α-converting enzyme-dependent amphiregulin shedding from human gastric carcinoma (NUGC4) cells, which can promote proliferation of NUGC4 cells in animal models. These experiments strongly imply the possibility that several cancer signaling pathways besides the CXCL12/CXCR4 axis are important for development of gastric cancer metastasis.

When they screened 40 gastric cancer patient samples, Zhao *et al*[124] found that CXCR4 mRNA levels were significantly higher in cases with lymph node metastasis than those without; they also found that the CXCR4 protein level was correlated with poorly differentiated lesions, more advanced tumor stage and lymph node metastasis. Further, they reported higher CXCL12 mRNA in lymph nodes in patients with metastatic gastric cancer. Ingold *et al*[125] used qRT-PCR to screen CXCL12/CXCR4 mRNA levels in 37 gastric carcinomas, and as well as screening protein levels in 347 gastric carcinomas and 61 matching lymph node metastases using tissue microarrays. They concluded that tumors expressing both CXCL12 in tumor cells and CXCR4 in adjacent microvessels showed a strong correlation with local tumor development and Union for International Cancer Control stages.

Another interesting study recently reported by Xu *et al*[126] describes the possible activation of lymphangiogenesis pathways in gastric cancer by CXCL1 secretion. A technique was established in an animal model for recovery of lymphatic endothelial cells (LECs) from afferent lymph vessels of sentinel lymph nodes, and the gene expression profile between normal LECs and LECs with lymph node metastasis was compared using microarray analysis. They found that CXCL1 stimulated LEC migration and tube formation through FAK-ERK1/2-RhoA activation and reorganization of F-actin. Importantly, it is well known that CXCR2 expression, which is a CXCL1 receptor, is positively correlated with tumor, node, and metastasis (TNM) stage and lymphatic vessel density[127-130]. Thus, this study may strongly imply a role for CXCL1 in metastasis mediated through effects on LECs. CXCL8 has also been well studied in gastric cancer development. Using the gastric cancer cell line SCG-7901, Ju *et al*[131] found that CXCL8 can enhance several tumor parameters, such as adhesion to endothelial cells, migration, and invasion. The expression of MMP9, intercellular adhesion molecule (ICAM)-1 and E-cadherin was upregulated in a dose-dependent manner. However, CXCL8 did not affect the proliferation of SCG-7901 cells under these conditions. Several groups have studied polymorphism of the CXCL8 251 allele. Wang *et al*[132] reported the CXCL8 251 allele AA genotype as a risk factor for gastric cancer in Asian groups but not in Caucasian or Mexicans. Furthermore, statistical analysis revealed that the TA and AA polymorphism is significantly associated with the diffuse type of gastric cancer, and the AA genotype was found to be a risk factor for gastric cardia cancer. Song *et al*[133] reported screening results of this allele in *Helicobacter pylori* (*H. pylori*)-infected Korean populations. They found a significant correlation between MMP9 and disease progression in the AA and AT genotype. Angiopoietin-1, which plays an important role in vascular development, showed upregulation, but not VEGF expression or disease progression in the AA genotype. They predicted that the CXCL8 251 AA genotype may be associated with angiogenesis in gastric carcinogenesis in the *H. pylori*-infected population. Vinagre *et al*[134] reported that interaction between CXCL8 251 (AA and AT) allele mutation carriers and the infection of *H. pylori* strain [phosphoinositide PI4, 5P(2) binding protein (s1m1)

cytotoxin-associated gene A product (*cagA*) positive] may have higher risk for development of gastric adenocarcinoma. In addition, Schneider *et al*[135] pointed out the complexity and difficulty in selecting *in vitro* epithelial study models to understand the molecular mechanisms of *H. pylori* infection. Moreover, a meta-analysis carried out by Liu *et al*[135] indicated that the variable results reported by many different studies can be affected by differences of histological type, tumor location, *H. pylori* infection, ethnicity and geographic location[136]. Another CXC chemokine, CXCL5, was also reported recently to be a potential molecular marker for cancer development, especially for late stage gastric cancer[137]. However, it is not yet confirmed if this observation is a consequence of secondary change as a result of gastric cancer progression, or whether it is one of the primary molecular events leading to tumor development. As CXCL5 is linked to tumor development in other organs[77,107,138], further confirmation is needed through the use of *in vitro* and *in vivo* model systems in the future. Yanagie *et al*[139] performed a comparative analysis of differential gene expression related to chemokines/chemokine receptors and cytokines in established gastric cancer cell lines using a cDNA microarray approach. They found that CC-chemokines CCL2, 5, 21 and CXC-chemokines CXCL1, 7, 8, 12, 14 and chemokine receptor CCR6 were upregulated, while CCL3 and CCL25 were downregulated. These chemokines and their receptors may be potential candidates for cancer diagnosis and/or treatment.

Collectively, in gastric cancer development and metastasis, multiple chemokines likely play important roles. Further studies are required to elucidate the many functional roles of these chemokines, especially in synergistic regulation of the signaling pathways that control development of gastric cancers. Another detailed review on the role of chemokines in esophageal and gastric cancer is available[140].

Liver

The liver is one of the major metastatic targets of colon cancer and this attributes directly to patient mortality. Many molecules have been proposed as being responsible for the development of hepatic metastases, and accumulated data suggest there are several important signaling pathways involved in the development of both primary and metastatic liver cancer. Among them, the CXCL12-CXCR4, CX3CL1-CX3CR1,and the CCL20-CCR6 axes have received much attention[141].

CXCR4 has been found to be a prognostic marker in various types of cancer as it plays an important role in normal stem cell homing. Cancer stem cells also express CXCR4, which implies that this axis may control the trafficking and metastasis of these cells to organs that express CXCL12, and the liver is one of these[142]. Recently, Li *et al*[143] reported that the expression of CXCR4 was higher in portal vein tumor thrombus tissue than hepatocellular carcinoma (HCC), and lentivirus-mediated siRNA knockdown of CXCR4 was shown to impair the potential invasiveness of tumor thrombus cells significantly. By screening tissues from 42 HCC patients, including tumor and adjacent regions, and comparing to tissues from cancer-free individuals, Liu *et al*[128] found that CXCR2 mRNA was significantly higher in HCC than in adjacent or normal liver tissues. Interestingly, TNM staging was not correlated to the level of CXCR2 mRNA but the protein level was relevant to staging, as protein was markedly higher in lesions classified as Stage Ⅲ/Ⅳ. CXCR2 mRNA and protein levels were correlated with intrahepatic metastasis, portal cancer embolus, and low differentiation. Other studies recently reported a possible therapeutic use of CCL2 for prevention of HCC metastasis. In a model of HCC, coupling adenovirus-based CCL2 and the thymidine kinase/ganciclovir expression was shown to prevent intrahepatic metastasis *in vivo*. This combination was also shown to induce an innate immune response involving monocytes/macrophages and NK cells, leading to prolongation of anti-metastatic effects[144-146]. CCL2 was also identified by Chen *et al*[147] as a potential target for development of future HCC treatment. Using the recombinant foot-and-mouth disease virus capsid protein VP1 (rVP1), they were able to induce apoptosis of HCC cell lines through deactivation of the AKT pathway and stimulation of caspase cascades *via* Bax. Furthermore, rVP1 downregulated the expression of CCL2 in an AKT-dependent manner, which can support the survival and migration of HCC tumor cell lines[147]. CXCR7, a CXCL12 receptor, has also been reported to be upregulated in HCC[148]. In another study, shRNA knockdown of CXCR7 expression was found to inhibit many facets of tumor development, such as cell invasion, adhesion, VEGF secretion, endothelial tube formation and tumor growth, although it did not affect metastasis *in vivo*[149]. CXCR4, an alternative receptor to CXCR7 for CXCL12, is known to play important roles in liver metastasis. CXCL12 is expressed by endothelial cells and likely by Kupffer cells lining the liver sinusoids. The binding of CXCL12 to CXCR4 activates Rho, Rac and Cdc42, enabling tumor cell extravasation without affecting cell adhesion[150].

In addition to direct roles in cancer development, chemokines make important contributions to chronic inflammatory diseases such as chronic hepatitis, which can be a possible precancerous condition. In chronic hepatitis, regulation of lymphocyte motility is controlled by several independent biochemical pathways. However, these signaling events are not well elucidated despite well-documented observations of lymphocyte recruitment to tissues *via* endothelium. Holt *et al*[151] suggested that activated human liver myofibroblasts (aLMF) affect the migration and accumulation of lymphocytes within the inflamed liver. Also, when cultured *in vitro*, aLMF from inflamed human livers and hepatic stellate cells from non-inflamed livers secrete a distinct profile of cytokines and chemokines. The aLMF-conditioned media had chemotactic activity for lymphocytes, which was partially inhibited by pertussis toxin, implying a requirement for GPCR signaling. Additionally, contribution of GPCR-independent lymphocyte chemotaxis by IL-6, hepatocyte growth factor, and VEGF was also reported[151].

Pancreas

As a multitude of studies expand our knowledge of pancreatic disease, the important role of chemokines in the development of pancreatic cancer is becoming evident. CCL20 is well known for its expression in various human cancers[152-155]. In pancreatic adenocarcinoma (PAC) patients, CCL20 mRNA and protein expression was found to be significantly associated with advanced T-stage[154]. It is interesting that CCR6, a canonical CCL20 receptor, is upregulated in chronic pancreatitis, pancreatic cystadenoma and pancreatic carcinoma compared to controls[154]. It has also been reported that, in metastatic pancreatic carcinoma, expression of the angiogenic chemokines CXCL5 and CXCL8 is highly elevated compared to pancreatic cystadenoma or chronic pancreatitis[138]. This observation suggests a potential contribution of these chemokines to the development of metastatic pancreatic cancer. Furthermore, another report suggests that CXCL5 may be a possible prognostic biomarker for pancreatic cancer. Li *et al*[156] reported that overexpression of CXCL5 is correlated with poorer tumor differentiation, advanced clinical stage, and shorter patient survival. These authors also performed xenograft assays in nude mice, in which they used shRNA downregulation of CXCL5 or antibody-mediated neutralization of CXCR2 and showed attenuation of pancreatic tumor cell growth. They also demonstrated that CXCL5 derived angiogenesis development was ERK, AKT and signal transducer and activator of transcription mediated signaling pathways[156]. Moreover, in a clinical study that analyzed 52 PACs and 52 pancreatic neuroendocrine tumors, Hussain *et al*[157] found that expression of CXCL8 and its receptors CXCR1/2 were significantly upregulated compared to normal pancreatic tissues, a finding confirmed by immunohistochemistry and qRT-PCR. This may suggest the existence of autocrine and/or paracrine loops that contribute to the development of these tumors. As in other organs, many reports have suggested the importance of the CXCL12-CXCR4 axis in the development of pancreatic cancers. Cui *et al*[158] compared expression of CXCL12 and CXCR4 between tumor and surrounding tissues. In tumor tissues, CXCL12 expression was significantly lower than that found in paracancerous tissues, normal pancreas, or lymph nodes. In contrast, CXCR4 expression in cancerous tissues was significantly higher than that in normal tissue. Furthermore, expression patterns of the CXCL12/CXCR4 and clinicopathological status showed a strong correlation, including lymph node metastasis. Additionally, CXCL12 expression was significantly associated with MVD but not with microlymphatic vessel density, while CXCR4 expression showed the opposite relationship[158]. These observations imply a significant role for CXCL12/CXCR4 signaling in the development and progression of metastatic pancreatic cancer. Also, in another study, samples from 249 PAC patients were screened by immunohistochemistry and tissue microarray for expression of innate CXCL12 receptors CXCR4 and CXCR7. Expression of CXCR7 was found to be associated with tumor grade, inversely associated with tumor size, and possibly associated with tumor progression and differentiation[159]. Interestingly, though, no significant correlation was found between CXCR4 expression and clinicopathological parameters, which may seem to be inconsistent with previous reports[158]. However, this may be a manifestation of different mechanisms of pathogenesis. Together with these observations, data from many studies suggest CXCL12-CXCR4 signaling may be a rational therapeutic target to prevent the development and metastasis of pancreatic tumors[160,161]. For example, the activation of this axis can be attenuated by suppressing NFκB activity[162]. In pancreatic cancer, CX_3CL1 and its cognate receptor CX_3CR1, is another possible ligand-receptor combination associated with the pathogenesis of pancreatic cancer. Marchesi *et al*[163,164] reported involvement of CX_3CR1 in perineural invasion and dissemination of neoplastic cells along intra- and extra-pancreatic nerves. Use of CX3CR1 as a possible therapeutic candidate is discussed in detail elsewhere[165]. Pancreatic cancer is one of the most aggressive and intractable malignancies amongst all cancers[166]. Therefore, finding molecular markers to facilitate accurate diagnosis at early stages of disease is an urgent need. From the evidence available, several chemokine-receptor pairs may be good candidates. A combination of several markers, together with these chemokines, may be promising diagnostic tools, such as CXCL17-ICAM2[167] and a classical molecular marker for pancreatic cancer, carbohydrate antigen 19-9, together with CXCL7[168].

Small and large intestine

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the colon and small intestine. It is now considered that chemokine-mediated chronic inflammation is a direct cause of colitis-associated cancer (CAC). However, the underlying mechanism of CAC is complex and not well elucidated. CC-chemokines and their receptors have long been recognized as key players in tumor promotion and progression during the course of chronic colitis. In several reports, expression of CC-chemokines, including CCL2, is highly upregulated in the colonic mucosa of IBD patients, as well as in the azoxymethane and dextran sulfate sodium experimental colitis model system[169]. Observations in the D6 receptor knockout mouse provide another important example of the role of chemokines in IBD. D6 is a silent receptor known to bind to a wide array of pro-inflammatory chemokines promiscuously, including CCL2, 5, 8, 7, and 13 in humans. Compared to wild type mice, D6 knockout mice were found to be more susceptible to chemically-induced colitis and failed to recover from the colitis. This may be due to the lack of the D6 receptor, which usually sequesters overexpressed chemokines, failing to stop the development of symptoms[170], and D6 plays a similar role in preventing liver injury[171]. Consistent with a suppressor role for D6, lymphatic vessels expressing D6 were demonstrated in epithelium and connective tissue of both small and large intestine[172]. Using a CCR2 knockout mouse or a CCL2 antagonist, Popivanova *et al*[173] showed that

CCL2 is a crucial mediator of colon cancer development, as mice lacking CCL2 had reduced intracolonic macrophage infiltration and COX-2 expression, attenuated neovascularization, and reduced numbers and size of colon tumors.

B-lymphocytes collected from patients with Crohn's disease (CD) express toll-like receptor 2 (TLR2) at the cell surface and secrete high levels of CXCL8, and the clinical disease course is well correlated with CXCL8 expression[174]. In contrast, ulcerative colitis (UC) patients also express TLR2, but do not secrete CXCL8 in large amounts. However, CXCL8 is inducible in UC by stimulating TLR2 and its clinical activity correlates inversely with levels of circulating TLR2-positive B cells, converse to CD[174]. In a model of experimental colitis, it was shown that infection with *Bacillus polyfermenticus* (*B. polyfermenticus*) affects the biological responses of human intestinal microvascular endothelial cells, including cell migration, tube formation, and permeability through an NFκB/CXCL8 signaling axis[175]. Interestingly, this study also reported that *B. polyfermenticus* can accelerate the healing process of colitis by stimulating mucosal angiogenesis. Lopez *et al*[176] found that *Lactobacillus rhamnosus* GG (LGG), a probiotic, can downregulate the flagellin-induced expression of CXCL8. Although the biological mechanisms of LGG in the maintenance of intestinal homeostasis are largely unknown, it is proposed that LGG may modulate induction of CXCL8 by tumor necrosis factor-α (TNF-α) in the intestinal epithelium[176,177]. In colorectal adenocarcinoma cells HCT-116 and HCT-8, immunohistochemistry revealed that diverse stimuli can upregulate CXCL8 expression[115], with upregulation of CXCL4L1 and synergistic CXCL8 and CXCL10 induction in carcinoma cells by IL-1 and TNF-α or immunoreactive fibronectin. In addition, full-length and N-terminally truncated (more active) CXCL8 was identified in HT-29 colorectal adenocarcinoma cells, as well as strong expression of CXCL4L1 and CXCL12 in patient samples[115]. Moreover, ERK2 and PI-3K/AKT have been identified as candidate pathways for induction of CXCL8 expression in HCT-15 colon cancer cells and MKN-45 gastric adenocarcinoma cells[178].

Arijs *et al*[179] reported that, in inflamed colonic IBD mucosa, many leukocyte/endothelial cell adhesion molecules (CAMs) and chemokines/chemokine receptors are upregulated, while E-cadherin gene expression was downregulated. Microarray analysis revealed that infliximab (an anti-TNF-α antibody) restores colonic gene expression of endothelial CAMs and most chemokines/chemokine receptors to normal levels of expression, with only CCL20 and CXCL1/2 expression remaining elevated after treatment. In addition to the previously identified 47 integrin-MADCAM1 axis, this study revealed a number of interesting targets for anti-adhesion therapy, including PECAM1, CXCL8, and CCL20, suggesting that anti-TNF-α therapy may work, at least in part, by downregulating certain CAMs[179]. Upregulation of CXCL1, CXCR1 and CXCR2 was reported by Oladipo *et al*[180] in tumor epithelium compared to normal adjacent tissue collected from patients with stage Ⅱ and Ⅲ CRC. In their analysis, no overall association between CXCL1, CXCR1 or CXCR2 expression and prognostic endpoints was found, although survival analysis demonstrated an inverse association between CXCL1 and recurrence-free survival in stage Ⅲ patients. Interestingly, CXCL8 positivity in the tumor infiltrate correlated with earlier disease stage and improved relapse-free survival in multivariate Cox regression analysis[180]. Schroepf *et al*[181] screened samples collected from 501 German patients with IBD (336 CD, 165 UC) including 258 children and 243 adults as well as 231 controls. They found CXCR3 pathway-related genes to be significantly overexpressed in inflamed colonic tissue of pediatric CD and UC patients. CXCL9, 10, and 11 are 3 innate ligands for CXCR3, and this study found a correlation between polymorphism in CXCL9 and pediatric CD, while carriers of the hetero- and homozygous genotype variants of CXCL11 rs6817952 were at increased risk for UC in all age groups. Thus, blockade of CXCR3 could be a possible therapeutic avenue in the future[181]. In a study using HT-29 colon cancer cells, Lee *et al*[182] reported multiple regulatory roles of IL-17 on chemokine expression. Their results indicated a positive effect of IL-17 on chemokines that recruit neutrophils (CXCL8 and CXCL1) and Th17 cells (CCL20). Contrary to this, IL-17 represses expression of CXCL10, CXCL11, and CCL5, three chemokines that selectively recruit Th1 lymphocytes.

Collectively, these findings suggest that synergistic targeting of critical proteins such as chemokines and their receptors may lead to improved treatment outcomes for inflammatory bowel disease and cancer. Table 2 is attached as a summary of published studies related to chemokine/chemokine receptors and gastrointestinal diseases according to their description in this review.

CHEMOKINE NETWORK AS A THERAPEUTIC TARGET

With the recent advances in understanding of the many and varied roles of chemokines and their receptors in tumor development and progression, together with the advent of targeted molecular therapies, excellent opportunities exist to develop novel approaches to treat cancer. This would appear to be of particular relevance for gastrointestinal malignancies, where radical improvements in clinical outcome have so far been elusive.

CONCLUSION

It is clear that chemokine networks play critical roles in inflammatory diseases and cancer progression, and tumor cells may influence their own proliferation as well as affecting stromal and immune system cells, and *vice versa*. Therefore, the chemokine network makes an attractive target for therapeutic intervention in many tumor types, including those of the gastrointestinal tract. However, we need to define more selective and specific targets, to minimize systemic side effects during treatment.

Table 2 Chemokines/chemokine receptors in this table appear sequentially according to their description in this review

Organ	Chemokines and receptors	Possible role/observed phenomenon
Oral cavity	CXCL1	Angiogenic activity[103]
	CXCL8	Proliferation, metastasis, tumor development and the induction of inflammation in periodontal disease[60,104-106]
	CXCL5	Proliferation, cell motility and invasion[107]
	CXCR4	Enhancement of invasiveness[108]
	CXCL12	Upregulation in metastasis[109]
	CCR6, CCR7	Involvement in metastatic activity[110]
	CCR7	Enhancement of invasion[111]
	CCL20	Upregulated with bacterial infection in OSCC cell lines[152]
Esophagus	CXCL8	Possible index of inflammation, upregulation in cancer-related cachexia[113,114]
	CXCR4	Positive regulator of HER[116]
Stomach	CXCL12, CXCR4	Metastasis through activation of AKT-mTOR pathway and MMPs, upregulation in lymph node metastasis, strong correlation with tumor development[117,118,124]
	CXCR4	Enhancement of metastasis through p38 signaling pathway[119]
	CXCL12	Acquisition of invasive/metastatic phenotype, enhancement of proliferation when coexpressed with other molecules[120,123]
	CXCL1	Activation of lymphangiogenesis by stimulating LECs[126]
	CXCR2	Strong correlation with TNM staging and lymphatic vessel density[127-130]
	CXCL8	Enhancement of tumor development factors, and a possible risk factor as mutant, association with angiogenesis, development of gastric adenocarcinoma[132-134,183]
	CXCL5	Marker for late stage gastric cancer[137]
	Other candidates	CC-chemokines (CCL2, 3, 5, 21, 25)/CXC-chemokines (1, 7, 8, 12, 14)/CCR6[139]
Liver	CXCR4	Metastasis, upregulation in PVTT[142-143]
	CXCR2	Upregulation in HCC, especially in late stage[128]
	CCL2	Application to prevent metastasis, application to prevent HCC by deactivating AKT pathway[144-147]
	CXCR7	Upregulation in HCC, functional in tumor development and angiogenesis but not in metastasis[148-149]
	CXCL12, CXCR4	Enhancement of tumor cell extravasation through upregulation of Rho/Rac/Cdc42[150]
	CCR6	Upregulation in metastasis[153]
	CCL20	Enhanced expression in HCC[155]
	D6 receptor	Prevention of liver injury[171]
Pancreas	CCL20	Associated with tumor staging[154]
	CCR6	Upregulated in chronic pancreatitis, pancreatic cystadenoma and pancreatic carcinoma[154]
	CXCL5, CXCL8	Upregulation in metastatic pancreatic carcinoma[140]
	CXCL5	Correlated with poorer tumor differentiation, advanced clinical stage, and shorter patient survival, and ERK, AKT and STAT mediated angiogenesis[156]
	CXCL8, CXCR1/2	Upregulation in adenocarcinomas and neuroendocrine tumors[157]
	CXCL12, CXCR4	Downregulation of CXCL12 and upregulation of CXCR4 in tumors. CXCL12 correlated with MVD but not with MLVD, while CXCR4 showed opposite pattern[158]
	CXCR4, CXCR7	CXCR7 associated with tumor grade, inversely associated with tumor size, and possibly associated with tumor progression and differentiation but not with CXCR4[159]
	CX3CL1,CX3CR1	Perineural invasion and dissemination of neoplastic cells along intra- and extra-pancreatic nerves[163,164]
	CXCL17 (+ ICAM2)	Diagnostic molecular marker[167]
	CXCL7 (+ CA19-9)	Diagnostic molecular marker[168]
Colon	CXCL4L1	Upregulation in colorectal cancer[115]
	CCL2	upregulation in mucosa of IBD[169]
	D6 receptor	Plays role of sequestering several chemokines (in mouse colitis model experiment), plays suppressive role in the development and growth of vascular tumors[170,172]
	CCL2, CCR2	important mediator in colon tumor development[173]
	CXCL8	Upregulation along with the development of Crohn's disease, affecting biological responses of human intestinal microvascular endothelial cells in colitis model, positvely correlated with earlier disease stage and improved relapse-free survival[164,175,180]
	CXCL10, CXCL41	Synergistic upregulation with CXCL8 by diverse stimuli, induction by ERK2 and PI-3K/AKT pathway *via* PAR2[175,178]
	CCL20, CXCL1/2, CXCL8	Remains high even after the treatment with anti-TNF antibody[179]
	CXCL1, CXCR1/2	Upregulation in stage II and III CRC, upregulation in stage Ⅱ and Ⅲ CRC[180]
	CXCR3 pathway	CXCL9-pediatric Crohn’s disease, CXCL11-UC in all age groups[181]
	Other chemokines	IL-17 affects CXCL8, CXCL1, CCL20, CXCL10, CXCL11 and CCR5 in colon cancer cells[182]

CXCL: CXC chemokine ligand; CXCR: CXC-chemokine receptor; CCR: CC-receptor; CX3CR: CX3C-receptor; CA19-9: Carbohydrate antigen 19-9; OSCC: Oral squamous cell carcinoma; HER: Human epidermal growth factor receptor; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; MMPs: Matrix metalloproteinases; LECs: Lymphatic endothelial cells; TNM: Tumor, node, and metastasis; PVTT: Portal vein tumor thrombus; HCC: Hepatocellular carcinoma; STAT: Signal transducer and activator of transcription; ERK: Extracellular signal-regulated kinase; MVD: Microvessel density; MLVD: Microlymphatic vessel density; IBD: Inflammatory bowel disease; PI-3K: Phosphatidyl inositide-3-OH kinase; PAR2: Protease-activated receptor-2; CRC: Colorectal cancer; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

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doi:10.3748/wjg.v19.i19.2864

World J Gastroenterol 2013 May 21; 19(19): 2864-2882
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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REVIEW

Herbal hepatotoxicity: Challenges and pitfalls of causality assessment methods

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Received: February 21, 2013 Revised: April 11, 2013
Accepted: April 17, 2013
Published online: May 21, 2013

Abstract

The diagnosis of herbal hepatotoxicity or herb induced liver injury (HILI) represents a particular clinical and regulatory challenge with major pitfalls for the causality evaluation. At the day HILI is suspected in a patient, physicians should start assessing the quality of the used herbal product, optimizing the clinical data for completeness, and applying the Council for International Organizations of Medical Sciences (CIOMS) scale for initial causality assessment. This scale is structured, quantitative, liver specific, and validated for hepatotoxicity cases. Its items provide individual scores, which together yield causality levels of highly probable, probable, possible, unlikely, and excluded. After completion by additional information including raw data, this scale with all items should be reported to regulatory agencies and manufacturers for further evaluation. The CIOMS scale is preferred as tool for assessing causality in hepatotoxicity cases, compared to numerous other causality assessment methods, which are inferior on various grounds. Among these disputed methods are the Maria and Victorino scale, an insufficiently qualified, shortened version of the CIOMS scale, as well as various liver unspecific methods such as the *ad hoc* causality approach, the Naranjo scale, the World Health Organization (WHO) method, and the Karch and Lasagna method. An expert panel is required for the Drug Induced Liver Injury Network method, the WHO method, and other approaches based on expert opinion, which provide retrospective analyses with a long delay and thereby prevent a timely assessment of the illness in question by the physician. In conclusion, HILI causality assessment is challenging and is best achieved by the liver specific CIOMS scale, avoiding pitfalls commonly observed with other approaches.

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Key words: Herbal hepatotoxicity; Herb induced liver injury; Herbs; Drug hepatotoxicity; Drug induced liver injury; Causality assessment

Core tip: This review focuses on diagnostic causality assessment algorithms that have been used so far in herb induced liver injury (HILI) cases. Detailed information of the various methods with their strengths and weaknesses is provided including their challenges and pitfalls that emerged during the assessing course. For the physician caring for a patient with suspected HILI, the Council for International Organizations of Medical Sciences (CIOMS) scale is the preferred tool for assessing causality compared to numerous other causality assessment methods, which are inferior on various grounds. CIOMS based assessment should start at the day HILI is suspected to ensure completeness of clinical data.

Teschke R, Frenzel C, Schulze J, Eickhoff A. Herbal hepatotoxicity: Challenges and pitfalls of causality assessment methods. *World J Gastroenterol* 2013; 19(19): 2864-2882 Available from:

URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2864.htm
DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2864

INTRODUCTION

A total of 60 herbs, herbal drugs, and herbal dietary supplements have been reported to cause herb induced liver injury (HILI), though convincing causality assessment rarely was provided[1]. Presented as a tabular compilation, these 60 different herbal products were based on a recent analysis of 185 case reports, spontaneous reports, review articles, and comments. The consideration of possible hepatotoxicity in various reports has been discussed by the National Institutes of Health (NIH) in their recently released LiverTox database, covering a selected group of herbal and dietary supplement (HDS) products[2,3]. Among these are: Aloe vera, Black cohosh (BC), Cascara, Chaparral, Chinese and other Asian herbal medicines (*Ba Jiao Lian*, Chi R Yun, Ephedra, *Jin Bu Huan*, Sho Saiko To and Dai Saiko To, *Shou Wu Pian*), Comfrey, Fenugreek, Germander, Ginkgo, Ginseng, Glucosamine, Greater Celandine, Green Tea, Hoodia, Horse Chestnut, Hyssop, Kava, Margosa Oil, Milk Thistle, Noni, Pennyroyal, St John's Wort, Saw Palmetto, Senna, Skullcap, Usnic acid, Valerian, and Yohimbine[2,3]. However, causality confirmation was surprisingly rare for individual cases of suspected herbal hepatotoxicity, which often were published as narrative and anecdotal reports without valid and transparent data collection[1-3] that require stringent efforts for causality attribution[4].

The focus of this review is on causality assessment methods for herbal hepatotoxicity with particular reference to liver specific evaluation methods. This approach gives insight into challenges and pitfalls of these methods with surprising clinical and regulatory issues. Valid causality assessment of assumed HILI cases is required for further case evaluations, otherwise speculations and fruitless discussions will emerge.

DATA BASIS FOR CAUSALITY ASSESSMENT

Herbal product essentials

Herbal product quality aspects are of primary concern, the respective evaluation should start at the day HILI is suspected. The products are destined for human use and must meet the highest possible quality based on specific standards (Table 1)[4-7]. Despite fulfilment of quality standards, batch and product variability is common[4,8-10]. Therefore, additional specific production quality standards have been described, for instance, as a proposal for a Kava Quality Standardization Code[8]. It details standardization of overall herbal quality and specifically addresses chemical, agricultural, manufacturing, nutritional, regulatory, and legislation standardizations. In addition, labelling and consumer leaflet of herbal drugs and herbal dietary supplements should mandatorily provide a clear definition and identification of the plant family, subfamily, species, subspecies, and variety as classical botanical description for any herb used as an ingredient of a herbal product (Table 1)[4,8].

As an example, several hundred kava varieties exist[8-11], but specific information on kava variety identification was missing in all spontaneous reports and case report publications of suspected hepatotoxicity. This leaves open which kava variety had to be incriminated[9-17]. On the other hand, the regulatory recommendation for kava drugs was to use its peeled rhizome[8,11,15]. In various HILI cases, it remained unclear, whether unpeeled rhizomes, peeled and unpeeled roots, and/or stem peelings were also used[8,11,16,17]. This again hampered any evaluation of the causative agent of kava hepatotoxicity[16,17]. For both the United States Food and Drug Administration and the Australian Therapeutic Goods Administration, peeled kava rhizomes were recommended for kava supplements[18,19].

Another point of interest focuses on solvents and solubilizers without regulatory advice[8,11,15,16], as well as on adulterants, impurities, contaminants, or misidentified herbs[4,7,8,11]. These key issues of herbal product quality are rarely addressed in publications related to herbal hepatotoxicity[1,4,8-17,20-33].

Clinical data requirements

Other concerns focus on incomplete clinical evaluation. Beginning at the day HILI is suspected, the physician has to gather all necessary information for an accurate diagnosis and the exclusion of alternative causes under relevant clinical aspects (Tables 1 and 2)[1,4,13,14,17,20-26,34-59]. Hepatotoxicity requires strict criteria, best defined by alanine aminotransferase (ALT) and/or alkaline phosphatase (ALP) values[4]. Its increases are expressed in multiples of the upper limit of their normal range as N[60-62]. For ALT, hepatotoxicity has been defined from > 2N[60,62], > 3N[63] or > 5N[64], while ALP values of > 2N are commonly considered diagnostic[60,62]. Restricting ALT increases to > 5N will eliminate false positive cases and substantiate causality at a higher level of probability[64]. Considering patients with ALT > 2N will include numerous cases with nonspecific increases, with higher requirements for thorough assessment and more stringent exclusion of causes unrelated to the herb(s) under discussion. Also for low threshold N values, the rate of alternative diagnoses must be higher[13,14,24-26,35-39], and missing a hepatotoxicity definition results in false high case numbers due to overdiagnosing and overreporting[17,23-26,38,39]. Special care is required for reporting of confounding variables[4,13,14,18,24,39]. For clinicians, a checklist with all clinical details is available for most alternative diagnoses (Table 2)[62].

Checklist

For a pragmatic approach to assess causality, special attention by the physician is of utmost importance. Only this physician can arrange collection and assessment of all data, thereby providing good data quality. To achieve this, a checklist with all important product and clinical items (Tables 1 and 2) and a valid causality assessment

Table 1 Essential steps of herbal hepatotoxicity assessments

Quality specifications
Herbal product quality
Good agricultural practices
Good manufacturing practices
Definition of plant family, subfamily, species, subspecies, and variety
Definition of plant part
Definition of solvents and solubilizers
Lack of impurities, adulterants, and misidentifications
Minimum of batch and product variability
Lack of variety to variety variability
Clinical assessment quality
Brand name with details of ingredients, plant parts, batch number, and expiration date
Identification as herbal drug or herbal supplement
Herb as an ingredient of a polyherbal product or an undetermined herbal product
Manufacturer with address
Indication of herbal use with dates of symptoms leading to herbal treatment
Daily dose with details of the application form
Exact date of herb start and herb end
Accurate dates of emerging new symptoms after herb start in chronological order
Accurate date of initially increased liver values
Timeframes of challenge, latency period, and dechallenge
Verification or exclusion of a temporal association
Provided temporal association is verified, evaluation of a causal relationship
Gender, age, body weight, height, body mass index
Ethnicity, profession
Past medical history regarding general diseases and specifically liver diseases
ALT value initially including normal range
ALT values during dechallenge at least on days 8 and 30, as well as later on
ALT values during dechallenge to exclude a second peak
ALT normalization with exact date and actual value
ALP value initially including normal range
ALP values during dechallenge up to 180 d, as well as later on
ALP values during dechallenge to exclude a second peak
ALP normalization with exact date and actual value
AST value initially including normal range
Laboratory criteria for definition of hepatotoxicity and its pattern
Definition of risk factors such as age and alcohol
Alcohol and drug use
Statement regarding actual treatment including steroids or ursodesoxycholic acid
Assessment of preexisting and coexisting liver unrelated diseases
Assessment of preexisting and coexisting liver diseases
Consideration of the several hundreds of other possible liver diseases
Providing details to exclude alternative diagnoses
Assessment and exclusion of hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis E virus, cytomegalovirus, Epstein-Barr virus, HSV, VZV
Liver and biliary tract imaging including color Doppler sonography of liver vessels
Specific evaluation of alcoholic, cardiac, autoimmune, and genetic liver diseases
Individual quantitative score of each alternative diagnosis
Comedicated synthetic drugs, herbal drugs, herbal and other dietary supplements
Definition of and search for accidental, unintended reexposure
Assessing of unintended reexposure
Search for evidence of prior known hepatotoxicity of the suspected herb
Assessing of known hepatotoxicity caused by the herb
Qualified data acquisition and documentation of complete data
Transparent presentation of all data
Causality assessment quality
Prospective assessment by the physician suspecting herb induced liver injury
Structured and quantitative method
Liver specific causality assessment method validated for hepatotoxicity
Use of the CIOMS scale
Gathering of all data required for the CIOMS scale item by item
Presentation of individual CIOMS items and of scores to regulatory agency
Gathering all clinical data and presentation to regulatory agency
Excluding all alternative causes and presentation to regulatory agency
Regulatory case assessment by skilled hepatologist with clinical experience
Regulatory assessment with assistance of external experts
Transparent presentation of regulatory verified causality assessment results

Required quality specifications of herbal products refer to herbs, herbal drugs, and herbal supplements including herbal mixtures. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; CIOMS: Council for International Organizations of Medical Sciences; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

Table 2 Check list for herb induced liver injury diagnosis

Items to be assessed	Information obtained		
	Yes	No	Partial
Brand name with batch number and expiration date	□	□	□
Indication of herbal use	□	□	□
Dates of symptoms leading to herbal treatment	□	□	□
Daily dose	□	□	□
Application form of herbal product	□	□	□
Exact date of herb start	□	□	□
Exact date of herb end	□	□	□
Accurate dates of emerging new symptoms after herb start in chronological order	□	□	□
Accurate date of initially increased liver values	□	□	□
Time frame of challenge	□	□	□
Time frame of latency period	□	□	□
Time frame of dechallenge	□	□	□
Verification of temporal association	□	□	□
Exclusion of temporal association	□	□	□
Gender, age, body weight, height, BMI	□	□	□
Ethnicity, profession	□	□	□
Past medical history and actual assessment regarding preexisting general diseases	□	□	□
Past medical history and actual assessment regarding preexisting liver diseases	□	□	□
Risk factors such as age and alcohol	□	□	□
Quantification of alcohol and drug use	□	□	□
Comedicated synthetic drugs, herbal drugs, herbal and other dietary supplements with all details of product, daily dose, exact dates of start and end of use, indication	□	□	□
ALT value initially including exact date and normal range	□	□	□
ALT values during dechallenge at least on days 8 and 30, and later on, with exact dates	□	□	□
ALT values during dechallenge to exclude a second peak, with exact dates	□	□	□
ALT normalization with exact date and actual value	□	□	□
ALP value initially including exact date and normal range	□	□	□
ALP values during dechallenge up to 180 d, and later on, with exact dates	□	□	□
ALP values during dechallenge to exclude a second peak, with exact dates	□	□	□
ALP normalization with exact date and actual value	□	□	□
AST value initially including normal range	□	□	□
Laboratory criteria for definition of hepatotoxicity	□	□	□
Laboratory criteria for injury pattern	□	□	□
Liver and biliary tract imaging including hepatobiliary sonography, CT, MRT, MRC	□	□	□
Color Doppler sonography of liver vessels	□	□	□
Unintended reexposure	□	□	□
Known hepatotoxicity caused by the herb	□	□	□
Consideration and exclusion of other possible causes	□	□	□
Hepatitis A	□	□	□
Anti-HAV-IgM			
Hepatitis B	□	□	□
HBsAg, anti-HBc-IgM, HBV-DNA			
Hepatitis C	□	□	□
Anti-HCV, HCV-RNA			
Hepatitis E	□	□	□
Anti-HEV-IgM, anti-HEV-IgG, HEV-RNA			
CMV	□	□	□
CMV-PCR, titer change for anti-CMV-IgM and anti-CMV-IgG			
EBV	□	□	□
EBV-PCR, titer change for anti-EBV-IgM and anti-EBV-IgG			
HSV	□	□	□
HSV-PCR, titer change for anti-HSV-IgM and anti-HSV- IgG			
VZV	□	□	□
VZV-PCR, titer change for anti-VZV-IgM and anti-VZV-IgG			
Other virus infections	□	□	□
Specific serology of Adenovirus, Coxsackie-B-virus, Echovirus, Measles virus, Rubella virus, Flavivirus, Arenavirus, Filovirus, Parvovirus, HIV, and others			
Other infectious diseases	□	□	□
Specific assessment of bacteria, fungi, parasites, worms, and others			
AIH type Ⅰ	□	□	□
Gamma globulins, ANA, SMA, AAA, SLA/LP, anti-LSP, anti-ASGPR			
AIH type Ⅱ	□	□	□
Gamma globulins, anti-LKM-1 (CYP 2D6), anti-LKM-2 (CYP 2C9), anti-LKM-3			
PBC	□	□	□
AMA, anti-PDH-E2			

PSC	□	□	□
p-ANCA, MRC			
AIC	□	□	□
ANA, SMA			
Overlap syndromes	□	□	□
See AIH, PBC, PSC, and AIC			
NASH	□	□	□
BMI, insulin resistance, hepatomegaly, echogenicity of the liver			
ALD	□	□	□
Patient's history, clinical and laboratory assessment, sonography			
DILI	□	□	□
Patient's history, clinical and laboratory assessment, sonography, use of the CIOMS scale			
Cocaine, ecstasy and other amphetamines	□	□	□
Toxin screening			
Rare intoxications	□	□	
Toxin screening for household and occupational toxins			
Hereditary hemochromatosis	□	□	□
Serum ferritin, total iron-binding capacity, genotyping for C2824 and H63D mutation, hepatic iron content			
Wilson's disease	□	□	□
Copper excretion (24 h urine), ceruloplasmin in serum, free copper in serum, Coombs-negative hemolytic anemia, hepatic copper content, Kayser-Fleischer-Ring, neurologic-psychiatric work-up, genotyping			
Porphyria	□	□	□
Porphobilinogen in urine, total porphyrines in urine			
α1-Antitrypsin deficiency	□	□	□
α1-Antitrypsin in serum			
Biliary diseases	□	□	□
Clinical and laboratory assessment, hepatobiliary sonography, endosonography, CT, MRT, MRC			
Pancreatic diseases	□	□	□
Clinical and laboratory assessment, sonography, CT, MRT			
Celiac disease	□	□	□
TTG antibodies, endomysium antibodies, duodenal biopsy			
Anorexia nervosa	□	□	□
Clinical context			
Parenteral nutrition	□	□	□
Clinical context			
Cardiopulmonary diseases with shock liver (cardiac hepatopathy, ischemic hepatitis)	□	□	□
Cardiopulmonary assessment of congestive heart disease, myocardial infarction, cardiomyopathy, cardiac valvular dysfunction, pulmonary embolism, pericardial diseases, arrhythmia, hemorrhagic shock, and various other conditions			
Addison's disease			
Plasma cortisol	□	□	□
Thyroid diseases			
TSH basal, T4, T3	□	□	□
Grand mal seizures			
Clinical context of epileptic seizure (duration > 30 min)	□	□	□
Heat stroke			
Shock, hyperthermia	□	□	□
Polytrauma			
Shock, liver injury	□	□	□
Systemic diseases			
Specific assessment of *M. Boeck*, amyloidosis, lymphoma, other malignant tumors, sepsis and others	□	□	□
Other diseases			
Clinical context	□	□	□

For each listed item, detailed results obtained for the individual patient are to be supplemented within the checklist. BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; CT: Computer tomography; MRT: Magnetic resonance tomography; MRC: Magnetic resonance cholangiography; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBsAg: Hepatitis B antigen; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; IgG: Immunoglobulin G; HIV: Human immunodeficiency virus; CMV: Cytomegalovirus; PCR: Polymerase chain reaction; EBV: Epstein Barr virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus; AIH: Autoimmune hepatitis; ANA: Anti-nuclear antibodies; SMA: Smooth muscle antibodies; AAA: Anti-actin antibodies; SLA: Soluble liver antigen; LP: Liver-pancreas antigen; LSP: Liver specific protein; ASGPR: Asialo-glycoprotein-receptor; LKM: Liver kidney microsomes; CYP: Cytochrome P450; PBC: Primary biliary cirrhosis; AMA: Antimitochondrial antibodies; PDH: Pyruvate dehydrogenase; PSC: Primary sclerosing cholangitis; p-ANCA: Perinuclear antineutrophil cytoplasmatic antibodies; AIC: Autoimmune cholangitis; NASH: Non alcoholic steatohepatitis; ALD: Alcoholic liver disease; DILI: Drug induced liver injury; CIOMS: Council for International Organizations of Medical Sciences; TSH: Thyroid stimulating hormone.

algorithm (Tables 3-6) should be applied early in the unfolding disease, beginning at the day HILI is suspected. Unless this is done in a stringent way, poor data quality will be provided to the scientific community, regulatory agencies, expert panels, and manufacturers, disabling reevaluation of the case. Initially poor data will produce poor results and is unacceptable. Complete and excellent case data including raw data provided by the physician

Table 3 Methods of causality assessments for suspected herbal hepatotoxicity

Methods of causality assessment	Specific criteria of various causality assessment methods					
	Expert based	Structured	Qualitative	Quantitative	Liver specific	Liver validated
Prospective evaluation						
CIOMS scale	No	Yes	No	Yes	Yes	Yes
MV scale	No	Yes	No	Yes	Yes	Yes
Naranjo scale	No	Yes	No	Yes	No	No
KL method	No	Yes	Yes	No	No	No
Ad hoc approach	No	No	No	No	No	No
Retrospective evaluation						
DILIN method	Yes	Yes	Yes	No	Yes	No
WHO method	Yes	Yes	No	No	No	No
Expert opinion	Yes	No	No	No	Yes	No

Compilation of details are derived from previous reports[2,3,60-62,76-79,81,89,102]. Council for International Organizations of Medical Sciences scale (CIOMS scale) refers to both the original scale[60] and its update (Tables 5 and 6)[62]. Liver-specific and liver-validated criteria reflect hepatotoxicity criteria. Expert based criterion refers to the requirement of several experts for the actual case under consideration. MV scale: Maria and Victorino scale; KL method: Karch and Lasagna method; DILIN method: Drug Induced Liver Injury Network method; WHO method: World Health Organization method.

Table 4 Details of the various causality assessment methods for herb induced liver injury

Assessed items with specific scores	CIOMS	MV	Naranjo	KL	*Ad hoc*	DILIN	WHO	Expert opinion
Time frame of latency period (score)	+	+	0	0	0	0	0	0
Time frame of challenge (score)	+	+	0	0	0	0	0	0
Time frame of dechallenge (score)	+	+	0	0	0	0	0	0
Recurrent ALT or ALP increase (score)	+	0	0	0	0	0	0	0
Definition of risk factors (score)	+	0	0	0	0	0	0	0
Verified alternative diagnoses (score)	+	+	0	0	0	0	0	0
Assessed HAV, HBV, HCV (score)	+	+	0	0	0	0	0	0
Assessed CMV, EBV, HSV, VZV (score)	+	+	0	0	0	0	0	0
Liver and biliary tract imaging (score)	+	0	0	0	0	0	0	0
Liver vessel Doppler sonography (score)	+	0	0	0	0	0	0	0
Assessed preexisting diseases (score)	+	0	0	0	0	0	0	0
Evaluated cardiac hepatopathy (score)	+	0	0	0	0	0	0	0
Excluded alternative diagnoses (score)	+	+	+	0	0	0	0	0
Comedication (score)	+	0	+	0	0	0	0	0
Prior known herbal hepatotoxicity (score)	+	+	+	0	0	0	0	0
Searched unintended reexposure (score)	+	+	+	0	0	0	0	0
Defined unintended reexposure (score)	+	+	0	0	0	0	0	0
Unintended reexposure (score)	+	+	0	0	0	0	0	0
Laboratory hepatotoxicity criteria	+	+	0	0	0	+	0	+
Laboratory hepatotoxicity pattern	+	+	0	0	0	+	0	+
Liver specific method	+	+	0	0	0	+	0	+
Structured, liver related method	+	+	0	0	0	+	0	0
Quantitative, liver related method	+	+	0	0	0	0	0	0
Validated method for hepatotoxicity	+	+	0	0	0	0	0	0

Items lacking specific scores were not considered, with the exception of the last six features. The data of the Drug Induced Liver Injury Network method are derived from the report of Rockey *et al*[102], references for the other methods are found in the text. Latency period indicates time from herb start to symptoms, alternatively to abnormal liver tests. The symbol + shows that this item is present and the symbol 0 indicates lack of this item. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus; CIOMS: Council for International Organizations of Medical Sciences scale; MV: Maria and Victorino scale; KL: Karch and Lasagna method; DILIN: Drug-Induced Liver Injury Network method; WHO: World Health Organization method.

are necessary to circumvent later investigative efforts, subsequent discussions, and speculative conclusions.

At each step of the evaluation, full transparency of all data is mandatory. This includes a complete narrative medical history, a causality assessment based on an established algorithm, and presentation of all data as item by item and raw data, ready for reevaluation by other scientists. This is also relevant for case publications and case series analyses, which is indeed feasible as shown in the past[13,14,25,35-39,58]. The same transparency is needed for statements and publications by regulatory agencies and expert panels. Neglecting full transparency will cause concern and uncertainty about the validity of the presented conclusions.

GENERAL ASPECTS OF CAUSALITY EVALUATION

Method categories

Some reservations exist about the best method for causal-

Table 5 Updated Council for International Organizations of Medical Sciences scale for the hepatocellular type of injury with items required for causality assessment in herb induced liver injury cases

Items for hepatocellular injury	Possible score	Patient's score
Time to onset from the beginning of the herb		
5-90 d (rechallenge: 1-15 d)	+2	
< 5 or > 90 d (rechallenge: > 15 d)	+1	
Alternative: Time to onset from cessation of the herb		
≤ 15 d (except for slowly metabolized herbal chemicals: > 15 d)	+1	
Course of ALT after cessation of the herb		
Percentage difference between ALT peak and N		
Decrease ≥ 50% within 8 d	+3	
Decrease ≥ 50% within 30 d	+2	
No information or continued herbal use	0	
Decrease ≥ 50% after the 30th day	0	
Decrease < 50% after the 30th day or recurrent increase	-2	
Risk factors		
Alcohol use (drinks/d: > 2 for women, > 3 for men)	+1	
No alcohol use (drinks/d: ≤ 2 for women, ≤ 3 for men)	0	
Age ≥ 55 yr	+1	
Age < 55 yr	0	
Concomitant herbs(s) and drug(s)		
None or no information	0	
Concomitant herb or drug with incompatible time to onset	0	
Concomitant herb or drug with compatible or suggestive time to onset	-1	
Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset	-2	
Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test)	-3	
Search for non drug causes		
Group Ⅰ (6 causes)		
Anti-HAV-IgM		
HBsAg, anti-HBc-IgM, HBV-DNA		
Anti-HCV, HCV-RNA		
Hepatobiliary sonography/colour Doppler sonography of liver vessels/endosonography/CT/MRC		
Alcoholism (AST/ALT ≥ 2 IU/L)		
Acute recent hypotension history (particularly if underlying heart disease)		
Group Ⅱ (6 causes)		
Complications of underlying disease(s)		
Infection suggested by PCR and titre change for		
CMV (anti-CMV-IgM, anti-CMV-IgG)		
EBV (anti-EBV-IgM, anti-EBV-IgG)		
HEV (anti-HEV-IgM, anti-HEV-IgG)		
HSV (anti-HSV-IgM, anti-HSV-IgG)		
VZV (anti-VZV-IgM, anti-VZV-IgG)		
Evaluation of group Ⅰ and Ⅱ		
All causes-groups Ⅰ and Ⅱ - reasonably ruled out	+2	
The 6 causes of group Ⅰ ruled out	+1	
5 or 4 causes of group Ⅰ ruled out	0	
Less than 4 causes of group I ruled out	-2	
Non herb cause highly probable	-3	
Previous information on hepatotoxicity of the herb		
Reaction labelled in the product characteristics	+2	
Reaction published but unlabelled	+1	
Reaction unknown	0	
Response to readministration		
Doubling of ALT with the herb alone, provided ALT below 5N before reexposure	+3	
Doubling of ALT with the herb(s) and drug(s) already given at the time of first reaction	+1	
Increase of ALT but less than N in the same conditions as for the first administration	-2	
Other situations	0	
Total score for patient		

The compilation of the individual items is adapted from the updated version of the Council for International Organizations of Medical Sciences (CIOMS) scale[62] and the original CIOMS scale[60]. The above items refer to the hepatocellular type of injury, whereas items for the cholestatic (± hepatocellular) type are presented in Table 6. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol[2,3,90]. Total score and resulting causality grading: ≤ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥ 9, highly probable. HAV: Hepatitis A virus; IgM: Immunoglobulin M; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CMV: Cytomegalovirus; CT: Computer tomography; EBV: Epstein Barr virus; HBc: Hepatitis B core; HBsAg: Hepatitis B antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E; HILI: Herb induced liver injury; HSV: Herpes simplex virus; MRC: Magnetic resonance cholangiography; N: Upper limit of the normal range; VZV: Varicella zoster virus.

Table 6 Updated Council for International Organizations of Medical Sciences scale for the cholestatic (± hepatocellular) type of injury with items required for causality assessment in herb induced liver injury cases

Items for cholestatic (± hepatocellular) injury	Possible score	Patient's score
Time to onset from the beginning of the herb		
5-90 d (rechallenge: 1-90 d)	+2	
< 5 or > 90 d (rechallenge: > 90 d)	+1	
Alternative: Time to onset from cessation of the herb		
⩽ 30 d (except for slowly metabolized herbal chemicals: > 30 d)	+1	
Course of ALP after cessation of the herb		
Percentage difference between ALP peak and N		
Decrease ⩾ 50% within 180 d	+2	
Decrease < 50% within 180 d	+1	
No information, persistence, increase, or continued herbal use	0	
Risk factors		
Alcohol use (drinks/d: > 2 for women, > 3 for men) and pregnancy	+1	
No alcohol use (drinks/d: ⩽ 2 for women, ⩽ 3 for men)	0	
Age ⩾ 55 yr	+1	
Age < 55 yr	0	
Concomitant herbs(s) and drug(s)		
None or no information	0	
Concomitant herb or drug with incompatible time to onset	0	
Concomitant herb or drug with compatible or suggestive time to onset	-1	
Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset	-2	
Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test)	-3	
Search for non drug causes		
Group Ⅰ (6 causes)		
Anti-HAV-IgM		
HBsAg, anti-HBc-IgM, HBV-DNA		
Anti-HCV, HCV-RNA		
Hepatobiliary sonography/colour Doppler sonography of liver vessels/endosonography/CT/MRC		
Alcoholism (AST/ALT ⩾ 2 IU/L)		
Acute recent hypotension history (particularly if underlying heart disease)		
Group Ⅱ (6 causes)		
Complications of underlying disease(s)		
Infection suggested by PCR and titre change for		
CMV (anti-CMV-IgM, anti-CMV-IgG)		
EBV (anti-EBV-IgM, anti-EBV-IgG)		
HEV (anti-HEV-IgM, anti-HEV-IgG)		
HSV (anti-HSV-IgM, anti-HSV-IgG)		
VZV (anti-VZV-IgM, anti-VZV-IgG)		
Evaluation of group Ⅰ and Ⅱ		
All causes-groups Ⅰ and Ⅱ - reasonably ruled out	+2	
The 6 causes of group Ⅰ ruled out	+1	
5 or 4 causes of group Ⅰ ruled out	0	
Less than 4 causes of group I ruled out	-2	
Non herb cause highly probable	-3	
Previous information on hepatotoxicity of the herb		
Reaction labelled in the product characteristics	+2	
Reaction published but unlabelled	+1	
Reaction unknown	0	
Response to readministration		
Doubling of ALP with the herb alone, provided ALP below 5N before reexposure	+3	
Doubling of ALP with the herb(s) and drug(s) already given at the time of first reaction	+1	
Increase of ALP but less than N in the same conditions as for the first administration	-2	
Other situations	0	
Total score for patient		

The compilation of individual items is adapted from the updated version of the Council for International Organizations of Medical Sciences (CIOMS) scale[62] and the original CIOMS scale[60]. The above items refer to the cholestatic (± hepatocellular) type of injury, whereas items for the hepatocellular type are presented in Table 5. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol[2,3,90]. Total score and resulting causality grading: ⩽ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ⩾ 9, highly probable. ALP: Alkaline phosphatase; N: upper limit of the normal range; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBsAg: Hepatitis B antigen; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CT: Computer tomography; MRC: Magnetic resonance cholangiography; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PCR: Polymerase chain reaction; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; IgG: Immunoglobulin G; VZV: Varicella zoster virus.

ity assessment in hepatotoxicity cases[1-4,13,14,17,21-26,34-39,59-64]. HILI case series reported in 23 publications with 573 HILI cases used various causality assessment methods[12-14,23,25,34-36,38,39,53,54,65-75]. These can be classified into prospective and retrospective analyses (Table 3).

The prospective evaluation focuses on the physician

caring for a patient with suspected liver injury. This setting requires a readily available and time efficient method to evaluate causation that can adapt to further clinical and causality approach necessities. Candidates are the Council for International Organizations of Medical Sciences (CIOMS) scale, also called Roussel Uclaf Causality Assessment Method scale[60-62], the Maria and Victorino (MV) scale[76], the Naranjo scale[77], the Karch and Lasagna (KL) method[78], and the *ad hoc* approach[79].

Retrospective evaluations are based on an expert panel evaluating reported or published case data, sometimes going back for months or years. Examples are the Drug Induced Liver Injury Network (DILIN) method[73,80], the World Health Organization global introspection method (WHO method) as defined by the WHO Collaborating Centre for International Drug Monitoring[81], and the expert opinion[2,3]. Major differences exist (Table 3), especially when assessing items that require score attribution (Table 4).

Usage frequency

Analyzing 23 publications of initially assumed causality but not necessarily confirmed later on[12-14,23,25,34-36,38,39,53,54,65-75] with HILI cases by BC, Greater Celandine, Green Tea extracts, some Herbalife products, Hydroxycut, kava, *Pelargonium sidoides*, and various herbs, the CIOMS scale was applied in 52.2%, the WHO method in 17.4%, the *ad hoc* approach in 13.1%, the Naranjo scale in 8.7%, and the KL and DILIN method each in 4.3% of these publications[82]. Similar results were obtained when analyzing the frequency for the 573 cases: the CIOMS scale was used in 275 cases (48.0%), the WHO method in 134 cases (23.4%), the Naranjo scale in 64 cases (11.2%), the *ad hoc* approach in 63 cases (11.0%), the KL method in 20 cases (3.5%), and the DILIN method in 20 cases (3.0%)[82]. For instance, the CIOMS scale was applied for Kava[13,14,67], BC[25,34,71,72], Greater Celandine[35,36], *Pelargonium sidoides*[38,39], and various herbs[75], the WHO method for Kava[65,68] and Herbalife products[53,54], the *ad hoc* approach for Kava[12,66] and Greater Celandine[69], the Naranjo scale for BC[23] and Green Tea extracts[70], the KL method for Herbalife products[74], and the DILIN method for Hydroxycut®[73].

A systematic analysis of causality methods is also available for DILI cases[83]. In 2008, 61 DILI publications in the PubMed database over the last decade were reviewed. It revealed that in 38 publications (62.3%) no specific causality assessment method was mentioned; presumably, the evaluation was based on the *ad hoc* approach. The CIOMS scale, Naranjo scale, and WHO method were used in 10, 8, and 2 publications, respectively[83]. Therefore, in HILI and DILI publications the CIOMS scale was the preferred specific causality assessment method if the unstructured *ad hoc* approach is excluded. Physicians are well advised to use the CIOMS scale for HILI causality evaluation, to err on the side of caution.

NIH PREFERENCE

The NIH LiverTox specifically addressed the item of causality in hepatotoxicity cases[2,3]. It focuses primarily on using the CIOMS scale, which is discussed in detail. Moreover, the MV and Naranjo scales, the Bayesian, and expert opinion assessment are referred to; details of the DILIN causality assessment also are presented. Some strengths and weaknesses of these methods are compiled (Tables 3 and 4).

PROSPECTIVE CAUSALITY ASSESSMENT METHODS

CIOMS scale

The method of choice for the causality assessment of suspected HILI is the CIOMS scale in its original form[60,61] or preferably its update (Tables 5 and 6)[62], with early starting of the evaluation at the day the physician assumes this diagnosis. The CIOMS scale is intended for prospective use at the time of manifestation; it does not require expert knowledge, is structured, quantitative, liver specific, and validated for hepatotoxicity (Table 3). Its items provide individual scores, which estimate causality levels for the agent(s) under consideration as highly probable, probable, possible, unlikely, and excluded (Tables 5 and 6). The CIOMS scale takes into account all core elements of hepatotoxicity and thereby has advantages over other algorithms (Table 4)[62]. Compared to the regulatory used *ad hoc* approach, assessment of HILI cases with the CIOMS scale leads to lower causality grades for the incriminated herb and/or for concomitant medications and to better reproducible results due to greater transparency[84].

CIOMS was developed by an international expert panel and validated by cases with positive reexposure tests serving as a gold standard[60,61]. CIOMS based assessment has shown good sensitivity (86%), specificity (89%), positive predictive value (93%), and negative predictive value (78%)[61]. The scales differ slightly for the hepatocellular and the cholestatic (± hepatocellular) type of injury (Tables 5 and 6)[62]. Differentiation between these types is feasible by comparing the ratio of the serum activities of ALT and ALP at diagnosis of suspected herbal hepatotoxicity[60,62]. Enzyme activity is expressed as a multiple of the upper limit of the normal range (N), and the ratio (R) of ALT/ALP is calculated. Liver injury is classified as: (1) hepatocellular, if ALT $> 2N$ alone or $R \geqslant 5$; (2) cholestatic, when there is an increase of ALP $> 2N$ alone or when $R \leqslant 2$; and (3) mixed cholestatic-hepatocellular, if ALT $> 2N$, ALP is increased, and R between 2 and 5.

Strengths and weaknesses of the CIOMS scale have been discussed extensively[2,3,62,73,79,82,85-91]. This scale clearly compiles liver specific criteria for challenge, dechallenge, risk factors, exclusion of unrelated diseases, and comedication, but does not use liver histology data (Tables 5 and 6)[60,62], agreed upon as less helpful criteria in most cases[90,91]. It considers unintentional reexposure results according to criteria as established by previous expert consensus meetings[92,93]. For reexposure results of the hepatocellular type of liver injury, ALT levels are as-

sessed before reexposure (designed as baseline ALT or ALTb), and at reexposure (designed as ALTr). The reexposure test is positive, if (1) ALTb is below 5N with N as the upper limit of the normal value, and (2) ALTr ≥ 2ALTb[92].

The test is negative, if only one or no criterion is fulfilled; it is uninterpretable, if ALT data are lacking for one or both times. For reexposure assessments of the cholestatic (± hepatocellular) type of liver injury, ALT has to be replaced by ALP. Criteria for positive reexposure tests are included in the updated CIOMS scale (Tables 5 and 6) and were not previously applied in cases with reported positive reexposure tests[40-57,59,91]. When these cases were submitted to retrospective analysis using the reexposure test criteria, a positive reexposure test could be confirmed in only 13/30 cases, the test was negative in 5/30 cases and uninterpretable in 12/30 cases[91]. In 8 cases of initially assumed Herbalife hepatotoxicity with a previously reported positive reexposure test result, retrospective evaluation applying the test criteria revealed that criteria for a positive reexposure were fulfilled in only 1/8 cases, whereas the reexposure test was classified as negative in another case or the data were considered as uninterpretable due to missing information to comply adequately with the criteria in the remaining six cases[94].

The CIOMS scale was widely used for hepatotoxicity assessments in epidemiological studies, clinical trials, case reports, case series, regulatory analyses, and genotyping studies[13,14,24,25,35,36,38,39,58,59,61,64,72,79,84,86,87,90,95-98]. Proposals for refinement and strengthening of the CIOMS scale focused on the weight of individual parameters and risk factors such as alcohol and age, and other shortcomings were addressed[24,87,89,90,98]. However, there is lack of valid data to verify improvements based on reassessing and reevaluating of published approaches[87,89,90,98], calling for new approaches.

Assessment of suspected HILI cases may be problematic in spontaneous reports with insufficient data. Evaluating these cases requires a sophisticated approach, as undertaken by EMA for 31 EU cases of suspected HILI by BC, using the CIOMS scale[34]. This series included 11/31 unassessable cases (35%) due to poor data quality, with causality assessment feasible in 20/31 cases (65%). Among these, EMA specified likely alternative causes in 8/20 cases with diagnoses such as autoimmune hepatitis, DILI, preexisting liver disease, alcoholic hepatitis, and preexisting liver cirrhosis with Stevens Johnson syndrome[34]. Causality for BC was unlikely or excluded in another 6/20 cases and 5/20 cases, respectively. In 1/20 cases, causality was judged as possible by EMA[34], but upon further evaluation this particular case with insufficient data quality was attributed with an excluded causality[71]. Consequently, in this EMA study group of 31 EU cases there was little evidence of liver injury by BC based on the use of the CIOMS scale, which was most helpful in this particular analysis and provided robust results[34]. The approach of EMA to apply the CIOMS scale in hepatotoxicity cases[34] should be highly appreciated and is in line with the corresponding recommendation by the NIH for their LiverTox database to prefer the CIOMS scale over other methods[2,3].

At present, we are far away from valid data and strict management in suspected HILI cases, which impedes description of classic HILI by the majority of herbs. Possible or likely alternative diagnoses were evident in 278/573 cases (48.5%) of suspected HILI cases; causality assessment was impeded in 165/573 patients (29.0%) due to missing case data or lack of a temporal association, resulting in diagnostic problems in 77.5% of all cases[82]. Given these limitations, actual discussions of validity of reported HILI cases are understandable[82,90,91,94,98-100], and uncertainty also extends to the validity of the type of liver injury reported for some cases lacking a probable or highly probable causality. Considering these restrictions, the hepatocellular type of injury was described for Indian Ayurvedic herbs[72,98], Chaparral (*Larrea tridentata*)[40,98], Dai Saiko To[47,98], Germander[98], Green Tea extract[98], Greater Celandine[37], Hydroxycut®[98], *Jin Bu Huan* (*Lycopodium serratum*)[45,98], Kava[13]; the cholestatic or mixed type for Chaparral[98], Germander[98], Green Tea extract[98], Greater Celandine[98], Hydroxycut®[98]; and the veno-occlusive disease for plants containing pyrrolizidine alkaloids such as *Senecio*, *Heliotropium*, *Crotalaria*, and *Symphytum* species[98].

In clinical practice, the physician will start at the day HILI is suspected with the CIOMS scale to arrive at an initial estimation and to exclude the most frequent alternative causes, provided point by point in the CIOMS questionnaire (Tables 5 and 6). The practical application of the CIOMS scale was published in various case series[13,25,35,36,38,39,71,72,94] and is shown by two single cases as examples, one for a case of hepatotoxicity by Indian Ayurvedic herbs (Table 7)[58], and another one for a case of liver injury by a dietary supplement[97]. For further refinement, specific information usually is necessary to rule out rare alternative causes (Table 2). This initial approach using the CIOMS scale ensures prospectively the collection of highly qualified case data and enables a sophisticated case evaluation currently and in the future. Information of individual CIOMS items (Tables 5 and 6), the checklist for HILI diagnosis (Table 2), all raw data, and a narrative case report should be presented to regulatory agencies, the scientific community, manufacturers, and expert panels to allow refined use of the CIOMS scale and all other case data, provided causality for the incriminated herb reached a probable or highly probable level.

MV scale

The MV scale[76] was developed in an attempt to improve the CIOMS scale by adding other clinical elements and by simplifying and changing the relative weight of assessment parameters, in detail discussed by the NIH LiverTox[2,3] and others[62,87], or briefly referenced[98]. As a shortened and modified version of the CIOMS scale[60], the MV scale[76] has fewer specific criteria than the original CIOMS scale (Table 4); due to major differences in test cases, however, the equivalency to CIOMS has been debated[2,3,62,84,87,89,96].

Specifically, the MV scale evaluates dechallenge as

Table 7 Council for International Organizations of Medical Sciences scale as an example with items required for causality assessment in a patient with herb induced liver injury by four Indian Ayurvedic herbs

Items for hepatocellular injury	Possible score	Psoralea corylifolia	Acacia catechu	Eclipta alba	Vetivexia zizaniodis
Time to onset from the beginning of the herb					
5-90 d (rechallenge: 1-15 d)	+2				
< 5 d or > 90 d (rechallenge: > 15 d)	+1	+1	+1	+1	+1
Alternative: Time to onset from cessation of the herb					
⩽ 15 d (except for slowly metabolized herbal chemicals: > 15 d)	+1				
Course of ALT after cessation of the herb					
Percentage difference between ALT peak and N					
Decrease ⩾ 50% within 8 d	+3	+3	+3	+3	+3
Decrease ⩾ 50% within 30 d	+2				
No information or continued herbal use	0				
Decrease ⩾ 50% after the 30th day	0				
Decrease < 50% after the 30th day or recurrent increase	-2				
Risk factors					
Alcohol use (drinks/d: > 2 for women, > 3 for men)	+1				
No alcohol use (drinks/d: ⩽ 2 for women, ⩽ 3 for men)	0	0	0	0	0
Age ⩾ 55 yr	+1	+1	+1	+1	+1
Age < 55 yr	0				
Concomitant herbs(s) and drug(s)					
None or no information	0				
Concomitant herb or drug with incompatible time to onset	0				
Concomitant herb or drug with compatible or suggestive time to onset	-1	-1			
Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset	-2		-2	-2	-2
Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test)	-3				
Search for non herb causes					
Group Ⅰ (6 causes)					
Anti-HAV-IgM		-	-	-	-
HBsAg, anti-HBc-IgM, HBV-DNA		-	-	-	-
Anti-HCV, HCV-RNA		-	-	-	-
Hepatobiliary sonography/colour Doppler sonography of liver vessels/endosonography/CT/MRC		-	-	-	-
Alcoholism (AST/ALT ⩾ 2 IU/L)		-	-	-	-
Acute recent hypotension history (particularly if underlying heart disease)		-	-	-	-
Group Ⅱ (6 causes)					
Complications of underlying disease(s)		-	-	-	-
Infection suggested by PCR and titre change for					
CMV (anti-CMV-IgM, anti-CMV-IgG)		-	-	-	-
EBV (anti-EBV-IgM, anti-EBV-IgG)		-	-	-	-
HEV (anti-HEV-IgM, anti-HEV-IgG)		-	-	-	-
HSV (anti-HSV-IgM, anti-HSV-IgG)		-	-	-	-
VZV (anti-VZV-IgM, anti-VZV-IgG)		-	-	-	-
Evaluation of group Ⅰ and Ⅱ					
All causes-groups Ⅰ and Ⅱ-reasonably ruled out	+2	+2	+2	+2	+2
The 6 causes of group Ⅰ ruled out	+1				
5 or 4 causes of group Ⅰ ruled out	0				
Less than 4 causes of group Ⅰ ruled out	-2				
Non herb cause highly probable	-3				
Previous information on hepatotoxicity of the herb					
Reaction labelled in the product characteristics	+2				
Reaction published but unlabelled	+1	+1			
Reaction unknown	0		0	0	0
Response to readministration					
Doubling of ALT with the herb alone, provided ALT below 5N before reexposure	+3				
Doubling of ALT with the herb(s) and drug(s) already given at the time of first reaction	+1				
Increase of ALT but less than N in the same conditions as for the first administration	-2				
Other situations	0				
Total score for each individual herb used by the patient		+7	+5	+5	+5

The data of the patient with severe hepatotoxicity by four different Indian Ayurvedic herbs are derived from a published report[58], using the updated Council for International Organizations of Medical Sciences scale for the hepatocellular type of liver injury (Table 5). The symbol - signifies that this particular item has been evaluated and no abnormality was found. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol[2,3,90]. For the four herbs, the total score was either 5 (possible causality) or 7 (probable causality). ALT: Alanine aminotransferase; N: Upper limit of the normal range; HBsAg: Hepatitis B antigen; HBc: Hepatitis B core; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CT: Computer tomography; MRC: Magnetic resonance cholangiography; AST: Aspartate aminotransferase; PCR: Polymerase chain reaction; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

the time necessary for ALT or ALP to fall below 2N, considers a shorter latency period, asks for less accurate exclusion criteria of drug-independent causes, ignores concomitant drug use, emphasizes drugs with more than 5 years marketing without published hepatotoxicity, and overestimates extrahepatic manifestations like hypersensitivity reactions[76]. The validation used real and fictive cases and as gold standard the opinion of three external experts[76,87] and not cases with verified results of positive reexposure tests[76]; for initial validation of the CIOMS scale, both a panel of experts and positive reexposure tests were used[60,61]. Compared to the CIOMS scale[60], the MV scale was equivalently accurate only in cases of hypersensitivity; otherwise, the CIOMS scale was superior to the MV scale[89,96]. A comparison of the two scales for hepatotoxicity cases demonstrated low consistency between the two systems, with agreement between the scales in only 18% of the cases; the CIOMS scale showed better discriminative power and produced assessments closer to those of specialists[87]. These limitations restrict the general use of the MV scale in hepatotoxicity cases[62].

A recent HILI study confirmed poor concordance between the MV and CIOMS scales for both the herb and concomitant medication assessment. The CIOMS scale found higher causality levels for the herb and concomitant medications than the MV scale; this was associated with considerably lower causality levels provided by the MV scale compared to the *ad hoc* approach[84]. The low MV scores were attributed to various parameters such as prolonged latency and dechallenge periods, the presence of several alternative herb independent causes for the observed liver disease, only partial exclusion of herb unrelated causes due to missing essential case data, and lacking consideration of extrahepatic manifestations like rash, fever, arthralgia, peripheral eosinophilia, and cytopenia. It therefore appeared that various confounders precluded a high level of causality for the herb in a setting of HILI cases assessed by the MV scale.

The MV scale may be useful in some selected hepatotoxicity cases. Nonetheless, little evidence is provided that this scale has advantages over the CIOMS scale and should be the preferred tool[2,3,62,87,89,95,96]. It has been criticized by the NIH LiverTox that the elements used in the MV scale and their relative weights were based upon the authors' expert opinion and not by prospective evaluation of a variety of possible elements and different cutoff values and weights[2,3]. Additional concern was expressed that the MV scale focuses on hypersensitivity features that are comparatively infrequent in hepatotoxicity cases; it performs poorly in atypical cases, such as unusually long latency periods or residual chronic symptoms after cessation of the culprit[87]. Another issue raised was the low numbers of experts and the low degree of validation[2,3] of the MV scale[76]. Thus, the MV scale is not commonly recommended for assumed HILI cases and certainly is no substitute for the CIOMS scale[2,3,87,98].

Naranjo scale

The NIH LiverTox summarized the arguments for and against the Naranjo scale[2,3]. In detail, while this scale includes all general features important in assessing causality, most critical elements are not weighed in judging the likelihood of liver injury, for example specific time to onset, criteria for recovery time, and list of critical diagnoses to exclude, limiting the use of this scale for assessing hepatotoxicity. The Naranjo scale includes testing for drug levels, which is rarely helpful in idiosyncratic drug induced liver disease. Finally, the scale was designed for use in clinical trials, and points are subtracted if the reaction reappears with administration of placebo, which does not apply to the usual case of drug induced liver disease. Direct comparisons to the CIOMS scale have shown that the Naranjo scale is easier to apply, but has less sensitivity and specificity in assigning causality to cases of liver injury. These statements of the NIH LiverTox[2,3] supported other views[87], confirming low sensitivity, and a lower prediction rate of the Naranjo scale in a careful comparison with the CIOMS scale for suspected hepatotoxicity cases[101]. These studies concluded that the Naranjo scale lacks validity and reproducibility when evaluating hepatotoxicity[86,93]; it was not recommended for hepatotoxicity assessment[87].

The Naranjo scale was designed to assess causality of any adverse drug reaction (ADR), independent from the affected organ[77]. It substantially differs from other causality algorithms for hepatotoxicity (Tables 3 and 4)[2,3,24-26,63,79,87,88,101]. This scale relates toxic drug reactions to general pharmacological drug actions rather than possibly to idiosyncratic reactions like rare hepatotoxicity[77]. Its items include drug concentrations and monitoring, dose relations such as decreasing dose, placebo response, cross-reactivity, and confirmation of ADRs using unidentified objective evidence, which is relevant only for toxic reactions[77,79,88]. The general use of the Naranjo scale in hepatotoxicity cases[23,79] created concern[2,3,24-26,63,70,87,88,101].

The use of the liver unspecific Naranjo scale[77] is unacceptable in suspected HILI cases[23,79], its results are heavily disputed[24-26,63,70,79,88]; this pertains especially to the shortened version used by the United States Pharmacopeia (USP)[23,79] with only 5 of the original 10 items[88]. Lack of liver specificity associated with the Naranjo algorithm is evident by lack of a definition of liver injury as ADR; an unclear time frame and latency period; undefined time frames for dechallenge; no definition of risk factors; insufficient evaluation of alternative diagnoses; inappropriate assessment of comedication; and lacking definition of a positive rechallenge test (Table 4)[77,88]. This scale also was considered too insensitive, allowing a possible causality even in the absence of essential data, by virtue of the patient simply having taken the suspected agent[63,70]. Most importantly, the modified Naranjo scale as used by USP[23,70] did not exclude relevant alternative causes such as idiopathic autoimmune hepatitis, alcoholic or cardiac hepatopathy, other preexisting liver

diseases, DILI, and drug-induced rhabdomyolysis[24-26]. Use of this method has raised concern about judgement validity by the USP[63,88]. Considering all shortcomings along with the lack of liver specificity and validation for hepatotoxicity, the Naranjo scale should be excluded from use in hepatotoxicity cases. It certainly is no substitute for the CIOMS scale.

KL method

The KL method[78] is neither liver specific nor validated for hepatotoxicity (Table 3), it also lacks important items for hepatotoxicity assessment (Table 4). It was recently applied for causality assessment of suspected hepatotoxicity for some Herbalife products[74]. Subjective judgement is needed for many steps, making this method more prone to bias[87]. Though commonly applied by the Spanish Pharmacovigilance Centres[74], the KL method is not used by the Spanish Group for the Study of Drug-induced Liver Disease[59,85,87,95], which applies the CIOMS scale as the preferred assessment tool. The KL method should not be used for assessment of hepatotoxicity cases.

Ad hoc approach

Numerous published HILI reports lack any causality method description and presumably are based on the *ad hoc* assessment with its relevant shortcomings (Tables 3 and 4). When using this approach, the physician notes the coincidence of herbal product and chemical drug use, and will estimate the likelihood of a hepatotoxic reaction[89].

After ruling out alternative causes, the *ad hoc* approach is often used to distinguish a probable, possible, or unlikely causality[89]. A probable causality is usually attributed when the manifestations of liver disease, temporal association, and dechallenge response seems to fit the typical signature pattern of the product in question. A possible attribution is assigned when one feature is not typical, the product not known to cause the reaction or so rarely that it is difficult to distinguish from background, or an alternative cause is less or equally plausible. An unlikely causality is assigned when most of the features are atypical or an alternative cause is more plausible[89].

Though relevant items such as signature of symptoms, latency period, dechallenge, definitive exclusion of alternative causes, risk factors, alcohol use, and track record of the product are used[79,89], no universally accepted description exists for either the method or its application[79]. Due to missing specific criteria (Tables 3 and 4), the *ad hoc* approach is obsolete to validly assess causality in HILI[79] or DILI cases[89].

With the *ad hoc* assessment applied prior to the liver specific CIOMS scale, the physician inevitably will postpone an assessment by such a procedure and thereby delay the diagnosis. Since the parameters of the *ad hoc* approach are liver unspecific and not validated (Tables 3 and 4), this method should be replaced by better alternatives. The NIH LiverTox does not even mention the *ad hoc* approach as a possible causality evaluation method for hepatotoxicity cases[2,3].

RETROSPECTIVE CAUSALITY ASSESSMENT METHODS

DILIN method

According to the NIH LiverTox, the DILIN method is based on a narrative summary and a compilation of clinical findings and sequential biochemical abnormalities[2,3]. These are extracted from clinical records and entered into a 65-page case report form, but a scoring system was lacking[102], as opposed to the CIOMS scale (Table 4). The DILIN causality adjunction process is outlined in a 12 step flow diagram, using three independently assessing experts in hepatotoxicity who grade the likelihood of a causal relationship between the drug and liver injury in one of five scores[102]: (1) Definite (> 95% assurance): the evidence for the drug causing the injury is beyond a reasonable doubt; (2) Highly likely (75% to 95% assurance): the evidence for the drug causing the injury is clear and convincing but not definite; (3) Probable (50% to 74% assurance): the preponderance of the evidence supports the link between the drug and the liver injury; (4) Possible (25% to 49% assurance): the evidence for the drug causing the injury is equivocal but present; and (5) Unlikely (< 25% assurance): there is evidence that an etiological factor other than the drug caused the injury.

While these causality grades appear vague, attempts are made to provide an objective and critical evaluation of the likelihood that the liver injury is due to the suspected agent[2,3]. In particular, cases are not considered "probable" merely because there is no other explanation. Similarly, cases are not considered "definite" if another diagnosis is possible. If two or three drugs are implicated, only one can be considered probable, highly likely or definite, the others are assigned "possible" or "unlikely", so that the total percent assurance does not exceed 100%[2,3]. The causality assessment is accepted as initially scored if the three expert reviewers completely agree; if there is disagreement, the reviewers meet to reconcile the differences and reach a final single score[2,3,102]. A complete summary of the definitions for each category is provided[102].

The DILIN method requires experts and has shortcomings (Tables 3 and 4)[2,3,73,80,86,102]; it is therefore not suitable for the physician who needs assessment results during the early disease. The DILIN method was used for retrospective assessments of case series where time to conclusion is not a crucial issue[73,86,102]. In combination with the CIOMS scale, this method is the basis for future DILIN group studies of clinical, genetic, environmental, and immunological risk factors[80]. To exclude alternative causes in retrospective analyses by the DILIN method, screening was required for previous liver disease, alcohol use, hepatitis A, B, or C infection, autoantibodies, ceruloplasmin, α_1-antitrypsin, ferritin, iron, and

imaging data; specific details or appropriate scores for each item were not provided (Table 4)[102]. Other possible causes were not considered (Table 2), including specific liver infections like hepatitis E or by cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV), and varicella zoster virus (VZV)[102]. At present, questions regarding the actual DILIN method validity remain, and transparent results of all diagnostic items from each individual patient would be preferred rather than a summarizing causality grade.

Another approach of the DILIN group targets a novel Causality Assessment Tool (CAT) specifically for HDS[103]. CAT was designed to retrospectively adjudicate multiple products as a single entity using structured causality assessment and expert opinion. The elements of the CAT considered the multiplicity of products consumed, implicated drugs, alternative diagnoses, and published DILI literature on the product or an ingredient[103]. In analogy to the scoring system, the DILIN method expresses causality levels as percentage assurance[102]; CAT also grades the likelihood of a causal relationship between HDS and liver injury from definitive to unlikely[103]. In this preliminary study, CAT was applied in 16 DILI cases, which were initially evaluated by the DILIN method and in which HDS are implicated as a potential cause. Overall agreement and reliability in this study of retrospective analysis requiring an expert panel was moderate[103]; this method needs further investigation and validation[98].

WHO method

In its recent statement, the NIH LiverTox does not mention the WHO method in connection with causality assessment methods for hepatotoxicity cases but rather discusses other methods[2,3]. Since the WHO method[81] was not developed for hepatotoxicity cases and therefore does not consider hepatotoxicity characteristics[79,104], this omission appears warranted. The shortcomings of the unspecific features of the WHO method (Tables 3 and 4) have been a matter of major concern[38,39,104-106] and led to the conclusion that this scale is not appropriate for causality assessment in suspected HILI cases[79,104].

The WHO method consists of two parts, one being the WHO scale to assess causality levels, the other one the global introspection by experts[81]. Though not validated for any ADR[103], global introspection surprisingly represented a popular strategy in evaluating the likelihood of drug causality for general ADRs of all organs[107]. As early as 1986, however, global introspection by experts has been shown to be neither reproducible nor valid[107]. In detail, the assessor considers factors that might support a causal link of one or more drugs to an observed ADR, lists all factors, weighs their importance, and estimates the probability of drug causation; no specific checklist or level of strength is given[107]. It has been recognized that both the questions and the answers are ambiguous[79]. Though these shortcomings are described for general ADRs, they certainly also apply even more to hepatic ADRs.

The WHO scale has not been based on a gold standard, is not quantitative, not liver specific, and has not been validated for hepatotoxicity (Tables 3 and 4)[4,38,39,79,104-106]. In particular, reliability, sensitivity, specificity, positive and negative predictive values are unknown, but likely are low[79,81,104-106]. Its scope is also limited since it cannot discriminate between a positive and a negative correlation, thereby resulting in overdiagnosing and overreporting[104].

The WHO method ignores relevant data like uncertainties in daily dose, temporal association, start, duration and end of herbal use, time to onset of ADR, and course of liver values after herbal discontinuation. Insufficiently considered or ignored are comedication, preexisting liver diseases, numerous alternative explanations, and exclusion of virus infections by hepatitis A, B, C and E, CMV, EBV, HSV, and VZV[38,39]. Since only a few raw data are evaluated, case duplications and retracted cases remain undetected by the WHO method to a higher degree than by other methods[38]. Despite these flaws, the WHO method was used for causality assessment[17,38,39,53,54]. Reevaluation often could not confirm causality in cases of two assessed reports[38,39]; therefore, the use of the WHO method in HILI cases has major limitations.

Causality assessment by the WHO method requires a panel of experts rarely available at a hospital or a family physician office. Consequently, analyses based on this method are retrospective; their results are available long after the patient problems of assumed HILI.

Expert opinion

Expert opinion as an assessment tool is poorly defined (Tables 3 and 4), except that a panel of specialists with clinical expertise in hepatology is available for causality assessment in HILI. For DILI, groups of skilled hepatologists exists without any doubt in most countries including Japan[108,109] and in expert projects like the international DILI Expert Working Group[90], the United States DILIN group[73,80,86,102,103], the Spanish Group for the Study of Drug-Induced Liver Disease[59,85,87,95,101], and the Spanish-Latin American network on drug induced liver injury[110]. For HILI, the Hong Kong Herb-Induced Liver Injury Network is of importance[75]. However, the qualification of assessors is sometimes crucial and may be problematic as discussed in detail[88,105,106]. Even with specialists, individual opinion often results in judgement bias.

RELEVANCE TO ACTUAL MEDICAL PRACTICE

For HILI case assessment, strategies need to be developed that are clinically useful and applicable in daily practice. These must meet the expectations of the scientific community, regulatory agencies, and manufacturers, provided the case is going to be reported. At the day when HILI is suspected and criteria of hepatotoxicity are fulfilled, the physician should explore through the internet and regulatory databases how frequently the suspected herb has been associated with hepatotoxic

adverse reactions both in the scientific literature and by regulatory notifications. Publication as an interesting case report should be encouraged, if there are few or even none hepatotoxicity reports of this particular herb. Consequently, the decision will depend on the physician's own interest and clinical experience, resulting in three different levels of assessment intensity. These include first a wait and see approach after cessation of the herbal product, second a strategy aimed at exclusion of the most frequent differential diagnoses, or third an exclusion of even rare alternative causes.

The first approach of wait and see requires little attention and few elements and is cost effective, at least initially but not necessarily in the further course. If for some reasons the correct diagnosis was missed, it will be costly and risky for the patient, the physician, or both. Submitting such an insufficiently documented case as suspected HILI case to scientific journals, regulatory agencies or manufacturers would be difficult to reconcile, leading to overreporting due to overdiagnosing[68,82,88,104,105,111]. In detail, diagnostic problems including alternative diagnoses as confounding variables were evident in 77.5% of 573 cases of initially suspected HILI, presented as spontaneous reports or as published case reports[82].

For the second strategy, the elements of the updated CIOMS scale are sufficient, starting with the evaluation of time to onset to verify at least a temporal association between the herbal use and the liver disease (Tables 5 and 6). For instance, if clinical assessment, hepatobiliary sonography, or serology of hepatitis A-C provides an alternative cause as the correct and final diagnosis, the costs will remain low since further diagnostic measures are not warranted. If diagnostic exclusion is unsuccessful so far, parameters of CMV, EBV, HEV, HSV, and VZV are needed (Tables 5 and 6), though in reality these elements are rarely reported in suspected HILI cases[13,14,17,23-26,38,39,94]. With complete or even some missing CIOMS elements, the CIOMS scale provided causality for various herbs with levels of probable and highly probable[35-37].

For the third level of evaluation, the physician will have to decide, which of the multiple other and rare differential diagnoses are worth of consideration. The checklist should be valuated as a reminder of possible alternatives and as a suggestion for further approaches, depending on the clinical phenotype. Clearly, the number of criteria set for ruling out alternative causes is not required for all cases, the checklist therefore asks selectively whether the information was completely, partially or not obtained (Table 2). A sophisticated strategy is needed, however, if the case is reported to regulatory agencies and the scientific community, which are overflooded by poorly documented suspected and often misdiagnosed HILI cases[26,34-36,38,39,82]. For optimum case presentation, the individual items of the updated CIOMS scale should be provided for a single case (Table 7)[58,97] as well as for case series. This is feasible as shown in numerous publications[13,25,35,36,38,39,71,72,94] for 26 cases[13], 22 cases[25], 22 cases[35], 21 cases[36], 15 cases[38], 13 cases[39], and 4-9 cases[71,72,94]. The presentation of the CIOMS items for the single case should be combined with a detailed report of all relevant case data[58,97] and a list of differential diagnoses that were excluded completely or partially, or were not considered[58], similar to the checklist for HILI diagnosis (Table 2). For a case series, basis data for each individual case are to be provided in a single table, focusing on details required for causality assessment; examples are presented in various publications[14,25,35,36,38,39]. Presentation of excellent data will lead to valid causality results and appropriate conclusions. This is prerequisite for well founded assessments of further HILI cases, with benefit for patients, physicians, the scientific community, regulatory agencies, and manufacturers.

FUTURE PERSPECTIVES

Future considerations will have to focus on improvements of causality assessment methods[90,98] to obtain prospectively valid HILI diagnoses at the time the patient experiences liver injury, corresponding efforts of retrospective causality assessments of HILI cases are promising and on the way with preliminary data[103]. Strategies are to be developed to characterize liver injury by various herbs with all facets. At the day HILI is suspected, causality assessment should be initiated in all cases using the CIOMS scale preferentially in its updated form (Tables 5 and 6). Supported by the checklist for HILI diagnosis (Table 2), this could provide HILI cases with a probable or highly probable causality for a special herb as basis for further evaluation. Overall, this will facilitate characterization of disease entities including phenotype standardization, retrospective reanalysis by expert panels, improvement of pharmacovigilance decisions, safety strategies of manufacturers, and studies directed to assess pathogenetic aspects of HILI.

Studies are needed in the future to assess factors leading to unpredictable HILI in few patients, who experience this disease with a probable or highly probable causality level. As for DILI, future issues for HILI cases with established causality are to define genetic, environmental, and immunological determinants of HILI susceptibility[80,90,112,113]. Overall, metabonomics, pharmacogenetics, proteomics, and transcriptomics are areas of potential interest in HILI, as detailed for DILI[112]. Since HILI is commonly an unpredictable disease[91], experimental studies dealing with predictive cellular systems as used to identify potentially hepatotoxic synthetic drugs[114] will be of limited if any relevance for herbs. Similarly, applying well-defined primary cultures of human hepatocytes and measuring a panel of signals directly linked to key mechanisms of liver injury to predict drugs, which can cause liver injury[114], will be restricted to drugs and not be applicable to herbs. Recent advances of the early pre-clinical assessment of the potential intrinsic hepatotoxicity of candidates drugs has been reviewed

in detail, focusing on cell-based models such as cell cultures with outcome and detection methods, on profiling technologies, and emerging technologies including stem cell technologies and 3D as compared to 2D culturing techniques[115]. However, it is unlikely that the results of these in vitro studies of intrinsic and predictable hepatotoxicity induced by synthetic drugs are transferable to a clinical setting of HILI that commonly represents the idiosyncratic and unpredictable form of liver injury by one or more herbs, each with multiple chemical constituents. More important seems the search for biomarkers in HILI patients with clearly established causality[116].

CONCLUSION

The rare liver injury by herbs, herbal drugs, and herbal supplements may present itself with numerous facets, providing challenging issues for causality assessment. The physician is responsible to make available all necessary data for a high quality judgement; otherwise, causality evaluation will be problematic. Timely causality assessment is mandatory when the disease is unfolding to base prospective diagnostic and therapeutic decisions. The most appropriate causality assessment method is the liver specific CIOMS scale, which should prospectively be applied by the physician. If used, other methods have pitfalls and cause ambiguous results debated on reasons of imprecision, liver unspecificity, and limitations to retrospective analyses, or they are unavailable due to requirements for expert panels.

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P- Reviewers Devarbhavi H, Helling TS, Taye A
S- Editor Wen LL **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2883

World J Gastroenterol 2013 May 21; 19(19): 2883-2893
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver

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Supported by Australian National Health and Medical Research Council Grant 512282 (to Gorrell MD); Rebecca L Cooper Foundation Equipment Grants (to Gorrell MD); University of Sydney International Scholarship (to Chen Y); Australian Postgraduate Scholarship (to Yao TW); and Grant NIH U19 AI066313 (to Schuppan D)

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Received: November 7, 2012 Revised: January 17, 2013
Accepted: February 2, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the expression of dipeptidyl peptidase (DPP) 8 and DPP9 in lymphocytes and various models of liver fibrosis.

METHODS: DPP8 and DPP9 expression were measured in mouse splenic CD4$^+$ T-cells, CD8$^+$ T-cells and B-cells (B220$^+$), human lymphoma cell lines and mouse splenocytes stimulated with pokeweed mitogen (PWM) or lipopolysaccharide (LPS), and in dithiothreitol (DTT) and mitomycin-C treated Raji cells. DPP8 and DPP9 expression were measured in epidermal growth factor (EGF) treated Huh7 hepatoma cells, in fibrotic liver samples from mice treated with carbon tetrachloride (CCl_4) and from multidrug resistance gene 2 (*Mdr2/Abcb4*) gene knockout (gko) mice with biliary fibrosis, and in human end stage primary biliary cirrhosis (PBC).

RESULTS: All three lymphocyte subsets expressed DPP8 and DPP9 mRNA. DPP8 and DPP9 expression were upregulated in both PWM and LPS stimulated mouse splenocytes and in both Jurkat T- and Raji B-cell lines. DPP8 and DPP9 were downregulated in DTT treated and upregulated in mitomycin-C treated Raji cells. DPP9-transfected Raji cells exhibited more annexin V$^+$ cells and associated apoptosis. DPP8 and DPP9 mRNA were upregulated in CCl_4 induced fibrotic livers but not in the lymphocytes isolated from such livers, while DPP9 was upregulated in EGF stimulated Huh7 cells. In contrast, intrahepatic DPP8 and DPP9 mRNA expression levels were low in the *Mdr2* gko mouse and in human PBC compared to non-diseased livers.

CONCLUSION: These expression patterns point to biological roles for DPP8 and DPP9 in lymphocyte activation and apoptosis and in hepatocytes during liver disease pathogenesis.

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Key words: Dipeptidyl peptidase; CD26; Lymphocytes; Liver fibrosis; Biliary fibrosis

Chowdhury S, Chen Y, Yao TW, Ajami K, Wang XM, Popov Y, Schuppan D, Bertolino P, McCaughan GW, Yu DMT, Gorrell MD. Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver. *World J Gastroenterol*

2013; 19(19): 2883-2893 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2883.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2883

INTRODUCTION

The four enzyme members of the dipeptidyl peptidase (*DPP*) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9*, have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes[1,2]. FAP is a potential cancer therapeutic target[2,3]. *DPP4*, the most well characterized family member, has ubiquitous cell surface and extracellular expression[2,4-7]. *DPP8* and *DPP9* are the most recently discovered members of the *DPP4* gene family[8-11]. *DPP4*, *DPP8* and *DPP9* are ubiquitously expressed cytosolic enzymes with *DPP4*-like activity[8,11,12]. They are expressed by major epithelial organs including liver, colon, small intestine, stomach, lung, skin, tongue, kidney, testis and the lymphoid cells of lymph node, blood, thymus, and spleen[13]. The biological functions of *DPP8* and *DPP9* are largely uncharacterized.

DPP4 is also known as *CD26* and has important roles in the immune system. It is a costimulatory molecule in T cell activation and proliferation and is critical in the development of T helper 1 responses to foreign antigens. It is expressed at detectable levels by some resting T cells but the cell surface expression increases 5-10 fold following stimulation with antigen or anti-CD3$^+$ interleukin-2 or with mitogens such as phytohaemagglutinin[14-19]. However, the costimulatory role of *DPP4*/*CD26* is mediated by extra-enzymatic activities[20-22]. Hence, some of the immunological effects observed in early *DPP4* inhibitor studies are now thought to be due to off-target non-selective inhibition of *DPP8* and *DPP9*[2,23,24]. In support of this viewpoint, there is some evidence that *DPP8* and *DPP9* are functionally significant in the immune system. Their mRNA levels are elevated in activated human leukocytes[25,26]. An inhibitor of *DPP8* and *DPP9* attenuates proliferation in *in vitro* models of human T-cell activation[23]. An inhibitor selective for *DPP8* and *DPP9 vs* related proteases can suppress DNA synthesis in mitogen-stimulated splenocytes from both wildtype *DPP4*$^+$/$^+$ and *DPP4*$^-$/$^-$ gene knockout (gko) mice[27]. Moreover, *DPP8* and *DPP9* have been implicated in hematopoiesis and in inflammatory diseases including arthritis[2,28,29]. Most importantly, *DPP8* and *DPP9* are involved in processing and degradation of peptides involved in antigen presentation by Major histocompatibility complex class I[30].

Inflammatory and immune responses are important in liver injury. Improved understanding of immune response, inflammation and fibrogenic progression is needed to advance the understanding of liver disorders. *DPP8* and *DPP9* are expressed in hepatocytes and lymphocytes of human cirrhotic liver[13]. Hepatocytes in the periseptal area of regenerative nodules and lymphocytes in the portal tracts are strongly positive for *DPP8* and *DPP9 in situ* hybridization (ISH)[13]. Bile ducts and ductular reactions are ISH positive for *DPP9* but not for *DPP8*[13]. However, the role of *DPP8* and *DPP9* in liver is unknown. Other members of this protease family, *DPP4* and *FAP*, are altered in liver diseases and are potential disease markers and therapeutic targets[31-36]. Despite the pleiotropic roles of *DPP4* and *FAP* in various biological processes, *DPP4* and *FAP* gko mice exhibit no spontaneous defects, suggesting that *DPP4* and *FAP* are not essential for normal functions, and hence, targeting them is likely to lack adverse side effects[37,38].

DPP8 and *DPP9* have interesting properties in cell biological processes that may contribute to disease pathogenesis, such as apoptosis and cell migration[39,40]. Their biological functions, especially in the immune system, are important considerations for the selectivity of *DPP4* inhibitors over *DPP8* and *DPP9* in clinical development of *DPP* antagonists. Here we studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury to elucidate their potential biological roles in the immune system and in liver diseases.

MATERIALS AND METHODS

Materials

Antibodies are detailed in Table 1. Other materials were from Sigma-Aldrich (St Louis, MO, United States) unless stated.

Animal studies

Mice were maintained in the Centenary Institute animal facility under specific pathogen-free conditions. The Animal Ethics Committee of the University of Sydney approved experimental procedures and housing arrangements. *FAP* gko[38] and *DPP4* gko mice[37] (C57BL/6J background) were bred at the Animal Resource Centre (Perth, Australia). Female multidrug resistance gene 2 (*Mdr2*/*Abcb4*) gko mice (FVB/N background) with targeted disruption of *Mdr2*, were obtained from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, United States)[35]. Liver samples from the *Mdr2* gko and wild type (wt) mice were obtained at 4, 8 and 12 wk after birth, the time points that span the most active fibrosis progression[35]. RNA were obtained as previously described[41]. Lymphocytes from wt, *DPP4* gko and *FAP* gko mouse spleen, liver and lymph nodes were isolated as previously described[42].

For the liver fibrosis mouse model, 8-wk-old female wt, *DPP4* gko and *FAP* gko mice were injected intraperitoneally with carbon tetrachloride (CCl_4) twice weekly for 3 wk. Each dose comprised 5.36 μL of 12% CCl_4 (in paraffin oil) per gram of initial weight of each mouse. Significantly elevated alanine aminotransferase (ALT) (68 ± 11.1 U/L *vs* untreated controls 32 ± 1.2 U/L) indicated liver injury. ALT was performed by an auto-analyzer at the Clinical Biochemistry Department of the Royal Prince Alfred Hospital. Organs were collected 3 d after the final CCl_4 treatment.

Table 1 Antibodies used in immunoblot and flow cytometry

Name	Isotype	Conjugate	Dilution	Supplier	Catalogue no.
Primary antibodies					
CD4	Rat IgG_{2b}	FITC	1:50	BD Pharmingen, NJ, United States	557307
B220	Rat IgG	PE	1:50	Caltag Laboratories, CA, United States	RM2604-3
CD8	Rat IgG_{2a}	APC	1:50	BD Pharmingen, NJ, United States	553035
Annexin V		APC	1:50	BD Pharmingen, NJ, United States	550474
V5	Mouse monoclonal IgG_{2a}		1:5000	Invitrogen	R960-25
DPP8-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc	Ab42077
DPP8-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc.	Ab42076
DPP9-catalytic domain	Rabbit polyclonal		1:2000	Abcam Inc.	Ab42080
GAPDH	Mouse monoclonal		1:1000	EnCor biotechnology	MCA-1D4
β-actin	Rabbit polyclonal		1:1000	Sigma	A2103
Secondary antibodies					
Anti-rabbit IgG	Goat	HRP	1:3000	DAKO, Carpinteria, CA, United States	PO448
Anti-mouse	Goat IgG	R-PE	1:400	Molecular Probes	P852

DPP: Dipeptidyl peptidase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IgG: Immunoglobulin G; FITC: Fluorescein; PE: Phycoerythrin; APC: Allophycocyanin; HRP: Horse radish peroxidase; R-PE: R-phycoerythrin.

Human liver samples

Human liver tissues were obtained from liver transplant recipients in accordance with National Health and Medical Research Council guidelines under Royal Prince Alfred Hospital Human Ethics Committee approvals. Non-diseased liver donors had an age range of 56-58 years and mixed genders. Cirrhotic livers were from primary biliary cirrhosis (PBC) patients of average age 51.7 ± 13.3 years (range 27-67 years; 10 females, 2 males) and end stage alcoholic liver disease patients of average age 49.3 ± 8 years (range 34-60 years, 9 males) as described previously[40].

In vitro stimulation assays

Human B lymphocyte Burkitt's lymphoma cell line (Raji) (ATCC, CCL-86) and human T cell leukemia cell line (Jurkat) (ATCC, TIB-153) were cultured in Roswell Park Memorial Institute (RPMI) Medium 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal calf serum (FCS) and Penicillin-Streptomycin (100 units of penicillin and 100 μg/mL of streptomycin) (1 × P/S) and human liver hepatocellular carcinoma cell line Huh7 were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% FCS and 1 × P/S.

Lymphocytes at 1×10^6 cells/mL RPMI were treated with either 5 μg/mL pokeweed mitogen (PWM), 20 μg/mL lipopolysaccharide (LPS), 50 μg/mL Mitomycin C or 10 mmol/L dithiothreitol (DTT). Human liver hepatocellular carcinoma cell line, Huh7 cells were serum starved for 20 h before stimulation with 0, 1, 10, 100 ng/mL epidermal growth factor (EGF; R-D Systems, MN, United States) for 4 h.

Apoptosis assay

To determine if *DPP9* overexpression induces apoptosis, Raji cells were transiently transfected with wt*DPP9*-V5-His, mut*DPP9*-V5-His or vector control (pcDNA3.1/V5-HisA; Invitrogen, Carlsbad, CA, United States) as described previously[39], then cultured. The lymphocytes were transfected by electroporation using Amaxa® Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) on a Lonza-amaxa Nucleofector device (Lonza). Forty hours post transfection, cells were washed with annexin binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4). Staining involved incubating cells with annexin V antibody (Table 1) for 30 min at room temperature in the dark followed by 4',6-diamidino-2-phenylindole (DAPI), Sigma Aldrich) at 100 ng/mL. Cells were enumerated using flow cytometry. Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OH, United States).

Fluorescence activated cell sorting

To isolate mouse lymphocyte subsets, 3×10^7 splenocytes were resuspended in primary antibody diluted in phosphate buffered saline (PBS) containing 1% FCS and incubated in the dark, on ice for 30 min. The primary antibodies used are listed in Table 1. Following antibody staining, cells were washed with PBS containing 1% FCS. Cells underwent a final resuspension of 2×10^7 cells/mL of PBS with 5% FCS and 2 mmol/L ethylene diamine tetraacetic acid (EDTA) to minimize clumping of cells. Twenty-five μL/mL of DAPI was added prior to cell sorting. Cell sorting was performed using the Fluorescence Activated Cell Sorting Vantage™ SE (BD Bioscience, NJ, United States). Cells were gated to exclude doublets and $DAPI^+$ (dead) cells. Three-way sort was performed to collect $CD4^+$ cells, $CD8^+$ cells and $B220^+$ cells into separate collection tubes.

Real time quantitative polymerase chain reaction

RNA from cells was extracted using the RNAqueous-Micro™ kit (Ambion, TX, United States) following manufacturer's instructions. Total RNA (1 μg) was then reverse-transcribed to cDNA using 10 pmol of oligo$(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA, United States), 10 mmol/L deoxyribonucleotide triphosphates and SuperScript Ⅲ reverse transcriptase (Invitrogen). Real time quantitative poly-

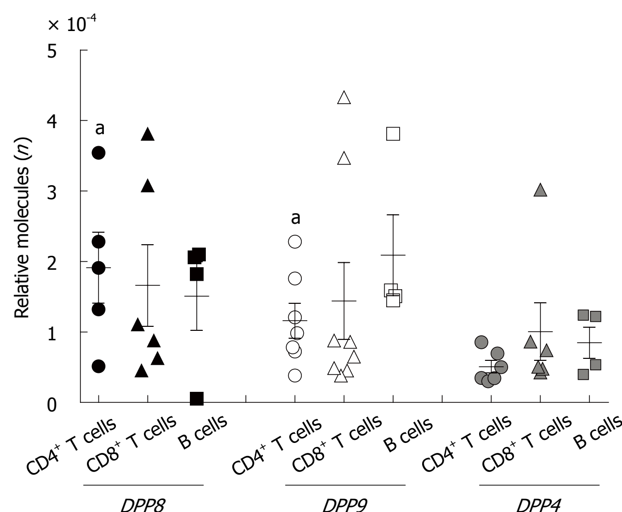

Figure 1 Dipeptidyl peptidase mRNA expression in C57BL/6 mouse splenic lymphocyte subpopulations. Number of molecules relative to 18S RNA (*n* = 4-6 mice). [a]*P* < 0.05 *vs* dipeptidyl peptidase (*DPP*) 4.

merase chain reaction (PCR) by Taqman® gene expression assays was performed using the Stratagene® Mx3000P™ System (La Jolla, CA, United States) according to manufacturer's recommendations. Taqman primers used for the assays were mouse *DPP4* (Mm00494548_mL), *DPP8* (Mm00547049_mL) and *DPP9* (Mm00841122_mL). The samples were run in duplicates. The gene expression level was analyzed using a standard curve of serially diluted known numbers of molecules of the same gene and then normalized relative to 18S (Hs99999901_s1). Quantitative PCR on human samples were performed using sequence detector (Prism, model 7700; Life Technologies, NY, United States) and were analyzed using sequence detector software (Prism, Version.1.6.3; Applied Biosystems Inc.). Primers used for human *DPP8* were forward: 5' CCAGATGGACCTCATTCAGACAG-3' and reverse: 5'GGTTGTTGCGTAAATCCTTGTGG-3' and for human *DPP9* were forward: 5'AGAAGCACCCCACCGTCCTCTTTG-3' and reverse: 5'AGGACCAGCCATGGATGGCAACTC-3'. The number of molecules was normalized with human aldolase B (forward: 5'-CCTCGCTATCCAGGAAAAC-3' and reverse: 5'TTGTAGACAGCAGCCAGGAC-3').

Immunoblotting assay

Cells were washed with ice-cold PBS three times and then lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1mmol/L $MgCl_2$, 300 μL of 150 mmol/L NaCl, 1% Triton-114, 10% glycerol and 1 × Roche complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and stored at -80 ℃. Protein concentration was determined using the micro BCA protein assay kit (Thermo Scientific, CA, United States) following the manufacturer's protocol. 50 μg total of each cell lysate in LDS sample buffer (catalogue No. NP0007, Invitrogen) with reducing agent (catalogue No. NP0004, Invitrogen) in conditions that retain *DPP8* and *DPP9* dimerization[8,9,40] was resolved on 3%-8% Tris-acetate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen) followed by immunoblotting. Antibodies for immunoblotting are listed in Table 1. Relative band intensities were quantified using Image J and normalized against control proteins as indicated[40].

Statistical analysis

Results are expressed as individual replicates. Horizontal lines represent mean and error bars represent standard error. Differences among groups were analyzed using Mann-Whitney *t*-test by GraphPad Prism 5 software. *P* values < 0.05 were considered significant.

RESULTS

To investigate which lymphocyte subsets express *DPP8* and *DPP9*, their transcript levels were quantified in the major lymphocyte subpopulations, $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells and $B220^+$ (B cells) from normal C57BL/6 mouse splenocytes. All three lymphocyte subsets expressed *DPP8* and *DPP9* mRNA (Figure 1). *DPP8* and *DPP9* transcripts were expressed to significantly greater levels than *DPP4* transcripts in $CD4^+$ T cell subpopulation (P = 0.02) and *DPP9* mRNA was significantly more abundant than *DPP4* mRNA in B cells (P = 0.03).

To examine whether, like *DPP4*[7], *DPP8* and *DPP9* are upregulated upon lymphocyte activation, mouse splenocytes were stimulated *in vitro* with PWM[43-45] and LPS[46,47]. *DPP8* and *DPP9* mRNA was markedly upregulated in PWM stimulated mouse splenocytes in a time dependent manner (Figure 2A). To examine whether the increased mRNA levels corresponded to protein expression, *DPP8* and *DPP9* proteins were measured in Jurkat (T cells) stimulated in-vitro with PWM. Both *DPP8* and *DPP9* were upregulated in a time dependent manner (Figure 2B and C).

Similarly, mRNA levels of *DPP8* and *DPP9* were upregulated in LPS stimulated mouse splenocytes (Figure 3A). Also, LPS stimulated Raji (B cells) had upregulated DPP8 and DPP9 protein expression in a time dependent manner (Figure 3B and C).

Immunoblots of *DPP8* exhibited a slow mobility band at 150-180 kDa, which probably represents dimer or processed dimer, in addition to the faster mobility bands at 95-100 kDa that are likely to be monomer and truncated or trimmed monomer (Figure 2B and 3B). *DPP9* showed a slow mobility band of monomer at 110 kDa and faster mobility bands, which are possibly truncated or trimmed monomers at 75-95 kDa (Figure 2C and 3C)[8,9,40,48]. The intensity of all three *DPP8* bands increased in a time dependent manner with PWM stimulation in Jurkat cells (Figure 2B). However, in LPS stimulated Raji cells the intensity of only the 150 kDa band increased in a time dependent manner (Figure 3B). The intensity of all the *DPP9* bands increased with time in both PWM stimulated Jurkat cells and LPS stimulated Raji cells (Figure 2C and 3C). In PWM stimulated Jurkat cells, *DPP8* and *DPP9* expression both peaked at 48 h (Figure 2B and C). In Raji cells, increased expression of *DPP8* was observed at 72 h post LPS stimulation (the longest time point of the

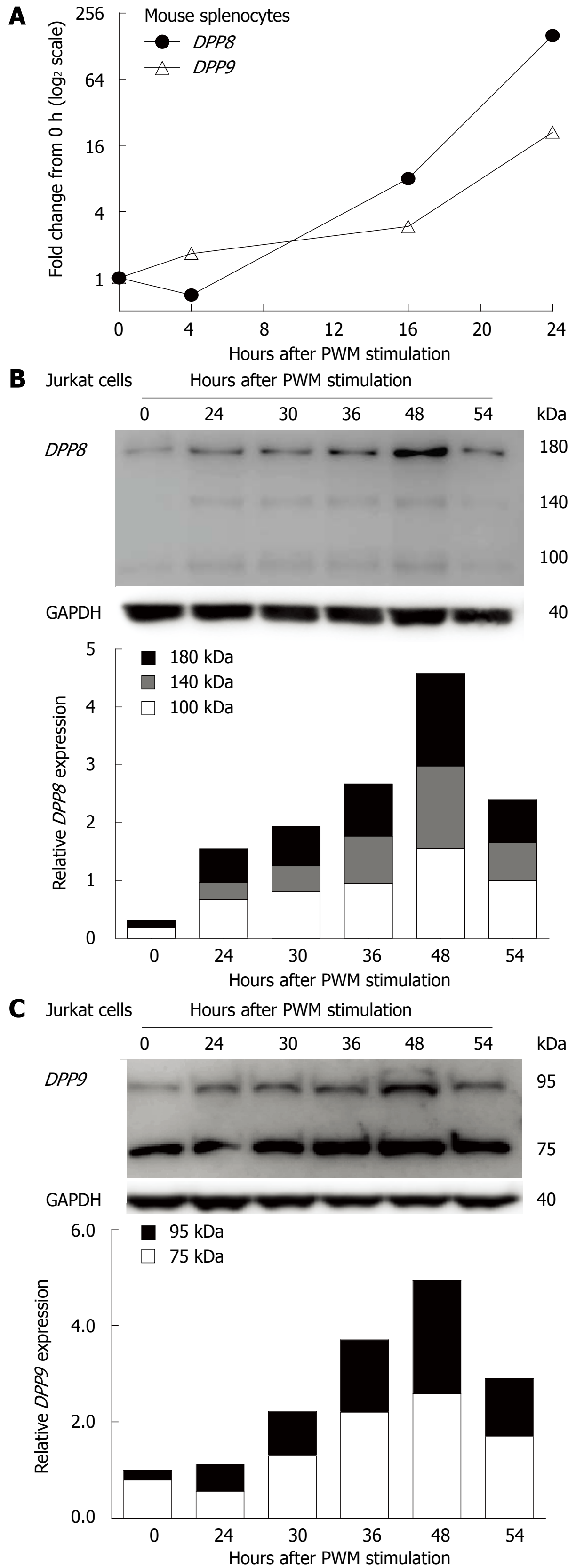

Figure 2 Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 upregulation in pokeweed mitogen stimulated lymphocytes. A: Dipeptidyl peptidase (*DPP*) 8 and *DPP9* mRNA in mouse splenocytes (representative data from one of three mice); *DPP8* and *DPP9* proteins from Jurkat cells; B: Immunoblot of DPP8 and densitometry analysis of *DPP8* bands; C: Immunoblot of *DPP9* and densitometry analysis of bands. Densitometry data shown are relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PWM: Pokeweed mitogen.

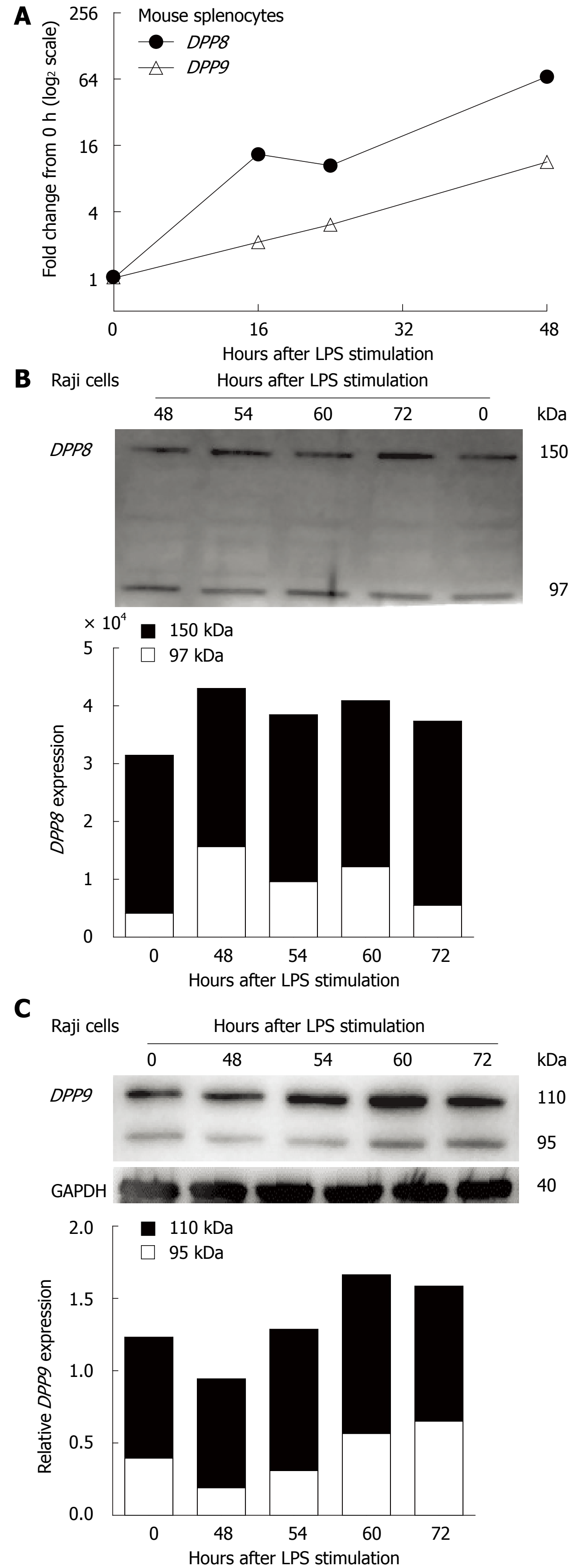

Figure 3 Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 upregulation in lipopolysaccharide stimulated lymphocytes. A: Dipeptidyl peptidase (*DPP*) 8 and *DPP9* mRNA in mouse splenocytes (representative data from one of three mice); B: Immunoblot of DPP8 and densitometry analysis of *DPP8* bands; C: *DPP9* immunoblot and densitometry analysis of *DPP9* bands relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). LPS: Lipopolysaccharide.

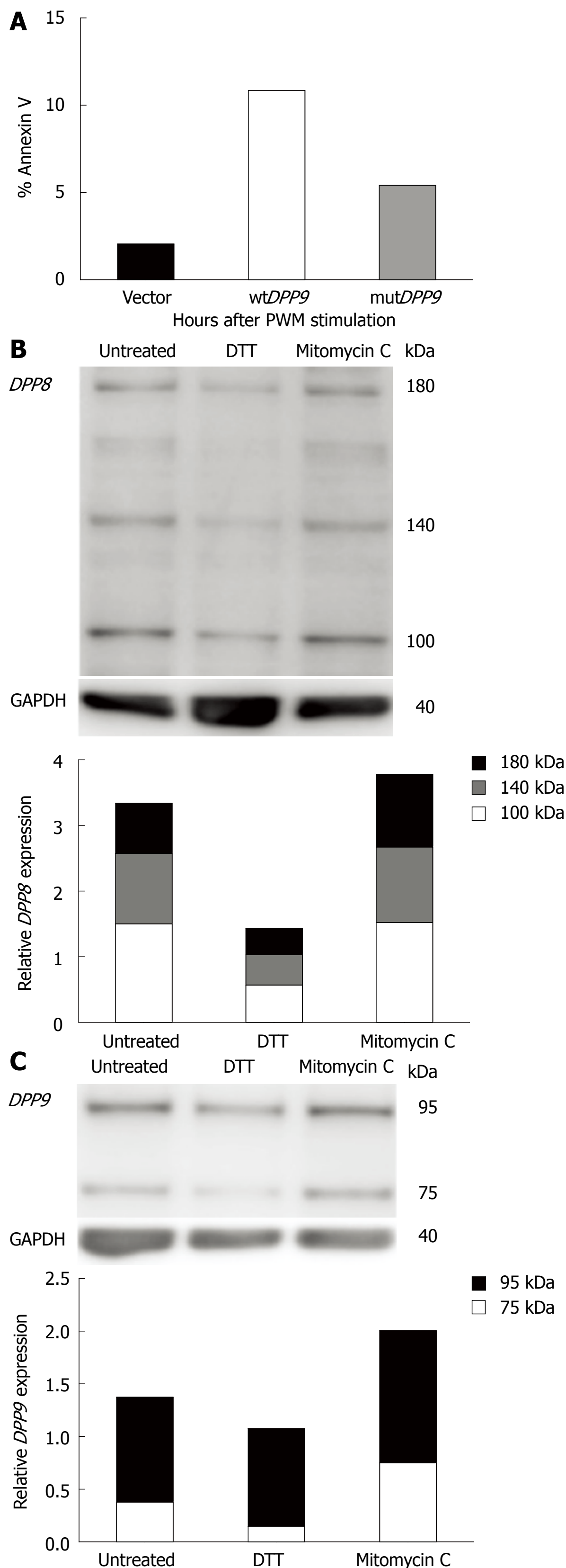

Figure 4 Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 were associated with lymphocyte apoptosis. A: Percentage of annexin V + Raji cells 40 h after transfection with vector, wild type (wt) dipeptidyl peptidase (*DPP*) 9-V5-His or enzyme-negative mutant (mut) *DPP9*-V5-His. Annexin V staining was enumerated by flow cytometry; B: Immunoblot of *DPP8* and its densitometry (C) immunoblot of *DPP9* and its densitometry in Raji cells untreated and treated with dithiothreitol (DTT) or mitomycin C for 24 h. Densitometry are shown as relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

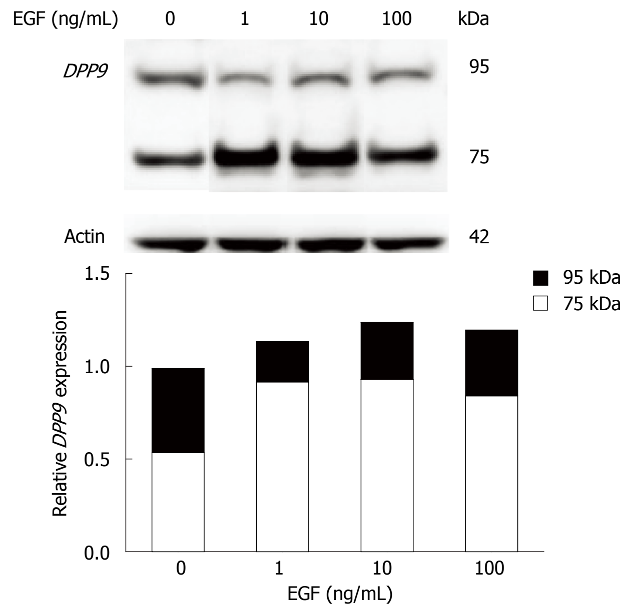

Figure 5 Dipeptidyl peptidase 9 upregulation in epidermal growth factor treated Huh7 cells. Dipeptidyl peptidase (*DPP*) 9 immunoblot of untreated and epidermal growth factor (EGF)-treated Huh7 cells at 4 h. Cells were serum starved overnight before EGF treatment. Densitometry of *DPP9* is shown relative to actin.

study) (Figure 3B), and *DPP9* expression peaked at 60 h (Figure 3C).

DPP8 and DPP9 in lymphocyte apoptosis

We have previously shown that *DPP9* overexpression induces intrinsic cell apoptosis in human hepatoma and embryonic kidney cell lines[39,40]. Similar to epithelial cells, *DPP9* overexpression induced increased cell death in Raji cells (Figure 4A). This effect was less pronounced when Raji cells were transfected with mutant *DPP9* lacking *DPP* activity (Figure 4A), suggesting that the enzyme activity of *DPP9* influences lymphocyte apoptosis.

Interestingly, Raji cells treated with DTT, an antioxidant that impairs cell apoptosis, had less *DPP8* and *DPP9* expression compared to untreated cells (Figure 4B and C). Conversely, treatment of Raji cells with mitomycin C, a lectin that impairs cell proliferation, resulted in increased *DPP8* and *DPP9* expression in Raji cells (Figure 4B and C). Intensities of all *DPP8* and *DPP9* band sizes were less with DTT treatment and greater with Mitomycin C treatment compared to untreated cells (Figure 4B and C).

DPP9 in EGF stimulated hepatocytes

EGF is a regulatory factor in cell survival, growth, proliferation and differentiation[49]. Previously, we have shown that *DPP9* overexpression impairs EGF-stimulated cell proliferation in HepG2 and Huh7 human hepatoma cell lines[40]. *DPP9* expression at 75 kDa was greater in Huh7 cells after EGF stimulation (Figure 5). This study expands the association of *DPP9* with EGF in this hepatoma cell line.

Intrahepatic DPP8 and DPP9 upregulation in CCl_4 induced liver injury

To examine *DPP8* and *DPP9* expression in liver injury,

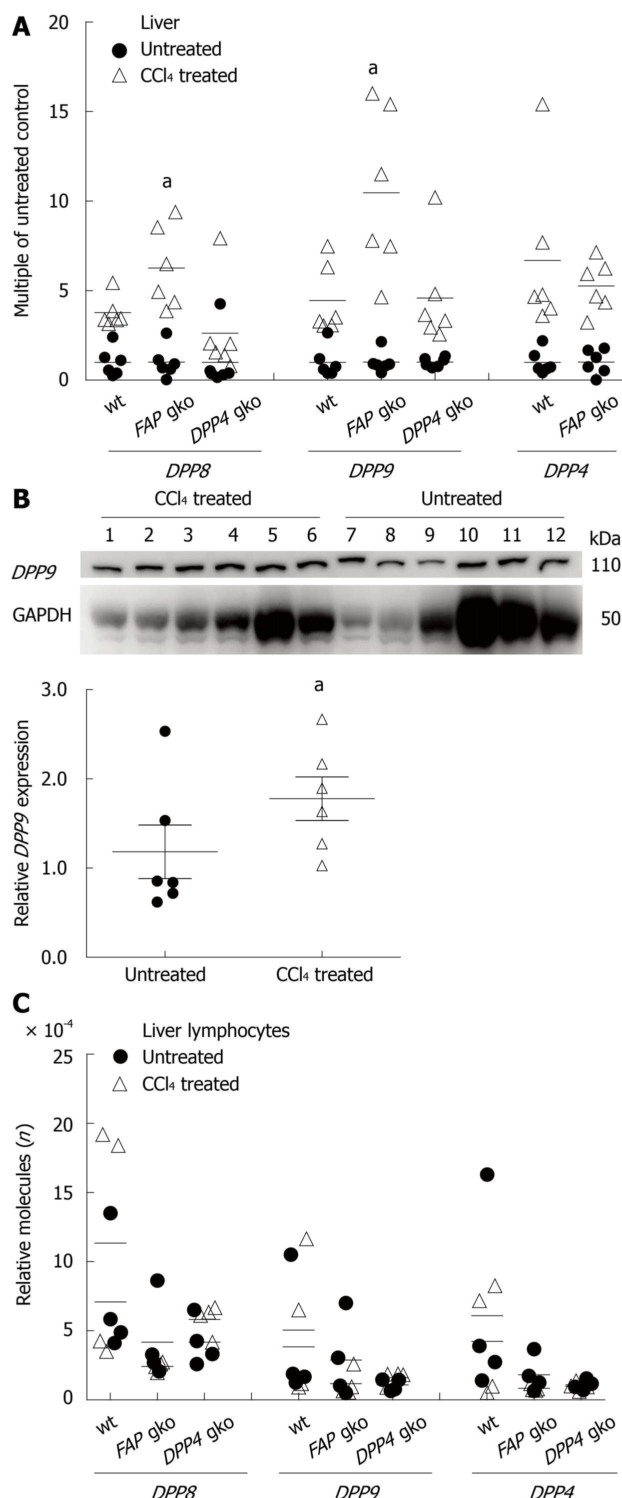

Figure 6 Dipeptidyl peptidase mRNA upregulation in carbon tetrachloride induced liver injury. A: Multiple of intrahepatic mRNA in carbon tetrachloride (CCl_4) treated mice to mean of untreated control mice; [a]P < 0.05 in CCl_4 treated fibroblast activation protein (*FAP*) gene knockout (gko) *vs* wild type (wt); B: Dipeptidyl peptidase (*DPP*) 9 immunoblot of livers from CCl_4 treated (lanes 1-6) and untreated mice (lanes 7-12) (n = 6 per group): Densitometry of intrahepatic *DPP9* relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). [a]P < 0.05 *vs* untreated controls; C: mRNA quantitation from isolated hepatic lymphocytes relative to 18S.

CCl_4 was used to induce liver fibrosis in wt, *DPP4* gko and *FAP* gko mice. *DPP4*, *DPP8* and *DPP9* mRNA were significantly more abundant in the livers from CCl_4 treated mice of all three genotypes compared to the untreated controls (Figure 6A). *DPP8* and *DPP9* mRNA expression in the CCl_4 treated livers were greater in the *FAP* gko mice compared to wt (*DPP8* P = 0.02; *DPP9* P = 0.02), suggesting that *DPP8* and *DPP9* might have compensatory roles in the absence of *FAP* (Figure 6A). The increase in *DPP9* mRNA in the fibrotic livers was consistent with protein expression in wt mice (P = 0.05) (Figure 6B). An appropriate antibody to mouse *DPP8* is not available.

Since *DPP8* and *DPP9* are expressed by human hepatic lymphocytes[13] and because there is an increase of infiltrating lymphocytes in liver fibrosis, we examined whether the mouse hepatic lymphocytes were likely to contribute to the observed upregulation of *DPP* expression. However, *DPP* mRNA in the mouse hepatic lymphocytes was similar in the fibrotic and normal livers (Figure 6C).

Intrahepatic DPP8 and DPP9 downregulation in biliary liver disease

The *Mdr2* gko mouse strain is deficient in the canalicular phospholipid flippase and is a model of periportal biliary fibrosis resembling primary sclerosing cholangitis[41]. These mice develop spontaneous hepatomegaly as early as 2 wk after birth and significant biliary fibrosis with a fivefold increased liver collagen content by 12 wk of age, when no further fibrosis progression occurs[41]. Measuring *DPP*s in these mice at 4, 8 and 12 wk of age showed that *DPP* mRNA expression was surprisingly very low at wk 4, significantly lower than in wt (*DPP8* P = 0.03; *DPP9* P = 0.03; *DPP4* P = 0.03) (Figure 7A). At 8 and 12 wk of age, *DPP* expression levels were similar to wt.

In human end-stage PBC livers, *DPP9* mRNA expression was significantly less than in the non-diseased livers (P = 0.03) (Figure 7B). This finding is consistent with the results in the *Mdr2* gko mice and with the human *DPP9* Western blot data[40]. *DPP8* mRNA expression levels in the non-diseased and PBC livers were not statistically different (P = 0.057).

DISCUSSION

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. We showed that *DPP8* and *DPP9* are widely expressed in lymphocyte subpopulations and upregulated in mitogen activated lymphocytes in a time dependent manner. Besides lymphocyte activation, we demonstrated their potential involvement in lymphocyte apoptosis. In liver, we showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of EGF in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration. The interestingly variable expression patterns of *DPP8* and *DPP9* in different conditions in lymphocytes and in liver injury suggest that these proteases may have important regulatory roles in the immune system and in liver disease pathogenesis.

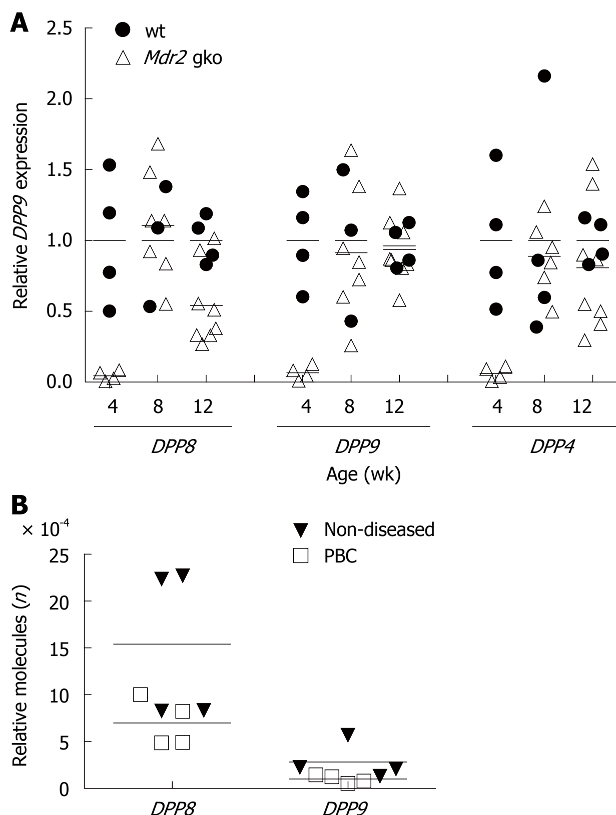

Figure 7 Dipeptidyl peptidase mRNA in mouse and human biliary liver diseases. A: Multiple of Intrahepatic dipeptidyl peptidase (*DPP*) mRNA in multidrug resistance gene 2 (*Mdr2*) gene knockout (gko) female mice to mean of wild type (wt) controls; B: Human end-stage primary biliary cirrhosis (PBC) and non-diseased control livers. Data from each individual is shown as the number of molecules relative to aldolase B (n = 4 per group).

DPP8/9 activity and expression in lymphocytes have been reported previously[8,30,50], but which lymphocyte subpopulations express *DPP8* and *DPP9* remained unknown. Here we show that all the lymphocyte subpopulations tested, $CD4^+$ T cells, $CD8^+$ T cells and $B220^+$ B cells express *DPP8* and *DPP9*. The wide expression of *DPP8* and *DPP9* in lymphocyte subpopulations suggests that these proteases have essential roles in the immune system. As it is now known that immune roles of *DPP4* are mainly extraenzymatic (such as protein-protein interaction), greater abundance of *DPP8* and *DPP9* compared to *DPP4/CD26* in the lymphocytes further supports the hypothesis that the immune effects with non-selective *DPP4* inhibitors in earlier studies were more likely due to *DPP8* and *DPP9* inhibition[2].

We demonstrated a quantitative time-dependent upregulation of *DPP8* and *DPP9* in mitogen-stimulated mouse splenocytes and human Jurkat $CD4^+$ T cells, as well as in polyclonally activated Raji B cells. Therefore, *DPP8* and *DPP9* might have roles in both T and B cell activation. *DPP8* and *DPP9* were upregulated in lymphocytes following acute mitogen stimulation, but with prolonged stimulation, they were downregulated. Hence, the role of *DPP8* and *DPP9* perhaps differ in recently activated lymphocytes compared to persistently activated lymphocytes.

DPP9 enzyme activity induces intrinsic cell apoptosis in epithelial cells through the phosphatidyl inositide-3-kinase/protein kinase B (Akt) signaling pathway[39,40]. Our data on Raji cells suggest that *DPP9* could similarly have a role in intrinsic lymphocyte apoptosis. Moreover the increase in *DPP8* and *DPP9* expression in mitomycin C treated cells is perhaps a hallmark of increased apoptosis in the absence of cell proliferation[51,52]. *DPP9* substrates and ligands involved in these processes have not been identified.

The modulation of *DPP8* and *DPP9* expression with varying lymphocyte activation, proliferation and apoptosis, implies that *DPP8* and *DPP9* have important regulatory roles in lymphocytes that deserve further investigation. Their role in lymphocyte activation is likely to differ from that of *DPP4*. While the role of cell surface *DPP4* in lymphocyte proliferation appears to be mainly extra-enzymatic[22], enzyme inhibition of intracellular *DPP8* and *DPP9* affects lymphocyte proliferation[23]. The observation of less annexin V staining in Raji cells overexpressing *DPP9* enzyme mutant compared to wild type *DPP9* suggests that enzyme activity of *DPP9* is important for its role in apoptosis. *DPP9* modulates Akt phosphorylation in hepatoma cell lines[40], so *DPP8* and *DPP9* might similarly modulate the activity of signaling molecules that are crucial in lymphocyte activation pathways. *DPP8* can cleave several chemokines, stromal cell-derived factor (SDF)-1α, SDF-1β, inflammatory protein 10 and interferon-inducible T-cell alpha chemoattractant, *in vitro*[12], however since *DPP8* is an intracellular protease, the biological relevance of this cleavage is unknown.

The association of *DPP4* and *FAP* with liver fibrosis is well documented[24,53]. Here we have demonstrated possible involvement of *DPP8* and *DPP9* in liver fibrosis, too. Treatment of mice with CCl_4 for 3 wk, which represents early fibrosis with mild hepatic injury, increased intrahepatic *DPP8* and *DPP9* expression. This association with early stage disease may suggest pro-fibrogenic roles of *DPP8* and *DPP9*. Though *DPP*s have been implicated in inflammation and inflammatory diseases[28,29,54], no change in *DPP* expression was observed in hepatic lymphocytes in this early stage fibrosis, suggesting that hepatocytes, which constitute more than 80% of the liver cell population, are probably the major source of upregulated *DPP8* and *DPP9* in this liver fibrosis model.

Unlike the CCl_4 induced liver fibrosis model, *DPP8* and *DPP9* were downregulated in end stage human PBC and in the *Mdr2* gko mice. This suggests that *DPP8* and *DPP9* expression varies with the pathophysiology of liver diseases. The mouse CCl_4 model represents zone 3 fibrosis whereas *Mdr2* gko represents a zone 1 fibrosis model[41,55]. *DPP8* and *DPP9* show a zonal distribution pattern, with stronger staining in zone 3, the periseptal hepatocytes and periportal lymphocytes[13]. Hence, the zonal injury pattern may be important for *DPP8* and *DPP9* expression. Another possibility could be that activated cholangiocytes downregulate *DPP8* and *DPP9* expression. In the *Mdr2* gko mice, *DPP8* and *DPP9* expression was least at week 4, when the cholangiocytes are

most active[41]. Hence, this could be the reason why *DPP8* and *DPP9* expression was downregulated in human PBC and *Mdr2* gko mice.

Alternatively, the differential expression of *DPP* in the different liver diseases could be due to acute *vs* chronic stimuli. CCl_4 induces acute liver injury with hepatocyte damage followed by a repair phase that involves increased collagen deposition[55]. Administration of CCl_4 twice per week for 3 wk leads to repeated cycles of injury and repair that results in fibrosis. We collected liver samples from the CCl_4 treated mice at day 3 after the last CCl_4 injection. At day 3, hepatocyte apoptosis is waning whereas fibrosis is developing[55]. In contrast, the *Mdr2* gko mice and human end stage PBC represent chronic liver injury, whereby there is persistent (mild) hepatocyte damage, a fibrogenic cholangiocyte/progenitor cell response and downregulation of collagenolytic activity resulting in continuing progression of biliary fibrosis until week 12 of age[41]. Thus, our data are consistent with the paradigm that *DPP8* and *DPP9* are upregulated in acute disease states then downregulated with progression to chronic disease states.

This distinctive *DPP* expression pattern in different liver diseases suggests that *DPP8* and *DPP9* have important regulatory roles in the pathogenesis of liver diseases, perhaps in modulating liver regeneration and apoptosis, which are important processes in liver disease progression. *DPP9* impairs EGF-stimulated hepatoma proliferation[40]. Our observation that *DPP9* is upregulated in the presence of EGF is perhaps part of a regulatory mechanism of *DPP9* in hepatocyte proliferation. *DPP9* can induce intrinsic apoptosis in hepatoma cell lines *via* the Akt signaling pathway[40]. Furthermore, *DPP8* and *DPP9* influence cell-extracellular matrix (ECM) interactions *in vitro*[39] and in liver fibrogenesis, cell-ECM interaction is responsible for disrupting wound healing and progressive scarring in liver disease[56].

The upregulated expression of *DPP8* and *DPP9* in acute conditions and less expression in chronic or persistent conditions in the immune system and in liver injury suggests that *DPP8* and *DPP9* are crucial for early cellular responses to stimuli. The mechanisms of *DPP8* and *DPP9* are yet to be elucidated. One obstacle in *DPP8* and *DPP9* studies is the poor availability of appropriate tools, such as monoclonal antibodies and selective inhibitors[57].

In conclusion, our study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in liver fibrogenesis. A better understanding of the biological functions of *DPP8* and *DPP9* could help reveal their therapeutic potential for liver diseases, cancer, inflammatory and autoimmune diseases.

ACKNOWLEDGMENTS

We thank the Royal Prince Alfred Hospital National Liver Transplant Unit for providing human liver samples, Michelle Vo for advice, Adrian Smith and Robert Salomon of the Centenary Institute Flow Cytometry Facility for their expert cell sorting.

COMMENTS

Background

The four enzyme members of the dipeptidyl peptidase (*DPP*) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9* have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes and FAP a potential cancer therapeutic target. *DPP8* and *DPP9* are the more recently discovered members of the *DPP4* gene family. They are ubiquitously expressed cytoplasmic enzymes with *DPP4* like enzyme activity. Many compounds intended to inhibit *DPP4* or *FAP* also inhibit *DPP8* and *DPP9*, but the compounds that became successful diabetes drugs are *DPP4*-selective.

Research frontiers

DPP4 is also known as CD26 T cell differentiation marker and has roles in T cell activation and proliferation. *DPP8* and *DPP9* are in lymphoid tissues and may have functional significance in the immune system. *DPP8* and *DPP9* are expressed in hepatocytes and expression is elevated in damaged hepatocytes near the septum of human cirrhotic liver. However, potential roles of *DPP8* and *DPP9* in liver disease are unknown. Here the authors studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury models to elucidate their potential biological roles in the immune system and in liver diseases. Models included hepatotoxicity from CCl_4, and the multidrug resistance gene 2 knockout mouse that spontaneously develops biliary fibrosis.

Innovations and breakthroughs

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. The authors showed that *DPP8* and *DPP9* were widely expressed in lymphocyte subpopulations and were upregulated in activated lymphocytes in a time dependent manner. The authors also demonstrated potential involvement of *DPP8* and *DPP9* in lymphocyte apoptosis. In liver, the authors showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of epidermal growth factor in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration.

Applications

This study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in chronic liver injury pathogenesis.

Terminology

DPP4 enzyme activity is a specialized proteolytic enzyme activity that cuts two amino acids from the N-terminus of each target peptide, usually cutting after a proline residue; Lymphocyte activation is a cellular process that leads to a radical shift in cell behavior to a more active and proliferative one. The activation of lymphocytes serves two purposes, augmenting the number of cells to respond to a particular antigen (clonal expression), and specializing to produce cytokines, and produce antibodies against a pathogen; cell apoptosis is the process of cell death mediated by an intracellular program. Apoptosis is important for normal cell turnover and organ remodeling.

Peer review

The manuscript deals with regulation of *DPP8* and *DPP9* expression in activated lymphocytes and injured liver. Here the authors focus on the expression levels of *DPP8* and *DPP9* in lymphocyte subpopulations in a time dependent manner. The authors have confirmed the altered expression level of *DPP8* and *DPP9* in liver injury and also confirmed their role in the regulation of epidermal growth factor in hepatocytes. The work has been carefully conducted and the experiments are clearly described in the vast majority of the cases.

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P- Reviewer Shembade ND **S- Editor** Jiang L
L- Editor A **E- Editor** Xiong L

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
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doi:10.3748/wjg.v19.i19.2894

World J Gastroenterol 2013 May 21; 19(19): 2894-2903
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats

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Supported by The Istanbul University Department of Scientific Research Projects, Grant No. 3101
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Received: November 14, 2012 **Revised:** January 23, 2013
Accepted: February 5, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the effects of long term pretreatment with low-, medium- and high-dose aspirin (acetylsalicylic acid, ASA) on a model of acute pancreatitis (AP) induced in rats.

METHODS: Forty male Wistar rats were used. Three experimental groups, each consisting of eight animals, received low- (5 mg/kg per day), medium- (150 mg/kg per day) and high-dose (350 mg/kg per day) ASA in supplemented pellet chow for 100 d. Eight animals, serving as the AP-control group, and another eight, serving as reference value (RV) group, were fed with standard pellet chow for the same period. After pretreatment, AP was induced in the experimental animals by intraperitoneal administration of cerulein (2 × 50 μg/kg), while the RV group received saline in the same way. Twelve hours after the second injection, the animals were sacrificed. Pancreatic tissue and plasma samples were collected. One part of the collected pancreatic tissues was used for histopathological evaluation, and the remaining portion was homogenized. Cytokine levels [tumor necrosis factor, interleukin (IL)-1β, IL-6], hemogram parameters, biochemical parameters (amylase and lipase), nuclear factor-κB, aspirin triggered lipoxins and parameters related to the antioxidant system (malondialdehyde, nitric oxide, hemeoxygenase-1, catalase and superoxide dismutase) were measured.

RESULTS: Cerulein administration induced mild pancreatitis, characterized by interstitial edema (total histopathological score of 5.88 ± 0.44 *vs* 0.25 ± 0.16, $P < 0.001$). Subsequent pancreatic tissue damage resulted in an increase in amylase (2829.71 ± 772.48 *vs* 984.57 ± 49.22 U/L, $P = 0.001$) and lipase (110.14 ± 75.84 U/L *vs* 4.71 ± 0.78 U/L, $P < 0.001$) in plasma, and leucocytes (6.89 ± 0.48 *vs* 4.36 ± 0.23, $P = 0.001$) in peripheral blood. Cytokines, IL-1β (18.81 ± 2.55 pg/μg *vs* 6.65 ± 0.24 pg/μg, $P = 0.002$) and IL-6 (14.62 ± 1.98 pg/μg *vs* 9.09 ± 1.36 pg/μg, $P = 0.04$) in pancreatic tissue also increased. Aspirin pretreatment reduced the increase in the aforementioned parameters to a certain degree and partially improved the histopathological alterations caused by cerulein. No evidence of side effects related to chronic ASA administration (*e.g.*, inflammation or bleeding) was observed in the gastrointestinal tract in macroscopic and histopathological examination.

CONCLUSION: Long term ASA pretreatment could prevent and/or ameliorate certain hematological, serological and histological alterations caused by cerulein-induced AP.

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Key words: Aspirin; Acute pancreatitis; Cerulein; Antioxidant; Cytokines

Core tip: Acute pancreatitis (AP) is an inflammatory and potentially life-threatening disease. An estimated 80000 cases of AP occur each year in the United States. There is no specific cure for AP; therefore, research interest has focused on prevention strategies. In the present study, the effects of a long-term pretreatment with different doses of aspirin, the oldest and most widely used non-steroidal anti-inflammatory drug, were investigated on a AP model in rats. Our results indicated that aspirin pretreatment dose-dependently prevents or ameliorates some hematological, serological and histological alterations caused by cerulein-induced AP.

Akyazi I, Eraslan E, Gülçubuk A, Ekiz EE, Çırakli ZL, Haktanir D, Bala DA, Özkurt M, Matur E, Özcan M. Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats. *World J Gastroenterol* 2013; 19(19): 2894-2903 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2894.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2894

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease with broad clinical variation, ranging from a mild and self-limiting condition to a severe, life-threatening necrotizing inflammation[1,2]. Furthermore, it can lead to the development of systemic inflammatory response syndrome (SIRS) and multisystem organ failure[3,4].

AP may have numerous causes, such as bile duct obstructions, alcohol abuse, metabolic abnormalities, various toxins and infections[5]. One of the aforementioned incidents may trigger pancreatic acinar cell injury and premature activation of pancreatic zymogens[6]. The initial acinar cell damage is followed by local activation of the immune system and induction of transcription factors, such as nuclear factor-κB (NF-κB)[4,7]. Activation of inflammatory cells and transcription factors leads to elaboration of various proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6[8]. If this proinflammatory response to acinar cell damage is balanced by an anti-inflammatory response, the pancreatitis and local inflammation resolve at this stage. However, in some cases, an overwhelming proinflammatory response upsets this balance and the proinflammatory mediators migrate into systemic circulation leading to a generalized inflammation and SIRS[4,8]. In this context, proinflammatory mediators and NF-κB, which play a key role in expression of these mediators, emerge as potential therapeutic targets[6,8].

Acetylsalicylic acid (ASA) exerts analgesic, antipyretic and antiplatelet effects, and is the oldest and most widely used nonsteroidal anti-inflammatory drug[9]. In addition to its conventional effects, ASA has a preventative effect on a wide range of diseases, including gastrointestinal cancer[10], ischemic stroke[11], myocardial infarction[12] and Alzheimer's disease[13]. The anti-inflammatory, analgesic and antipyretic efficacy of ASA is attributed mainly to its inhibitory impact on the enzymatic activity of cyclooxygenases (COX), which convert arachidonic acid to prostaglandins (PGs)[14]. On the other hand, it has been speculated that simple inhibition of PG production cannot fully account for the wide spectrum of effects of ASA[9,15]. Indeed, substantial data have been gathered, indicating that COX-independent mechanisms play a significant role in ASA's efficacy[15]. Kopp *et al*[16] discovered that ASA inhibits NF-κB activation, which is a pivotal transcription factor in cytokine network. NF-κB regulates the expression of proinflammatory enzymes, cytokines, chemokines, immunoreceptors, acute phase proteins and cell adhesion molecules; therefore, it has often been termed a "central mediator of the immune system"[15,17,18]. In this regard, it has been stated that even partial inhibition of NF-κB by ASA could have a substantial effect on inflammation[16]. Another major finding was the discovery that acetylation of COX-2 by ASA can lead to transcellular biosynthesis of a new class of eicosanoids, the 15-epi-lipoxins or so-called aspirin-triggered lipoxins (ATL), which promote the resolution of inflammation[19,20]. Lipoxins have potent counter-regulatory effects *in vivo* and *in vitro* on proinflammatory mediators such as TNF-α, IL-1β, IL-6 and IL-4[19,21,22]. Furthermore it has been speculated that ASA's unique ability to trigger the synthesis of ATLs causes an increase in nitric oxide (NO) synthesis and this aspirin-elicited NO exerts anti-inflammatory effects[23]. Grosser *et al*[24] found that ASA stimulates the expression and enzymatic activity of hemeoxygenase-1 (HO-1) protein in a COX-independent manner. HO-1 is a crucial mediator of the cellular antioxidant defense system and has anti-inflammatory, anti-apoptotic, and antiproliferative effects[25,26]. Recent data[27] elucidated the underlying mechanism of HO-1 expression stimulated by ASA: ATL is mainly responsible for the aforementioned stimulation.

Taken together, this wide spectrum of therapeutic effects of ASA is a consequence of its efficacy in regulating a network of biochemical and cellular events in a more complex manner than was initially thought[9,28].

The significant role of proinflammatory mediators (*e.g.*, TNF-α, IL-1β, IL-6 and platelet activating factor) and transcription factors (*e.g.*, NF-κB and AP-1) in the pathogenesis and complications of AP are well documented in the literature[6,8]. Considering the inhibitory efficacy of ASA on these agents, it would be reasonable to suggest that ASA may be efficient in preventing or at-

Table 1 Grouping and experimental design

Group No.	*n*	Group name	ASA pretreatment (mg/kg)	AP induction
1	8	Reference value	No	No
2	8	Acute pancreatitis control	No	Yes
3	8	Low-dose ASA	5	Yes
4	8	Medium-dose ASA	150	Yes
5	8	High-dose ASA	350	Yes

ASA: Acetylsalicylic acid; AP: Acute pancreatitis.

tenuating AP and its subsequent complications. Furthermore, ASA's antioxidant efficacy exerted *via* the stimulation of HO-1 expression and the anti-inflammatory efficacy of ATL supports and strengthens the aforementioned hypothesis that ASA may be a therapeutic agent for the prevention and/or treatment of AP. However, to the best of our knowledge, there are no studies investigating the preventive and/or therapeutic effects of ASA on AP. Therefore, this study aimed to investigate the effects of ASA pretreatment on experimental AP in rats. By designing an experimental study with a long-term pretreatment, we focused on the preventive effects of ASA, rather than the curative ones, because the multiple and diverse mechanisms of action of ASA seem to be most effective on the initial proinflammatory progress in the pathogenesis of AP.

MATERIALS AND METHODS

Animals and grouping

Studies were performed on 40 male Wistar rats weighing 350-400 g. Animals were housed in polycarbonate cages (four rats/cage) with wood chip bedding and fed standard laboratory chow (supplemented with ASA for treatment groups) and tap water *ad libitum*. They were maintained in a climate-controlled animal room (temperature: 22 ± 3 ℃; relative humidity: 60% ± 5%) with a 12 h/12 h light/dark cycle.

The Istanbul University's Local Ethics Committee approved all the experimental procedures. The animals were randomly allocated to five groups as shown in Table 1.

ASA pretreatment and dosing

Low, medium and high doses of ASA pretreatment were performed as diet supplements for 100 d[29]. Doses of 80 mg/d, 2-4 and 6-8 g/d ASA have been regarded as low, medium and high doses for humans, respectively[30]. Based on average human body weight of 70 kg, these doses correspond to 1.1, 28-56 and 86-114 mg/kg per day, respectively[31]. These human doses were scaled to rats according to Kleiber's rule[32] using the following equation: dose (rat)/dose (human) = $BW^{0.25}$ (rat)/$BW^{0.25}$ (human) (BW= body weight)[33]. Based on the dose intervals derived from the above equation, the following doses were chosen for ASA pretreatment: 5 mg/kg (low-dose), 150 mg/kg (medium-dose) and 350 mg/kg (high-dose). Considering the daily food consumption of rats, standard rat chow material was supplemented with the corresponding amounts of ASA before pelleting to achieve the aforementioned low, medium and high doses. Groups 3-5 were fed these ASA supplemented pellets, while the other groups (Groups 1 and 2) received standard chow during the 100-d pretreatment.

Induction of AP

After pretreatment, all the animals, except those in the reference value (RV) group (Group 1), received two intraperitoneal injections of cerulein in 0.9% NaCl at an hourly interval at a dose of 50 μg/kg to induce AP[34]. Animals in the RV group received injections of the same volume of sterile saline solution (0.9% NaCl) in the same way.

Sample collection and preparation

Twelve hours after the induction of AP, all animals were anesthetized (xylazine/ketamine, 10/75 mg/kg) and exsanguinated *via* cardiac puncture. Blood samples were collected into ethylene diamine tetraacetic acid-coated tubes and plasma samples were separated *via* centrifugation after performing a complete blood count. The plasma samples were aliquoted and frozen at -80 ℃. After sacrificing the animals, necropsies were performed and pancreatic tissues were removed. One part of the pancreas of each animal was used for homogenization, while the remaining portion was fixed in formol-saline (10%) for histopathological examination.

Pancreas samples were homogenized in a 20 mmol/L Tris-HCl buffer (pH 7.4) containing 0.5 mol/L sucrose, 25 mmol/L KCl and 5 mmol/L $MgCl_2$ using a rotor-stator homogenizer. The homogenates were centrifuged at 1000 *g* for 10 min at 4 ℃, and the supernatants containing the cytosolic fraction were removed, aliquoted and frozen at -80 ℃ until assayed. Sedimented pellets containing the nuclear fraction were used to obtain nuclear protein extracts using a commercial protein extraction kit (Intron Biotechnology Inc., Sungnam, South Korea).

Statistical analysis

TNF-α, IL-1β and IL-6 levels were determined in tissue homogenates containing the cytosolic fraction and in plasma samples using commercial enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Camarillo, CA, United States). NF-κB levels were measured in nuclear protein extractions, using a commercial ELISA kit (USCN Life Science Inc., Wuhan, Hubei Province, China).

Catalase (CAT) activities (Cell Biolabs, San Diego, CA, United States), superoxide dismutase (SOD) activities (Assay Designs, Ann Arbor, MI, United States), and malondialdehyde (MDA) levels (Cell Biolabs) were measured in pancreas homogenates and in plasma using commercial test kits. HO-1 levels were determined in pancreas homogenates and plasma using commercial ELISA kits (Assay Designs).

Amylase and Lipase levels in plasma were measured using an automated analyzer (Architect 16200, Abbott,

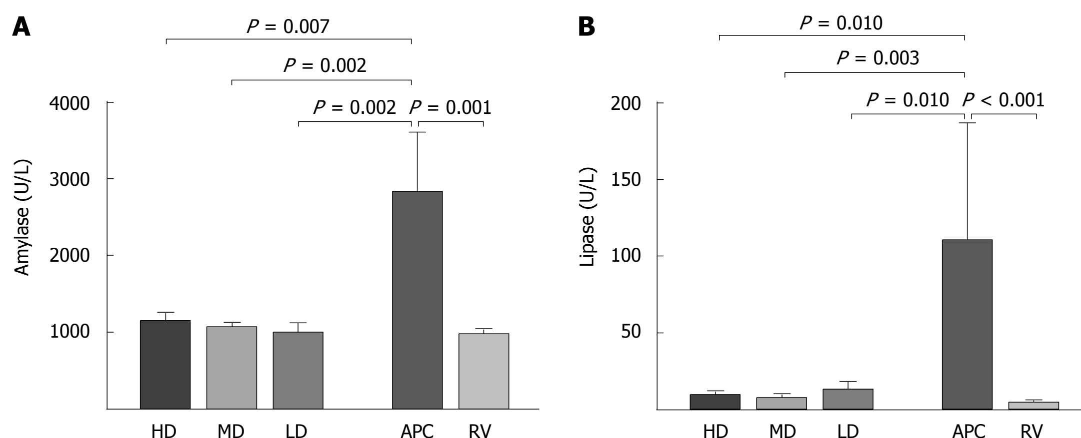

Figure 1 Plasma amylase (A) and lipase (B) activities. Columns show the mean and the error bars represent SEM. All groups are compared with the acute pancreatitis control (APC) group and the statistical significance is expressed as a vertical *P* value over the column. LD: Low-dose; MD: Medium-dose; HD: High-dose; RV: Reference values.

IL, United States). Total NO levels were determined in plasma and pancreas homogenates using commercial test kits (Assay Designs). Plasma ATL levels were measured using commercial ELISA kits (Neogen, Lexington, KY, United States). Total protein contents of homogenates were determined using the method described by Lowry *et al*[35] and all parameters measured in homogenates were proportioned to the total protein content of the homogenate in mg.

Tissue samples fixed in formol-saline were embedded in paraffin blocks, sectioned using a microtome and stained with hematoxylin-eosin. Histopathological scoring was performed as described by Gülçubuk *et al*[36], graded on a score of 0 to 3.

Statistical analysis of the obtained data was performed using the SPSS-software package (Version 11.5.2.1, SPSS Inc., Chicago, IL, United States). Results are expressed as mean ± SEM. Data for all groups were first tested for normality using the Shapiro-Wilk test. Data of groups found to be normally distributed were than compared using one-way analysis of variance. If the normality assumption was found to be violated, data were analyzed using the non-parametric Kruskal-Wallis test. Planned (a priori) contrasts and Mann Whitney *U* tests were used for pairwise comparisons following parametric and non-parametric tests, respectively. The ordinal data of histopathological scoring were analyzed using aforementioned non-parametric tests.

RESULTS

Intraperitoneal administration of cerulein (2 × 50 μg/kg) caused AP in all tested rats, as indicated by the marked increase in serum amylase and lipase levels (Figure 1) and histopathological changes (Figure 2).

Cerulein induced AP caused almost 3- and 23-fold increases in plasma amylase and lipase levels, respectively. ASA pretreatment significantly decreased these levels to close to those of the RV group (Figure 1). Cerulein-induced AP increased the peripheral white blood cell (WBC) count significantly compared to the RV group (6.89 ± 0.48 and 4.36 ± 0.23, respectively, $P = 0.001$). This increase was abolished by medium- and low-dose ASA.

Cytokine levels, lipid peroxidation and WBCs

Columns show the mean and error bars represent SEM in all figures. All groups were compared with the APC group and statistical significance was expressed as a vertical *P* value over the column.

The histopathological scores are shown in Table 2. Marked interstitial edema was observed in the APC group, with a score of 2.75 ± 0.16. In contrast, the edema score of the RV group was 0.25 ± 0.16 and the difference was significant ($P < 0.001$). Concerning the total score (Figure 2), which indicates the overall level of pathological changes, a marked difference was found between the APC and RV group scores (5.88 ± 0.44 and 0.25 ± 0.160, respectively) with a high level of significance ($P < 0.001$). Considering the histopathological scores, ASA pretreatment generally improved the histopathological changes. However, only the effect of the medium-dose was statistically significant ($P < 0.001$ for the total score). No evidence of side effects related to chronic ASA administration (*e.g.*, inflammation or bleeding) for any of the three doses was observed in the gastrointestinal tract by macroscopic and histopathological examinations.

Histopathological scores

All groups were compared with the APC group and statistical significance was expressed as *P* values under the corresponding mean ± SEM values.

As shown in Figure 3A, cerulein-induced AP caused a marked elevation of the IL-1β level in pancreatic tissue compared to that of the RV group (18.81 ± 2.55 pg/μg and 6.65 ± 0.24 pg/μg, respectively, $P = 0.002$). This elevation was suppressed significantly by ASA in all treatment groups. A similar increase was observed in the pancreatic IL-6 level (14.62 ± 1.98 pg/μg *vs* 9.09 ± 1.36 pg/μg, $P = 0.04$, Figure 3B); however, the low-dose could not prevent this increase, whereas the medium- and

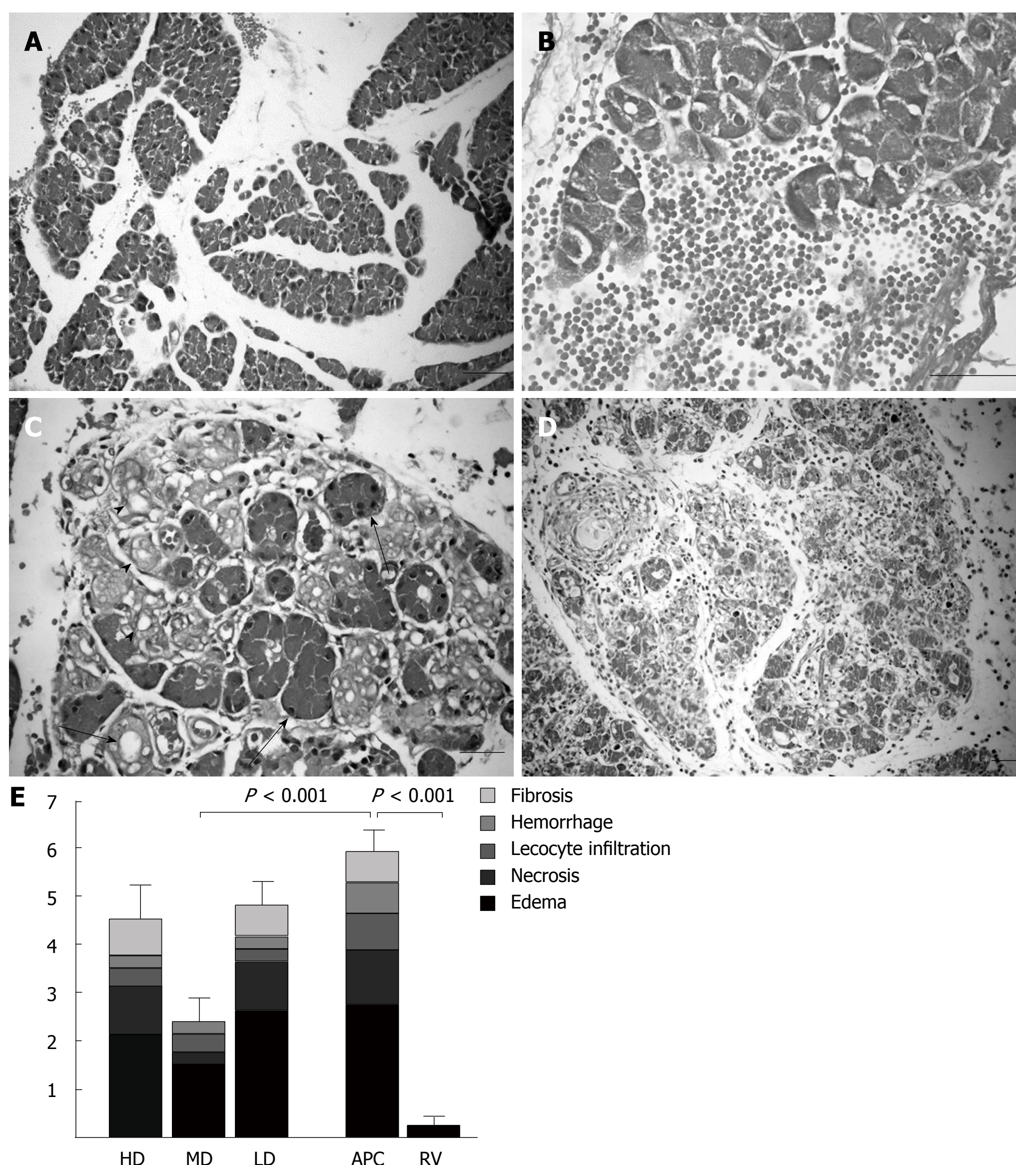

Figure 2 Histopathological alterations in rat pancreas caused by cerulein-induced acute pancreatitis and histopathological scores. A: Pancreatic acini were separated because of interlobular edema in cerulein treated animals (bar = 100 μm); B: Occasionally mild hemorrhages were observed (bar = 50 μm); C: Several acinar cells lost their zymogen granules (arrows) and ductus-like structures (arrowheads) occurred (bar = 50 μm); D: In some animals, leucocyte, fibrocyte and fibroblast infiltrations and collagen bands were detected (bar = 200 μm); E: Histopathological scores of each group are shown as stacked columns representing means. The whole column corresponds to the mean of the total score and the error bars represent the SEM of the total score. All groups are compared with the APC group and the statistical significance is expressed as a vertical *P* value over the column. HD: High-dose; MD: Medium-dose; LD: Low-dose; APC: Acute pancreatitis control; RV: Reference values.

Table 2 Histopathological scores

	HD-ASA (*n* = 8)	MD-ASA (*n* = 8)	*P* value	LD-ASA (*n* = 8)	APC (*n* = 8)	RV (*n* = 8)	*P* value
Edema	2.13 ± 0.30	1.50 ± 0.19	0.002	2.63 ± 0.18	2.75 ± 0.16	0.25 ± 0.16	0.001
Hemorrhage	0.25 ± 0.16	0.25 ± 0.16		0.25 ± 0.16	0.63 ± 0.18	0.00 ± 0.00	0.01
Leukocyte infiltration	0.38 ± 0.18	0.38 ± 0.18		0.25 ± 0.16	0.75 ± 0.16	0.00 ± 0.00	
Necrosis	1.00 ± 0.19	0.25 ± 0.16	0.007	1.00 ± 0.19	1.13 ± 0.13	0.00 ± 0.00	0.001
Fibrosis	0.75 ± 0.25	0.00 ± 0.00		0.63 ± 0.26	0.63 ± 0.18	0.00 ± 0.00	
Total score	4.50 ± 0.68	2.38 ± 0.50	0.001	4.75 ± 0.49	5.88 ± 0.44	0.25 ± 0.16	0.001

ASA: Acetylsalicylic acid; RV: Reference values; APC: Acute pancreatitis control; LD: Low-dose; MD: Medium-dose; HD: High-dose.

high-dose ASA pretreatments could suppress the IL-6 elevation. There were no statistical differences between the groups regarding the TNF-α and NF-κB levels.

Cerulein-induced AP increased MDA levels in both plasma and pancreatic tissue compared to that of the RV group (Figure 3C and D). ASA pretreatment at all three

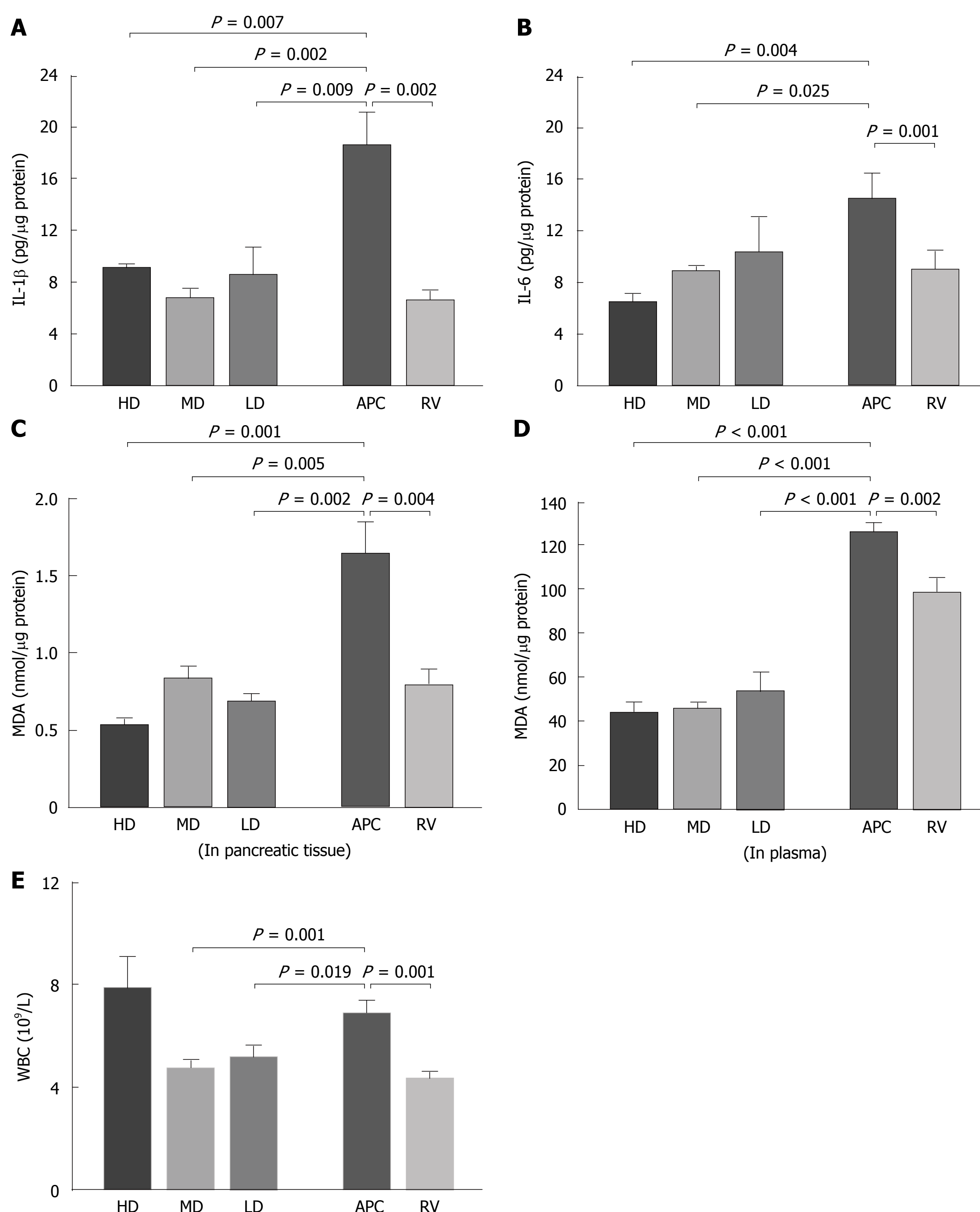

Figure 3 Cytokine levels, lipid peroxidation and white blood cells. A: Cerulein induced acute pancreatitis (AP) caused a marked elevation of interleukin (IL)-1β level in pancreatic tissue compared to that of the reference values (RV) group (18.81 ± 2.55 and 6.65 ± 0.24 pg/μg, respectively, P = 0.002); B: This elevation was suppressed significantly by acetylsalicylic acid (ASA) in all treatment groups. A similar increase was observed in the pancreatic IL-6 level. However, this time, the low-dose seemed to be ineffective against it, while medium- and high-dose ASA pretreatments suppressed the IL-6 elevation. There was no statistical difference between the groups regarding the tumor necrosis factor-α and nuclear factor-κB levels; C, D: Cerulein induced AP increased malondialdehyde (MDA) levels in both pancreatic tissue and plasma compared to that of the RV group; E: Cerulein induced AP increased the peripheral white blood cell (WBC) count significantly compared to the RV group. HD: High-dose; MD: Medium-dose; LD: Low-dose; APC: Acute pancreatitis control.

doses inhibited this increase. Cerulein-induced AP increased the peripheral WBC count (Figure 3E). Other antioxidant system parameters, including NO, SOD, HO-1 and CAT, were not affected by cerulein-induced AP and ASA treatment; there were no significant differences between the treatment, APC and RV groups regarding these parameters.

DISCUSSION

To the best of our knowledge, the present study is the first investigation of the effect of long-term ASA pretreatment on a cerulein-induced pancreatitis model. Our findings indicate that long term ASA pretreatment dose-dependently prevents or ameliorates certain hematologi-

cal, serological and histological alterations caused by cerulein-induced AP.

Cerulein-induced pancreatitis is the most preferred animal model of AP, because it is non-invasive, easily applicable and highly reproducible[37]. The similarity of the cerulein-induced histopathology to human AP, especially in the early phase, has substantially increased the preference for this model[38]. Administration of cerulein, a cholecystokinin (CCK) analog, stimulates the pancreatic acinar cells *via* CCK receptors, which leads to prematuration of proteolytic enzymes[39]. The activation of proteases triggers an autodigestion of pancreatic tissue, causing vascular damage, edema, fibrosis and necrosis, which constitute the histopathological profile of AP[6]. The markedly higher edema, hemorrhage, necrosis and the total histopathological scores of the APC group compared to the RV group, observed in our present study, are the expected results of the cerulein-induced AP model and are consistent with the literature.

The serum amylase level has been the most widely used parameter for the diagnosis of AP[40], since 1929, when its diagnostic value was demonstrated for the first time[41]. The serum lipase level, another widely accepted marker of AP, rises after the onset of AP in parallel with the amylase level, although its rise starts slightly later and lasts longer than that of amylase[42]. The plasma levels of both enzymes have substantial sensitivity and specificity for the diagnosis of AP[43]. As expected, in the present study, both the amylase and lipase levels rose markedly in the AP group. In the pretreatment groups, ASA prevented the elevation of both enzyme levels. This observation constitutes additional evidence supporting the preventive effect of ASA against cerulein-induced AP.

There is a positive correlation between the severity of AP and the increase in the peripheral WBCs[44] and the WBC count is one of the parameters included in most of the scoring systems used for the assessment of the severity of AP[45]. The increased WBC in the AP group is an expected result of the inflammation induced by cerulein. Our observation that the WBC count of medium- and low-dose groups was significantly lower than that of the AP group and close to that of the RV animals, suggests that ASA pretreatment ameliorates the inflammation induced in the pancreas.

Cytokines are a group of low-molecular weight proteins that play a crucial role in induction and progression of inflammatory processes, including AP. Thus, they have been subjected to a wide range of studies in this context[8,46,47]. Consequently there is no doubt about the constitutive role of many cytokines in progression of local tissue damage and distant complications in AP[46].

Cytokines can be functionally divided into two groups: pro- and anti-inflammatory cytokines[46]. In the proinflammatory group TNF-α and IL-1β are especially prominent and are regarded as "first-line" cytokines[48]. IL-1β levels are elevated in the cerulein-induced models of AP[4,8,49]. Furthermore, a strong, positive correlation was found between the increase in IL-1β level and the severity of inflammation[46,50]. In the present study, the IL-1β level in pancreatic tissue increased nearly threefold in the AP group compared with the RV group, whereas the difference between plasma levels showed no statistical significance. This rise in the IL-1β level in the AP group is an expected result of cerulein-induced AP. In addition, the contrast observed between the tissue and plasma levels of IL-1β is consistent with previous reports[50,51]. Considering the tissue levels of IL-1β in the pretreatment groups, ASA pretreatment had a significant diminishing effect. This finding is consistent with the amylase, lipase and MDA levels. Thus, this represents further evidence of the protective effect of ASA pretreatment against AP. IL-6 is another proinflammatory cytokine that increases in cerulein-induced pancreatitis[52]. IL-6 levels correlate with the clinical scenario and severity of AP; therefore, IL-6 has been attributed as a marker of the disease[4,53]. Thus, the increase in the IL-6 level in the APC group in the present study is an expected result of cerulein-induced AP. ASA pretreatment in the medium- and high-dose groups decreased the pancreatic IL-6 level significantly. This effect is consistent with the other findings of our study. The numerical decrease in the low-dose group was not statistically significant.

Sanfey *et al*[54] suggested a possible involvement of reactive oxygen species (ROS) in the pathogenesis of AP and since then, observations from increasing numbers of experimental studies have supported this suggestion[55,56]. Consequently, there is currently no doubt about the detrimental role of oxidative stress in the pathogenesis of AP and this makes it a therapeutic target. Yu *et al*[52] reported that, in the cerulein-induced AP model, administration of cerulein increased ROS formation and oxidative stress, and caused an increase in IL-1β expression. In the present study, the MDA level, an indicator of oxidative stress, was elevated in the cerulein administered AP group, both in plasma and in pancreatic tissue. This high level of MDA in the AP group, taken together with increased pancreatic IL-1β expression, is a consequence of cerulein-induced AP and these data are consistent with the findings of Yu *et al*[52]. ASA reduces oxidative stress by exerting free radical scavenging activity and antioxidant efficacy[57-60]. The findings of Shi *et al*[61] support ASA's free radicals scavenging efficacy and also suggest that it is more potent than several well established antioxidants, such as ascorbate, glutathione and cysteine, with respect to the reaction rate. In the present study, oxidative stress, indicated by the MDA levels, decreased in all the ASA treated groups compared with the AP-induced groups, in both the plasma and pancreatic tissue homogenates. Moreover, considering the plasma, these levels were even below the levels of the reference group, which was fed with commercial diet without ASA supplementation. These findings can be explained by potent antioxidant effect of ASA and are in accordance with the other results presented in this study. Nevertheless, parameters related to the enzymatic antioxidant system, including NO, SOD, HO-1 and CAT, showed no significant changes. Thus,

the reduced oxidative stress induced by ASA in the treatment groups seems not to involve the classic enzymatic antioxidant system and could be attributed to alternative mechanisms[60].

When examining the histopathological scores numerically, the ASA pretreatment generally attenuated the alterations caused by AP. Nevertheless, these numerical changes could not be confirmed statistically for all treatment doses or for all histopathological parameters. Only the reducing effect of the medium dose (150 mg/kg) on edema, necrosis and total score values was found to be statistically significant (P = 0.002, P = 0.007 and P < 0.001, respectively). These histopathological scoring results seem to be inconsistent with the previously discussed data for amylase, lipase, MDA and IL-1β, which were reduced significantly by ASA pretreatment at all three doses. Although there is evidence showing that serum amylase and lipase levels do not correlate with the histopathological alterations and severity of AP[62-64], we believe the aforementioned inconsistency resulted from the high variance of our data set and the small sample size and should be considered as a limitation of our study.

The analgesic, antipyretic, antiplatelet and antiinflammatory effects of ASA have been known for a long time, and most of these effects have been attributed to its COX-inhibitory activity[14]. However, it has been speculated that the therapeutic potential of ASA cannot be completely explained by COX inhibition[15,65]. Previous results from a wide variety of studies revealed different mechanisms of action of ASA, including inhibition of proinflammatory cytokines, such as IL-1β[65], scavenging of ROS[59], triggering the production of antiinflammatory mediators, such as ATL[21,22], and inhibition of transcription factors, such as NF-κB[66].

In conclusion, our findings indicate that the ASA pretreatment exerted preventive and/or ameliorating effects against AP by normalizing some of the hematological and biochemical indicators of the disease to close to those of the reference value group. These beneficial effects of the pretreatment were confirmed partially by the histopathological findings.

Our findings suggest that these beneficial effects of ASA can be explained by its free radical scavenging efficacy and the inhibitory effect on proinflammatory cytokines IL-1β and IL-6. The ATL pathway, involving the stimulation of NO and HO-1 expression, seemed not to play a role in this preventive effect, as there was no difference between groups with respect to the ATL levels.

Beside its conventional use as an analgesic, antiinflammatory and antipyretic agent, daily intake of ASA is recommended by a large group of physicians as a preventive therapy against cardiovascular diseases[67,68]. Furthermore, several studies have indicated the efficacy of long-term ASA use in prevention of colorectal cancer[69], and the long-term use of ASA as a chemopreventive agent against other cancer types has attracted substantial research interest[70]. Our results may provide another perspective on the effects of long-term ASA pretreatment.

ACKNOWLEDGMENTS

We thank Turgay Cakmak, Gizem Kutun, Dilara Yilmaz and Hayriye Guler Tarinci for their assistance in laboratory work.

COMMENTS

Background

Acute pancreatitis (AP) is an inflammatory disease with broad clinical variation, ranging from a mild and self-limiting condition to a severe, life-threatening necrotizing inflammation. Aspirin (acetylsalicylic acid, ASA) is the oldest and most widely used non-steroidal anti-inflammatory drug. In addition to its conventional effects, ASA is effective in the prevention of a wide range of diseases, including gastrointestinal cancer, ischemic stroke, myocardial infarction and Alzheimer's disease. This broad range of effectiveness has led to the daily intake of aspirin being recommended by a wide group of physicians as a preventive therapy for the aforementioned diseases. Considering the early events in pathophysiology of AP and the broad variety of aspirin's mechanisms of action, it is reasonable to hypothesize that long term aspirin pretreatment can effectively prevent AP.

Research frontiers

An estimated 80000 cases of AP occur each year in the United States. Much research interest has focused on prevention strategies because there is no specific cure for AP.

Innovations and breakthroughs

In the present study, the effects of ASA pretreatment on a pancreatitis model were investigated for the first time. The findings indicate that long term ASA pretreatment dose-dependently prevents or ameliorates some hematological, serological and histological alterations caused by cerulein-induced AP.

Applications

The experimental data obtained in the present study point out another aspect of aspirin's preventive effectiveness and could be used in further studies of preventive strategies against AP.

Peer review

This is a very interesting, well-structured and original study, being the first reported study on this topic. It is methodologically well planned and performed with well-designed cohorts. The paper is well written and clear.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2904

World J Gastroenterol 2013 May 21; 19(19): 2904-2912
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa

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Received: November 5, 2012 **Revised:** December 13, 2012
Accepted: January 11, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the role of growth hormone (GH), hyperbaric oxygen therapy (HBOT) and combined therapy on the intestinal neomucosa formation of the gastric serosa.

METHODS: Forty-eight male Wistar-albino rats, weighing 250-280 g, were used in this study. The rats were divided into four groups (n = 12): Group 1, control, gastric serosal patch; Group 2, gastric serosal patch + GH; Group 3, gastric serosal patch + HBOT; and Group 4, gastric serosal patch + GH + HBOT. Abdominal access was achieved through a midline incision, and after the 1-cm-long defect was created in the jejunum, a 1 cm × 1 cm patch of the gastric corpus was anastomosed to the jejunal defect. Venous blood samples were taken to determine the insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP-3) basal levels. HBOT was performed in Groups 3 and 4. In Groups 2 and 4, human GH was given subcutaneously at a dose of 2 mg per kg/d for 28 d, beginning on the operation day. All animals were sacrificed 60 d after surgery. The jejunal segment and the gastric anastomotic area were excised for histological examination. The inflammatory process, granulation, collagen deposition and fibroblast activity at the neomucosa formation were studied and scored. Additionally, the villus density, villus height, and crypt depth were counted and recorded. The measurements of villus height and crypt depth were calculated with an ocular micrometer. New vessel growth was determined by calculatingeach new vessel in a 1 mm^2 area.

RESULTS: In the histological comparison of groups, no significant differences were observed between the control group and Groups 2 and 3 with respect to epithelialization, granulation, fibroblastic activity and the inflammatory process, but significant differences were present between the control group and all others groups (Groups 2-4) with respect to angiogenesis (P < 0.01) and collagen deposition (P < 0.05, P < 0.01). Significant differences between the control group and Group 4 were also observed with respect to epithelialization and fibroblastic activity (P < 0.01 and P < 0.05, respectively). There were significant differences in villus density in all of groups compared with the control group (P < 0.05). Crypt depth was significantly greater in Group 4 than in the control group (P < 0.05), but no other groups had deeper crypts. However, villus height was significantly longer in Groups 2 and 4 than in

the control group ($P < 0.05$). The comparison of groups revealed, significant difference between control group and Groups 2 and 4) with respect to the levels of IGF-1 and IGFBP-3 ($P < 0.01$) 3 wk after the operation.

CONCLUSION: HBOT or GH and combined therapy augmented on neomucosal formation. The use of combined therapy produced a synergistic effect on the histological, morphological and functional parameters.

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Key words: Growth hormone; Hyperbaric oxygen; Neomucosa; Short bowel syndrome; Hypoxia

Adas G, Adas M, Arikan S, Sarvan AK, Toklu AS, Mert S, Barut G, Kamali S, Koc B, Tutal F. Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa. *World J Gastroenterol* 2013; 19(19): 2904-2912 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2904.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2904

INTRODUCTION

Short bowel syndrome (SBS) is a significant problem in clinical medicine that emerged at the beginning of the last century, when the first resections of the gastrointestinal tract were performed[1]. SBS a malabsorptive disorder characterized by the loss of intestinal length, occurs when patients have < 200 cm of the post-duodenal small intestine, resulting in inadequate digestion and/or nutrient absorption[2-5]. Depending on the extent, degree, and location of the intestinal resection, patients may experience severe malabsorption of fluids, electrolytes, and other nutrients. Many become dependent on long-term parenteral nutrition, which has been a life maintenance therapy[6]. Even in the best hands, this treatment can be associated with nutritional deficiencies, septic complications and life-threatening organ failure[7-9]. Another important factor is the time allowed for post-enterectomy or in utero bowel loss adaptation, which is the compensatory process in the remnant small intestine that includes mucosal regeneration, villous hypertrophy, bowel dilatation and lengthening, and delayed motility[10]. Growth hormone (GH), glutamine, and dietary modification have been proposed as a regimen to enhance bowel adaptation[3,6,7]. GH administration produced a positive nitrogen balance at all levels of energy intake[4,5,11,12]. Evidence supporting the use of GH in the SBS includes the observation that exogenous GH stimulates structural and functional intestinal adaptation[13,14].

The treatment consists of surgery to slow intestinal transit or increase the area of absorption. Reconstructive procedures on the remnant bowel and intestinal transplantation are areas of special interest to surgeons working in this field[4,10,15]. Another potential technique for increasing the intestinal surface area is the growth of new intestinal mucosa, which takes advantage of the regenerative capability of the intestine[10]. Some researchers made gastric anastomosis and, colonic and abdominal wall flaps between intestinal defects in experimental SBS to expand the mucosal surface. The regenerated intestine develops by lateral ingrowth from the surrounding mucosa and becomes functionally normal intestinal mucosa[16-19].

In gastrointestinal surgery, if the degree of hypoxia is sufficient to interfere with tissue viability, the tissues become necrosed, resulting in delayed wound healing[20]. Ischemic wounds heal poorly and become infected. Tissue hypoxia can be reversed using hyperbaric oxygen therapy (HBOT). The effects of HBOT result from increased pressure and hyperoxia. Several studies have shown that increased oxygen tension with HBOT not only prevents the adverse effects of ischemia but also accelerates healing in different types of wounds[21].

The serosal patch technique is one of the most popular methods. However, in many cases, only short segments of the small intestine can be patched because of the limited serosal surface and anatomical factors. In this experimental study, we used a gastric serosal patch to form neomucosa in ileum defects. Our aim in the present work was to investigate the role of GH, HBOT and combined therapy on the intestinal neomucosa formation of the gastric serosa.

MATERIALS AND METHODS

Animals

Forty-eight male Wistar-albino rats (Istanbul University, Institute of Experimental Medicine and Research, Turkey), weighing 250-280 g, were used in the study. The study was approved by the ethics committee of Istanbul University, Istanbul Medical School. All animals were housed incages in a room at a constant temperature of 22 ± 2 ℃. The rats were fed a standard chow diet and tap water.

Study design

The rats were divided into four groups (n = 12): Group 1, control, gastric serosal patch; Group 2, gastric serosal-patch + GH; Group 3, gastric serosal patch + HBOT; Group 4, gastric serosal patch + GH + HBOT.

Surgical procedure

After one night of fasting, the animals were anaesthetized with an intramuscular injection of ketamine hydrochloride (50-100 mg per kg of body weight). Abdominal access was achieved through a midline incision, and the jejunum was incised at 1 cm longitudinally. After the 1-cm-long defect was created in the jejunum, a 1 cm × 1 cm patch of the gastric corpus was anastomosed tothe jejunal defect with interrupted 6/0 polypropylene sutures (Figure 1A and B). During the operation, venous blood samples (portal vein) were taken to determine the insulin-like growth factor 1 (IGF-1) and insulin like growth factor binding protein 3 (IGFBP-3) basal levels. After the bleeding control had been performed, 2 cc of 0.9% NaCl was injected into the intraperitoneal area, and the abdo-

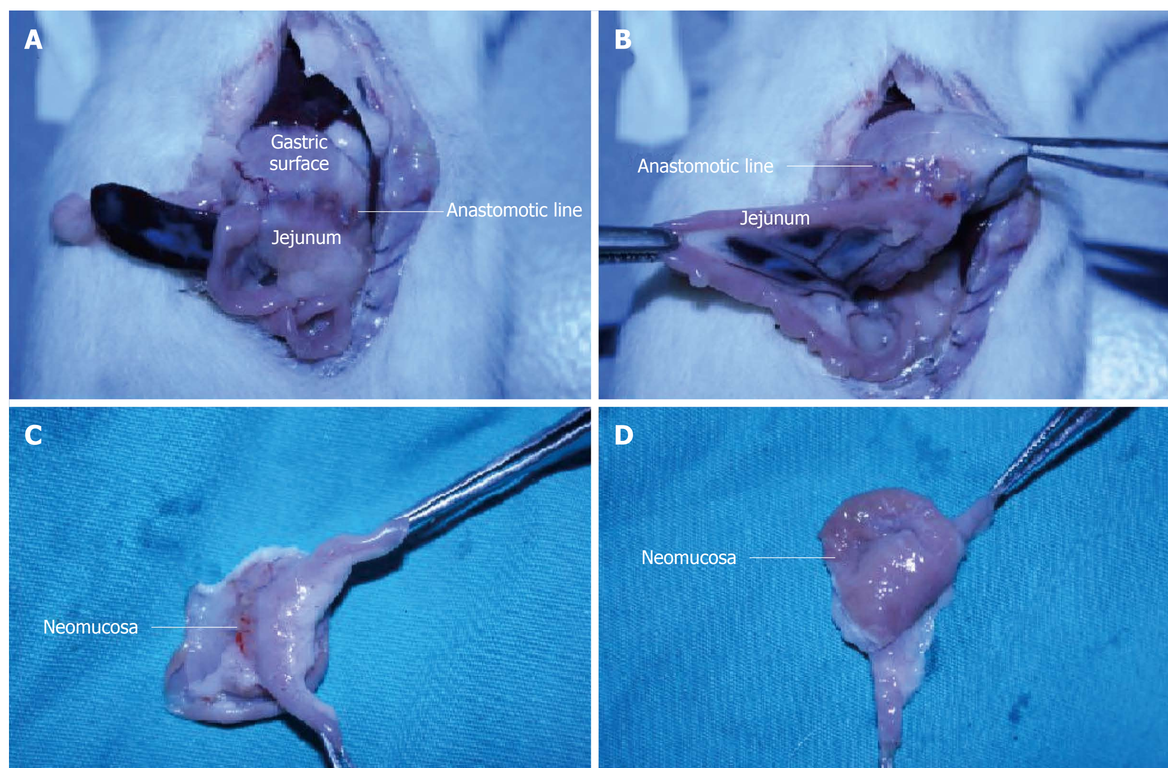

Figure 1 Surgical procedure and histopathological assessment. A, B: Anastomotic line is shown between the gastric surface and jejunum, C: Outer surface of the neomucosa formation is shown with the jejunal segment; D: Inner surface of the neomucosa is shown, and the neomucosa has a typical small intestinal phenotype.

men was closed with 3/0 continuous silk sutures. Twelve hours after the surgery, water was given; twenty-four hours after the surgery, food was given.

Treatment

HBOT was started 12 h after the surgery and completed after 12 d. The HBOT was performed 3 times per day for the first 4 d, 2 times per day for the following 4 d and then once per day for the remaining 4 d. This therapy was applied in the Istanbul Faculty of Medicine, Department of Underwater and Hyperbaric Medicine. The total number of HBOT sessions was 24. The treatments were conducted in a small research chamber (0.4 m^3). The chamber was flushed with oxygen for 10 min to vent the air inside before compression, and thus the animals could be pressurized with 100% oxygen. The HBOT sessions consisted of 10 min of compression to 2.5 atmosphere absolute (ATA), 60 min at 2.5 ATA and 10 min of decompression to the surface pressure.

In Groups 2 and 4, human GH was given [Norditropin 4 IU (1.3 mg), Novo Nordisk-Denmark] subcutaneously at a dose of 2 mg per kg/d[22-25] for 28 d, beginning on the operation day.

Histopathological assessment

All of the rats were sacrificed 60 d after the operation. The anastomosis, including the jejunal segment and the gastric anastomotic area, was excised (Figure 1C and D). To clean the fat tissues, the anastomotic area was washed with distilled water. The edges of the anastomotic area were determined by following the subject line of the non-absorbablesuture. Then, the area was fixed in 10% formalin for approximately 24 h and embedded in a paraffin block. Transverse sections of the embedded tissue, 4 μm in thickness, were stained with hematoxylin and eosin, and the histological assessment was performed in a blinded manner. The intestinal neomucosa, inflammatory process, granulation and fibroblast activity at the neomucosa formation were studied and scored (from 0 to 3; 0, none; 1, slight; 2, moderate; 3, dense). Masson's trichrome staining was performed to distinguish the cells from the surrounding connective tissue. Three dyes were employed, and solution C was used to stain for collagen. Collagen deposition in the neomucosa was scored according to the density in the tissue (from 0 to 3; 0, none; 1, slight; 2, moderate; 3, dense). Small intestinal epithelial cell lineages (goblet cells and enteroendocrine cells) were identified within the regenerated intestinal mucosa. The goblet cells were stained by the periodic acid-Schiff stain, andmucin was identified with alcian blue staining, pH 2.5. The alcian blue staining at pH 2.5 was used because the acid mucins of the small intestine are primarily sialo mucins[26]. The enteroendocrine cells were identified by immune histochemicalstaining.

Additionally, the villus density, villus height, and crypt depth were determined and recorded. The villus density was scored (from 0 to 3). The measurements of villus height and crypt depth were calculated with an ocular micrometer. New vessel growth was determined by calculating each new vessel in a 1 mm^2 area.

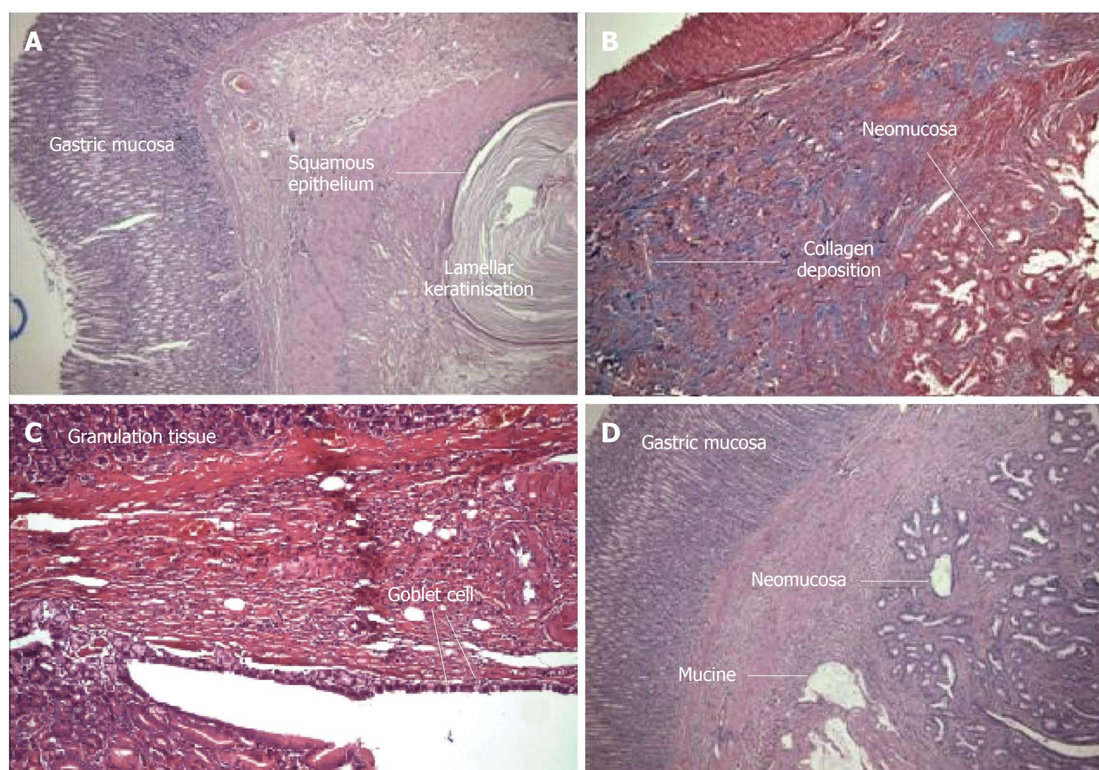

Figure 2 Histological and morphologic evaluation. A: Unexpected neomucosal formation. The gastric corpus mucosa can be seen. The squamous epithelium and lamellar keratinization formed from the anastomosis [Hematoxylin and eosin (HE) × 100] (Group 3); B: Granulation tissue and newly formed neomucosa. The blue area is connective tissue (Masson Trichrome × 100) (Group 4); C: In the gastric mucosa of the large granulation tissue, newly formed goblet cells can be seen (HE × 100) (Group 4); D: The left side shows the gastric mucosa, and the right side shows newly formed neomucosa that contains mucin. The granulation tissue is reduced (HE × 100) (Group 4).

GH assessment

To determine the GH efficacy, we assessed the venous IGF-1 and IGFBP-3 levels at the beginning of treatment and on postoperative day 21. Blood samples were taken from the tail vein of the rats. The analyses were performed in a biochemistry laboratory, at the Cerrahpasa Medical Faculty, University of Istanbul. The levels of IGF-1 and IGFBP-3 were measured by double-antibody, immune-radiometric assays. The IGF-1 antibody was from Immunotech France, and the IGFBP-3 antibody was from Diagnostic Systems Laboratories. The mean intra batch coefficients of variation calculated from the quality-control samples in this study were 5.6% and 2.7% for IGF-1 and IGFBP-3, respectively.

Statistical analysis

The statistical analysis was performed using SPSS 16.0 for Windows. Spearman's test was used for the intergroup correlations. Differences in the histological parameters between the treatment and control groups were analyzed non-parametrically with the Student-*t* test. All data were expressed as the mean ± SD, and $P < 0.05$ was accepted as significant.

RESULTS

Mortality analysis

Seven rats died in the early phase (in the first week) of the study. Upon post-mortem examination, we discovered that two had anastomotic leakage and sepsis, two had ileus, and three had pneumothorax.

Histological evaluation

In the histological comparison of the groups, no significant differences were observed between the control group and Groups 2 and 3 with respect to epithelialization, granulation, fibroblastic activity and the inflammatory process ($P > 0.05$), but significant differences were observed between the control group and all other groups (Groups 2-4) with respect to angiogenesis ($P < 0.01$) and collagen deposition ($P < 0.05$, $P < 0.01$) (Figure 2). We also found significant differences between the control group and Group 4 with respect to epithelializationand fibroblastic activity ($P < 0.01$ and $P < 0.05$, respectively). Two histological parameters were significantly different in Groups 2 and 3, and four histological parameters were significantly different in Group 4. These parameters in Group 4 were epithelialization, fibroblastic activity, angiogenesis and collagen deposition. The histological features and results are given in Table 1.

Morphologic evaluation

For the morphological evaluation, we measured the villus density (mm^2), villus height and crypt depth in the neomucosa. By 60 d, the luminal surface of the neomucosa tissue was nearly covered by mucosal epithelium. The

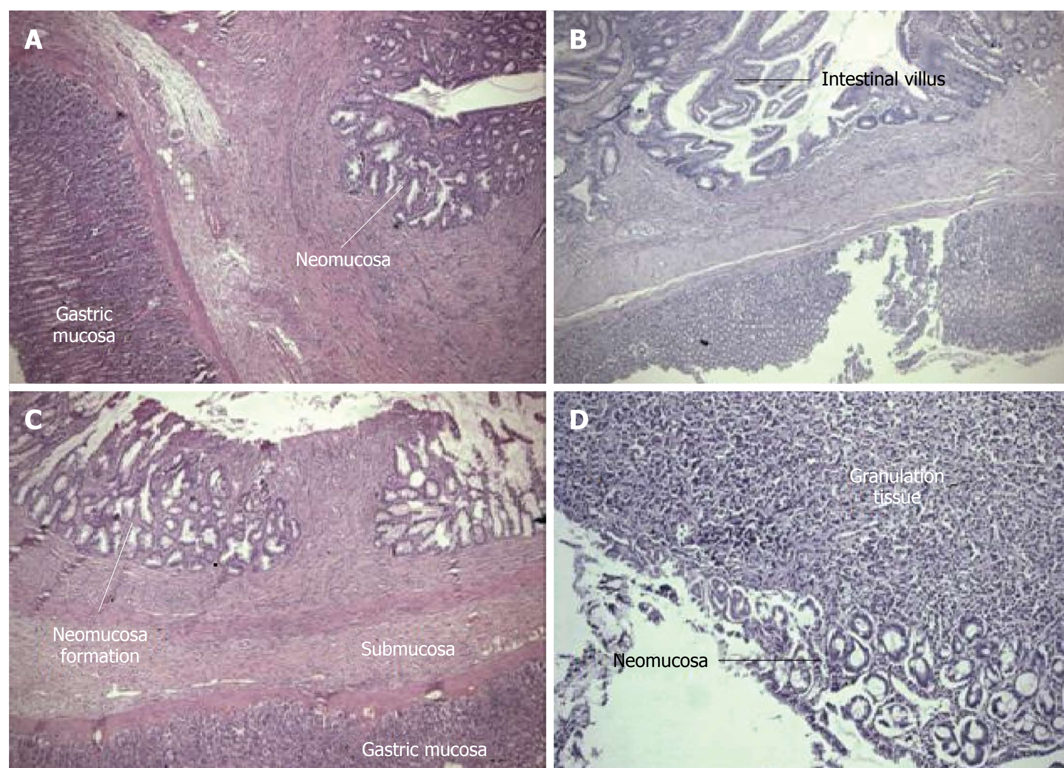

Figure 3 There were significant differences in the villus density in all of the groups compared with the control group. A: Gastric corpus mucosa on the left, newly formed thin neomucosa on the right. The muscularispropria has not yet formed. The granulation tissue regressed [Hematoxylin and eosin (HE) × 100] (Group 3); B: Neomucosa formation is observed at the bottom of the gastric mucosa (HE × 125) (Group 4); C: The granulation tissue is in the middle, with newly formed neomucosa on either side. At the bottom, the stomach tissue is visible (HE x 100) (Group 2); D: The early development of the mucosal layer and granulation tissue (HE x 125) (Group 1).

Table 1 Histological features of healing, morphological and functional findings and neomucosa formation

	Group 1 (control)	Group2 (GH)	Group 3 (HBOT)	Group 4 (GH + HBOT)	P^1 (Groups 1-2)	P^2 (Groups 1-3)	P^3 (Groups 1-4)
Histological findings of wound healing							
Epithelialization	0.3	1.3	1.0	1.7	> 0.05	> 0.05	< 0.01
Granulation	2.4	1.4	1.9	1.1	> 0.05	> 0.05	> 0.05
Fibroblast	1.7	1.8	2.0	2.4	> 0.05	> 0.05	< 0.05
Inflammation	1.4	1.1	1.2	1.2	> 0.05	> 0.05	> 0.05
Angiogenesis	4.4	7.5	7.2	8.9	< 0.01	< 0.01	< 0.01
Collagen deposition	0.9	2.2	1.6	2.6	< 0.01	< 0.05	< 0.01
Morphological and functional findings							
Villus density	0.2	1.3	1.1	2.0	< 0.01	< 0.01	< 0.01
Villus height (μm)	101 (70-109)	135 (98-153)	118 (81-129)	153 (119-181)	< 0.05	> 0.05	< 0.05
Crypt depth (μm)	83 (74-88)	102 (82-114)	93 (76-101)	120 (101-133)	> 0.05	> 0.05	< 0.05
Goblet cells	0.2	1.4	1.1	2.2	< 0.01	< 0.01	< 0.01
Mucin	0.2	1.7	1.2	2.2	< 0.01	< 0.01	< 0.01

All parameters scored from 0 to 3, angiogenesis counted within 1 mm^2, villus density, goblet cells, mucin secretion and collagen deposition, scored from 0 to 3. The P^1 value compares Groups 1 and 2, P^2 value compares Groups 1 and 3, and P^3 value compares Groups 1 and 4. All values are expressed as the mean ± SD. GH: Growth hormone; HBOT: Hyperbaric oxygen therapy.

neomucosa had a typical small intestinal phenotype (Figure 1C and D). There were significant differences in the villus density in all of the groups compared with the control group (P < 0.05). The crypt depth was significantly greater in group 4 than in the control group (P < 0.05), but no other groups had deeper crypts. However, the villus height was significantly longer in Groups 2 and 4 than in the control group (P < 0.05) (Figure 3). The comparison of the morphological characteristics and activation of the neomucosa between the groups revealed significant differences between the control group and Groups 2-4, with respect to the number of goblet cells and mucin secretion, respectively (both P < 0.01) (Figure 2C and D). When we analyzed all of the groups, we obtained the highest significant differences in Group 4. The morphological and functional features in all groups are given in Table 1. We could not make any comparisons between in groups, because the immunohistochemical staining was too weak.

Table 2 The per-operative and 3-wk post-operative venous levels of like growth factor and insulin like growth factor binding protein 3 (nmol/L)

	Group 1 (control)	Group 2 (GH therapy)	Group 4 (GH + HBO therapy)	P^1 (Groups 1-2)	P^2 (Groups 1-4)
Before the surgery					
IGF-1 (nmol/L)	445 (402-482)	421 (405-460)	431 (395-470)	NS	NS
IGFBP-3 (nmol/L)	2250 (2100-2350)	2170 (2000-2300)	2147 (2025-2350)	NS	NS
After the surgery (3 wk)					
IGF-1 (nmol/L)	445 (415-500)	799 (755-875)	813 (775-900)	< 0.05	< 0.05
IGFBP-3 (nmol/L)	2290 (2050-2500)	3300 (3000-3600)	3328 (3000-3650)	< 0.05	< 0.05
P	NS	< 0.05	< 0.05		

The *P* value compares the per- and post-operative levels within the same group, P^1 value compares the levels between Groups 1 and 2, and P^2 value compares the levels between Groups 1 and 4. All values are expressed as the mean ± SD. NS: Non-significant; GH: Growth hormone; HBOT: Hyperbaric oxygen therapy; IGF-1: Like growth factor; IGFBP-3: Insulin like growth factor binding protein 3.

GH determination

To determine the effect of the GH, we measured the IGF-1 and IGFBP-3 blood levels in the rats. The comparisons revealed no significant differences between the control group and Groups 2 and 4 in the blood levels of IGF-1 and IGFBP-3 ($P > 0.05$) per-operatively but significant differences between the control group and the other groups (Groups 2 and 4) in the levels of IGF-1 and IGFBP-3 ($P < 0.01$) 3 wk after the operation (Table 2). We also compared the per- and post-operative results within each group. There were significant differences in Groups 2 and 4 ($P < 0.05$, Table 2).

DISCUSSION

The use of serosal patching to grow new intestinal mucosa is a technique for enlarging the intestinal surface. In the literature, different animal models have been utilized to study the growth of intestinal neomucosa in full thickness defects patched with a variety of surfaces, including colonic serosa, abdominal wall, pedicle flaps, and prosthetic material. The serosal patch technique is one of the most popular methods. However, in many cases, only short segments of small intestine can be patched because of the limited serosal surface and anatomical factors[10,27]. The peritoneal surface has also been utilized to develop epithelial cells. In a rat model, researchers folded the colon to form a seromuscular tunnel and sutured the two ends to the transacted ileum. After 6-12 wk, a pouch in which a single layer of cylindrical epithelium had developed showed evidence of disaccharidase activity[10]. Erez *et al*[28] showed that using pigs and rats, one could successfully enlarge the small bowel surface by growing new mucosa on the parietal peritoneum following entero-peritoneal anastomosis. Some important advantages of this technique include the absorption of fluids and electrolytes through the peritoneum, and slowing of the bowel transit time. Bragg *et al*[29] used colonic serosa for patching, and by 8 wk, the defects were completely covered by neomucosa. In this study, we used the gastric serosal surface as a patch. An extensive literature search did not reveal a previous report of the use of the gastric serosal surface for this technique. We selected thegastric serosal-surface because the gastric serosa constitutes a large area and because it is anatomically close to the small intestine. Furthermore, the stomach tissue is thick and has a wide network of collateral blood vessels. The fitness for surgery is also influenced by the interaction of the main vein with the easily protected neomucosa structure, the ability of the serosal flap of the small bowel to easily move to the surface, and the quick self-renewal and large surface area of the gastric surface, enabling the surgical operation to be repeated multiple times. In our study, we demonstrated that neomucosal formation occurred two months after the operation. To prevent SBS, it is important that the surface area of the bowel increases rapidly after the surgery to allow the absorption of nutrients to occur. To increase the rate of neomucosal formation, we used GH and HBOT together. To our knowledge, this study is the first to report the use of both these techniques together to encourage neomucosal formation.

There are a number of peptide growth factors, such as IGF-1, or general growth factors, such as GH, that are used as promoting factors for intestinal hyperplasia and adaptation[1,2,6,14,30,31]. GH is expressed throughout the intestinal epithelium and in the lamina propria, muscularis mucosa, submucosa, and muscularispropria, indicating the potential for direct GH action within the intestine. GH has been shown to directly promote wound healing and growth of the intestine by increasing cell proliferation and collagen deposition[14]. In neomucosa formation, Thompson investigated the effect of epidermal growth factor (EGF), and octreotide-induced enterocyte apoptosis. Compared with the controls, the EGF group had decreased apoptosis in the crypt and villus[32]. Circulating GH binds to the GH receptor in the target cells and stimulates the production of IGF-1 in the liver and other target tissues, including the intestine[14]. Clinical trials of GH treatment have reported significantly increased levels of IGF-1 and IGF-binding protein-3[14,33-35]. In the GH treatment group, to control the efficacy of the GH, we assessed the level of the IGF-1 and IGF-binding protein at the end of 3 wk. Groups 2 and 4 had significantly higher levels of these hormones at the end of week three compared with the control group ($P < 0.01$).

Oxygen is an essential material for cell metabolism,

and reparative processes, such as cell proliferation and collagen synthesis[21,36,37], have an especially increased demand for oxygen. Evidence from animal and cell line studies has shown that HBOT, the administration of pure oxygen at pressures greater than 1 ATA, results in increased growth factor production, such as platelet-related growth factor, transforming growth factor-β1 and vascular endothelial growth factor, and improved wound healing[38,39]. Hyperoxygenation can also increase collagen production, enabling these rapidly migrating fibroblasts to lay down larger, stronger beds of collagen for the advancing capillary beds, leading to increased granulation tissue formation and enhanced overall healing[40]. A search of the literature did not reveal any data on HBOT regarding neomucosa formation. In plastic surgery, hyperbaric oxygen has also been used for the management of wounds requiring skin grafting and for the treatment of ischemic flaps[39]. Additionally, HBOT has been reported to improve, the healing of foot ulcers and glucose metabolism in patients with diabetes mellitus[36]. Angiogenesis is a process in which new blood vessels originate by budding or spouting from pre-existing vessels. HBOT increases angiogenesis, which is an important step in wound healing[21]. Huddy *et al*[41] published the results of giving HBOT to a patient who had suffered from SBS with stomal complications. After the therapy, the patient rapidly made natural adaptations. Neovascularization has been suggested as the mechanism by which HBOT acts. In our study, angiogenesis was significantly different in Group 3, which was given HBOT, compared with the control group ($P < 0.01$). Moreover, collagen (Masson trichrome) was significantly different in Group 3 compared with the control group ($P < 0.05$). This situation is consistent with other data reported in the literature in which HBOT increased the formation of collagen and angiogenesis. The exact mechanism by which HBOT enhances neomucosal formation is not known. There is no direct evidence regarding the mechanism by which HBOT improved neomucosa formation in our study. We thought our use of HBOT directly increased angiogenesis and collagen formation. Furthermore, HBOT may have increased the levels of some growth factors. To further examine the role of HBOT in wound healing, a more in-depth analysis of growth factors is warranted. In our study, we found some adverse side effects of HBOT. Three animals died of pneumo-thorax with the use of HBOT at the beginning. We believe that during the HBOT therapy, the tension pneumo-thorax complication can occur rarely in experimental animals; this condition was a cause of animal deaths in our study. The pneumothorax, could be caused directly by the high pressure of the HBOT. Murphy *et al*[42] showed that tension pneumothorax occurred in 3 patients who underwent HBOT. The GH and HBOT combined treatment had positive effects in all 4 groups.

Studies on the management of SBS have continued worldwide. Early attempts at increasing the surface area by serosal patching with regeneration from the margins of the wound were limited by the marked contraction of the defects created, and thus only a modest gain in the surface area was achieved. The use of stem cells isolated from the intestine should lead to further progression of intestinal regeneration[43]. The current techniques for experimental intestinal tissue engineering employ artificial biodegradable scaffolds in a 3-dimensional structure in which organoid units are seeded[43,44]. Although these results have been reported as experimental studies, they have not been tested in clinical studies yet. Our study is a pilot study and also an experimental study model. We suggest that using this method in appropriate cases is easy. These adaptations will be increased with the combined use of GH and HBOT.

In conclusion, this study demonstrated that intestinal neomucosa can be successfully produced on a gastric serosal surface. In addition, HBOT, GH and their combined therapy augmented neomucosal formation. The combination therapy appears to be more effective. The simultaneous use of both therapies produced a synergistic effect on the histological, morphologic and functional parameters.

COMMENTS

Background

Short bowel syndrome (SBS) is a malabsorptive disorder characterized by loss of intestinal length and occurs when patients have < 200 cm post-duodenal small intestine, resulting in inadequate digestion and/or nutrient. Treatment consists of surgery to slow intestinal transit or to increase the area of absorption. Reconstructive procedures on the remnant bowel and intestinal transplantation are areas of special interest to surgeons working in this field. Another potential technique for increasing the intestinal surface area is the growth of new intestinal mucosa, which takes advantage of the regenerative capability of the intestine. The regenerated intestine develops by lateral in growth from the surrounding mucosa and is functionally to normal intestinal mucosa.

Research frontiers

The serosal patch technique is one of the most popular technique to treat SBS. In this experimental study, authors used gastric serosal patch to form neomucosa in ileum defects. In literature search did not reveal a previous report of the use of the gastric serosal surface for this technique. Moreover in the present work is to investigate the role of growth hormon, hyperbaric oxygen therapy (HBOT) and combined therapy on intestinal neomucosa formation of the gastric serosa. Evidence supporting the use of growth hormone (GH) in the SBS includes the fact that exogenous GH stimulates structural and functional intestinal adaptation. Several studies have shown that increased oxygen tension with HBOT not only prevents adverse effects of ischemia but also accelerates healing in different types of wounds.

Innovations and breakthroughs

In this experimental study, 1 cm-long defect was created in the jejunum, after that a 1 cm × 1 cm patch of the gastric corpus was anastomosed tothe jejunal defect with interrupted 6/0 polypropylene sutures. HBOT was started 12 h after the surgery and completed to 12 d. GH were given subcutaneously at a dose of 2 mg per kg/d for 28 d beginning on the operation day. In order to increase the rate of neomucosal formation, we used the GH and HBOT together. This is the first study to report using these two techniques together to encourage neomucosal formation.

Applications

Their study is a surface expander research and also is an experimental study model. Authors suggest that it is easy to use of this method in the appropriate cases. These adaptation will be increased with the togetherness use of GH and HBOT.

Terminology

SBS, also short gut syndrome or simply short gut, is a malabsorption disorder caused by the surgical removal of the small intestine, or rarely due to the complete dysfunction of a large segment of bowel.

Peer review

This study demonstrated that intestinal neomucosa can be successfully raised on a gastric serosal surface. In addition, HBOT or GH and combined therapy augmented on neomucosal formation. Combination therapies seem to be more effective. Simultaneous use of combined therapy produced synergistic effect on histological, morphologic and functional parameters.

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P- Reviewer Yang Z **S- Editor** Gou SX
L- Editor A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2913

World J Gastroenterol 2013 May 21; 19(19): 2913-2920
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma

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Supported by The Beijing Natural Science foundation, No. 5102018; National Bio-Tech 86-3, No. 2006AA02A402 and No. 2012AA02A504

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Received: January 20, 2013 Revised: April 2, 2013
Accepted: April 9, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the association of p42.3 expression with clinicopathological characteristics and the biological function of p42.3 in human hepatocellular carcinoma (HCC).

METHODS: We used reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR and western blotting to detect p42.3 mRNA and protein expression in hepatic cell lines. We examined primary HCC samples and matched adjacent normal tissue by immunohistochemistry to investigate the correlation between p42.3 expression and clinicopathological features. HepG2 cells were transfected with a pIRES2-EGFP-p42.3 expression vector to examine the function of the *p42.3* gene. Transfected cells were analyzed for their viability and malignant transformation abilities by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation assay, and tumorigenicity assay in nude mice.

RESULTS: p42.3 is differentially expressed in primary HCC tumors and cell lines. Approximately 69.6% (96/138) of cells were p42.3-positive in hepatic tumor tissues, while 30.7% (35/114) were p42.3-positive in tumor-adjacent normal tissues. Clinicopathological characteristics of the HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation (P = 0.031). However, p42.3 positivity was not related to tumor tumor-node-metastasis classification, hepatitis B virus status, or hepatoma type. Regarding p42.3 overexpression in stably transfected HepG2 cells, we discovered significant enhancement of cancer cell growth and colony formation *in vitro*, and significantly enhanced tumorigenicity in nude mice. Western blot analysis of cell cycle proteins revealed that enhanced p42.3 levels promote upregulation of proliferating cell nuclear antigen, cyclin B1 and mitotic arrest deficient 2.

CONCLUSION: p42.3 promotes tumorigenicity and tumor growth in HCC and may be a potential target for future clinical cancer therapeutics.

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Key words: p42.3; Hepatocellular carcinoma; HepG2; Overexpression; Tumorigenicity

Core tip: p42.3 is a novel tumor-specific and mitosis phase-dependent expression gene. It is believed to be involved in tumorigenesis in gastric and colorectal can-

cer. To the best of our knowledge, this is the first study to investigate the expression and function of p42.3 in hepatocellular carcinoma (HCC). We found that p42.3 promotes tumorigenicity and tumor growth in HepG2 cells and is overexpressed in HCC. These results suggest that p42.3 may act as a novel tumor biomarker and aid in the development of improved therapeutic strategies.

Sun W, Dong WW, Mao LL, Li WM, Cui JT, Xing R, Lu YY. Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(19): 2913-2920 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2913.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2913

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. The annual number of new HCC cases worldwide is over one million, making it the 5th most common cancer and the 3rd leading cause of cancer-related deaths[1], accounting for more than 1 million deaths annually[2]. Despite improvements in monitoring and clinical treatment strategies, HCC prognosis remains poor[3,4]. Discovering novel biomarkers that correlate with HCC development or progression may present opportunities to reduce the severity of this disease through early and novel therapeutic interventions.

In our previous research, we cloned the full-length cDNA of the *p42.3* gene by using mRNA differential display in a synchronized gastric cancer (GC) cell lines. We found that p42.3 expression is frequently upregulated in primary tumors and embryonic tissues but not in normal tissues from adult organs. Moreover, stable silencing of p42.3 in BGC823 cells suppresses tumorigenicity and cell proliferation with accumulation of cells at G2/M stage of the cell cycle[5]. In addition, Jung *et al*[6] reported that the expression of p42.3 mRNA was significantly elevated in colorectal cancer (CRC) tissues compared to normal tissues. All these data indicate that p42.3 plays an important role in tumorigenesis, suggesting that it may be a potential tumor biomarker. In order to elucidate the role of p42.3 in tumorigenesis, we characterized p42.3 expression and validated its biologic significance in HCC.

MATERIALS AND METHODS

Patients and tissues

HCC specimens (*n* = 138) were collected from 98 men and 40 women (age, 31-74 years; mean ± SD, 52.6 ± 8.7 years) who were inpatients at Beijing Cancer Hospital, Beijing, China, from January 2006 to September 2009. Patient data are shown in Table 1. All patients underwent a radical resection with curative intent and had sufficient clinical information available. No patients had received chemotherapy or radiation therapy. Moreover, 114 adjacent normal hepatic tissues (at least 5 cm distant from the tumor edge) were also collected from HCC patients. Tumor stage was classified according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification. The investigation project and its informed consent have been examined and certified by the Ethics Committee of Beijing Cancer Hospital.

Table 1 p42.3 status in relation to clinicopathological features in patients with hepatocellular carcinoma (*n* = 138) *n* (%)

Tissues parameters	No. of cases	Positive	Negative	*P* value
Gender				NS
Male	98 (71.0)	42 (42.9)	56 (57.1)	
Female	40 (29.0)	23 (58.0)	17 (42.0)	
Age at diagnosis (yr)				NS
< 60	117 (84.8)	52 (44.4)	63 (55.6)	
≥ 60	21 (15.2)	12 (57.1)	9 (42.9)	
Carcinoma and adjacent tissue				0.0008
Carcinoma tissue	138 (54.8)	96 (69.6)	42 (30.4)	
Adjacent tissue	114 (45.2)	35 (30.7)	79 (69.3)	
Degree of differentiation				0.031
Well	42 (30.4)	11 (26.2)	31 (73.8)	
Moderate	87 (63.0)	39 (44.8)	48 (55.2)	
Poor	9 (6.5)	6 (66.7)	3 (33.3)	
TNM classification				NS
Stage Ⅰ/Ⅱ	101 (73.2)	43 (42.6)	58 (57.4)	
Stage Ⅲ/Ⅳ	37 (26.8)	19 (51.4)	18 (48.6)	
HBV				NS
Negative	41 (29.7)	15 (36.6)	26 (63.4)	
Positive	97 (70.3)	47 (48.5)	50 (51.5)	
Type of hepatoma				NS
Nodular	94 (68.1)	44 (46.8)	50 (53.2)	
Massive	35 (25.4)	13 (37.1)	22 (62.9)	
Diffuse	9 (6.5)	5 (55.6)	4 (44.4)	

TNM: Tumor-node-metastasis; NS: Not significant; HBV: Hepatitis B virus.

Tissue microarray immunohistochemistry

The hepatic tissue microarray was constructed using a tissue array instrument as previously described[7]. For immunohistochemistry studies, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 solution for 10 min at room temperature. After blocking with 5% skim milk, sections were incubated with specific murine p42.3 mAb (1:1000, our lab) at 4 ℃ overnight, followed by the incubation with the peroxidase-based EnVision TM kit (Dako Cytomation, Cambridgeshire, United Kingdom) for 30 min at room temperature. The reaction product was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark) for 5 min at room temperature. Sections were counterstained with hematoxylin.

Purified IgG from normal mouse sera was used as a negative control. The number of tumor cells or normal hepatic cells was evaluated by two independent pathologists. A specimen with more than 20% immunostained cells was classified as a positive case.

Cell lines and cell culture

The 6 human HCC cell lines MHCC97L, MHCC97M3,

BEL7402, Huh7, HepG2, and SMMC7721 and the immortal human hepatocyte line HL7702 were routinely maintained as previously described[8]. HL7702 was cultured in Roswell Park Memorial Institute medium (RPMI 1640; Gibco, Grand Island, NY, United States), supplemented with 20% fetal bovine serum (FBS; Gibco). BEL7402 and SMMC7721 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. The remaining cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS. All media contained 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained at 37 ℃ in 5% CO_2.

Reverse transcription-polymerase chain reaction and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using TRIzol (Qiagen, United States). The prepared RNA (5 μg) was mixed with oligo-dT primers and reverse-transcribed with moloney murine leukemia virus reverse transcriptase (Promega, United States) for 60 min at 37 ℃, followed by polymerase chain reaction (PCR) amplification with specific primers for p42.3 (forward: 5'-TGGACTGCGGCCTGCTGAA-3'; reverse: 5'-ACTCCATCGCTGTGTTTCAAT-3'). PCR amplification was performed in 20 μL using a thermocycler (Biometra, Germany) with the following PCR program: pre-denaturation for 5 min at 94 ℃, denaturation for 45 s at 94 ℃, annealing for 45 s at 61 ℃, extension for 45 s at 72 ℃, and a final elongation at 72 ℃ for 10 min. β-Actin served as an internal positive control (forward: 5'-TCACCCACACTGTGCCCATCTACGA-3'; reverse: 5'-CAGCGGAACCGCTCATTGCCAATGG-3'). PCR was performed for 24 or 32 cycles (β-actin 24 cycles; p42.3 32 cycles). PCR products were separated by electrophoresis on a 1.5% agarose gel. Quantitative real-time reverse transcription-PCR (Q-RT-PCR) using SYBR-Green Master PCR mix (Applied Biosystems, Carls-bad, CA) was performed in triplicate (p42.3 forward: 5'-CCTGGCATCTTTACTGGACTGGA-3'; p42.3 reverse: 5'-GTGCCAGCCTGTCTCACATTTC-3'). Quantification was normalized to the endogenous control β-actin (forward: 5'-TTAGTTGCGTTACACCCTTTC-3'; reverse: 5'-ACCTTCACCGTTCCAGTTT-3').

Western blotting

Cell lysates were prepared by incubating cells at 4 ℃ for 1 h in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 2 mmol/L dithiothreitol, 5 mmol/L ethylene diamine tetraacetic acid, 100 mmol/L NaCl, and 2 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of protein were electrophoresed on a 12% sodium dodecylsulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using standard techniques. We used four specific antibodies obtained from Santa Cruz Biotechnology: proliferating cell nuclear antigen (PCNA) (diluted 1:300; F-2), cyclin B1 (diluted 1:500; H-433), cell division cycle 25 A (Cdc25A) (diluted 1:500; DCS-122), and cell division cycle 25 homolog C (Cdc25C) (diluted 1:500; C-20). The following specific antibodies were also used: mitotic arrest deficient 2 (MAD2) (diluted 1:1000; Ab70383; Abcam, United Kingdom), actin (diluted 1:10000, AC-15; Sigma, United States), and p42.3 (diluted 1:1000; our lab). Nonspecific binding was blocked using a 5% fat-free milk solution. Signals were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Plasmid construction and cell transfection

The whole coding region of p42.3 was cloned into the pIRES2-EGFP vector at the *Bam*HI and *Hin*dⅢ sites. Nucleotide sequences of the subcloned cDNAs were verified by sequencing. HepG2 were selected and cultured at 60%-70% confluence in 35 mm plates. Cells were transfected with recombinant p42.3 plasmids or an empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, United States). At 48 h post-transfection, cells were seeded for 21 d in selection medium containing 400 μg/mL G418 to screen for stable clones. To confirm the transfection efficiency, RT-PCR and Western blot analysis were performed.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and soft agar colony formation assay

Stably transfected cells in were seeded (2×10^3) in duplicates into each well of a 96-well culture plate and grown in 200 μL DMEM with 5% FBS; 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Genview, Florida, United States) (5 mg/mL) was added at 0, 24, 48, 72, 96 and 120 h. The MTT was removed after 4 h incubation; 100 μL of dimethylsulfoxide (Amresco, Cochran, United States) was added to each well, then incubated for 30 min. Absorbency was measured at 570 nm using an iMark Microplate Reader (Bio-Rad, CA, United States).

For the soft agar assay, cells (2×10^3) were trypsinized and resuspended in 4 mL of 0.3% agar in DMEM containing 10% FBS, and overlaid with 0.6% agar in 60-mm culture dishes. The dishes were incubated routinely for 21 d. Colonies were stained with 0.2% *p*-iodonitrotetrazolium violet, then photographed and counted.

Tumorigenicity assay in nude mice

Stably transfected cells were washed twice and resuspended in 1 × Hank's buffer at a concentration of 1×10^6 cells/mL. A 100-μL cell suspension of HepG2-p42.3 was then injected subcutaneously into the left dorsal flank of 10, 4-wk-old female nude mice. As a control, the right side was inoculated with HepG2-vector. Tumor diameters were checked every 3 d, and tumor volume was calculated according to $ab^2/2$ ($a > b$). Tumor specimens were collected at 15 d after injections and split. Immunohistochemistry (IHC) analysis was used to detect p42.3 protein expression. Three independent experiments were performed and yielded similar results.

Statistical analysis

To evaluate the possible differences of p42.3 expression in different hepatic specimens, we performed Pearson's

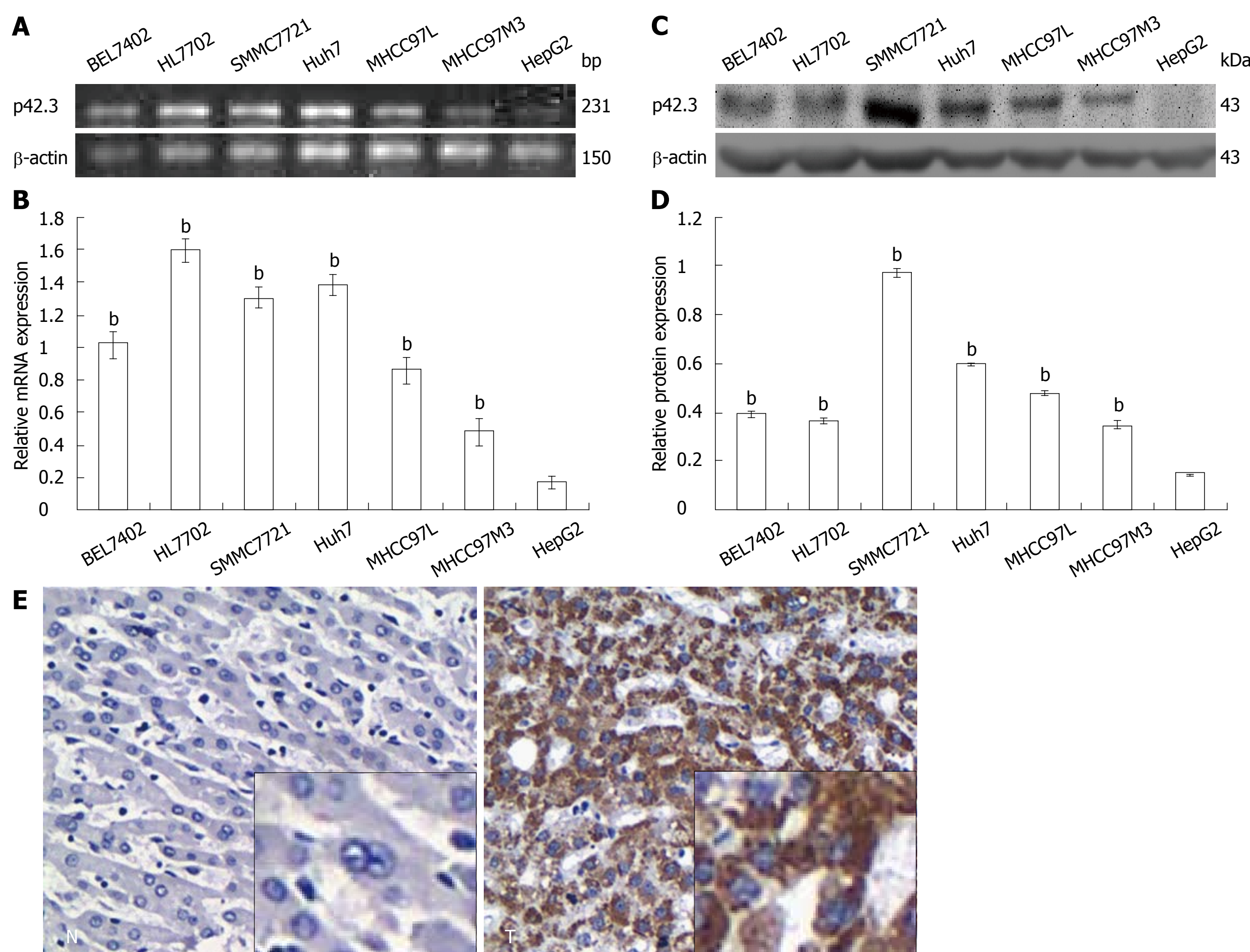

Figure 1 Detection of p42.3 in hepatic cell lines and hepatocellular carcinoma tissues. A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that p42.3 was detectable in all 7 cell lines, and the lowest expression was in HepG2 cells; B: Relative expression of p42.3 mRNA in seven hepatic cell lines using quantitative real-time RT-PCR. Data are shown as the mean ± SD, endogenous references was β-actin (^bP < 0.01 *vs* HepG2); C and D: Expression of p42.3 protein in hepatic cell lines analyzed by Western blotting (C) and shown as mean ± SD (D) (^bP < 0.01 *vs* HepG2); E: Negative staining of p42.3 in hepatocellular carcinoma-adjacent normal tissue (left), positive staining of p42.3 in tumor (right). Original magnification, × 100; the inset boxes are at original magnification × 200.

χ^2 test. The Student's two-sided *t*-test was used to compare test and control sample values in MTT assay, soft agar colony formation assay and tumorigenicity assay. All statistical analyses were carried out using the SPSS statistical software package 16.0 (SPSS Inc., United States). *P* values < 0.05 were considered statistically significant.

RESULTS

p42.3 protein expression in human tumor cell lines

p42.3 mRNA and protein expression were examined in 6 human HCC cell lines and the immortal human hepatocyte HL7702. RT-PCR and Q-RT-PCR showed that p42.3 mRNA was expressed in all of 7 cell lines (7/7, 100%), and the lowest expression was found in HepG2 cells (Figure 1A and B). Consistent with mRNA expression levels, p42.3 protein was expressed at high levels in all cell lines except HepG2 cells (6/7, 85.7%) (Figure 1C and D). Thus, we confirmed that the HepG2 cell line is a p42.3-deficient line and could therefore be used as a model to investigate p42.3 protein function.

p42.3 protein levels in human primary tumors

To characterize p42.3 expression in HCC specimens, IHC was performed on tumor tissues and tumor-adjacent normal tissues. We found p42.3 protein was detected in 69.6% (96/138) of hepatic tumor tissues. However, p42.3 expression was less apparent, with significantly less positive cells (30.7%, 35/114) in tumor-adjacent normal tissues (*P* < 0.001, Table 1 and Figure 1E). The results indicate that p42.3 protein is highly expressed in primary HCC tissues rather than tumor-adjacent normal tissues. Analysis of the clinicopathological characteristics of the 138 HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation (*P* = 0.031, Table 1). However, we found no relationship between p42.3 positivity and tumor TNM classification, hepatitis B virus status, or type of hepatoma.

Overexpression of p42.3 induces PCNA, cyclin B1 and MAD2 expression in HepG2 p42.3-deficient cells

To examine the gene function of *p42.3* overexpression on HCC cells, we stably transfected the pIRES2-EGFP-

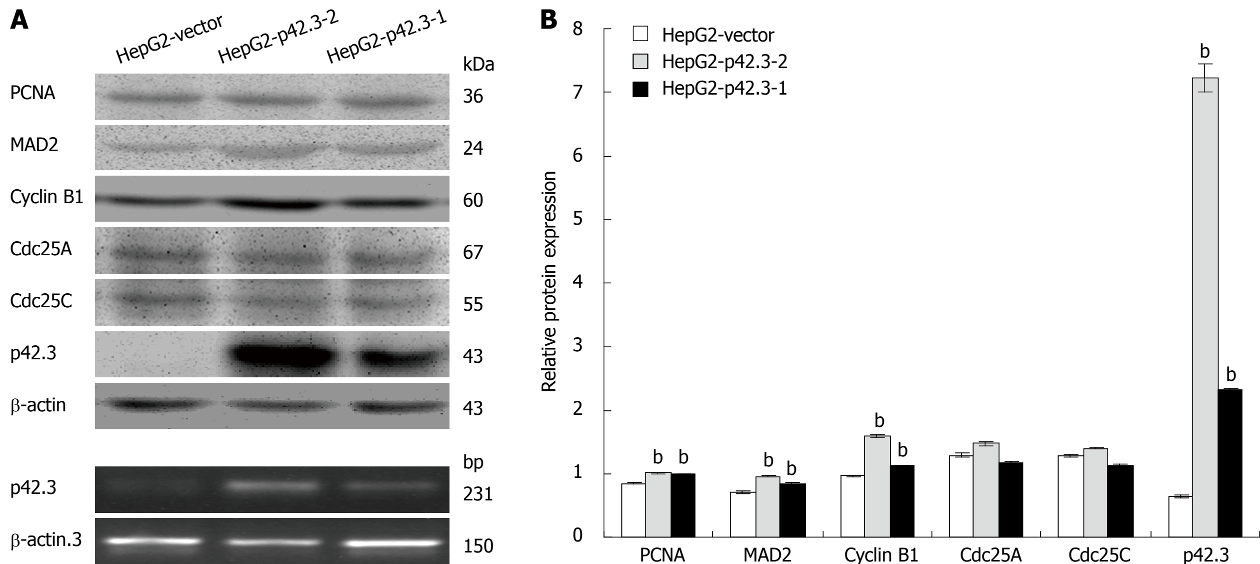

Figure 2 The effect on molecular by overexpression of p42.3 in HepG2 cells. A: Reverse transcription-polymerase chain reaction and Western blotting were performed to confirm p42.3 overexpression in a stable single colony of HepG2-p42.3-1 and HepG2-p42.3-2 cells. p42.3 expression was deficient in the HepG2-vector control cells. β-actin served as an internal control; B: Expression of proteins shown as mean ± SD. Consistent with p42.3 protein expression, proliferating cell nuclear antigen (PCNA), mitotic arrest deficient 2 (MAD2) and cyclin B1 expression were significantly upregulated. The protein levels of cell division cycle 25 A (Cdc25A) and cell division cycle 25 homolog C (Cdc25C) hardly changed following p42.3 expression ($^bP < 0.01$ *vs* HepG2-vector).

p42.3 expression vector into HepG2 cells. A cell line that stably expresses p42.3 (HepG2-p42.3) was generated and analyzed by western blotting. As shown in Figure 2, p42.3 protein was not detected in cells stably transfected with the empty vector. However, p42.3 protein was significantly increased in the p42.3 overexpressing cells, HepG2-p42.3-1 and HepG2-p42.3-2. These results indicated that the eukaryotic vector for p42.3 used in this study sufficiently upregulates p42.3 expression in HepG2 cells.

Since p42.3 is a novel cell cycle-dependent protein, we investigated cyclin B1 and other M phase-related proteins in p42.3-expressing HepG2 cells and control cells (HepG2-vector). We found that p42.3 expression resulted in a significant upregulation in PCNA, cyclin B1 and MAD2 protein levels. However, Cdc25A and Cdc25C protein levels only slightly changed with p42.3 expression (Figure 2).

Overexpression of p42.3 promotes growth and colony formation in HepG2 cells

The effects of p42.3 overexpression on the viability of HepG2 cells were measured using an MTT colorimetric assay. We found that transfection with pIRES2-EGFP-p42.3 promotes HepG2 cell growth. A stable single clone of HepG2-p42.3-1 and HepG2-p42.3-2 cells grew much faster over a 6-day period when compared to parental HepG2-vector cells, indicating that p42.3 may confer a strong growth capability in HepG2 cells ($P < 0.01$, Figure 3A).

The colony formation assay was used to evaluate the ability for anchorage-independent growth of cells in soft agar medium. Our data showed a significant increase in HepG2-p42.3-1 and HepG2-p42.3-2 colony formation in both number and size; however, the HepG2-vector cells only formed a few small colonies ($P < 0.01$, Figure 3B and C). This suggests that p42.3 confers anchorage-independent growth to cells.

Overexpression of p42.3 promotes HepG2 cell tumorigenicity

We tested p42.3 tumorigenicity in nude mice. HepG2-p42.3 cells were injected subcutaneously into the left dorsal flank of female nude mice (BALB/c), the right side was injected with HepG2-vector cells as a control. Of the 5 animals injected with HepG2-p42.3-1 or HepG2-p42.3-2 cells, tumors appeared faster and were larger than in the controls (HepG2-vector) ($P < 0.01$, Figure 4A and B). After the animals were sacrificed, the xenografts were removed and collected for immunohistochemistry analysis. The results showed that p42.3 protein was found in all HepG2-p42.3 xenografts, but that p42.3 protein was not found in HepG2-vector xenografts (Figure 4C). These results further confirmed that the p42.3 overexpression promotes tumorigenicity of HepG2 cells.

DISCUSSION

p42.3 is a highly conserved mammalian gene and strong G2 induction[5,9-11]. Moreover, p42.3 is involved in Chromosome segregation[12], it may play a key role in tumorigenicity. Our previous studies have shown that p42.3 was overexpressed in GC tissue and its expression is cell cycle dependent in the BGC823 cell line, expression peaked at early G1 phase[5,13]. Additionally, reduced proliferation and tumorigenic properties were detected in the BGC823 cell line that lacked p42.3[5], suggesting that p42.3 is involved in gastric carcinogenesis. While most studies have focused on the roles of p42.3 in GC development[5,11,14],

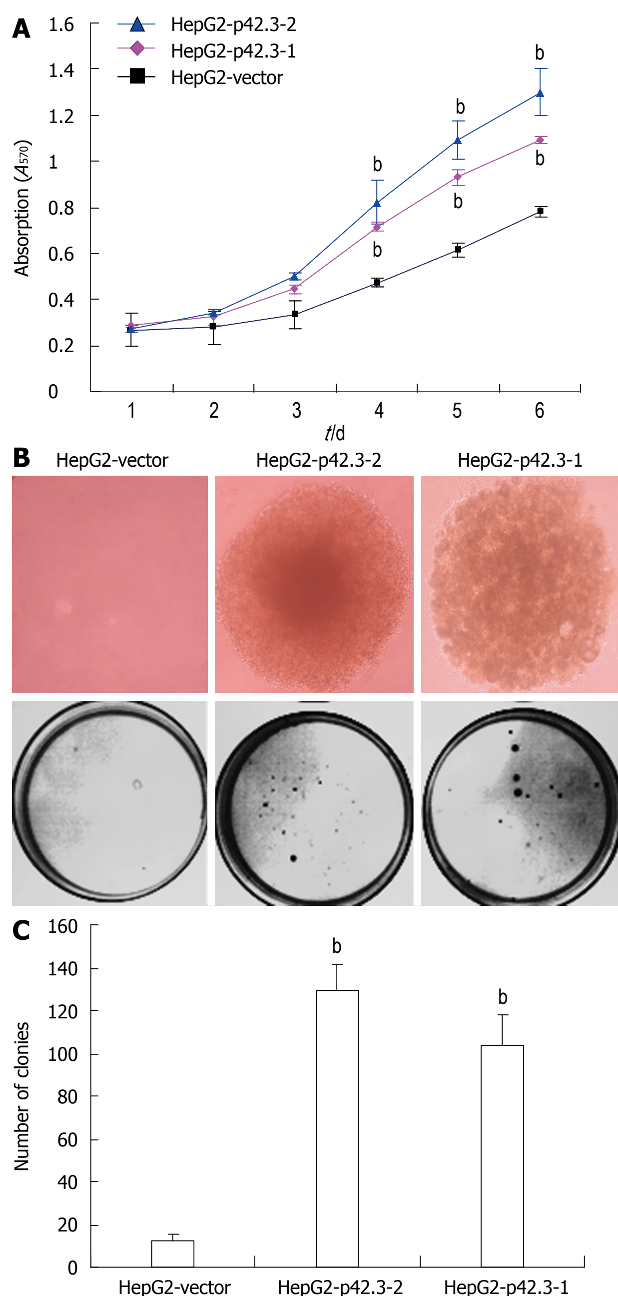

Figure 3 Promotion of cell growth and colony formation with p42.3 overexpression in HepG2 cells. A: Promotion of cell growth after overexpression of p42.3 in HepG2. Growth curve comparing HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector cells over a 6-d time course. Data are shown as the mean ± SD of three independent experiments (^bP < 0.01 *vs* HepG2-p42.3-1); B: The colonies of HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector formed on soft agar. The colonies grew faster and were larger in HepG2 cells that overexpressed p42.3-2 than in the HepG2-vector control cells; C: Raw value indicating colony number. Data revealed that the colony-forming activities of HepG2-p42.3-1 and HepG2-p42.3-2 were significantly promoted on soft agar. The data represent the mean ± SD of three independent experiments (^bP < 0.01 *vs* HepG2-vector).

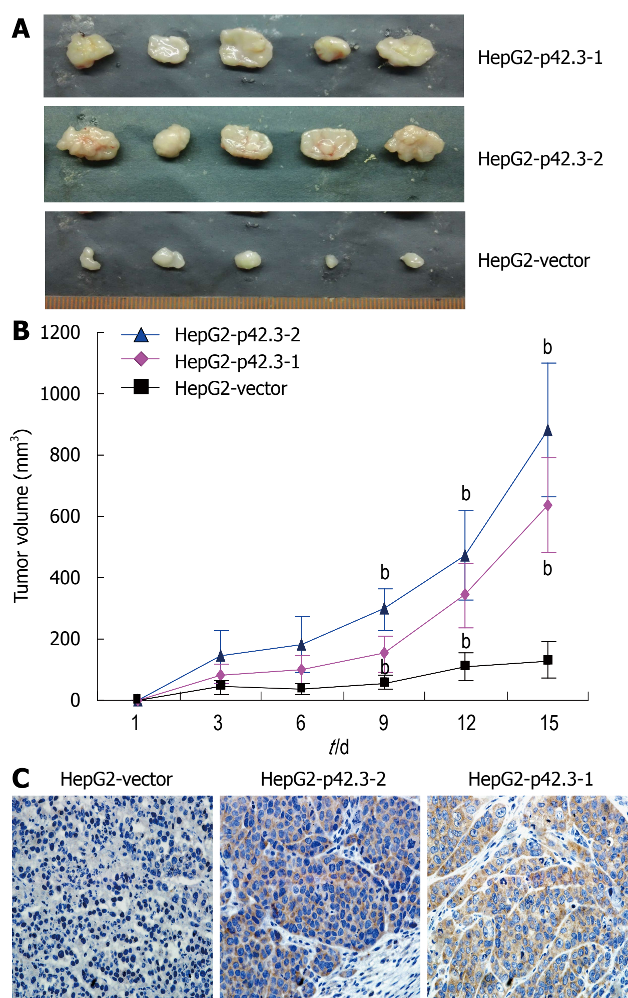

Figure 4 Promotion of tumorigenesis by overexpression of p42.3 shows statistical significance compared with the control in HepG2 cells. A: The tumor induced by HepG2-p42.3-1 and HepG2-p42.3-2 was much larger than that in the control; B: Over the course of 15 d, the tumor growth curve comparing HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector cells revealed that p42.3-overexpressing cells grew faster (^bP < 0.01 *vs* HepG2-p42.3-1); C: Immunohistochemistry staining was performed in xenograft tissues from tumors. p42.3 protein was detectable in xenografts that were formed by injection of p42.3-overexpressing cells, but not in the tumors developed from HepG2-vector cells.

the roles of p42.3 in other cancer remain to be elucidated. Therefore, we characterized p42.3 expression in HCC tissues from patients. Moreover, we investigated p42.3 functions and potential mechanisms of action in HepG2 cells.

In 7 human HCC cell lines, consistent with the mRNA expression, p42.3 protein was highly expressed with the exception of the HepG2 line. In concert with our previous study in GC cells, we found that the *p42.3* gene was highly expressed in the majority of the tested tumor cell lines. This suggests that the *p42.3* gene is overexpressed in tumor cells. In previous research, data showed that the *p42.3* gene is closely related to GC and CRC[5,6]. Similarly, our current data revealed that the p42.3 protein was expressed in 64.7% of hepatic tumor tissues compared to only 35.3% in tumor-adjacent normal tissues. The clinical p42.3 data in GC, CRC and HCC tissues suggest that upregulation of p42.3 may be a common feature in a variety of tumors.

Our results further support the hypothesis that p42.3 might stimulate cellular viability and malignant transformation since overexpression of p42.3, by stable transfection of the pIRES2-EGFP-p42.3 into HepG2 cells, significantly promotes cancer cell growth by MTT colo-

rimetry and colony formation *in vitro*, and significantly induced tumorigenicity in nude mice. Thus, these findings provide evidence that p42.3 plays an important role in tumorigenesis. Therefore, we investigated the molecular mechanism responsible for stimulating cell growth and malignant transformation. Since the expression of p42.3 is cell cycle dependent and G2/M checkpoint abrogated[5,11,13,15], we analyzed the effects of elevated p42.3 levels on a series of cell cycle proteins. Our results indicate that p42.3 expression significantly upregulates PCNA, cyclin B1 and MAD2 protein levels. However, Cdc25A and Cdc25C protein levels hardly change with p42.3 expression. Cyclin B1 is one of the key genes involved in M-phase regulation[16-20]. Together with Cdc2, cyclin B1 forms a complex that controls M-phase entry and exit[17,21]. Cyclin B1 can promote the G2-M transition, and even leads to a loss cell proliferation control, thus leading to malignant transformation[22,23]. The alteration of cyclin B1 protein levels shown here is consistent with our previous study[5], it shows p42.3 may a regulator of cyclin B1. Furthermore, MAD2 is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate[24-29], and MAD2 is involved in mediating the upregulation of cyclin B1 proteins[30,31]. Our results showed that the significant increase in expression of cyclin B1 and Mad2 may correlation with G2/M checkpoint abrogation. On the other hand, though Cdc25 phosphatases involved in cell cycle checkpoints as key regulators of normal cell division and the cell's response to DNA damage[32-35], our data did not reveal any obvious change in total Cdc25A and Cdc25C levels with p42.3 overexpression, Cdc25 phosphatases may have no role in the cell's response to induced p42.3 expression.

In summary, the data obtained in this study demonstrate that p42.3 protein is upregulated in primary HCC tissues but not tumor-adjacent normal tissues, and that cyclin B1 might be responsible for the cellular proliferation and malignant transformation induced by p42.3. These insights may help to identify p42.3 as a potential target for improved cancer therapies or as a diagnostic marker in clinical cancer treatment.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. Discovering novel biomarkers that correlate with HCC may present opportunities to reduce the severity of this disease. As a novel tumor-specific and mitosis phase-dependent expression gene, *p42.3* is involved in cell proliferation and tumorigenesis in gastric cancer (GC). Previous data also indicate that p42.3 expression is significantly elevated in GC and colorectal cancer (CRC), thus raising the possibility that it may act as a potential tumor biomarker.

Research frontiers

p42.3 is a novel tumor-specific and mitosis phase-dependent expression gene. It is involved in tumorigenesis in GC and CRC. However, the research concerning p42.3 in HCC is lacking. In this study, the authors investigate p42.3 expression and function in primary HCC. These results suggest that p42.3 may act as a novel disease biomarker and aid in the development of improved therapy strategies.

Innovations and breakthroughs

Recent reports have highlighted that p42.3 is involved in GC and CRC. This is the first study to investigate the expression and function of p42.3 in HCC. The authors found that p42.3 promotes tumorigenicity and tumor growth in HepG2 cells and is overexpressed in HCC.

Applications

In understanding the expression and function of p42.3 in HCC, this study may represent a future strategy as a therapeutic target and/or improve clinical cancer HCC treatment.

Peer review

The authors examined the expression of p42.3 and its function in HCC. These data revealed that p42.3 was increased in HCC and in all HCC cells with the exception of HepG2 cells. Moreover, p42.3 expression was correlated with tumor differentiation. p42.3 promotes tumorigenicity and tumor growth in HCC; therefore, it may be used as a potential target to improve the clinical treatment of HCC.

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P- Reviewers Butterworth J, Grassi G, Yu DY
S- Editor Wen LL **L- Editor** A **E- Editor** Xiong L

WJG World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2921

World J Gastroenterol 2013 May 21; 19(19): 2921-2934
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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ORIGINAL ARTICLE

Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation

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Supported by Grants from the National Natural Science Foundation of China, No. 30970886; The Science and Technology Project of Dalian, No. 2010E15SF179; and the Initial Doctoral funding of Liaoning Province, No. 20121110
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Received: January 13, 2013 Revised: March 15, 2013
Accepted: March 28, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the effects of the heme oxygenase (HO)-1/carbon monoxide system on iron deposition and portal pressure in rats with hepatic fibrosis induced by bile duct ligation (BDL).

METHODS: Male Sprague-Dawley rats were divided randomly into a Sham group, BDL group, Fe group, deferoxamine (DFX) group, zinc protoporphyrin (ZnPP) group and cobalt protoporphyrin (CoPP) group. The levels of HO-1 were detected using different methods. The serum carboxyhemoglobin (COHb), iron, and portal vein pressure (PVP) were also quantified. The plasma and mRNA levels of hepcidin were measured. Hepatic fibrosis and its main pathway were assessed using Van Gieson's stain, hydroxyproline, transforming growth factor-β1 (TGF-β1), nuclear factor-E2-related factor 2 (Nrf2), matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1).

RESULTS: Serum COHb and protein and mRNA expression levels of HO-1 and Nrf2 were increased in the BDL group compared with the Sham group and were much higher in the CoPP group. The ZnPP group showed lower expression of HO-1 and Nrf2 and lower COHb. The levels of iron and PVP were enhanced in the BDL group but were lower in the ZnPP and DFX groups and were higher in the CoPP and Fe groups. Hepcidin levels were higher, whereas superoxide dismutase levels were increased and malonaldehyde levels were decreased in the ZnPP and DFX groups. The ZnPP group also showed inhibited TGF-β1 expression and regulated TIMP-1/MMP-2 expression, as well as obviously attenuated liver fibrosis.

CONCLUSION: Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and regulating PVP.

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Key words: Heme oxygenase-1; Hepcidin; Iron accumulation; Oxidative stress; Portal vein pressure; Carboxyhemoglobin; Bile duct ligation

Core tip: In this study, inhibiting heme oxygenase-1 (HO-1)/carbon monoxide (CO) system by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation, the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the nuclear factor-E2-related factor 2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining portal vein pressure.

Wang QM, Du JL, Duan ZJ, Guo SB, Sun XY, Liu Z. Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation. *World J Gastroenterol* 2013; 19(19): 2921-2934 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2921.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2921

INTRODUCTION

Iron is an essential nutrient for growth and survival, but excessive iron accumulation in cells can result in cell injury[1,2]. Iron overload is not uncommon in many patients with end-stage liver cirrhosis, and it can also occur in patients with a history of multiple blood transfusions[3,4].

Research has shown that in cultured hepatocytes, iron activates hepatic stellate cells and increases the secretion of latent transforming growth factor-β (TGF-β) due to hepatocytes being injured by iron in the pathogenesis of iron-induced liver fibrosis[5]. In mice, iron overload enhanced the development of carbon tetrachloride-induced hepatic fibrosis[6]. In clinical studies, approximately half of patients with hereditary iron accumulation (hemochromatosis) developed liver fibrosis[7]. Moreover, a significant reduction of fibrosis in the liver was demonstrated in a number of thalassemia patients treated with deferasirox[8].

Clinically, repeated large-volume blood transfusions are sometimes necessary for cirrhotic patients with massive upper gastrointestinal bleeding; in most cases, patients are transfused with packed red blood cells (RBCs), which results in iron overload as the human body cannot excrete iron. Each unit of RBCs contains approximately 250 mg of iron, and after 10-15 RBC transfusions, iron typically accumulates in the liver, heart, skin, and endocrine organs[9]. However, how iron overload affects the pathogenesis and treatment of patients with hepatic fibrosis is not yet well understood.

Heme oxygenase-1 (HO-1) is the primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme into free iron, carbon monoxide (CO), and biliverdin[10,11].

Previous reports have recently shown HO-1 to be protective in liver cells in various liver diseases such as acute liver injury, alcoholic liver disease, liver fibrosis and ischemia/reperfusion injury through multiple pathways[12-15]. Other reports have indicated that this protection might be restricted to a narrow threshold of HO-1 over-expression[13,16]. Our previous studies showed that over-expression of HO-1 could be harmful to the liver functioning of rats with cirrhosis induced by bile duct ligation (BDL)[17,18], which was also reported by Froh *et al*[19], but whether this effect was related to iron accumulation and CO release was not clear.

In normal Sprague-Dawley (SD) rats, increased HO activity as a pro-oxidant mechanism resulted in iron accumulation in the liver; in contrast, decreased HO activity reduced intracellular iron levels and oxidative stress[20].

In this study, we investigated the effect of HO-1 on iron accumulation and CO release by inhibiting or inducing HO-1 expression with zinc protoporphyrin (ZnPP) or cobalt protoporphyrin (CoPP) in fibrotic rat models induced by BDL, and we further studied whether regulating HO-1 expression could improve liver fibrosis by reducing hepatic iron accumulation and portal vein pressure (PVP).

MATERIALS AND METHODS

Animal care

The experimental protocols were approved by the Animal Care and Use Committee of Dalian Medical University (Liaoning Province, China), in accordance with the guidelines established by the Canadian Council on Animal Care.

BDL and treatment in rat

Fifty-three healthy male SD rats, weighing 200-220 g, were obtained from the Laboratory Animal Center of Dalian Medical University and were randomly divided into six groups: a Sham group (n = 6), BDL group (n = 10), CoPP treatment group (n = 12), ZnPP treatment group (n = 8), Fe treatment group (n = 9) and DFX treatment group (n = 8). The rats were housed in a specific pathogen-free (SPF) center, at room temperature of 24-26 ℃ and relative humidity of 60%-65%. Water was provided ad libitum.

The rats were well fed and housed for 3 d before any experimental protocols. Biliary cirrhosis was induced by BDL[21,22]. Five groups underwent BDL together with Sham-operated animals as a healthy control. The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with 4-0 silk sutures, and the antibiotic gentamicin (0.3 mL) was injected intramuscularly. Rats in the Sham group underwent laparotomy with the bile duct isolated but not ligated. After surgery, the Sham and BDL groups received an intraperitoneal injection of saline. Other groups received an intraperitoneal injection consisting of CoPP, ZnPP, iron-dextran (ID) and deferoxamine (DFX) (5, 5, 50, 100 mg/kg body weight) three times per week, respectively. After the establishment of the rat models, the number of rats was reduced to 6 in each group because of deaths during the study process.

ZnPP and CoPP (Sigma, St Louis, MO, United States) were dissolved in 0.2 mol/L of NaOH, adjusted to a pH of 7.4, were diluted in 0.85% NaCl, with a final concentration of 1 mg/mL as previously described, and were used for inhibiting and inducing HO-1 expression, respectively[23]. DFX mesylate salt and ID (Sigma, St Louis, MO, United States) were diluted in 0.85% NaCl with final concentrations of 40 and 20 mg/mL, respectively. Histostain™ - Plus Kits (SP9001) (Zhongshan Goldenbridge

Biological Technology, Beijing, China); hydroxyproline (HYP), malonaldehyde (MDA) and superoxide dismutase (SOD) (Key GEN Biotech Nanjing, China); a hepcidin enzyme-linked immunosorbent assay (ELISA) Kit (EIAab Science, Wuhan, China); and a TaKaRa RNA polymerase chain reaction (PCR) kit (avian myeloblastosis virus), version 3.0 (TaKaRa Biotechnology, Dalian, China), were used in this study.

Sample collection and examination

Four weeks after surgery, a catheter connected to a pressure transducer (BL-420F biological experimental system, Chengdu Technology and Market Co. Ltd., China), was placed in the portal vein to measure PVP. Subsequently, 1 mL of arterial blood was withdrawn to measure carboxyhemoglobin (COHb), using a Rapid Lab 1245 Blood Gas Analyzer (Siemens, New York, NY, United States). Then, blood samples were collected from the abdominal aorta to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and serum iron, using a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co, Tokyo, Japan). The levels of liver SOD and MDA were determined with a UV-2100 spectrophotometer (Chemito Instruments Pvt. Ltd.).

Liver iron content measurement

Liver iron content was determined by atomic absorption spectrometry with acetylene-air flame atomization. The analysis was performed with a Varian atomic absorption spectrometer (Mulgrave) with deuterium background correction. Measurements were obtained with a 248.3 nm analytical line in the spectral interval of 0.2 nm. Iron concentration was determined by the standard addition method. Sample digestion was accomplished with the MDS 2000 microwave sample preparation system (CEM) in Teflon cartridges, using a mixture of nitric acid (5 mL) and hydrogen peroxide (2 mL) (both from Merck, ultrapure grade) for 20 min at a pressure of 120 psi. The resulting product was analyzed directly in the Teflon cartridges.

Histology and immunohistochemistry

Part of liver lobe was excised, and the tissue was fixed in 10% neutral formalin solution and embedded in paraffin. Hematoxylin and eosin staining and Van Gieson's (VG) staining were performed according to standard procedures. The severity and degree of lesions were graded according to methods previously described[24,25]. Briefly, tissue sections (4 μm thick) were treated with HCl (5%) to liberate ferric ions. The samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. The slides were counterstained with Neutral red. For immunohistochemical examination, deparaffinized sections were incubated with HO-1 antibodies (1:1000 dilution) and appropriate biotinylated secondary antibodies, followed by the avidin-biotin-peroxidase complex. The immunoreactive signal was developed by color deposition, using diaminobenzidine as a substrate. Yellow material in the cytoplasm was considered to indicate positive cells. Cell staining was assigned 4 scores, as previously described[26]. The final score was defined as staining intensity × percentage of positive cells. The mean score of five fields was used to compare the six groups.

Hepatic HYP content

Liver tissue (100 mg) was prepared for HYP determination, according to a modification of the method previously described[27]. The HYP content of the liver, as an indirect measurement of tissue collagen content, was expressed in microgram per gram of wet weight (μg/g).

Measurement of plasma hepcidin

Plasma hepcidin was measured by ELISA and was determined using 96-well microtiter plates coated with the recombinant peptide and a polyclonal antibody (Santa Cruz Biotechnology, INC, 1:3000 dilution). Assay procedures were performed according to the manufacturer's instructions, and absorbance of each well was determined at a 450 nm wavelength. The process was performed as described previously[28].

Western blot analysis

The resected hepatic tissues were extracted with lysis buffer (1% Triton X-100; 50 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; and 1% protease inhibitor cocktail). The protocols for western blot analyses have been described previously[29]. Western blot analyses were performed with liver homogenates (30 μg protein) using anti-nuclear factor-E2-related factor 2 (Nrf2) antibody (Boster Biological Technology, Wuhan, China, 1: 100 dilution), anti-TGF-β1 antibody (Boster Biological Technology, Wuhan, China, 1:100 dilution), anti-HO-1 antibody (Abcam, Cambridge, MA, United States, 1:2000 dilution), anti-β-actin antibody (Zhongshan Goldenbridge Biological Technology, Beijing, China, 1:500 dilution), and secondary antibody anti-rabbit and anti-mouse immunoglobulin G (Biosynthesis Biotechnology, Beijing, China, 1:500 dilution). The intensity of each signal was corrected using the values obtained from the immunodetection of β-actin, and the relative protein intensity was expressed as multiples of the content in the normal group.

RNA isolation and gene expression analysis

Total RNA was extracted from the livers following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm (A_{260} = 1 for 40 μg/mL RNA), and the purity of RNA was assessed by determining the ratio of the optical density obtained at 260 and 280 nm (pure RNA: A_{260}/A_{280} = 2.0) using a UV-1206 spectrophotometer (Shimadzu, Japan). An aliquot of each mixture was used for reverse-transcription (RT)-PCR amplification, using reagents purchased from Takara Bio Inc. (Dalian, China). PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were

Table 1 Primers used for the reverse transcription-polymerase chain reaction and polymerase chain reaction analysis

Gene	Gene ID	Forward/reverse	Sequences 5'-3'	Product size (bp)
HO-1	NM012580	Forward	ATATCTATACGGCCCTGGAA	350
		Reverse	GATGCTCGGGAAGGTGAA	
Nrf2	AF304364	Forward	GACGGCAACACTGATTCCA	345
		Reverse	CATCCGCCACTCATTCCT	
TGF-β1	NM021578	Forward	CCGCAACAACGCAATCTA	437
		Reverse	TGAGGAGCAGGAAGGGTC	
Hepcidin	NM053469	Forward	GCTGCCTGTCTCCTGCTT	159
		Forward	GGTGTCTCGCTTCCTTCG	
TIMP-1	NM053819	Forward	CTCTGGCCTCTGGCATCCT	300
		Reverse	ACTCCTCGCTGCGGTTCT	
MMP-2	NM031054	Forward	CTGGGCAACAAGTATGAGA	430
		Reverse	CTGTCCGCCAAATAAACC	
β-actin	NM031144	Forward	GAGGGAAATCGTGCGTGAC	445
		Reverse	CTGGAAGGTGGACAGTGAG	

HO-1: Heme oxygenase-1; Nrf2: Nuclear factor-E2-related factor 2; TGF-β1: transforming growth factor-β1; TIMP-1: Tissue inhibitor of metalloproteinase-1; MMP-2: Matrix metalloproteinase-2.

photographed, and the density of each product band was quantified. The results are expressed as the ratios of the band density for target mRNA to that of β-actin mRNA. The primers utilized for PCR and RT-PCR are listed in Table 1.

Statistical analysis

All of the data are presented as the mean ± SD. Statistical testing was performed with SPSS software, version 16.0. The groups were compared using one-way analysis of variance with Dunnett's multiple comparison test (where applicable). Correlative comparison of two non-hierarchical variances with normal distribution was evaluated by Pearson's test, whereas Spearman's test was used for non-normally distributed data. $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of biochemical indicators in liver fibrosis induced by BDL

Four weeks postoperatively, common bile duct dilatation was observed in the BDL group, and ascites and jaundice also developed in the BDL group, suggesting that the BDL model was successfully established in our experiments.

The serum levels of AST, ALT and TBIL in the BDL group were much higher than those in the Sham group ($P < 0.01$). These levels were much lower in the ZnPP group and DFX group, but the levels in the CoPP group and Fe group were significantly higher CoPP than those in the BDL group ($P < 0.01$) (Figure 1G and H). The serum levels of AST were decreased in the DFX group compared with those in the ZnPP group ($P < 0.05$) (Figure 1G). These data indicated that inhibiting HO-1 expression and further reducing iron accumulation could improve liver function; in contrast, inducing HO-1 expression aggravated liver injury.

Inhibiting HO-1 expression reduced liver fibrosis and PVP induced by BDL

Liver damage was analyzed by hematoxylin and eosin staining. The livers in the Sham group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation were found in the interlobular and central venous regions in the livers in the BDL and CoPP groups (Figure 1B and C). Compared with the BDL group, fibrous hyperplasia was significantly reduced around the central veins in the ZnPP group (Figure 1D). The histopathological scores for fibrosis in the livers of BDL rats were improved in the ZnPP group (Figure 1I). Collagen type I was observed with VG staining (Figure 2A-F). In the BDL group, collagen type I in the portal area and bile duct wall was much thicker than in the Sham group ($P < 0.01$) (Figure 2A and B). Compared with the BDL group, there was a decrease in type I collagen in the ZnPP group. The extent of fibrosis was much higher in the CoPP group than in the BDL group ($P < 0.01$) (Figure 2G). The change in HYP content in liver tissue was in accordance with type I collagen. It was observed that HYP was significantly decreased in the ZnPP group compared with the BDL group (Figure 2H). These data showed that inhibiting HO-1 expression reduced collagen deposition in liver fibrosis.

The COHb levels in arterial blood were significantly higher in the BDL group compared with the Sham group Sham ($P < 0.01$), and they were much lower following ZnPP and DFX treatment, while they were higher in the CoPP- and Fe-treated groups than in the BDL group ($P < 0.01$) (Figure 3A). PVP was significantly higher in the BDL group compared with the Sham group ($P < 0.01$). Compared with the BDL group, PVP decreased in the ZnPP and DFX groups ($P < 0.01$) and was enhanced in CoPP and Fe rats ($P < 0.01$). Moreover, PVP decreased following ZnPP treatment relative to DFX treatment ($P < 0.05$) (Figure 3B).

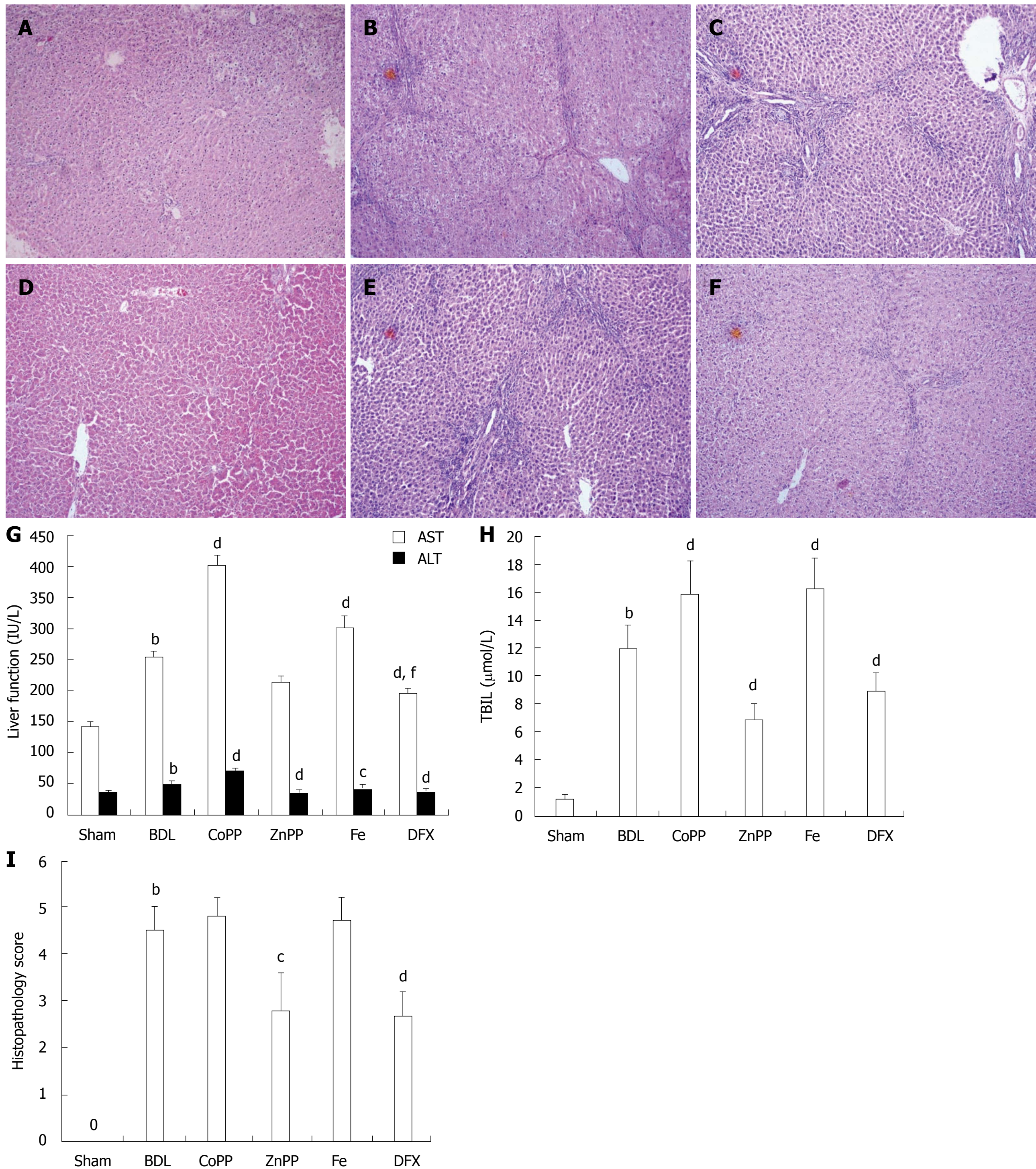


Figure 1 Pathological features of rat liver tissue detected by hematoxylin and eosin staining and serum index. A: Normal lobular architecture in the Sham group; B, C, E: Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D and F: Less fibrous hyperplasia and fibrosis in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; G, H: Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (TBIL); I: Histopathological scores for fibrosis (magnification × 100). Values are expressed as mean ± SE (n = 6). $^bP < 0.01$ *vs* Sham group; $^cP < 0.05$, $^dP < 0.01$ *vs* BDL group; $^fP < 0.01$ *vs* ZnPP group.

Levels of TGF-β1 were significantly enhanced in the BDL group compared with the Sham group ($P < 0.01$). These levels were lower in the ZnPP group and higher in the CoPP group compared with the BDL group (Figure 4A and B). The mRNA expression levels of MMP-2 and TIMP-1 were much higher in the BDL group than in the Sham group ($P < 0.01$). These levels were significantly lower in the ZnPP group and higher in the CoPP group than in the BDL group ($P < 0.01$) (Figure 4C). These results showed that down-regulated HO-1 expression reduced extracellular matrix (ECM) deposition and fibrosis.

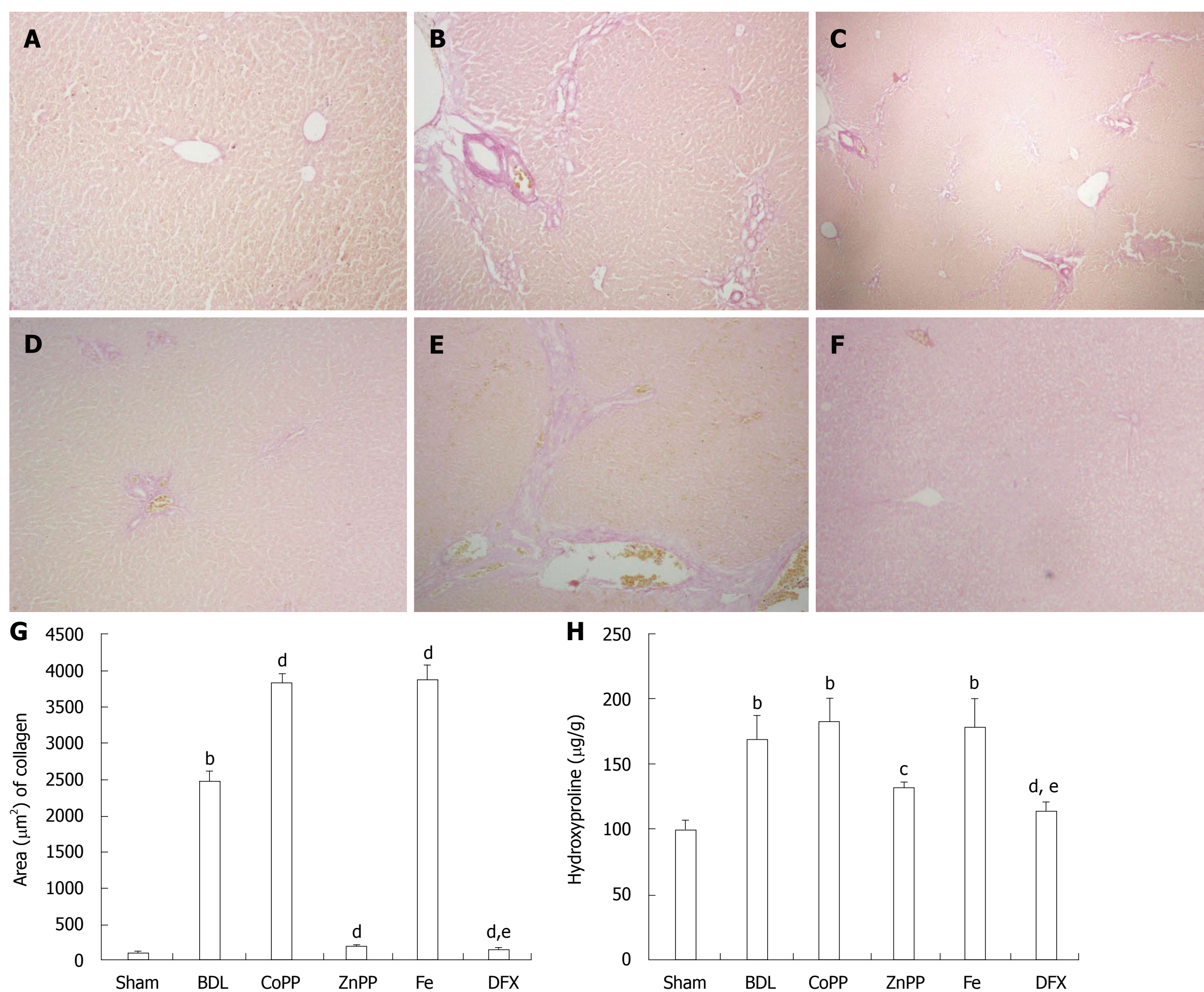


Figure 2 Van Gieson's staining of collagen Ⅰ for liver sections and liver hydroxyproline content. A-F: Collagen type Ⅰ was deposited in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group (B, C and E) and was rarely found in the Sham group, zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group (A, D and F); G: The area of collagen type Ⅰ; H: Hydroxyproline content of liver tissue (magnification, × 100). Values are expressed as mean ± SE (n = 6). ^bP < 0.01 *vs* Sham group; ^cP < 0.05, ^dP < 0.01 *vs* BDL group; ^eP < 0.05 *vs* ZnPP group.

HO-1 mediated iron accumulation and oxidative stress in liver

The mRNA and protein expression levels of HO-1 were significantly higher in the BDL group than in the Sham group (P < 0.01). These levels were obviously lower in the ZnPP group and higher in the CoPP group than in the BDL group (P < 0.01) (Figure 4A and B). Hepatic immunostaining showed that HO-1 was mainly expressed in the liver cells and partly in the mesenchymal cells and Kupffer cells. Localization of HO-1 occurred mainly around the centrilobular veins (Figure 5A-F). The values of HO-1 expression were consistent with the above data (Figure 5G).

The serum levels of iron in the BDL group were significantly higher than in the Sham group (P < 0.01). These levels were greatly lower in the ZnPP group than in the BDL group (P < 0.01) (Figure 6G). The change in liver iron content was in accordance with serum iron levels (Figure 6H).

The mRNA and plasma levels of hepcidin were significantly lower in the BDL group than in the Sham group (P < 0.01). These levels were higher in the ZnPP group and lower in the CoPP group compared with the BDL group, and they were higher in the DFX group than in the ZnPP group (Figure 4C and 6I).

We used Prussian blue staining to localize iron accumulation in liver tissue and found that iron obviously accumulated in the BDL and CoPP groups. Iron was strongly stained mainly in Kupffer cells in these groups (Figure 6B and C). However, iron staining was rarely found in the Sham and ZnPP groups (Figure 6A and D). These results indicate that inhibiting HO-1 expression could reduce iron production, resulting in decreased iron accumulation in the liver. In contrast, enhanced HO-1 expression led to increased hepatic accumulation of iron.

Levels of SOD were obviously lower in the BDL group than in the Sham group (P < 0.01), and they were significantly higher in the ZnPP group and lower in the CoPP group than in the BDL group (P < 0.01) (Figure 3C). The MDA change tendency was opposite that of

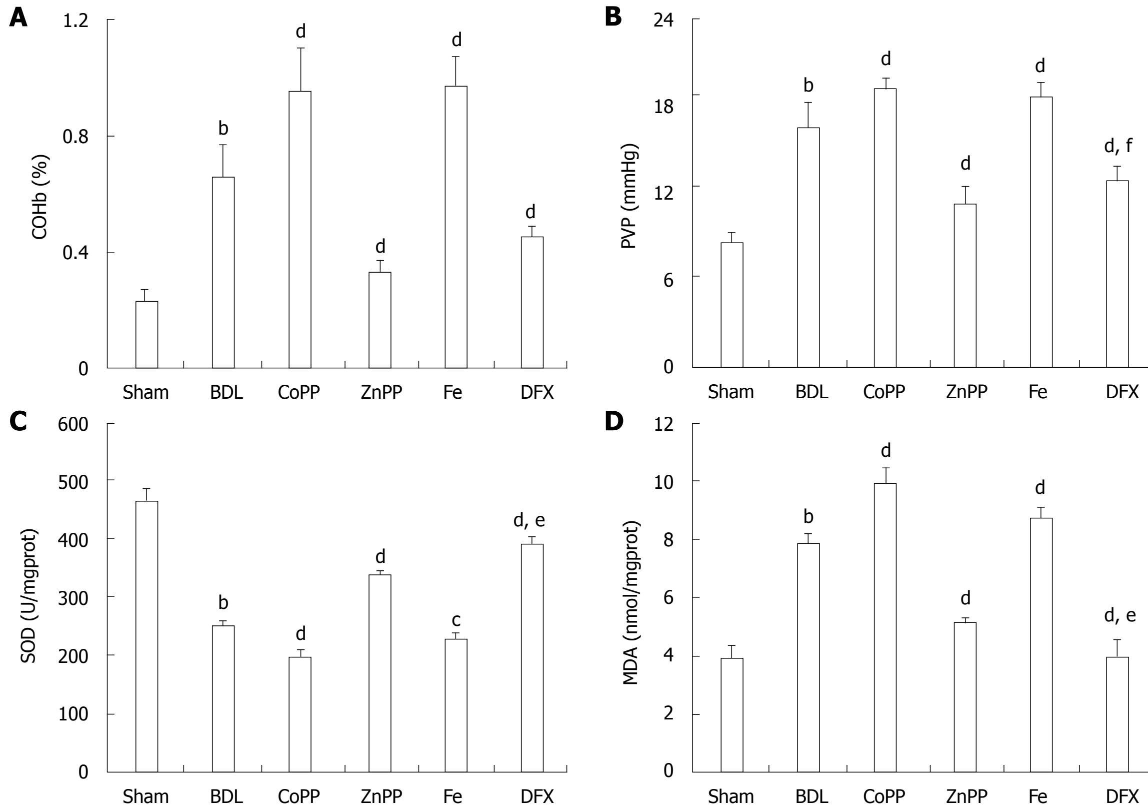
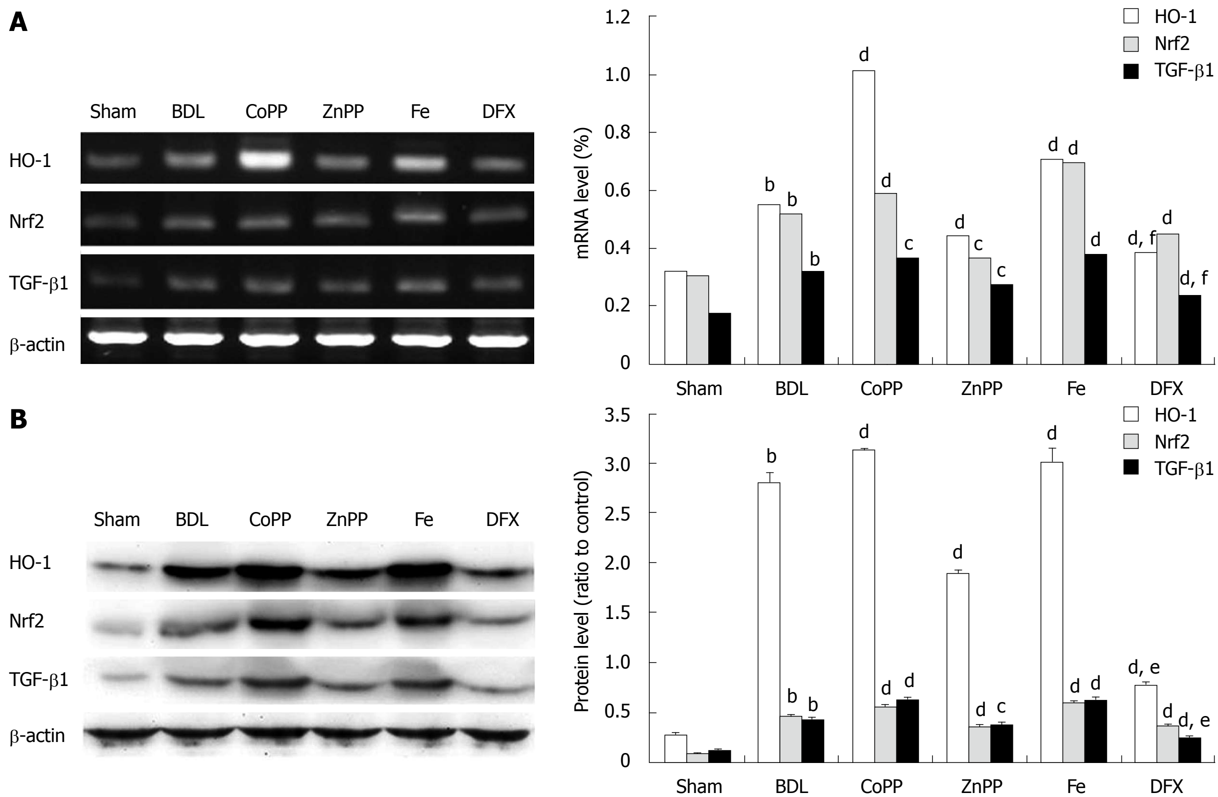


Figure 3 The levels of carboxyhemoglobin, portal vein pressure malonaldehyde and superoxide dismutase. A, B: The levels of carboxyhemoglobin (COHb) were accordance with heme oxygenase-1 expression, and portal vein pressure (PVP) levels were measured; C, D: The levels of superoxide dismutase (SOD) and malonaldehyde (MDA) was detected. Values are expressed as mean ± SE (n = 6). ^{b}P < 0.01 *vs* Sham group; ^{d}P < 0.01 *vs* BDL group; ^{e}P < 0.05, ^{f}P < 0.01 *vs* zinc protoporphyrin (ZnPP) group. BDL: Bile duct ligation; CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.



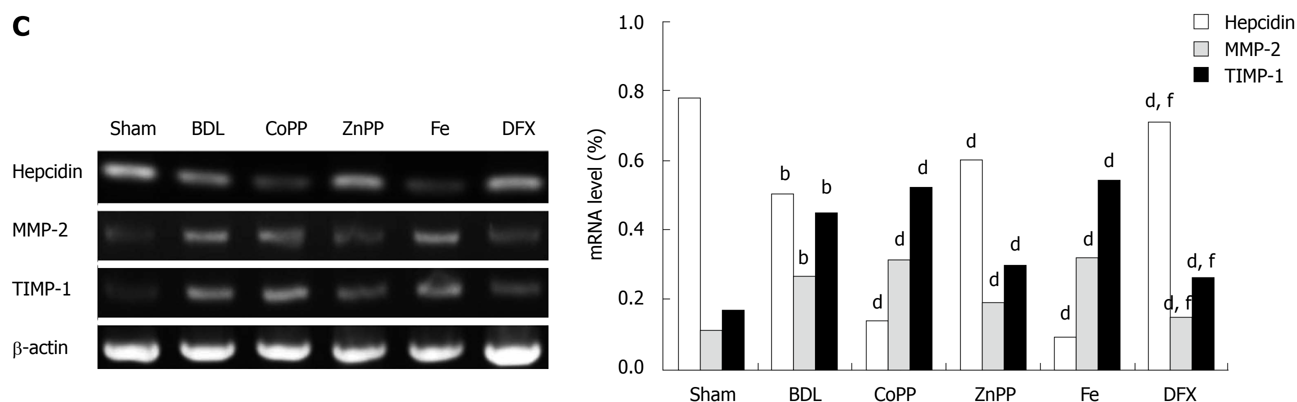


Figure 4 The heme oxygenase-1, transforming growth factor-β1, nuclear factor-E2-related factor 2, hepcidin, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression were detected by Western blot and reverse transcription-polymerase chain reaction. A, B: The mRNA and protein levels of heme oxygenase-1 (HO-1), transforming growth factor-β1 (TGF-β1) and nuclear factor-E2-related factor 2 (Nrf2); C: The mRNA levels of hepcidin, matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1). Values are expressed as mean ± SE. $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* bile duct ligation (BDL) group; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* zinc protoporphyrin (ZnPP) group. CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.

Figure 5 Liver sections were stained with heme oxygenase-1 antibody. A: Heme oxygenase-1 (HO-1) expression was less around the central veins in the Sham group; B, C, E: Much more HO-1 expression was found around the central veins in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D, F: Less staining was observed in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; H: Immunohistochemical staining scores (magnification ×400). Values are expressed as mean ± SE (n = 6). $^{b}P < 0.01$ *vs* Sham group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* BDL group; $^{f}P < 0.01$ *vs* ZnPP group.

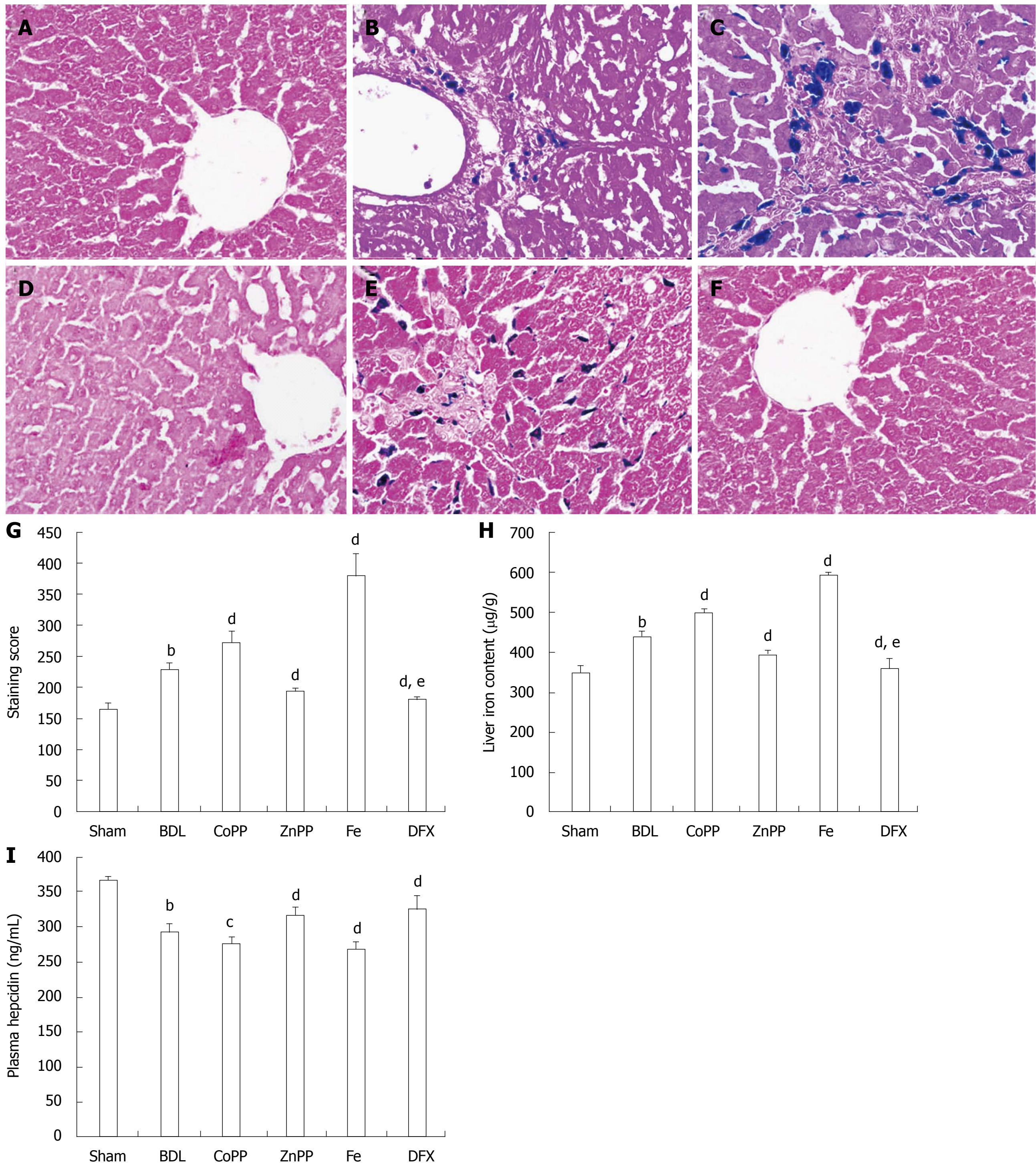


Figure 6 Perl's Prussian blue staining, levels of hepcidin, serum and liver iron. A: No iron accumulated in the Sham group; B: A small amount of iron mainly accumulated on Kupffer cells in the bile duct ligation (BDL) group; C: Much more iron accumulation was found in interlobular and macrophagocytes in the cobalt protoporphyrin (CoPP) group; D, F: Almost no iron accumulation was detected in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; E: Massive iron accumulation was observed in the Fe group; G, H: There were no differences in the hepatic and serum iron content of these six groups; I: Plasma hepcidin also was measured by enzyme-linked immuno sorbent assay (magnification × 400). Values are expressed as mean ± SE (n = 6). $^bP < 0.01$ *vs* Sham group; $^cP < 0.05$, $^dP < 0.01$ *vs* BDL group; $^eP < 0.05$ *vs* ZnPP group.

SOD (Figure 3D). In the BDL model, inhibiting HO-1 expression reduced oxidative stress.

Iron induced oxidative stress and Nrf2 expression

Both the serum iron and liver iron content were higher in the Fe group but lower in the DFX group compared with the BDL group (Figure 6G and H). Prussian blue staining showed more iron accumulation in the Fe group and less in the DFX group (Figure 6E and F). The ZnPP group showed low iron levels (Figure 6D and F-H). The levels of plasma hepcidin were obviously lower in the Fe group but higher in the DFX group compared with the

BDL group (Figure 6I). The expression of HO-1 was significantly higher in the Fe group but was lower in the DFX group compared with the BDL group ($P < 0.01$). It was also lower in the DFX group than in the ZnPP group (Figure 4A and B).

The mRNA and protein expression levels of Nrf2 were enhanced in the BDL group compared with the Sham group. Additionally, these levels were significantly higher in the CoPP and Fe groups than in the BDL group, and they were lower in the ZnPP and DFX groups ($P < 0.01$) (Figure 4A and B). We found the levels of SOD in the Fe group to be slightly lower than in the BDL group ($P < 0.05$); however, they were much higher than in the DFX group ($P < 0.01$) (Figure 3C). The levels of MDA were significantly increased in the Fe group but were reduced in the DFX group compared with the BDL group ($P < 0.01$) (Figure 3D). The levels of SOD were much higher, and MDA was lower in the DFX group compared with the ZnPP group ($P < 0.01$) (Figure 6C and D). These data indicate that iron accumulation in the liver increased the oxidative stress reaction and caused further damage to the liver.

Hematoxylin and eosin staining showed more fibrous hyperplasia in the Fe group and less in the DFX group compared with the BDL group (Figure 1E and F). The content of HYP was significantly higher in the Fe group than in the Sham group ($P < 0.01$), and it was lower in the DFX group than in the BDL group ($P < 0.01$) (Figure 2H). Compared with the BDL group, collagen I was increased in the Fe group and decreased in the DFX group (Figure 2E-G). The mRNA and protein expression levels of α-smooth muscle actin and TGF-β1 were significantly enhanced in the Fe group and decreased in the DFX group compared with the BDL group (Figure 4A and B). The levels of MMP-2 and TIMP-1 mRNA were much higher in the Fe group and were lower in the DFX group compared with the BDL group ($P < 0.01$). Compared with the ZnPP group, TGF-β1 expression and ECM were lower in the DFX group (Figure 4C).

Correlation between oxidative stress and liver fibrosis

Correlation analysis revealed that both SOD and MDA were significantly correlated with HYP levels (R = -0.912, 0.887, respectively, $P < 0.01$). These data also showed that oxidative stress could result in ECM deposition in the liver and could further aggravate liver fibrosis.

DISCUSSION

Many chronic liver diseases progress to hepatic fibrosis[30]. Iron overload in the liver increased the risk of developing fibrosis, as well as subsequent morbidity and mortality[31]. HO-1 catalyzes heme into iron, and it plays an important role in iron homeostasis. A previous study showed that HO-1 was associated with hepatocellular damage and had multiple mechanisms to influence liver fibrosis progression. In this study, we aimed to investigate how iron and CO, the product affected by HO-1 activity, affected hepatic fibrosis and PVP. We found that lower HO-1 expression could reduce iron accumulation and PVP and improve fibrosis.

In several chronic liver diseases, HO-1 plays a protective effect in the liver against oxidative stress-dependent damage[32-34]. However, its protective effects in inflammation and fibrosis have been disputed. Some studies have shown that HO-1 over-expression increases liver injury in rats under conditions of experimental chronic cholestasis[19]. Low HO-1 induction was shown to be cytoprotective, and high levels of HO-1 could result in the accumulation of free divalent iron, thus increasing oxidative injury in fibroblast cell cultures[35]. We found that lower HO-1 expression could benefit end-stage liver cirrhosis by reducing iron accumulation, which is accordance with the findings of the above studies. Surprisingly, induction of HO-1 interfered with chronic inflammation and prevented progression of liver fibrosis in Mdr2-knockout mice, and it further might delay progression to hepatocellular carcinoma[33]. Our previous study indicated that induction of HO-1 could ameliorate immune liver fibrosis[36]. The reason why the above studies are different from this study could be that HO-1 plays a diverse role in different stages during the progression of liver fibrosis. In early stages of liver fibrosis, inducing HO-1 could have a protective effect, but it could increase liver injury in end stages *via* liver hypertension. Moreover, the different animal models for inducing fibrosis could constitute another explanation of these results.

The majority of endogenous CO is catalyzed by inducible expression of HO-1. CO can modulate blood flow and maintain the integrity of the vessel wall[37]. COHb levels can be used to estimate HO activity in experimental animals. Interestingly, we observed that upregulated COHb resulted from increased HO-1, which aggravated PVP in BDL rats. Moreover, lower levels of COHb can decrease the PVP found in the ZnPP and DFX treatment groups. HO/CO plays a role in the pathophysiology of portal hypertension, and CO can regulate the intrahepatic vascular resistance of cirrhotic rats[38]. Tarquini *et al*[39] indicated that the HO/CO system is activated in patients with liver cirrhosis, and CO contributes to the hyperdynamic circulatory syndrome. CO might improve intrahepatic microcirculation in early stage hepatic fibrosis, and excessive CO could be harmful, leading to an unbalanced nitric oxide/CO system in end-stage hepatic fibrosis. It therefore seems best to reduce PVP by decreasing CO.

Normally, HO-1 is only slightly expressed in hepatocytes and Kupffer cells. In hepatic cirrhosis, the expression of HO-1 is increased. Khan *et al*[20] reported that an increase in HO-1 expression is associated with iron accumulation. The study of Kartikasari *et al*[40] showed that iron is derived from intracellular heme degradation, and HO-1 activity contributes to increased levels of intracellular labile iron. Other research has shown that non-heme iron increases are associated with the induction of HO-1 in neurons, microglia and capillary endothelial cells, whereas HO-2 levels remain unchanged, implying that the non-heme iron increases might be the result

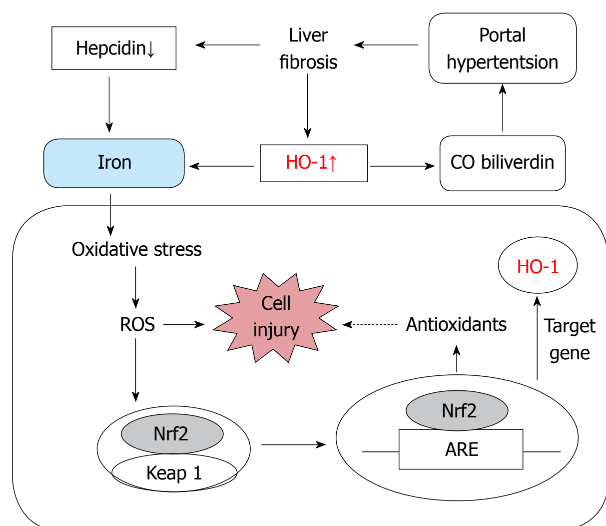


Figure 7 Iron is involved in the heme oxygenase-1 cycle *via* the nuclear factor-E2-related factor 2/Keap1 pathway, and heme oxygenase-1 regulates portal pressure in liver cirrhosis. Low hepcidin and heme oxygenase-1 (HO-1) over-expression could mediate iron accumulation, which accelerates oxidative stress, leading to cell injury, and it also increases HO-1 expression through the nuclear factor-E2-related factor 2 (Nrf2)/Keap1 pathway. Nrf2 protects cells against oxidative stress, but this effect is limited, and Nrf2 contributes to induction of HO-1 expression, which can produce iron. HO-1 can increase carbon monoxide (CO) production, and an unbalanced CO/nitric oxide system could play a role in portal pressure. ROS: Reactive oxygen species. ARE: Antioxidant response element.

of HO-1-mediated heme degradation[41]. These results showed that HO-1 played a central role in maintaining iron homeostasis *in vivo*. In this study, we found that serum iron and liver iron contents all increased in the CoPP group, and inhibiting HO-1 activity with ZnPP reduced iron accumulation in the liver and further attenuated liver fibrosis in liver fibrosis induced by BDL.

Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum. It was also noted that hepcidin was abnormally low in alcoholic patients with associated iron overload[42]. Iron was accumulated in the liver and pancreas of hepcidin-deficient mice[43]. It also was found that serum pro-hepcidin concentrations were lowered in liver cirrhosis, which could be the result of impaired liver functioning[44]. Hepcidin is down-regulated during progressive cholestasis in biliary atresia[45-47]. Furthermore, Huang *et al*[48] showed that iron loading down-regulates hepcidin by inhibiting both inflammatory and iron-sensing pathways and inhibiting transducers and activators of transcription 3 and SMAD4 signaling *in vivo*. These findings are consistent with the results of our experiment. Under physiological conditions, hepcidin expression is stimulated by iron overload and inflammation and is suppressed by anemia and tissue hypoxia[49,50]. However, levels of hepcidin were decreased in the iron accumulation group and were increased in the ZnPP and DFX groups in our study. The reason for this finding might have been the various signals affecting hepcidin production. Up-regulation of hepcidin by inhibiting HO-1 expression could be beneficial for cholestasis in cirrhosis.

It is now commonly accepted that HO-1 plays an important role in the control of inflammation and oxidative stress[51]. HO-1 protected primary human hepatocytes from ethanol-derived oxidative stress *via* the MAPK/Nrf2 pathway[52]. Surprisingly, however, in this study, we found that inducing HO-1 expression increased MDA and decreased SOD. Further, these results indicate that HO-1 could not reduce the oxidative stress reaction. Other studies have shown the pro-oxidant nature of the released cellular low-molecular-mass iron and the antioxidant effect of the released bilirubin[53]. In this study, we demonstrated that the pro-oxidant activities of iron accumulation were much stronger than the antioxidant effects of bilirubin.

Iron primarily accumulates in the reticuloendothelial cells. Previously, it was shown that increased deposition of iron in the liver often triggered oxidative stress and inflammation and induced liver cell damage[54]. It can participate in Fenton and Haber-Weiss chemistry, and excessive redox-active iron might lead to oxidative stress, with damage to membranes, proteins and DNA[55]. It was also shown that increased deposition of iron in the liver induced liver cell damage and cirrhosis by triggering oxidative stress and inflammation[56]. In fact, signs of iron-catalyzed lipid peroxidation and oxidative stress have been found by many investigators during chronic iron overload in rodents[57,58]. In this study, iron intoxication dramatically enhanced MDA adducts, decreased antioxidant enzyme SOD and aggravated liver injury in the BDL, CoPP and Fe groups. Our results revealed that iron accumulation exacerbated the oxidative stress reaction, leading to the aggravation of liver cirrhosis.

Previous reports have shown that elevated hepatic iron can activate Nrf2 in 3,5,5-trimethyl-hexanoyl-ferrocene-treated mouse models[59]. In our study, Nrf2 was up-regulated in the BDL, CoPP and Fe groups, in which iron accumulation in the liver was found. Nuclear translocation of activated Nrf2 is an important upstream contributor to the induction of HO-1 expression[60]. Up-regulation of Nrf2 increased HO-1 gene transcription in the CoPP and Fe groups. The pathway of iron-dependent HO-1 induction involves Nrf2/Keap. Nrf2 also plays a key role in the protection of cells against oxidative stress[61]. However, in our study, Nrf2's protective effect was limited, and HO-1, which is its target gene, increased iron production, resulted in oxidative stress.

Previous studies have demonstrated that oxidative stress significantly contributes to hepatic fibrogenesis from various liver injuries[62]. Reactive oxygen species (ROS) can stimulate the production of type Ⅰ collagen and could act as intracellular signaling mediators of TGF-β[63,64]. Our study showed that preventing oxidative stress by inhibiting HO-1 expression could attenuate liver fibrosis through regulation of the TGF-β1 pathway and reducing collagen deposition.

In conclusion, HO-1 played a pivotal role in iron accumulation and portal pressure in the livers in our study

(Figure 7). In the clinic, many end-stage cirrhosis patients with upper gastrointestinal bleeding treated with multiple massive transfusions run the risk of iron overload and further liver injury. Today, iron chelation therapy is often utilized to remove excess stored iron in some diseases[65,66]. In our study, iron accumulation induced hepatic fibrogenesis, indicating that cirrhotic patients with massive stored RBC transfusions would benefit from iron removal therapy. Our research provided a new way to reduce liver iron and portal pressure by inhibiting HO-1 expression. However, we must find a proper model for the simulation of upper gastrointestinal bleeding and transfusion. In clinical experiments, we will attempt to include cirrhotic patients with bleeding complications, who would eventually receive transfusions, to investigate the effects of iron transfusions on liver cirrhosis.

Removing iron and reducing portal pressure by inhibition of HO-1 improves liver fibrosis in bile duct-ligated rats. In addition, iron is also closely related to another complication of cirrhosis: hepatocellular carcinoma[67]. Regulation of iron homeostasis, by interfering with HO-1, could effectively treat hepatic cirrhosis and also prevent hepatocellular carcinoma.

ACKNOWLEDGMENTS

The authors thank Li Lv for providing the clinical and experimental equipment. The authors also thank Qin Zhou for technical support.

COMMENTS

Background

Iron overload in the liver is a very common phenomenon in many chronic liver diseases. Heme oxygenase-1 (HO-1) and its by-products, iron and carbon monoxide (CO), play crucial roles in hepatic fibrosis. The underlying molecular mechanisms of HO-1, regarding iron deposition and portal vein pressure (PVP) in hepatic fibrosis, remain unknown.

Research frontiers

HO-1 and degradation products are important to cytoprotection in many types of liver injury, but protection can be restricted to a narrow threshold. Iron overload often triggers oxidative stress and inflammation and induces liver cell damage, and the CO/nitric oxide system could be harmful to portal pressure. Iron can activate nuclear factor-E2-related factor 2 (Nrf2) and increase HO-1 expression. Inhibiting HO-1 activity is necessary for reducing iron and PVP.

Innovations and breakthroughs

In this study, by inhibiting HO-1 expression by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation (BDL), the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity though the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining PVP.

Applications

Removing iron and reducing CO by inhibiting HO-1 activity provides a new strategy for treating liver fibrosis, and further, it could help prevent liver carcinoma.

Terminology

HO-1 is a primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme to free iron, CO, and biliverdin. Nrf2 is an important upstream contributor to the induction of HO-1 expression, and it has a protective effect on cells against oxidative stress. Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum.

Peer review

This study analyzed the role of HO-1 inhibition in rat liver fibrosis using an experimental model of BDL. The results are interesting, and they suggest that regulation of iron homeostasis and CO production could effectively treat liver cirrhosis and PVP and even prevent hepatocellular carcinoma.

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P- Reviewers Bustos M, Boscá L, Montecucco F, Okoshi S
S- Editor Huang XZ **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2935

World J Gastroenterol 2013 May 21; 19(19): 2935-2940
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Long-term follow-up study of gastroduodenal lesions after radioembolization of hepatic tumors

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Received: November 6, 2012 Revised: January 18, 2013
Accepted: February 2, 2013
Published online: May 21, 2013

Abstract

AIM: To evaluate the long-term natural history of the gastroduodenal lesions secondary to extrahepatic embolization with Ytrium 90 (^{90}Y) spheres.

METHODS: From September 2003 to January 2012, 379 procedures of liver radioembolization (RE) using resin microspheres loaded with ^{90}Y were performed in our center. We have retrospectively compiled the data from 379 RE procedures performed in our center. We report a comprehensive clinical, analytical, endoscopic and histologic long-term follow-up of a series of patients who developed gastroduodenal lesions after the treatment.

RESULTS: Six patients (1.5%) developed gastrointestinal symptoms and had gastrointestinal lesions as shown by upper endoscopy in the next 12 wk after RE. The mean time between RE and the appearance of symptoms was 5 wk. Only one patient required endoscopic and surgical treatment. The incidence of gastrointestinal ulcerations was 3.75% (3/80) when only planar images were used for the pre-treatment evaluation. It was reduced to 1% (3/299) when single-photon emission computed tomography (SPECT) images were also performed. The symptoms that lasted for a longer time were nausea and vomiting, until 25 mo after the treatment.

CONCLUSION: All patients were free from severe symptoms at the end of follow-up. The routine use of SPECT has decreased the incidence of gastrointestinal lesions due to unintended deployment of ^{90}Y particles.

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Key words: Radioembolization; Liver neoplasms; Gastroduodenal ulcer; Single-photon emission computed tomography; Liver

Rodríguez-Lago I, Carretero C, Herráiz M, Subtil JC, Betés M, Rodríguez-Fraile M, Sola JJ, Bilbao JI, Muñoz-Navas M, Sangro B. Long-term follow-up study of gastroduodenal lesions after radioembolization of hepatic tumors. *World J Gastroenterol* 2013; 19(19): 2935-2940 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2935.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2935

INTRODUCTION

Radioembolization (RE) with microspheres embedded with Yttrium 90 (^{90}Y) is a therapeutic option for primary and secondary liver tumors. Prior to treatment, an angiography is performed to identify the hepatic vasculature and to mimic the actual procedure by injecting ^{99}Tc-radiolabeled macroaggregated albumin (99Tcm-MAA)[1]. During this procedure, guidelines recommend the embolization of the gastroduodenal artery, right gastric, and other extrahepatic arteries to isolate the hepatic circulation[2]. Unintended extrahepatic deployment of particles is a rare but well known complication of this procedure, that may result in cholescystitis[3], pancreatitis[4], radiation pneumonitis[5] and gastrointestinal ulcerations[6,7]. Radiation injury is likely to be the main pathogenical factor of these lesions[6]. However, although the small diameter of the particles (20-30 μm) does not induce a significant macroembolic effect, it has been shown in animal models that microspheres may sometimes aggregate and occlude small size vessels[8].

Radiation-induced gastrointestinal ulcerations may have a significant negative impact in quality of life due to decreased oral intake, pain or anemia, and the even need to perform gastric surgery has been described[9]. However, most case reports or series only describe the appearance and immediate consequences and very little is known about the long-term outcome of this complication. We describe for the first time a case series of six patients with gastrointestinal lesions secondary to RE and their clinical, endoscopic and histologic long-term follow-up.

MATERIALS AND METHODS

From September 2003 to January 2012, 379 procedures of liver RE using resin microspheres loaded with ^{90}Y (SIR-Spheres, Sirtex Medical, Sidney, Australia) were performed in our center. Six patients (1.5%) developed gastrointestinal symptoms and had an upper endoscopy performed in the next 12 wk after the procedure that showed gastrointestinal lesions. Findings in the initial and the subsequent gastroduodenoscopies and their histopathologic studies were compiled retrospectively. Although the follow-up was not structured, at least two endoscopies were available from all but one patient. The maximum endoscopic follow-up time was 78 mo. We have gathered from the medical reports and thoroughly analyzed all clinical, analytical, endoscopic and histopathological findings.

RESULTS

Patient characteristics

The characteristics of the six patients included in the study are shown in Table 1. Mean age was 53.1 years and a range of primary tumors were treated including cholangiocarcinoma and liver metastases from different primary tumors. None of the patients had any significant gastrointestinal disease before RE and only two patients were receiving concomitant anticancer treatments, namely the combination of 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX, patient 1), and the combination of gemcitabine and oxaliplatin (GEMOX, patient 4).

RE procedure

From the day of RE and for the following 2 mo, all patients received pantoprazole (40 mg daily) and a tapered regimen of corticosteroids.

A selective embolization of any extrahepatic vessel that could be included in the treated volume was performed prior to microsphere injection. Regarding variant anatomies, patient 2 presented a complete occlusion of the celiac axis and reversed blood flow of the pancreatoduodenal artery and in patient 4 the right hepatic artery originated from the superior mesenteric artery. The mean activity of ^{90}Y administered was 1.02 GBq. In 4 patients (66%) the spheres were infused in the common or proper hepatic artery. In one case a bilobar treatment was performed by injecting the microspheres into the left and right branches of the hepatic artery (patient 4). In this patient only half the prescribed dose could be injected into the left hepatic artery due to spasm, while the entire prescribed dose was injected into the right hepatic artery uneventfully. This was the only case in which premature occlusion of the vessel occurred. In the remaining case the site of injection was the left hepatic artery (lobar infusion, patient 2).

99Tcm-MAA scans obtained prior to the procedures consisted in planar scintigraphic images in 3 patients and planar plus single-photon emission computed tomography (SPECT) images in the 3 other patients. When considered relative to the number of RE procedures, the incidence of gastrointestinal ulcerations was 3.75% (3 out of 80 patients) in the early period when planar images were used, and only 1% (3 out of 299 patients) in the late period when SPECT was also performed. We have retrospectively reviewed the 99Tcm-MAA scans of these 6 patients and no activity was observed in the gastrointestinal area prior to RE.

Endoscopic and histological findings

Patients had their first upper endoscopy performed 1 to 12 wk after RE (Table 2) but none of them required an urgent procedure. At diagnosis, all of them showed ulcers and erosions in the stomach and/or duodenum (Figure 1). Gastrointestinal lesions were found only in the stomach in 3 cases, while the duodenum and the pyloric channel were also affected in 1 and 2 patients, respectively. Mucositis (gastritis or duodenitis) was present in 5 patients (83%) and a friable mucosa was described in 2 patients (33%). The mean endoscopic follow-up was 29.6 mo and the end of follow-up was usually due to clinical improvement (5 patients, 83%). One patient (case 6) died 2.5 mo after the procedure due to tumor progression. No patient showed neoangiogenesis, scars or antral deformities at diagnosis but these findings were lately seen in 4 patients (67%) after a mean period of 7.5 mo.

Table 1 Patient and treatment characteristics

Patient	Age (yr)	Primary tumor	Concomitant treatment	Gastroduodenal artery embolization	Site of injection	Gastrointestinal activity on 99Tcm-MAA scan	Dose administrated	Pain during infusion
1	60	Colorectal	FOLFOX	Yes	Common hepatic artery	No	75%	Yes
2	39	Pancreatic NET	No	No	Left hepatic artery	No	100%	No
3	53	Ileal carcinoid	No	Yes	Proper hepatic artery	No	100%	Yes
4	65	Cholangiocarcinoma	GEMOX	No	Right and left hepatic artery	No	75%	Yes
5	54	Renal	No	Yes	Proper hepatic artery	No	100%	Yes
6	48	Colorectal	No	Yes	Proper hepatic artery	No	60%	Yes

99Tcm-MAA scan: ^{99}Tc-radiolabeled macroaggregated albumin; FOLFOX: Folinic acid, fluorouracil and oxaliplatin; GEMOX: Gemcitabine and oxaliplatine; NET: Neuroendocrine tumour.

Table 2 Endoscopic and histologic findings

Patient	Time from RE to first endoscopy (wk)	Findings in first endoscopy (CTCAE 4.02 grading scale)	Histology	Endoscopic follow-up time (mo)	Total number of endoscopies	Endoscopic treatment
1	6	Ulcers in cisura angularis and gastric antrum (20 mm); Multiple erosions in duodenal bulb (2)	Microspheres in lamina propia; No *H. pylori* bacilli	No	1	No
2	5	Severe mucositis in gastric fundus, body and antrum, with mucosal friability and superficial ulcers (2)	Microspheres; No *H. pylori* bacilli	15	3	No
3	8	Severe gastritis (cisura angularis, antrum and pylorus) and duodenitis with ulcers (3)	Microspheres; No *H. pylori* bacilli	53	8	Argon plasma coagulation (after 13 mo of RE)[1]
4	7	Mucositis in gastric body; Extense ulcer in antrum (2)	Microspheres; No *H. pylori* bacilli	1	2	No
5	12	Deep ulcer in pyloric channel and severe mucositis in gastric antrum; Superficial ulcer in duodenal bulb (2)	Microspheres in lamina propia; No *H. pylori* bacilli	78	11	No
6	4	Severe mucositis in gastric antrum, with mucosal friability; Ulcer in pyloric cannel (2)	Microspheres in lamina propia; No *H. pylori* bacilli	1	2	No

[1]A gastroenteroanastomosis was later performed (25 mo after the diagnosis of gastrointestinal ulcers). RE: Radioembolization; CTCAE: Common Terminology Criteria for Adverse Events.

Microspheres were detected in all the biopsy specimens, mainly located in the vessels of the *lamina propria*. Granulation tissue was frequently present next to the ulcers, along with a variable grade of linfoplasmocytic and eosinophilic inflammatory infiltrate. In some samples, focal atypia (patient 1) or anaplasia (patient 3) were present. None of the samples from the gastric mucosa showed bacilli suggestive of *Helicobacter pylori* (*H. pylori*) infection and none of the patients had a previous documented *H. pylori* infection.

Treatment

Only one patient (patient 3) required endoscopic treatment because of late anemia and duodenal stenosis. Thirteen mo after RE, argon plasma coagulation was successfully used to treat the antral and duodenal mucosa. He required this endoscopic treatment because of significant and progressive anemia (hemoglobin dropped from 13.7 g/dL at baseline to 8 g/dL prior to endoscopic treatment and the patient had already received transfusion of 6 units of packed red blood cells) and weight loss. Anemia resolved but his nutritional status worsened over the next mo due to a duodenal stenosis. Finally a gastroenteroanastomosis to the first jejunal loop was performed 25 mo after the diagnosis of gastrointestinal ulcers with antropyloric deformity.

All patients referred abdominal pain as the initial symptom, mainly located in the epigastric region (Table 3). Other symptoms were nausea and vomiting (5 patients, 83%) and anorexia (3 patients, 50%). The mean time between RE and the appearance of symptoms was 5 wk (range 2 to 12 wk). Patients were treated with multiple combinations of proton pump inhibitors, Sucralfate, Almagate, Domperidone, Misoprostole, Pentoxifylline, Cinitapride and Metoclopramide. A slow but progressive improvement was seen in all but one of the patients described above (83%). The majority of these symptoms were mild and graded 1 or 2 in the Common Terminology Criteria for Adverse Events grading scale. Abdominal pain had a maximum grade of 1 or 2, which means a mild pain that only in some patients limited activities of daily living. Nausea and vomiting was graded with a maximum of 1 in all but one patient. This correlates with one or two episodes of emesis a day in the worst period with symptoms during the follow-up.

Table 3 Clinical follow-up and treatment

Patient	Time from RE to symptoms (wk)	Clinical follow-up time (mo)	Symptoms (CTCAE v4.02 grading scale)			Treatment			Weight loss[1] (kg)	Hemoglobin[2] (g/dL)	Reason for end of endoscopic follow up
			Pain	Nausea and vomiting	Anorexia	Treatment used	Time on treatment (mo)	Clinical response			
1	4	6	Epigastric pain (1)	No	No	Pantoprazole, domperidone and almagate	4	Yes	6	-1.5	Improvement
2	4	29	Epigastric pain (1)	Yes (1)	No	Esomeprazole, cinitapride sucralfate and ranitidine	8	Yes	7	-0.6	Improvement
3	4	88	Epigastric pain (2)	Yes (1)	Yes (3)	Pantoprazole, metoclopramide, sucralfate and cinitapride	10	Yes	17	-6.9	Improvement
4	2	9	Left subcostal pain (2)	Yes (1)	No	Pantoprazole, sucralfate and almagate	5	Yes	4	-3.1	Improvement
5	12	88	Epigastric pain (1)	Yes (1)	Yes (2)	Pantoprazole, pentoxifylline, esomeprazole and almagate	5	Yes	4	-1.6	Improvement; still on follow-up
6	4	3	Epigastric pain (2)	Yes (2)	Yes (2)	Esomeprazole, pentoxifylline and misoprostole	1	No	4.1	-2.4	Exitus

[1]Maximal loss of weight over 4 mo; [2]Maximal change in hemoglobin over 4 mo. CTCAE: Common Terminology Criteria for Adverse Events; RE: Radioembolization.

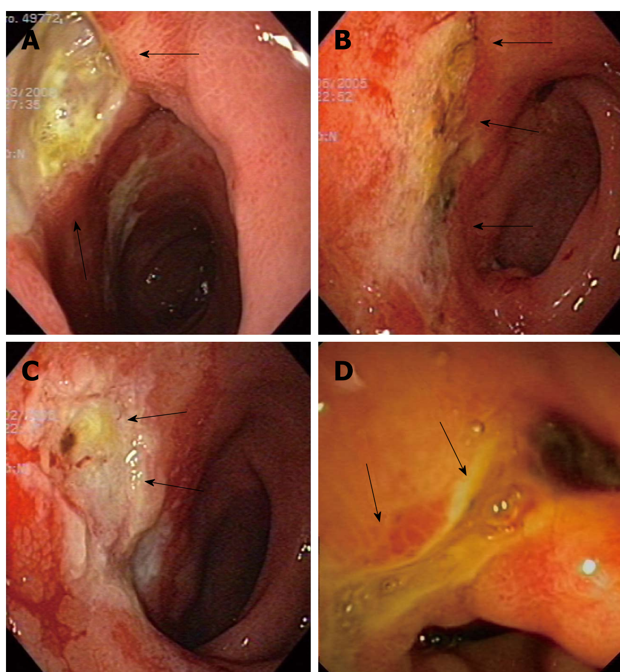

Figure 1 Figures of patient 5. A: A big and deep ulcer was seen in the pyloric channel and duodenal bulb at diagnosis (arrows); B: The same ulcer as in (A) in the same location seen 3 mo later, partially healed (arrows); C: Ulcer located in the duodenal bulb (arrows) with an irregular and friable mucosa after 11 mo; D: Endoscopical view of antral, pyloric and duodenal bulb deformity (arrows) seen with endoscopic ultrasound scope 78 mo after diagnosis.

DISCUSSION

RE is an accepted therapeutic technique for advanced primary and secondary liver tumors. It seems to be better tolerated than transarterial chemoembolization in terms of abdominal pain[10], length of hospital stay[11] and post-embolization symptoms[12]. Main complications do not result from the microembolic effect of the spheres, even in patients with portal vein occlusion, but rather from an excessive irradiation of non-target tissues, including the liver[13].

We describe in this case series an incidence of symptomatic gastroduodenal lesions after RE of 1.5% (6 out of 379 patients). Other authors have reported an incidence between 0% and 28%, with an average 4.8% calculated from the data previously published in 23 studies[7]. The two largest series of patients with secondary liver tumors from colorectal cancer or other malignancies reported rates of grade 3 gastrointestinal ulceration of 1% to 2%[14,15]. A higher incidence of 3.7% was found among 325 patients with hepatocellular carcinoma treated in 8 different European institutions[16].

A decrease in the incidence of gastroduodenal lesions was observed when SPECT images were used for evaluation the RE procedures compared with planar images. These data suggest that SPECT imaging is an important tool to minimize the risk of gastrointestinal adverse events secondary to RE treatment. Our results arise from a retrospective single center study. If they are further confirmed by other series, a possible change in the current guidelines could be considered in favor of the use of SPECT imaging.

In most patients (4 out of 6 patients, 66%) the spheres were injected from the common or proper hepatic artery.

This site of injection leads to a greater chance of non-target embolization of the spheres in extrahepatic vessels. However, we have retrospectively reviewed all the angiographies and none of them showed collaterals or had extrahepatic deposits of MAA in the subsequent scans. In one of the patients treated by bilobar injection, a spasm in the left hepatic artery could have contributed to this complication.

In our case series, two patients were receiving combined concomitant treatment with the combination of oxaliplatin and 5-fluorouracil (FOLFOX) or gemcitabine (GEMOX). None of these drugs produce gastrointestinal lesions that could act as a confounding factor. In a literature search, we have found only one case of gastroduodenal ulceration with FOLFOX therapy and the patient was concurrently treated with Bevacizumab[17]. Saif *et al*[18] described a case of a patient treated with gemcitabine and warfarine who presented with gastrointestinal haemorrhage secondary to gastric and duodenal ulcers (with an international normalized ratio of 8.0). The lesions in our series are not likely to have been caused by these drugs, but we can not exclude a certain contribution.

The most common presenting symptoms in our series were abdominal pain, nausea/vomiting and anorexia. The intensity was usually mild and did not limit daily activities. Many drugs have been used, alone or in combination, demonstrating a slow but effective relieve of symptoms of this complication. However, more studies are needed to determine the best medical treatment. Only one patient required elective surgical treatment due to a late (> 1 year) duodenal stenosis. No urgent endoscopic or surgical treatment was performed. All but one patient are free of symptoms at the end of follow-up. All these data suggest that unintended gastrointestinal deployment of particles should be considered as a potential but transient cause of abdominal pain after the procedure. As shown in our series, the largest so far reported, a conservative approach must be considered as the primary treatment option. Nevertheless, the long-standing duration of symptoms (maximum 25 mo) in association with other factors (anemia, tumor progression and other comorbidities) certainly impacts the quality of life of affected patients. A common approach between oncologists, hepatologists and endoscopists would help improving the management and prognosis of the RE procedure and its complications.

COMMENTS

Background

Primary and secondary liver neoplasms are an important cause of morbidity and mortality worldwide. Radioembolization is a treatment indicated in those patients not eligible for curative resection or liver transplantation but still have their disease confined to the liver. It is considered a liver-directed therapy in which implanted radioactive microspheres are delivered into the arteries that feed the tumors. Secondary to this procedure some of the spheres can get into the arteries that feed some extrahepatic territories as the gastrointestinal tract.

Research frontiers

As the unintended extrahepatic deployment of Ytrium 90 spheres is a rare complication of radioembolization little is know about its management and long-term prognosis. The authors have reviewed the clinical, endoscopic, analytical and histologic long-term follow-up of patients treated with this liver-directed therapy. They also describe how the introduction of new image techniques has decreased the incidence of this complication.

Innovations and breakthroughs

In this retrospective single-center study, the authors have found an incidence of 1.5% of gastrointestinal lesions secondary to the inadvertent deployment of radioactive microspheres. The incidence of this complication decreased from 3.75% to 1% when single photon emission computed tomography images where included in the routine planification of the treatment. The most frequent symptoms referred at diagnosis where abdominal pain and nausea and vomiting.

Applications

The authors have shown the good long-term prognosis of this rare complication. It should be considered as a potential but transient cause of abdominal pain after the procedure. As it may be associated with long-standing symptoms the authors should take into account this complication to increase the patients' quality of life. It seems like the inclusion of new image techniques have decreased the incidence of this complication, but more research is needed to confirm these promising results.

Terminology

Radioembolization: Treatment procedure in which radioactive microspheres loaded with Ytrium 90 are delivered into the hepatic artery.

Peer review

This paper is well written and adds to understanding of outcomes of patients with ulcers following radioembolization.

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P- Reviewers Çil BE, Nosher J **S- Editor** Jiang L
L- Editor A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2941

World J Gastroenterol 2013 May 21; 19(19): 2941-2949
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Predominant mucosal *IL-8* mRNA expression in non-*cagA* Thais is risk for gastric cancer

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Supported by JSPS Ronpaku (Dissertation PhD) program (No. NRCT 10726) award by Japan Society for the Promotion of Scince and in collaboration with Kobe University School of Medicine, Kobe, Japan; JSPS Asian CORE Program 2012, Nippon Medical School, and the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (in part)
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Received: December 28, 2012 Revised: April 1, 2013
Accepted: April 9, 2013
Published online: May 21, 2013

Abstract

AIM: To study gastric mucosal interleukine-8 (*IL-8*) mRNA expression, the cytotoxin-associated gene A (*cagA*) mutation, and serum pepsinogen (PG) Ⅰ/Ⅱ ratio related risk in Thai gastric cancer.

METHODS: There were consent 134 Thai non-cancer volunteers who underwent endoscopic narrow band imaging examination, and 86 Thais advance gastric cancer patients who underwent endoscopic mucosal biopsies and gastric surgery. Tissue samples were taken by endoscopy with 3 points biopsies. The serum PG Ⅰ, Ⅱ, and *Helicobacter pylori* (*H. pylori*) immunoglobulin G (IgG) antibody for *H. pylori* were tested by enzyme-linked immunosorbent assay technique. The histopathology description of gastric cancer and non-cancer with *H. pylori* detection was defined with modified Sydney Score System. Gastric mucosal tissue *H. pylori* DNA was extracted and genotyped for cagA mutation. Tissue *IL-8* and cyclooxygenase-2 (COX-2) mRNA expression were conducted by real time relative quantitation polymerase chain reaction. From 17 Japanese advance gastric cancer and 12 benign gastric tissue samples, all were tested for genetic expression with same methods as well as Thai gastric mucosal tissue samples. The multivariate analysis was used for the risk study. Correlation and standardized *t*-test were done for quantitative data, P value < 0.05 was considered as a statistically significant.

RESULTS: There is a high non *cagA* gene of 86.8 per cent in Thai gastric cancer although there are high yields of the East Asian type in the positive cagA. The *H. pylori* infection prevalence in this study is reported by combined histopathology and *H. pylori* IgG antibody test with 77.1% and 97.4% of sensitivity and specificity, respectively. The serum PG Ⅰ/Ⅱ ratio in gastric cancer is significantly lower than in the non-cancer group, P = 0.045. The serum PG Ⅰ/Ⅱ ratio of less

than 3.0 and IL-8 mRNA expression ⩾ 100 or log_{10} ⩾ 2 are significant cut off risk differences between Thai cancer and non-cancer, P = 0.03 and P < 0.001, respectively. There is a significantly lower PGI/II ratio in Japanese than that in Thai gastric cancer, P = 0.026. Serum PG Ⅰ/Ⅱ ratio at cut off less than 3.0 and IL-8 mRNA expression Raw RQ > 100 or log_{10} > 2 are significantly difference between Thai cancer group when compared to non-cancer group, P = 0.013 and P < 0.001, respectively. In the correlation study, low PG Ⅰ/Ⅱ ratio does not associate with chronic atrophic gastritis severity score in Thais non-cancer cases. However, there is a trend, but not significant convert correlation between IL-8 mRNA expression level and low PG Ⅰ/Ⅱ ratio in Thai positive *H. pylori* infection. The high expression of IL-8 gene demonstrates a poorer prognosis by stage and histology.

CONCLUSION: Predominant gastric mucosal IL-8 mRNA expression level, *H. pylori* infection, and low PG Ⅰ/Ⅱ ratio are relative risks for Thai gastric cancer without correlation with cagA mutation.

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Key words: Gastric cancer; CagA mutation; Interleukine-8 mRNA expression; *Helicobacter pylori*; Pepsinogen Ⅰ/Ⅱ ratio

Core tip: A high level of interleukine-8 (*IL-8*) mRNA expression was detected in more than eighty percent of Thai gastric cancer patients and nearly two fold in the normal Thai population. The majority of northern Thai gastric cancer patients who had negative *cagA* gene Helicobacter pylori infection even with or without its mutation, still have a high *IL-8* mRNA expression level. The pathogenesis of Thai gastric cancer may primarily involve another gate-way besides the bacterial factor. The results show that there is a predominantly cancer inflammation state regulated by *IL-8* mRNA expression level that can be detected in Thai gastric cancer patients.

Yamada S, Kato S, Matsuhisa T, Makonkawkeyoon L, Yoshida M, Chakrabandhu T, Lertprasertsuk N, Suttharat P, Chakrabandhu B, Nishiumi S, Chongraksut W, Azuma T. Predominant mucosal *IL-8* mRNA expression in non-*cagA* Thais is risk for gastric cancer. *World J Gastroenterol* 2013; 19(19): 2941-2949 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2941.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2941

INTRODUCTION

Gastric cancer pathogenesis is a well-known worldwide multifactorial condition. The gastric cancer incidence rate in Thailand ranks ninth by 4.1:100000 in males and 2.1:100000 in females. Despite being a low incidence country, northern Thailand has a higher gastric cancer incidence rate with 6.6:100000 in males that ranks fifth of overall cancer in the northern Thai region, and 4.5:100000 in females[1]. The author was interested in the carcinogenesis of gastric cancer in Thais, and why the incidence in Thais is much different from other East Asian countries. The interleukin-8 (*IL-8*) gene is one of the principal mediators for the inflammatory response gate way that was first reported in 1970s, and it is one of factors that are possible to affect gastric cancer carcinogenesis[2]. A recent case-controlled surveillance study in northern Thailand on cytokine gene *IL-1b-511* mutations in three East Asian populations showed no predominantly correlated specific causative factor responsible for differences among ethnics and histologic types[3,4]. Therefore, the author proposed the study on other gate-ways of cytokine expression in the human gastric mucosal cell.

Recently, an *in vitro* study showed the association of the mucosal tissue *IL-8* mRNA expression related to the *Helicobacter pylori* (*H. pylori*), positivity cytotoxin-associated gene A (*cagA*) gene. The East Asian genotype was reported in Japanese gastric cancer in about 85% of the cases. Many *in vitro* studies showed this toxicity gene related to gastric mucosal cell injury, inflammation, and oncogenic potential[5-7]. The *cagA*, East Asian genotype is commonly detected in chronic gastritis and gastric cancer of the Japanese[8,9]. There is reported data that a low serum pepsinogen (PG) Ⅰ/Ⅱ ratio of less than 3.0 with a PG Ⅰ level of less than 70 ng/dL was considered as a high risk factor for Japanese gastric cancer[10,11]. There is no recent *in vivo* study reporting a correlation among these above factors, especially *IL-8* and cyclooxygenase-2 (COX-2) mRNA expression level in Thais.

The author hypothesized that gastric mucosal tissue *IL-8* mRNA expression may be different among ethnicities, and it may correlate to other reference pathogenesis factors. This study aimed to look for the risk and correlation of these factors in Thai gastric cancer. The level of *IL-8*, COX-2 mRNA expression, and *cagA* gene mutation distribution were also to be the first report in Thai gastric cancer.

MATERIALS AND METHODS

Research methodology was considered and permitted by Thai and Japanese local ethical committees, the NRCT and Japan Society for the Promotion of Science code ID-NRCT 10726.

Patient characteristics and volunteer selection

An experimental based cross-sectional study was conducted in the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital from 2007 through 2010. Informed consents were obtained from 86 Thai gastric cancer patients who underwent narrow band imaging (NBI) endoscopy and gastric surgery during year 2007-2010, and 134 Thai non-cancer volunteers who underwent NBI endoscopic examination from 2006 to 2008. All gastric cancer patients in this study had locally

advanced gastric cancer, and underwent examinations by endoscopy before curative gastric resection. Seventeen advanced stage Japanese gastric cancer and 12 non-cancer surveillance patients were recruited. Peptic ulcer disease was excluded in this study. Gastric mucosal tissue samples were taken by endoscopy with three biopsy sites for pathology and bimolecular genetic tests before surgical treatment. In cancer cases, biopsy points were specified from non-necrotic areas of the tumor. The histopathology description of the tumor and histologic type were defined. For pathological examination in both groups, chronic gastritis and metaplasia with *H. pylori* detection were classified with a modified Sydney Score System.

Serum PG Ⅰ and Ⅱ level, and H. pylori immunoglobulin G antibody test

A 5 cc sample of venous blood was collected from each study participant. The red blood cell and serum separation was done, and preserved at -20 ℃. The serum PG Ⅰ, Ⅱ, and immunoglobulin G (IgG) antibody for *H. pylori* were tested by the standard enzyme-linked immunosorbent assay technique. The standard cut off value used was a PG Ⅰ level of more than 70 ng/mL or PG Ⅰ/Ⅱ ratio more than 3.0 for no atrophy or positive Grade 1, PG Ⅰ < 70 ng/mL and PG Ⅰ/Ⅱ ratio < 3.0 excluding severe atrophy for moderate atrophy or positive Grade 2, and PG Ⅰ < 30 ng/mL and PG Ⅰ/Ⅱ ratio < 2.0 for severe atrophy or positive Grade 3, respectively[10,11]. All samples were tested twice for reliability confirmation (Toyobo, co, Ltd., Japan)

Tissue H. pylori DNA extraction and cagA genotypting method

The tissue *H. pylori* DNA extracted from the lower antral position in the stomach was examined by the polymerase chain reaction method, and genotyped for *cagA* mutation in all samples by the author (Samples were also examined by double blinded test by Toyobo, co, Ltd). The *H. pylori* positive control of *cagA* positive strain number 11638 (Western), 26695 (Western), and F57 (East Asian) were provided by the collaborative institute. The bacterial tissue DNA and genotyping method with primers used in this study were conducted as recently described. The specific oligonucleotide primers forward (5’-AAAAGCGACCTTGAAAATTCC-3’; nucleotides 2299-2319), reverse-1 (5’-CTTCATTTTTTTGAGCTTGTTGAGC-3’; nucleotides 2488-2463) and reverse-2 (5’-ATTAATGCGTATGTGGCTGTTAGTAGC-3’; nucleotides 3222-3195, were originally described by Azuma *et al*[12].

Cell line culture and gastric mucosal total mRNA extraction with reverse transcriptase reaction for cDNA synthesis

The AGS cell line was grown before cell collection for mRNA extraction at a cell count of 2×10^6-4×10^6. They underwent a total mRNA extraction protocol. The technique followed was a reverse transcriptase reaction using a commercial high capacity RNA-to-cDNA kit (Applied Biosystems)[13].

Gastric mucosal IL-8 and COX-2 mRNA expression by relative quantification real time reverse transcription-polymerase chain reaction

We conducted the experiment from three positions of gastric mucosal biopsies in all Thais and Japanese study participants. All of gastric mucosal tissue samples were transformed to cDNA after total mRNA extraction. The analysis was substantially correctable by analysis both in raw relative quantitation (RQ) and $\log_{10}$ value for adjusted normal distribution curve. All Human TaqMan probe primer express that was used in this study had 81-base pairs (bp) *IL-8* specific human primer assay ID number Hs99999034_m1, 111- base pairs (bp) COX- 2 assay ID number Hs01573471_m1, and 121- base pairs (bp) specific human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Hs99999905_m1 those designed and supplied by Applied Biosystems, United States. The internal control was performed by GAPDH of a matched number template. The real-time relative quantitation value was measured by comparing to the base line value of AGS cell line subject control before making the analysis.

Statistical analysis

A student *t*-test was used for quantitative data, *IL-8* and COX-2 mRNA expression level, and PG level. The χ^2 test was used for qualitative data. The correlation study for pair factors was done in subgroup analysis for defined groups of ethnic, cancer and non-cancer populations. The multivariate analysis was used for risk study for both non-normal distribution and normal distribution curve data bases. STATA 11.0, United States and SPSS 16, United States were used for statistical analysis, and the *P* value of less than 0.05 was considered statistically significant.

RESULTS

There were 86 cases of advanced gastric cancer and 45 (33.8%) normal control cases, 46 (34.6%) non-peptic disease benign lesions without recent history of any treatment, and 42 (31.6%) chronic gastritis cases among 134 non-cancer control cases who were included in the genetic expression experiment. Thai male and female cancer incidences are 60.5% (52/86) and 34.0% (39/86), respectively. Males are also the predominant gender in Japanese. Both nations have significantly high incidence of gastric cancer at age 40 years old or above.

The *H. pylori* infection prevalence is reported by combined histopathology, *H. pylori* IgG antibody level, and 23S rDNA results that have 77.1% and 97.4% of sensitivity and specificity, respectively. Among Thai cancer patients and non-cancer volunteers, *H. pylori* prevalence was 72.1% and 71.6%, respectively. Meanwhile, Thai gastric cancer cases had a *cagA* genotype demonstrated in only 7/62 (12.3%) in positive *H. pylori* infection cases by 23S rDNA that yields six cases of East Asian type and one case of Western type. In non-cancer volunteers, there were 62/98 (63.9%) of positive *cagA* and 34/98 (36.1%) of negative *cagA* genotyping in positive *H. pylori* infec-

Table 1 Characteristics of 220 Thais examined for interleukine-8 mRNA expression *n* (%)

Variable	Cancer (*n* = 86)	Benign (*n* = 134)
Sex		
Male	52 (60.5)	41 (30.6)
Female	34 (39.5)	93 (69.4)
Age (yr)		
< 40	5 (5.8)	28 (20.9)
⩾ 40	81 (94.2)	106 (79.1)
mean ± SD	56 ± 11.3	48.5 ± 11.2
Alcohol drinking		
No	50 (58.1)	80 (59.7)
Yes	36 (41.9)	54 (40.3)
Smoking		
No	62 (72.1)	122 (91.0)
Yes	24 (27.9)	12 (9.0)
Diseases		
Normal	-	45 (33.8)
Benign lesion (polyps, erosion, mild superficial gastritis)	-	46 (34.6)
Chronic active gastritis	-	42 (31.6)
Cancer	86 (100.0)	-

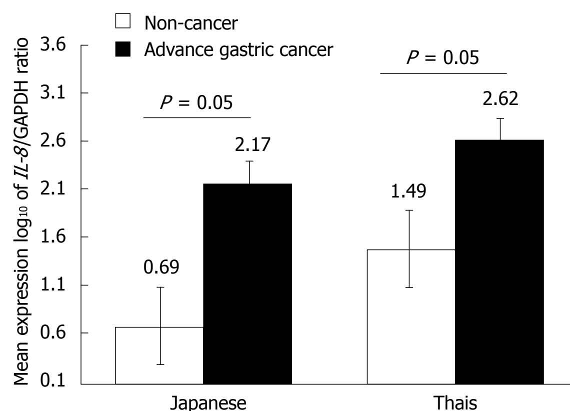

Figure 1 Mean interleukine-8 mRNA expression level measurement of relative quantitation by real-time reverse transcription-polymerase chain reaction study in gastric cancer comparing with non-cancer population both Japanese and Thais. In Japanese and Thais, gastric mucosal interleukine-8 (*IL-8*) mRNA expression in cancer is higher than in non-cancer with $P = 0.05$. The mean level of *IL-8* mRNA expressions in Thai cancer and Japanese cancer were 9615.65 ($\log_{10} = 2.62$) and 1509.11 ($\log_{10} = 2.17$), respectively, $P = 0.014$. The mean level of *IL-8* mRNA expression in non-cancer Thais is 2262 ($\log_{10} = 1.49$) while that in non-cancer Japanese is 10.79 ($\log_{10} = 0.69$), $P < 0.001$.

tion cases that yielded 47.7% of East Asian, 27.4% of Western, and 24.9% of Mixed genotype. For the six year follow up of 18 cases of high grade chronic atrophic gastritis (CAG group) in non-cancer Thais who had long term *H. pylori cagA* East Asian type infection, no one has developed gastric cancer.

The enzyme PG results, showed a significantly lower PG Ⅰ/Ⅱ ratio with a mean of 3.3 ± 1.7 in gastric cancer patients than one in non-cancer volunteers, $P = 0.045$, and of other CAG, $P = 0.002$. There is a significantly lower PG Ⅰ/Ⅱ ratio in Japanese gastric cancer than in Thai gastric cancer, $P = 0.026$.

For *IL-8* and *COX-2* mRNA expression results, 86 Thai gastric cancers were tested successfully in comparison with 134 Thai non-cancer volunteers. The detection rates of *IL-8* mRNA expression were 77/86 (89.5%) in Thai gastric cancer and 102/134 (74.6%) in Thai non-cancer volunteers. Thai population characteristic data that was examined for *IL-8* mRNA expression is demonstrated in Table 1. Serum enzyme PG Ⅰ, Ⅱ level, and *H. pylori* infection status are demonstrated in the cancer population and non-cancer volunteers in Thais is demonstrated in Table 2. We found a remarkable number of Thai gastric cancers with a negative *cagA*; therefore, *IL-8* mRNA expression was examined and the cut-off point of expression value difference is demonstrated in Table 3. Serum PG Ⅰ/Ⅱ ratio at cut-off point of less than 3.0 and raw RQ ⩾ 100 or $\log_{10} \geqslant 2$ of *IL-8* mRNA expression level showed the significantly different between the Thai gastric cancer group and the non-cancer group, $P = 0.045$ and $P < 0.001$, respectively. In the multivariate analysis application, the four co-factors related to gastric cancer risk including *IL-8* mRNA expression in Thais are shown in Table 4.

At the same stage of advanced gastric cancer, the mean levels of *IL-8* mRNA expression in Thai cancer and Japanese cancer were 9615.65 ($\log_{10} = 2.62$) and 1509.11 ($\log_{10} = 2.17$), respectively, $P = 0.014$. For gastric cancer risk at cut-off *IL-8* expression level by $\log_{10}$ greater than two 2 in Thais and Japanese, odds ratio (OR) = 7.97 (95%CI: 3.75-16.97, $P < 0.001$) and OR = 4 (95%CI: 1.29-12.40), respectively. In the non-cancer group, we found that the *IL-8* mRNA expression level was lower than cancer population with a significant difference, $P < 0.001$. The total mean *IL-8* mRNA expression in non-cancer Thais was 2262 ($\log_{10} = 1.49$) while that in Japanese non-cancer was 10.79 ($\log_{10} = 0.69$), $P < 0.001$. In comparison within the same ethnic group, the mean levels of *IL-8* mRNA expression in Thai and Japanese cancer were higher than those in non-cancer, $P = 0.05$ as showed in Figure 1.

The COX-2 mRNA expression did not indicate significant rising level with detection rate of 65% in Thai and Japanese gastric cancer. In comparison with *IL-8* mRNA expression, although the level of COX-2 mRNA expression was slightly higher in gastric cancer than normal gastric mucosal tissue, there were much lower levels than those of *IL-8* mRNA expression.

In the correlation study, low PG Ⅰ/Ⅱ ratio was not associated with the CAG severity score in Thai non-cancer cases because of a few number of CAG in both Thai gastric cancer and non-cancer populations in this study. There was no significant difference for the *IL-8* mRNA expression level in cancer between positive and negative *H. pylori* infection. There was no direct correlation of *IL-8* mRNA expression level and serum *IgG* levels. In subgroup analysis, there was a significant difference of higher levels in groups of poorly differentiated histopathology in comparing both nations. For the diffuse histologic type, the *IL-8* mRNA expression level is about 1.5 times higher than that of intestinal histologic type with a statistically significant difference in Japanese.

Table 2 Serum enzyme pepsinogen Ⅰ, Ⅱ level and *Helicobacter pylori* infection detection results in Thais *n* (%)

Variable	Cancer (*n* = 86)	Benign (*n* = 134)	*P* value
PG Ⅰ/Ⅱ, (ng/μL), mean ± SD			
Ⅰ	57.39 ± 46	54.86 ± 68.5	0.780
Ⅱ	19.42 ± 21	15.42 ± 11.7	0.090
PG Ⅰ/Ⅱ ratio			
≤ 3	28 (39.4)	34 (27.9)	0.045[1]
> 3	43 (60.6)	88 (70.1)	
H. pylori pathology			
Negative	31 (36.8)	60 (44.8)	0.001
Positive	55 (63.2)	74 (55.2)	
Serum IgG			
Negative	31 (46.3)	57 (44.2)	0.820[1]
Positive	36 (53.7)	72 (55.8)	
CagA genotyping in positive 23S rDNA			
Negative	55 (88.7)	62 (64.6)	< 0.001[1]
Positive	7 (12.3)	34 (35.4)	
H. pylori Infection status			
True negative	20 (24.4)	37 (36.0)	0.120[1]
True positive	62 (75.6)	96 (64.0)	

[1]Some numbers are not included in the analysis due to missing laboratories entries. The statistical analysis was performed by χ^2 of each factor. IgG: Immunoglobulin G; CagA: Cytotoxin-associated gene A; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

Table 3 Molecular genetic results of cyclooxygenase-2 and interleukine-8 mRNA expression in Thais *n* (%)

Variable	Cancer (*n* = 86)	Benign (*n* = 134)	*P* value
COX2 raw RQ			
No expression detection	30 (34.90)	43 (53.10)	< 0.001[1]
Expression detection	56 (65.10)	38 (46.90)	
COX2 raw RQ			
mean ± SD	41.69 ± 4.90	5.37 ± 4.20	< 0.001[1]
COX2 $\log_{10}$ (N, %)			
mean ± SD	1.62 ± 0.96	0.73 ± 0.62	< 0.001[1]
IL-8 raw RQ			
No expression detection	9 (10.47)	32 (25.37)	< 0.001
Expression detection	77 (89.53)	102 (74.63)	
IL-8 raw RQ			
≤ 100 or undetected	33 (38.37)	105 (78.36)	< 0.001
> 100	53 (61.63)	29 (21.64)	
mean ± SD	9615.64 ± 49715.00	2262.29 ± 10454.60	< 0.010
IL-8 $\log_{10}$			
≤ 2 or undetected	32 (37.21)	105 (78.36)	< 0.001
> 2	54 (62.79)	29 (21.64)	
mean ± SD	2.62 ± 1.10	1.49 ± 1.20	< 0.010

[1]Some numbers are not included in the analysis due to missing laboratory data. RQ: Relative quantity; COX2: Cyclooxygenase-2; *IL-8*: Interleukin-8.

Table 4 Multivariate risk analysis for Thai gastric cancer

Variable	OR	95%CI	*P* value
Male	4.32	2.06-9.04	< 0.001
H. pylori infection status	0.98	0.96-0.99	0.020
PG Ⅱ/Ⅰ ratio ≤ 3	2.06	0.94-4.47	0.060
IL-8 mRNA expression	7.97	3.75-16.97	< 0.001

OR: Odds ratio; *H. pylori*: *Helicobacter pylori*; PG: Pepsinogen; *IL-8*: Interleukin-8.

Table 5 Comparative means interleukine-8 mRNA expression level detection between Thai and Japanese Cancer populations divided by histopathology *n* (%)

Histopathology	Thai (*n* = 77)	Japanese (*n* = 17)	*P* value
Diffuse type	55 (71.4)	4 (23.5)	0.01
mean ± SD	2.85 ± 1.10	2.55 ± 0.52	0.95
Intestinal type	22 (28.6)	13 (76.5)	
mean ± SD	2.52 ± 1.11	1.56 ± 1.06	0.01

Statistical difference between groups (*P* = 0.04). *IL-8*: Interleukin-8.

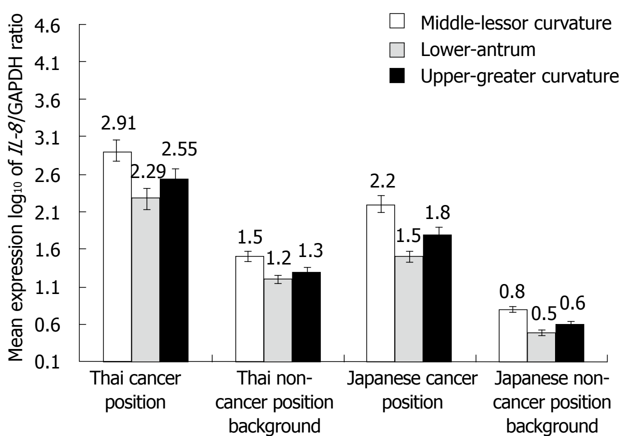

Figure 2 The mean interleukine-8 mRNA expression in Thais divided by histology and cancer position. Middle is the lessor curvature and non-cancer position, lower is the antrum, and Upper is the greater curvature. *IL-8*: Interleukine-8.

High *IL-8* mRNA expression was primarily found in the non-*cagA* Thai gastric cancer population. There was a significantly different mean *IL-8 mRNA* expression level between groups of negative *cagA* by $\log_{10}$ = 2.46 (± 1.04) and positive *cagA* by $\log_{10}$ = 3.29 (± 1.68) in the Thai gastric cancer group. However, there were few numbers of Thai gastric cancers with positive *cagA*. In other subgroup analysis of 18 Thais who had high grade CAG, some level of *IL-8* mRNA expression in the 12 Japanese non-cancer patients appeared which an equivalently lower level.

Gastric cancer mucosal tissue *IL-8* mRNA expression in the cancer position had a significantly higher mean level than its level at the non-cancer background position in both Thai and Japanese shown in Figure 2. There was significantly different *IL-8* mRNA expression level between intestinal (favorable) and diffuse (unfavorable) histologic cell types. In Thai gastric cancer, the poorly differentiated gastric adenocarcinoma and signet ring cell were predominantly found in this study. The $\log_{10}$ *IL-8* mRNA mean expressions in unfavorable cell type were 2.55 and 2.85 in Japanese and Thais, respectively, as shown in Table 5. There is a significantly higher level of *IL-8* mRNA expression in diffuse cell type than that in a differentiated histologic cell type, *P* = 0.04. The differentiated histologic cell type in Thais has higher expression level than that in Japanese with a statistically significant difference, *P* = 0.013 The RT-PCR results of gastric mucosal tissue and AGS

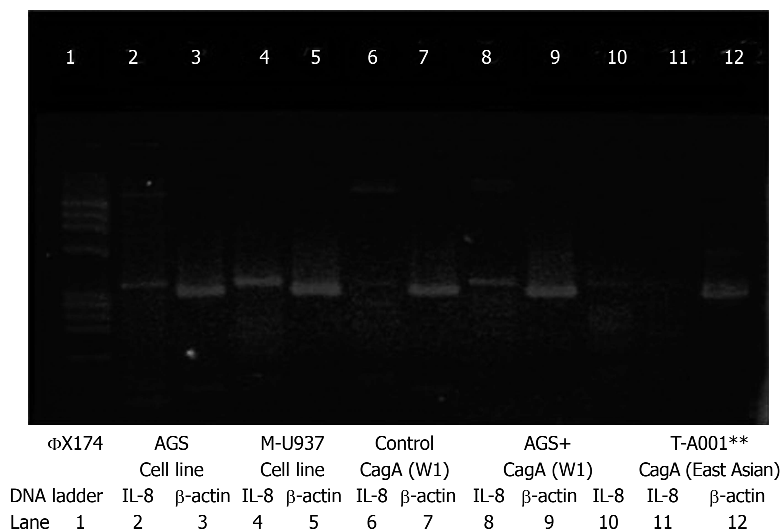

Figure 3 Basic experiment result on real-time reverse transcription-polymerase chain reaction of interleukine-8 mRNA expression with AGS, macrophage cell line, normal gastric mucosal cell, AGS cancer cell line co-culture with two strains of cagA *Helicobacter pylori*, and positive cagA, Thai non-cancer samples sequences showed on 12 lanes. M: Marker.

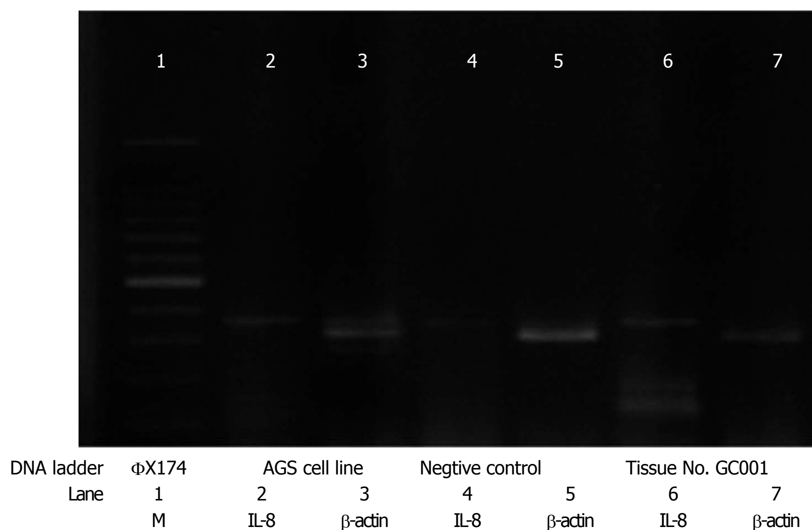

Figure 4 Real-time reverse transcription-polymerase chain reaction result of interleukine-8 mRNA expression from AGS, negative control, and Thai gastric cancer mucosal tissues. The positive results of interleukine-8 (*IL-8*) mRNA expression appeared at 320 bp band comparing with 300 bp band of marker (M) in lane 1.

cell line *IL-8* mRNA expression were demonstrated in Figure 3. and Figure 4. However, there was no significant difference of *IL-8* mRNA expression level between positive and negative *H. pylori* infection in subgroup analysis of non-cancer background positions.

In summary, *IL-8* mRNA expression level is predominantly found, and trend toward an inverted correlation to PG Ⅰ/Ⅱ low ratio in Thai gastric cancer patients. There is no direct correlation of *IL-8* mRNA expression level with the *cagA* gene mutation in Thai gastric cancer.

DISCUSSION

In the present *in vivo* study, there is a significant risk of *IL-8* mRNA expression level predominantly found rising up more than 80% of northern-Thai gastric cancer. There is significantly higher level of *IL-8* mRNA expression in poorly differentiated than in well differentiated carcinoma in both Thailand and Japan. There is a trend of converted independent correlation with the very low PG Ⅰ/Ⅱ ratio in gastric cancer as well as a few numbers of Thai severe CAG, but no direct correlation with positive *H. pylori* infection or *cagA* genotypes.

Higher level *IL-8* mRNA expression in non-cancer Thais comparing with non-cancer Japanese demonstrated the difference of gastric mucosal defense and genetic expression between the two nations. Thai gastric cancer has less background of CAG. In this study, there is no evidence that showed a direct correlation of *IL-8* mRNA expression with *cagA* mutation genotype in *H. pylori* positive cases. Predominant *IL-8* mRNA expression level resulted in non-atrophic mucosa of both gastric cancer and non-cancer Thais. The result is different from previous *in vitro* or some *in vivo* studies in high incidence gastric cancer countries, such as Japan, China, and South Korea.

In Thai gastric cancer patients, *IL*-8 mRNA expression level at the lessor curvature is the most represented location. Its predominantly high level may represent relatively vascular invasion as well as major gastric mucosal inflammation. However, the area of gastric antrum in Thai gastric cancer patients has less activity than the lessor curvature because atrophy occurs more frequently.

Also, a high level of *IL-8* mRNA expression is matched with the poor prognosis by histopathology cell type and tumor stage. Long-term bacterial infection has less effect to change the gastric mucosa into CAG. In our

recent six year followed up study in non-cancer cases controlled with positive *H. pylori* infection and without eradication of 500 new non-cancer cases, a few people developed severe gastritis and still have a high PG Ⅰ/Ⅱ ratio. In this study, the number of high PG Ⅰ/Ⅱ ratio in Thai cancer is still about 45% which is nearly the same percentage in our recently published study[4].

A few gastric cancer preventive models on natural Thai products were reported[14]. There is one study on diet consumption in Thais showing a linkage of gastric cancer risk. The factors which were found to be a higher risk but not statistically significant were low intake of vegetables and fruits (OR = 1.2, 95%CI: 0.74-1.96) and Jeaw prik (mainly chilly with Plara broth or pickled fish), a kind of preserved food in North and North-eastern regions of Thailand (OR = 1.2, 95%CI: 0.76-2.01)[15]. The consensus of the Asian Pacific guideline on gastric cancer prevention is still debated in some experts' opinions[16]. *H. pylori* infection screening in a low risk gastric cancer population is not recommended, but serum PG may be helpful to screen the high risk population in northern Thailand. We reported its different characteristics that rely on *H. pylori IgG* antibody and PG Ⅰ/Ⅱ ratio in our cancer populations comparing to the data in a Japanese report[17].

The Japanese study primarily reported the relation of *cag*A genotype and a low PG Ⅰ/Ⅱ ratio. Nevertheless, approximately half of the Thai cancer population demonstrates a low ratio of PG Ⅰ/Ⅱ similar to the Japanese. There are still a small number of Thais who had a severe atrophy score related *to H. pylori* infection though in the positive *cagA* Thai population.

In this study, the negative *cagA* gene is found in the majority of Thai gastric cancer unlike the Japanese. In Thais, the poorly differentiated cell type gastric adenocarcinoma occurred mainly in the negative *cagA* gene *H. pylori* infection, not in Western type *cagA*.

There is a small number of the Thai population who had a severe atrophy score of gastric mucosa found in our recent and present collaborative study[18]. Reduction of the fundic gland in chronic gastritis was also related to the low level of PG Ⅰ/Ⅱ ratio in Japanese[19,20]. In our recent study of subgroup analysis on the PG Ⅰ/Ⅱ ratios, there was no significant difference between CAG and gastric cancer group, P = 0.12. However, the low PG Ⅰ/Ⅱ ratio was significantly related to gastric cancer when compared to normal population in a recent match-case control study by OR of 2.3 (95%CI: 1.10-4.80), P = 0.025[4]. The tumor location demonstrated locations mainly at the upper portion and corpus in both of our studies. In the present study, the author found risk for cancer by OR 2.06 (95%CI: 0.94-4.47), P = 0.059 that seems to be close to the result of our recent study. The low PG Ⅰ/Ⅱ ratio was not found to be a high percentage in Thais unlike in Japanese gastric cancer[9].

For other genetic host factors, in one interesting study, two of the four gastric carcinoma cell lines expressed vascular endothelial growth factor (VEGFR-3) mRNA. In 17 of 36 gastric carcinoma specimens, VEGFR-3-specific immune activity was detected in tumor cells. These angiogenesis and lymphangiogenesis were also detected in VEGF-C-transfected tumors than in control tumors[21]. *IL-8* mRNA expression is found to be the gate way mechanism of the vascular epithelial growth factor. For *IL-1* gene, it is a pro-inflammatory cytokine, and the T/T genotype of IL-1β-511 is suspected as the risk factor of both hypochlorhydria related *H. pylori* infection and gastric cancer in a case-control population in the United States[22]. The author reported that *c/c* genotype was a risk in Japanese, and a lower number of *c/c* genotype was found as a minor risk related to Thai gastric cancer[4].

Recent *in vivo* animal models studies showed the expressions of *IL*-8 and COX-2 had linkage to the epithelial cell which was co-infected with *H. pylori*. However, our preliminary study reported that there was no difference of *IL-8* mRNA expression level between a cell line which was co-infected with *H. pylori* and the Thai gastric mucosa tissues which had positive or negative *H. pylori*[23,24]. The toleration, remarkable host response to cancer inflammatory process, healing of stomach mucosal turnover rate, and re-healing process of ulcer in Thais may be different and caused by host susceptible differences to the virulence bacteria.

The theory regarding inflammatory cytokine's influence on cancer development was first contributed by Rudolph Virchow around 150 years ago[25]. Many studies regarding *IL-8* gene expression remarkably found significant relation with *H. pylori* infection and many *cag* pathogenicity island both *in vitro*[26,27] and in some number in *in vivo* study of Japanese cancer[28,29]. However, no study has demonstrated differences of expression level in the individualized host[30].

In this study, Japanese gastric cancer has a lower *IL-8* mRNA expression on average than that in Thai gastric cancer patients at the same stage of disease and *H. pylori* infection status. However, Japanese gastric adenocarcinoma cases are mostly an intestinal type and infected by positive *cagA* strain *H. pylori*. In contrast with northern Thai gastric adenocarcinoma cases, they are mostly diffuse histologic cell type, and infected by negative *cagA* strain *H. pylori*. Therefore, the authors speculate that the predominant level of *IL-8* mRNA expression found in non-*cagA* gene *H. pylori* infection is not directly related to atrophic gastritis mucosa in Thai gastric cancer.

Some results in this study are unexpected outcomes and different from our recent knowledge of *in vitro* and *in vivo* study in Japanese atrophic gastric mucosa which almost easily infected by positive *cagA H. pylori* infection. The *cagA* was also the suspected cause of the *IL-8* gene expression rising.

This result in Thais showed that gastric mucosal tissue *IL-8* mRNA expression has a higher level in the advanced stage and poorer differentiated cell type than in favorable histology or differentiated cell type. The author was suspicious that the less atrophic background of Thai stomach cancer and non-cancer gastric mucosa may be caused by non-*cagA H. pylori* infection. However, the high level of mRNA *IL-8* gene expression in Thai gastric can-

cer cases may be explained by the cancer inflammation carcinogenesis that may not be directly related to only *H. pylori* infection in Thais.

The level of *IL-8* mRNA expression in Thai gastric cancer or poorly differentiated gastric carcinoma may be regulated by other factors besides of *H. pylori* infection. Also, unknowns remain regarding how long *IL-8* mRNA expression has been high before the occurrence of gastric cancer or after becoming a more advanced stage. Although the author analyzed the level of *IL-8* and *COX-2* mRNA expression level in normal mucosa and of advanced gastric cancer, this occurrence could not be shown in early gastric cancer. The environmental factors and bacterial virulence effect cannot be excluded.

In this study, the signet ring cell type is predominantly found in Thai gastric cancer population. The poor prognostic histological cell type may have different disease carcinogenesis related to gastric mucosal tissue *IL-8* mRNA high expression level and severity of the disease. The *COX-2* mRNA expression level is directly correlated with only the *H. pylori* infection, and tended to be suppressed unlike *IL-8* mRNA expression. The author supposed that extremely high gastric mucosal *IL-8* expression level may relate to other factors, such as VEGF that could not be demonstrated in this study and should be explored further.

In conclusion, this present *in vivo* study shows results of new factual data on predominant gastric mucosal *IL-8* mRNA expression level in Thai gastric mucosal biopsy tissues in both non-cancer and gastric cancer volunteers. In the present study, the northern Thai gastric cancer population has a high incidence of signet ring cell by the nature of histologic type. The positive *H. pylori* may be one of the co-factors, though the host is infected with non-*cagA* gene and still has an extremely high *IL-8* mRNA expression level. This cytokine expression may represent the individual host defense in both high and low incidence gastric cancer ethnics. The factual results in an experimental based study demonstrated how prevalence of the northern Thai gastric cancer host had active co-infection or had been recently infected by non-*cagA* gene *H. pylori* infection. The *IL-8* mRNA expression level does not directly correlate to non-*cagA H. pylori* infection in Thai gastric cancer. However, there is a trend of converted correlation between *IL-8* mRNA expression and low ratio PG Ⅰ/Ⅱ without statistical significance, and it seems to be an independent correlation. By these preliminary results, the author expected to do further study on *IL-8* mRNA expression that may act as one of prognostic genetic biomarkers in clinical practice and for chemotherapy application in the nearby future. A study on the current cancer chemotherapy with northern Thai gastric cancer population is on-going.

ACKNOWLEDGMENTS

This study with preliminary results was presented in a free oral paper symposium session of Oncology and Stomach session, International Surgical Week 2011, in Yokohama during August 28-September 1.

COMMENTS

Background

The incidence of gastric carcinoma is very low although the incidence of *Helicobacter pylori (H. pylori)* seemed not to be low in Thais. However, the northern Thai population still has the highest gastric cancer incidence in Thailand. Currently, the disease incidence is rising faster than in the past and still the second cause of cancer death worldwide. The cause and risk factors for gastric cancer carcinogenesis in Thais is unclear especially the risk related with *H. pylori* infection or other cofactors.

Research frontiers

Interleukine-8 (*IL-8*) mRNA expression is a common event found in some epithelial malignancies and in gastric adenocarcinoma either due to *H. pylori* caused chronic inflammation or other causes by unrelated carcinogens. It is not clear how the level of this expression related to gastric adenocarcinoma. The authors demonstrated that the predominant overexpression of *IL-8* mRNA could be a potential relative risk for gastric adenocarcinoma in Thais and demonstrated the difference of its level in relationship with the histologic type of gastric cancer.

Innovations and breakthroughs

Recent reports have highlighted the importance of cytotoxin-associated gene A (*cagA*) *H. pylori* infection and its mutation type that is shown predominantly in Japanese gastric cancer carcinogenesis. Particularly in the well differentiated histologic type, the *IL-8* mRNA expression level in the Japanese seems to be much lower than in Thais. However, its level in poorly differentiated cell type of Thai gastric adenocarcinoma has less atrophic background and a higher level of expression than in well differentiated gastric adenocarcinoma. This is the first study to report how measurement of *IL-8* mRNA expression level demonstrates risk in *non-cagA H. pylori* infection of Thai gastric cancer and the trend of differences in carcinogenesis related to *H. pylori* infection between high and low incidence ethnics. Furthermore, their *in vivo* studies would suggest that the *IL-8 mRNA* expression level yields high prevalence detection in gastric adenocarcinoma and may be a useful tool for gastric cancer prognostic or therapeutic study.

Applications

By understanding different *IL-8* mRNA expression levels, this study may represent a future study with a tissue molecular biomarker for gastric cancer.

Terminology

IL-8 mRNA expression is pro-inflammatory cytokines that is detected in gastric epithelial mucosa and gastric cancer cell lines, such as Kato Ⅲ and AGS cells.

Peer review

The authors examined the expression of *IL-8 and* cyclooxygenase-2 in AGS cell line, normal gastric mucosa, gastritis, and gastric adenocarcinoma tissues. It revealed that *IL-8* mRNA expression predominantly increased in poorly differentiated or signet ring cell gastric adenocarcinoma that showed the trend of a poorer prognosis. The expression was not directly correlated to *cagA H. pylori* infection and its mutation type. The results are interesting and may represent a different carcinogenesis of Thai gastric cancer in comparison to recent Japanese studies.

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P- Reviewer Aoyagi K **S- Editor** Wen LL
L- Editor A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2950

World J Gastroenterol 2013 May 21; 19(19): 2950-2955
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Current application situation of gastrointestinal endoscopy in China

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Received: December 24, 2012 Revised: February 18, 2013
Accepted: March 22, 2013
Published online: May 21, 2013

Abstract

AIM: To study the current application situation of gastrointestinal (GI) endoscopy in mainland China.

METHODS: From 12 August, 2011 to 15 February, 2012, draft questionnaires were sent by e-mail to 289 hospital-based GI endoscopy units, including units with three levels (provincial, prefecture and county level) in mainland China. All the surveyed GI endoscopy units were state-owned and hospital-based. Proportions were compared using χ^2 tests. Comparisons between groups were performed using the Mann-Whitney *U* test. A probability of $P < 0.05$ was considered to represent a statistically significant difference.

RESULTS: Based on satisfactory replies, 169/279 (60.6%) of units were enrolled in the survey, which covered 28 provinces (90.3%, 28/31) in mainland China. Compared with published survey data, the number of GI endoscopes per unit has increased by nearly three times (from 2.9 to 9.3) in the past decade. About 33 of 169 (19.5%) endoscopy units possessed an X-ray machine, which was mainly owned by provincial endoscopy units (43.2%, 19/44). Video capsule endoscopes, which were almost unavailable ten years ago, were owned by 20.7% (35/169) of GI endoscopy units. Endoscopic submucosal dissection could be performed by 36.4% (19/44) of the provincial units, which was significantly higher than the prefecture level (9.9%, $P < 0.01$) and county level (0.0%, $P < 0.01$) units, respectively.

CONCLUSION: Rapid development in GI endoscopy has been made in mainland China, and major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly used in large endoscopy units.

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Key words: Application situation; Gastrointestinal endoscopy; Video capsule endoscopy; Endoscopic submucosal dissection

Core tip: Rapid developments in gastrointestinal (GI) endoscopy have taken place in China in the past decade. Major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly confined to large endoscopy units, whereas small and medium units, often perform fewer endoscopic procedures and have less equipment, and are mostly restricted to diagnostic endoscopy. In addition to improvement in GI endoscopy equipment, standard procedures including the standard reprocessing for endoscopy will be the focus in the future in China.

Zhang XL, Lu ZS, Tang P, Kong JY, Yang YS. Current application situation of gastrointestinal endoscopy in China. *World J Gastroenterol* 2013; 19(19): 2950-2955 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2950.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2950

INTRODUCTION

Endoscopy is a universally popular, minimally invasive intervention for gastrointestinal (GI) and pancreatico-biliary disorders[1]. It is reported that > 10 million GI endoscopies are performed every year in the United States[2], and the number of procedures worldwide, although there are no exact figures, is believed to be increasing yearly due to the rapid increase in popularity of GI endoscopy. Although GI endoscopy services have become a routine procedure in western countries[3], in most developing countries GI endoscopy services are sometimes available in so-called centres of excellence[4].

In the past decade, the emergence and application of a variety of novel endoscopic techniques and equipment, *e.g.*, video capsule endoscopy (VCE) and double/single balloon enteroscopy (DBE/SBE) have substantially promoted the diagnostic value for GI tract lesions[5-7]. Additionally, also in the past decade, GI endoscopy has experienced rapid evolution from a diagnostic medical procedure to a minimally invasive therapeutic procedure, *e.g.*, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) for removal of mucosal lesions[8-10]. All these advances in GI endoscopy have ushered in a new era in digestive medicine. However, the systemic data concerning the current status and development of GI endoscopy in China is still lacking. Here, we conducted a survey of GI endoscopy procedures and equipment in hospital-based GI endoscopy units, in order to demonstrate the rapid development of GI endoscopy in mainland China in the past decade.

MATERIALS AND METHODS

Survey design

According to the scale (bed number) and location, hospitals in mainland China are traditionally divided into three levels: provincial (bed numbers > 1000/hospital, usually located in the provincial capital city), prefecture (bed numbers 500-1000/hospital, usually located in the prefecture capital city) and county (bed numbers < 500/hospital, usually located in county city) level; or large scale (*i.e.,* provincial) and small-to-medium scale (*i.e.,* prefecture and county level). The endoscopy units from three levels of hospitals are regarded as provincial, prefecture and county level endoscopy units, respectively.

From 12 August, 2011 to 15 February, 2012, 289 GI endoscopy units, which are official members of the Chinese Society of Digestive Endoscopy, were included in the present study. All the surveyed GI endoscopy units were state-owned and hospital-based. They were required to fill in a questionnaire. The questionnaires were sent by e-mail to the physician in charge of the unit, and if an e-mail address was unavailable or invalid, a phone call was made to complete the questionnaire. The draft comprised 21 questions about the endoscopy equipment and procedures performed in the units. The queries pertained to: the number of GI endoscopy procedures performed per year; the number and brands of all kinds of GI endoscopes; the number of separate purpose-designed rooms for endoscopy procedures; the number of the full-time GI endoscopy physicians and nurses; the major auxiliary endoscopy equipment (*e.g.*, X-ray machine); which endoscopy procedure they could perform [*e.g.*, endoscopic retrograde cholangiopancreatography (ERCP), EMR and ESD].

Ethical considerations

The study was approved by the Ethics Committee of Chinese PLA General Hospital.

Statistical analysis

Proportions were compared using χ^2 tests. Comparisons between groups were performed using the Mann-Whitney U test. A probability of $P < 0.05$ was considered to represent a statistically significant difference. Statistical analysis was performed using SPSS version 13.0 (Chicago, IL, United States).

RESULTS

Responding GI endoscopy units and their locations

Based on provision of a satisfactory reply, 169/289 (58.5%) GI endoscopy units from three levels of hospital were enrolled in our study, which covered 28 provinces (90.3%, 28/31) in mainland China. Of these, 44 (26.0%, 44/169) units were from provincial hospitals, 91 (53.8%, 91/169) from prefecture level hospitals, and 34 (20.1%, 34/169) from county level hospitals.

Number of endoscopy procedures per year

All 44 provincial endoscopy units performed ≥ 5000 procedures, which was significantly higher than the prefecture-level (16.5%, $P < 0.05$) and county-level (0.0%, $P < 0.05$) endoscopy units (Table 1).

Number of endoscopes per unit

The average number of gastrointestinal endoscopes (including all types of GI endoscope) for the 169 endoscopy units was 9.3/unit (1568/169). The average number of endoscopes in the provincial endoscopy units was 22.4 ± 5.5, which was significantly higher than that in the prefecture level (5.4 ± 1.4, $P < 0.05$) and county level (2.7 ± 1.2, $P < 0.05$) units. Moreover, 59.1% (26/44) of the provincial endoscopy units had at least 10 endoscopes, which was also significantly higher than that of the prefecture level (6.6%, $P < 0.05$) and county level (0.0%, $P < 0.05$) units (Table 1).

All of the 169 endoscopy units possessed gastroscopies. The possession rate of colonoscopies in provincial and prefecture level units was 100% and 97.8%, respectively, which was significantly higher than that of county level units (88.2%, $P < 0.05$). Enteroscopies (DBE/SBE) were available only in provincial units (40.9%, 18/44) and a VCE was possessed by 20.7% (35/169) of all GI units (Table 1).

Endoscope manufacturers

The most frequently used GI endoscopes were manufac-

Table 1 Comparison of endoscopy items at three levels of endoscopy units *n* (%)/(mean ± SD)

Endoscopy unit	Provincial (*n* = 44)	Prefecture-level (*n* = 91)	County-level (*n* = 34)	Total (*n* = 169)
Procedures per year				
> 5000	44 (100.0)	15 (16.5)	0 (0.0)	59 (34.9)
3000-5000	0 (0.0)	65 (71.4)	3 (8.8)	69 (40.2)
< 3000	0 (0.0)	11 (12.1)	31 (91.2)	42 (24.9)
Average number of endoscopes	22.4 ± 5.5[a]	5.4 ± 1.4	2.7 ± 1.2	9.3 ± 3.2
≥ 10 endoscopes/unit	26 (59.1)[a]	6 (6.6)	0 (0.0)	32 (18.9)
Gastroscope	44 (100.0)	91 (100.0)	34 (100.0)	169 (100.0)
Colonoscope	44 (100.0)[a]	89 (97.8)[c]	30 (88.2)	163 (96.4)
DBE/SBE	18 (40.9)[a]	0 (0.0)	0 (0.0)	18 (10.7)
EUS	31 (70.5)[a]	7 (7.7)	0 (0.0)	38 (22.5)
VCE	28 (63.6)[a]	7 (7.7)	0 (0.0)	35 (20.7)
Average number of procedure rooms	4.9 ± 1.4[a]	2.6 ± 1.3[c]	1.6 ± 0.8	3.6 ± 1.8
Average number of full-time physicians	3.0 ± 1.3[a]	1.6 ± 1.2	1.2 ± 0.9	1.9 ± 1.4
Average number of full-time nurses	5.4 ± 1.3[a]	2.2 ± 1.1	1.3 ± 0.8	2.8 ± 1.3
X-ray machine	19 (43.2)[a]	14 (15.4)[c]	0 (0.0)	33 (19.5)
Polypectomy	44 (100.0)[a]	71 (78.0)[c]	14 (41.2)	129 (76.3)
ERCP	34 (77.3)[a]	39 (42.9)[c]	4 (11.8)	77 (45.6)
EMR	30 (68.2)[a]	20 (22.0)[c]	2 (5.9)	52 (30.8)
ESD	16 (36.4)[a]	9 (9.9)	0 (0.0)	25 (14.8)
EVS/EVL	33 (75.0)[a]	37 (40.7)[c]	3 (8.8)	73 (43.2)

[a]P < 0.05 *vs* prefecture-level or county-level endoscopy units; [c]P < 0.05 *vs* county-level endoscopy units. EUS: Endoscopic ultrasonography; VCE: Video capsule endoscope; DBE/SBE: Double balloon/single balloon enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EVS: Endoscopic variceal sclerotherapy; EVL: Endoscopic variceal ligation.

tured by Olympus (140/169, 82.8%), followed by Fujinon (52/169, 30.8%) and Pentax (28/169, 16.6%).

Number of full-time staff in GI endoscopy units

The average number of full-time physicians in each provincial endoscopy unit was 3.0 ± 1.3, which was significantly higher than that in prefecture level (1.6 ± 1.2, P < 0.05) and county level (1.2 ± 0.9, P < 0.05) endoscopy units. A similar trend was found for the numbers of full-time nurses in these three levels of endoscopy units (Table 1).

Possession of X-ray machine in GI endoscopy units

Thirty three of the 169 (19.5%, 33/169) endoscopy units possessed an X-ray machine. Furthermore, 43.2% of the provincial endoscopy units owned an X-ray machine, which was significantly higher than that of prefecture level (15.4%, P < 0.01) and county level (0.0%, P < 0.01) endoscopy units (Table 1).

Endoscopy procedures performed

Polypectomy could be performed by all the provincial units (100%), 78.0% of the prefecture level units and 41.2% of county level units. ERCP could be performed by 77.3% of the provincial units, which was significantly higher than in the prefecture level (42.9%, P < 0.05) and county level (11.7%, P < 0.01) units. A similar trend was also found for EMR, ESD and endoscopic variceal sclerotherapy (EVS)/endoscopic variceal ligation (EVL) (Table 1).

Table 2 Comparison of main endoscopy items in three independent surveys

Endoscopy items	Shanghai survey (2001)	Qinghai survey (2003)	Present survey (2011)
Number of units	138.0	37.0	169.0
Average number of endoscopes/unit	3.3	1.4	9.3
Possession rate			
Gastroscope	100.00%	100.00%	100%
Colonoscope	70.30%	-	97.60%
Enteroscope	4.30%	0.00%	10.60%
EUS	7.20%	0.00%	22.50%
VCE	-	2.70%	20.70%
X-ray machine	5.70%	-	19.50%
Procedures			
Polypectomy	54.30%	-	76.30%
EVS/EVL	40.20%	-	43.20%
ERCP	38.40%	-	45.60%

EUS: Endoscopic ultrasonography; VCE: Video capsule endoscope; ERCP: Endoscopic retrograde cholangiopancreatography; EVS: Endoscopic variceal sclerosis; EVL: Endoscopic variceal ligation.

Comparisons of gastrointestinal endoscopy between current survey and previously published data

From January 2000 to November 2010, only two regional endoscopy surveys (*i.e.*, Shanghai survey in 2001 and Qinghai provincial survey in 2003) in mainland China were available by searching the Chinese national (Wanfang bases) and international databases (Medline), and they were both published in Chinese[11,12]. The major GI endoscopy items mentioned by these two regional surveys were compared with the current survey results, and a rapid development of GI endoscopy items, including the average number of GI endoscopes per unit, was shown in Table 2.

DISCUSSION

In 1973, GI endoscopy was first introduced from Japan into China. Since then, much progress in GI endoscopy has been made by Chinese endoscopists, and the efforts and achievements of Chinese endoscopists in GI endoscopy have gradually gained international recognition[13-16]. For example, with regard to the latest endoscopy techniques, EUS, ERCP, VCE and DBE have been the focus for Chinese endoscopists, and they have accounted for 66% of all the international publications[17].

Although the Shanghai survey in 2001 and the Qinghai provincial survey in 2003 were from single and different areas in China, which means lower comparability with the present survey here we use them to demonstrate the progress of GI endoscopy facilities in the past decade in mainland China because these are the only journal publications available[11,12].

The total number of GI endoscopes per unit is an important and determinant factor for what kinds of and how many endoscopy procedures they can perform. In the present survey, the average number of GI endoscopes per unit increased nearly threefold (9.3/unit) compared with that of previously published data ten years ago (the average number for Shanghai and Qinghai surveys was 2.9/unit). This is major progress in mainland China in the past decade.

VCE was first reported in 2000 by an Israeli company, Given Imaging[5], and it is predicted that major developments in endoscopy over the next 10-20 years will centre on this technique[18]. In the past eight years, the possession rate of VCE has markedly increased from 2.7% (Qinghai survey, 2003) to 20.7% in the present survey.

EUS and small-intestine enteroscopy have been used in clinical practice for many years[19,20], but in the past decade, these two types of endoscopy have developed rapidly. For example, DBE, which has evolved from conventional push enteroscopy, was first introduced into clinical practice in 2001 by Yamamoto *et al*[21]. DBE is a completely new technique that allows complete visualization, biopsy and treatment of small-bowel diseases. In the Shanghai survey, the rates for EUS and push-type enteroscopy were 4.3% and 7.2%, respectively, but in the present survey, the possession rates had increased to 10.6% and 22.4%, respectively. However, these two types of endoscope were still mainly owned by provincial hospitals in mainland China, which was largely due to their high cost and fewer indications for such procedures in small-to-medium endoscopy units (*i.e.,* prefecture and county level units).

Another important piece of endoscopy equipment, especially for large scale units, is a dedicated X-ray machine, which is necessary for a variety of GI interventions, including ERCP, percutaneous trans-hepatic catheter drainage, luminal stent placements and dilation[22]. In the Shanghai survey, the rate of possession of a dedicated X-ray machine (not mentioned in the Qinghai survey) was 5.7% (8/138), and the rate has increased to 19.5% in the present survey. Our data also demonstrated that Olympus was the leading manufacturer of GI endoscopes in mainland China, which is in accordance with the fact that Tokyo-based Olympus is the world's largest manufacturer and provider of conventional endoscopes[23].

Staffing requirements for GI procedures should be based on what is needed to ensure safe and proficient performance of the individual procedure[24]. As the number of procedures carried out and the complexity of the procedures and equipment have increased, the need for specialised staff has become apparent. Our survey indicated that most full-time endoscopy staff were found in provincial hospitals in mainland China.

Compared with the Shanghai survey in 2001, easily performed therapeutic procedures, such as polypectomy, have become more popular in the past 10 years; even in small-to-medium endoscopy units in China. Another conventional procedure is ERCP, which was first performed in 1968 and is usually regarded as the representative endoscopic intervention in pancreaticobiliary disorders[25]. In 1973, ERCP was introduced to China, and since then it has been extensively used. It is estimated that Chinese physicians perform nearly 60000 ERCP procedures annually, including therapeutic ERCP[17]. Our survey showed that, in China, ERCP (diagnostic and/or therapeutic) was a frequently performed procedure not only in provincial hospitals (77.3%), but also in prefecture level hospitals (42.9%). In the Shanghai survey in 2001, the overall rate of capability of performing ERCP was 38.4%, but now the nationwide rate has increased to 45.6%.

EVS or EVL is an effective endoscopic procedure for treatment of oesophageal varices[26], and it has been frequently performed in China because of the large number of patients with oesophagogastric variceal bleeding[27]. The Shanghai survey showed that 40.2% of the surveyed endoscopy units can perform an EVS procedure. According to the present survey, EVS/EVL is now frequently carried out in provincial (75.0%) and prefecture level (40.7%) units (Table 2); but in county level units (9.0%), there is still room to increase the popularity of this procedure, with regard to its simple operation without special devices. EMR and ESD were developed in the past decade as a novel therapeutic endoscopic procedure to remove the mucosal lesions, including early malignant lesion[8,9]. Our survey demonstrated that EMR or ESD is also predominantly confined to the large units (provincial level) in mainland China, which may be due to the shortage of special devices in small-to-medium endoscopy units, and the procedure itself is technically demanding.

In conclusion, rapid developments have taken place in GI endoscopy in China in the past decade. Major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly confined to large endoscopy units (*i.e.*, provincial hospitals), whereas small-to-medium units, often perform fewer endoscopic procedures and have less equipment, and are mostly restricted to diagnostic endoscopy. In addition to improvements in GI endoscopy equipment, standard procedures including the standard reprocessing for endoscopy will be the focus in the future in China[28]. Therefore, there is still much room for improvement in GI endoscopy in China, and our results may provide crucial information needed for the national level GI endoscopy planning.

ACKNOWLEDGMENTS

We thank Professor Yan-Qing Li (Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan, Shandong Province), Professor Ming-Jun Sun (Department of Gastrointestinal Endoscopy, the First Affiliated Hospital, China Medical University, Shenyang, Liaoning Province), Professor Xiao-Ping Wu(Department of Gastroenterology, the Second Hospital, Central South University, Changsha, Hunan Province), Professor Xue-Hong Wang (Department of Gastroenterology, the Affiliated Hospital, Qinghai University, Xining, Qinghai

Province), Hai Xing Jiang (Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region), Yu-Xiu Yang (Department of Gastroenterology, the People's Hospital of Henan Province, Zhengzhou, Henan Province) and Professor Yang Li (Department of Gastroenterology, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region) for their commitment to the survey, and we are indebted to all our colleagues who have taken the time to answer this survey.

COMMENTS

Background

Endoscopy is a universally popular, minimally invasive intervention for gastrointestinal (GI) and pancreatico-biliary disorders. Although GI endoscopy services have become a routine procedure in western countries, in most developing countries GI endoscopy services are sometimes available in so-called centres of excellence.

Research frontiers

In the past decade, the emergence of a variety of novel endoscopic equipment and techniques, *e.g.*, video capsule endoscopy (VCE), double/single balloon enteroscopy, endoscopic mucosal resection and endoscopic submucosal dissection (ESD) have substantially promoted the application value for GI tract lesions worldwide. GI endoscopy is experiencing rapid evolution from a diagnostic medical procedure to a minimally invasive therapeutic procedure.

Innovations and breakthroughs

The authors conducted a survey of GI endoscopy procedures and equipment in hospital-based GI endoscopy units, in order to demonstrate the current status of GI endoscopy in mainland China in the past decade. Based on provision of a satisfactory reply, 169/289 (58.5%) GI endoscopy units from three levels of hospital were enrolled in their study, which covered 28 provinces (90.3%, 28/31) in mainland China.

Applications

The authors found that the number of GI endoscopes per unit increased by nearly three times (from 2.9 to 9.3) in the past decade. The VCE, which was almost completely unavailable ten years ago, was possessed by 20.7% (35/169) of GI endoscopy units. ESD could be performed by 36.4% (19/44) of the provincial units, which was significantly higher than the prefecture level (9.9%) and county level (0.0%) units, respectively.

Terminology

The survey of GI endoscopy included equipment and procedures performed in each GI endoscopy unit. The questionnaires comprised 21 questions which were sent by e-mail to the physician in charge of the unit.

Peer review

This is a very important survey reflecting the development of GI endoscopy in mainland China in the past decade.

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P- Reviewers Dinis-Ribeiro M, Dumonceau JM
S- Editor Zhai HH **L- Editor** O'Neill M **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2956

World J Gastroenterol 2013 May 21; 19(19): 2956-2962
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Intraperitoneal perfusion of cytokine-induced killer cells with local hyperthermia for advanced hepatocellular carcinoma

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Received: January 23, 2013 Revised: March 24, 2013
Accepted: April 10, 2013
Published online: May 21, 2013

Abstract

AIM: To study the effect and tolerance of intraperitoneal perfusion of cytokine-induced killer (CIK) cells in combination with local radio frequency (RF) hyperthermia in patients with advanced primary hepatocellular carcinoma (HCC).

METHODS: Patients with advanced primary HCC were included in this study. CIK cells were perfused intraperitoneal twice a week, using 3.2×10^9 to 3.6×10^9 cells each session. Local RF hyperthermia was performed 2 h after intraperitoneal perfusion. Following an interval of one month, the next course of treatment was administered. Patients received treatment until disease progression. Tumor size, immune indices ($CD3^+$, $CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$), alpha-fetoprotein (AFP) level, abdominal circumference and adverse events were recorded. Time to progression and overall survival (OS) were calculated.

RESULTS: From June 2010 to July 2011, 31 patients diagnosed with advanced primary HCC received intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia in our study. Patients received an average of 4.2 ± 0.6 treatment courses (range, 1-8 courses). Patients were followed up for 8.3 ± 0.7 mo (range, 2-12 mo). Following combination treatment, $CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ cells increased from 35.78% ± 3.51%, 24.61% ± 4.19% and 5.94% ± 0.87% to 45.83% ± 2.48% (P = 0.016), 39.67% ± 3.38% (P = 0.008) and 10.72% ± 0.67% (P = 0.001), respectively. AFP decreased from 167.67 ± 22.44 to 99.89 ± 22.05 ng/mL (P = 0.001) and abdominal circumference decreased from 97.50 ± 3.45 cm to 87.17 ± 4.40 cm (P = 0.002). The disease control rate was 67.7%. The most common adverse events were low fever and slight abdominal erubescence, which resolved without treatment. The median time to progression was 6.1 mo. The 3-, 6- and 9-mo and 1-year survival rates were 93.5%, 77.4%, 41.9% and 17.4%, respectively. The median OS was 8.5 mo.

CONCLUSION: Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia is safe, can efficiently improve immunological status, and may prolong survival in HCC patients.

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Key words: Cytokine-induced killer cell; Radio frequency hyperthermia; Primary hepatocellular carcinoma; Intraperitoneal perfusion; Clinical observation

Core tip: Intraperitoneal perfusion of cytokine-induced killer (CIK) cells in combination with local radio frequencyc hyperthermia can result in a high concentration of

CIK cells. This treatment can efficiently improve immunological status, and attack small lesions in the abdominal wall, which can reduce ascites and relieve abdominal distention. This comprehensive treatment may prolong survival time and improve quality of life in patients with advanced hepatocellular carcinoma.

Wang XP, Xu M, Gao HF, Zhao JF, Xu KC. Intraperitoneal perfusion of cytokine-induced killer cells with local hyperthermia for advanced hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(19): 2956-2962 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2956.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2956

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and has a poor prognosis. Surgical resection at an early stage is still the best remedy[1]. As the onset of HCC is occult, it is typically diagnosed in stage Ⅲ-Ⅳ when patients present with clinical symptoms. However, the surgical resection rate is only 10%-30%. Currently, there is still no standard treatment for advanced HCC, but minimally invasive therapy and targeted therapy are favored. Patients with HCC may have poor therapeutic outcomes due to multiple local lesions or excessive tumor load. Consequently, integrative therapy is now a useful treatment for patients with advanced HCC[2].

Adoptive cellular immunotherapy has been applied in the clinical treatment of advanced HCC due to the close relationship between the pathogenesis of HCC and the autoimmune system, and the persistence of pathogenic factors such as hepatitis and cirrhosis[3-5]. Cellular immunotherapy includes several types of immunological cells, such as lymphokine-activated killer cells, tumor infiltrating lymphocytes and cytokine-induced killer (CIK) cells. CIK cells have been confirmed to have potential for immunotherapy against residual tumor cells. In recent years, several authors have reported that CIK cells were a heterogeneous population, and the major population to express both T cell marker monoclonal antibody and natural killer cell marker monoclonal antibody[6]. Cells with this phenotype are rare (1%-5%) in natural peripheral blood mononuclear cells. CIK cells are able to expand nearly 1000-fold when cultured in a cytokine cocktail, comprising interleukin (IL)-1α, interferon-γ (IFN-γ), IL-2 and mAbs against CD3, and have characteristics which are more effective in the treatment of tumors with a non-major histocompatibility complex (MHC)-restricted mechanism[7]. Thus, CIK cells may have some benefit in potential immunotherapeutic treatments in patients with HCC.

Hyperthermia treatment of tumors refers to tumor tissue which is treated with a continuous direct current through two or more electrodes placed outside the tumor. Some researchers have suggested that hyperthermia can normalize cell growth, and accelerate cell division after inhibiting cell division when it becomes abnormally accelerated[8,9]. Hyperthermia is an alternative treatment for HCC patients and has a positive effect on tumors. This study aimed to evaluate the safety and efficacy of the combination of CIK cell therapy and local radio frequency (RF) hyperthermia in patients with advanced HCC.

MATERIALS AND METHODS

General information

From June 2010 to July 2011, 31 patients with advanced HCC in the Oncology Center of Clifford Hospital were included in this study. Seven patients were identified by pathological diagnosis and the remaining patients all met the clinical diagnostic criteria[10]. Staging of disease was by tumor-node- metastasis staging according to the American Joint Committee on Cancer staging criteria[11]. All patients were stage Ⅲ-Ⅳ without metastases outside the abdomen. Reasons for being unable to undergo surgery and transcatheter arterial chemoembolization (TACE) were as follows: multiple tumors with obscure boundaries, extremely small remnant liver, lesions close to or involving great vessels, extreme damage to the cardiovascular system or other organs, liver functional lesion and rejection of surgery or TACE. Written informed consent was obtained before treatment.

Instrument and reagents

The instrument used was the FACS Calibur Flow cytometer (manufactured by BD Biosciences, United States). The reagents required for incubation of CIK cells were as follows: IL-1α (10 μg/vial, PeproTech Inc., Rocky Hill, NJ, United States), monoclonal mouse anti-human CD3 (500 μg/vial, PeproTech Inc.), IFN-γ (1000000 U/vial, PeproTech Inc.), IL-2 (PeproTech Inc.), complete Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, RPMI 1640 + gentamicin + 5% human type AB serum), lymphocyte separation medium (Tianjin Biological Products Company, Tianjin, China), human type AB serum (Tianjin Biological Products Company) and normal saline.

Preparation of CIK cells

CIK cells were prepared by the Cell Laboratory as follows: mononuclear cells were isolated from 50 mL peripheral blood from each patient using a blood cell separator, and then incubated in medium containing 1000 U/mL IFN-γ at 37 ℃; 24 h later 100 ng/mL monoclonal mouse anti-human CD3, 1000 U/mL IL-2 and 1000 U/mL IL-lα were added; the culture medium was renewed every 3 d and rhIL-2 was replenished. After 7-10 d, CIK cells were collected when the cell count reached 3.2×10^9 to 3.6×10^9, centrifuged, washed with normal saline, dispersed in 100 mL normal saline (containing 5 mL 20% human serum albumin) and retained as a sample.

Abdominocentesis

Routine abdominocentesis was performed before intraperitoneal perfusion of CIK cells and a tube was left

Table 1 Clinical characteristics of the patients included in this study (*n* = 31)

Characteristics	*n* (%)
Patient	
Gender (M/F)	22/9
Age, yr (range)	47.6 ± 8.8 (28-61)
Stage status	
Ⅲ	18 (58.06)
Ⅳ	13 (41.93)
Complications	
Seroperitoneum	14 (45.16)
Hypoproteinemia	17 (54.83)
Choloplania	8 (25.80)
ECOG performance status	
0	3 (9.67)
1	18 (58.06)
2	10 (32.25)
Past history before treatment	
Surgery	4 (12.90)
TACE	7 (22.58)
Chemotherapy	1 (3.22)
Surgery + TACE	8 (25.80)
Surgery + chemotherapy	2 (6.45)
TACE + chemotherapy	3 (9.67)
Initial treatment	6 (19.35)

M: Male; F: Female; ECOG: Eastern Cooperative Oncology Group; TACE: Transcatheter arterial chemoembolization.

in the abdomen for perfusion. After abdominocentesis, 100-200 mL physiologic saline was perfused into the abdomen and observations on defecation sensation, discomfort, and whether the tube was smooth and in the correct position for the preparation of intraperitoneal perfusion of CIK cells were carried out. Patients with a recent history of surgery or suspicious ankylenteron required ultrasonic guidance during abdominocentesis.

Local RF hyperthermia

The machine used was an EHY-2000 local RF Hyperthermia Machine, made in Hungary. Health education and psychological preparation were carried out prior to treatment. Patients were evaluated for dysmetabolism and disturbed perception of temperature. Patients were asked to lie on the treatment water bed in the correct body position according to treatment requirements. An appropriate electrode plate size was chosen to cover the body surface projection area of the tumor. Treatment time was 60 min per session and power was 100-150 W (according to the tolerance level of patients).

Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia

Intraperitoneal perfusion of CIK cells was performed twice a week (Monday and Thursday), with 3.2×10^9 to 3.6×10^9 cells each session. Local RF hyperthermia was carried out 2 h after intraperitoneal perfusion. In one treatment course consisting of 4 sessions, 1.2×10^{10} to 1.5×10^{10} cells were perfused. After an interval of one month, the next treatment course was administered, resulting in a time period of 1.5 mo for one cycle. Patients received treatment until disease progression or intolerant adverse effects.

Follow-up

Patients were followed up for 1 year. The treatment process, adverse reactions, and lost cases were recorded. A computed tomography (CT) or magnetic resonance imaging scan of the liver was performed every 2 mos. The size and number of tumors before and after treatment were compared. The therapeutic effect was evaluated and recorded. Levels of peripheral blood T lymphocyte subgroups ($CD3^+$, $CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$), abdominal circumference (patients with seroperitoneum) and AFP level were determined every 2 wk. Time to progression (TTP) and overall survival (OS) were calculated.

Statistical analysis

Results were analyzed using SPSS 17.0 software. The data were expressed as mean ± SD. The survival curves were calculated using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

RESULTS

Patient profile

From June 2010 to July 2011, a total of 31 patients aged 28-61 years (mean 47.6 ± 8.8 years) were included in this study. The characteristics of the patients are shown in Table 1.

Efficacy evaluation

According to the Response Evaluation Criteria In Solid Tumors, none (0%) of the patients had a complete response, 10 (32.25%) patients had a partial response (PR), 11 (35.48%) patients had no change (SD), and 10 (32.25%) patients had progressive disease. After treatment, AFP level decreased from 167.67 ± 22.44 to 99.89 ± 22.05 ng/mL (P = 0.001) and seroperitoneum in 14 patients significantly decreased, with abdominal circumference decreasing from 97.50 ± 3.45 to 87.17 ± 4.40 cm (P = 0.002).

Changes in cell immunity indices

The immunologic markers examined included the serum levels of $CD3^+$, $CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ T cells. After CIK cells were transfused back into the patients, all of these immune parameters increased, but not all of them increased significantly (Table 2).

Adverse events

No serious adverse events were observed in this study. Several mild adverse events were observed, which rapidly resolved without treatment (Table 3).

TTP and OS

TTP and OS were assessed in all 31 eligible patients. The median follow-up was 8.3 ± 0.7 mo (range, 2-12 mo). At the time of the analysis, 25 patients were dead and 6 patients were alive. The median TTP was 6.1 mo (95%CI:

Table 2 Immunity indices before and after treatment

Group	Pre-treatment	Post-treatment	*P* value
$CD3^+$	70.44% ± 6.68%	72.67% ± 6.22%	0.55
$CD4^+$	35.78% ± 3.51%	45.83% ± 2.48%	0.016
$CD3^+CD8^+$	24.61% ± 4.19%	39.67% ± 3.38%	0.008
$CD3^+CD56^+$	5.94% ± 0.87%	10.72% ± 0.67%	0.001

Table 3 Treatment-related adverse events (*n* = 31)

Side effects	*n* (%)
Abdominal side effects	
Abdominal pain	3 (9.67)
Abdominal erubescence	5 (16.12)
Abdominal lesser tubercle	1 (3.22)
Systemic side effects	
Slight fever	4 (12.90)
Dizziness	3 (9.67)
Debilitation	6 (19.35)
Nausea or vomiting	1 (3.22)
Diarrhea	2 (6.44)
Tachycardia	1 (3.22)

4.8-7.2). The 3-, 6- and 9-mo and 1-year survival rates were 93.5%, 77.4%, 41.9% and 17.4%, respectively. The median OS was 8.5 mo (Figure 1).

Typical case 1

A 57-year-old male was diagnosed with primary HCC by CT-guided aspiration biopsy of the liver in July 2010. Diffuse lesions in the liver with portal vein encroachment were observed on the CT scan. The patient also had complications of abdominal distention, debilitation, lack of appetite and emaciation. Eastern Cooperative Oncology Group (ECOG) was 2. AFP level was 201 ng/mL, alanine aminotransferase (ALT) 70 U/L, and aspartate aminotransferase (AST) 120 U/L. T lymphocyte subgroups were $CD3^+$ 76%, $CD4^+$ 22%, $CD3^+CD8^+$ 17%, and $CD3^+CD56^+$ 5.6%, respectively. The patient received 3 cycles of intraperitoneal CIK cells in combination with local RF hyperthermia. The patient achieved a PR. AFP fell to 98 ng/mL and ECOG increased to 1. ALT and AST fell to normal levels. T lymphocyte subgroups were $CD3^+$ 77%, $CD4^+$ 34%, $CD3^+CD8^+$ 27% and $CD3^+CD56^+$ 9.1%, respectively (Figure 2A and B).

Typical case 2

A 51-year-old male was admitted to hospital due to abdominal distention and pain. The patient was diagnosed with primary hepatic carcinoma in December 2012, and underwent surgery in a local hospital with postoperative Sorafenib therapy. The patient had been experiencing obvious abdominal distention with abdominal pain, poor appetite, nausea, and emesis for 1 mo. Enhanced CT scanning showed multiple metastases in the liver, metastases in the portal lymph nodes and significant seroperitoneum. Due to the advanced stage of hepatic carcinoma, further surgical treatment and interventional therapies were inappropriate. Considering the failure of targeted drug treatment, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia was administered to the patient. Before treatment, abdominal circumference was 97.5 cm. Ultrasound examination showed that the deepest portion of seroperitoneum was 8.16 cm. After 1 cycle of treatment with intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia, the patient noted that abdominal distension was significantly reduced, with a decrease in abdominal circumference to 93.0 cm and seroperitoneum decreased to 5.03 cm under ultrasound examination (Figure 2C and D).

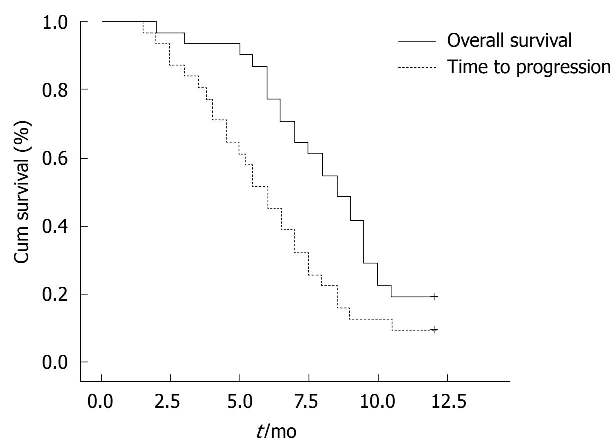

Figure 1 Time to progression and overall survival after treatment.

DISCUSSION

Recently, targeted therapy and palliative chemotherapy have been the main treatment approaches for patients with advanced HCC. New targeted drugs such as sorafenib and sunitinib have improved clinical efficacy. However, the serious side effects and high costs of these drugs make it impossible for many patients to complete the entire treatment course[12,13]. Thus, it is necessary to investigate alternative therapies for patients with advanced HCC.

Tumor killing ability of CIK cells

The special biological behavior of primary HCC, such as multiple lesions, existing hepatitis and cirrhosis, continuously suppresses cellular immunity and results in immune dysfunction. Autoimmune disorders in patients with HCC is manifested by lower levels of $CD4^+$ and $CD3^+CD56^+$ cells and significantly higher levels of $CD8^+$ cells[14,15]. The ability to efficiently kill tumor cells is the ultimate requirement in immune effector candidates for adoptive immunotherapy. CIK cells have a MHC-independent tumor killing capacity in both solid and hematologic malignancies. The antitumor activity is mainly associated with a high percentage of the $CD3^+CD56^+$ subset[16,17]. The exact mechanism involved in tumor recognition and killing is not completely known, but mainly involves the secretion of a cytokine to inhibit the growth of tumor cells[18-20].

It has been demonstrated that CIK cells are effective against some solid malignancies including lung, gastro-

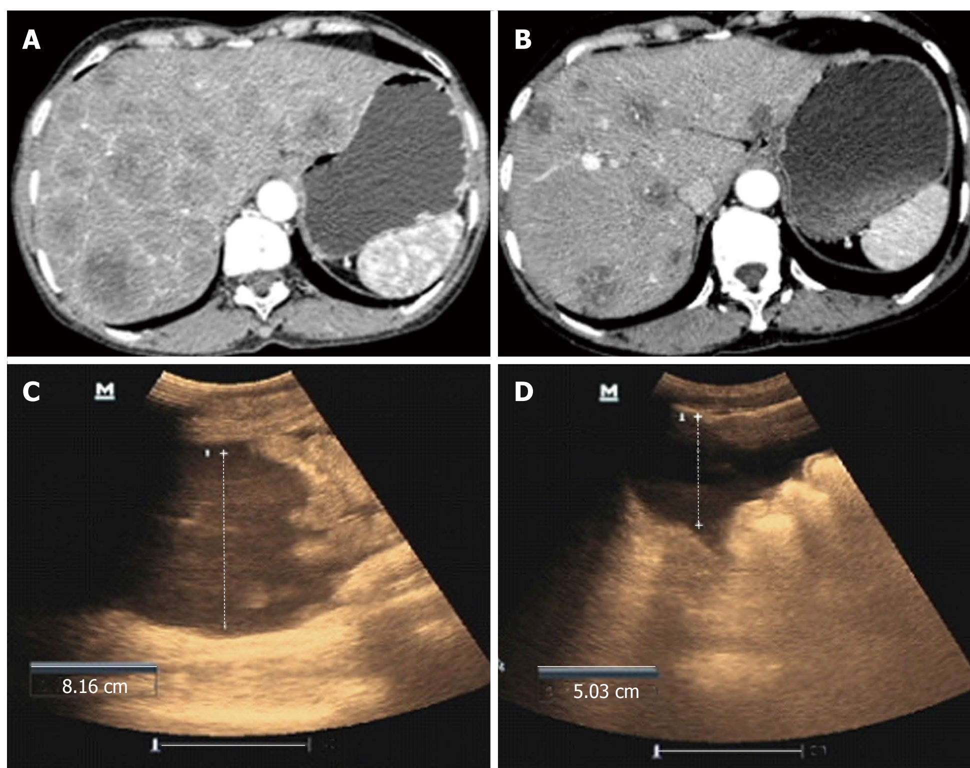

Figure 2 Computed tomography scan. A: Typical case 1 with hepatocellular carcinoma before treatment; B: Typical case 1 with hepatocellular carcinoma after treatment; C: Typical case 2 with hepatocellular carcinoma before treatment; D: Typical case 2 with hepatocellular carcinoma after treatment.

intestinal and mesenchymal tumors both *in vitro* and *in vivo*[21,22]. Olioso *et al*[23] reported 12 patients (6 advanced lymphomas, 5 metastatic kidney carcinomas and 1 HCC) who were intravenously transfused with 28 × 10^9 (range, 6 × 10^9 to 61 × 10^9) CIK cells per patient during one cycle. The treatment schedule consisted of three cycles of CIK cell infusions at an interval of 3 wk. After treatment, the absolute median number of lymphocytes, $CD3^+$, $CD8^+$ and $CD3^+CD56^+$ cells significantly increased in peripheral blood. In our study, $CD4^+$, $CD3^+CD8^+$ and $CD3^+CD56^+$ cells increased after treatment from 35.78% ± 3.51%, 24.61% ± 4.19% and 5.94% ± 0.87% to 45.83% ± 2.48% (P = 0.016), 39.67% ± 3.38% (P = 0.008) and 10.72% ± 0.67% (P = 0.001), respectively. Our results were similar to those of Olioso *et al*[23].

Intraperitoneal perfusion with local hyperthermia

The most common causes of death in patients with advanced HCC are liver failure, ascites and obstruction. Control of the abdominal tumor is the principal goal of treatment, which can prolong survival and improve quality of life. Recently, intravenous infusion of CIK cells has been widely used in the treatment of HCC. However, the concentration of CIK cells is low in tumor tissues following intravenous infusion, and the anti-tumor effect is low. In this study, in order to achieve a high concentration of CIK cells in the abdomen, CIK cells were perfused intraperitoneally instead of intravenously. Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia can improve efficacy in clinical practice. The primary reason for this may be that hyperthermia accelerates the conjugation of CIK cells and tumor cells, which improves the reaction rate of CIK cells. Local RF hyperthermia after high-capacity perfusion of CIK cells improves the sensitivity of tumor cells, allowing CIK cells to permeate into tumor cells more effectively.

Local RF hyperthermia kills tumor cells by heating using physical energy and the temperature of tumor tissue rises to an effective treatment temperature. Studies have shown that high temperature can damage the membranes of mitochondria, lysosomes and endoplasmic reticulum and triggers the massive release of acid hydrolase from lysosomes, resulting in membranolysis, outflow of cytoplasm and death of cancer cells[24,25]. In addition, some studies have reported that hyperthermia improves immune function by stimulating the development of the anti-tumor immune effect and resolving the inhibition of blocking factors in the immune system[26]. The core points behind this treatment were to administer intraperitoneal perfusion of CIK cells to improve immune function to kill the tumor, and local hyperthermia to enhance the concentration of CIK cells which directly affect liver tumor tissues and small lesions on the abdominal wall, which can damage cancer cells and effectively reduce abdominal dropsy. Local hyperthermia plays an important role in enhancing the concentration of CIK cells which directly affect these parameters.

Adverse events

The most common adverse events in our study were low fever and slight abdominal erubescence, which resolved without treatment. No serious side effects were observed.

During the treatment process, 4 patients experienced low fever ranging from 37.5-38 ℃, which resolved without treatment. This fever might have been caused by the application of IL-2. Some other symptoms, including erubescence and lesser tubercle of abdominal subcutaneous fat, were observed, which were caused by high temperature and were relieved by simple symptomatic treatment. Intraperitoneal perfusion of autologous CIK cells can avoid immune rejection triggered by foreign cell infusion.

In conclusion, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia is safe and effective for advanced HCC. More clinical trials with a large sample size are warranted to provide evidence for further applications. The addition of palliative chemotherapy or targeted therapy is worthy of further investigation.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Recently, integrative therapy has become a useful treatment for patients with advanced HCC. Due to the close relationship between the pathogenesis of HCC and the autoimmune system, cellular immunotherapy has been used in the clinical treatment of advanced HCC. In addition, local hyperthermia increases the role of cytokine-induced killer (CIK) cells in killing tumor tissues.

Research frontiers

Intraperitoneal perfusion of CIK cells in combination with local radio frequency (RF) hyperthermia can improve immune function in HCC patients, shrink the tumor, and relieve discomfort such as debilitation and abdominal distention.

Innovations and breakthroughs

Recently, intravenous infusion of CIK cells has been widely used in the treatment of HCC. However, the concentration of CIK cells is low in tumor tissues following intravenous infusion, and the anti-tumor effect is low. In their research, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia resulted in a high concentration of CIK cells in the abdominal cavity and effective killing of tumor tissues.

Applications

The authors have been using this treatment strategy for more than 1 year. Forty-two patients accepted this treatment, of whom 31 were selected for this study. There were no serious adverse events following this treatment. A few patients showed low-grade fever or slight chest distress which resolved after symptomatic treatment. Following intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia, abdominal distention resolved, tumor size diminished, and survival time was prolonged in some patients. This treatment strategy is worthy of further investigation.

Terminology

CIK cells are alloplasmic cells with non-major histocompatibility complex (MHC) restriction anti-tumor activity which is acquired by multiple-cell factor-cultivation *in vitro* from human peripheral blood mononuclear cells. $CD3^+CD56^+$ T is the main effector cell of the CIK cell group, and is also called nature killer cell-like T lympholeukocyte. These cells have powerful anti-tumor activity and MHC restriction of T lympholeukocytes. Local RF hyperthermia is used in the non-invasive treatment of malignant tissues. The difference between the complex dielectric constant (complex impedance) of malignant and healthy tissues makes it possible to select malignant tissues.

Peer review

In this study, 31 patients with advanced HCC who could not be treated by surgical treatments or interventional therapies, were treated by CIK cell intraperitoneal perfusion in combination with RF local hyperthermia. The result showed that this treatment is safe and reliable, and worthy of further clinical studies of a larger sample. It is a new option of treatment for patients with advanced HCC.

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P- Reviewers Han SB, Introna M, Zhong H
S- Editor Gou SX **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2963

World J Gastroenterol 2013 May 21; 19(19): 2963-2968
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Effect of Danzhijiangtang capsule on monocyte chemoattractant protein-1 mRNA expression in newly diagnosed diabetes subclinical vascular lesions

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Received: December 25, 2012 Revised: February 6, 2013
Accepted: February 28, 2013
Published online: May 21, 2013

Abstract

AIM: To investigate the effect of Danzhijiangtang capsule (DJC) on monocyte chemoattractant protein-1 (MCP-1) mRNA expression in newly diagnosed type 2 diabetes mellitus (T2DM) subclinical vascular lesions.

METHODS: Sixty-two patients with newly diagnosed T2DM subclinical vascular lesions were randomly divided into a control group and treatment group of 31 cases each. Oral antidiabetic therapy with routine western medicine was conducted in both groups, and the treatment group was additionally treated with DJCs. The treatment course for both groups was 12 wk. Before and after treatment, the total efficiency and traditional Chinese medicine (TCM) syndrome score were calculated. The fasting plasma glucose (FPG), 2-h plasma glucose (2hPG), fasting insulin (FINS), insulin resistance index (IRI), hemoglobin (Hb)A1c, blood lipids, and hemorheology indices were determined. In addition, the levels of vascular endothelial growth factors including thrombomodulin (TM), von Willebrand factor (vWF), P-selectin and MCP-1 mRNA were determined.

RESULTS: After 12 wk of treatment, the TCM syndrome score was significantly decreased compared to before treatment in both groups. After treatment, FPG, 2hPG, HbA1c, FINS, IRI, total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, whole blood low shear specific viscosity, plasma specific viscosity, TM, vWF, P-selectin and MCP-1 mRNA were significantly improved compared to before treatment in both groups. After treatment, the total efficiency and TCM syndrome score in the treatment group were better than in the control group. FINS, IRI, whole blood high shear specific viscosity, plasma specific viscosity, TM, vWF, P-selectin and MCP-1 mRNA level in the treatment group were significantly reduced after treatment compared with control group.

CONCLUSION: DJCs are efficacious in supplementing qi, nourishing yin and invigorating blood circulation, and upregulate MCP-1 mRNA expression in patients with T2DM subclinical vascular lesions.

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Key words: Danzhijiangtang capsule; Type 2 diabetes mellitus; Subclinical vascular lesions; Monocyte chemoattractant protein-1

Core tip: The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key mediator of inflammation. MCP-1 also belongs to the chemokine superfamily. T2DM subclinical vascular lesions are a type of mild inflammation. En-

dothelial cell MCP-1 can be increased markedly. Danzhijiangtang capsule is a traditional Chinese medicine compound preparation. The technique can restrain mononuclear cell chemotactic activity by supplementing qi, nourishing yin and invigorating blood circulation, thereby preventing and treating subclinical vascular lesions.

Fang ZH, Liu Y, Bao TT, Ni YQ, Liu J, Shi GB, Wu JP, Yang JP, Zhang H. Effect of Danzhijiangtang capsule on monocyte chemoattractant protein-1 mRNA expression in newly diagnosed diabetes subclinical vascular lesions. *World J Gastroenterol* 2013; 19(19): 2963-2968 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2963.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2963

INTRODUCTION

The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response, which then promotes the process of T2DM. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response. MCP-1 also belongs to the chemokine superfamily[1]. As shown in a large number of experimental studies, MCP-1 is a risk factor for the occurrence and development of insulin resistance (IR), T2DM and related vascular complications[2].

Danzhijiangtang capsule (DJC) is a traditional Chinese medicine (TCM) compound preparation, and is efficacious in supplementing qi, nourishing yin and invigorating blood circulation. In this study, we investigated the effects of DJCs on general indexes, including fasting plasma glucose (FPG), 2-h postprandial plasma glucose (2hPG), fasting insulin (FINS), insulin resistance index (IRI), hemoglobin (Hb)A1c, blood lipids, and hemorheology indicators in newly diagnosed T2DM subclinical vascular lesions, and the effects on expression of vascular endothelial growth factors including thrombomodulin (TM), von Willebrand factor (vWF), P-selectin and MCP-1 mRNA. The objective was to investigate preliminarily the mechanism of TCM on delaying vascular lesions. This study has importance for achieving a comprehensive understanding of the efficacy of DJCs and illuminating the multichannel and multitarget regulating effects of TCM on newly diagnosed T2DM subclinical vascular lesions.

MATERIALS AND METHODS

General data

A total of 62 outpatients and inpatients with T2DM in the Department of Metabolism and Endocrinology, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine were enrolled in this study from 2009 to 2011. All patients conformed to the diagnostic criteria of diabetes according to World Health Organization (1999), without clinical manifestations of heart, brain or kidney vascular lesions. Color Doppler ultrasound showed that the intima-media thickness of the common carotid, femoral and iliac arteries was > 0.6 cm. The exclusion criteria were as follows: patients with diabetes duration > 1 year; pregnant or lactating women; patients with metabolic disorders (*e.g.*, ketoacidosis) and complicated severe infection in the preceding month; patients complicated with a vascular lesion of the heart, brain, kidney or other region, or other severe primary diseases; patients taking anticoagulant, antiplatelet or antifibrinolytic drugs during the preceding month.

Patients were randomly divided into a control group (31 cases, 7 male and 14 female, mean age 53.67 ± 9.32 years) and treatment group (31 cases, 16 male and 15 female, mean age 51.90 ± 10.13 years). There was no significant differences in age, sex, FPG, 2hPG, HbA1c, FINS, body mass index and IRI between the two groups ($P > 0.05$).

Experimental methods

Basic treatments including diabetes education, alimentary control, and regular exercise were conducted in both groups. According to actual conditions, patients were treated with one or more antidiabetic drugs, with uniform administration in both groups. The antidiabetic drugs were as follows: metformin hydrochloride tablets (Glucophage; 0.5 g; Shanghai Squibb Pharmaceutical Co. Ltd., China), Acarbose (Glucoba; 50 mg; Bayer Pharmaceutical Co. Ltd., Leverkusen, Germany), pioglitazone hydrochloride (Kasiping; 15 mg; East China Pharmaceutical Group, Zhejiang, China), gliclazide sustained release tablets (Gliclazide tablets; 30 mg; Servier Pharmaceutical Company, Paris, France), and repaglinide tablets (NovoNorm; 1 mg; Novo Nordisk Pharmaceutical Industries, Bagsvaerd, Denmark). In the treatment group, in addition to the oral administration with the above western medicines, the patients were treated with DJCs (provided by the Pharmaceutical Formulations Centre, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine; 1.8 g of effective extract in each capsule) at a dose of five capsules, three times daily (a total daily dose of 15 capsules).

Observational indexes

Blood glucose and blood lipid: Blood glucose was determined by the glucose hexokinase method. Total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) with fasting for > 12 h were determined using a Hitachi-7600 fully automatic biochemical analyzer.

Hemorheology indices: High shear specific viscosity and low shear specific viscosity of whole blood, plasma specific viscosity, hematocrit and fibrinogen were determined using a rotary whole blood viscometer and plasma viscometer in the Experimental Centre, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine.

Vascular endothelial growth factors: Fasting venous

Table 1 Comparison of fasting plasma glucose, 2-h plasma glucose, haemoglobin A1c, fasting insulin, insulin resistance index, total cholesterol, triglycerides, low density lipoprotein, high density lipoprotein, haemorheology indices, thrombomodulin, von Willebrand factor, P-selectin levels and monocyte chemoattractant protein-1 mRNA levels

Index	Control group		Treatment group	
	Before treatment (n = 31)	After treatment (n = 29)	Before treatment (n = 31)	After treatment (n = 30)
FPG (mmol/L)	8.74 ± 1.42	8.01 ± 1.09[b]	9.02 ± 1.14	7.94 ± 0.91[b]
2hPG (mmol/L)	12.42 ± 1.76	10.03 ± 1.39[b]	11.96 ± 1.53	9.81 ± 1.34[b]
HbA1c (%)	7.98 ± 0.71	7.11 ± 0.69[b]	8.03 ± 0.68	6.98 ± 0.65[b]
FINS (uIU mL^{-1})	12.05 ± 2.93	8.68 ± 1.83[b]	11.93 ± 2.45	7.58 ± 1.82[b,c]
IRI	1.81 ± 0.24	1.60 ± 0.19[b]	1.79 ± 0.23	1.48 ± 0.19[b,c]
TC (mmol/L)	5.36 ± 0.76	4.55 ± 0.59[b]	5.27 ± 0.69	4.32 ± 0.49[b]
TG (mmol/L)	2.01 ± 0.38	1.78 ± 0.36[a]	1.99 ± 0.42	1.76 ± 0.31[a]
LDL (mmol/L)	3.15 ± 0.31	3.06 ± 0.29[a]	3.24 ± 0.28	3.07 ± 0.28[a]
HDL (mmol/L)	1.41 ± 0.19	1.66 ± 0.17[b]	1.37 ± 0.21	1.77 ± 0.15[b,c]
High shear specific viscosity (mPa·s)	5.37 ± 0.78	5.10 ± 0.71	5.43 ± 0.65	4.73 ± 0.54[b,c]
Low shear specific viscosity (mPa·s)	12.65 ± 0.89	11.91 ± 1.02	12.51 ± 0.92	11.84 ± 0.94[b]
Hematocrit (%)	42.95 ± 3.69	42.89 ± 3.51	43.15 ± 4.31	41.98 ± 3.01
Plasma specific viscosity (mPa·s)	1.93 ± 0.35	1.72 ± 0.37	2.01 ± 0.31	1.54 ± 0.24[b,c]
Fibrinogen (mg/L)	4.52 ± 0.48	4.36 ± 0.37	4.61 ± 0.38	4.39 ± 0.35
TM (μg/L)	25.68 ± 6.54	23.27 ± 4.06	25.41 ± 6.28	19.32 ± 4.29[a,c]
vWF (U/L)	1540.17 ± 74.70	1480.66 ± 72.32	1551.92 ± 82.23	1146.70 ± 94.45[b,d]
P-selectin (ng/mL)	25.03 ± 6.06	22.57 ± 5.56	25.39 ± 6.81	17.08 ± 5.82[a,c]
MCP-1 mRNA (%)	0. 537 ± 0. 136	0.473 ± 0.134[a]	0. 541 ± 0. 148	0.421 ± 0.132[b,c]

[a]P < 0.05, [b]P < 0.01 *vs* before treatment; [c]P < 0.05 *vs* control group. FPG: Fasting plasma glucose; 2hPG: 2-h plasma glucose; HbA1c: Haemoglobin A1c; FINS: Fasting insulin; IRI: Insulin resistance index; TC: Total cholesterol; TG: Triglycerides; LDL: Low density lipoprotein; HDL: High density lipoprotein; TM: Thrombomodulin; vWF: von Willebrand factor; MCP-1: Monocyte chemoattractant protein-1.

blood was obtained during the early morning. After anticoagulation with ethylenediaminetetraacetic acid and centrifugation (4 ℃, 3000 *g*, 10 min), the plasma was separated and stored at -70 ℃. TM was quantitatively determined using an enzyme-linked immunosorbent assay (ELISA) kit (ADL Company, CA, United States). vWF was quantitatively determined by the double antibody sandwich ELISA method (Shanghai Sun Biotechnology). P-selectin was quantitatively determined by the ABC-ELISA double antibody sandwich method (R and D Systems, Minneapolis, MN, United States).

MCP-1 mRNA: Expression of serum MCP-1 mRNA was determined using the reverse transcriptase polymerase chain reaction (Promega, Madison, WI, United States) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical software. Non-normally distributed data were logarithmically transformed to normally distributed data. Normally distributed measurement data are expressed as mean ± SD. Analysis of variance was performed for comparisons of multiple means. Student's t tests were used to analyze the differences between two samples. χ^2 tests were conducted to compare the enumerated data. P < 0.05 or P < 0.01 was considered to be statistically significant.

RESULTS

Comparison of blood glucose and blood lipid levels

Before treatment, there was no significant difference in FPG, 2hPG, HbA1c, FINS, IRI, TC, TG, LDL and HDL level between the two groups (P > 0.05 for all comparisons). After treatment with western medicines, the above indexes were significantly improved in both groups (P < 0.05 or P < 0.01). In addition, after treatment, FINS and IRI levels in the treatment group were significantly lower than in the control group (P < 0.05), and HDL in the treatment group was significantly higher than in the control group (P < 0.05) (Table 1).

Comparison of hemorheology indices

Before treatment, there was no significant difference in high shear specific viscosity and low shear specific viscosity of whole blood, plasma specific viscosity, hematocrit, and fibrinogen level between the two groups (P > 0.05 for all comparisons). After treatment, the whole blood high shear specific viscosity, plasma specific viscosity and hematocrit in the treatment group were significantly lower than in the control group (P < 0.05) (Table 1).

Comparison of TM, vWF and P-selectin levels

In the control group, after treatment, TM, vWF and P-selectin levels decreased, but the differences compared with baseline values before treatment were not significant (P > 0.05). In the treatment group, the above indexes were significantly reduced after treatment (P < 0.05 or P < 0.01) and compared with the control group (P < 0.05 or P < 0.01) (Table 1).

Comparison of MCP-1 mRNA levels

Before treatment, there was no significant difference in MCP-1 mRNA levels between the two groups (P > 0.05). After treatment, the MCP-1 mRNA level in the treatment group was significantly lower than before (P < 0.01) and

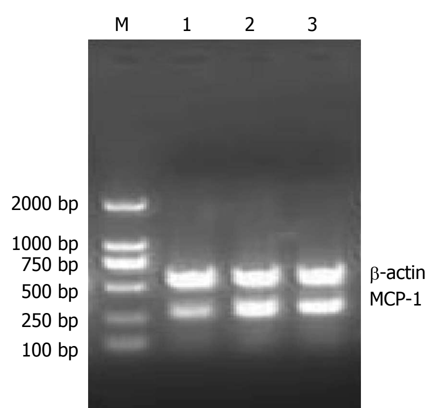

Figure 1 Electropherogram of monocyte chemoattractant protein-1 mRNA obtained by reverse transcription-polymerase chain reaction. MCP-1: Monocyte chemoattractant protein-1; M: Marker; 1: Normal group; 2: Control group; 3: Treatment group.

compared with control group (P < 0.05) (Figure 1 and Table 1).

DISCUSSION

The disease course of newly diagnosed T2DM is < 1 year. There are different degrees of vascular lesions in 50% of patients with newly diagnosed T2DM. Subclinical vascular lesions exist in some patients with newly diagnosed T2DM, but the clinical symptoms are atypical[3-5]. They are the pathological basis of diabetic complications, with an extensive scope. Once the subclinical vascular lesions occur, they develop rapidly and both large and small vessels, arteries, capillaries and veins may be involved, with complicated lesions in many organs, such as angiocarpy, brain, kidney, eyeground, nerve and skin. T2DM subclinical vascular lesions are caused by many factors including abnormal glucose metabolism, IR, lipid metabolism disorders, inflammatory factors, and oxidative stress[6,7]. Vascular endothelial injury is the initiating step of T2DM subclinical vascular lesions, and the anatomical endothelial layer is also the first involved site. During T2DM, continued hyperglycemia and high capillary pressure can cause vascular endothelial cell injury and dysfunction, changing angiostasis and hemodynamics, activate the coagulation system and platelets, and increase the vascular permeability, thus leading to a series of pathological changes[8]. Therefore, studying the change in endothelial cell function is beneficial for further preventing the occurrence and development of T2DM subclinical vascular lesions.

In recent years, TM has been confirmed as a molecular marker of endothelial cell injury[9,10]. In addition, it can be used as a marker for judging the severity of atherosclerosis of peripheral arteries and coronaries, and a sensitive detection index for the occurrence of vascular complications[11]. As found in prospective studies, vWF is involved in blood coagulation, and can promote platelet adhesion[12]. It is an important marker for predicting early subclinical endothelial cell injury in T2DM and development of urinary albumin excretion rate[13]. In addition, it is an important risk factor for new microvascular lesions. P-selectin has been a research hotspot for vascular lesions in recent years. It is an important marker of endothelial cell injury and platelet activation, and is considered to be an important factor in the initial pathological process of atherosclerosis[14].

MCP-1 is one member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response[15]. In T2DM, high-level blood glucose, non-enzymatic glycated protein, angiotensin, oxidative stress, interleukin-1, and tumor necrosis factor-α can all up-regulate MCP-1 expression[16-19]. MCP-1 can specifically induce blood monocytes to enter the endothelium of the lesion. This is an important mechanism in the occurrence and development of T2DM vascular lesions[20]. MCP-1 mainly attracts monocytes and T lymphocytes, and induces monocytes and endothelial cells to express adhesion molecules. It can promote various inflammatory cells, especially monocytes, to aggregate in the lesion sites, and respond to stimulation of the inflammatory cytokines[21]. In addition, MCP-1 can promote peripheral blood mononuclear cells to adhere, chemoattract and migrate to the intima, and then phagocytose lipids and transform into foam cells[22]. The activated leukocytes and vascular wall cells can release a variety of growth factors, which promote vascular smooth muscle cell proliferation, stimulate inflammation[23], and participate in the occurrence and development of atherosclerosis. It has been found that MCP-1 plays an important role in atherosclerosis in diabetes, and can be used as a useful index for predicting T2DM subclinical atherosclerosis[24]. At the same time, MCP-1 is considered to be a potential biochemical marker for reflecting early atherosclerotic changes and prognosis[25].

Therefore, MCP-1 is closely related to the occurrence and development of T2DM vascular lesions. Further study of MCP-1 will enrich the theoretical basis for pathogenesis of T2DM vascular lesions. Analysis of the chemokine dynamic change, regulatory mechanism and interactions, and treatment aimed at chemokines and receptors is a new method for therapy of T2DM vascular complications. The deepening awareness of the complexity of the chemokine network has provided new methods and means for prevention and treatment of T2DM vascular lesions.

TCM believes that the prethrombotic status of T2DM is similar to the blood stasis syndrome of emaciation-thirst disease. The pathogenesis of emaciation-thirst disease is that the dryness-heat, impairment of yin, and body fluid scorching will cause blood viscosity and blood stagnation. The long-term yin deficiency injures the healthy qi, leading to qi and yin deficiency and weakness, which aggravate the blood stasis. Deficiency of yin affects yang, and insufficiency of yang brings about cold syndrome. The cold coagulation causes blood stasis. The blood stasis is present throughout the disease course, and is a common pathological product and pathogenic factor of emaciation-thirst disease. In the treatment of this dis-

ease, supplementing qi, nourishing yin, invigorating blood circulation and dissolving stagnant blood should be carried out, and the treatment emphasis should be focused on regulating qi-blood circulation and improving blood circulation. DJCs are composed of Radix Pseudostellariae, Radix Rehmanniae, Cortex Moutan, Dodder, Alisma, leech and others. Radix Pseudostellariae can invigorate the qi of the spleen and kidney. Radix Rehmanniae can nourish the yin of the spleen and kidney. Dodder can tonify the kidney to arrest spontaneous emission. Cortex Moutan and leech can promote qi circulation, invigorate blood circulation, dissolve stagnant blood, and resolve kidney collateral obstruction. Alisma can clear heat and purge phlegm. The whole prescription can supplement qi and nourish yin, invigorate blood circulation and dissolve stagnant blood, thus achieving the efficacy of balancing yin and yang, dispelling blood stasis, and making the kidney pulse unobstructed. Modern pharmacological research shows that Radix Pseudostellariae, Radix Rehmanniae and Alisma have certain antidiabetic and antihypertensive effects. Moutan bark can inhibit a cyclooxygenase reaction, thus inhibiting platelet aggregation. The hirudin contained in leech can inhibit the effect of thrombin on fibrinogen, impede blood coagulation, and prevent thrombus formation. In addition, Leech also secretes a histamine-like substance, which can directly expand the blood vessels, activate plasmin, and inhibit collagen synthesis, thus reducing the blood viscosity and improving the hemorheology.

Results of this study have shown that, after treatment with DJCs, FINS and IRI levels are significantly lower than those obtained from basic treatment, with an increase in HDL level. In addition, after treatment with DJCs, the whole blood high shear specific viscosity, plasma specific viscosity and hematocrit, as well as TM, vWF and P-selectin expression levels are significantly reduced, with downregulated peripheral blood MCP-1 mRNA expression. This indicates that DJCs can alleviate lipid metabolism disorders, downregulate expression of TM, vWF, P-selectin and MCP-1 mRNA, lower whole blood viscosity and plasma specific viscosity, reduce hematocrit, antagonize platelet activation, inhibit platelet aggregation, and protect vascular endothelial cells, thus intervening in the newly diagnosed T2DM subclinical vascular lesions. It is believed that this therapy, by supplementing qi, nourishing yin and invigorating blood circulation, can enhance the anticoagulant and fibrinolytic activity, intervene in the hypercoagulable state, and improve the function of impaired endothelial cells. This has important significance in delaying vascular complications associated with T2DM.

COMMENTS

Background

The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response, which in turn promotes the process of T2DM. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response. MCP-1 also belongs to the chemokine superfamily. As shown in a large number of experimental studies, MCP-1 is a risk factor for the occurrence and development of insulin resistance, T2DM and related vascular complications.

Research frontiers

The disease course of newly diagnosed T2DM is < 1 year. There are different degrees of vascular lesions in 50% of patients with newly diagnosed T2DM. Subclinical vascular lesions exist in some patients with newly diagnosed T2DM, but the clinical symptoms are atypical. They are the pathological basis of diabetic complications, with an extensive scope.

Innovations and breakthroughs

This study has important significance for comprehensive understanding of the efficacy of Danzhijiangtang capsules (DJCs) and illuminating the multichannel and multitarget regulating effects of traditional Chinese medicine on newly diagnosed T2DM subclinical vascular lesions.

Applications

DJCs are efficacious in supplementing qi, nourishing yin and invigorating blood circulation, and can upregulate MCP-1 mRNA expression in patients with T2DM subclinical vascular lesions.

Terminology

In recent years, thrombomodulin has been confirmed as a molecular marker of endothelial cell injury. In addition, it can be used as a marker for judging the atherosclerosis severity of peripheral arteries and coronaries, and a sensitive detection index for the occurrence of vascular complications.

Peer review

This manuscript describes a traditional Chinese medicine DJCs for treatment of T2DM subclinical vascular lesions, and it has significant research interest.

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P- Reviewers Assy N, Ismail-Beigi F **S- Editor** Song XX
L- Editor Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2969

World J Gastroenterol 2013 May 21; 19(19): 2969-2973
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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BRIEF ARTICLE

Theory of mind deficits in patients with esophageal cancer combined with depression

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Received: November 10, 2012 Revised: March 5, 2013
Accepted: March 22, 2013
Published online: May 21, 2013

Abstract

AIM: To characterize the two components of theory of mind (ToM) in patients with esophageal cancer combined with depression.

METHODS: Sixty-five patients with esophageal cancer combined with depression (depressed group) and 62 normal controls (control group) were assessed using reading the mind in the eyes test, faux pas task, verbal fluency test, digit span test and WAIS IQ test. The depressed group was divided into two subgroups including psychotic depressed (PD) group (32 cases) and nonpsychotic depressed (NPD) group (33 cases). The clinical symptoms of patients were assessed using Beck depression inventory version II and brief psychiatric reacting scale (BPRS).

RESULTS: There was a significant difference between the depressed group and the control group on tasks involving ToM social perceptual components (mind reading: t = 7.39, P < 0.01) and tests involving ToM social cognitive components (faux pas questions: t = 13.75, P < 0.01), respectively. A significant difference was also found among the PD group, the NPD group and the control group on mind reading (F = 32.98, P < 0.01) and faux pas questions (χ^2 = 78.15, P < 0.01), respectively. The PD group and NPD group performed worse than normal group controls both on mind reading and faux pas questions (P < 0.05). The PD group performed significantly worse than the NPD group on tasks involving ToM (mind reading: F = 18.99, P < 0.01; faux pas questions: F = 36.01, P < 0.01). In the depressed group, there was a negative correlation between ToM performances and BPRS total score (mind reading: r = -0.35, P < 0.01; faux pas questions: r = -0.51, P < 0.01), and between ToM performances and hostile suspiciousness factor score (mind reading: r = -0.75, P < 0.01; faux pas questions: r = -0.73, P < 0.01), respectively.

CONCLUSION: The two components of ToM are both impaired in patients with esophageal cancer combined with depression. This indicates that there may be an association between ToM deficits and psychotic symptoms in clinical depression.

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Key words: Esophageal cancer combined with depression; Theory of mind; Social perceptual component; Social cognitive component

Core tip: In this study, the theory of mind deficits in patients with esophageal cancer combined with de-

pression was investigated, and the relation between ToM deficits and psychotic symptoms was discussed.

Cao Y, Zhao QD, Hu LJ, Sun ZQ, Sun SP, Yun WW, Yuan YG. Theory of mind deficits in patients with esophageal cancer combined with depression. *World J Gastroenterol* 2013; 19(19): 2969-2973 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2969.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2969

INTRODUCTION

Depression exists in about 24% of patients with esophageal cancer[1]. It is found that there are theory of mind (ToM) deficits in patients with depression[2-4]. In cognitive neuropsychiatry, the ToM is divided into a social perceptual component and a social cognitive component[5,6], in terms of information processing theories. In recent years, the social cognitive impairment caused by depression and its potential cognitive neuropsychological mechanisms have become a research hotspot[2-4].

It is reported that the occurrence of esophageal cancer combined with depression is closely related with cognition in disease[7-9]. Whether the cognitive biases include ToM deficits or not is not clear. In this study, the ToM deficits in patients with esophageal cancer combined with depression were investigated, and the relation between ToM deficits and psychotic symptoms was discussed.

MATERIALS AND METHODS

Research objects

Sixty-five patients with esophageal cancer combined with depression in Changzhou Second People's Hospital Affiliated to Nanjing Medical University (Changzhou, China) were studied from January to December in 2011. Their beck depression inventory version Ⅱ (BDI-Ⅱ)[10] scores were not less than 5. All patients had an education background of junior middle school and above. They were right-handed and had normal eyesight and hearing. There was no patient with a medical history of head trauma, central nervous system disease, metastatic brain tumor, mental illness or substance dependence. No patient had been treated with chemotherapy.

The patients were divided into a psychotic depressed (PD) group (32 cases) and a nonpsychotic depressed (NPD) group (33 cases), according to whether or not patients had psychotic symptoms [the brief psychiatric reacting scale (BPRS)[11] total score was > 35]. Psychotic symptoms were distinguished from schizophrenia. The ages of patients were 28-60 years, with an average age of 48.50 ± 4.53 years. They had obtained 9-16 years of education, with an average education duration of 12.57 ± 1.64 years. The average age of the NPD group and the PD group was 45.00 ± 5.02 and 46.28 ± 4.19 years, respectively. The average duration of education in the NPD group and the PD group was 12.18 ± 1.06 and 11.37 ± 1.25 years, respectively.

The control group consisted of 62 cases of college students, physicians and people with normal physique. They had no medical history of neurological disease, mental illness, substance abuse, or family psychiatry. They had an education background of junior middle school and above. They were right-handed and had normal eyesight and hearing. Their ages were 27-62 years, with an average age of 47.65 ± 4.64 years. The duration of education was 9-16 years, with an average education duration of 11.59 ± 2.01 years.

All research participants had signed informed consent before enrolling in this study. The patients had obtained the agreement of guardians. There was no significant difference in gender, age, or duration of education between the depressed group and the control group ($P > 0.05$) or between the PD group, the NPD group and the control group ($P > 0.05$). The difference in disease course between the NPD group and the PD group was not statistically significant ($t = 0.69$, $P > 0.05$).

Reading the mind in the eyes test

The reading the mind in the eyes test reflected the social perceptual component of ToM. It required the participants to conduct a perceptual processing of the mental activity status of the character in the eye area, and select one word from four alternatives which could most accurately reflect the character's mental activity status. The gender recognition task was used as a control task, which required the participant to recognize the gender of the character in the eye area. It reflected the perceptual processing of general social cues. Five min of learning of alternative vocabulary annotations was performed before test. One score was obtained for each correct answer. For a total of 34 questions (17 male and 17 female), there was a total score of 34 for reading the mind in the eyes test and the gender recognition task, respectively[12-14].

Faux pas task

The faux pas task (faux pas questions) belonged to the social cognitive component of ToM. It required the research object to plot the mental activity status of the character in the story and judge whether or not the character had said the words which should not be said or the embarrassing words, according to the storyline. The understanding of general text content in story was used as a control question. It reflected the understanding of the story content, and comprised 20 little stories (10 faux pas stories and 10 stories without faux pas). There were 2 faux pas questions and 2 control questions for each story. One score was obtained for each correct answer. The total score for faux pas questions and control questions was 20 and 40, respectively[13-15].

Clinical evaluation and neuropsychological test

The clinical symptoms of patients were assessed using BDI-Ⅱ and BPRS ($K = 0.83$). The WAIS-IQ test[16], digit

Table 1 General data of patients with esophageal cancer combined with depression

Groups	n	BDI-Ⅱ score	BPRS total score	Anxiety depression factor score	Anergia factor score	Thought disturbance factor score	Activation factor score	Hostile supiciousness factor score
PD group	23	33.00 ± 4.62[1]	43.72 ± 7.11[1]	14.28 ± 2.21[1]	6.13 ± 3.14	6.34 ± 3.35	4.99 ± 2.36	10.31 ± 2.95[1]
NPD group	33	20.22 ± 3.28	27.13 ± 6.12	10.75 ± 2.69	5.12 ± 3.46	5.91 ± 3.29	4.97 ± 3.01	2.21 ± 1.95

PD: Psychotic depressed; NPD: Nonpsychotic depressed. [1]t test, P < 0.01 *vs* NPD group.

Table 2 Comparisons of neuropsychological test and theory of mind test performance

Groups	n	IQ score	VFT score	DST score	Mind reading score	Gender recognition score	Faux pas questions score	Control questions score
Depressed group	65	103.12 ± 5.18	32.11 ± 2.38[1]	13.02 ± 1.08	24.12 ± 2.19[1]	30.11 ± 1.02	15.02 ± 1.63[1]	37.25 ± 0.68
PD group	32	103.42 ± 3.58	30.02 ± 2.16[2]	12.68 ± 1.02	22.89 ± 2.07[2,3]	30.12 ± 0.99	13.16 ± 1.71[2,3]	37.31 ± 0.71
NPD group	33	103.77 ± 4.30	34.21 ± 2.08	13.79 ± 1.01	25.38 ± 2.32[2]	30.22 ± 0.95	15.82 ± 1.13[2]	37.52 ± 0.62
Control group	62	104.11 ± 3.22	35.05 ± 2.01	13.88 ± 0.98	27.89 ± 2.05	30.57 ± 1.01	19.92 ± 1.01	37.51 ± 0.65

[1]t test, P < 0.01 *vs* NPD group; [2]Bonferroni test or Mann-Whitney U test, P < 0.05 *vs* control group; [3]t test, P < 0.01 or 0.05 *vs* NPD group. VFT: Verbal fluency test; DST: Digit span test; PD: Psychotic depressed; NPD: Nonpsychotic depressed.

span test (DST)[17] and verbal fluency test (VFT)[18] were conducted on all research objects. The DST included the recitation and inverted recitation of digits. The total score was expressed as the sum of two recitation scores. The VFT required the participant to say as many names as possible of vegetables, fruits and animals. One score was obtained for each correct answer. There was no score for duplicated names.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software. The χ^2 test, independent samples t test, single factor analysis of variance (using Bonferroni correction for multiple comparisons) and Kruskal-Wallis test (using Mann-Whitney U test for multiple comparisons) were used to compare data according to the different types of variables. In the depressed group, the analysis of covariance was conducted on ToM performance using the BDI-Ⅱ score as a covariant, and the partial correlation analysis was conducted between ToM performance and BPRS total score, and between ToM performance and other factor scores, respectively.

RESULTS

Comparisons of general data

There was no significant difference in IQ score between the depressed group and the control group (t = 0.52, P > 0.05). It was the same with IQ score among the NPD group, PD group and control group (F = 0.12, P > 0.05). The BDI-Ⅱ score (t = 6.77, P < 0.01), BPRS score (t = 6.78, P < 0.01), anxiety-depression factor score (t = 3.56, P < 0.01) and hostile suspicion factor score (t = 10.95, P < 0.01) in the PD group were significantly higher than those in the NPD group. The differences in other factors scores were not significant between the two groups (P > 0.05). Results were shown in Table 1.

Comparisons of neuropsychological test performance

As shown in Table 2, the VFT score in the depressed group was significantly lower than that in the control group (t = 4.34, P < 0.01), but there was no significant difference in DST score between the two groups (t = 0.75, P > 0.05). The differences in VFT score among the NPD group, PD group and control group were statistically significant (F = 15.56, P < 0.01). The VFT score in the PD group was significantly lower than that in the control group and the NPD group (P < 0.05), respectively, and there was no significant difference between the NPD group and the control group (P > 0.05).

Comparisons of ToM test performances

There was a significant difference between the depressed group and control group on tasks involving a ToM social perceptual component (mind reading: t = 7.39, P < 0.01) and tests involving a ToM social cognitive component (faux pas questions: t = 13.75, P < 0.01), respectively. But there was no significant difference for the gender recognition score and control questions score (t = 0.47, P > 0.05; t = 0.52, P > 0.05), respectively. A significant difference was also found among PD group, NPD group and control group on mind reading (F = 32.98, P < 0.01) and faux pas questions (χ^2 = 78.15, P < 0.01). Results of multiple comparisons showed that the PD group was worse than the NPD group and the NPD group was worse than the control group on mind reading (P < 0.05 for both). In addition, on faux pas questions, the PD group performed worse than the control group and the NPD group, respectively, (Mann-Whitney U = 153.08, Z = -7.38, P < 0.05; Mann-Whitney U = 127.95, Z = -4.26, P < 0.05), and the NPD group performed worse than the control group (Mann-Whitney U = 153.13, Z = -6.81, P < 0.05). In addition, the PD group performed significantly worse than the NPD group on tasks involving ToM (mind reading: F = 18.99, P < 0.01; faux pas questions: F = 36.01, P < 0.01).

Partial correlation analysis between ToM performance and BPRS score

In the depressed group, the faux pas questions score was positively correlated with the mind reading score ($r = 0.56$, $P < 0.01$). There was a negative correlation between ToM performances and BPRS total score (mind reading: $r = -0.35$, $P < 0.01$; faux pas questions: $r = -0.51$, $P < 0.01$), and between ToM performances and hostile suspiciousness factor scores (mind reading: $r = -0.75$, $P < 0.01$; faux pas questions: $r = -0.73$, $P < 0.01$), respectively. There was no correlation between ToM performances and other factors score related to BPRS ($P > 0.05$).

DISCUSSION

This study aims to investigate the components of ToM in patients with esophageal cancer combined with depression. Results show that the depressed group performed worse than the control group on tasks involving ToM social perceptual components and test involving ToM social cognitive components, respectively. This suggests that the ToM deficits exist in patients with esophageal cancer combined with depression. These results are in accordance with the studies of Inoue *et al*[3,4] and Lee *et al*[19], in which ToM deficits are found in patients with depression in ToM tests.

In this study, there are impairments in the social perceptual component and social cognitive component of ToM. The social perceptual component of ToM occurs mainly in the right cerebral hemisphere, and the left cerebral hemisphere is mainly responsible for the social cognitive component[6,12]. This indicates that there are bilateral brain impairments in patients with esophageal cancer combined with depression. These results are similar to those of the study by Rotenberg[20] which suggests that depression is related not only to right hemisphere dysfunction, but also to left hemisphere prefrontal hypofunction. This indicates that there is a cognitive neuropsychology mechanism of bilateral brain impairments for depression.

In this study, the ToM performances in the PD group are significantly worse than those in the NPD group. Results of partial correlation analysis show that there was a negative correlation between ToM performances and BPRS total score, and between ToM performances and hostile suspiciousness factor score, respectively. This indicates that there is a positive correlation between ToM deficits and psychiatric symptoms in clinical depression. Therefore, early detection and intervention of ToM deficits and psychiatric symptoms in patients with esophageal cancer combined with depression is helpful for reducing the perniciousness of disease.

There is no metastatic brain tumor or chemotherapy in these patients, which has purified the samples to a certain extent. But the remote effects of tumors on neuropsychiatric function can not be excluded. Therefore, the follow up is very important for enriching and improving the final results.

COMMENTS

Background

Twenty-four percent of esophageal cancer patients also have depression, which may aggravate the disease condition and affect the treatment and rehabilitation. Previous studies suggest that there are a variety of cognitive disorders including theory of mind (ToM) deficits in patients with depression. The occurrence of esophageal cancer complicated with depression is closely related to cognition on disease. Whether cognitive biases include ToM deficits is not clear.

Research frontiers

ToM deficits are social cognitive disorders. In this study, ToM deficits in esophageal cancer patients complicated with depression are observed using "Reading the Mind in the Eyes" test and Faux pas Task.

Innovations and breakthroughs

Previous studies on cognitive impairment in esophageal cancer patients complicated with depression mainly focus on episodic memory impairment, executive function decline, psychomotor slowing and attention deficits, except ToM deficits. In this study, ToM deficits in esophageal cancer patients complicated with depression have been investigated. This contributes to prevention and treatment of esophageal cancer with depression, and improvement of patient's social adaptability.

Applications

Results of this study can be applied to preventing and treating esophageal cancer with depression, and improving the rehabilitation level and social adaptability of patients.

Terminology

ToM refers to the knowledge system on speculation of psychological state, namely the cognitive system on judgment of psychological state such as belief, intention, wish, need, motive and emotion. It is one of the most basic abilities of an individual for adapting into society. In cognitive neuropsychiatry, ToM is divided into a social perceptual component and social cognitive component, in terms of information processing theory.

Peer review

This is good research. It finds ToM deficits in esophagus cancer patients complicated with depression, and has analyzed the damage characteristics of two subcomponents. This study is helpful for clinical assessment of cognitive disorders in esophageal cancer patients complicated with depression, and prevention and treatment of disease. In addition, it can provide new approaches for improving the social adaptabilities of patients.

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P- Reviewers Inoue Y, Kataria K **S- Editor** Wen LL
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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v19.i19.2974

World J Gastroenterol 2013 May 21; 19(19): 2974-2978
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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CASE REPORT

Successful liver resection in a giant hemangioma with intestinal obstruction after embolization

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Received: October 9, 2012 Revised: January 17, 2013
Accepted: March 28, 2013
Published online: May 21, 2013

Abstract

Hepatic hemangiomas are the most common benign tumor of the liver. Most hepatic hemangiomas remain asymptomatic and require no treatment. Giant hepatic hemangiomas with established complications, diagnostic uncertainty and incapacitating symptoms, however, are generally considered an absolute indication for surgical resection. We present a case of a giant hemangioma with intestinal obstruction following transcatheter arterial embolization, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. A 21-year-old Asian man visited our hospital for left upper quadrant pain. Examinations at the first visit revealed a left liver hemangioma occupying the abdominal cavity, with a maximum diameter of 31.5 cm. Embolization of the left hepatic artery was performed and confirmed a decrease in its size. However, the patient was readmitted to our hospital one month after embolization for intestinal obstruction. A left hepatectomy was completed through a herringbone incision, and safely removed a giant hemangioma of 26.5 cm × 19.5 cm × 12.0 cm in size and 3690 g in weight. Pre-operative arterial embolization is effective for reducing tumor size, but a close follow-up to decide the time for hepatectomy is important.

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Key words: Hepatic hemangioma; Transcatheter arterial embolization; Intestinal obstruction; Complications; Hepatectomy

Core tip: Hepatic hemangiomas are the most common benign tumor of the liver. Most hepatic hemangiomas remain asymptomatic and require no treatment; giant hepatic hemangiomas with established complications, diagnostic uncertainty and incapacitating symptom, however, are generally considered an absolute indication for surgical resection. We present a case of a giant hemangioma with intestinal obstruction following transcatheter arterial embolization, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. Our experience indicates the effectiveness of pre-operative arterial embolization to reduce tumor size, and the importance of a close follow-up to decide when to perform the surgery.

Zhou JX, Huang JW, Wu H, Zeng Y. Successful liver resection in a giant hemangioma with intestinal obstruction after embolization. *World J Gastroenterol* 2013; 19(19): 2974-2978 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i19/2974.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i19.2974

INTRODUCTION

Liver hemangiomas are the most common benign tumors of the liver, with an estimated prevalence of 3%-20%[1-3]. Most of them are small in size (< 4 cm in diameter) and asymptomatic and are discovered incidentally during

screening tests by modern diagnostic procedures. Giant hemangiomas are defined as tumors with a diameter > 4 cm, and symptoms rarely appear unless the tumor size exceeds 4 cm[4-6]. Although the majority of hepatic hemangiomas remain asymptomatic, symptomatic hepatic hemangiomas can present with abdominal pain, hemorrhage, biliary compression, or a consumptive coagulopathy.

A range of treatment options exists for liver hemangiomas, from observation to various radiological and surgical procedures. When treatment is needed, surgical excision of the hemangioma is most effective, and associated with low morbidity and mortality[7,8]. Other treatments, including transcatheter arterial embolization (TAE)[9], arterial ligation[10], radiotherapy[11], radiofrequency ablation[12], corticosteroid therapy[13], and liver transplantation[14,15], have been employed for large unresectable lesions. Apart from liver transplantation, however, the long-term effect of these methods usually cannot be anticipated.

Here, we report a case of a giant hemangioma with intestinal obstruction following TAE treatment, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy.

CASE REPORT

A 21-year-old Asian man visited a local hospital for left upper quadrant pain as the chief complaints. Ultrasonography, performed during the visit, detected huge hyperechoic lesions, and under the diagnosis of a giant hemangioma, he was recommended for surgery and transferred to our hospital. The patient's past or family medical history was unremarkable. Physical examination revealed a grossly distended abdomen without a fluid wave, and tenderness and bounce pain in the left upper quadrant.

On admission, the patient's laboratory values were notable for an international normalized ratio of 1.16, a decreased fibrinogen level of 1.99 g/L (normal range, 2.0-4.0) and D-dimer levels of 8.12 mg/L fibrinogen equivalent unit (normal range, < 0.55 mg/L). The results of blood routine and liver function tests were normal, including a total bilirubin level of 0.99 mg/dL, an albumin level of 40.2 g/L, and an indocyanine green retention rate at 15 min of 5.6%. Serum tumor markers were all within normal range.

Multi-detector computed tomography (MDCT) on admission revealed a huge hemangioma, 31.5 cm × 24.8 cm × 11.1 cm, located on the left liver, and replacing the parenchyma of the left liver. His abdomen was distended by the huge hemangioma extending to the pelvis. The non-contrast phase showed a homogenous hypodense lesion contrasted with the surrounding liver parenchyma. On arterial phase images, the lesion remained hypodense relative to normal liver, but early central enhancement was detected. On delayed phase images, the lesion showed progressive fill-in. These findings indicated a giant hemangioma. The left hepatic artery and its branches were extremely stretched, and the left portal vein was compressed and occluded by the tumor. The left hepatic duct was slightly dilated as a result of compression by the hemangioma. The left hepatic vein was completely occluded, while the hepatic vena cava and the middle and right hepatic veins remained patent (Figure 1A-C). The gastrointestinal tract was compressed with no sign of intestinal obstruction. Volumetric analysis revealed a tumor volume of 6503 mL and a right hemiliver volume of 1140 mL. The findings of MDCT were further confirmed by magnetic resonance imaging (Figure 2).

TAE of the left hepatic artery was performed with lipiodol. Thereafter, we planed to calculate and investigate the tumor volume, anatomical positions, and recanalizations by dynamic MDCT once a month, not to misjudge the timing of operation. However, the patient was readmitted to our emergency department one month after TAE, with abdominal pain for two days and clinical characteristics of intestinal obstruction. MDCT on admission revealed the thickening and swelling of the small bowel, dilatation and multiple liquid gas level of the lower part of esophagus, stomach and small bowel, and the colon collapsed. Volumetric analysis revealed that the tumor volume had decreased to 3988 mL (Figure 1D-F). The patient was initially managed conservatively by gastrointestinal decompression and intravenous therapy. Close observation and timely treatment were conducted. The patient's condition improved in 7 d.

The remarkable volume reduction of the tumor allowed for a safe approach to the portal vein and hepatic artery. Through a herringbone incision, a left hepatectomy was safely conducted after the ligation of left hepatic artery, middle hepatic artery and left branch of portal vein. There were extensive adhesions formed between the hemangioma and the jejunum, ileum, sigmoid colon, peritoneum and omentum. No dilated intestinal loops were found. The duration of operation was 280 min and intraoperative blood loss was 400 mL. The resected tumor was 26.5 cm × 19.5 cm × 12.0 cm in size and 3690 g in weight (Figure 3). Histologically, it was diagnosed as a cavernous hemangioma with local subcapsular necrosis, calcification and fibrous tissue proliferation.

The patient's post-operative course was uneventful and he was discharged from the hospital 16 d after surgery. At 6 mo following surgery, he enjoys an improved quality of life with normal liver function.

DISCUSSION

Cavernous hemangiomas of the liver are benign, and usually small (< 4 cm) in size, but when they are larger than 4 cm in diameter, they are classified as giant cavernous hemangiomas[16]. They arise from the mesoderm and are composed of blood-filled cavernous spaces of varying size lined with a single layer of flat endothelial cells, which may be separated by fibrous septa of variable thickness[16,17]. Hemangiomas show specific features in imaging diagnosis; therefore most cases can be diagnosed preoperatively. As a hemangioma increases in size, it can cause congestion, bleeding, thrombosis and infarc-

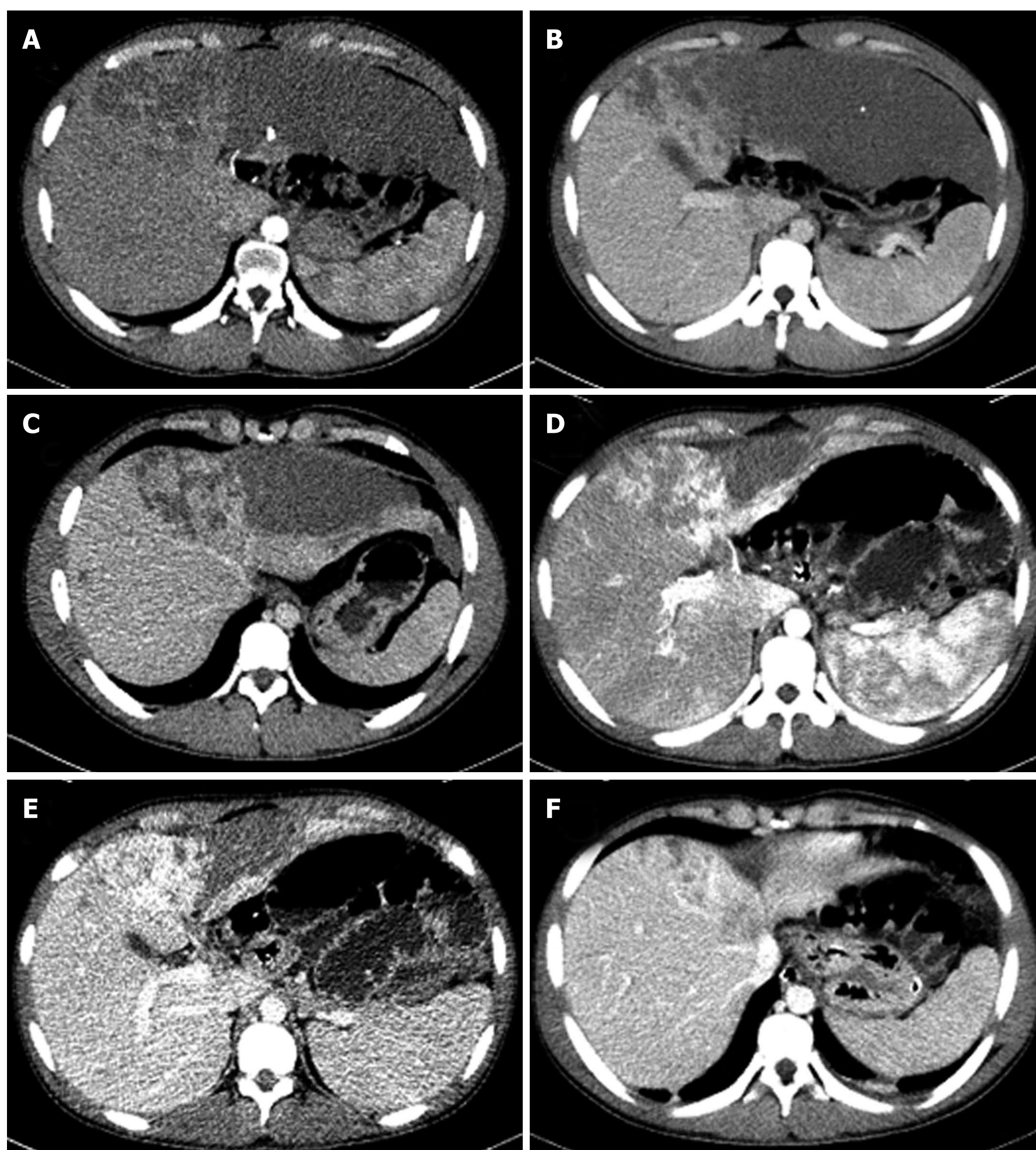

Figure 1 Axial images of multi-detector computed tomography. Multi-detector computed tomography (MDCT) images at the first visit (A-C), and corresponding MDCT slices just before the operation (*i.e.*, one month after transcatheter arterial embolization) (E-G).

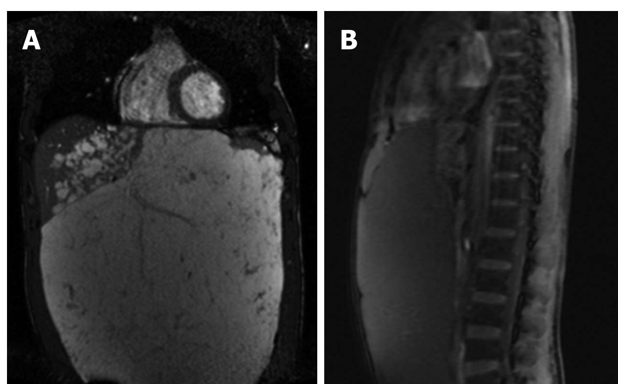

Figure 2 Coronal (A) and sagittal (B) views of the hemangioma from magnetic resonance imaging.

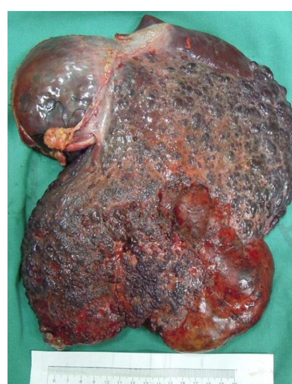

Figure 3 Intra-operative photograph of the tumor.

tion (and consequent stomachache), Kasabach-Merritt syndrome, and spontaneous rupture. Out of the mass effect, it might produce symptoms such as obstructive jaundice, biliary colic, and gastric outlet obstruction[18].

A recent major argument in the treatment of liver hemangiomas is the indications of operation. Considering the benign and non-progressive nature of the dis-

ease, it is currently accepted that a giant hemangioma is not necessarily an indication for surgery just because of its size, and continued observation in asymptomatic patients or patients with minimal abdominal symptoms seems to be justified[19,20]. Surgery remains the only consistently effective curative treatment for giant hemangiomas and should be considered for patients with established complications, diagnostic uncertainty and incapacitating symptoms, where operative risk is acceptable, or where the diagnosis remains uncertain despite appropriate specialist investigation[21]. Various other treatment methods have been reported but their long-term results have been poor[20].

In our case, the patient complained of abdominal pain. For the treatment of a giant hemangioma accompanied with symptoms, surgical resection is primarily recommended. Based on the liver function tests and remnant liver volume, urgent primary tumor resection seemed possible. But we considered that an urgent resection at the first admission would be dangerous because it seemed difficult to approach the bifurcation of hepatic artery and portal vein behind the tumor. Liver transplantation was also considered an option, but the patient strongly refused because of donor shortage and high expenses for transplantation. Although some authors reported that symptomatic giant liver hemangiomas can be managed successfully and non-invasively by TAE with a satisfactory decrease in symptoms and tumor volume[22], the effect of TAE generally seems to be variable and sometimes even results in a volume increase[14,15]. However, there were reports that TAE for giant hemangiomas, performed prior to surgical resection, facilitated the mobilization of the liver by shrinking the hemangioma and, consequently, decreased intraoperative hemorrhage[9,23]. We also performed TAE before surgery to ensure the safety of the future radical resection of the tumor, which resulted in a decrease in the size of hemangioma, as shown in the previous report.

Considering the various complications and vascular recanalization after TAE which might postpone the operation and result in the loss of an opportunity for the radical resection, some authors recommend urgent operation after TAE[9,24]. In the present case, the patient was readmitted to our hospital one month after TAE with intestinal obstruction. To our knowledge, intestinal obstruction after embolization of a giant hepatic hemangioma has not previously been reported in the English literature, we speculated that it might be related to the tumor shrinkage, and inflammatory adhesions formed between the tumor and small bowel. Based on this speculation and the remarkable volume reduction of the tumor, we decided to perform a left hepatectomy. Fortunately, the operation was safely conducted without any complication, and our speculation was confirmed by the laparotomy and histological examination. However, when to operate should be decided on a case-by-case basis, with close follow-up and meticulous assessment by skillful surgeons and radiologists, not to misjudge the appropriate timing for the radical surgery.

We report a case of a single huge hemangioma with intestinal obstruction following TAE treatment, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. The outcome in our case indicates the importance of pre-operative management to reduce tumor size before surgery, and surgeons should pay attention to the complications of TAE.

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Online Submissions: http://www.wjgnet.com/esps/
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World J Gastroenterol 2013 May 21; 19(19): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
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Name of journal
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Launch date
October 1, 1995

Frequency
Weekly

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Production center
Beijing Baishideng BioMed Scientific Co., Limited
Room 903, Building D, Ocean International Center,
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Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2011 Impact Factor: 2.471 (32/74 Gastroenterology and Hepatology).

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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