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REVIEW

Etiology and consequences of thrombosis in abdominal vessels

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Abstract

The thrombophilia which can be either congenital or acquired in adult life has major implications in the abdominal vessels. The resulting portal vein thrombosis, Budd-Chiari syndrome and mesenteric vein thrombosis have a variety of consequences ranging from acute abdomen to chronic hepatomegaly and even totally asymptomatic patient in whom the only finding is pancytopenia. The complications like esophageal varices, portal gastropathy, ascites, severe hypersplenism, liver failure requiring liver transplantation are well known. Interesting features of collateral venous circulation showing itself as pseudocholangiocarcinoma sign and its possible clinical reflection as cholestasis are also known from a long time. The management strategies for these complications of intraabdominal vessel thrombosis are not different from their counterpart which is cirrhotic portal hypertension, but the prognosis is unquestionably better in former cases. In this review we presented and discussed the abdominal venous thrombosis, etiology and the resulting clinical pictures. There are controversial issues both in nomenclature, and management including anticoagulation problems and follow up strategies. In light of the current knowledge, we discussed some controversial issues in literature and presented our experience and our proposals about this group of patients.

Key words: Portal vein thrombosis; Pseudocholangiocarcinoma sign; Thrombophilia; Budd-Chiari syndrome; Mesenteric vein thrombosis

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INTRODUCTION

The thrombosis in the major vessels of abdomen causes a wide spectrum of clinical pictures ranging from a totally asymptomatic patient to a patient with acute abdominal pain and even impending liver failure in patients with underlying chronic liver disease. As liver is the main organ of synthesis of many essential proteins in the body, it bears the burden of thrombosis of major vessels in the abdomen whether this may be a partial or total thrombosis with resulting liver disease, portal hypertension and cirrhosis requiring different management strategies including liver transplantation.

In this clinical setting, the thrombosis of the abdominal vessels has a special place in medicine since the clinicopathological pictures and courses of these processes are usually heterogenous, protean and fluctuant in nature. In general practice physicians pay extra attention to the thrombosis of coronary, pulmonary, mesenteric or cerebral circulation but not to abdominal venous circulation. We believe that the thrombotic occlusion of all major vessels of abdominal cavity has severe clinical consequences and chronic complications.

In this review we will describe thrombophilic conditions and discuss the potential consequences of thrombosis in the major abdominal vessels with potential clinical implications.

THROMBOPHILIA

Normal coagulation hemostasis involves the interaction of an initial vascular reaction (vasoconstriction), thrombocytic activation (white clot formation) and formation of thrombin via activation of coagulation cascade. The balance between the forces favoring formation of a clot and forces against it is the normal state which is controlled with very delicate systems. Although very complicated and several mechanisms play a role in the vascular and thrombocytic steps of clot formation, these steps are beyond the scope of this review and will not be discussed further.

Thrombophilia is a term which is proposed as an opposite term against hemophilia. Thrombophilia can be defined as a disturbed state of the coagulation-fibrinolysis balance in favor of thrombosis formation (congenital or acquired in adult life) in which thrombosis (in arterial and/

or venous vasculature) is observed more frequently than normal population. For the gastroenterologists and surgeons, the congenital and acquired causes of the thrombophilia are important not only due to their potential risks to patients' lives but also their preventability with the advent of genetic tests and surveillance and their treatability with new invasive techniques and new anticoagulant drugs.

Thrombophilia can be grouped under two major headlines: inherited and acquired.

CAUSES OF INHERITED THROMBOPHILIA

Factor V Leiden mutation (FVLM)

The most common cause of inherited thrombophilia is FVLM. This mutation was firstly defined by Bertina *et al*[1] in 1994, after studies of Dahlback and colleagues[2] who showed a resistance factor against activated protein C (APC). The prevalence of this mutation differs between populations throughout the world. The prevalence of this mutation is very heterogenous that some native populations of Africa, America and Asia show no mutation whereas in some districts of Scandinavian peninsula the prevalence rises to 15%. In Turkey, the prevalence is about 8-9% and this prevalence rate decreases going west from Asia Minor towards Europe to 2-4%[3].

Factor V (FV) is synthesized in liver and megakaryocytes which must be activated into form of FVA to play a crucial role in prothrombinase complex along with activated factor X to produce thrombin. The mutation involves a point at the 1691st nucleotide of the exon 10 in first chromosome making G-C change. This results in a change in aminoacid sequences which is depicted as A506G (Arginine at 506 changed by Glutamine). The mutation containing FVA is resistant to degradation by protein C (which is a natural anticoagulant protein) and undegraded factor rises over time increasing the risk of uncontrolled thrombin and therefore thrombus formation. The diagnosis of FVLM depends partially on the activated protein C resistance (APCR) as a screening tool but direct PCR test to detect mutation can also be applied. APCR is a standard test which is widely available in most laboratories but modified APCR has a very high level of sensitivity and specificity and should be applied to patients who are on warfarin treatment, anti-phospholipid syndrome patients, pregnant women, protein S (PS) deficiency and patients with high levels of factor VIII[4].

When compared with normal population, the impact of FVLM on thrombosis formation is a risk about 3-7 times higher in heterozygotes and 50-100 times higher in homozygotes.

Prothrombin mutation (PM)

PM is firstly defined in 1996 by Poort *et al*[5]. The mutation involves a base pair change in the 20210th position (guanosine vs. adenosin) resulting in excessive generation of prothrombin which forms excessive procoagulant accumulation[6]. This mutation has a general population prevalence of about 2% compared to the prevalence increased to 6% in patients who had their first attack of venous thrombosis. Like FVLM, the genetic basis is also important for the prevalence. In African and Asian populations the PM is rare when compared to Caucasians[7].

Protein C (PC) and protein S (PS) deficiency

PC is synthesized in liver by a vitamin K dependent mechanism. During coagulation cascade, this protein is activated by thrombin into its active form, namely activated PC (APC). PC is encoded in chromosome 2 and up to date about 160 mutations have been defined[4,8-10]. The PC activity is usually determined by functional tests. The heterozygote PC deficiency reveals itself by PC activity less than 50%, whereas in homozygote deficiency PC activity is below 5%. The prevalence of PC deficiency is about 0.2% in general population and in the population who had first venous thrombosis attack the prevalence is found to be about 3%[11]. An important factor to point out is that the acquired PC deficiency occurs in patients with liver disease (due to decreased synthetic capacity), oral anticoagulation treatment and acute thrombosis cases (due to utilization).

PS is naturally a cofactor of activated PC during inactivation processes of activated factors V and VIII. This protein is synthesized from liver, endothelial cells, megakaryocytes and in testis by vitamin K dependent reactions. There are many mutations responsible for PS deficiency so that genotyping and analysis for a suspected PS deficiency is not practical. The screening for PS deficiency is functional activity testing. The protein S deficiency is accepted as a weak risk factor for thrombosis formation which is about 2 times more than normal population. The importance of liver disease in assessing these natural anti-coagulant deficiencies is discussed later.

Antithrombin (AT) deficiency

AT is a natural serine protease inhibitor with a very potential controlling functions over coagulation cascade. AT inhibits the steps of coagulation depending on mainly thrombin and less importantly activated factors IX, X, XI, and XII. In the presence of heparin or heparin like molecules the inhibitor function is 1000 times more potent[12]. The prevalence of deficiency of AT in the general population is about 0.02-0.2%, while it accounts for 1% of venous thrombosis etiologies[11,13].

CAUSES OF ACQUIRED THROMBOPHILIA

In young adults, the annual risk of venous thrombosis is 1/10000 for event/person but this figure increases to 1/100 for event/person in patients over 70 years of age[14]. Age is considered as an independent risk factor for venous thrombosis mainly due to inactivity (related with venous congestion), comorbid illnesses related with age and degenerative changes in the vasculature that occur with aging[15].

Malignant disease is also an important cause of thrombosis. Cancer patients have a crude risk of 10-20% to have venous thrombosis in the rest of their lives. The thrombosis related deaths row in the second place in all causes of deaths in this patient population[16-18]. Myeloproliferative disorders (MPD) merit a special discussion in this section. MPD forms a group of special blood disorders which may be termed as half-malignant due to their natural course

Table 1 Relative risks and comparison of common thrombophilic conditions[21,22].

Thrombophilic condition	Relative risk of thrombosis
Inherited	
Heterozygote deficiency of natural anticoagulants (PC, PS, AT)	10-fold
Heterozygote FVLM	5-8 fold
Homozygote FVLM	50-80 fold
Heterozygote PM	2-4 fold
Homozygote PM	10-fold
Acquired	
Oral contraceptive use	4-fold
Surgery	6-fold
Immobilization	11-fold
Anti-phospholipid syndrome	10-fold
Combined	
Oral contraceptive use + Heterozygote FVLM	35-fold

and this group of patients frequently suffer from venous thrombosis. Thrombocytosis and the increased hematocrit which are natural characteristics of this disorders which also cause thrombosis in the venous systems[19].

Other well known acquired thrombophilic conditions are surgery (orthopedic and neurosurgery), drugs (i.e., oral contraceptives, hormone replacement treatment, tamoxifen, L-Asparaginase), antiphospholipid syndrome, pregnancy and Behcet's disease[20].

The relative risks of common thrombophilic conditions[21,22] are shown in Table 1.

PORTAL VENOUS THROMBOSIS: ETIOLOGY, CLINICAL CONSEQUENCES

Portal vein thrombosis (PVT) refers to the total or near-total obstruction of blood flow secondary to thrombus formation. This thrombus may extend towards liver involving intrahepatic portal veins or may extend distally to involve splenic veins or mesenteric veins. On some occasions extensive involvement of all of these vessels may occur with an increased risk of intestinal ischemia. Therefore, the involved segment of portal venous system and the degree of compensatory mechanisms determine the clinical presentation. Although PVT has been observed most commonly in the setting of cirrhosis, discussion presented in the text from this point specifies chronic, non-cirrhotic PVT unless otherwise specified.

PVT has considerable consequences for the liver. Upon cessation of blood flow liver loses about two thirds of its blood supply. Interestingly, while the acute arterial blockage usually results in severe hepatic failure or death, this condition is tolerated well and the patients are almost asymptomatic due to compensatory mechanisms. First compensatory mechanism is the well known "arterial vasodilation" of the hepatic artery (which is a vascular reflex seen in every dual-vessel supplied organ) also observed during portal vein clamping in liver surgery[23]. This "arterial rescue" stabilizes the liver functions at a normal level in the very acute stages of PVT. The second rescue mechanism is the "venous rescue" involving rapid development of collateral vessels to bypass the obstructed segment. These vessels begin to form in a few days after the obstruction and organize into a cavernomatous transformation (Figures 1 and 2) (see later section for detailed discussion) in 3-5 wk[24,25]. We believe that when symptomatic, acute PVT rather be called "acute mesenteric thrombosis" due to extensive co-involvement of the superior mesenteric vein and branches. This condition has very acutely developing deleterious effects over intestinal circulation compromising the patient's life before development of portal hypertension and its consequences.

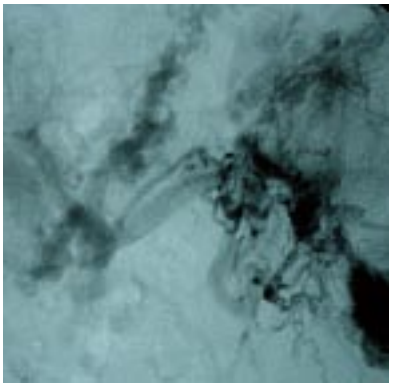

Figure 1 Conventional splenoportography showing a case of portal cavernomatous transformation with portosystemic collaterals, and extensive esophageal-gastric varicose veins.

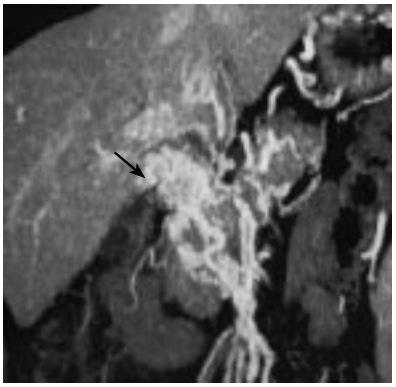

Figure 2 CT-angiography of portal system. Arrow shows the portal cavernomatous transformation with portosystemic collaterals.

Besides these compensatory mechanisms, liver bears the burden of decreased blood to some extent. The decreased portal blood flow stimulates apoptosis in hepatocytes of rats when portal vein is obliterated in a gradual fashion[26] and increases mitotic activity of hepatocytes in the unaffected lobe. The latter effect is well known from selective pre-surgery portal vein obliteration performed in an intention to stimulate the hypertrophy of the opposite lobe of the liver used in cancer surgery. Gradual loss of hepatic mass may be responsible for the occurrence of mild to moderate degree of hepatic synthetic dysfunction observed in advanced stages.

Etiology

PVT has various causes. The design of the studies and the parameters evaluating the cause of PVT have changed in parallel to the evolution in medical and genetic technology. In previous studies between 1979-1997, PVT were mostly attributed to trauma (5-17%), intra-abdominal sepsis (5-36%), umbilical sepsis (5-12%)[27] (important cause of PVT in children), pancreatitis (4-5%) and prothrombotic

Table 2 Role of genetic thrombophilic factors in development of PVT

Thrombophilic factor	Chamourad *et al*[41]	Egesel *et al*[40]	Janssen *et al*[39]	Denninger *et al*[33]	Primignani *et al*[38]
n	10	32	92	36	65
FVLM	10	30	8	2,8	3
PM	40	NS[1]	3	14	22
PC deficiency	NS	26	7	0	0
PS deficiency	NS	43	2	30	2
AT deficiency	NS	26	1	4,5	3

All values except number of patients (*n*) indicate percentage of patients.
[1]NS, not studied.

disorders (2-28 %), but in nearly 50% of the patients etiology remained unidentified[28-32]. But with the advent of better medical care, potent antibiotic treatment, advanced medical technology, discovery of various causes of genetic thrombophilia and with better understanding of the coagulation system, this profile has also changed.

Identification of etiology usually starts by exclusion of local factors such as cirrhosis, primary or metastatic cancer of liver, pylephlebitis, liver cysts, vascular abnormalities (like webs or aneurysms) and pancreatitis. Besides routine investigations such as liver biopsies or Doppler-USG studies, magnetic resonance or CT based (3 dimensional angiographic images also available) techniques are very helpful at this stage. If no local lesion is found, then the studies are directed for a possible thrombophilic condition. After all, if no factor can be found, then the condition should be named as "idiopathic PVT".

In a study by Denninger *et al*[33], the underlying prothrombotic condition was identified in 72 % of PVT patients. There was one or more prothrombotic condition in 26 out of 36 patients studied and primary myeloproliferative disorders were the leading cause with a prevalence of 30 % in this study. Other studies by Valla *et al*[34-36] and De Stefano *et al*[37] report the similar figure that as a cause of PVT, myeloproliferative disorders (overt or occult) rank first in the thrombophilic conditions. Valla *et al* also proposed that overt or latent myeloproliferative disorders may be responsible for thrombophilic milieu for PVT in 48% of patients.

The importance and impact of genetic thrombophilic factors in PVT are investigated in various studies. Table 2 shows the recent and most comprehensive studies about this topic published between 1999-2005. One of the most interesting findings about the results of these studies is the differences between them. Although all of the studies investigated the same patient population, the results are totally different from each other. The explanations for this situation may be the patient selection bias which is the problem of the tertiary care facilities, small number of patients, non-standard evaluation of the same parameters and genetic differences of the patient population.

The investigation of PC, PS and AT deficiencies in patients with PVT is a challenge for the clinician to intepret when the results are found to be lower than normal. There are studies investigating whether the low values are result of liver dysfunction or indicate a frank deficiency. One of the most important studies is performed in children. Mack *et al*[42] studied the coagulation parameters (including factors synthesized exclusively in liver) in 11 children with PVT who underwent a surgical correction (mesentericoleft portal vein bypass: Rex Shunt) of the portal venous system before and after surgery. The investigators found a significant correction of both coagulation factors (factors II, V and VII) indicated also by improvement in prothrombin time and of PC and PS levels after surgery. The same parameters studied in other 2 children receiving distal splenorenal shunt in this study and 7 children in another study receiving H-type mesocaval shunt[43] did not improve as the Rex shunting supporting the importance of lower portal vein flow in the development of secondary PC and PS deficiencies. Fisher *et al*[44] studied the same topic by combining the family investigation of PC and PS deficiencies in patients with PVT. They found out that there were only 3 familial cases of natural anticoagulant deficiencies out of 18 patients indicating a secondary finding. Another support to this finding and the previous results is that they have found significant reductions in AT, PC and PS concentrations after distal splenorenal shunt surgery (3 patients). All of the mentioned studies confirm that the lower portal blood flow has a great impact over the synthesis of natural anticoagulant and coagulant proteins. The shunt procedures directing the blood from portal system towards systemic circulation may worsen this as evidenced from comparison of results of splenorenal shunt vs. Rex shunt. In our opinion this phenomenon of secondary loss of coagulant and anti-coagulant proteins must be considered carefully in clinical practice when anticoagulation with warfarin is considered.

Secondary deficiencies of genetic thrombophilia factors like AT, PC and PS is still a matter of diagnotic challenge. To overcome this problem, there seems to be a very narrow range of solutions such as family study (first degree relatives) of deficient proteins in an attempt to detect familial aggregation of mutant genes and primary genetic studies of the patient to detect the mutant gene(s). These studies are important but not practical and not applicable in every facility. A practical screening method for detection of natural anticoagulant deficiencies in patients with liver disease was proposed by Pabinger *et al*[45] and used firstly by Janssen *et al*[39] in a clinical study which utilizes the ratio of natural anti-coagulant factor to coagulation factors (synthesized exclusively in liver). The formula is the ratio of PS or PC or AT (whichever tested) to [(factor II + factor X) /2]. If the result of this ratio is lower than 70 %, then this may indicate a deficiency of the tested natural anti-coagulant protein significantly disproportionate to decreased synthesis of proteins from liver. This formula is easy to use in clinics and is practical to determine a possible genetic deficiency.

The concept of PVT can not be solely attributed to one factor. It is now widely accepted that the PVT occurs from both a primary thrombophilic milieu and a factor that triggers the formation of the pathological thrombus in portal circulation. The aforementioned factors are currently considered as thrombophilic background rather than the primary etiologic factor according to the concept of multifactorial theory of thrombogenesis. The other co-

factors required to develop a thrombus can be grouped as local and systemic factors most of which are potentially curable or preventable factors.

Clinical consequences

The consequences of the PVT vary from one patient to another and depend on some important factors. The condition may present itself as one of the complications but due to lack of convincing evidence in the literature, the most common type of initial presentation of PVT (not related with cirrhosis) is still not known. In our personal experience, most common type of presentation is variceal bleeding followed by pancytopenia due to hypersplenism. Undefined cholestasis related with pseudocholangiocarcinoma sign (without icterus) is also one of the rare presentations.

With the advent and widely distribution of USG and Doppler-USG, the condition is becoming to be diagnosed very earlier and a patient presenting with ascites (which is a very late finding in course of PVT) is almost not seen. Webb and Sherlock[27] reported in 1979 that out of 97 patients with PVT 13 presented with ascites which seems to be a very high rate (13.5%). There are still some PVT cases presenting as ascites in underdeveloped parts of the world related with absence of early diagnosis with similar rates around 13%[46].

Varices Esophageal and gastric varices related bleeding contribute to the most important cause of morbidity and hospitalization in this group of patients. When the characteristics of variceal bleeding are considered, major differences between cirrhosis *vs* PVT related bleeding must be pointed out. Firstly, the risk of variceal bleeding in cirrhosis is approximately 80-120 times more than PVT (non-cirrhotic) related variceal bleeding[47-49] and esophageal varices (irrespective of size) are observed in about 90% of patients. Gastric varices are mostly seen concomitantly with esophageal varices in about 40% of the patients in PVT while portal gastropathy is also a rare feature of this condition[49, 50]. Compared to portosystemic varices observed in cirrhotic patients, the varices in PVT patients have some special charateristics: (1) "varice-on-varice" finding is less commonly observed; (2) the sizes of the varices are smaller; (3) associated portal gastropathy is less commonly present[50]. The retrospective study (investigated the determinants of survival in a heterogenous group of patients including 124 non-cirrhotic *vs* 48 cirrhotic PVT cases) of Janssen *et al*[47] showed that either the presence of varices or bleeding episodes of varices had no impact on survival rates and the most common cause of death was malignancy related causes. Twenty four percent of the study population had malignancies like hepatocellular cancer, pancreatic cancer, or other malignancy elsewhere metastatic to the liver which presented itself in liver as PVT indicating end stage malignancies.

Although the risk of variceal bleeding is low in non-cirrhotic patients, the management of bleeding varices is not different from cirrhotic patients. Unfortunately there is not any study in the literature comparing endoscopic procedures *vs* surgical procedures in acutely bleeding varices and about the topic of primary prophylaxis. We believe that primary prophylaxis with endoscopic procedures in an effort to clear varices should be applied with careful monitoring and secondary prophylaxis with combination of endoscopic procedures and medical treatment despite beta-blockers may work although there is no objective and sufficient evidence.

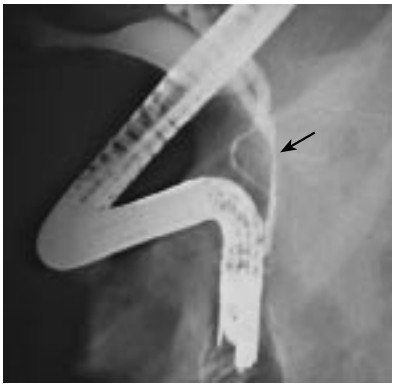

Figure 3 ERCP of a patient with portal cavernomatous transformation. Arrow shows the site of depression in the main bile duct.

In contrast, beta-blockers may result in more sluggish portal flow as is the same issue for nitrates and terlipressin increasing the risk of progression of thrombosis and even worsening of portal hypertension. This decision must be adjusted with the patient's condition rather than a standard medical care.

Pseudocholangiocarcinoma sign One of the interesting and misleading clinical conditions that may occur during the course of the PVT is "pseudocholangiocarcinoma sign" (PSCS). After the obstruction in the portal system, the "venous rescue" begins to occur immediately which takes about 5 wk[24] forming new vessels around intrahepatic, extrahepatic biliary tracts and around gallbladder (majorly, vascular plexus of Saint and Petren enlarge and dilate to become large serpentine vessels) named as "portal cavernomatous transformation". Sometimes these vessels can be very small in caliber to visualize depending on the extent of portal flow and capacity of new vessel formation, but if a careful ERCP is performed (Figure 3) the direct effects of these vessels on biliary ducts or main bile duct as strictures, displacements, thumprinting effects or irregularity can be seen in at least 80% of the patients[49]. This condition mimics a cholangiocellular cancer in ERCP and therefore called PSCS[51, 52].

The clinical impact of these changes results in a wide spectrum of findings ranging from totally normal biochemical and physical findings to overt cholestasis with elevated cholestatic enzymes and liver injury (very rarely observed). Although a term called "portal biliopathy" and a classification system are proposed[53], we believe that this term and classification system are impractical to use because: (1) new collaterals form depending on the site and level of portal vein obstruction, (2) wide range of anatomical variations between human beings in this anatomical site resulting in different types of cavernous transformations, (3) the nature of new vessel formation is unpredictable and not standard in all portal vein thrombosis patients, (4) the classification system does not have impact over treatment or follow up strategy. We propose that only defining the presence or absence of PSCS is a satisfactory practice not to complicate the picture further because the clinically evident cholestasis and/jaundice is observed in only about

5%[54]. Therefore this physiological compensatory response is not a "-pathy" but just an adaptive change with a low degree of clinical importance.

The clinically evident cholestasis either presenting itself as repeating biliary stones or cholangitis or liver injury can be treated with conventional methods including stenting, papillotomy or even surgery.

3Hypersplenism and issue of anticoagulation treatment Hypersplenism is a finding of latent or chronic PVT and this complication is important when anticoagulation treatment is considered. Hypersplenism is almost always present except in the rare patient with a partial thrombus in one branch of the portal vein with the other branch remaining intact presenting with a mild degree of portal hypertension. The levels of all blood elements begin to decrease as the condition worsens and severe hypersplenism is now considered one of the most important indications in this group of patients to undergo a shunt surgery combined with or without a splenectomy. In the clinical setting of hypersplenism with low platelet counts combined with esophageal varices it raises concerns about the safety of anticoagulation. Unfortunately, the literature lacks information about the safety and long-term results of anticoagulation in well designed prospective controlled studies. A study to clarify this controversial topic was a retrospective analysis. In this study Condat *et al*[48] included 136 PVT patients in whom 84 received anticoagulant treatment. The study has some limitations like heterogenous groups (pretreatment endoscopies of all patients and standardization of a homogenous varice distribution between two groups were not accomplished) and lack of information about comorbid conditions of the participating patients, but the study has a low statistical degree of evidence to favor anticoagulation treatment. Anticoagulation was not found to be a risk factor for bleeding in this study while non-anticoagulation resulted in more thrombotic recurrences as expected. In our personal experience it may be a good practice to select patients according to their co-morbid conditions, degree of hypersplenism (low platelet counts may be a relative contraindication for anticoagulation) and the condition of varices (eradication combined with or without medical prophylaxis before start of treatment may be considered to decrease bleeding related risks).

BUDD-CHIARI SYNDROME: ETIOLOGY, CLINICAL CONSEQUENCES

Budd-Chiari syndrome (BCS) is a disorder which (besides its rarity and heterogenous clinical presentations) can potentially result in mortality and severe morbidities including liver failure requiring transplantation and even hepatocellular cancer. Veno-occlusive liver disease (also defined as sinusoidal obstruction syndrome) is a different pathology and therefore not included in this discussion.

There are various classifications of BCS in the literature. Classification systems include; 1) differrences of etiology (primary: related with thrombophilia and secondary: due to tumor or a mass occupying lesion), 2) differences of anatomical involvement (site of obstruction: small and/or large hepatic veins, isolated inferior vena cava involvement, or combination of all), 3) progression of disease (acute/fulminant and chronic/indolent). The forthcoming discussion about BCS involves primary BCS unless otherwise specified.

Etiology

Major underlying mechanism resulting in BCS is thrombosis. The etiology of thrombosis is very similar to PVT as discussed earlier, myeloproliferative disorders are the leading cause in 20-50% of all causes which may be overt or latent in presentation[33,39]. The latent myeloproliferative disorders (for instance; endogenous erythroid colonies) are not easily detectable and requires an expert laboratory and sensitive tests (bone marrow is cultured *in vitro* to observe whether there is spontaneous erythroid colonies formed in the absence of erythropoietin stimulation). The mirror image of relation between myeloproliferative diseases and BCS is also interesting. In an autopsy study by Wanless *et al*[55], the hepatic vein thrombosis was found in 6% of all subjects having a history of myeloproliferative disease. Paroxysmal nocturnal hemoglobinuria is also an acquired thrombophilic disorder characterized by occasional hemoglobinuria, venous thrombosis and anemia. It poses a high risk for development of BCS, about up to 10 % of all paroxysmal nocturnal hemoglobinuria patients have thrombosed hepatic veins[56].

Behcet's disease is a subtype of large vessel vasculitis observed mostly in Turkish population which is related with hepatic vein thrombosis very frequently. Behcet's disease is associated with recurrent oral and genital ulcers with skin findings (erythema nodosum, pathergy reaction), neurologic involvement and eye inflammation. BCS occuring in Behcet's disease must be treated with an appropriate combination of colchicine, thalidomide, penicillins and anti-coagulating agents[20,57,58].

Pregnancy and use of oral contraceptives are also related with BCS but studies in the literature have conflicting results due to study designs and the number of subjects included. The studies will not be discussed in detail but the exact relation of both conditions must be studied with adequate number of patients, defining other concurrent acquired and inherited thrombophilic conditions, estrogen content of the oral contraceptive studied. Nevertheless, both of these conditions have a theoretical risk for BCS.

Inherited causes of thrombophilia like FVLM, AT, PC and PS deficiencies account for a considerable percent age in the list of etiology of BCS. FVLM is found to be associated with 25-30% of all cases[59,60], whereas PC deficiency (accounts for 9-20%) leads in all natural anticoagulant deficiencies[33,39].

Other acquired causes of BCS include oral contraceptive use, pregnancy, Behcet's disease and inflammatory bowel disease.

Clinical consequences

The obstructed hepatic veins present to clinic in variable forms. The most common findings are ascites, hepatomegaly, pain in the abdomen and less frequently jaundice. Although the clinical findings are more florid with drastic consequences in fulminant forms, indolent

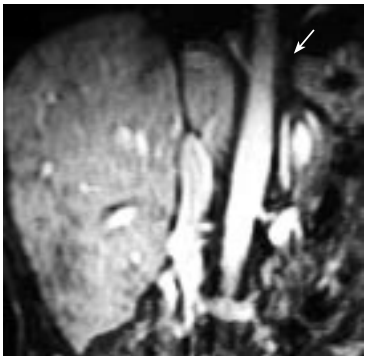

Figure 4 An MRI venography of hepatic veins and inferior vena cava. Arrow shows the site of obstruction in vena cava and unvisible hepatic veins.

BCS is an insidious process in which development of findings mimic chronic liver disease of any other kind. The latter course results in development of portal hypertension and eventually cirrhosis combined with classical complications like variceal bleeding, intractable ascites and hypersplenism. Indolent course of BCS is characterized by pathological changes involving a variety of histological patterns like veno-portal fibrosis, veno-centric cirrhosis and nodular regenerative hyperplasia.

The studies describing the pathological changes unique to liver in chronic BCS have found that 1) the liver has an evolving change in terms of vascular dynamics to eventually result in changes in portal system (either intrahepatic or extrahepatic portal system becomes affected in time which shows itself as micro- or macro-thrombosis), 2) the liver is heterogenous, and there is great variability in random sampling (indicating that the liver biopsy must be multiple in number to clearly define the pathology and histological stage in BCS), 3) the regenerative hyperplasia with nodule formation of changing sizes are common which represents the areas with increased arterial supply with a patent venous drainage[61,62].

Portal venous system must always be kept in mind when clinical presentation and management is considered. Portal system is almost always affected in patients with BCS. This results from various reasons: 1) perfusion of the portal system has a profound decrease since the sinusoidal pressure abruptly increases after initiation of BCS, 2) the enlarged and hypertrophied caudate lobe in response to BCS has a mass effect over main portal branches and intrahepatic portal venules resulting in stasis and vulnerability to thrombosis and further depleting liver perfusion. In patients with chronic BCS, the portal system is found to be involved in nearly 50% of patients when adequately studied either confined to the liver or as PVT.

The diagnosis of BCS can be made by utilizing Doppler-USG with a sensitivity of more than 85% in most cases[63]. We believe that when combined with clinical presentation, Doppler-USG is readily the diagnostic tool of option when compared with CT or MRI techniques. One option that can be applied is directly having a non-invasive venography with CT or MRI (Figure 4) to clear out lesions in inferior vena cava, hepatic veins and portal veins. But this is expensive and not widely available. The portal vein involvement must always be investigated during initial evaluation for further follow-up and management decisions since portal vein thrombosis has been shown to be a poor prognostic finding[64]. The liver biopsy must also be a part of routine evaluation of BCS since it may be related with concomitant liver diseases, but there is no consensus in literature for the definition of an adequate biopsy in BCS. We believe there must be multiple biopsies from both lobes to clearly define, diagnose and estimate prognosis of BCS due to heterogenous nature of liver involvement.

Chronic liver disease eventually develops in nearly all BCS patients depending on the severity of liver involvement, potential of collateral development, co-morbid conditions (especially renal involvement, congestive or ischemic heart disease), thrombosis of other organs, progression of thrombosis in liver. Independent factors predicting survival include Child-Pugh score, age of the patient (younger age predicts better prognosis), renal functions and presence of portal vein involvement.

Management of the patients is directed towards prevention of recurrence of thrombosis and activate the natural thrombolytic potential (anticoagulation and antiplatelet treatment), treatment of complications of liver dysfunction (ascites, infections, renal failure) and relief of hepatic venous obstruction. In this sense, medical treatment has been proved efficacious in patients with an indolent, non-progressive (both histologically and biochemically) course[65,66]. The repeated biopsies (in every 4-5 years) combined with biochemical follow-up usually suffices for this purpose. Doppler USG also must be a part of controls for both hepatic and portal venous system evaluations. The risk of anticoagulation and potential bleeding elsewhere including esophageal varices will not be discussed in detail. But we recommend that anticoagulation must be tailored for every patient according to the presence and stage of varices, history of previous bleeding, presence of thrombophilic condition and presence of portal vein thrombosis.

A failing liver with signs of decompensation indicated by refractory ascites, deterioration of Child-Pugh scores and renal failure preventing adequate medical therapy is definite and urgent indications for the need of relief of hepatic venous obstruction by means of vascular interventions including angioplasty, transjugular intrahepatic portosystemic shunt (TIPS) or surgery. TIPS and hepatic vein angioplasty are cost-effective techniques with a potential risk of recurrence of thrombosis[67,68]. The effectiveness of vascular interventions has decreased the need for a shunt surgery which is rarely performed now. In selected patients with refractory, progressive disease despite adequate medical therapy and vascular interventions liver transplantation should be an option for absolute treatment. Sometimes presence of portal venous thrombosis itself precludes an angiographic intervention and results in need for a liver transplantation.

Liver tansplantation performed for BCS has a five-year survival rate as high as 95%[69]. Complications of transplantation are recurrence of thrombosis and development of secondary malignancies due to long-term immunesuppression. The presence of a myeloproliferative disorder underlying the BCS is not accepted as a contraindication for transplantation[70].

MESENTERIC VEIN THROMBOSIS: ETIOLOGY, CLINICAL CONSEQUENCES

Mesenteric vein thrombosis (MVT) is an acute thrombotic disorder of vessels before formation of main portal vein. Some authors proposed to use the time-dependent definition for MVT, namely acute, subacute and chronic. We believe this classification should be abandoned and the term "MVT" be used for only acute venous obstruction of superior and/or inferior mesenteric veins, whereas chronic MVT itself is already PVT. Subacute thrombosis is an intermediary form of thrombosis in which the patient has abdominal pain and other intestinal symptoms. Therefore, the term MVT is used for acute mesenteric venous thrombosis in the rest of the discussion.

MVT presents itself mostly as a severe abdominal pain located mostly at periumbilical area with a blunt and colicky nature. It may be associated with nausea, vomiting, increased bowel movements and sometimes bloody diarrhea. With progression in time, the clinical picture converts itself into an acute abdomen with findings of rebound tenderness, fever, septicemia and finally a full blown peritonitis which is an inevitable consequence of erroneous diagnosis and treatment. Therefore, early diagnosis and rapid initiation of treatment is mandatory for a good clinical outcome.

Etiology

With the improving diagnostic tests in thrombophilia research, the number of idiopathic cases has decreased to 20 %. We believe this percentage will also decrease in time with advent of new techniques and development of new concepts of research. Currently the most important risk factors are considered as presence of a large spleen, cirrhosis, surgery, abdominal inflammation (pancreatitis, abcess, inflammatory bowel disease, diverticulitis), intraabdominal cancer, thrombophilic conditions (acquired or inherited). The importance of inherited thrombophilia is difficult to evaluate (sparing mutations like FVLM and PM) since the measurements of PC, PS and AT in the acute thrombosis stage and in the anti-coagulation treatment stage of the disease is not useful. The literature is lacking convincing evidence about the importance and prevalence of these disorders in the setting of MVT. One good example for this topic is the study by Kumar *et al*[71]. In this study, the risk factors for MVT (comparing isolated MVT *vs* combined MVT with portosplenic venous thrombosis) are studied and they reported the inherited thrombophilic conditions (PC, PS and AT deficiencies, FVLM) in 27% and 5% of isolated MVT and combined MVT with portosplenic thrombosis, respectively. Interestingly, they found out that the risk of inherited thrombophilia was lower in patients with more extensive vessel involvement. Although the method and criteria of diagnosis of deficiencies of natural anticoagulants are not explained in detail due to retrospective design, this study is important to point the etiology and clinical characteristics of MVT in detail.

Although myeloproliferative disorders play a great role both in PVT and BCS, it does not seem to play the same role in case of MVT. This difference in presentation between MVT and PVT has important clinical implications for further investigations.

Consequences

The mortality rate of MVT is about 20-50%, increasing in parallel to the older age and presence of comorbid clinical conditions (cirrhosis, cardiac failure, etc.). We believe the most important factor to determine the morbidity and mortality is the prompt diagnosis and rapid initiation of treatment. Clinical suspicion has utmost importance in early diagnosis due to the obscure nature of the initial clinical presentation. History of venous thrombosis, older age, use of oral contraceptives, presence of other thrombophilic conditions are indications to rule out a possible MVT. Although the gold standard diagnostic modality is a conventional angiography, CT establishes diagnosis in nearly 90 % of patients[72,73].

After diagnosis the treatment must be initiated with anti-coagulation unless the patient has peritoneal irritation findings in which surgery is indicated. The most extensive analysis including 45 studies (a total of 3692 patients) about survival in mesenteric ischemia was performed by Schoots *et al*[74]. They found that MVT has a better survival (including study populations requiring surgical resection of bowel and conservative treatment groups) compared to arterial or non-thrombotic ischemia of the bowel. This finding seems to be related with a limited involvement of bowel in MVT.

CONCLUSION

Thrombophilia has a wide range of clinical presentations but abdominal organs like liver and intestines bear the most important and interesting sequelae. The consequences of thrombophilia like PVT, BCS and MVT are potentially treatable if only diagnosed early and prompt treatments are initiated. With the advent of new technologies in research of thrombophilia, clinicians will be able to diagnose the idiopathic thrombosis group of patients which is a topic of controversy. Although anticoagulant treatment is mandatory, some limitations and contraindications may prevent their use. Lastly, these group of patients must be followed with a consulting surgeon who has experience in this field to decide the best timing for a shunt surgery and for possible acute operation indications.

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REVIEW

Serum tumor markers for detection of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors and is the second most common cause of cancer death in China. Therefore, it is very important to detect this disease and the recurrence at its earlier period. Serum tumor markers, as the effective method for detecting hepatocellular carcinoma for a long time, could be divided into 4 categories: oncofetal antigens and glycoprotein antigens; enzymes and isoenzymes; genes; and cytokines. Serum alpha fetoprotein (AFP) is the most widely used tumor marker in detecting patients with hepatocellular carcinoma, and has been proven to have capability of prefiguring the prognosis. However, it has been indicated that AFP-L3 and DCP excel AFP in differentiating hepatocellular carcinoma from nonmalignant hepatopathy and detecting small hepatocellular carcinoma. Some tumor markers, such as human cervical cancer oncogene and human telomerase reverse transcriptase mRNA, have also been indicated to have higher accuracies than AFP. Furthermore, some other tumor markers, such as glypican-3, gamma-glutamyl transferase II, alpha-l-fucosidase, transforming growth factor-beta1, tumor-specific growth factor, have been indicated to be available supplementaries to AFP in the detection. AFP mRNA has been shown to correlate with the metastasis and recurrence of HCC, and it may be the most useful marker to prefigure the prognosis. Some other markers, such as gamma-glutamyl transferase mRNA, vascular endothelial growth factor, and interleukin-8, could also be used as available prognostic indicators, and the simultaneous determination of AFP and these markers may detect the recurrence of HCC at its earlier period.

Key words: Hepatocellular carcinoma; Serum tumor markers; Sensitivity; Specificity; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most frequent malignant tumors, is the second common cause of cancer death in China, where its mortality rate is 20.37/100 thousand[1]. Surgical resection is the most effective method for curing this disease, but a large amount of cases are not adapted to surgery because of their intrahepatic or distant metastases at the time of diagnosis. Furthermore, the long-term survival of postoperative HCC patients is unsatisfactory for the high incidence of recurrence. Therefore, it is very important to detect HCC and the recurrence at its earlier period. By reasons of convenience, inexpensiveness, and the satisfactory accuracy, serum tumor markers have been used as an effective method for detecting malignant tumors for a long time, and they could be valuable supplementaries to ultrasonography and computer tomography in the diagnosis of HCC. Using the appropriate single or combination of tumor markers may improve the effectiveness in screening HCC patients.

ONCOFETAL ANTIGENS AND GLYCOPROTEIN ANTIGENS

Alpha-fetoprotein and alpha-fetoprotein-L3

Alpha fetoprotein (AFP) is a fetal specific glycoprotein produced primarily by the fetal liver. Normally, its serum concentration falls rapidly after birth and its synthesis in adult life is repressed. However, greater than 70% of HCC patients have a high serum concentration of AFP because of the tumor excretion. Forty years after its discovery, serum AFP remains a most useful tumor marker in screening HCC patients. The serum concentration of 20 ng/mL is the most commonly used cut-off value to differentiate HCC patients from healthy adults in clinical researches. However, some investigations have showed that the cut-off value is fluctuant in different ethnic groups.

The best cut-off value of AFP has been reported to be 30 ng/mL (sensitivity of 65%, specificity of 89%) in Sicilian population compared with 200 ng/mL (sensitivity of 70%, specificity of 100%) in Burman population[2,3]. One of possible reasons for this difference is the diverse living circumstance which has a great influence on epidemiology. Besides the purpose of screening HCC, serum and tissues AFP could also be used as prognostic indicators[4]. HCC patients with a high AFP concentration ($\geqslant$ 400 ng/mL) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis, and a lower median survival rate[5,6]. This is partially caused by the expression of ephrin-A1 (an angiogenic factor) and the ability of AFP to elicit the escape of carcinoma cells from the host's lymphocytes immune surveillance[7,8]. Though the measurement of AFP serves as an important tool in screening HCC patients, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for its high false-positive and false-negative rates, and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased AFP levels[9]. Using the cut-off value of 20 ng/mL to differentiate HCC from HCV-infected patients, sensitivities merely range from 41% to 65% with specificities of 80% to 94% correspondingly[10]. Moreover, the positive predictive value (PPV) of AFP is significantly lower in detecting HCC patients with viral etiology than that in detecting HCC patients with non-viral etiology (70% *vs* 94%, P <0.05), and it will not reach 100% in HCC patients with viral etiology unless their serum concentration of AFP is greater than 400 ng/mL[2,11]. Therefore, AFP is more useful in detecting HCC patients with non-viral etiology.

Total AFP can be divided into three different glycoforms, namely AFP-L1, AFP-L2 and AFP-L3, according to their binding capability to lectin lens culinaris agglutin (LCA). AFP-L1, as the non-LCA-bound fraction, is the major glycoform of AFP in the serum of nonmalignant hepatopathy patients. On the contrary, AFP-L3, as the LCA-bound fraction, is the major glycoform of AFP in the serum of HCC patients, and it can be detected in approximately 35% of patients with small HCC (< 3 cm), especially when the tumor mass is supplied by the hepatic artery. At the cut-off level of 15%, sensitivities of AFP-L3 in detecting HCC range from 75% to 96.9% with specificities of 90% to 92.0% correspondingly[3,12]. Furthermore, some clinical researches have indicated that the high percentage of AFP-L3 is closely related to poor differentiation and biologically malignant characteristics (especially portal vein invasion) of HCC[12,13], and HCC patients with positive AFP-L3 would have worse liver function, poorer tumor histology, and larger tumor mass[14]. Compared with those with serum concentration of des-gamma-carboxyprothrombin (DCP) over 100 mAU/mL, HCC patients with percentage of serum AFP-L3 over 15% also showed a higher incidence of infiltrative-type HCC with an irregular margin (P<0.05) and a higher frequency of poorly differentiated HCC (P<0.01)[15]. Therefore, it could be used as a valuable indicator of poor prognosis.

Glypican-3

Glypican-3 (GPC3) is a heparan sulfate proteoglycan anchored to the plasma membrane. It has been demonstrated to interact with growth factors and modulate their activities. The expression of GPC3 (at both mRNA and protein levels) in the serum of HCC patients is significantly higher than that in the serum of healthy adults (P<0.001) or patients with nonmalignant hepatopathy (P<0.01), and it can be detected in 40-53% of HCC patients and 33% of HCC patients with seronegative for both AFP and DCP[16-18]. Some clinical researches have indicated that the simultaneous determination of GPC3 and AFP could significantly increase the sensitivity in the diagnosis of HCC[16]. Furthermore, it has been shown that soluble GPC3 (sGPC3), the NH2-terminal portion of GPC3, is superior to AFP in the sensitivity of detecting well or moderately differentiated HCC, and the simultaneous determination of both markers improves overall sensitivity from 50% to 72%[17]. Thus, it can be seen that GPC3 could be a good supplementary to AFP in the detection. Some other investigators have reported that GPC3 mRNA is upregulated significantly in tumor tissues of HCC compared to paraneoplastic tissues of HCC, liver tissues of healthy adults and liver tissues of patients with nonmalignant hepatopathy, thus it could also be a good molecular marker for HCC[16,19-21].

ENZYMES AND ISOENZYMES

Gamma-glutamyl transferase

Serum gamma-glutamyl transferase (GGT) in healthy adults is mainly secreted by hepatic Kupffer cell and endothelial cell of bile duct, and its activity increases obviously in tissues of HCC and fetal liver. Total GGT can be divided into 13 isoenzymes (Ⅰ, Ⅰ', Ⅱ, Ⅱ', β, δ, ε, φA, Ⅶ, B, φC, γA, γB) by using polymeracrylamide gradient gel electrophoresis, and some of them (Ⅰ', Ⅱ, Ⅱ') can only be detected in the serum of HCC patients. Sensitivities of GGTII have been reported to be 74.0% in detecting HCC and 43.8% in detecting small HCC[22]. Furthermore, the simultaneous determination of GGTII, DCP, and AFP can significantly improve the sensitivity over AFP alone[22]. It should be a valuable tumor marker in detecting small HCC and a good supplementary to AFP in the diagnosis of HCC.

Alpha-l-fucosidase

Alpha-l-fucosidase (AFU) is a sort of enzyme to hydrolyze fucose glycosidic linkages of glycoprotein and glycolipids. Its activity increases obviously in the serum of HCC patients (1 418.62 ± 575.76 nmol/mL per hour) compared with that in the serum of healthy adults (504.18 ± 121.88 nmol/mL per hour, P <0.05), patients with cirrhosis (831.25 ± 261.13 nmol/mL per hour), and patients with chronic hepatitis (717.71 ± 205.86 nmol/mL per hour)[23,24]. It has been reported that the sensitivity and specificity of AFU at the cut-off value of 870 nmol/(mL per h) are 81.7% and 70.7%, respectively, in contrast with 39.1% and 99.3% of AFP at the cut-off value of 400 ng/mL, and the

simultaneous determination of both markers can improve the sensitivity to 82.6%[23]. This indicates that AFU could serve as a valuable supplementary to AFP in the detection. Furthermore, it has been indicated that HCC will develop within a few years in 82% of patients with liver cirrhosis, if their serum AFU activity exceeds 700 nmol/(mL per h), and the activity of AFU is already elevated in 85% of patients at least 6 months before the detection of HCC by ultrasonography[24]. Thus, it can be seen that AFU could be a good tumor marker in detecting HCC at the earlier period.

Des-gamma-carboxyprothrombin

DCP, also known as a protein induced by vitamin K absence or antagonist II (PIVKA-II), is an abnormal product from liver carboxylation disturbance during the formation of thrombogen, and acts as an autologous mitogen for HCC cell lines[25]. Its mean serum concentration, which is not correlated to serum levels of AFP, is obviously elevated in HCC patients compared with that in healthy adults and patients with nonmalignant hepatopathy[26,27]. Though a few researches have an opposite result[28], serum and tissues DCP have been proved to be more useful than AFP in differentiating HCC from nonmalignant hepatopathy and in detecting patients with small HCC[6,26,27,29]. Cui *et al*[26] reported that the sensitivity and specificity of serum DCP (at the most commonly used cut-off value of 40 mAU/mL) in discriminating HCC from cirrhosis were 51.7% and 86.7%, respectively, which were much better than those of AFP at the cut-off value of 20 ng/mL, and 36.84% of patients with small HCC had serum DCP values above this level. Marrero *et al* [27] reported that the sensitivity and specificity of serum DCP (at the cut-off value of 125 mAU/mL) in discriminating HCC from nonmalignant hepatopathy were 89% and 86.7%, respectively, which were much better than those of AFP at the cut-off value of 11 ng/mL. Furthermore, the simultaneous determination of DCP and other tumor markers, such as AFP and AFP-L3, may have a greater accuracy than the determination of each of them alone[26,30-32]. It has been reported that the electrochemiluminescence enables measurement of low-concentration of DCP (high-sensitive DCP) in the serum, and the simultaneous determination of high-sensitive DCP (at the cut-off value of 40 mAU/mL), AFP (at the cut -off value of 20 ng/mL), and AFP-L3 (at the cut -off value of 10%) gives the highest accuracy (sensitivity of 82.1%, specificity of 82.4%, and accuracy of 82.2%)[32]. Besides the purpose of screening HCC, serum DCP could also be used as a clinicopathological or prognostic indicator for HCC patients, and may be more useful than AFP in reflecting the invasive characteristics of HCC[28,33,34]. It has been reported that patients with DCP seropositive and AFP seronegative have a higher frequency of HCC with a distinct margin, large nodule more than 3 cm, few nodules, and moderately to poorly differentiation[33,34]. Moreover, the simultaneous determination of serum DCP levels and tissue DCP expression is more valuable than either factor alone in predicting the prognosis of HCC patients[35].

GENES

Alpha-fetoprotein mRNA

Though a few researches have an opposite result[36], HCC cells spread into blood circulation and become the source of recurrence after operation, which may be the primary reason for the unsatisfactory long-term survival after surgery, and the presence of circulating HCC cells may be indicative of metastasis if AFP mRNA is detected in peripheral blood[37]. A large number of clinical researches have indicated that serum AFP mRNA detected by reverse-transcription polymerase chain reaction (RT-PCR) may be a valuable indicator of poor prognosis for HCC patients, and its expression is correlated with portal thrombosis, nodules of tumor, tumor diameter, and TNM stage ($P<0.05$)[38-42]. The recurrence-free interval of HCC patients with postoperative serum AFP mRNA positivity has been reported to be significantly shorter than that of HCC patients with postoperative negativity (53% *vs* 88% at 1 year, 37% *vs* 60% at 2 years, $P=0.014$)[42], and the recurrence-free survival rates of HCC patients with postoperative serum AFP mRNA positivity have been reported to be significantly lower than those of HCC patients with preoperative positivity (52.6% *vs* 81.8% at 1 year, 15.6% *vs* 54.5% at 2 years, and 0% *vs* 29.2% at 3 years, $P<0.001$)[43]. From the result of meta-analysis, the expression of AFP mRNA one week after surgery has also been showed to be correlated with the recurrence of HCC[44]. Moreover, the simultaneous determination of AFP mRNA and melanoma antigen gene (MAGE-1) mRNA may have a higher sensitivity and specificity[41].

Gamma-glutamyl transferase mRNA

GGT mRNA can be detected in the serum and liver tissues of healthy adults or patients with HCC, nonmalignant hepatopathy, hepatic benign tumor, and secondary carcinoma of liver. It can be divided into three types: fetal liver (type A), HepG2 cells (type B), and placenta (type C). Type A is predominant in normal liver tissues or liver tissues with nonmalignant hepatopathy, benign tumor, and secondary carcinoma ($P<0.05$). On the contrary, type B is predominant in cancerous tissues of HCC ($P<0.05$)[45-48]. During the development of HCC, the expression of GGT mRNA in liver tissues may shift from type A to type B[48]. It has been indicated that HCC patients with positive type B would have a worse outcome, earlier recurrence, and more post-recurrence death ($P=0.0107$)[49]. Therefore, the expression of tissues type B may be a valuable indicator of poor prognosis for HCC patients. The same as in liver tissues, the serum levels of type B have also been reported to be significantly higher in HCC patients than in healthy adults ($P<0.05$)[46]. Therefore, serum type B may be an available supplementary to AFP in the diagnosis of HCC.

Human telomerase reverse transcriptase mRNA

Human telomerase reverse transcriptase (hTERT) mRNA has been reported to be detectable in the serum of patients with breast cancer. Furthermore, it has also been demonstrated to be a novel and available marker for HCC diagnosis. The expression of hTERT mRNA in the serum

of HCC patients is significantly higher than that in the serum of healthy adults or patients with nonmalignant hepatopathy[50, 51], and the use of newly developed real-time quantitative reverse transcription polymerase chain reaction may improve the effectiveness of determination[50]. It has been reported that the sensitivity and specificity of hTERT mRNA in detecting HCC are 88.2% and 70.0%, respectively, which excel those of conventional tumor markers, such as AFP mRNA, AFP and DCP[50]. Moreover, it has been indicated that the expression of serum hTERT mRNA, which is associated with the serum concentration of AFP, tumor size, and tumor differentiation degree ($P < 0.001$, each), may be a valuable indicator of poor prognosis for HCC patients[50, 51].

There are some other markers in this category, which could be used as diagnostic or prognostic indicators for HCC. It has been reported that the simultaneous determination of p53 antigen and anti-p53 antibodies has a sensitivity of 41.1% in the diagnosis of HCC[52], and the over-expression of p53 in the serum or liver tissues of HCC patients prefigures the poorer prognosis and a shorter survival time ($P = 0.0014$)[52-56]. HCC patients with positive MAGE-1 or MAGE-3 mRNA die earlier because of metastasis or recurrence[57]. The sensitivity and specificity of serum human cervical cancer oncogene (HCCR) at the cut-off value of 15 μg/mL in detecting HCC are 78.2% and 95.7%, respectively. Moreover, its sensitivities could achieve 76.9% in detecting HCC patients with seronegative for AFP and 69.2% in detecting HCC patients with tumor size less than 2 cm[58].

CYTOKINES

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a secreted homodimeric cytokine that positively regulates tumor neovascularization[59]. Recent researches have suggested that angiogenesis is essential in tumor growth and progression, including that of HCC, which are typically characterized by a high level of vascularization[60-62]. In fact, it has been shown that the expressions of VEGF in cancerous tissues of HCC and HCC with microscopic venous invasion are significantly higher than that in normal liver tissues and HCC without microscopic venous invasion ($P < 0.05$), and HCC patients with over-expression of VEGF have a lower survival rate ($P < 0.05$)[63, 64]. Platelets have been reported to act as transporters of tumor-originated VEGF. It has been indicated that serum VEGF per platelet count, as an indirect theoretical estimate of VEGF in platelets, in HCC patients is significantly higher than that in healthy adults and patients with nonmalignant hepatopathy ($P < 0.01$), and the high serum VEGF per platelet count (>1.4 pg/10^6) is associated with advanced stage of HCC, portal vein thrombosis, poor response to treatment, and shorter overall survival ($P < 0.01$)[65]. Therefore, it may be an available diagnostic or prognostic indicator for HCC.

Interleukin- 8

Interleukin-8 (IL-8) is a multifunctional CXC chemokine that affects human neutrophil functions, including chemotaxis, enzyme release, and expression of surface adhesion molecules. It has direct effects on tumor and vascular endothelial cell proliferation, angiogenesis, and tumor migration. Recent researches have indicated that IL-8 regulates tumor cell growth and metastasis in liver[66]. It has been reported that the preoperative serum IL-8 levels in HCC patients are significantly elevated compared with those in healthy adults (17.6 pg/mL *vs* 1.0 pg/mL, $P = 0.046$), and its high serum levels correlate with a large tumor size (> 5 cm), absence of tumor capsule, presence of venous invasion, advanced pathological tumor-node-metastasis stage, and a poorer disease-free survival[67]. Therefore, it may be an available diagnostic or prognostic indicator for HCC.

Transforming growth factor-beta 1

Transforming growth factor-beta1 (TGF-β1) is a negative growth factor which correlates with cellular immunosuppression during the progression of HCC, and its serum levels in HCC patients have been shown to be obviously elevated compared with those in healthy adults and patients with nonmalignant hepatopathy ($P < 0.0001$)[68,69]. At the cut-off value of 800 pg/mL, the specificity of serum TGF-β1 in detecting HCC has been reported to be over 95% which is similar to AFP at the cut-off value of 200 ng/mL, but the sensitivity of serum TGF-β1 is 68% which excels AFP with the sensitivity of 24%[68]. Moreover, the elevated serum TGF-β1 can be detected in 23% of HCC patients with normal serum AFP values[69]. These researches have indicated that TGF-β1 may be a good supplementary to AFP in the diagnosis of HCC.

Tumor-specific growth factor

Malignant tumor can release tumor-specific growth factor (TSGF), which results in blood capillary amplification surrounding the tumor, into peripheral blood during its growing period. Therefore, the serum levels of TSGF can reflect the existence of tumor. It has been indicated that TSGF can be used as a diagnostic marker in detecting HCC, and its sensitivity can reach 82 % at the cut-off value of 62 U/mL[70]. Furthermore, the simultaneous determination of TSGF and other tumor markers has been shown to give a higher accuracy. It has been reported that the simultaneous determinations of TSGF, AFP, CEA, TSA, and serum ferritin have a sensitivity of 97.5%[70], and the simultaneous determinations of TSGF (at the cut-off value of 65 U/mL), AFP (at the cut-off value of 25 ng/mL) and serum ferritin (at the cut-off value of 240 μg/mL) have a sensitivity of 98.4% and specificity of 99%[71].

There are some other markers, which could be used as diagnostic or prognostic indicators for HCC, in this category. It has been reported that the determination of serum insulin-like growth factor-II (IGF-II) (at the cut-off value of 4.1 mg/g, prealbumin) has a sensitivity of 63%, specificity of 90%, and accuracy of 70% in the diagnosis of small HCC. Moreover, the simultaneous determination of IGF-II and AFP (at the cut-off value of 50 ng/mL) can improve the sensitivity to 80% and accuracy to 88%[72]. The over-expression of granulin-epithelin precursor (GEP) in cancerous tissues of HCC is associated with venous infiltration and early intrahepatic recurrence ($P < 0.05$)[73].

CONCLUSION

Serum AFP is the most widely studied screening test for detecting HCC. The normal range for serum AFP levels is 10-20 ng/mL and a level > 400 ng/mL is usually regarded as diagnostic. Furthermore, some reports have indicated that the high serum concentration of AFP correlates with the poor prognosis of HCC patients. However, two thirds of HCC patients with the nodule less than 4 cm have serum AFP levels less than 200 ng/mL and up to 20% HCC patients do not produce AFP[74]. Moreover, it has limited utility of differentiating HCC from benign hepatic disorders for the high false-positive and false-negative rates. Serum AFP-L3 and DCP are also widely used as tumor markers for HCC, and have been indicated to be more valuable than AFP in differentiating HCC from nonmalignant hepatopathy, detecting small HCC, and predicting the prognosis. Considering the large population with cirrhosis and chronic hepatitis in our country, AFP-L3 and DCP may be more useful than AFP in the diagnosis of HCC. hTERT mRNA and HCCR have been shown to have a higher accuracy than AFP in detecting HCC, but there are not enough researches to manifest their superiority. Therefore, they may not be the first choice in the detection of HCC. IGF-II has been reported to be more valuable than AFP in the diagnosis of small HCC, however, more studies are needed to demonstrate its superiority. There are some serum markers, such as GPC3, GGT II, AFU, TGF-β1, and TSGF, that have been indicated to be available supplementaries to AFP and DCP in the detection of HCC, and some of them even can be detected in HCC patients with seronegative for both AFP and DCP, the simultaneous determination of these markers may improve the accuracy. Serum AFP mRNA, which has been shown to be correlated with the metastasis and recurrence of HCC, may be the most useful marker to prefigure the prognosis of HCC patients. Some other markers, such as p53, MAGE-1, MAGE-3, GGT mRNA, VEGF, GEP, and IL-8, have also been indicated to be able to serve as prognostic indicators of HCC patients, the simultaneous determination of AFP and these markers may discover the recurrence of HCC at earlier period. In addition, there are some tumor markers, such as CYFRA 21-1[75], activin-A[76] and proliferating cell nuclear antigen[54,77,78], which do not belong to each of the categories above, but they can also be used as prognostic or screening indicators for HCC patients, especially when combined with AFP.

In a word, AFP, AFP-L3 and DCP are the most useful serum tumor markers for the detection of HCC, and the simultaneous determination of these markers could improve the accuracy, especially in differentiating HCC from nonmalignant hepatopathy. Other tumor markers, which have been mentioned in our review, could be used as supplementaries to AFP and DCP in the diagnosis of HCC, but each of them has no satisfactory accuracy in detecting HCC or prefiguring the prognosis when used alone.

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GASTRIC CANCER

Prognostic factors in patients with node-negative gastric carcinoma: A comparison with node-positive gastric carcinoma

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Abstract

AIM: To identify the clinicopathological characteristics of lymph node-negative gastric carcinoma, and also to evaluate outcome indicators in the lymph node-negative patients.

METHODS: Of 2848 gastric carcinoma patients, 1524 (53.5%) were lymph node-negative. A statistical analysis was performed using the Cox model to estimate outcome indicators.

RESULTS: There was a significant difference in the recurrence rate between lymph node-negative and lymph node-positive patients (14.4% *vs* 41.0%, $P < 0.001$). The 5-year survival rate was significantly lower in lymph node-positive than in lymph node-negative patients (31.1% *vs* 77.4%, $P < 0.001$). Univariate analysis revealed that the following factors influenced the 5-year survival rate: patient age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis. The Cox proportional hazard regression model revealed that tumor size, serosal invasion, and curability were independent, statistically significant, prognostic indicators of lymph node-negative gastric carcinoma.

CONCLUSION: Lymph node-negative patients have a favorable outcome attributable to high curability, but the patients with relatively large tumors and serosal invasion have a poor prognosis. Curability is one of the most reliable predictors of long-term survival for lymph node-negative gastric carcinoma patients.

Key words: Gastric carcinoma; Survival; Tumor size; Serosal invasion; Curability

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INTRODUCTION

The presence or absence of lymph node metastasis is one of the most important prognostic indicators among several clinicopathological factors that influence the prognosis of patients with gastric carcinoma[1-4]. Several studies have been conducted to identify prognostic indicators in patients with lymph node-negative gastric carcinoma, but these studies have involved small numbers of patients[5-9]. In the present study, we compared lymph node-negative patients with lymph node-positive ones to identify the clinicopathological characteristics of lymph node-negative gastric carcinoma. We also evaluated outcome indicators for lymph node-negative carcinoma.

MATERIALS AND METHODS

Patients and specimens

From 1986 to 2000, 2848 patients with gastric carcinoma were treated in the Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Gwangju, Korea. Of these, 1524 (53.5%) were in the lymph node-negative group.

The clinicopathologic features of these patients with lymph node-negative gastric carcinoma were retrospectively reviewed. Information on the patient's age, sex, tumor size, tumor location, macroscopic appearance, depth of invasion, hepatic metastasis, peritoneal dissemination, stage at the initial diagnosis, operative type, recurrence pattern, curability, and survival rate was obtained from the hospital records. The American Joint Committee on Cancer (AJCC) TNM Staging system was used for clinical and pathologic staging[10]. Histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology[11].

Statistical analysis

The survival rates of the patients were calculated using the Kaplan-Meier method and the relative prognostic

Table 1 Clinical features of patients with node-negative and -positive gastric carcinoma

Variables	Node-negative (*n* = 1524) (%)	Node-positive (*n* = 1324) (%)	*P* value
Age (mean, yr)	56.9±11.1	57.1±11.6	NS
Gender			NS
Male	988 (64.8)	889 (67.1)	
Female	536 (35.2)	435 (32.9)	
Extent of lymph node dissection			NS
<D2	262 (17.2)	246 (18.6)	
⩾D2	1,262 (82.8)	1,078 (81.4)	
Operative type			<0.001
Total gastrectomy	251 (16.5)	383 (28.9)	
Subtotal gastrectomy	1,226 (80.4)	902 (68.1)	
Proximal gastrectomy	12 (0.8)	3 (0.3)	
Others	35 (2.3)	36 (2.7)	
Curability			<0.001
Curative	1,492 (97.9)	1,024 (77.3)	
Non-curative	32 (2.1)	300 (22.7)	
Recurrence			<0.001
Locoregional	29 (13.2)	46 (8.5)	
Peritoneum	163 (74.1)	480 (88.4)	
Others	28 (12.7)	17 (3.1)	

NS, not significant.

Table 2 Histopathologic features of node-negative and -positive gastric carcinoma

Variables	Node-negative (*n* = 1524) (%)	Node-positive (*n* = 1324) (%)	*P* value
Tumor size (mean, cm)	2.9±2.0	5.0±2.7	<0.001
Location			NS
Upper	123 (8.1)	141 (10.7)	
Middle	443 (29.0)	358 (27.0)	
Lower	946 (62.1)	783 (59.1)	
Whole	12 (0.8)	42 (3.2)	
Macroscopic appearance			
EGC			NS
Elevated	161 (20.0)	27 (24.1)	
Depressed	572 (71.2)	69 (61.6)	
Flat	71 (8.8)	16 (14.3)	
Borrmann type			<0.001
1	47 (6.5)	54 (4.5)	
2	183 (25.4)	218 (18.0)	
3	441 (61.3)	812 (67.0)	
4	49 (6.8)	128 (10.5)	
Depth of invasion			<0.001
T1	804 (52.8)	112 (8.5)	
T2	343 (22.5)	170 (12.8)	
T3	338 (22.1)	856 (64.7)	
T4	39 (2.6)	186 (14.0)	
Hepatic metastasis			<0.001
H (-)	1,519 (99.7)	1,269 (95.8)	
H (+)	5 (0.3)	55 (4.2)	
Peritoneal dissemination			<0.001
P (-)	1,509 (99.0)	1,179 (89.0)	
P (+)	15 (1.0)	145 (11.0)	
Histologic type			<0.001
Well-differentiated	355 (23.3)	165 (12.5)	
Moderately differentiated	352 (23.1)	339 (25.6)	
Poorly differentiated	536 (35.1)	642 (48.5)	
Signet ring cell	189 (12.4)	59 (4.5)	
Mucinous	53 (3.5)	95 (7.1)	
Others	39 (2.6)	24 (1.8)	
Stage			<0.001
I	1,145 (75.1)	91 (6.9)	
II	324 (21.3)	124 (9.4)	
III	30 (2.0)	689 (52.0)	
IV	25 (1.6)	420 (31.7)	

NS, not significant

importance of the parameters was investigated using the Cox proportional hazards model. The x^2 was used to evaluate the statistical significance of differences and *P* values less than 0.05 were considered statistically significant.

RESULTS

Table 1 summarizes the clinical findings in 1524 (53.5%) patients with lymph node-negative gastric carcinoma and 1324 (46.5%) patients with lymph node-positive gastric carcinoma. There was no significant difference in the mean age between lymph node-negative and lymph node-positive patients (56.9 *vs* 57.1 years, respectively). Of the 1524 patients with lymph node-negative gastric carcinoma, 988 (64.8%) were male and 536 (35.2%) were female. There were 889 males (67.1%) and 435 females (32.9%) in the group of 1324 lymph node-positive patients. Although there were more males than females in each group, the gender ratio was the same in both groups. Dissection above the D2 lymph node was performed in most patients in each group (82.8% and 81.4% of the lymph node-positive and lymph node-negative patients, respectively). Subtotal gastrectomy was the procedure performed most frequently (80.4%) in patients with lymph node-negative gastric carcinoma. The curative resection rate was significantly higher (97.9%, 1492/1534) in lymph node-negative patients than in node-positive patients (77.3%; 1,024/1,324; $P<0.001$), and the recurrence rate was significantly lower (14.4%) in lymph node-negative than in lymph node-positive patients (41.1%; $P<0.001$). Peritoneal recurrence was the predominant type of recurrence in both groups.

The histopathological features are listed in Table 2. The mean tumor size in patients with lymph node-negative gastric carcinoma (2.9 cm) was significantly smaller than that in lymph node-positive patients (5.0 cm; $P<0.001$). Most gastric carcinomas were located in the lower third of the stomach in both lymph node-negative (946 cases; 62.1%) and lymph node-positive patients (783 cases; 59.1%). There were no significant differences between the groups with respect to the locations of the carcinomas. An invasion depth limited to T2 was found more frequently in patients with lymph node-negative (75.3%) than in lymph node-positive patients (21.3%; $P<0.001$). Hepatic metastases were found in 0.3% of the lymph node-negative patients and in 4.2% of the lymph node-positive patients. Peritoneal dissemination was present in 1.0% of the lymph node-negative patients and in 11.0% of the lymph node-positive patients. There was a significant difference between the groups in the number of cases with

Table 3 Prognostic significance by univariate analysis of variables for patients with lymph node-negative gastric carcinoma

Variables	5-year survival (%)	*P* value
Age (yr)		< 0.01
< 65	79.1	
⩾ 65	70.7	
Gender		NS
Male	74.4	
Female	81.3	
Tumor size (cm)		< 0.001
< 2.0	88.5	
2 - 4.9	79.2	
⩾ 5	53.6	
Depth of invasion		< 0.001
T1, T2	88.4	
T3, T4	55.4	
Location		< 0.001
Upper third	49.9	
Middle third	67.9	
Lower third	62.3	
Histologic type		NS
Differentiated	78.4	
Undifferentiated	75.7	
Operative type		< 0.001
Total	61.5	
Subtotal	80.9	
Extent of lymph node dissection		NS
< D2	67.6	
⩾ D2	77.3	
Stage		< 0.001
I	88.4	
II	61.5	
III	17.9	
IV	22.3	

NS, not significant

Table 4 Multivariate analysis of significant prognostic factors for survival in lymph node negative gastric carcinoma patients using Cox proportional hazard regression model

Variables	Risk ratio	95% CI	*P* value
Age(yr) (< 65 *vs* ⩾65)	1.354	0.95-1.92	NS
Gender (male *vs* female)	0.923	0.66-1.28	NS
Location (upper *vs* distal)	0.731	0.49-1.09	NS
Tumor size (mm) (< 50 *vs* ⩾50)	1.513	1.06-2.21	< 0.05
Histologic type (differentiated vs. undifferentiated)	0.749	0.55-1.02	NS
Serosal invasion (negative *vs* positive)	3.409	2.42-4.80	< 0.001
Extent of lymph node dissection (< D2 *vs* ⩾ D2)	1.188	0.56-2.50	NS
Curability (curative *vs* non-curative)	3.84	2.11-7.00	< 0.001
Esophageal invasion (negative *vs* positive)	1.007	0.34-2.97	NS

CI, confidence interval; NS, not significant.

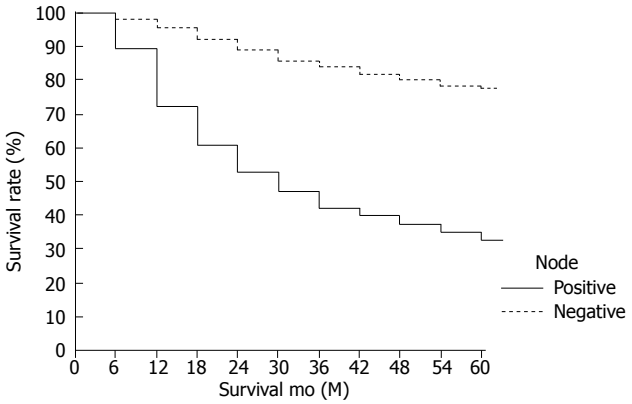

Figure 1 Survival curves of patients with lymph node-negative and node-positive gastric carcinoma (node-negative, 77.4% *vs* node-positive, 31.1%) ($P < 0.001$).

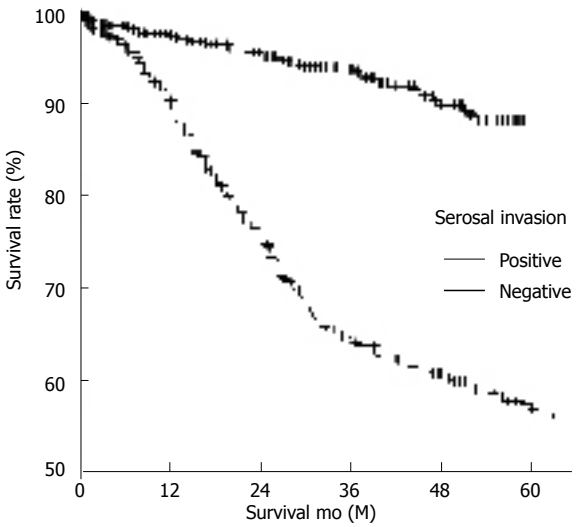

Figure 2 Survival curves of lymph node-negative gastric carcinoma according to serosal invasion (positive, 55.4% *vs* negative, 88.4%) ($P < 0.001$).

hepatic metastases and peritoneal dissemination. Poorly differentiated adenocarcinoma was found more frequently in patients with lymph node-positive gastric carcinoma than in lymph node-negative patients (48.5% *vs* 35.1%, $P<0.001$). Of the lymph node-negative patients, 1470 (96.4%) were classified as either stage I or II at the time of initial diagnosis.

The overall survival rate of the lymph node-negative patients (77.4%) was higher than that of the lymph node-positive patients (31.1%; $P<0.001$) (Figure 1). The 5-year survival rate of patients with early lymph node-negative gastric carcinoma was significantly higher than that of patients with early lymph node-positive gastric carcinoma (93.3% *vs* 84.3%, $P=0.0147$). Patients with advanced lymph node-negative gastric carcinoma also had a significantly higher 5-year survival rate than that of patients with advanced lymph node-positive gastric carcinoma (66.9% *vs* 33.1%, $P<0.001$). The clinicopathological variables tested by univariate analysis are shown in Table 3. The factors that influenced the 5-year survival rate were patient's age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis. Using the Cox proportional hazard regression model, tumor size, presence of serosal invasion, and curability emerged as independent, statistically significant, prognostic indicators(Table 4). The survival curves according to serosal invasion, tumor size, and curability for patients with lymph node-negative gastric carcinoma are presented in Figures 2-4.

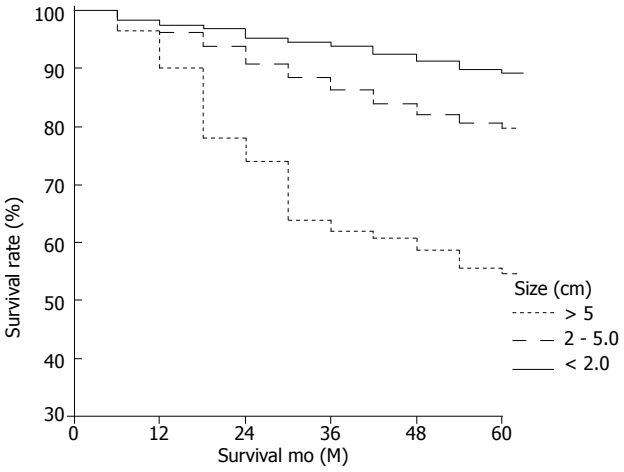

Figure 3 Survival curves of lymph node-negative gastric carcinoma according to tumor size (< 2cm, 88.5% *vs* 2-5 cm, 79.2% *vs* ⩾5 cm, 53.6%) ($P < 0.001$).

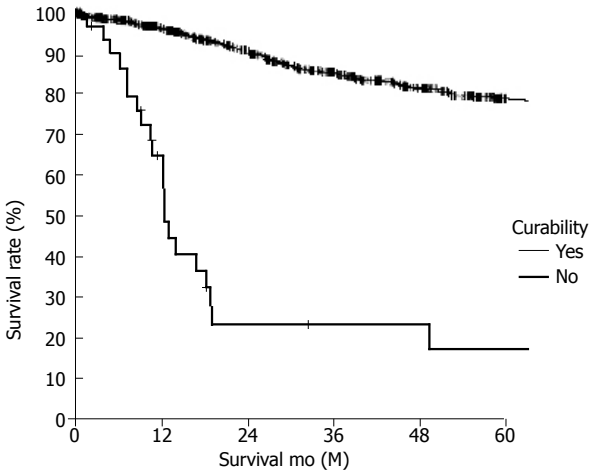

Figure 4 Survival curves of lymph node-negative gastric carcinoma according to curability (curative, 78.9% *vs* non-curative, 17.4%) ($P < 0.001$).

DISCUSSION

In Korea, gastric carcinoma is the leading cause of death as a result of a malignant neoplasm. Although most patients with lymph node-negative gastric carcinoma have a better prognosis than lymph node-positive ones, some lymph node-negative patients have recurrence and poor survival. The identification of factors associated with poor survival in patients with lymph node-negative gastric carcinoma is important. In this study, we compared node-negative and node-positive patients in order to identify the clinicopathological characteristics of lymph node-negative gastric carcinoma. We also evaluated outcome indicators in lymph node-negative patients.

In addition to lymph node invasion, the depth of wall invasion emerged as one of the most important prognostic indicators of lymph node-negative gastric carcinoma[12]. Adachi *et al*[13] reported that the depth of wall invasion provided useful prognostic information in patients with gastric carcinoma. In the present study, serosal invasion also emerged as a statistically significant independent prognostic indicator using the Cox proportional hazard regression model.

Whether age is a prognostic indicator for lymph node-negative gastric carcinoma is controversial[3,14]. Moriguch *et al*[15] reported that age at operation was a significant prognostic indicator in patients with early gastric carcinoma. Similarly, Adachi *et al*[13] reported that patient's age was the second most important prognostic indicator in patients with lymph node-negative gastric carcinoma. Furthermore, some investigators have reported that survival rates were lower in elderly patients with gastric carcinoma[16,17]. Contrary to the aforementioned reports, our study of patients with lymph node-negative gastric carcinoma revealed that age did not affect the survival rate.

Whether tumor size independently correlates with the prognosis for gastric carcinoma is also controversial. In the present study, there was a significant difference in tumor size between lymph node-negative and lymph node-positive patients (2.9cm *vs* 5.0cm), and node-negative patients with large tumors had poor survival. Some investigators have stressed that tumor size is an independent prognostic indicator for gastric carcinoma, whereas others believe that tumor size does not independently influence survival. Adachi *et al*[13] reported that tumor size served as a simple prognostic indicator for gastric carcinoma. By contrast, Yokota *et al*[18] reported that the presence of lymph node metastasis, depth of invasion, and tumor location were more important prognostic indicators than tumor size. Maruyama[2] reported findings that supported the conclusions of Yokota *et al*.

Surgical resection is the only potentially curative modality for localized gastric carcinoma. Adachi *et al*[13] stated that it was reasonable to conclude that the extent of lymph node dissection did not influence the survival of patients without lymph node metastasis. They stressed that the macroscopic diagnosis of lymph node involvement was unreliable and recommended D2 lymph node dissection for curative treatment of node-negative gastric carcinoma. Harrison *et al*[19] also recommended extended lymph node dissection for the improvement of survival of patients with lymph node-negative gastric carcinoma. We concurred with the aforementioned recommendations, and performed dissection above the D2 lymph node in most patients with lymph node-negative gastric carcinoma. In accordance with most reports, curative resection offered the only chance of long-term survival. In our series, this approach allowed us to achieve a high rate of curative resection (97.0%) and favorable outcomes in the lymph node-negative patients.

In this study, 5-year survival rates were different between patients with lymph node-negative and lymph node-positive gastric carcinoma. The 5-year survival rate was significantly lower for lymph node-positive patients than for lymph node-negative ones (35.8% *vs* 79.2%). Bruno *et al*[8] and Harrison *et al*[19] reported overall survival rates of 72% and 79%, respectively, for patients with lymph node-negative gastric carcinoma. Many factors influence the 5-year survival rate. Yokota *et al*[20] reported that tumor size, vascular microinvasion, and the cancer-stromal relationship were the most reliable predictors of 5-year survival for patients with lymph node-negative gastric carcinoma, while Adachi *et al*[21] reported that depth of wall invasion and patient's age were the most important prognostic

indicators. Bruno *et al*[8] found that serosal invasion, residual disease, and poor differentiation were independent prognostic indicators of gastric carcinoma. Our univariate analysis revealed that age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis were prognostic indicators of lymph node-negative gastric carcinoma. In addition, in the present study, the Cox proportional hazard regression model revealed that tumor size, presence of serosal invasion, and curability were prognostic indicators of lymph node-negative gastric carcinoma.

In conclusion, we found that lymph node-negative gastric carcinoma is associated with a favorable outcome. We also found that tumor size, serosal invasion, and curative resection are the most reliable predictors of long-term survival for patients with lymph node-negative gastric carcinoma.

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P120ctn overexpression enhances β-catenin-E-cadherin binding and down regulates expression of survivin and cyclin D1 in BEL-7404 hepatoma cells

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Abstract

AIM: To understand the role of P120ctn in E-cadherin-mediated cell-cell adhesion and signaling as well as in hepatoma cell biological function.

METHODS: We stably overexpressed p120ctn isoform 3A in BEL-7404 human hepatoma cells and studied the effect of p120ctn on β-catenin and E-cadherin binding as well as p120ctn and β-catenin subcellular localization using immunoprecipitation, Western blotting and confocal microscopy. We also investigated the inhibitory effect of p120ctn transfection on the expression of apoptotic protein survivin survivin and cell cycle regulator cyclin D1 in the cells.

RERULTS: Western blotting indicated that p120ctn expression increased after cells were transfected with p120ctn isoform 3A. The protein was located mainly at membrane under immunofluorescent microscope. β-catenin nuclear expression was reduced after overexpression of p120ctn isoform 3A. The p120ctn-E-cadherin binding increased after transfection of p120ctn isoform 3A. Furthermore, overexpression of p120ctn down regulated the expression of apoptotic protein survivin and cell cycle regulator cyclin D1. These effects led to reduction of cell proliferation.

CONCLUSION: Our results indicate that p120ctn plays an important role in regulating the formation of E-cadherin and -catenin complex, cell apoptosis, cell cycle and cancer cell biological function.

Key words: P120ctn; β-catenin; Hepatoma; Cyclin D1; Survivin

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INTRODUCTION

P120ctn is a catenin family member[1,2]. Studies indicated that p120ctn plays an important role in E-cadherin-mediated epithelial cell adhesion and cell signaling[3-6]. Recent studies demonstrated that p120ctn plays a critical role in certain cancerous diseases[7-13]. Nuclear translocation of β-catenin is correlated with tumor progression and has been defined as an oncogene[14]. P120ctn acts as a modulator in E-cadherin-mediated cell-cell adhesion and signaling including β-catenin turnover[11,15]. However, the exact role of p120ctn in cell biological function remains unclear. Tyrosine phosphorylation of p120ctn enhances its nuclear translocation and displays hepatoma cell malignant features[16,17]. In order to further understand the role of p120ctn in E-cadherin-mediated cell biological function and its mechanism, we studied the effects of p120ctn overexpression on β-catenin subcellular localization, regulation of cell cycle and apoptotic proteins as well as cell proliferation in hepatoma cells.

MATERIALS AND METHODS

Cell culture

BEL-7404 human hepatoma cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in Shanghai. Cells were maintained in RPMI 1640 medium (Gibcol BRL, Grand Island, NY, USA) supplemented with 100 mL/L of fetal bovine serum, 10000 U/L of penicillin and 10000 μg/L of streptomycin, in humidified atmosphere containing 50 mL/L of CO_2 at 37 ℃.

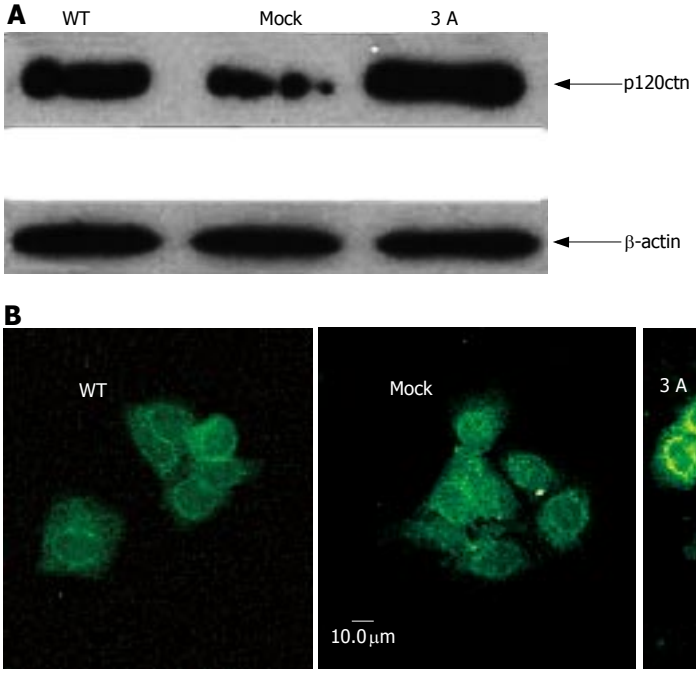

Figure 1 Western blotting (**A**) and confocal microscopy (**B**) showing increased expression of p120ctn and its subcellular localization after cells transfected with p120ctn isoform 3A.

DNA transfection

P120ctn isoform 3A plasmid and empty vector were a generous gift from Dr. Albert B. Reynolds in the department of Cancer Cell Biology, Vanderbilt University, USA. Plasmid DNA was propagated using a conventional protocol. Lipofectamine transfection reagent (Gibcol BRL) was used following the manufacturer's instructions. G418 (Gibcol BRL) was used as a selection antibiotic.

Immunoprecipitation and Western blot

Cells were lysed in lysis buffer containing 40 mmol/L Na_3PO_4 (pH 7.2), 250 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mL/L Triton X-100, 10 mL/L deoxycholate (Sigma-Aldrich, St. Louis, MO, USA)) for 20 min on ice. Cellular debris and nuclei were removed by centrifugation at 13 000 r/min for 15 min at 4 ℃. Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), 1 000 μg of total protein from each sample was immunoprecipitated with 10 μg of anti-E-cadherin monoclonal antibody (BD Pharmingen, San Diego, CA, USA), and 50 μL of protein G agarose (Gibcol BRL) was added and samples were mixed by rotation at 4 ℃ for 1 h. The beads were pelleted and washed four times, then 50 μL of 2×SDS sample buffer was added and boiled for 5 min, and 30 μL of supernatant was loaded to SDS-PAGE gel for protein resolving. Proteins were then transferred to PVDF membrane (Bio-Rad) and incubated with anti-p120ctn (BD Pharmingen), anti-β-catenin (BD Pharmingen) or anti-survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-cyclin D1 (Santa Cruz) for Western blotting at 4 ℃ overnight. Membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h, and illuminated by ECL solution (Amersham Biosciences, Piscataway, NJ, USA). Protein bands were visualized using Kodak X-ray film and processed by Kodak film processor (Kodak, Rochester, NY, USA).

Confocal microscopy

Cells were grown on coverslips coated with poly lysine or laminin. The cover slips were rinsed with PBS, then fixed with 12 g/L of formaldehyde at room temperature for 15 min, followed by blocking with 30 mL/L BSA at room temperature for 30 min, the cover slips then were incubated with primary antibodies for 1 h at room temperature and were washed with TBS-T and incubated with FITC-conjugated secondary antibody for 1 h at room temperature. The cover slips were mounted on slides and examined under a Leica laser confocal microscope.

Cell proliferation assay

A total of 200 000 of different types of cells were seeded into 60 mm dishes and grown for 3 d. Cells were trypsinized and trypan blue exclusion assay was used in cell counting. Experiment was performed in triplicate.

RESULTS

P120ctn expression after transfection

BEL-7404 cells were stably transfected with p120ctn isoform 3A, and the transfection efficiency was determined by Western blotting using mouse anti-p120ctn antibody. The result showed that the expression of p120ctn increased after transfection (Figure 1A). Confocal microscopy result indicated that membranous and cytoplasmic expression of the protein increased mainly at cell-cell contact region (Figure 1B).

Effect of transfection on the binding of p120ctn to E-cadherin

In order to understand the effect of p120ctn overexpression on the binding of p120ctn to E-cadherin, we immunoprecipitated E-cadherin and immunoblotted it with anti-p120ctn. The result showed that the level of p120ctn in E-cadherin-p120ctn complex was increased (Figure 2).

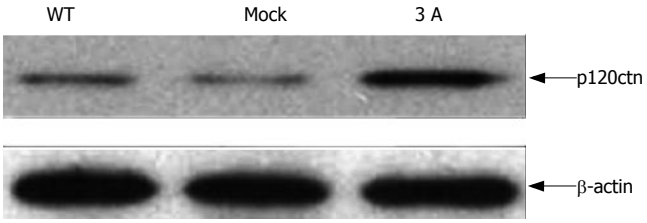

Figure 2 Binding of p120ctn to E-cadherin after cells transfected with p120ctn isoform 3A.

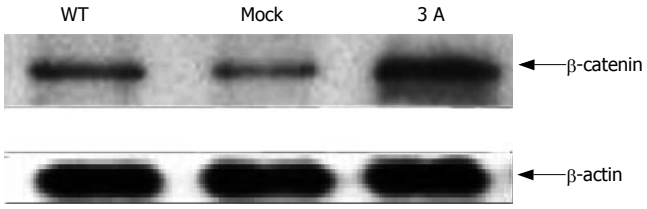

Figure 4 Binding of β-catenin to E-cadherin after cells transfected with p120ctn isoform 3A.

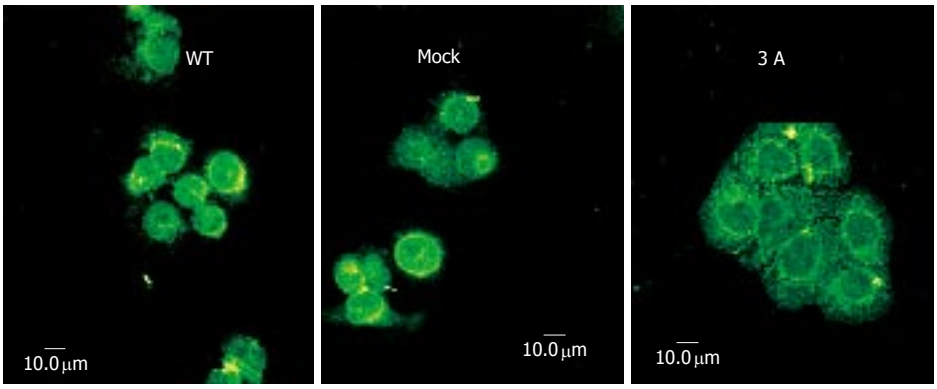

Figure 3 Changes of β-catenin subcellular localization after cells transfected with p120ctn isoform 3A.

Subcellular relocalization of β-catenin after overexpression of p120ctn

In order to understand the relationship between p120ctn and β-catenin in E-cadherin-mediated cell adhesion and signaling, we examined the alteration of β-catenin subcellular localization after overexpression of p120ctn isoform 3A under confocal microscope. Figure 3 shows the changes of β-catenin expression pattern, namely the obvious expression of membranous protein and the reduction of nuclear expression.

Effect of p120ctn overexpression on the binding of β-catenin to E-cadherin

Since overexpression of p120ctn reduced the nucleic but increased membranous β-catenin expression, we immunoprecipitated E-cadherin and immunoblotted it with anti-β-catenin. The result showed that the expression level of β-catenin in E-cadherin and β-catenin complex increased after p120ctn isoform 3A transfection (Figure 4).

P120ctn transfection down regulated survivin expression

Survivin could be highly expressed in cancer but undetectable in nonproliferating normal adult tissues[18-20]. Our Western blot result showed that overexpression of p120ctn reduced survivin expression, in some extent, in the cells (Figure 5A).

P120ctn expression down regulated cyclin D1 expression

We performed Western blotting to detect cyclin D1 expression after cells were transfected with p120ctn isoform 3A. The result indicated that cyclin D1 was down regulated after transfection (Figure 5B).

Effect of p120ctn transfection on cell proliferation

Our above results showed that overexpression of p120ctn could reduce β-catenin nuclear expression, enhance binding of p120ctn and β-catenin to E-cadherin. Moreover, overexpression of p120ctn isoform 3A could down regulate survivin and cyclin D1 expression in cells. Thus, the effect of p120ctn overexpression on the proliferation of BEL-7404 hepatoma cells was examined. Using trypan blue exclusion cell counting method, we found that transfection of p120ctn decreased cell proliferation (Figure 6).

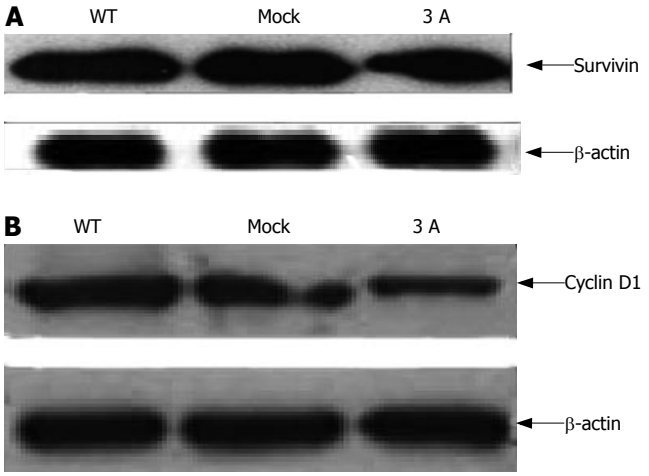

Figure 5 Down regulation of survinin (**A**) and cyclin D1 (**B**) expression after transfection of p120ctn.

DISCUSSION

P120ctn binds to the cytoplasmic tail of E-cadherin in epithelial cells[1,4]. Studies indicated that perturbing p120ctn-E-cadherin binding can lead to nuclear translocation and affect cell biological behavior[21-23],

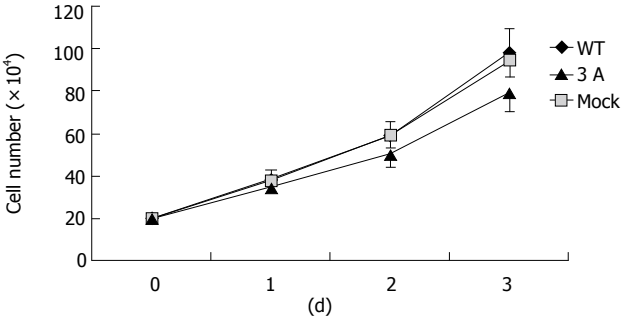

Figure 6 Effect of p120ctn transfection on cell proliferation.

suggesting that p120ctn acts as an oncogenic protein like β-catenin. On the other hand, alterations in E-cadherin and its cytoplasmic regulators, catenins, have been implicated as central to this process, and p120-catenin is frequently altered and/or lost in tumors of the colon, bladder, stomach, breast, prostate, lung, and pancreas[5,11]. Moreover, loss of p120ctn appears to be an early event in tumor progression, possibly preceding loss of E-cadherin, suggesting that p120 plays a role as a "dual modulator" in different cell types[5,7].

In our study, overexpression of p120ctn isoform 3A could enhance its binding to E-cadherin in BEL-7404 human hepatoma cell line, the expression was mainly found at membrane, and cytoplasmic expression could be seen as a minor event. Our results are consistent with a previous report[17], indicating that p120ctn binds to E-cadherin, forming an E-cadherin-p120ctn adhesion complex.

In order to better understand the relationship between p120ctn and β-catenin in E-cadherin-mediated cell-cell adhesion and signaling in liver cancer cells, we examined β-catenin subcellular localization and its binding to E-cadherin after transfection of p120ctn isoform 3A. Our results showed that β-catenin translocation could be seen after transfection of p120ctn. β-catenin became nuclear "exporting", namely its nuclear expression was reduced while membranous and cytoplasmic expression was increased. Transfection of p120ctn also increased β-catenin binding to E-cadherin, demonstrating that p120ctn can recruit β-catenin from nucleus to cytoplasm and strengthen the formation of E-cadherin-catenin complex. Since β-catenin is an oncoprotein, it binds to the TCF/LEF transcription factor and triggers cell cycle progression, thus influencing cell biological function. Our results are consistent with a previous report[22]. We were unable to detect other pathways of the turnover of nuclei-exported β-catenin, such as APC or ubiquitous degradation pathway due to certain study limitations.

It was reported that β-catenin and p120ctn play an important role in cell proliferation, and TCF/beta-catenin signaling participates in regulation of survivin transcription in colon cancer[23,24]. Monoclonal anti-Wnt-2 antibody induces melanoma cell apoptosis by inactivating survivin[25]. Pizem *et al*[26] reported that the expression of survivin is associated with aberrant activation of the WNT (wingless) pathway in medulloblastoma patients. Our results indicated that overexpression of p120ctn could down regulate survivin expression, which could be explained by the fact that survivin is reduced due to the recruitment of β-catenin to E-cadherin-β-catenin complex and the free portion of β-catenin could be degradated by APC and ubiquitization after p120ctn overexpression, leading to decrease of nuclear β-catenin and transcription factor inactivation.

β-catenin plays an important role in cell biological function[27,28]. It was reported that cyclin D1 transcription involves Wnt signaling in gastric cancer cell line[29]. In skeletal myocytes, beta-catenin overexpression increases proliferation and cyclin D1 expression while decreases apoptosis and induces hypertrophy[30]. It has been shown that in HeLa and squamous cells, differentiation-inducing factor-1 inhibits tumor cell proliferation and reduces the expression of cyclin D1 mRNA and the amount of beta-catenin[31], suggesting that there is a close correlation between β-catenin and cyclin D1 in tumor cell biology. In our study, transfection of p120ctn isoform 3A could reduce cyclin D1 expression, resulting in the decrease of hepatoma cell proliferation.

In conclusion, overexpression of p120ctn in hepatoma cells can recruit β-catenin from nucleus to membrane and cytoplasm, enhanced E-cadherin-catenin adhesion complex, down regulated apoptosis related protein survivin and cell cycle related protein cyclin D1 expression, and finally, inhibited cell proliferation.

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COLORECTAL CANCER

Pedigree and genetic analysis of a novel mutation carrier patient suffering from hereditary nonpolyposis colorectal cancer

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Abstract

AIM: To screen a suspected Hungarian HNPCC family to find specific mutations and to evaluate their effect on the presentation of the disease.

METHODS: The family was identified by applying the Amsterdam and Bethesda Criteria. Immunohistochemistry was performed, and DNA samples isolated from tumor tissue were evaluated for microsatellite instability. The identification of possible mutations was carried out by sequencing the hMLH1 and hMSH2 genes.

RESULTS: Two different mutations were observed in the index patient and in his family members. The first mutation was located in exon 7, codon 422 of hMSH2, and caused a change from Glu to STOP codon. No other report of such a mutation has been published, as far as we could find in the international databases. The second mutation was found in exon 3 codon 127 of the hMSH2 gene, resulting in Asp→Ser substitution. The second mutation was already published, as a non-pathogenic allelic variation.

CONCLUSION: The pedigree analysis suggested that the newly detected nonsense mutation in exon 7 of the hMSH2 gene might be responsible for the development of colon cancers. In family members where the exon 7 mutation is not coupled with this missense mutation, colon cancer appears after the age of 40. The association of these two mutations seems to decrease the age of manifestation of the disease into the early thirties.

Key words: Hereditary nonpolyposis colon cancer; Bethesda criteria; Mutation; hMLH1; hMSH2

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INTRODUCTION

According to published data, about 15-20 % of colorectal carcinomas (CRC) follow a familial pattern. Familial adenomatous polyposis coli syndrome (FAP) and its subtypes are responsible for only about 1 % of all CRCs, while hereditary nonpolyposis colon cancer (HNPCC) accounts for approximately 3-8 % of cases[1-3]. The characteristic presentation of HNPCC is frequently right-sided localization, the presence of synchronous and metachronous CRCs, and its association with other HNPCC-related extracolonic tumors, including gastric, endometrial, and urinary and biliary tract cancers in afflicted families[2,3]. Compared to sporadic colorectal carcinomas, HNPCC has an earlier age of onset, Crohn's disease-like lymphocytic infiltration in tumor tissues, increased mucin production, and a lower degree of histological differentiation[3-5]. The classic adenoma-carcinoma sequence can be observed in HNPCC patients as well, but the conversion of polyps to malignant proliferation is accelerated, taking only 1-3 years, as opposed to sporadic adenomas, where this can take as long as 10-15 years[1]. The number of polyps in the colon is not so high as in cases of FAP. The genetic background of HNPCC has been identified in the germline defect of DNA mismatch repair genes (MMR). The syndrome is in-

herited in an autosomal dominant fashion, with an estimated penetrance of 80%[1-3,6]. At present, seven MMR genes are known (hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1, hPMS2); however, the influence of a specific mutation on the pathogenesis of the disease shows great diversity[7-10]. In about 90-95% of the cases, germline mutations of the hMLH1 or hMSH2 genes can be demonstrated, while only a low percentage is caused by the mutations of hMSH6, hPMS1 and hPMS2[1]. The detection of afflicted families must be initiated by taking a thorough family history, embracing at least three generations, applying the widely accepted Amsterdam and Bethesda Criteria[6]. If the diagnosis of HNPCC is further supported by the immunohistology of the MMR proteins, the microsatellite status must be determined. The hallmark of mismatch repair deficiency is microsatellite instability (MSI), variability of the lengths of short repetitive DNA stretches scattered in the genome (microsatellites) compared to normal tissue DNA[11-14]. Finally, sequencing of the MMR genes can accurately identify mutations and makes screening of other family members possible[6]. It is emphasized in the literature that only around 40% of the families identified by the Amsterdam Criteria will actually present detectable mutations. This fact influences both therapeutic plans and the required long-term follow-up[3,13,15,16].

In this study, our goal was to perform the genetic analysis of a young male patient and the members of his family who had been selected by the Amsterdam Criteria, and to identify the genetic alterations in the MMR genes hMLH1 and hMSH2. We also attempted to establish a correlation between the occurrence of mutations and disease pattern.

MATERIALS AND METHODS

Patient and surgery

A 32-year-old male patient (index person) with recurrent hematochesia was received at an outpatient clinic in North-Eastern Hungary. Colonoscopy was performed and a friable, bleeding lesion was found at the lienal flexure of the large bowel with circular narrowing of the lumen. The histopathological report of the biopsy showed a mucinous adenocarcinoma infiltrating the muscularis propria, pT2, Grade 2. Ultrasound and computer tomography ruled out evidence of liver metastases, enlarged lymph nodes in the mesocolon or spread to neighboring organs. Since the disease was localized, a left hemicolectomy was performed with an end-to-end transverso-rectostomy. The family and the index person were evaluated using the Amsterdam and Bethesda Criteria[2]. Based on the family history, the possible diagnosis of HNPCC was suspected and, following discussions about the disease, the patient and his family agreed to further genetic evaluation.

Immunohistochemistry

Routine 5 μm, paraffin-embedded tissue sections were dewaxed, rehydrated, and treated in a microwave oven in 10 mmol/L citrate buffer (pH 6.4) for 20 min, in order to retrieve antigenecity. Unspecific protein binding was blocked with 1% bovine serum albumin containing PBS for 30 min at 37 ℃, then slides were incubated overnight with the primary antibodies (clone 2 MSH01, mouse monoclonal anti-MSH2, 1:100 and clone M074581 mouse monoclonal anti-MLH1, Labvision Corp., Fermont, CA, USA), respectively. Primary antibodies were detected by a biotin-streptavidine detection kit (LSAB, Dako, Carpinteria, CA, USA) using VIP chromogen. Negative controls were stained with the omission of the primary antibodies.

DNA extraction

DNA was extracted from a paraffin-embedded cancerous tissue sample of the index patient following deparaffinization and proteinase K digestion, according to the protocol of the High Pure PCR Template Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA samples of the patient and family members were also isolated from whole blood using the Wizard DNA Purification System (Promega Corporation, Madison WI, USA) according to Promega's Technical Manual (TM050).

Microsatellite analysis

DNA samples isolated from the tumor tissue were tested for microsatellite instability. Two mononucleotide markers (BAT25 and BAT26) were evaluated using the technique described by Dietmaier *et al*[17]. A multiplex PCR was performed using sequence specific hybridization probes (HyProbes) labeled with LightCycler dyes, LCRed640 and LCRed705. Amplification of microsatellites by LightCycler (Roche Diagnostics GmbH, Germany) was followed by melting point analysis to display alterations in the length of repetitive sequences.

Heteroduplex and single strand conformation polymorphism (SSCP) analysis

All exons of hMLH1 and hMSH2 genes were analyzed in the blood sample of the index patient. Primers and PCR conditions were used as previously described[18]. Primers are available upon request. After denaturation or heteroduplex formation, the PCR products were loaded to electrophoresis on MDE gel (Cambrex Bio Science Rockland Inc., Rockland, MA, USA) according to manufacturer's instructions and visualized by silver staining.

Sequencing of hMSH2 and hMLH1 genes

PCR products showing altered migration patterns on MDE gel were purified and sequenced in both directions. Sequencing reactions were performed using a BigDye thermocycler sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA). The semi-automated fluorescence analysis was performed with an ABI-PRISM 310 Genetic Analyzer (Perkin Elmer, Boston, MA, USA).

RESULTS

After a comprehensive evaluation of three generations, the index person's family was found to fulfill several points of the Amsterdam and Bethesda Criteria. In the maternal line of the pedigree, seven persons carry the exon 7 mutation. In this line, four cases of colorectal cancer and one case of breast cancer were recognized spanning three generations among first degree relatives. Analyzing the history of the paternal line, pulmonary carcinoma and gastric carcinoma

Table 1 Details of the pedigree analysis

Classification	Degree of relationship	7 exon mutation	3 exon mutation	Type of the tumor, age at tumor detection	Age at investigation
I/1	Sister of the mother of the index person's mother			Breast cancer, 61 yr	Died
I/2	Mother of the index person's mother				76 yr
I/3	Brother of the father of the index person's mother	+		CRC, 56 yr	68 yr
I/4	Sister of the father of the index person's mother	+			61 yr
I/5	Husband of the person No I/4		+		64 yr
I/6	Mother of the index person's father			Gastric cancer, 55 yr	Died
I/7	Father of the index person's father			Lung cancer, 75 yr	Died
II/1	Index person's mother	+		CRC, 43 yr	54 yr
II/2	Index person's father		+		58 yr
II/3	Sister of index person's father		+		48 yr
II/4	The middle son of person No I/4	+	+	CRC, 34 yr	36 yr
II/5	The youngest son of person No I/4	+			32 yr
II/6	The oldest son of person No I/4				39 yr
III/1	Index person	+	+	CRC, 31 yr	33 yr
III/2	Index person's younger brother	+	+		28 yr
IV/1	Older son of the index person				3 yr
IV/2	Younger son of the index person				1 yr

CRC:colorectal carcinoma.

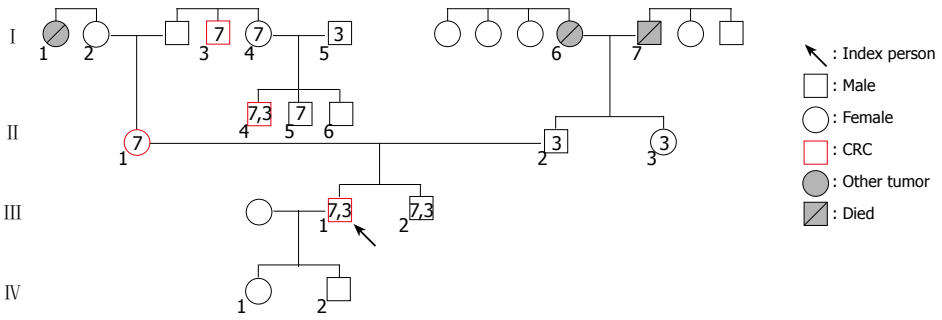

Figure 1 Pedigree of the index person.

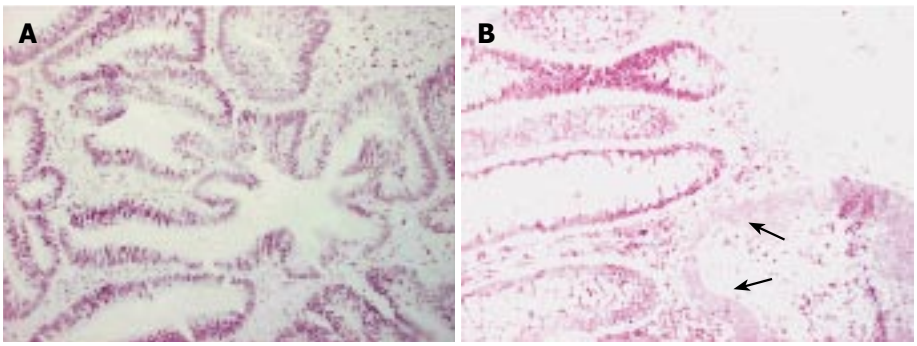

Figure 2 Immunostaining for hMLH1 and hMSH2. Nuclei of tumor and normal colon mucosa cells show reactivity for hMLH1 (left), whereas hMSH2 staining is missing from the tumor cells (right, arrows). LSAB, original magnification, x200.

(one case each) were detected (Figure 1 and Table 1). We found two generations (I./5 and II./2) in the maternal line, where exon 3 mutations join from two different families. In both cases of mutation associations, the tumor manifestation could be observed at a younger age than in the other family members, who carried the exon 7 mutation only. Numbers in icons mean the location of hMSH2 exon mutations. Numbers below icons identify the investigated family members. The younger brother of the index person (III./2.) was 28 years old at the time of the investigation and was disease-free.

Immunohistochemistry of the index person's tumor tissue showed a complete loss of hMSH2 reactivity. The hMLH1 staining was retained in the nuclei of the tumor cells (Figure 2). Furthermore, this tumor tissue was also proved to be MSI-High, as was demonstrated by melting point analysis of both mononucleotide markers, BAT25 and BAT 26 (Figure 3).

Sequencing of all the exons of hMSH2 and hMLH1 genes from the same tumor tissue showed two mutations of the hMSH2 gene. We were not able to demonstrate any alterations in the hMLH1 gene. The first mutation was a

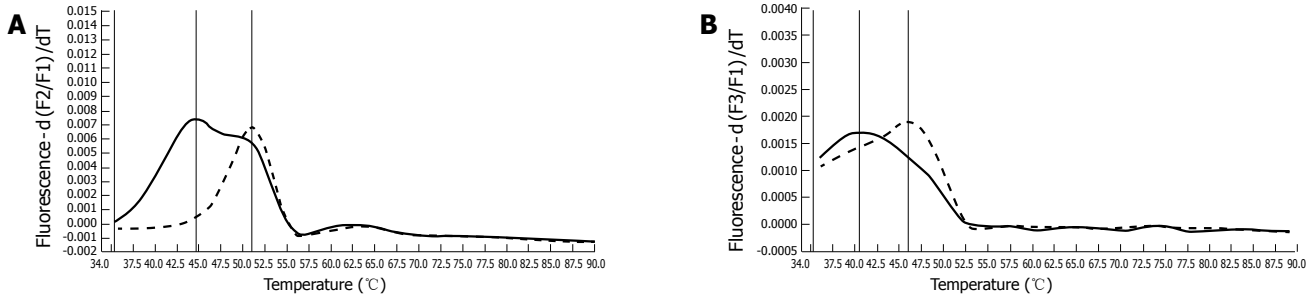

Figure 3 LightCycler Tm estimation of Bat26 (**A**) and Bat25 (**B**) microsatellite markers. Dotted lines represent MSS control. Tm is 51℃ for Bat26 and 46℃ for Bat25. The solid lines show decreased Tm values corresponding to the MSI-H status of tumor tissue. Tm is 44.6℃ for Bat26 and 40.5℃ for Bat25 respectively.

Table 2 Type and location of detected mutations

Gene	Exon	Codon	Mutation	Type
hMSH2	7	422	gaa→taa Glu→STOP	Nonsense
hMSH2	3	127	aat→agt Asn→Ser	Missense

nonsense mutation, located in exon 7, codon 422 causing a glutamine→STOP codon change (Table 2). The second one was a missense mutation, located in exon 3, codon 127 resulting in substitution of Asp for Ser.

Targeted sequencing of these two identified mutations was performed on DNA samples isolated from the peripheral blood leukocytes of fourteen other family members. On the paternal side, the missense mutation of exon 3 was identified in the index patient's younger brother and his father, as well as in the sister of the father. Interestingly, the exon 3 mutation was also found in two members of the maternal line. The nonsense mutation of exon 7 was found in seven members of the maternal side and four of these subjects have been diagnosed as CRC (Table 1).

DISCUSSION

A characteristic feature of HNPCC is a tendency to develop metachronous tumors in affected individuals. In a 15-year follow-up study, Jarvinen and coworkers demonstrated convincingly that recurrence of CRC can be eliminated effectively by regular interval colonoscopies and polypectomies. Out of the 133 individuals who underwent regular interval follow-up examinations, 18% developed colon cancer, but no patients died. Whereas, among the 119 patients who deferred colonoscopy at three-year intervals, 41% were diagnosed as CRC and 9 deaths occurred[19]. Since the correct identification of HNPCC families is still an unsolved issue, considerable effort is dedicated to the task of uncovering further details of the disease. The most widely applied initial screening tools are the Amsterdam and Bethesda Criteria[2,3,6]. Unfortunately, neither of them is accurate enough to identify all HNPCC families with complete certainty. The most reliable, currently available technique is the demonstration of mutations in the MMR genes of suspected index persons, followed by a comprehensive search for the specific mutation in their family members. A promising way to reach an effective screening tool is to find and describe the nature and frequency of population specific mutations and to test these first in patients from a particular geographic location. Currently, more than 300 different MMR gene mutations have been published from different parts of the world. Some of these are recurrent, and have been described as founder mutations in particular populations[20-24] .

Our group, applying the previously suggested protocol, initiated an investigation in Hungary. A 31-year-old male patient was selected by the Bethesda criteria and genetic testing identified him as having HNPCC. A double mutation of the hMSH2 gene was found and subsequently compared with the available international databases (The Human Gene Mutation Database, Cardiff, International Society for Gastrointestinal Hereditary Tumors). The nonsense mutation of exon 7 has not been published before; therefore, it may potentially be characteristic in Hungarian families. The genetic error clearly appears to be pathogenic, since all the family members with CRC carry this mutation. The newly identified mutation causes development of a STOP codon, leading to a non-functional, truncated protein, which could not be detected by immunohistochemistry. The human MSH2 protein interacts with MSH6 forming the complex called hMutSα and with hMSH3 protein forming the complex named hMutSβ. Each subunit of these heterodimers is formed by five flexible domains which are defined as I. mismatch bindig, II. connector, III. core, IV. DNA clamp, and V. ATPase domains. The mismatch binding and DNA clamp domains act in binding to mismatched DNA whereas the core and connector domains constitute the backbone of each subunit. The latter two transmit allosteric information of bound DNA co-factor to the ATPase domain. Our detected mutation at codon 422 causing Glutamine→STOP change results in the loss of more than half of the expressed MSH2 protein (truncated by 513 aminoacids from 934), involving core, DNA clamp and ATPase domains. This nonfunctional truncated protein cannot take a part in forming normal heterodimers and disintegrated quite soon after expression. To the best of the authors' knowledge there was only one publication in Hungary of a novel hMSH2 germline mutation causing truncated protein expression. Czakó *et al.* investigated a 62 year old female patient who suffered from colon cancer at the age of 46, rectum cancer at the age of 60, endometrial cancer at the age of 56 years, basosquamous and squamous cell cancer of the face at the ages of 53, 54, 62, and 58 years respectively. This patient's family fulfilled

the Amsterdam criteria. The DNA sequencing analysis revealed a mutation as a single nucleotide change at codon 2292 in exon 14 of the hMSH2 gene. This guanin to adenin change altered the 764 amino acid, the tryptophan to STOP codon. Thus the hMSH2 protein was truncated by 171 aminoacids[25].

The missense mutation of exon 3 has already been described by several authors as a single amino acid change, and represents a non-pathogenic polymorphism of the gene. Samowitz *et al* published data showing 3 cases of this polymorphism in 1066 screened subjects. One of the 3 affected carriers was also found to have another missense mutation (exon 6, codon 328, Ala→Pro change, of hMSH2 gene), and in another carrier it was coupled with a pathogenic frameshift mutation of the hMLH1 gene (exon 16, codon 626).[26] De la Chapelle *et al* published another interesting double mutation, where this polymorphism was associated with an MSH2, exon 6, codon 333 mutation (International Society for Gastrointestinal Hereditary Tumors). The allele frequency of this polymorphism, according to the database of The National Institute of Environmental Health Sciences Genome Project, is 0.02, which would lead to as much as a 4% prevalence of heterozygous carriers in the general population.

Both mutations identified in this study are single nucleotide changes of the hMSH2 gene. Large genomic deletions and/or rearrangements, on the other hand, can account for 36% of all hMSH2 mutations according to the Dutch HNPCC mutation analysis study.[27] Unlike the hMSH2 mutations, the majority of hMLH1 mutations are single nucleotide changes, but interestingly, a 3.5 kb deletion encompassing exon 16 of hMLH1 has been observed in Finland, as a founder mutation[23].

The presence of the exon 7 mutation alone resulted in a tumor manifestation at an age of 43 and 56 years (Table 1). When the missense and nonsense mutations were inherited together, the age of manifestation shifted to a younger age of 31 and 34 years.

It is well known that the penetrance of the MMR genes is less then 100%, which might explain why two of the older family members carrying the pathogenic mutation have not acquired the disease yet. Young age may answer the same question in the case of the younger brother of the index person, who was 28 years old at the time of the investigation (Table 1). On the other hand, all these family members must be considered "high-risk" patients, and be placed under close regular interval surveillance. To date, three family members carrying only the exon 3 mutation are disease-free. Three other members of the family, who suffered from breast, gastric and pulmonary cancers, respectively, could not be investigated because of their deaths before the initiation of the study.

Both the paternal and maternal lines of the evaluated family in this study came from the same small village in Hungary, which may explain the high prevalence of the exon 3 mutation of MSH2 in the tested individuals. It will be interesting to see whether the exon 7 mutation is truly characteristic for Hungarian families, or whether this mutation can be found in different populations elsewhere.

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VIRAL HEPATITIS

Distinct toll-like receptor expression in monocytes and T cells in chronic HCV infection

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Abstract

AIM: Hepatitis C virus often establishes chronic infections. Recent studies suggest that viral and bacterial infections are more common in HCV-infected patients compared to controls. Pathogens are recognized by Toll-like receptors (TLRs) to shape adaptive and innate immune responses.

METHODS: In this study, to assess the ability of HCV-infected host to recognize invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells by real-time PCR.

RESULTS: We determined that RNA levels for TLRs 2, 6. 7, 8, 9 and 10 mRNA levels were upregulated in both monocytes and T cells in HCV-infected patients compared to controls. TLR4 was only upregulated in T lymphocytes, while TLR5 was selectively increased in monocytes of HCV-infected patients. MD-2, a TLR4 co-receptor, was increased in patients' monocytes and T cells while CD14 and MyD88 were increased only in monocytes.

CONCLUSION: Our data reveal novel details on TLR expression that likely relates to innate recognition of pathogens and immune defense in HCV-infected individuals.

Key words: Hepatitis C virus; Toll-like receptors; T cells; Monocytes

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INTRODUCTION

Hepatitis C virus (HCV) infection is the cause of chronic hepatitis in more than 4 million people in the USA[1]. Patients with chronic HCV infection experience a various severity of liver diseases ranging from mild hepatitis to cirrhosis and hepatocellular carcinoma. Liver cirrhosis that appears decades after the initial infection often predisposes to spontaneous bacterial peritonitis and sepsis[2]. HCV is the most frequent indication for liver transplantation, and transplant recipients suffer from recurrent HCV and infections associated with immunosuppression[3]. HCV, a blood borne pathogen, is frequently associated with human immunodeficiency virus (HIV) infection acquired through the same route of invasion[4]. According to clinical data, HCV is not only the cause of morbidity due to liver disease, but it is also associated with other infections. A recent study by El-Sarag *et al*[5] suggested that infections with other viral and bacterial pathogens were more common in HCV-infected patients who otherwise had no immunocompromise compared to non-HCV infected controls. The pathogens causing infections at a higher rate in HCV patients included cytomegalovirus, cryptococcus, Mycobacterium tuberculosis and those causing sexually transmitted diseases[5]. It remains to be evaluated whether recognition of pathogens and/or mounting appropriate immune responses is impaired in HCV infection.

Bacterial and viral pathogens are recognized as "danger" signals by the family of Toll-like receptors (TLRs) that alert innate immunity against the invading pathogens[6]. The known 11 mammalian TLRs have distinct extracellular domains but share common intracellular mechanisms of cell activation that culminate in production of inflammatory cytokines. Each TLR has specific ligands, which allow the host to sense a wide diversity of pathogens. TLR1, TLR6 and possibly TLR10 form heterodimers with TLR2 to recognize fungi (Sarcomyces cereviciea, Candida albicans, and Aspergillus fumigatus), parasites (Trypanosoma cruzi), Gram positive bacteria-derived peptidoglycan (PGN) and bacterial lipoproteins, lipoarabinomannan from *Mycobacterium tuberculosis*, Treponema pallidum-derived glycolipid

Table 1 Patient characteristics

Parameter		Value
Gender (male/female)		12/2
Age (yr)		44 ± 12
Duration of disease	>10 yr	5
	Unknown	9
Viral load (by QUNT BDNA, IU/ml)		$1 \times 10^7 \pm 0.78 \times 10^7$
Viral genotype (by PCR)	1a	5
	1b	4
	3a	3
	4a	2
Plasma SGOT (ALT) levels (IU/mL)		110 ± 86
Plasma SGPT (AST) levels (IU/mL)		102 ±60

Table 2 Real-time PCR primers

Primer identification		Sequence
TLR1	Forward	5'-GGG TCA GCT GGA CTT CAG AG-3'
	Reverse	5'-AAA ATC CAA ATG CAG GAA CG-3'
TLR2	Forward	5'-GCC TCT CCA AGG AAG AAT CC-3'
	Reverse	5'-TCC TGT TGT TGG ACA GGT CA-3'
TLR3	Forward	5'-GTG CCA GAA ACT TCC CAT GT-3'
	Reverse	5'-TCC AGC TGA ACC TGA GTT CC-3'
TLR4	Forward	5'-AAG CCG AAA GGT GAT TGT TG-3'
	Reverse	5'-CTG AGC AGG GTC TTC TCC AC-3'
TLR5	Forward	5'-TTG CAT CCA GAT GCT TTT CA-3'
	Reverse	5'-TTC AAC TTC CCA AAT GAA GGA-3'
TLR6	Forward	5'-GAA CAT GAT TCT GCC TGG GT-3'
	Reverse	5'-GCT GTT CTG TGG AAT GGG TT-3'
TLR7	Forward	5'-AAT GTC ACA GCC GTC CCT AC-3'
	Reverse	5'-GCG CAT CAA AAG CAT TTA CA-3'
TLR8	Forward	5'-TGT GAT GGT GGT GTC TCA AT-3'
	Reverse	5'-ATG CCC CAG AGG CTA TTT CT-3'
TLR9	Forward	5'-ATT CTG ACT TTG CCC ACC TG-3'
	Reverse	5'-GCT GAG GGA CAG GGA TAT GA-3'
TLR10	Forward	5'-GGC CAG AAA CTG TGG TCA AT-3'
	Reverse	5'-AAA TGA CTG CAT CCA GGG AG-3'
MyD88	Forward	5'-GAG CGT TTC GAT GCC TTC AT-3'
	Reverse	5'-CGG ATC ATC TCC TGC ACA AA-3'
MD2	Forward	5'-ATT CCA AGG AGA GAT TTA AAG CAA TT-3'
	Reverse	5'-CAG ATC CTC GGC AAA TAA CTT CTT-3'
CD14	Forward	5'-CGC TCC GAG ATG CAT GTG-3'
	Reverse	5'-TTG GCT GGC AGT CCT TTA GG-3'

and lipoteichoic acid[6,7]. TLR2 can also recognize certain viruses such as cytomegalovirus, measles virus and core and NS3 proteins of HCV[8-10]. TLR3 recognizes poly(I-C) and double-stranded viral RNA, whereas TLR4 agonists include Gram-negative bacterial LPS, respiratory syncytial virus, Cryptococcus neoformans and the plant product Taxol[6,7,11]. Bacterial flagellin has been identified as a TLR5 ligand, synthetic components imiquimod and resiquimod 848 and single stranded RNA stimulate TLR 7 and 8, while unmethylated CpG-containing DNA and herpes simplex virus have been identified as TLR9 agonists[6,7,12].

The presence of TLRs was originally discovered in innate immune cells[13]. Monocytes express all TLRs of which TLRs 1, 2, and 4 are present at high levels[14]. Latest research, however, indicates that TLR expression is not restricted to innate immune cells and TLRs can be detected in adaptive immune cells as well as in parenchymal cells. In particular, T cells express all TLRs at low levels with the exception of TLR5, which is present abundantly[14, 15].

In order to dissect the ability of HCV-infected host to respond to invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells. We determined that TLR2, 7, 8, 9 and 10 were upregulated in both immune compartments in HCV-infected patients compared to controls. In addition, TLR4 was upregulated in lymphocytes and TLR6 was upregulated in monocytes only. Our data reveal new insights into immune defense of HCV-infected individuals.

MATERIALS AND METHODS

Blood donors

Healthy individuals (controls, $n = 14$) and treatment-naïve patients chronically infected with hepatitis C virus (HCV patients, $n = 14$) were enrolled in the study. All individuals were free of acute infections. The study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School and informed consent was obtained. The clinical characteristics of the patients are detailed in Table 1. The blood was collected from cubital vein with anti-coagulant (heparin sodium) and processed immediately. Controls and patients were matched for age and gender where possible.

Cells

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll gradient. In order to separate monocytes from lymphocytes, the PBMCs were plated (10^7/well in 2 mL of RPMI1640 media supplemented with 10 % FBS) in 6 well plates (Corning, Corning, NY) and incubated at 37 ℃ in 50 ml/L CO2 atmosphere for 3 h. Monocytes were separated based on their adherence to plastic and the purity of the population, based on CD14 expression, was greater than 95% as determined by flow cytometry (data not shown). Non-adherent cells were washed and T lymphocytes were purified using T cell negative isolation kit (Dynal Biotech Inc, Lake Success, NY), as manufacturer recommended. Briefly, non-adherent cells were incubated with a cocktail of antibodies for CD14, CD16a, CD16b, CD56, HLA class II DR/DP and CD235A, followed by incubation with depletion magnetic Dynabeads. The non-T cells bound to the Dynabeads and were separated in a strong magnetic field while T cells were washed and subjected to RNA extraction.

Real-time PCR

Total cellular RNA was extracted using RNeasy kits (Qiagen, Valencia, CA), according to manufacturer's recommendations. All samples were co-processed to eliminate technical variations. Equal amounts of RNA from controls and HCV-infected patients were analyzed. Reverse transcription of 1 μg of RNA into cDNA was performed using reverse transcription System (Promega, Madison, WI). The real-time PCR primers were synthesized by IDT (Coralville, Iowa), except for 18S (Quantum RNA Classic II 18S Internal Standard, Ambion, Austin, TX). The real-time PCR was performed using the ICycler (Biorad Laboratories, Hercules, CA). The PCR primers are described in Table 2. The reaction mixture contained 12.5 μL qPCR

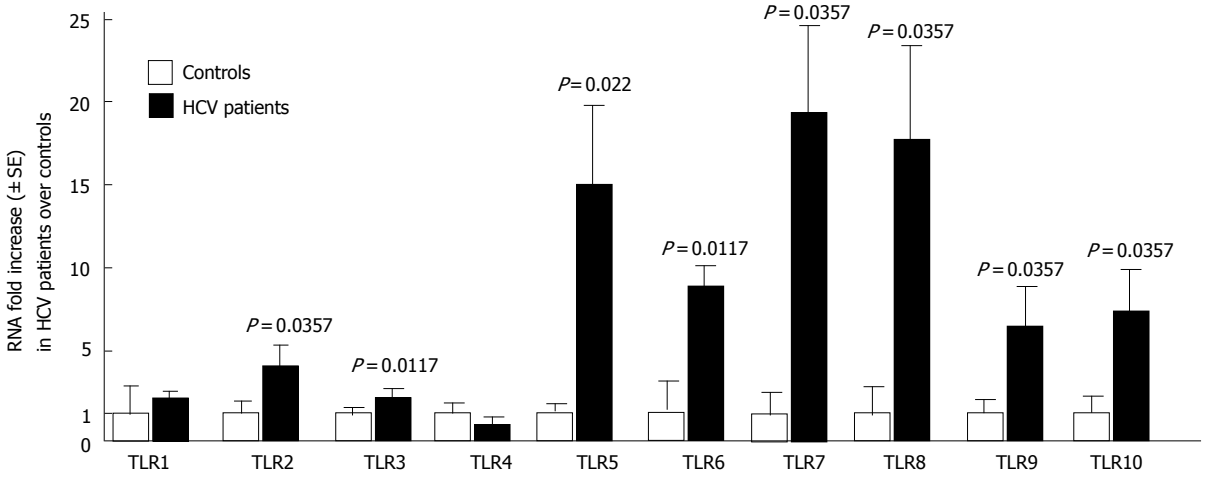

Figure 1 Higher TLRs 2, 5, 6, 7, 8, 9 and 10 RNA expression by monocytes of HCV-infected patients compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for TLRs 1-10 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR-coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each TLR in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

MasterMix Plus with 2 x SYBR Green (Eurogentec, Seraing, Belgium), 0.5 μmol/L of forward and reverse primer and 1 μL of cDNA (corresponding to 50 ng of RNA) in a total volume of 25 μL. All amplifications and detections were carried out in a BioRad optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by dsDNA-binding SYBR Green. After the PCR was performed, a dissociation/melting curve was constructed in the range of 55°C to 95°C. Data were analyzed using the Biorad ICycler software. As a first step in data analysis 18S was used for normalization of all experiments (normalizer) and it was closely compared between controls and HCV patients. For second step in data analysis we used comparative Ct method (ΔΔCt method) with the following formula: ΔCt = Ct (target, TLR) - Ct (normalizer, 18S). The comparative ΔΔCt calculation involved finding the difference between ΔCt of HCV patient and the mean value of the ΔCt from normals for each analyzed molecule. Fold increase in the expression of specific mRNA in HCV patients compared to normal controls was calculated as $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

The non-parametric Wilcoxon test from StatView on a G4 Machintosh platform (Cuppertino, CA) was employed to determine the statistical difference between controls and HCV patients.

RESULTS

HCV-infected patient selection and their characteristics

Our study population included patients with chronic HCV infection but without cirrhosis or cancer, thus, HCV infection-specific imprints on the immune system were present but cirrhosis or cancer-related non-specific immune alterations could be excluded. All patients in the study had viremia and elevated levels of liver transaminases consistent with chronic hepatitis. Patients infected with different viral genotypes (1A, 1B, 3A and 4A) were included, as viral genotype could affect host immune response[16]. Our patients had never been treated with anti-viral therapy, thus possible influence of therapy on TLR expression was eliminated. This is important because Ribavirin was shown to augment signaling in TLR-transfected cells[17].

Monocytes of HCV-infected patients express higher levels of TLRs 2, 3, 5, 6, 7, 8, 9 and 10 RNA compared to controls

Chronic HCV infection is associated with increased serum levels of inflammatory cytokines[18]. Blood monocytes and tissue macrophages are major sources of these cytokines. We recently reported increased monocyte production of TNFα in patients with chronic HCV infection[10]. In this study, we used real-time PCR to evaluate TLR expression in monocytes of HCV-infected patients and healthy controls. Monocytes both from controls and HCV-infected patients expressed TLRs 1-10 (Figure 1), as expected based on previous studies[14]. We found that monocytes from patients with chronic HCV infection expressed significantly higher levels of RNA coding for TLR2, TLR5, TLR6 and TLR10. Different TLRs reside at different locations in the cell. TLRs 1, 2, 4, 5, 6 and 10 are expressed mostly on the surface of the cellular membrane, while TLRs 3, 7, 8 and 9 are localized inside the cells[6,7]. Surprisingly, we found that not only some outer cellular membrane-associated TLRs, but also the intracellularly localized TLRs, TLRs 7, 8 and 9, were upregulated in HCV patients' monocytes. The level of TLR3 mRNA was also significantly higher in HCV-infected patients compared to controls.

Ligand recognition and signaling of TLR2 and TLR4 is augmented by co-receptors[6,7]. Thus, we further analyzed the expression of TLR co-receptors and adaptors in both controls and HCV-infected patients. We found that the mRNA level of CD14, a co-receptor for TLR2 and TLR4, was upregulated in HCV patients compared to controls (Figure 2). In addition to CD14, MD-2 is another accessory protein of the TLR4 that is necessary

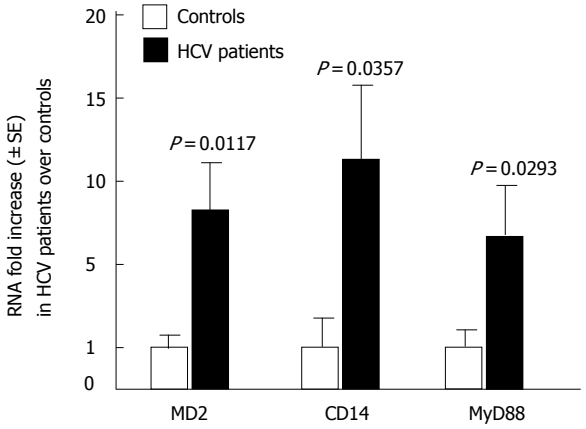

Figure 2 Monocytes of HCV-infected patients express higher levels of TLR co-receptors MD2 and CD14, and TLR adaptor MyD88 compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for MD2, CD14, MyD88 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual protein of interest-coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each protein of interest in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

for assembling the TLR4-containing receptor complex to sense low quantities of lipopolysaccharide[19]. We found that MD2 mRNA was expressed at significantly higher levels in monocytes of HCV infected patients compared to controls. The mRNA for MyD88, an intracellular adaptor molecule that is recruited to the intracellular domain of TLRs 2, 4, and 9 upon engagement with specific ligands, was also higher in patients compared to controls. These results suggest that increased RNA expression of various TLRs and their co-receptors may predispose monocytes to increased TLR-mediated activation in chronic HCV.

Lymphocytes of HCV-infected patients express higher levels of TLRs compared to controls

We next investigated the expression of TLRs in CD3+ T cells of the adaptive immune cells. T cells from controls and HCV infected patients expressed mRNA for TLRs1-10 (Figure 3) as expected based on previous publications[14,15]. There was no CD14 mRNA expressed in T lymphocytes of either controls or HCV-infected patients, confirming the purity of the cell population (data not shown). We found that levels of RNA coding for membrane-localized TLR1, 2, 4, 6 and 10 as well as the TLR4 co-receptor, MD2, were significantly higher in HCV infected patients compared to controls. No differences in levels of RNA coding for MyD88 or TLR3 and TLR5 (Figure 3) between controls and patients were identified. In contrast, the RNA levels of intracellularly localized TLRs 7, 8 and 9 were significantly higher in T lymphocytes of HCV infected patients compared to controls.

DISCUSSION

Monocytes and lymphocytes, respectively, are representative populations of innate and adaptive immunity and their cooperation is required to recognize, limit and eliminate invading microbes[20]. Here we show that patients infected with HCV have a unique pattern of TLR expression in both the innate and adaptive immune compartments and have elevated RNA expression of both membrane-associated and intracellularly localized TLRs. Recent reports demonstrate that HCV-infected patients have a higher prevalence of infections with cytomegalovirus, Cryptococcus, Mycobacterium tuberculosis and sexually transmitted diseases compared to controls[5]. Recognition of these pathogens requires TLR-mediated signals. For example, sensing of Cryptococcus by the innate immune system requires TLR2, TLR4, MyD88 and CD14[1], while TLR2 and TLR4 are instrumental in the host's response against Mycobacteria[21]. The pathogens implicated in sexually-transmitted diseases, found more often in HCV-infected patients, are also recognized by TLR. Chlamydia elicits an unusual set of inflammatory responses via TLR2 and TLR4 *in vivo*, and TLR2 is essential for development of oviduct pathology in chlamydial genital tract infection[22], while Neisseria gonorrhoeae stimulates cytokine release and NF-κB activation in epithelial cells in a TLR2-dependent manner[23]. In addition, some TLRs may play a protective or even therapeutic role in defense against sexual disease pathogens: TLR3 agonists protect against genital herpes infection[24] and Imiquimod, a TLR7&8 ligand and the first FDA-approved imidazoquinoline, has been approved for the therapy of genital warts[25]. Increased expression of TLR2 and TLR4 is a bad prognostic factor in patients with sepsis[26], while low TLR expression may protect the host against excessive inflammation and tissue damages[27], thus, differential expression of TLRs in HCV infected patients may contribute to susceptibility to infections as well as to the underlying disease progression and this remains to be determined.

The observation of increased expression of TLR2 in patients was not totally unexpected, since chronic HCV infection is associated with elevated levels of circulating LPS, which upregulates the expression of TLR2 in different cell types, including monocytes[1,21,22]. We have previously shown that HCV core and non-structural protein 3 (NS3), activate cells through TLR2[10]. We found elevated levels of the TLR co-receptors, CD14 and MD2, and adaptor molecule MyD88, in HCV-infected patients compared to controls, suggesting that MyD88-mediated TLR signal transduction may also be affected in patients. Indeed, we have previously found that monocytes from HCV infected patients are hyper-responsive to stimulation with TLR4 and TLR2 ligands[10,31]. Although we did not find a role for CD14 in TLR2-mediated cell activation by HCV-derived core and NS3 proteins (unpublished data), increased CD14 expression may be relevant to cell activation mediated by other TLR2 and TLR4 ligands, as previously reported for LPS, lipoteichoic acid and even viruses[9].

Intracellularly-localized TLR 3, 7, 8 and 9 are of specific interest in patients with chronic viral infections since these receptors can recognize virus-derived molecular patterns[6,7]. We found that TLR3 expression was higher in monocytes of HCV-infected patients compared to controls. TLR3 is a sensor for double-stranded RNA and it has been implicated in pathogenesis of viral infections[32,33]. Synthetic RNA compounds, such as polyI:C activate cells expressing TLR3[34]. In a recent study by Edelmann *et al*[35] the role of TLR3 was investigated in four differ-

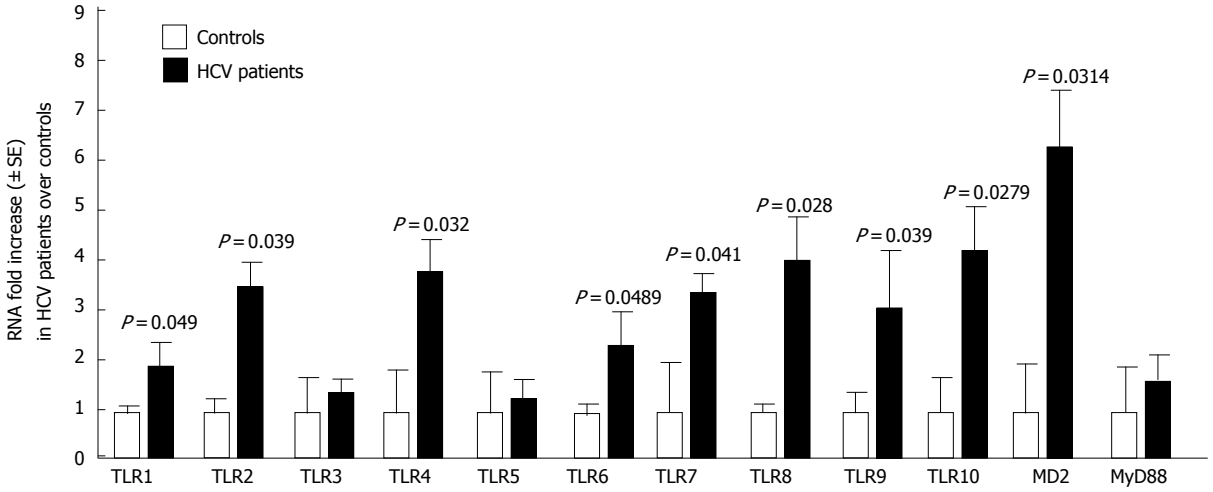

Figure 3 Higher TLRs 1, 2, 4, 6, 7, 8, 9 and 10 and MD2 RNA expression by lymphocytes of HCV-infected patients compared to controls. RNA was isolated from lymphocytes and real-time PCR for TLRs 1-10, MD2 and 18S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR- or MD2-coding RNA expression was normalized to corresponding 18S RNA. Results are expressed as fold increase of each TLR or MD2 in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

ent infectious viral models [lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus in TLR3$^{-/-}$ mice]. The investigators found that TLR3 is not always required for the generation of effective antiviral responses, as the absence of TLR3 did not alter either viral pathogenesis or host's generation of adaptive antiviral responses to those viruses. Interestingly, intracellular transduction of poly(I:C) initiates activation of an IFN response in a TLR3-independent manner, thus limiting the role of TLR3 in the IFN pathway[34]. A recent report by Li *et al*[33] indicates that HCV may use TLR3 pathway to evade immune surveillance via HCV NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. Furthermore, HCV NS3/4A interferes with retinoic acid-inducible gene-I (RIG-I), a key factor in TRIF-independent signaling[34]. Our finding of increased TLR3 mRNA levels in monocytes of HCV patients awaits further investigation. It is tempting to speculate that HCV RNA may trigger cellular activation via TLR3, and indeed, all our patients had high levels of viremia, however no clear relationship between HCV RNA levels and TLR3 expression has been proven to date.

TLR7 and TLR8 are stimulated by synthetic compounds imiquimod and resiquimod 848[6,7,34]. In a recent publication, Lund *et al*[12] showed that TLR7 recognizes the single-stranded RNA viruses, vesicular stomatitis virus and influenza virus. The recognition of these viruses by plasmacytoid dendritic cells and B cells through TLR7 results in cellular activation and the production of cytokines. However, the specific role of TLR7 and TLR8 in innate immune response to HCV is yet to be understood. HCV is a single stranded RNA virus, thus, it could theoretically act as a ligand for TLR7. Our data show that TLR7 mRNA is significantly upregulated in HCV patients compared to controls, and all patients had detectable levels of HCV RNA in their plasma, thus suggesting that TLR7 may be implicated in the pathogenesis of HCV infection.

We found that both monocytes and T cells of HCV-infected patients expressed elevated levels of TLR9 mRNA compared to controls. CpG-containing DNA acts as a stimulatory ligand for TLR9[6,7,28,3]. TLR9 is regulated at the transcriptional level by multiple nuclear regulatory factors, co-repressors and co-activators, including CREB1, Ets2, Elf1, Elk1, and C/EBP and HCV may regulate these transcription factors[36,37]. Toll-like receptors 9 and 3 are essential components of innate immune defense against cytomegalovirus infection in mice[38]. While we found that TLR9 mRNA was upregulated in patients, others have shown that infections with cytomegalovirus are more frequent in HCV-infected patients compared to controls[5]. It remains to be elucidated if the increased TLR9 RNA levels in monocytes or T cells have a role in increased susceptibility to infections with CMV, as seen in HCV patients.

To date little is known about the role of TLR expression in T cells of the adaptive immune compartment. Caramalho *et al*[39] recently reported that LPS, a TLR4 ligand, promotes survival of activated CD4+ cells and stimulates regulatory T cell (T reg) activity. Here we found that TLR4 mRNA expression was higher in HCV-infected patients compared to controls only in lymphocytes, but not in monocytes. It is tempting to speculate that elevated levels of TLR4 and its co-receptor, MD-2, in patients' T cells may provide ongoing activation in the presence of serum LPS found in chronic HCV and provide preferential survival signals for T regs[29]. Recent data suggest a role for T regs in immune alterations associated with chronic HCV. Cabrera *et al*[40] reported that Tregs appear to play a role in viral persistence by suppressing HCV-specific T cell responses in a cell-cell contact manner, while MacDonald *et al*[41] showed that Tregs could be induced against the same epitopes on the HCV core protein.

A limitation of our study is that it did not distinguish between TLR expression in CD4+ and CD8+ T cells. Differential CD4$^+$ and CD8$^+$ T-cell responsiveness in hepatitis C virus infection has been reported, thus, leading to the hypothesis that if TLRs are of any functional role in T cells, there may be differences in TLR expression between CD4+ and CD8+ T cells in HCV-infected patients. Due to

the presence of anti-CD56 mAbs in the separation cocktail, we also lost $CD56^+$ T cells, known as gamma delta, with an important role in HCV infection[42].

In conclusion, we showed that patients with chronic HCV infection express elevated levels of selected TLRs in both adaptive and innate compartments of the immune system. Our data may suggest additional therapeutic targets.

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Autoimmune thrombocytopenia in response to splenectomy in cirrhotic patients with accompanying hepatitis C

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Abstract

AIM: To estimate the contribution of autoimmune thrombocytopenia to hepatitis C virus-related liver cirrhosis (type C cirrhosis), we evaluated the influence of splenectomy upon platelet-associated immunoglobulin G (PAIgG) levels and platelet numbers.

METHODS: PAIgG titers and immune markers were determined in 24 type C cirrhotic patients with an intact spleen, 17 type C cirrhotic patients submitted to splenectomy, and 21 non-C cirrhosis with an intact spleen.

RESULTS: Thrombocytopenia (PLT<$15\times10^4/\mu L$) in type C cirrhosis was diagnosed in all patients with an intact spleen, 8 patients submitted to splenectomy, and in 19 non-C cirrhosis with intact spleen. Elevated titers of PAIgG at more than 25.0 ng/10^7cells were detected in all cirrhotic patients except for one splenectomized patient. PAIgG titers (ng/10^7cells) were significantly higher in the type C cirrhosis with an intact spleen (247.9 ± 197.0) compared with the splenectomized patients (125.6±87.8) or non-C cirrhosis (152.4 ± 127.4). PAIgG titers were negatively correlated with platelet counts in type C cirrhotic patients with an intact spleen. In comparison with the type C cirrhosis with an intact spleen, the splenectomized patients had a reduced CD4/CD8 ratio and serum neopterin levels. The spleen index (cm^2) was negatively correlated with platelet counts in the non-C cirrhosis, but not in the type C cirrhosis.

CONCLUSION: Our data indicate that the autoimmune mechanism plays an important role in thrombocytosis complicated by HCV-positive cirrhosis. In addition, splenectomy may impair T cells function through, at least in part, a reduction of CD4/CD8 ratio, consequently suppressing PAIgG production.

Key words: Platelet-associated immunoglobulin G; Autoimmune thrombocytopenia; Liver cirrhosis; Hepatitis C virus; Splenectomy; CD4/CD8 ratio

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INTRODUCTION

Accompanying thrombocytopenia in patients with liver cirrhosis was initially attributed to an increase in platelet sequestration in the enlarged spleen secondary to portal hypertension[1]. Elevated platelet counts after procedures, such as transjugular intrahepatic portosystemic shunt[2,3], partial splenic embolization[4,5], splenectomy[6], and shunt operation[7,8], confirmed this theory. Nevertheless, controversial results on the relief of portal hypertension by surgical means have shown a reduction in spleen size with no concomitant persistent rise in platelet counts unless accompanied by liver transplantation[9]. Thus, the clinical significance of portal hypertension in the pathogenesis of thrombocytopenia in liver cirrhosis remains to be elucidated. Recently, not only impaired production of thrombopoietin, which regulates megakariocyte development[10,11], but also immune disturbance is thought to be responsible for thrombocytopenia associated with cirrhosis. In autoimmune-mediated thrombocytopenia, patients with chronic liver disease had a high incidence of platelet-associated immunoglobulin G (PAIgG)[12-16]. Pereira *et al*[12] detected autoantibodies that reacted specifically with platelet membrane glycoprotein in chronic liver disease, suggesting that an immune mechanism may participate in the induction or aggravation of thrombocytopenia.

Hepatitis C virus (HCV) has been identified as a major causative agent for parenterally transmitted non-A non-B hepatitis throughout the world[17]. Approximately 85% of HCV-infected individuals fail to clear the virus and develop persistent chronic hepatitis which leads to cirrhosis[18]. Chronic HCV infection is associated with an increased risk of liver cancer, and most cases of HCV-

related hepatocellular carcinoma occur in the presence of cirrhosis. In addition, HCV infection is often associated with immune disturbance, including the development of autoantibodies and an increased frequency of immune-mediated disease[19]. We already hypothesized that chronic HCV infection may cause autoimmune thrombocytopenia because of prevalence of elevated levels of PAIgG and an inverse correlation between PAIgG titers and platelet counts[20]. On the other hand, Kosugi *et al*[21] reported that hepatitis C patients had a high incidence of PAIgM but relatively low levels of PAIgG, insisting that thrombocytopenia in hepatitis C patients was not due to anti-platelet autoantibodies. Substantial evidence exists that PAIgG is frequently identified in HCV-positive patients[20,22], but whether this immunoglobulin acts as a specific anti-platelet antibody remains unclear.

The role of spleen in PAIgG production and modification of cell-mediated immunity was previously reported in patients with idiopathic thrombocytic purpura (ITP), in whom the prevalence of PAIgG was found to contribute to the development of autoimmune thrombocytopenia[23]. Chronic ITP patients characteristically had varied PAIgG levels post-splenectomy[24], but showed immediate normalization in splenectomy-responders[25]. In ITP patients, CD4+ and HLA-DR-restricted T cells to platelet membrane GPIIb-IIIa were found to involve in the production of anti-platelet autoantibodies[26]. These results suggest that splenectomy may modify PAIgG production and immune mediators in patients with chronic HCV infection. In the present study, we, in order to elucidate the role of autoimmune thrombocytopenia in the development HCV-positive liver cirrhosis, retrospectively evaluated the influence of splenectomy on PAIgG levels and platelet counts, as well as cellular and humoral immune mediators.

MATERIALS AND METHODS

Patients

In the present study, 41 patients with type C cirrhosis, who were referred to our hospital, were enrolled. They were found to be seropositive for HCV antibodies detected a second or third generation enzyme-linked immunosorbent assay (Ortho Diagnostic System Co., Ltd., Tokyo, Japan) and/or recombinant immunoblot assay (Chiron RIBA-2 and/or RIBA-3). Seventeen patients had undergone splenectomy in association with surgery for esophageal varices (the splenectomies). The mean duration of follow-up after splenectomy was 11.5±4.7 years (range, 2 to 19 years). Twenty-four consecutive cirrhotic patients had an intact spleen (the non-splenectomies). In addition, 20 cirrhotic patients including 10 hepatitis B, 4 autoimmune hepatitis, 3 alcoholic and 3 idiopathic cirrhosis served as the non-C cirrhosis. All the non-splenectomies and non-C cirrhosis had esophageal varices complication, and eight of the non-splenectomies and five of the non-C cirrhosis received treatment for the varices by endoscopic sclerotherapy and/or band ligation.

Morphological diagnosis of cirrhosis was performed in all the splenectomies, 15 non-splenectomies and 12 non-C cirrhosis. The others were diagnosed clinically, and they had typical laboratory findings of liver cirrhosis and characteristic liver imaging on computed tomography or ultrasonography. Patients' characteristics are shown in Table 1. Clinical features were similar among the three groups. Informed consent was obtained from each patient according to the Helsinki Declaration.

Table 1 Characteristics of the patients (*n*, mean±SD)

	Type C cirrhosis	Type C cirrhosis submitted to splenectomy	Non-C cirrhosis
Number	17	24	20
Age (yr)	62.6±10.6	63.0±7.1	61.8±9.5
Gender			
Female	10	15	10
Male	7	9	10
Child's classification			
A	11	13	9
B	4	6	7
C	2	4	4
Association of HCC	4	7	5

Non-C cirrhosis consisted of 10 hepatitis B, 4 autoimmune hepatitis, 3 alcoholic and 3 idiopathic cirrhosis. HCC: hepatocellular carcinoma.

Assays

Quantitative HCV-RNA was analyzed from serum samples by a combined reverse transcription PCR assay (Amplicor-HCV monitor). Peripheral blood counts and liver function parameters were measured by an autoanalyzer.

Quantity of PAIgG was determined by a competitive micro enzyme-linked immunosorbent assay (ELISA) as described by Kawaguchi *et al*[27]. Briefly, platelets were separated from whole blood collected in EDTA, and washed with phosphate-buffered saline containing 11 g/L bovine serum albumin. The ELISA was performed in 96-well microplates coated with purified human IgG. A horseradish peroxidase-conjugated anti-human IgG antibody was incubated simultaneously with the samples, and visualized with o-phenylenediamine. PAIgG titers from 49 healthy individuals were within 25.0 ng/10^7 cells (mean, 16.4±4.2 ng/10^7 cells)[20].

The proportion of CD4 and CD8 subsets of peripheral bloods was assayed by flow cytometry. The levels of neopterin and soluble interleukin-2 receptor (sIL2R) were measured using HPLC and Cell Free IL-2R EIA kit, respectively. Immunological markers and PAIgG were determined using fresh blood samples.

Spleen size in cirrhotic patients was evaluated as spleen index using ultrasonography as previously reported[28], in which spleen index correlated well with the volumes of resected spleens. Briefly, the spleen index was calculated as the product of the transverse diameter and its perpendicular diameter measured on the maximum cross-sectional image of the spleen, and expressed in cm^2. Values obtained from healthy individuals were below 20 cm^2, and the mean values of 28 healthy controls were 15±7 cm^2[28].

Statistical analysis

Values were expressed as mean±SD. Comparisons of the serum measurements among patients of the splenectomies, non-splenectomies and non-C cirrhosis were carried out using analysis of variance (ANOVA), and analyses of

Table 2 Laboratory findings in the type C cirrhotic and non-C cirrhotic patients (*n*, mean±SD)

	Type C cirrhosis	Type C cirrhosis submitted to splenectomy	Non-C cirrhosis
RBC ($\times10^4/\mu L$)	379±61	374±65	381±63
WBC ($/\mu L$)	3 652±1 080	5 855±1 595[1]	3 832±1 048
Platelet ($\times10^4/\mu L$)	8.2±2.9	16.1±4.9[1]	9.2±3.4
>15.0	0	9	1
10.0-14.9	5	6	6
<9.9	19	2	13
ALT (IU/L)	58.2±25.3	51.1±35.2	40.6±23.8
Total bilirubin (mg/dL)	1.4±1.2	0.9±0.3[1]	2.3±1.8
Albumin (g/L)	37±4	37±6	34±8
r-globulin (g/L)	21±0.5	22±6	20±9
IgG (mg/ dL)	2 454±693	no assay	2 220±877
HCV RNA (kcopies/mL)	523±403	198±251[2]	
Splenic index (cm^2)			
- 19	6 cases		5 cases
20 - 29	11 cases		9 cases
30 - 39	6 cases		5 cases
40 -	1 case		3 cases

ALT: alanine aminotransferase. 1: Significant difference compared with the type C cirrhosis with an intact spleen and non-C cirrhosis. 2: Significant difference compared with the type C cirrhosis with an intact spleen.

Table 3 Immunological markers in patients with type C cirrhosis (mean±SD)

	Type C cirrhosis	Type C cirrhosis submitted to splenectomy
Neopterin (pmol/mL)	8.3±3.2	5.2±2.5[b]
sIL2R (U/mL)	850.7±175.9	877.2±458.6
CD4 (%)	43.1±8.6	33.4±7.6
CD8 (%)	27.6±7.9	29.6±9.6
CD4/CD8 ratio	1.8±0.9	1.3±0.4[a]

sIL2R: Soluble interleukin-2 receptor. [a]$P<0.05$ *vs* type C cirrhosis with an intact spleen; [b]$P<0.01$ *vs* type C cirrhosis with an intact spleen.

non-parametric data between two groups were carried out by Mann-Whitney *U* test. A correlation was calculated with the Spearman's rank correlation coefficient.

RESULTS

Laboratory findings and spleen index

Thrombocytopenia (PLT<$15\times10^4/\mu L$) was diagnosed in all the non-splenectomies (100%), eight of the splenectomies (47.1%), and 19 of the non-C cirrhosis (95.0%) (Table 2). Marked thrombocytopenia at less than $10\times10^4/\mu L$ was obviously found in the non-splenectomies (79.2%) and the non-C cirrhosis (65.0%) as compared with the splenectomies (11.8%). Peripheral platelet and white blood cells were significantly higher in the splenectomies than that in the non-splenectomies and non-C cirrhosis ($P<0.01$). Platelets and white blood cells were similar in the non-splenectomies and non-C cirrhosis. Red blood cell counts were not changed among the three groups. No difference was found among the three groups for liver function parameters except for total bilirubin levels and HCV-RNA titers.

Spleen index ranged widely from 9.6 to 42.0 cm^2. The mean spleen index was above the upper limit of normal individuals (20 cm^2) in both type C cirrhosis (24.6±8.0 cm^2) and non-C cirrhosis (23.5±8.0 cm^2).

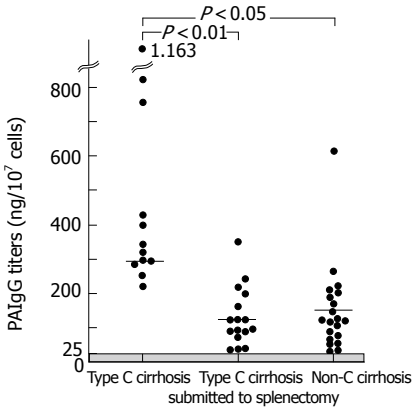

Figure 1 PAIgG titers in the type C cirrhosis with an intact spleen, type C cirrhosis submitted to splenomegaly, and non-C cirrhosis. The type C cirrhosis with an intact spleen had significant higher titers of PAIgG as compared with those of the splenectomized patients with type C cirrhosis and non-C cirrhosis.

Immunological markers

An elevation of PAIgG at more than 25.0 ng/10^7cells was observed in all cirrhotic patients except for one splenectomized patient. The mean PAIgG titers were significantly higher in the non-splenectomies as compared with the splenectomies (297.9±197.0 *vs* 125.6±87.8 ng/10^7cells, $P<0.05$) and non-C cirrhosis (297.9±197.0 *vs* 152.4±127.4 ng/10^7cells, $P<0.05$). There was no significant difference in PAIgG titers between the splenectomies and the non-C cirrhosis (Figure 1). In comparison with the type C cirrhotic patients, the splenectomies had reduced percentage of CD4 and similar percentage of CD8, resulting in a lower ratio of CD4/CD8 than those of the non-splenectomies. In addition, neopterin levels were significantly lower in the splenectomies as compared with the non-splenectomies, while sIL2R levels were similar between the two groups (Table 3).

Correlation between peripheral blood cells, immunological markers and spleen index

In the non-splenectomies, PAIgG titers were negatively correlated with platelet counts, and positively correlated with IgG and γ-globulin levels (Figure 2). In the non-C cirrhosis, PAIgG titers were neither correlated with platelet counts nor with IgG and γ-globulin levels. A significant negative correlation between the spleen index and platelet counts was found in the non-C cirrhosis, whereas not in the type C cirrhosis (Figure 3). In addition, the spleen index was negatively correlated with white blood cell counts in both type C and non-C cirrhosis. In the splenectomies, PAIgG titers were not correlated with platelet counts, γ-globulin levels, or the duration of follow-up after splenectomy.

DISCUSSION

Our data demonstrated that the HCV-positive cirrhosis (type C cirrhosis) with an intact spleen had a significant rise

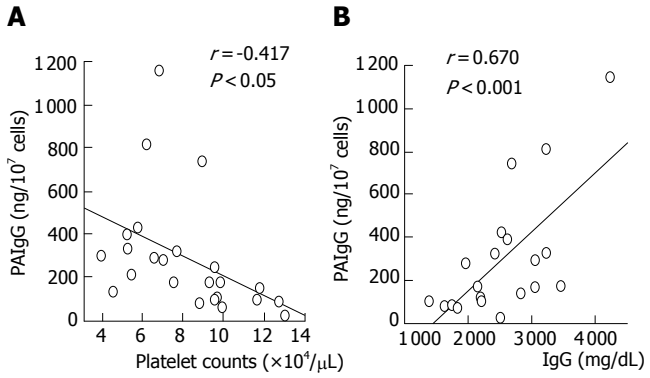

Figure 2 Relationships among PAIgG titers, platelet counts, and IgG levels in the type C cirrhosis with an intact spleen. PAIgG titers are negatively correlated with platelet counts, and positively correlated with IgG levels.

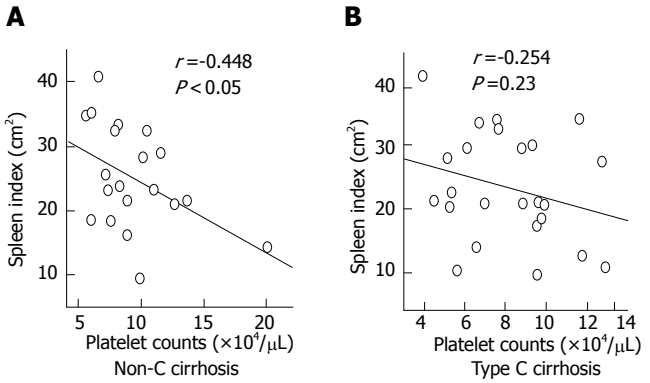

Figure 3 Relationship between the spleen index and platelet counts. A significant negative correlation is shown in the patients with non-C cirrhosis, but not in the type C cirrhosis with an intact spleen.

of PAIgG as compared with the non-C cirrhosis, including hepatitis B infection, autoimmune hepatitis, alcohol abuse, and idiopathic cirrhosis. PAIgG titers were negatively correlated with platelet counts in the type C cirrhosis with an intact spleen. The type C cirrhotic patients submitted to splenectomy showed a significant elevation of platelet counts and reduction in PAIgG titers compared with those of the patients with an intact spleen. Similar PAIgG and platelet levels following splenectomy are commonly found in ITP patients, in whom the thrombocytopenia is responsible for the autoimmune mechanism mediated by a specific IgG bound to platelet membrane proteins[24,25]. There exist controversies regarding the clinical significance of PAIgG in pathogenesis of thrombocytopenia in the patients with liver disease[7,14,29,30]. Two studies on partial splenic artery embolization in patients with hypersplenism clearly confirmed an immunological mechanism mediated by PAIgG-induced thrombocytopenia accompanying liver cirrhosis[7,14]. These studies reported a significant rise in platelet numbers and a significant fall in PAIgG levels after partial splenic artery embolization. In the present study, the changes in PAIgG levels and platelet numbers among the type C cirrhotic patients with or without an intact spleen are practically similar to those previous results. These data support a key role of spleen in PAIgG production and that an autoimmune mechanism plays an important role in the development of thrombocytopenia associated with HCV-positive cirrhosis. On the contrary, a non-specific adsorption of elevated γ-globulin by platelets was suspiciously reported on chronic liver disease[27]. Our data found a negative correlation between PAIgG titers and platelet counts in the type C cirrhosis with an intact spleen, but not in the non-C cirrhosis. Presumably, the elevated PAIgG in the type C cirrhosis may act as antiplatelet autoantibodies. However, we found a positive correlation between PAIgG titers and IgG levels in the type C cirrhosis with an intact spleen. This result is in agreement with that of de Noronha *et al*[31] in the patients with various liver diseases. The methods used for quantitating PAIgG in their study as well as in our study appear to measure total PAIgG, which is not equivalent to platelet-specific autoantibodies. They suggested that increased platelet IgG was due to enhanced retention of plasma IgG in the platelets. In our study, despite similar γ-globulin levels, a relationship between PAIgG and γ-globulin was found neither in the splenectomized patients with type C cirrhosis nor in the non-C cirrhotic patients. Consequently, the theory of 'enhanced retention of IgG in the platelets' proposed by de Noronha *et al*[31] is deniable in the present study.

Semple *et al*[32] indicated that CD4+ T-helper cells from patients with ITP are stimulated by normal platelet antigen(s) to secrete IL-2 and may modulate the enhanced anti-platelet autoantibody response. The underlying mechanisms for the production of anti-platelet autoantibodies require CD4+ and HLA-DR-restricted T cells to GPIIb-IIIa in ITP patients[26]. GPIb+ cells isolated from spleens of patients with chronic ITP strongly expressed HLA-DR, and splenic T cells had a high level of *in vitro* platelet-stimulated IL-2 secretion as compared with the controls[33]. Thus, the spleen is such an important site for antibody production in patients with chronic ITP that the levels of PAIgG rapidly normalize in response to a splenectomy[24,34]. Our data showed that compared with the type C cirrhosis with an intact spleen, the type C cirrhosis submitted to splenectomy had lower CD4/CD8 ratio because of a reduced percentage of CD4+ T cells. Neopterin is secreted by macrophages when stimulated for helper functions in the proliferation and activation of T cells. This immune marker was significantly decreased in the type C cirrhosis submitted to splenectomy compared to the type C cirrhotic patients with an intact spleen, supporting impaired T cell function following splenectomy. Taken together, previous and present results indicate that splenectomy may impair T cell function in HCV-positive liver cirrhosis through, at least in part, a reduction in CD4/CD8 ratio, which thereby suppresses PAIgG production. The data from the present study provide some indications that the spleen induces helper T-cell proliferation, but a further research is necessary to clarify the role of spleen in PAIgG production and T helper lymphocyte cytokine production.

Hypersplenism due to portal hypertension is believed as a major cause of thrombocytopenia complicated with liver cirrhosis; however, studies on the relief of portal hypertension have yielded the controversial results that observable reduction in spleen size is not associated with a concomitant persistent rise in platelet counts, unless accom-

panied by liver transplantation[8,9]. Thus, the role played by immune disturbance as well as thrombopoietin is an attractive possibility in the pathogenesis of thrombocytopenia concomitant with liver cirrhosis. Noguchi *et al*[5] observed the improved numbers and survival time of platelets and reduction in the raised PAIgG levels by partial splenic artery embolization in cirrhotic patients with hypersplenism. Owing to a negative correlation between platelet numbers and splenic volume before and after the procedure, they concluded that partial splenic artery embolization may not only reduce the increased platelet pool in the spleen, but also improve the autoimmune thrombocytopenia induced by cirrhosis. In the present study, the elevated platelets and reduced PAIgG levels in the splenectomized patients support their results. In addition, the present study showed that spleen size was correlated with platelets counts in the non-C cirrhotic patients. The data suggested that hypersplenism due to portal hypertension is assumed to be a major cause of thrombocytopenia observed in the non-C cirrhotic patients. If no significant relationship is found between the spleen index and platelet counts in HCV-positive cirrhotic patients, autoimmune disturbance may be the most likely principal mechanism for thrombocytopenia rather than sequestration and/or destruction of platelets secondary to hypersplenism.

In the present retrospective study, we confirmed that the autoimmune disturbance induces thrombocytopenia associated with liver cirrhosis which is related to HCV-infection. Prospective pre- and post-splenectomy studies should elucidate the role of spleen in PAIgG production, platelet sequestration and destruction, as well as cellular and humoral immunity.

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BASIC RESEARCH

Smad3 knock-out mice as a useful model to study intestinal fibrogenesis

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Abstract

AIM: To evaluate the possible differences in morphology and immunohistochemical expression of CD3, transforming growth factor β1(TGF-β1), Smad7, α-smooth muscle actin (α-Sma), and collagen types I-VII of small and large intestine in Smad3 null and wild-type mice.

METHODS: Ten null and ten wild-type adult mice were sacrificed at 4 mo of age and the organs (esophagus, small and large bowel, ureters) were collected for histology(hematoxylin and eosin, Masson thrichrome, silver staining), morphometry and immunohistochemistry analysis. TGF-β1 levels of intestinal tissue homogenates were assessed by ELISA.

RESULTS: No macroscopic intestinal lesions were detected both in null and wild-type mice. Histological and morphometric evaluation revealed a significant reduction in muscle layer thickness of small and large intestine in null mice as compared to wild-type mice. Immunohistochemistry evaluation showed a significant increase of CD3+T cell, TGF-β1 and Smad7 staining in the small and large intestine mucosa of Smad3 null mice as compared to wild-type mice. α-Sma and collagen I-VII staining of small and large intestine did not differ between the two groups of mice. TGF-β1 levels of colonic tissue homogenates were significantly higher in null mice than in wild-type mice. In preliminary experiments a significant reduction of TNBS-induced intestinal fibrosis was observed in null mice as compared to wild-type mice.

CONCLUSION: Smad3 null mice are a useful model to investigate the *in vivo* role of the TGF-β/Smad signalling pathway in intestinal inflammation and fibrosis.

Key words: Transforming growth factor; TGF-β; Fibrosis; Smad proteins

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INTRODUCTION

Smads are a family of eight related proteins which function as signalling intermediates for the transforming growth factor β (TGF-β) superfamily of ligands[1-3]. Upon ligation and activation of TGF-β with its receptors (I, II and III), the phosphorylated Smad2 and 3 form a complex with the common mediator Smad4. The Smad2/3- Smad4 complex can translocate into the nuclei where they enhance specific TGF-β target genes. The inhibitory Smad7 antagonizes TGF-β signalling by interfering with the ligation of Smad2/3 with the activated receptor complex. Experimental evidence from several research groups suggests that disruption of the TGF-β/Smad signalling pathway plays a central role in sustaining both chronic tissue inflammation and fibrosis[4-6].

TGF-β is a multifunctional polypeptide hormone influencing different functions in a variety of cells including regulation of cell proliferation, differentiation and apoptosis, immunoregulation, regulation of the inflammatory response, restitution and healing[5,7,8]. At cellular level, TGF-β affects virtually all stages of the chronic inflammatory and fibrotic disease processes. The effects of TGF-β on the extracellular matrix are more complex than those of any other growth factor and are central to its effects in increasing the maturation and strength of wounds, as well as in the pathological matrix accumulation, characteristic of fibrotic disease[4,9,10]. TGF-β regulates the transcription

of a wide spectrum of matrix proteins including collagen, fibronectin, glycosaminoglycans, and matrix-degrading proteases (metalloproteinases) and their inhibitors.

TGF-β/Smad signalling plays an important role in chronic inflammatory diseases, especially in Crohn's disease (CD)[5,7,11,12]. The transmural infiltrate of CD is responsible for initiating and maintaining a series of connective tissue changes not only involving the mucosa but also the submucosa and *muscularis mucosae* and *propria* where a marked increase of type I, III and V collagens and RNA transcripts are observed[13-15]. In CD, there is a marked overexpression of TGF-β1 and TGF-β receptors in the colonic mucosa[16-18]. Fibrosis in CD can therefore be viewed as an aberrant healing response to injury[19]. In addition, TGF-β appears to be involved in intestinal fibrosis present in other enteropathies, such as radiation enteritis, collageneous colitis and intestinal graft-versus-host disease[20-22].

Experimental transgenic animal models are useful tools to study the *in vivo* function of individual molecules[23-25]. TGF-β knock-out mouse model is characterized by the loss of a critical regulator of immune function which leads to an excessive inflammatory response with massive infiltration of leukocytes in several organs[26]. This condition develops rapidly with onset during the first week of life and results in severe wasting and death by the fourth week of life[27,28]. Unlike the targeted disruptions of Smad2 and 4 which are lethal[29,30], the disruption of Smad3[31] results in the birth of mice which are viable and can survive to adulthood (up to 8 mo of age). Since the Smad3 knock out model provides pivotal information concerning cutaneous wound healing[32-34], it is thought that this model might also be useful to investigate the *in vivo* role of the TGFβ/Smad signalling pathway in intestinal inflammation and fibrosis.

The present study was to evaluate the small bowel and colonic morphology as well as the immunohistochemical expression of collagens I-VII, α-smooth muscle actin (α-SMA), TGFβ1, Smad7, and CD3 in Smad3 wild-type and null mice.

MATERIALS AND METHODS

Animals

Colonies of Smad3 wild-type, heterozygous and null mice (black Swiss strain) were bred in our laboratory. These animal colonies were developed using pairs of Smad3 heterozygous mice kindly provided by A. Roberts (NCI, Bethesda, MD, USA). Smad3$^{ex8/ex8}$ mice were generated by targeted disruption of the Smad3 gene by homologous recombination. Targeted embryonic stem-cell clones were injected into a C57BL blastocyst to obtain germline transmission. Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offsprings[31].

All mice were maintained in a specific pathogen-free (SPF) facility and routinely monitored. Mice were kept in microisolator cages and allowed free access to food and water. All mice were examined 4 times a week for signs of colitis including weight loss, diarrhea, rectal bleeding and prolapse[35], as well as signs of systemic inflammation such as piloerection, lethargy and periorbital exudates[36].

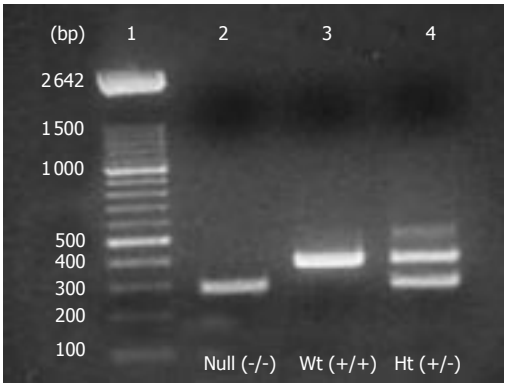

Figure 1 Genotyping of animal offsprings by PCR of cDNA (tail extracts). Lane 1= molecular weight ladder of 100 bp; lane 2= null mice; lane 3= wild-type (Wt) mice; lane 4 = heterozygous(Ht) mice.

DNA extraction and genotype analysis

Mouse tail DNA extraction was performed according to the protocol reported elsewhere[37].

Genotype analysis was carried out by the polymerase chain reaction (PCR) method in which the wild type Smad3 allele was detected using primer 1 (5'-CCAGACTGCCTT GGGAAAAGC-3') and primer 2 (5'-CCCGAACAGTTG GATTCACACA-3'). Primer 1 is located 5' to the deletion and primer 2 is located within the deletion. This primer pair amplified a fragment of ~ 400 bp from wild-type and Smad3 ex$^{8/+}$ mice, but not from Smad3$^{ex8/ex8}$ mice (Figure 1). DNA was also amplified using primer 1 and primer 3 (5' -CCAGACTGCCCTTGGGATGCCCCTG-3'), which is located in the pLoxpneo, to detect the mutant Smad3 allele. In this case, a 250 bp fragment was detected in mice, heterozygous or homozygous for the mutant Smad3 allele, while no signal was detected in wild-type mice.

Sample recovery and preparation

A total of 20 mice (10 wild-type and 10 null for the Smad3 allele) were sacrificed at 4 mo of age. Laparotomy was performed under ether anesthesia. The esophagus, small bowel, colon and ureters were visualized, rapidly excised and placed in a Petri dish containing sterile saline solution. Tissue samples from the esophagus, small bowel, colon, and ureters were processed for structural and immunohistochemical studies.

Histology and morphometry

Specimens obtained from the esophagus, small bowel, colon and ureters of all animals were washed and immediately immersed in 10% buffered formalin in phosphate buffer saline (PBS) (pH 7.4) for 3 h at room temperature followed by the standard procedure for paraffin embedding. Serial 3 μm sections were stained with hematoxylin and eosin (HE) to assess the degree of inflammation and with Masson trichrome and silver stain to detect connective tissue. The stained sections were then observed under an Olympus BX51 light microscope (Olympus, Optical Co. Ltd., Tokyo, Japan). The degree of intestinal inflammation was scored as absent, mild, moderate or severe according to the density and extent of both acute and chronic inflammatory infiltrate, loss of goblet cells and

Table 1 Muscle layer thickness of gastrointestinal and urinary tract segments from Smad3 wild-type and null mice (mean±SD)

	Wild-type (*n*)	Null (*n*)	*P* value
Colon	221.79±99.78 (6)	104.68±50.73 (7)	0.02
Small bowel	95.55 ±38.45 (6)	46.44±25.96 (6)	0.03
Esophagus	189.36±42.60 (5)	188.49±43.94 (6)	NS
Ureters	32.85±3.74 (5)	30.31±1.91 (5)	NS

(*n*) = Number of mice evaluated. Muscle layer thickness is expressed in micron. NS = not significant.

bowel wall thickening[35,38]. Intestinal fibrosis was scored as mild, moderate or severe depending on the density and extent of trichrome-positive connective tissue staining and disruption of tissue architecture[38]. A quantitative estimate of mucosa, submucosa and muscular layer thickness of distal esophagus, proximal and distal small bowel, proximal and distal colon, and distal ureters was performed. Morphometric analysis was done in all animals by ten measurements randomly taken in 4 different fields (x 40) in a double blind fashion by two independent pathologists with an agreement always higher than 90%. Light microscopic and IHC microphotographs were taken by Olympus BX-51 Light Microscopy (Olympus, Optical Co. Ltd., Tokyo, Japan) with a videocam (Spot Insight, Diagnostic Instrument, Inc., Sterling Heights, MI, USA) and processed with an image analysis system (IAS-Delta Sistemi, Rome, Italy) software.

Immunohistochemistry

Samples from small bowel and colon obtained as previously described, were also promptly fixed with 10% buffered formalin in PBS (pH 7.4) for 3 h, dehydrated in graded ethanols and embedded in low-temperature-fusion paraffin. Serial 3 μm sections were incubated for 40 min in methanol and 3% hydrogen peroxide solution and then rinsed in PBS. Thereafter, sections were incubated overnight at 4 ℃ with monoclonal antibodies to CD3, TGFβ-1, Smad7 and α-SMA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), used at a dilution of 1:100, 1:250, 1:100 and 1:400 respectively in PBS. Additional sections were incubated with a cocktail of monoclonal antibodies to collagen types (I, III, IV, V, VII) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in order to demonstrate the morphological and topographic features of collagen distribution in different layers of small bowel and colonic wall.

Samples were then rinsed with PBS for 5 min and incubated with a labeled streptavidin-biotin-peroxidase conjugate kit (Dako LSAB, cod. K0675, Dako-Cytomation, Milan, Italy). After rinsed in PBS for 10 min, the sections were incubated with 3,3-diaminobenzidine-tetrahydrochloride (DAB, Sigma Aldrich, Milan, Italy) for 1-3 min.

To control specificity of the immune reaction, sections were incubated omitting the primary antibody, i.e., incubated with the secondary antibody alone. Finally, the samples were counterstained with Mayer's hematoxylin and observed under photomicroscope (Olympus BX51 light microscope; Olympus, Optical Co. Ltd, Tokyo, Japan).

Figure 2 Morphology of Smad3 null and wild-type mice. The majority of Smad3 null mice exhibited a reduced size compared to littermate controls. Severe bending of forepaw joints was present in Smad3 null mice.

Measurement of colonic TGF-β1 protein levels

TGF-β1 protein was determined by ELISA. Briefly, tissue was homogenized in the presence of a mixture of protease inhibitors with a broad specificity for the inhibition of serine, cysteine, aspartic proteases and aminopeptidases (1 mL/20 g). The mixture contained 4, (2-aminoethyl) benzenesulphonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin and aprotinin without metal chelators. For determination of TGF-β1 levels, an aliquot of the supernates was treated with 1 mol/L HCl to activate TGF-β1, followed by neutralization with 1 mol/L NaOH using a standard ELISA procedure (Quantikine, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All statistical analyses were performed in a double-blind fashion and results were computed using an appropriate program (SAS/STAT software). Results were expressed as mean±SD. Statistical significance was performed by the two-tailed Student's *t* test for paired data and $P < 0.05$ was considered statistically significant.

RESULTS

Animal gross phenotype

The phenotype of Smad3 null mice at birth was identical to that in heterozygous and wild-type littermate controls. Nevertheless, the Smad3 null mice at three weeks of age were characterized by the following hallmarks: short body length compared to the tail and a predominant deformity of the anterior paws (Figure 2). About 20% of Smad3 null animals developed a wasting disease (Figure 3) and died between 3-6 mo but none of them developed diarrhea or hematochezia, and only a marked dilatation of the colon was observed in two Smad3 null mice.

About 80 % of null mice survived up to 6 mo of age, while a mortality rate of 6% and 2% was observed at 18 mo of age in heterozygous and wild-type mice, respectively. No serosal or mucosal macroscopic lesions of the small or large intestine were detected either in wild-type mice or in null mice.

Histologic and morphometric evaluation

HE staining of the small and large bowel showed a normal morphology both in wild-type mice and in Smad3 null mice (Figure 4). Masson trichromic staining and silver

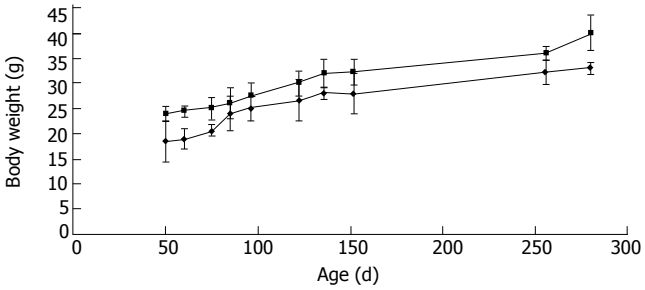

Figure 3 Body Weight changes of wild-type and Smad3 null mice. Each point represents mean weight data pooled from 10 mice. Standard deviations are indicated. Plot of weight (g) *vs* age (days). Wild-type are indicated as □ (squares), and null as ◊ (diamonds).

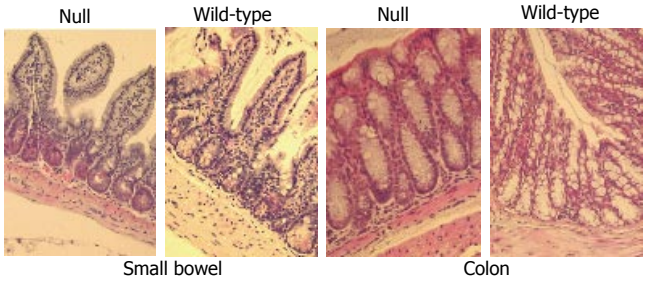

Figure 4 Haematoxylin and eosin-stained sections (x 20) analysis of small and large bowel from wild-type and Smad3 null mice shows normal morphology.

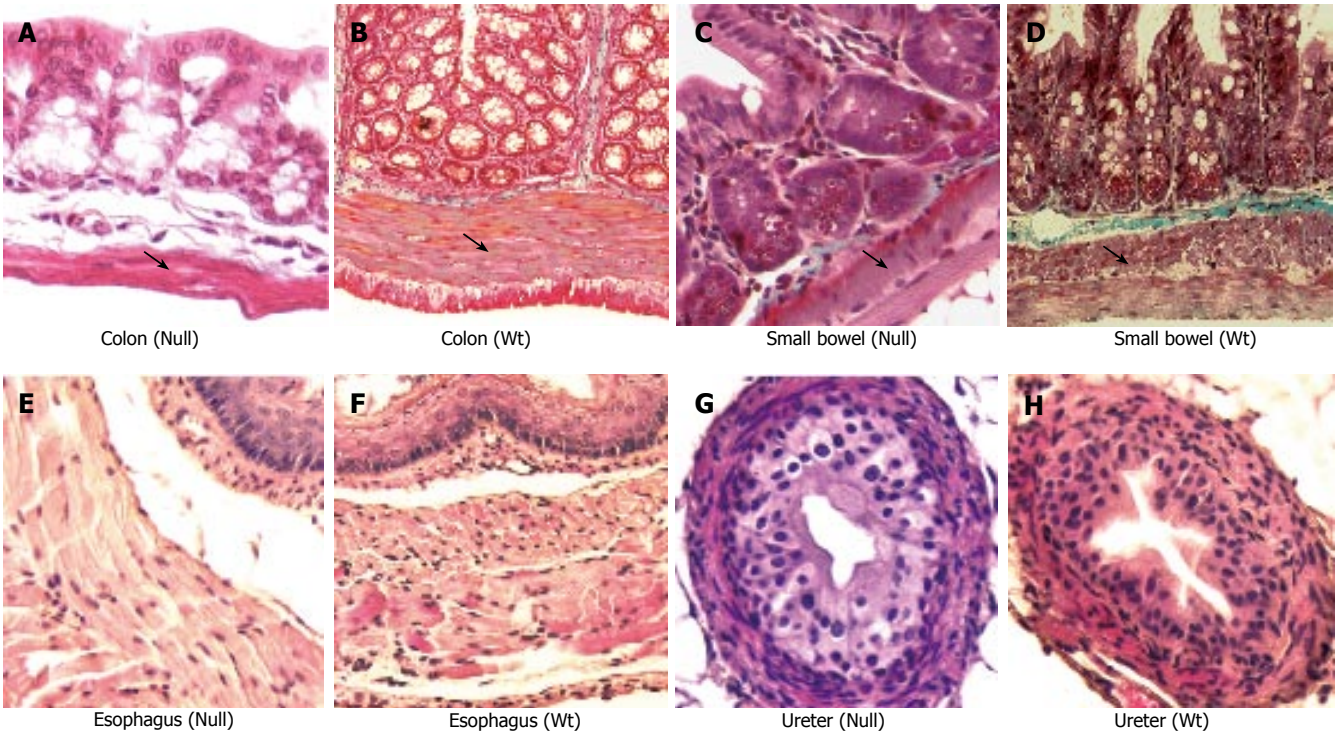

Figure 5 Masson trichrome staining (x 20) of small and large bowel from Smad3 mice. Significant reduction of muscular layer of descending colon of Smad3 null (**A**) is observed compared to colon from wild-type (WT) mice (**B**), reduction of muscle layer in cross sections of the proximal small bowel from Smad3 null (**C**) as compared to wild-type mice (**D**). Haematoxylin and eosin staining (x 20) of ureter and esophagus of Smad3 mice. Cross section of esophagus from Smad3 null (**E**) and wild-type mice (**F**) shows no differences in muscle layer. Cross section of ureter from Smad3 null (**G**) and wild-type mice (**H**) shows no differences in muscle layers.

staining of the colon and small intestine showed a similar collagen distribution in all intestinal layers both in wild-type mice and in Smad3 null mice (Figure 5).

A significant reduction in muscle layer thickness confined to the colon and small intestine was observed in Smad3 null mice compared to wild-type mice, while the mucosa and submucosa layers were similar in the two groups (Table 1, Figure 5). No differences in the thickness of the mucosa, submucosa and muscle layers of the ureter and esophagus were observed either in wild-type mice or in Smad3 null mice (Figure 5).

Immunohistochemistry evaluation

In the colonic and small intestine mucosa of Smad3 null mice, a significant staining of CD3+ T cells, TGF-β1 and Smad7 was observed compared to the intestinal mucosa of wild-type mice (Figure 6). TGF-β1 and Smad7 staining was localized mainly in lymphocytes of the intestinal lamina propria from Smad3 null mice. In small intestine and colon, α-SMA staining was limited to the smooth muscle cells of muscularis mucosa, muscularis propria as well as myocytes of the median vessel layer, with a comparable pattern both in wild-type mice and in null mice (Figure 7). Staining of collagens I-VII of the small intestine and colon was localized mainly in the submucosa and muscularis propria connective tissue and did not differ between the wild-type and null mice (Figure 7).

TGF-β1 levels in colon homogenates

TGF-β1 levels in colonic tissue homogenates were significantly higher in null mice than in wild-type mice (Figure 8).

DISCUSSION

In this study, we characterized the changes in intestinal structures which may occur in a Smad3 knock out mouse

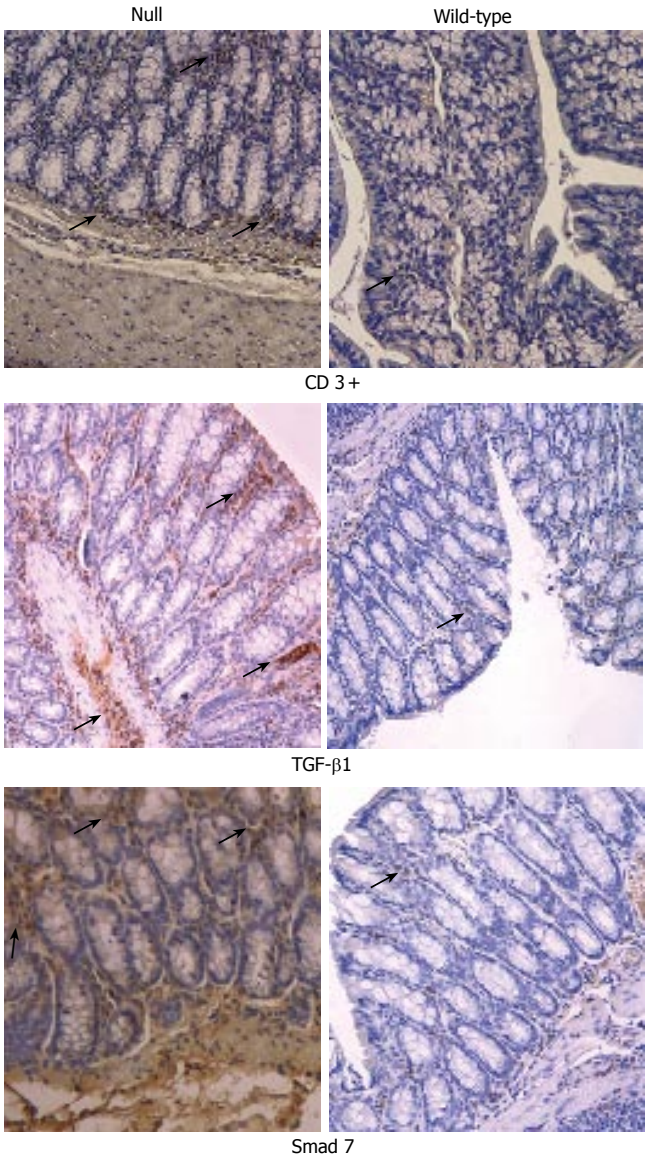

Figure 6 Immunohistochemical analysis (x 20) of CD3+ T cells, TGF-β1 and Smad7 in colon obtained from Smad3 null and wild-type mice. CD3+ T cells were significantly increased within large intestine of Smad3 null mice as compared to the wild-type mice. TGF-β1 and Smad7 were significantly increased within large intestine of Smad3 null mice compared to wild-type mice.

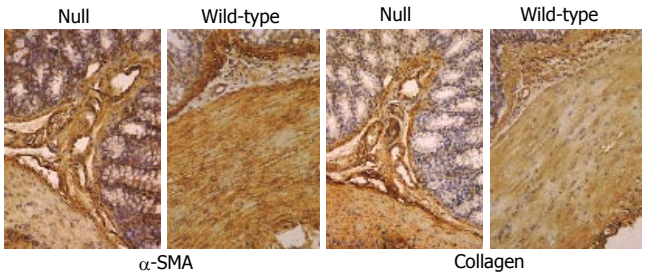

Figure 7 Immunohistochemical analysis (x 20) of α-SMA and collagens I-VII in colon from Smad3 null and wild-type mice. A similar localization of α-SMA antibody was found in miocytes of muscularis mucosae, muscle layer and vessels of both groups of animals. Staining of collagens I-VII in large intestine of Smad3 null and wild-type mice was localized mainly within connective tissue of submucosa and muscularis propria showing identical staining pattern between the two groups of mice.

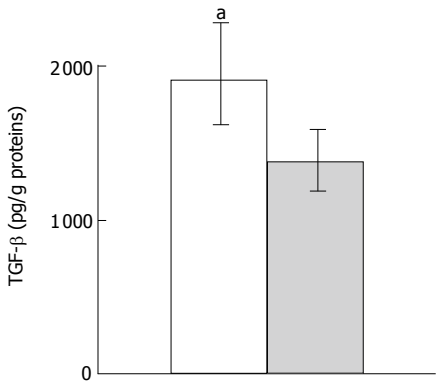

Figure 8 TGF-β1 ELISA of colon homogenates from Smad3 null (solid column) and wild-type mice (dashed column). Data are given as mean±SD. $^{a}P<0.05$ *vs* wild type mice.

model compared to the littermate wild-type controls. In particular, attention was focused on evaluation of intestinal alterations present in healthy adult animals that could be used as a model to investigate the role of the TGFβ1/Smad3 pathway in the development of chronic intestinal inflammation and fibrosis. The animals studied did not develop any signs of colitis, but Smad3 null mice had a deficit in body weight gain as compared to their controls. Smad3 null mice were smaller than wild-type littermates and about 40% of them showed the presence of medially torqued forepaws associated with kyphosis sometimes. Some aspects of this phenotype are similar to those of mice expressing a transgenic dominant negative type II TGF-β receptor[39]. We did not observe any significant macroscopic intestinal lesions except for colonic dilatation in 20% of Smad3 null mice.

Histopathological analysis of small and large intestine specimens did not reveal any neoplastic lesions, significant intestinal mucosa inflammation (i.e., chronic abscesses or marked neutrophil/monocyte infiltrate) or changes in intestinal connective tissue distribution. On the contrary, mice that died prematurely (1-3 mo of age) often showing signs of systemic inflammation, presented severe histologic lesions of the intestinal mucosa (data not shown) similar to the findings described earlier[31]. These animals were not included in the present study since the aim of the investigation was to evaluate the intestine of healthy adult mice in which an intestinal fibrosis could be experimentally induced.

A significant reduction in smooth muscle layer thickness of small and large intestine was present in Smad3 null mice as compared to wild-type mice. These alterations of colonic smooth muscle layers could account for the colonic dilation observed in Smad3 null mice. In this respect, immunostaining of the colonic mioenteric plexus was also performed which showed a normal appearance (data not shown). Smooth muscle layer thickness from the esophagus and ureters was normal and similar in the two groups of mice. The reason why the intestinal muscle layer thickness was reduced in Smad3 null mice is unknown. Nevertheless, several lines of evidence suggest that TGF-β/Smad3 signalling plays an important role in the development of smooth muscle cells from totipotent or multipotent embryonic stem cells[40,41]. Furthermore, TGF-β/Smad3 signalling is also involved in the differentia-

tion and proliferation of smooth muscle cells [42,43].

A number of phenotypic changes as observed in our mice with a targeted disruption of Smad3 in exon 8[31], are similarly present also in mice with a disruption of Smad3 in exon 1 or 2[44,45]. In fact, in all these three models a decrease in size and growth rate, and the presence of skeletal abnormalities have been observed. They also show a decreased survival. The deletions of both exon 8 and 1 are associated with different degrees of intestinal inflammation [31,44], while the deletion of exon 2 accompanies the development of metastatic colorectal cancer[45]. The reason for this discrepancy is unknown. This discrepancy could be related to differences in genetic background of the Smad3 null animals used. It is possible that in mice a differential activation of downstream targets exists with a disruption in exons 1, 2 and 8. This hypothesis is supported by *in vitro* studies indicating that different domains of the Smad3 protein may be involved in activation of diverse downstream pathways[46,47].

Although no evident intestinal mucosa inflammation was found, the immunohistochemical analysis showed an increase in CD3+ T cells within the intestinal mucosa of Smad3 null mice, compared to wild-type mice, which is consistent with previous data[31]. In addition, increased TGF-β1 and Smad7 staining was observed in the intestinal mucosa of Smad3 null mice. The constitutively high TGF-β expression in the intestine could account for the positive counteractive mechanism due to the loss of intracellular transduction signals in Smad3 null mice. TGF-β overexpression could not be attributed to monocytes and macrophages not increased in the intestinal mucosa[31,32], nor to TGF-β autoinduction under the control of Smad3[32,33]. On the other hand, increased TGF-β expression could be attributed to the increased T cells in the intestinal mucosa or to the platelet degranulation not assessed in this study. The mechanism that may upregulate Smad7 expression is not clear. Moreover, Smad7 is strongly and rapidly induced by TGF-β itself[48]. Efficient expression of Smad7 appears to depend upon the cooperation of Smad, Sp1 and AP-1 transcription factors[49]. In Smad3 null mice, the overexpression of TGF-β1 may induce Smad2 phosphorylation which in turn could upregulate Smad7 intracellular expression even in the absence of Smad3. In some cell types, Smad7 expression is induced by other signalling pathways, such as Jak1/Stat1 pathway following stimulation with interferon (INF)-γ and activated nuclear factor (NF)-kB following stimulation with tumor necrosis factor (TNF)-α [50,51]. Whatever the main inducible Smad7 factor is, high Smad7 expression levels interfere with activation of Smad2 and Smad3 or accelerate degradation of TGF-β receptors, inhibiting TGF-β/Smad signalling.

The lack of significant neutrophil/monocyte infiltrate in intestinal mucosa of Smad3 null mice could be related to the impaired chemotactic response toward TGF-β shown by mutant neutrophils and monocytes[32,33]. In contrast, mutant-activated T cells are resistant to the suppressive effect of TGF-β leading to their significant increase in the intestinal mucosa[31]. Furthermore, a reduced number of IgA+ plasma cells has been detected in the intestine of severely affected Smad3 null mice[31]. These data suggest that Smad3 plays a pivotal role in TGF-β-mediated regulation of mucosal immunity and local inflammatory response. Loss of these functions may also be responsible for infection and the high mortality rate of Smad3-null mice.

It has been reported that mice lacking Smad3 show accelerated healing of cutaneous incisional wounds with reduced inflammation and accumulation of matrix[32,33], and decreased cutaneous lesions and fibrosis after exposure to ionizing radiation[34] or subcutaneous injection of bleomycin[52]. Furthermore, loss of Smad3 could attenuate bleomycin-induced lung fibrosis[53], CCl4-induced liver fibrosis[54], and glomerular fibrosis induced by different methods[55] in mice, suggesting that Smad3 plays a pivotal role during tissue injury that leads to skin, lung, liver and kidney fibrosis[56].

Recently, it has also been reported that reduction of Smad3 accelerates re-epithelization in a murine model of colitis[57]. Amelioration of cutaneous radiation-induced fibrosis has also been obtained by halofuginone, which inhibits the formation of phospho-Smad2 and Smad3, increases inhibitory Smad7 expression, and decreases cytosolic and membrane TGF-β type II receptors[58]. There is increasing evidence that Smad3 may take part in recruitment of fibroblasts to the site of injury, differentiation of fibroblasts to myofibroblasts and regulation of collagen synthesis[33,34,59]. Since loss of Smad3 interferes with the effects of TGF-β on chemotaxis and autoinduction in inflammatory cells, and as well as induction of many ECM genes by TGF-β is also Smad3-dependent, this may explain why Smad3 null mice are resistant to cutaneous[33,34,52], pulmonary[53], hepatic[54] and renal fibrosis[55]. Based on these observations, we may hypothesize that mice lacking Smad3 would be also resistant to chronic intestinal inflammation and fibrosis. In fact, by inducing intestinal fibrosis with TNBS according to the protocol of Lawrance *et al*[38], we observed a significant reduction of intestinal fibrosis in knock out mice as compared to the wild-type mice in preliminary experiments (data not shown).

In conclusion, about 80% of Smad3 knock-out mice survive up to 6 mo of age without developing significant macroscopic and histological lesions of the small and large intestine, with the exception of a reduction in intestinal muscle layer thickness. Preliminary data shown that Smad3 knockout mice are resistant to intestinal fibrosis. This model could be a useful tool to unravel the molecular mechanisms of chronic intestinal inflammation and fibrosis.

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BASIC RESEARCH

Neutrophil depletion-but not prevention of Kupffer cell activation-decreases the severity of cerulein-induced acute pancreatitis

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Abstract

AIM: To determine whether neutrophil depletion and Kupffer cell inhibition might combine their protective effects to decrease the severity of acute pancreatitis.

METHODS: Mice had cerulein administration to induce acute pancreatitis and were pretreated with either anti-mouse neutrophil serum or gadolinium chloride ($GdCl_3$) to prevent Kupffer cell activation, or both treatments. Injury was assessed in pancreas and lungs. Myeloperoxidases (MPO) assessed neutrophil infiltration. Interleukin-6 (IL-6) and IL-10 were measured in serum, pancreas, lungs and liver.

RESULTS: In mice with acute pancreatitis, neutrophil depletion reduced the severity of pancreatitis and pancreatitis-associated lung injury. Kupffer cell inactivation by $GdCl_3$ had less protective effect, although IL-6 and IL-10 concentrations were significantly decreased. The protective treatment brought by neutrophil depletion was not enhanced by Kupffer cell inactivation and both treatments did not combine their protective effects.

CONCLUSION: Our results confirm the role of activated neutrophils in aggravating organ injury in acute pancreatitis while the role of Kupffer cell activation is less obvious.

Key words: Acute pancreatitis; Cytokines; Neutrophils; Kupffer cells; Pulmonary injury

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INTRODUCTION

During acute pancreatitis, the pathophysiology of pancreatic injury includes inflammatory processes and, following early activation of trypsinogen in acinar cells, recruitment of inflammatory cells aggravates pancreatic damage[1,2]. Upon activation, circulating inflammatory cells adhere to vascular endothelial cells and transmigrate across the endothelial barrier within injured areas in pancreas as well as in remote organs. Thus, mice deficient in adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1)[3] on endothelial cells or animals treated with an antineutrophil serum (ANS)[3,4] are protected from pancreatitis and remote injuries. However, the protection remained incomplete[3,4].

In contrast to the severe lung injury frequently associated with pancreatitis, hepatic injury is minor during acute pancreatitis[5]. Nevertheless, hepatic functions are modified by pancreatitis. Detoxification is altered early in the evolution of the disease[6,7] and this decreased hepatic detoxification is compensated by an increased detoxification in blood, lungs, and intestine macrophages[7]. Moreover, mediators released from the damaged pancreas directly activate Kupffer cells and promote important inflammatory responses in the liver.

Elastase administration, either in the peritoneum[8] or in perfused livers[9] mimics this important inflammatory response. The role of the liver in acute pancreatitis is then to propagate pancreatic injury to lungs. Portocaval shunts (that dilute harmful pancreatic mediators in the systemic circulation)[5,10] and inhibitors of Kupffer cell activation such as gadolinium chloride ($GdCl_3$)[11] and liposome-encapsulated dichloromethylene diphosphonate (DMDP)[12] decrease acute pancreatitis-associated-lung injury.

Because neutrophil depletion or prevention of Kupffer cell activation is only partially effective in decreasing the severity of acute pancreatitis, the aim of our study was to determine whether both treatments might combine their protective effects.

MATERIALS AND METHODS

Animals

Breeding pairs of C57BL6 mice were purchased from Charles River (Saint-Germain sur l'Arbreles, France) and bred and housed in temperature-controlled (23 ± 2 ℃) cages with a 12 h light/dark cycle. They were fed with standard laboratory chow, given water *ad libitum*, and randomly assigned to control or experimental groups. The experimental protocol was reviewed and approved by the Ethics Committee for Animal Research of the University of Geneva and the Veterinary Office in Geneva and followed the guidelines for care and use of laboratory animals.

Induction of acute pancreatitis

Male mice (20-22 g) were intraperitoneally injected hourly for 10 h (10 injections) with a supramaximally stimulating dose of cerulein (CER, 50 μg/kg in 0.2 mL saline solution) to elicit a secretagogue-induced pancreatitis. Cerulein, the analog of the pancreatic secretagogue cholecystokinin was purchased from Research Plus (Bayonne, NJ, USA). Control (CONT) mice were injected with saline solution. One hour after the final injection, mice were sacrificed by a lethal intraperitoneal injection of pentobarbital.

Neutrophil depletion

Twelve hour before the start of cerulein administration, mice were treated with 0.2 mL rabbit anti-mouse neutrophil serum (ANS, ip injection, Accurate Chemical and Scientific Corp, Westbury, NY) as previously described[3].

Kupffer cell inactivation

To prevent Kupffer cell activation, gadolinium chloride ($GdCl_3$, 1 mg/100 g of body weight, Sigma, Basel, Switzerland) was injected into mice tail vein 12 h and 1 h before the start of cerulein or saline administration. Another experimental group received both treatments.

Experimental groups

Five experimental groups were studied: CONT mice had saline injection and no treatment; CER mice had cerulein administration and were treated with saline; CER + ANS mice had cerulein administration and were treated with anti-mouse neutrophil serum; CER + $GdCl_3$ mice had cerulein administration and were treated with gadolinium chloride; CER + ANS + $GdCl_3$ mice had cerulein administration and were treated with ANS and $GdCl_3$.

Quantification of cerulein-induced injuries

At the time of sacrifice (1 h following the last cerulein injection), blood was collected from the heart, centrifuged for 10 min, and the serum was kept at -80 ℃ until assayed. Serum amylase activity was measured using 4,6-ethylidene (G1)-p-nitrophenyl (G1)-αD-malto-heptoside (Sigma Chemical Company, St. Louis, Missouri) as substrate.

To quantify pancreatic edema, part of the pancreas was removed to measure tissue water content. Pancreatic tissue was weighed before and after desiccation at 95 ℃ during 24 h. The difference between the wet and dry tissue weights was calculated and expressed as a percent of tissue wet weight. The other parts of pancreatic tissues were frozen in liquid nitrogen for cytokine measurements.

Neutrophil sequestration within pancreas was detected by measuring tissue myeloperoxidase activity (MPO)[13]. Pancreatic samples were thawed, homogenized in 1 mmol/L in 20 mmol/L phosphate buffer (pH 7.4), centrifuged (10000 g, 10 min), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecylmethylammonium bromide. The suspensions were subjected to four cycles of freezing and thawing and sonicated. The samples were centrifuged (10000 g, 5 min) and the supernatants were used to measure MPO activity. The reaction solution included 1.6 mmol/L tetramethylbenzidine (Sigma, Switzerland), 80 mmol/L sodium phosphate buffer (pH 5.4) and 0.3 mmol/L hydrogen peroxide (Sigma, Switzerland). Samples in solution were incubated at 37 ℃ for 2 min and the absorbance was read by an autoanalyzer. MPO activity was expressed as U/mg tissue dry weight.

Pulmonary microvascular permeability was evaluated by quantitating the leakage of intravenously injected fluorescein isothiocyanate (FITC)-labeled bovine albumin (0.5 mg/kg in 0.2 mL) into the bronchoalveolar space (BAL)[14]. Immediately after sacrifice, the trachea was exposed and the lungs were injected three times with saline solution (1 mL). The lavage fluid was collected and FITC fluorescence was measured in the lavage fluid and serum using a Hitachi fluorospectrophotometer (excitation wave: 494 nm; emission wave: 520 nm). The ratio of fluorescence between BAL and blood was calculated and expressed the pulmonary permeability index.

Measurements of cytokines

Interleukin-6 (IL-6) and interleukin-10 (IL-10) were quantified in serum, pancreas, lung and liver using the commercially available enzyme linked immunosorbent assay kits (R&D, Abingdon, UK). Freshly isolated tissues were homogenized in 1.5 mL phosphate buffer (20 mmol/L, pH 7.4). After centrifugation (14000 g for 5 min at 4 ℃), IL-6 and IL-10 concentrations were measured in the supernatant according to the manufacturer's recommendations and expressed as pg per mg proteins in tissues and pg/mL in serum. The mean concentrations of IL-6 and IL-10 mea-

Table 1 Pancreatic injury in control (CONT) mice and mice treated with cerulein (CER)

	CONT	CER	
Serum amylase (UI/L)	1582 [1236 - 1850]	19528 [16369 - 26457]	$P < 0.0001$
Pancreatic water content (% wet weight)	0.74 [0.71 – 0.76]	0.80 [0.78 - 0.83]	$P < 0.0001$
Pancreatic MPO activity (U/mg tissue dry weight)	322 [125 - 456]	2738 [1978 - 3245]	$P = 0.0002$
Pancreatic necrosis (%)	1.26 [0.5 – 2.3]	27.7 [24.5 – 35.0]	$P = 0.0001$

MPO = myeloperoxidase. n ⩾ 5 in each group. Data in CER and CONT groups were compared with Mann-Whitney test.

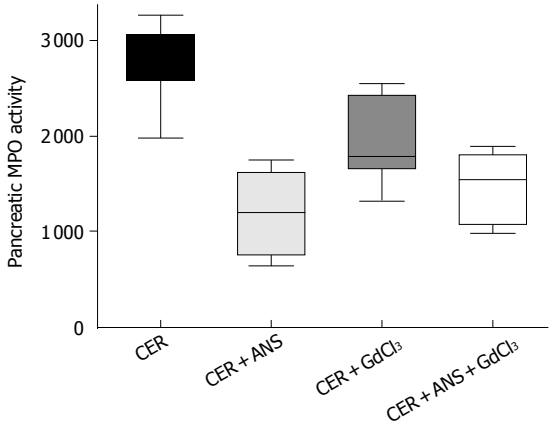

Figure 1 Pancreatic myeloperoxidase (MPO, U/mL tissue dry weight) activity in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + $GdCl_3$), CER + ANS + GdCl3. n ⩾ 5 in each group. Kruskall Wallis analysis with Dunn posthoc test found a significant difference between CER and CER + ANS or CER + ANS + GdCl3 mice.

sured in CER mice served as the 100% baseline values.

Morphology

At the time of sacrifice, pancreatic tissues were rapidly removed, fixed in 4% neutral phosphate-buffered formalin, embedded in paraffin, and sectioned (5 μm). After staining with hematoxylin-eosin, sections were examined by two morphologists who were not aware of the sample identity. The extent of acinar cell necrosis was quantified by computer-assisted morphometry and expressed as a function of total acinar tissue. Acinar cell necrosis included destruction of normal pancreatic architecture in combination with inflammation. After exclusion of non-acinar cells (islets of Langerhans and perivascular and periductular adventitial tissue), the amount of acinar necrosis were morphometrically quantified using a computerized image analysis video unit (Zeiss Camera, Zeiss, Bern, Switzerland).

For pulmonary morphology, a polyvinyl catheter was inserted into the trachea and used to instill 4% neutral buffered formalin into the lungs with a hydrostatic pressure of 30 cm H_2O. Formalin-distended lungs were harvested, fixed, paraffin-embedded, sectioned (5 μm) and stained.

Statistical analysis

Data are median [minimum - maximum] and differences between groups were analyzed by Mann-Whitney test (comparison between CONT and CER mice) or Kruskal-Wallis and Dunn posthoc tests (comparisons between CER, CER + ANS, CER + $GdCl_3$, and CER + ANS + $GdCl_3$). A P value < 0.05 was considered significant.

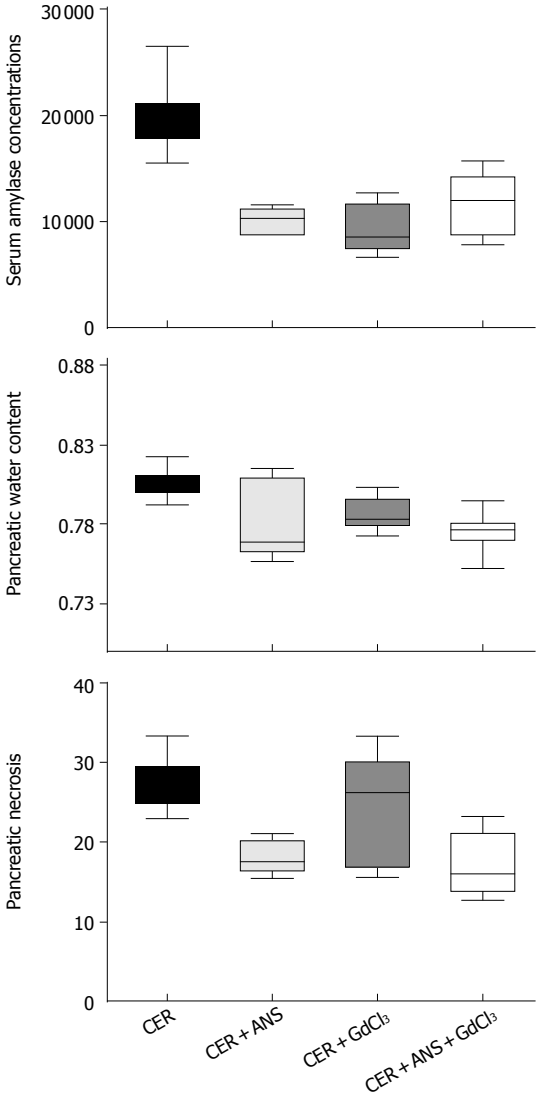

Figure 2 Serum amylase concentration (UI/L), pancreatic water content (% wet weight), and pancretic necrosis (%) in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + $GdCl_3$), CER + ANS + $GdCl_3$. $n \geqslant 5$ in each group. Kruskall Wallis analysis with Dunn posthoc test found a significant difference between CER and CER + ANS, CER + $GdCl_3$, and CER + ANS + $GdCl_3$ (amylase concentrations) and CER and CER + ANS or CER + ANS + $GdCl_3$ mice (pancreatic water content and pancreatic necrosis).

RESULTS

Acute pancreatitis

Cerulein administration induced severe acute pancreatitis. Serum amylase concentrations and pancreatic water content markedly increased in CER mice in comparison to CONT mice (Table 1). Pancreatic injury was also characterized by an increase in acinar cell necrosis and MPO activity.

Pre-treatment with ANS before acute pancreatitis induction significantly decreased pancreatic MPO activity ($P < 0.001$, Figure 1) with a concomitant decrease in

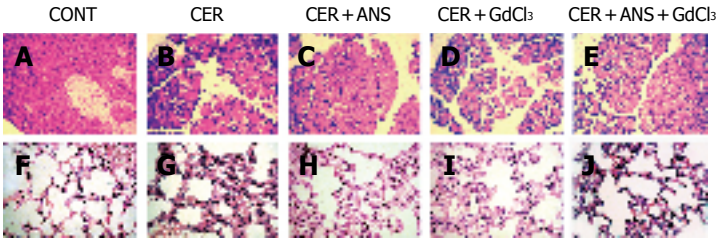

Figure 3 Representative sections of pancreas (upper panels) and lung (lower panels) stained with hematoxylin and eosin. Mice were injected with saline solution (CONT, **A** and **F**), cerulein (CER, **B** to **E** and **G** to **J**) and treated with antineutrophil serum (CER + ANS, **C** and **H**), gadolinium chloride (CER + $GdCl_3$, **D** and **I**) or both treatments (CER + ANS + $GdCl_3$, **E** and **J**). These illustrations represent sections from ⩾ 3 mice. Magnification x400.

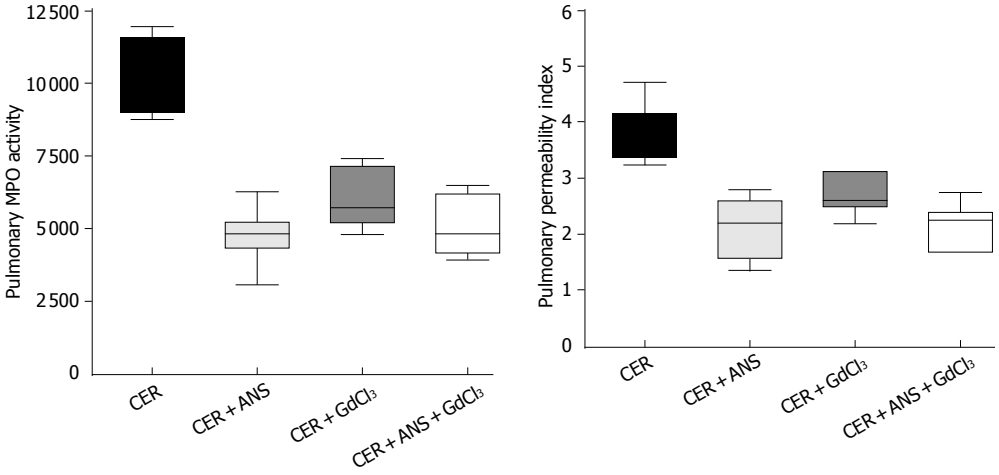

Figure 4 Pulmonary myeloperoxydase activity (MPO, U/mg tissue dry weight) and pulmonary permeability index in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + $GdCl_3$), CER + ANS + $GdCl_3$. $n \geqslant 5$ in each group. Kruskall Wallis analysis with Dunn posthoc test found a significant difference between CER and CER + ANS or CER + ANS + $GdCl_3$ mice (pulmonary MPO and permeability index).

Table 2 Pulmonary injury in control (CONT) mice and mice treated with cerulein (CER)

	CONT	CER	
Pulmonary MPO activity (U/mg tissue dry weight)	947 [782 - 1145]	10173 [8710 - 12035]	$P < 0.0001$
Pulmonary permeability index	0.57 [0.5 – 0.8]	3.9 [3.1 - 5.1]	$P = 0.0002$

MPO = myeloperoxidase. Pulmonary permeability index: ratio of FITC-labeled bovine albumin between bronchoalveolar lavage and serum. $n \geqslant 5$ in each group. Pulmonary MPO activity and pulmonary permeability index were compared by Mann-Whitney test.

Table 3 IL-6 and IL-10 concentrations in serum, pancreas, lungs, and livers in control (CONT) mice and mice treated with cerulein (CER)

	CONT	CER	
Serum IL-6 (pg/mL)	8.9 [8.7 - 11.4]	267.5 [98.0 - 312.0]	P = 0.02
Serum Il-10 (pg/mL)	11.8 [8.9 - 14.7]	18.7 [10.9 - 21.6]	P = 0.23
Pancreatic IL-6 (pg/mg protein)	1.2 [0.5 - 1.9]	8.4 [6.4 - 13.2]	P = 0.02
Pancreatic IL-10 (pg/mg protein)	0.08 [0.03 - 0.20]	0.32 [0.20 - 0.79]	P = 0.03
Pulmonary IL-6 (pg/mg protein)	1.5 [0.5 - 2.1]	3.0 [2.4 - 6.9]	P = 0.004
Pulmonary IL-10 (pg/mg protein)	0.10 [0.01 - 0.20]	0.87 [0.42 - 3.80]	P = 0.06
Hepatic IL-6 (pg/mg protein)	1.15 [0.80 - 1.60]	8.20 [5.60 - 11.52]	P = 0.006
Hepatic IL-10 (pg/mg protein)	0.12 [0.05 - 0.20]	0.43 [0.33 - 0.48]	P = 0.06

$n \geqslant 5$ in each group. Data in CER and CONT groups were compared by Mann-Whitney test.

pancreatic injury: serum amylase concentration ($P < 0.01$, Figure 2), pancreatic water content ($P < 0.01$, Figure 2), and acinar cell necrosis significantly decreased ($P < 0.01$, Figure 2). However, pre-treatment with ANS did not fully protect against pancreatic injury. Morphologic studies confirmed these findings (Figure 3).

Prevention of Kupffer cells activation by $GdCl_3$ decreased serum amylase concentration ($P < 0.05$) but did no change significantly pancreatic necrosis and pancreatic water content (Figure 2). $GdCl_3$ had no effect on pancreatic MPO activity (Figure 1). The decreased pancreatic injury was similar CER + ANS and CER + ANS + $GdCl_3$ mice.

Acute pancreatitis-associated lung injury

Because acute pancreatitis is frequently associated with injury in remote organs, we determined the consequences of both treatments on lung injury. CER administration increased pulmonary MPO activity and the pulmonary permeability index (Table 2). Lung injury is also evidenced by a marked thickening of the alveolar-capillary membrane (Figure 3).

Pre-treatment with ANS significantly decreased MPO activity ($P < 0.001$) and the pulmonary permeability ($P < 0.01$) induced by CER, while $GdCl_3$ did not (Figure 4). Pulmonary injury was similarly prevented in CER + ANS and CER + ANS + $GdCl_3$ mice.

Cytokine expression

CER administration greatly increased IL-6 concentrations in serum but not that of IL-10 (Table 3). CER administration increased expression of both cytokines in liver, pancreas, and lungs.

$GdCl_3$ pretreatment significantly attenuated IL-6 and IL-10 concentrations in liver ($P < 0.01$ for both IL),

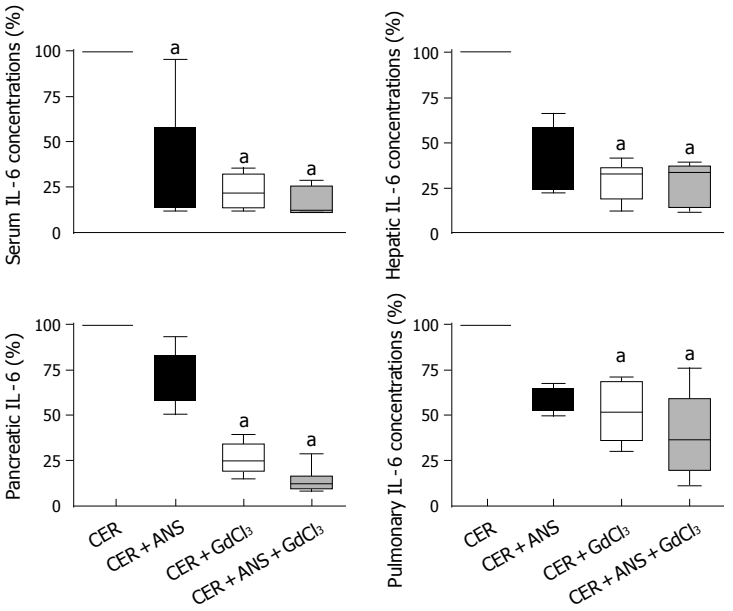

Figure 5 Interleukin-6 (IL-6, %) concentrations in serum, pancreas, lung and liver the 100% value corresponding to the mean value measured in mice injected with cerulein (CER). Mice were injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + $GdCl_3$), and CER + ANS + $GdCl_3$. $n \geqslant 5$ in each group. $^aP < 0.05$ *vs* CER.

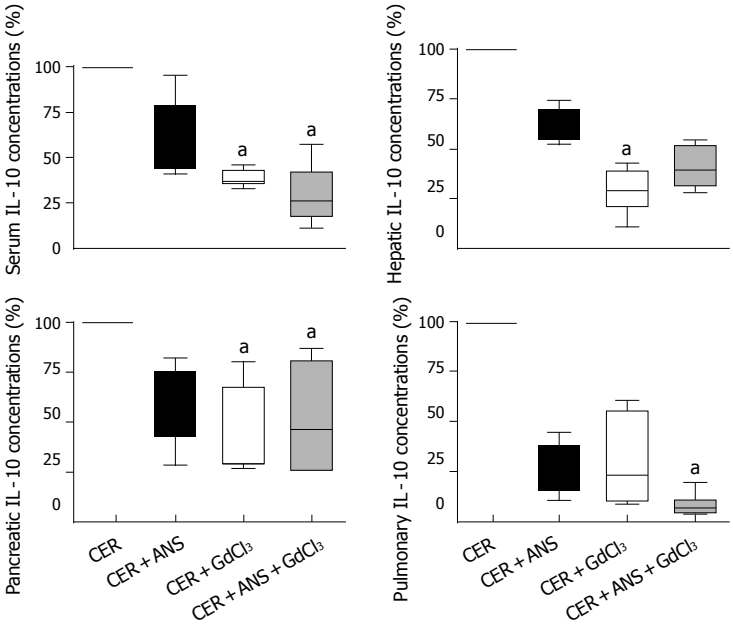

Figure 6 Interleukin-10 (IL-10, %) concentrations in serum, pancreas, lung and liver the 100% value corresponding to the mean value measured in mice injected with cerulein (CER). Mice were injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + $GdCl_3$), and CER + ANS + $GdCl_3$. $n \geqslant 5$ in each group. $^aP < 0.05$ *vs* CER.

pancreas ($P < 0.05$ and $P < 0.01$ respectively), and serum ($P < 0.05$ for both IL-6 and IL-10) (Figures 5 and 6). IL-6 concentrations in lungs were decreased by $GdCl_3$ ($P < 0.05$) but IL-10 concentrations were not. ANS did not significantly modify IL-6 and IL-10 concentrations in liver, pancreas, and lungs. IL-6 concentrations in serum was decreased ($P < 0.05$) but serum IL-10 concentrations were not modified by ANS pretreatment.

DISCUSSION

In mice with acute pancreatitis, neutrophil depletion reduced the severity of pancreatitis and pancreatitis-associated lung injury. Kupffer cell inactivation by $GdCl_3$ had less protective effect, although IL-6 and IL-10 concentrations in tissues were significantly decreased. The protective treatment brought by neutrophil depletion was not enhanced by inhibition of Kupffer cell activation and both treatments did not combine their protective effects.

Neutrophil infiltration in pancreas and lungs

Following cerulein injections in mice, neutrophil infiltration (assessed by MPO activity) was observed 12 h after the first injection. However, we previously showed that MPO activity peaks later by 24 h in pancreas and 36 h in lungs after the first injection and remains elevated by d 7[14]. This neutrophil infiltration in tissues plays an important role in the development of acute pancreatitis and pancreatitis-associated lung injury. Thus, mice deficient in adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on endothelial cells[3] or animals treated with an antineutrophil antibody[3,4] are protected from pancreatitis and remote injuries. However, the protection remained partial[3, 4]. This incomplete protection suggests that either the dose of ANS used is insufficient to completely remove all neutrophils or that additional pathways must be targeted to obtain a full protection. We previously showed that ANS injection (0.2 mL) in mice reduced the circulating neutrophils by 85%[3] while 0.5 mL/100 g in rats decreased

the circulating neutrophils to below 200 cells/mL[4]. Thus, neutrophil depletion with ANS is correct and the residual MPO activities in lungs and pancreas after ANS treatment (Figures 2 and 4) might reflect the presence of local macrophages. An incomplete protection by ANS has already been shown in mice[3] or rats[4] treated with cerulein. Consequently, an additional independent pathway, Kupffer cell inactivation, was targeted to further prevent acute pancreatitis.

Role of the liver in acute pancreatitis

The role of the liver in propagating pancreatic injury to lungs has been evidenced by inactivating Kupffer cell or diverting blood from the pancreatic vein to the systemic circulation. Thus, portocaval shunts (that dilute harmful pancreatic mediators in the systemic circulation)[5,10] and inhibitors of Kupffer cell activation such as gadolinium chloride ($GdCl_3$)[11] and liposome-encapsulated dichloromethylene diphosphonate (DMDP)[12] significantly decrease acute pancreatitis-associated-lung injury. As expected, we showed that $GdCl_3$ pretreatment decreased IL-6 and IL-10 concentrations in the liver (Figures 5 and 6). In isolated rat livers, perfusion of pancreatic elastase activates Kupffer cells with subsequent NF-$\varkappa$B activation and TNF-α overproduction and this effect is abolished by $GdCl_3$ pre-treatment[9]. $GdCl_3$ is specifically taken up by Kupffer cells and is not toxic by itself (when the injected dose is 100 mg/ kg, iv), as previously published by Gloor *et al*[15]. However, in our study, the pulmonary permeability index and MPO activity were not significantly decreased in comparison to CER mice (Figure 4). Pulmonary IL-6 concentrations in CER + $GdCl_3$ mice were lower than in CER mice but the IL-10 concentration decrease did not reach significance.

$GdCl_3$ pretreatment also decreased serum IL-6 and IL-10 concentration as previously published[15]. In contrast to Gloor *et al*[15] we found that $GdCl_3$ also decreased pancreatic IL-6 and IL-10 concentrations, as well as serum amylase concentration. Pancreatic necrosis, water content, and pancreatic MPO were not different between CER + $GdCl_3$ mice and CER mice, as previously described[15]. Our results either question the specific inactivation of hepatic macrophages by $GdCl_3$ or suggest that the decreased cytokine concentrations in pancreas reflect a decreased systemic inflammatory response. In a severe form of acute pancreatitis, $GdCl_3$ pretreatment was able to reduce the mortality rate[15]. The beneficial effect of $GdCl_3$ on the mortality rate remains puzzling because $GdCl_3$ administration decreases IL-10 concentrations, a cytokine which has a protective effect on the evolution of acute pancreatitis[16,17].

Kupffer cell inactivation has also been induced by other drugs such as liposome-encapsulated dichloromethylene diphosphonate which decreases apoptosis and TGF-β production in liver[12] or CNI-1493 which reduced pancreatitis-associated liver injury through TNF-α and IL-1 expression[18].

In conclusion, our results confirm the role of activated neutrophils in aggravating organ injury in acute pancreatitis while the role of Kupffer cell activation is less obvious. Another more early event should probably be targeted in combination with neutrophil depletion to fully prevent the initiation of the disease.

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BASIC RESEARCH

Effect of hypercholesterolemia on experimental colonic anastomotic wound healing in rats

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Abstract

AIM: To evaluate the mechanical and biochemical parameters of colonic anastomotic healing in hypercholesterolemic rats.

METHODS: Sixty rats were divided into two groups of 30 each according to their dietary regimens. The test group was fed with a high cholesterol-containing diet for two months while the control group had standard diet. These two groups were further divided into three subgroups consisting of ten rats each. After hypercholesterolemia was established, left colon resection and anastomosis were performed in both groups and samples from liver and abdominal aorta were taken to evaluate the systemic effects of hypercholesterolemia. Anastomotic wound healing, blow-out pressures and tissue hydroxyproline levels were evaluated.

RESULTS: The test group had a significant weight gain in two months. Microscopic examination of the abdominal aorta revealed no atherosclerotic change in none of the groups, but liver tissue specimens showed significant steatosis in the test group. Tissue hydroxyproline levels and anastomotic blow-out pressures were significantly lower in the test group than in the controls.

CONCLUSION: Hypercholesterolemia not only increases hydroxyproline levels and blow-out pressures but also worsens anastomotic wound healing.

Key words: Hypercholesterolemia; Colonic anastomosis; Anastomotic wound healing

INTRODUCTION

Hypercholesterolemia is the major etiologic factor for atherosclerosis in Western countries[1]. However, the effects of hypercholesterolemia on anastomotic wound healing have not been studied.

Leakage in colonic anastomosis has a higher incidence of morbidity and mortality rate compared with that in small intestinal anastomosis. The overall leakage incidence of intestinal anastomosis is 2-35%[2]. Most of them are minimal and can be limited by the host defense mechanisms. Systemic and local factors play a role in anastomotic wound healing. Anemia, hypovolemia, low arterial PO_2, neutropenia, low O_2 saturation, malnutrition, vitamin deficiencies, zinc deficiency, jaundice, uremia and high dose corticosteroids are some of the systemic factors, while local factors include infection, intestinal contents, prophylactic antibiotics, technique of suturing and suture material, radiation and mesenteric vascular occlusion[2-4].

In experimental studies, microscopic healing in small intestine is better than that in the colon[5,6]. Intestinal anastomotic wound healing can be evaluated mechanically, biochemically and histopathologically. Mechanical evaluation depends on the blow-out pressure of the anastomotic line. This pressure can change with the inflation rate of the intestine or the in situ procedure[2,7-9]. Continuous suturing technique has no effect on this pressure[10].

Biochemically, collagen production rate and amount in the anastomosis line are the factors to be measured. Since the majority of the collagens are hydroxyproline, all measurements are made by detecting the hydroxyproline content[7,11,12]. Histopathologically, the only evidence is the infiltration of several kinds of inflammatory cells[2,7].

In this study, the mechanical and biochemical parameters of colonic anastomotic wound healing were evaluated in rats fed with a high cholesterol containing diet (2% cholesterol + 1% colic acid: Sigma Co., Manchester, UK) for two months[13]. Abdominal aortic and liver tissue samples were also obtained to evaluate the systemic effects of hypercholesterolemia.

Table 1 Pre- and post-feeding body weights of hypercholesterolemic rats

	Pre-feeding weight (g)	Post-feeding weight (g)
Rat 1	250	325
Rat 2	255	330
Rat 3	245	350
Rat 4	240	310
Rat 5	260	325
Rat 6	250	335
Rat 7	275	360
Rat 8	280	355
Rat 9	270	350
Rat 10	240	340
Mean	256.5	338

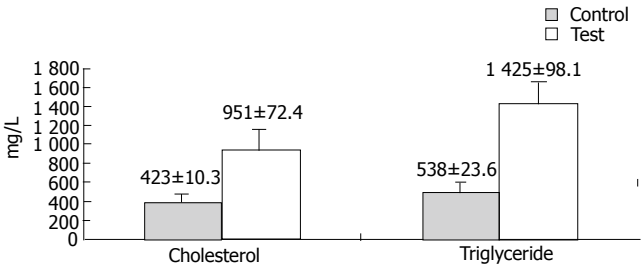

Figure 1 Mean cholesterol and triglyceride level in test and control groups.

Figure 2 Insertion of an infusion pump into the colon.

Figure 3 Anastomosis line.

MATERIALS AND METHODS

Experimental animals and grouping

Sixty rats of both sexes weighing 240-300 g used in this study were divided into two groups according to their dietary regimens. The test group consisted of 30 rats fed with a high cholesterol diet for two months while the control group had standard diet for the same period. The test group had a significant weight gain at the end of this period (Table 1).

These two groups were further divided into three subgroups, ten rats each group. Ten milliliter blood was taken from each rat for measurement of serum total cholesterol and triglyceride levels. In the test group, serum total cholesterol and triglyceride levels were significantly higher than in the control group (Figure 1). After hypercholesterolemia was established, left colon resection and anastomosis were performed in both groups and tissue samples were taken from liver and abdominal aorta.

Surgical technique

After an overnight fasting, intramuscular ketamin HCl (Ketalar, Parke Davies) was administered (50 mg/kg). One percent povidon-iodine was used for local cleansing. A midline laparotomy was performed and a segment of left colon (1 cm) was resected. Colo-colonic anastomosis was made using 7/0 polyproene (Prolene, Ethicon Inc., UK) with 8-10 stitches for a layer. There was no postoperative mortality.

In both groups, 10 rats underwent relaparotomy on the third day and 10 rats on the seventh day. Colon lumen was obliterated 10 cm distal to the anastomosis using 3/0 silk ligature. Colon was transected 10 cm proximal to the anastomotic line and an infusion set was inserted into the lumen. This set was connected to an infusion pump (Life Care 5000 Infusion System, Abbot, Illinois, USA) and isotonic sodium chloride was given at a rate of 2 mL/min into the lumen (Figure 2). The pressure at the moment of the first leakage observed from the suture line was recorded as the blow-out pressure. All measurements were done *in situ*. After the measurements, tissue samples were taken from the liver and abdominal aorta for histopathological examination. The experiment was terminated by creating a pneumothorax.

In all groups, one centimeter segment of colon (including 0.5 cm proximal and 0.5 cm distal to the anastomotic line) was resected (Figure 3). The suture material was removed and the samples were kept in liquid nitrogen until the assay day.

Biochemical measurements were done as described previously[14]. The volume of hydroxyproline was defined as micromoles of hydroxyproline per gram of wet weight.

Statistical analysis

Mann Whitney U test was used for statistical analysis compare the blow-out pressures and hydroxyproline contents between groups. $P < 0.05$ was considered statistically significant.

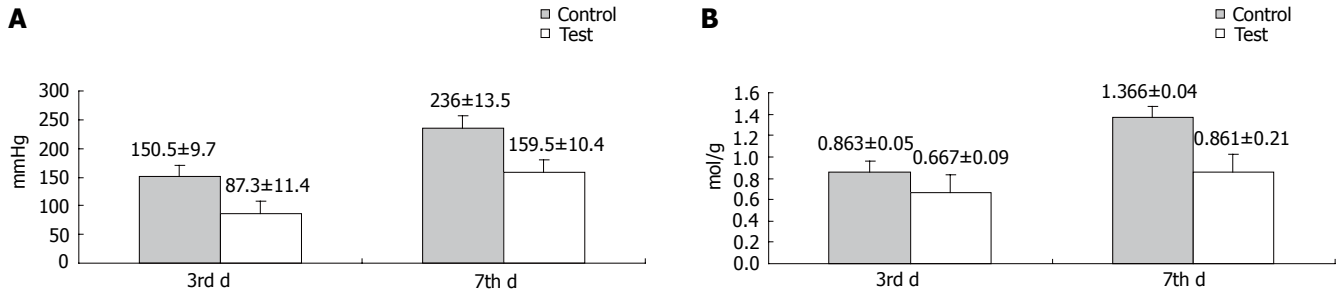

Figure 4 Mean blow-out pressure (**A**) and hydroxyproline level (**B**) in test and control groups.

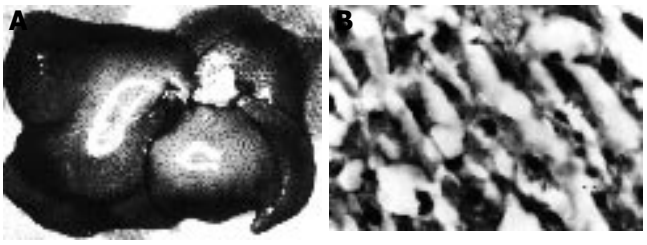

Figure 5 Macroscopic (**A**) and microscopic (**B**) steatosis in liver.

RESULTS

The rats in the test group gained significant weight (Table 1). The weight difference between the pre and post-feeding periods was statistically significant ($P < 0.05$).

After feeding with a high cholesterol containing diet for two months, a statistically significant difference serum cholesterol and triglyceride levels between the test and control groups was observed ($P < 0.05$, Figure 1). In the test group, serum total cholesterol level was 951 ± 72.4 mg/L and triglyceride level was 1425 ± 98.1 mg/L, while they were 423 ± 10.3 mg/L and 538 ± 23.6 mg/L in the control group, respectively.

On the third postoperative day, the mean blow-out pressure was 87.3 ± 11.4 mmHg in the test group and 150.5 ± 9.7 mmHg in the control group (Figure 4A). This difference was statistically significant ($P < 0.05$). The mean hydroxyproline level was 0.667 ± 0.09 in test group and 0.863 ± 0.05 in control group ($P < 0.05$) (Figure 4B).

On the seventh postoperative day, the blow-out pressure was 159.5 ± 10.4 mmHg in the test group and 236 ± 13.5 mmHg in the control group, while the hydroxyproline level was 0.861 ± 0.21 in the test group and 1.366 ± 0.06 in the control group, respectively. The difference in the parameters between the two groups was statistically significant ($P < 0.05$) (Figures 4A and 4B).

Liver tissue samples of the test group had a significant macroscopic steatosis compared to the control group (Figure 5A). In microscopic examination with hematoxilen-eosin staining, significant steatosis was also found in the test group (Figure 5B). No difference in microscopic results of the aortic specimens was found between the two groups.

DISCUSSION

In this study, left colon anastomosis was performed in hypercholesterolemic rats and the mechanical and biochemical parameters of anastomotic wound healing were investigated. Anastomotic wound healing in the test group was worse than that in the control group in terms of hydroxypline levels and blow-out pressures. Significant steatosis was found in the liver specimens but no atherosclerotic changes in the aortic specimens. This finding is in accordance with several studies[15-17].

Factors influencing colonic healing have been widely studied[2,4]. Since blood flow is the major component of wound healing, hypercholesterolemia-induced atherosclerosis has a detrimental effect on intestinal anastomotic wound healing. However, the effects of hypercholesterolemia start long before the onset of atherosclerosis[16-20]. There are also reports on how hypercholesterolemia worsens vascular functions using different pathways of microendocrine system inside the endothelium[21-23]. Ross *et al*[24] have shown that monocytes accumulate on endothelium and damage it. Besides the macrovascular system, hypercholesterolemia also plays an important role in microvascular endothelial injury.

The cause of hypercholesterolemia-induced endothelial damage is predominantly determined by loss of endothelium-mediated relaxation. The mechanisms are as follows: decrease in the relaxing effect of NO/EDRF, ADP, thrombin, Ca^{++}-ionophore A 23187 on endothelium[2,16,21,25,26]; increase in platelet aggregation and turnover as well as the sensitivity of platelets to aggregating substances due to the modification of effects of PGI_2 and TXA_2[27-29]; increase in the release of endothelium-derived vasoconstrictor substances such as serotonin and TXA_2 through the cyclooxygenase pathway[22,27].

The above effects have been shown *in vivo* and *in vitro* in experimental models of rabbit aorta, monkey iliac artery, pig coronary artery and human coronary arteries[28]. Our study did not investigate these effects, but the effect of hypercholesterolemia on hydroxyproline level and blow-out pressure was emphasized.

Why hypercholesterolemia worsens anastomotic wound healing is still unknown. Luminal narrowing is the net result of atherosclerosis in both macro and microvascular systems. Consequently, ischemia and related injuries occur. Hypercholesterolemia is the major risk factor for atherosclerosis. In early phase of hypercholesterolemia, no morphological change takes place in arterial wall but vasospasm occurs due to neurohumoral mechanisms[30]. Atherosclerosis is an irreversible process but hypercholesterolemia is reversible and these effects are preventable.

In our study, anastomotic wound healing in the test group was worse than that in the control group. Since hypercholesterolemia predominantly affects the endothelium, this can partly be explained as a result of endothelial damage-induced vasospasm. But we did not measure the anastomotic blood flow or assess the endothelial injury. Although a relationship between hypercholesterolemia and wound healing was demonstrated, our results could not fully explain the mechanisms. Further studies are needed to reveal the relationship between hypercholesterolemia and anastomotic wound healing.

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BASIC RESEARCH

Cloning of α-β fusion gene from *Clostridium perfringens and its expression*

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Abstract

AIM: To study the cloning of α-β fusion gene from *Clostridium perfringens* and the immunogenicity of α-β fusion expression.

METHODS: Cloning was accomplished after PCR amplification from strains NCTC64609 and C58-1 of the protective antigen genes of α-toxin and β-toxin. The fragment of the gene was cloned using plasmid pZCPAB. This fragment coded for the gene with the stable expression of α-β fusion gene binding. In order to verify the exact location of the α-β fusion gene, domain plasmids were constructed. The two genes were fused into expression vector pBV221. The expressed α-β fusion protein was identified by ELISA, SDS-PAGE, Western blotting and neutralization assay.

RESULTS: The protective α-toxin gene (cpa906) and the β-toxin gene (cpb930) were obtained. The recombinant plasmid pZCPAB carrying α-β fusion gene was constructed and transformed into BL21(DE3). The recombinant strain BL21(DE3)(pZCPAB) was obtained. After the recombinant strain BL21(DE3)(pZCPAB) was induced by 42℃, its expressed product was about 22.14% of total cellular protein at SDS-PAGE and thin-layer gel scanning analysis. Neutralization assay indicated that the antibody induced by immunization with α-β fusion protein could neutralize the toxicity of α-toxin and β-toxin.

CONCLUSION: The obtained α-toxin and β-toxin genes are correct. The recombinant strain BL21(DE3)(pZCPAB) could produce α-β fusion protein. This protein can be used for immunization and is immunogenic. The antibody induced by immunization with α-β fusion protein could neutralize the toxicity of α-toxin and β-toxin.

Key words: *Clostridium perfringens*; α-β fusion gene; Cloning and expression

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INTRODUCTION

Clostridium perfringens is an anaerobe, forming a part of normal intestinal flora in humans and animals[1]. This organism is the causative agent of diseases such as gas gangrene, hemorrhagic enteritis, enterotoxemia[2]. It is also a secondary pathogen in various diseases, such as necrotic enteritis[3]. *Clostridium perfringens* can produce extra cellular toxins and enzymes including α-toxin and β-toxin produced by *Clostridium perfringens* types A and C respectively. These toxins are significant virulence factors in case of gas gangrene or clostridial myonecrosis[4,5].

In this study, α-toxin and β-toxin protective antigen genes were amplified by PCR from *Clostridium perfringens* type A strain NCTC64609 and type C strain C58-1 respectively. The recombinant plasmid pZCPAB carrying α-β fusion gene was constructed by transforming it into BL21(DE3). The fusion protein that could produce α-β was obtained. The expressed α-β fusion protein could resist the attack of α-toxin or β-toxin.

MATERIALS AND METHODS

Materials

Clostridium perfringens type A strain NCTC64609 and type C strain C58-1 were purchased from China Institute of Veterinary Drug Control. Plasmid pBV221 and *E coli* DH5α, BL21(DE3) were kept in our Laboratory. Plasmid pMD18-T was purchased from TaKaRa Company. Taq DNA polymerase, T4 DNA ligase, restriction endonuclease and DNA markers DL2000 were purchased from TaKaRa Company. DNA fragment recover kit, SDS, IPTG, Rnase A and lysozyme were purchased from Promega Company. Other reagents if not described, were of analytic purity.

Extraction of genomic DNA of clostridium perfringens

Clostridium perfringens strains were incubated in TYG me-

dium at 37℃ overnight. Bacterium cells were harvested by centrifugation, dissolved in TE buffer containing lysozyme(2 g/L) and incubated at 37℃ for 1 h. Then SDS(final concentration was 1%), EDTA(final concentration was 50 mmol/L) and proteinase K(final concentration was 150 mg/L) were added. The reaction mixture was incubated at 58℃ for 4 h and extracted three times with equal volume of saturated phenol. The aqueous phase was transferred to an Eppendorf tube. Then 0.2 volume of ammonium acetate(10 mol/L) and 2 volumes of ice-cold absolute alcohol were added, mixed well and deposited for 10 min at room temperature. Chromosome DNA deposition like clew was picked with a glass stick to another Eppendorf tube. Appropriate TE was added to dissolve DNA and stored at 4℃.

PCR amplification of α-toxin and β-toxin protective antigen genes

Chromosome DNA of *Clostridium perfringens* was amplified by PCR from genomic DNA using forward primers designed from the available sequence.

One set of primers of α-toxin protective antigen gene (cpa906) was synthesized. The *Eco*R Ⅰ and *Bam*H Ⅰ sites were inserted to the 5' and 3' ends of α-toxin genes via PCR. The sequence of α-toxin primers is as follows:

Up-stream primer:

P1: CCGGAATTCATGGGTCGGGATCCTGAT

*Eco*R Ⅰ

Down-stream primer:

P2: GGCGGATCCCTTAATTTTATATTATA

*Bam*H Ⅰ

Another set of primers of β-toxin protective antigen gene was synthesed. The *Bam*H Ⅰ and *Pst* Ⅰ sites were inserted to the 5' and 3' ends of β-toxin genes via PCR. The sequence of β-toxin primers is as follows:

Up-stream primer:

P1: 5'-CGCGGATCCAATGATATAGGTAAAACT-3'

BamH Ⅰ

Down-stream primer:

P2: 5'-CCGCTGCAGCTACTTAATAGCTGT-3

Pst Ⅰ

The following components were added into a 500 μL microcentrifuge tube. Each PCR contained 0.1 μmol/L primer1, 0.1 μmol/L primer2, 0.25 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 10 μL 10×PCR buffer and appropriate template DNA. Sterile distilled water was added into a 100 μL reaction system. After the tubes were heated at 95℃ for 5 min, 5 u *Taq* DNA polymerase was added into each tube. Thirty cycles of PCR were performed (denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min). The amplified α- and β-toxin genes were purified separately by agarose gel electrophoresis to remove primers and extraneous amplification products.

Cloning and sequencing of α-toxin and β-toxin protective antigen genes

The α-toxin and β-toxin protective antigen genes were separated and purified by agarose gel electrophoresis alongside DNA marker DL2000 and sequentially ligated to the pMD18-T cloning vector. Sequencing was performed with ABI 377 sequencer from Takara Company.

Construction of expression vector carrying α-β fusion gene

The α-toxin protective antigen gene and expression plasmid pBV221 were digested by restrictive enzymes *Eco*R I and *BamH* I respectively to generate cohesive ends. The α-toxin gene was inserted into the vector pBV221 and the recombinant vector pZBVA1 containing α-toxin protective antigen genes of *Clostridium perfringens* was constructed. Then the β-toxin protective antigen gene and the recombinant vector pZBVA1 were digested by restrictive enzymes *BamH* I and *Pst* I respectively to generate cohesive ends. The β-toxin gene was inserted into the vector pZBVA1 and recombinant expression vector pZCPAB containing α-β fusion gene was obtained. The sequence of inserted fragment was confirmed by DNA sequencing.

Growth condition for α-β fusion gene expression

The recombinant was transformed into *E. coli* BL21(DE3) and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping. *E. coli* BL21(DE3) cells transformed with the plasmids described above were grown in LB medium with 50 mg/mL ampicillin (1/50) at 30℃ to OD_{600}=0.4~0.6. The growth was conditioned at 42℃ for 5 h in order to express α-β fusion protein.

Analysis of bacterial samples

The cultured cells were collected by centrifugation at 5000 r/min for 10 min at 4℃. The optimum time of maximum expression of proteins was analyzed through SDS-PAGE. The expressed protein was tested through Western blot with specific antiserum. SDS-PAGE and Western blotting were performed as previously described[6, 7]. The antigen-binding activity of the α-β fusion protein was determined by ELISA as previously described[8]. For determination of protein expression, the absorption value at 560 nm was measured by thin-layer chromatogram scanner type CS-9000.

Protective effect of α-β fusion protein on mice

The 8-wk old Kunming mice were divided into 8 groups randomly, 10 mice in each group. In treatment groups, each mouse was inoculated with inactive recombinant strains that could express α-β fusion protein. In control groups, each mouse was inoculated with strains that could not express α-β fusion protein. After 4 wk, each mouse of 4 groups was treated with one minimum lethal dose (MLD) of α-toxin or β-toxin. Another 4 groups were treated with 2 MLD of α-toxin or β-toxin. The survived mice/total mice were counted in a week. The segments of liver, lung, heart, spleen and intestines of mice in treatment group and control group were tested by LM assay.

RESULTS

Gene cloning and identification of α-toxin gene from clostridium perfringens type A

The α-toxin gene from *Clostridium perfringens* type A strain NCTC64609 was isolated from genomic DNA-extracted template by PCR amplification. The reaction yielded a single product. Electrophoresis of PCR products confirmed

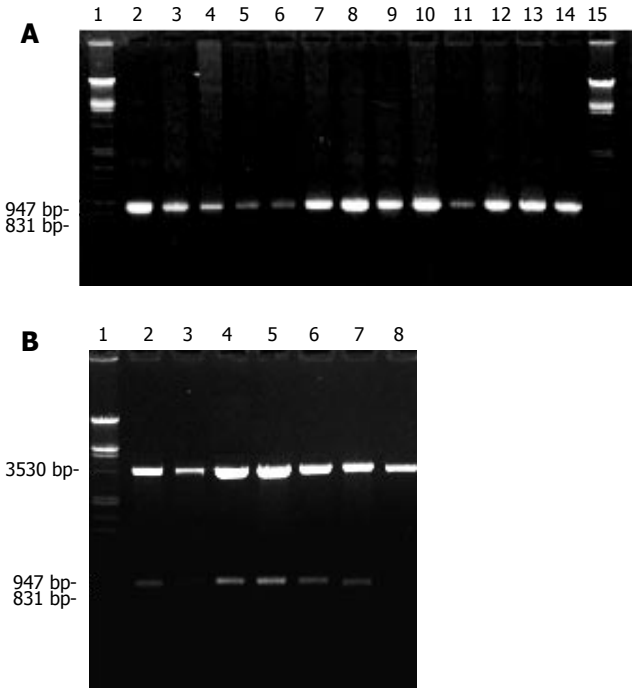

Figure 1 Identification of α-toxin gene product amplified by PCR (**A**) and recombinant plasmid by enzyme digestion (**B**). In A, lanes 1, 15: DNA markers; lanes 2-14: product of α-toxin gene amplified by PCR; In B, lane 1: DNA markers; lanes 2-7: pMD 906/ EcoR I + BamH I; lane 8: pMD 906/ EcoR I.

ATGGGTCGGGATCCTGATACAGATAATTTCTCAAAGGATAATAGTTGGTATTTAGCT
TATTCTATACCTGACACAGGGGAATCACAAATAAGAAAATTTTCAGCATTAGCTAGA
TATGAATGGCAAAGAGGAAACTATAAACAAGCTACATTCTATCTTGGAGAGGCTATG
CACTATTTTGGAGATATAGATACTCCATATCATCCTGCTAATGTTACTGCCGTTGATA
GCGCAGGACATGTTAAGTTTGAGACTTTTGCAAGAGGAAAGAAAAGAACAGTATAA
AATAAACACAGCAGGTTGCAAAACTATGAGGCTTTTTATACTGATATCTTAAAAAACA
AAGATTTTAATGCATGGTCAAAAGAATATGCAAGAGGTTTTGCTAAAACAGGAAAAT
CAATATACTATAGTCATGCTAGCATGCATAGTTGGGATGATTGGGATTATGCAGCAA
AGGTAACTTTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATTCTTACA
CGATGTATCAGAGGGTAATGATCCATCAGTTGGAAAGAATGTAAAAGAACTAGTAG
CTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGATGACTACATGTATTTTG
GAATCAAAACAAAGGATGGAAAAACTCAAGAATGGGAAATGGACAACCCAGGAAAT
GATTTTATGACTGGAAGTAAAGACACTTATACTTTCAAATTAAAAGATGAAAATCTAA
AAATTGATGATATACAAAATATGTGGATTAGAAAAGAAAATATACAGCATTCTCAGAT
GCTTATAAGCCAGAAAACATAAAGATAATAGCAAATGGAAAAGTTGTAGTGGACAAA
GATATAAACGAGTGGATTTCAGGAAATTCAACTTATAATATAAAATAA

Figure 2 Nucleotide sequence of α-toxin gene from Clostridium perfringens type A strain NCTC64609.

the length of PCR fragment, which was approximately 900 bp (Figure 1A). The α-toxin gene was purified with DNA fragment recover kit and evaluated by agarose gel electrophoresis. The α-toxin gene fragment was ligated to the cloning vector pMD18-T with T4 DNA ligase. The cloning vector containing the α-toxin gene was introduced into competent *E. coli* DH5 α cells by $CaCl_2$ transformation. Transformed *E. coli* were grown at 37℃ in medium containing X-gal/IPTG and ampicillin. The positive plasmids were identified via sequential digestion with *Eco*R I and *BamH* I and α-toxin protective antigen gene about 900 bp was obtained (Figure 1B). Sequence of inserted DNA was analyzed with automatic sequence analyzer and about 906 bp in size and encoded 302 amino acid residues (Figure 2).

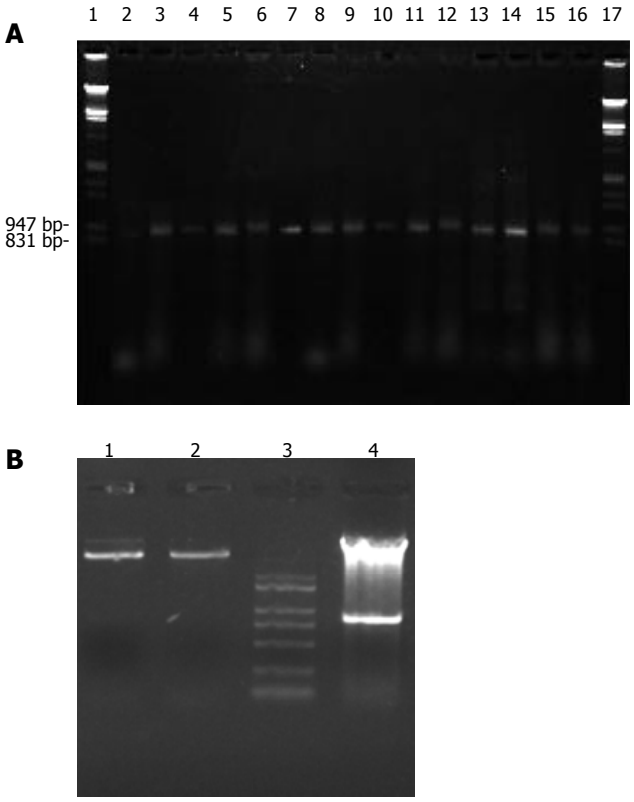

Figure 3 Identification of β-toxin gene product amplified by PCR (**A**) and recombinant plasmid by enzyme digestion (**B**). In A, lanes 1,17: DNA markers; lanes 2-16: product of β-toxin gene amplified by PCR; In B, lane 1: pMD930/EcoR I; lane 2: pMD930/ Pst I; lane 3: DNA Markers; lane 4: pMD930/BamH I+Pst I

The recombinant plasmid was named pMD 906.

Cloning and identification of β-toxin gene from clostridium perfringens type A

The β-toxin protective antigen genes were amplified from *Clostridium perfringens* type C and strain C58-1 by PCR. The PCR products were approximately 930 bp in length (Figure 3A). The β-toxin gene was purified and ligated to the cloning vector pMD18-T as α-toxin gene. The positive plasmids were identified via sequential digestion with *Pst* I and *BamH* I and about 930 bp β-toxin genes were obtained (Figure 3B). Sequence of inserted DNA was analyzed with automatic sequence analyzer and bout 930 bp in length and encoded 310 amino acid residues (Figure 4). The recombinant plasmid was named pMD 930.

Construction of recombinant expression vector pzcpab

The recombinant expression plasmids containing α-β fusion gene were constructed. The positive plasmids were identified via sequential digestion with *Eco*R I and *Pst* I and about 1.8 kb α-β fusion genes were obtained (Figure 5). Sequence of α-β fusion genes was analyzed with automatic sequence analyzer and about 1842 bp in length and encoded 614 amino acid residues. The recombinant plasmid was named pZCPAB.

Expression of α-β fusion gene in E coli BL21 (DE3) (PZCPAB)

The recombinant plasmid pZCPAB carrying α-β fusion gene was transformed into *E coli* BL21(DE3) and the

AATGATATAGGTAAAACTACTACTATAACTAGAAATAAGACATCAGATGGCTATACT
ATAATTACACAAAATGATAAACAGATAATATCATATCAATCTGTTGACTCTTCAAGT
AAAAATGAAGATGGTTTTACTGCATCTATAGATGCTAGATTTATCGATGATAAATAT
TCATCTGAAATGACAACTTTAATAAACTTAACTGGATTTATGTCTTCAAAAAAAGAA
GATGTTATAAAAAAATACAATTTGCATGATGTTACTAATTCTACTGCAATTAATTTTC
CGGTTAGATACTCGATTTCTATTTTAAATGAAAGTATTAATGAAAATGTAAAAATAG
TTGATAGTATTCCTAAAAATACAATTTCTCAAAAAACTGTATCCAATACAATGGGAT
ACAAAATAGGAGGTTCAATTGAAATAGAAAAAAATAAACCTAAAGCTTCAATTGAAA
GCGAATATGCTGAATCATCTACAATAGAATATGTCCAACCTGATTTTTCTACTATACA
GACAGATCATTCAACCTCTAAAGCTTCATGGGATACAAAATTTACAGAAACTACTCG
TGGTAATTATAATTTAAAATCAAACAACCCTGTATATGGAAATGAAATGTTTATGTAC
GGAAGATATACTAATGTTCCTGCAACTGAAAATATAATTCCAGATTATCAAATGTCA
AAATTAATAACAGGTGGTTTAAACCCTAATATGTCTGTAGTTCTAACTGCTCCTAAT
GGTACTGAAGAATCTATAATAAAAGTTAAAATGGAGCGTGAAAGAAACTGTTATTA
TCTTAATTGGAATGGTGCTAACTGGGTAGGACAAGTCTATTCCAGGCTAGCTTTTGA
TACCCCAAATGTAGATAGTCATATATTTACATTCAAAATAAATTGGCTTACTCACAAA
GTAACAGCTATTAAGTAG

Figure 4 Nucleotide sequence of β-toxin gene from Clostridium perfringens type C strain C58-1

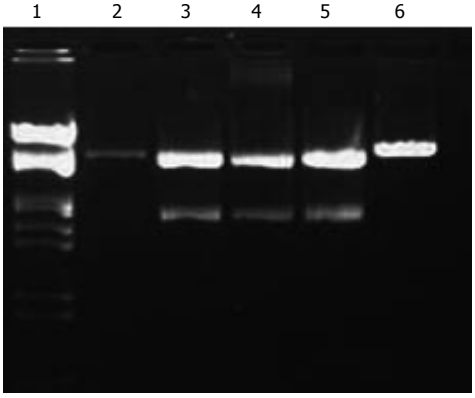

Figure 5 Agarose gel electrophoresis of restriction endonucleases-digested plasmid pZCPAB. Lane 1: DNA markers; lane 2: pZCPAB / EcoR I; lanes 3-5: pZCPAB / EcoR I+Pst I; lane 6: pZCPAB / BamH I.

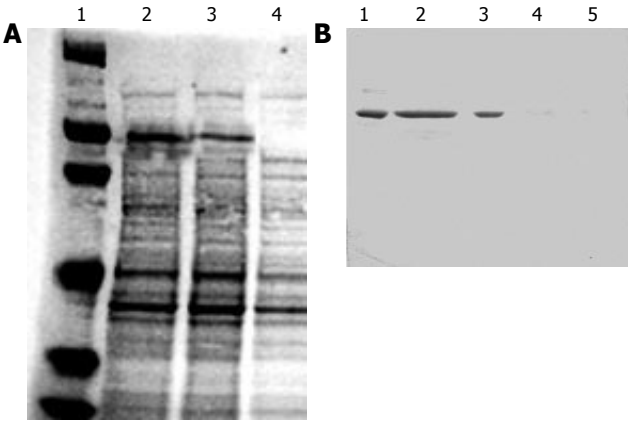

Figure 6 Analysis of expressed products of α-β fusion gene in E.coli by SDS-PAGE (**A**) and Western blot (**B**) In A, lane 1: protein markers (97400, 66200, 43000, 31000, 20100, 14400); lanes 2, 3: BL21(DE3)(pZCPAB); lane 4: BL21(DE3)(pBV221); In B, lane 1: BL21(DE3)(pZCPAB)/α; lane 2: BL21(DE3)(pZCPAB)/α+β; lane 3: BL21(DE3)(pZCPAB)/β; lanes 4, 5: BL21(DE3)(pBV221).

Table 1 Detection of the fusion protein by ELISA assay

Antibody	pZCPAB	pBV221	pXETA	pXETB
Anti-α-toxin	0.66±0.08	0.001	0.92±0.13	
Anti-β-toxin	0.72±0.12	0.002		0.97±0.19

Table 2 Expressed α-β fusion protein in mice

Challenge dose	α-toxin(survived mice/total mice)		β- toxin(survived mice/total mice)	
	Control group	Treatment group	Control group	Treatment group
1 MLD	0/10	10/10	0/10	10/10
2 MLD	0/10	8/10	0/10	9/10

recombinant strain BL21(DE3)(pZCPAB) was obtained. SDS-PAGE and Western blotting of the bacterial protein showed that a specific protein band with a molecular weight of 66 kD emerged in the bacterial protein of *E coli* BL21(DE3)(pZCPAB). SDS-PAGE gel-scanning showed that the expressed α-β fusion protein accounted for 22.14% of the total bacterial protein (Figure 6). ELISA results indicated that the expressed fusion protein could be recognized by anti-α toxin antibody and anti-β toxin antibody respectively (Table 1).

Bioactivity assay of expressed α-β fusion protein

The expressed α-β fusion protein yielded in bacteria could provide protection of mice against the challenge of lethal doses of α-toxin or β-toxin of *Clostridium perfringens* (Table 2).

Pathological changes in different organs

The segments of liver, lung, heart, spleen and intestine were sampled from the control group in which mice died of toxicosis and the treatment group in which survived mice were immunized with α-β fusion protein. Then they were observed by LM assay.

In treatment group, the structure of liver was seen clearly except that a few central veins of hepatic lobules were full of blood. The liver cytoarchitectures were intact (Figure 7A). In control group, the structure of hepatocytes was grain-like metamorphic. The nuclei of hepatocytes were concentrated and collapsed. Swollen hepatic sinusoids, veins of hepatic lobules and central veins were full of red cells or red thrombi, swollen Kupffer cells in the margin of hepatic sinusoids (Figure 7B).

The capillaries in alveolar septum of the lung in treatment group were slightly bloodshot with no other abnormalities (Figure 7C). The swollen capillaries in alveolar septum were full of red cells. Some of capillaries were full of red thrombi (Figure 7D).

The structure of cardiac muscle fibers in treatment group was intact (Figure 7E). The capillary vessels and cardiac muscle fibres were swollen and bloodshot (Figure 7F).

There was no abnormal structure of spleen in treatment group (Figure 7G). The splenic cord was extruded and could not be seen clearly (Figure 7H).

The structure of duodenum and jejunum in treatment group was intact (Figure 7I). The capillary vessels of lamina propria of tunica mucosa had little bleeding. The epithelia of tunica mucosa were metamorphic and

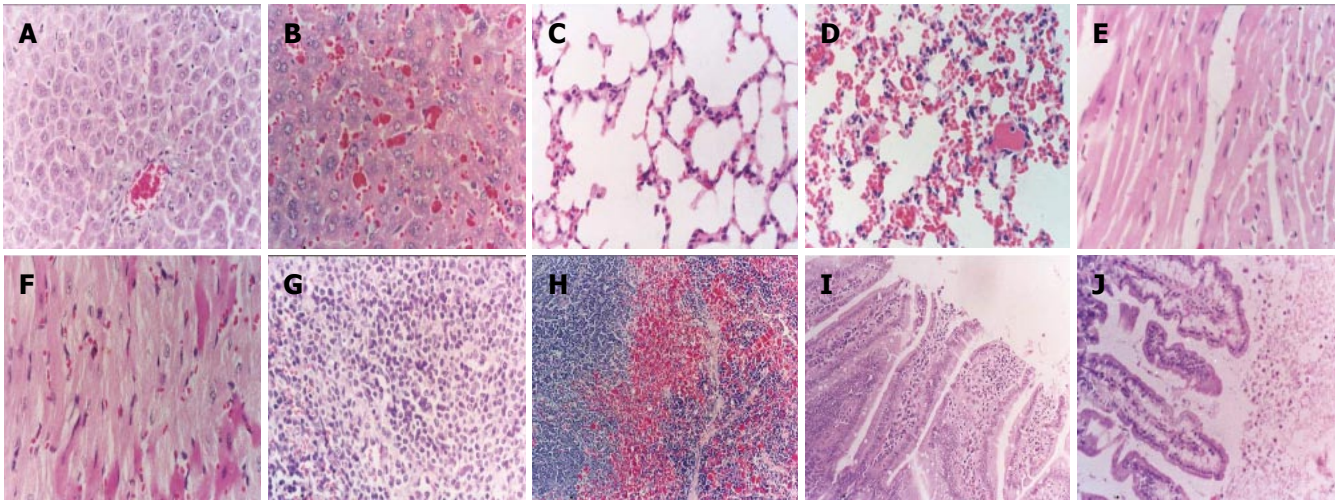

Figure 7 Pathological changes in livers of survived mice (**A**) and dead mice (**B**), in lung of survived mice (**C**) and dead mice (**D**), in heart of survived mice (**E**) and dead mice (**F**), in spleen of survived mice (**G**) and dead mice (**H**), in intestine of survived mice (**I**) and dead mice (**J**).

necrotic, one or many of intestinal villi fell into the enterocele (Figure 7J).

DISCUSSION

Clostridium perfringens is ubiquitous in the environment and has been found in soil, decaying organic matter and a member of the gut flora in humans and animals. Different strains of *C perfringens* can be classed into one of the five biotypes (A-E) according to the spectrum of toxins produced[9]. *Clostridium perfringens* is a Gram-positive anaerobic spore-forming bacterium which is widely found in the environment and also in the intestinal tract of humans and animals. Due to the production of several exotoxins, *C perfringens* is of great importance as a pathogen in humans, domestic animals and wildlife[10]. The production of four major toxins alpha (*α*), beta (*β*), epsilon (*ε*) and iota (*ι*) allows the discrimination into five types from A to E. In veterinary medicine, each of these types has been linked to specific diseases[11]. *Clostridium perfringens* enterotoxin (CPE) serves as an additional virulence factor which is believed to cause enteritis in pigs, cats and dogs[12]. The Gram-positive pathogen *Clostridium perfringens* is a major cause of human and veterinary enteric disease largely because this bacterium can produce several toxins when present inside the gastrointestinal tract[13].

Biotype A strains of *Clostridium perfringens* are of particular importance as the aetiological agents of gas gangrene in humans. Gangrenous disease is of increasing significance in elderly and diabetic population, especially in those who have undergone lower limb surgery, where impaired blood supply to tissues can lead to anoxic conditions suitable for proliferation of bacteria[14]. The disease can also arise in patients who have undergone surgery of the gastrointestinal tract when contamination of damaged tissues with gut contents can result in the establishment of disease[15]. *Clostridium perfringens* β-toxin is known to be the primary pathogenic factor of necrotic enteritis in type C strains that produce β-toxin. Necrotizing enteritis is believed to be due to the production of β-toxin by Clostridium perfringens type C. *Clostridium perfringens* β-toxin can also cause dermonecrosis and oedema in the dorsal skin of animals[16-18].

This paper describes the successful isolation and cloning of α-toxin and β-toxin genes from the strains NCTC64609 and C58-1 of *Clostridium perfringens* respectively. The construction and expression of α-β fusion gene, and the biological activities of α-β fusion protein have a significant impact on mice. To our knowledge, this is the first successful experiment of the development of a recombinant α-β fusion protein. We believe that it could serve as a model for development and construction of novel fusion protein for α-toxin, β-toxin and other potential toxins. In this study, the expressed α-β fusion protein could provide protection of mice against the challenge of lethal dose of α-toxin and β-toxin of *Clostridium perfrigens*. These findings indicate that the expressed α-β fusion protein can be applied in preventing toxicosis of α-toxin and β-toxin of *Clostridium perfringens*.

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CLINICAL RESEARCH

Prior appendectomy and the phenotype and course of Crohn's disease

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Abstract

AIM: To determine whether prior appendectomy modifies the phenotype and severity of Crohn's disease.

METHODS: Appendectomy status and smoking habits were specified by direct interview in 2838 patients consecutively seen between 1995 and 2004. Occurrence of complications and therapeutic needs were reviewed retrospectively. Additionally, annual disease activity was assessed prospectively between 1995 and 2004 in patients who had not had ileocecal resection and of a matched control group.

RESULTS: Compared to 1770 non-appendectomized patients, appendectomized patients more than 5 years before Crohn's disease diagnosis (n=716) were more often females, smokers, with ileal disease. Cox regression showed that prior appendectomy was positively related to the risk of intestinal stricture (adjusted hazard ratio, 1.24; 95% confidence interval, 1.13 to 1.36; P=0.02) and inversely related to the risk of perianal fistulization (adjusted hazard ratio, 0.75; 95% confidence interval, 0.68 to 0.83; P=0.002). No difference was observed between the two groups regarding the therapeutic needs, except for an increased risk of surgery in appendectomized patients, attributable to the increased prevalence of ileal disease. Between 1995 and 2004, Crohn's disease was active during 50% of years in appendectomized patients (1318 out of 2637 patient-years) and 51% in non-appendectomized patients (1454 out of 2841 patient-years; NS).

CONCLUSION: Prior appendectomy is associated with a more proximal disease and has an increased risk of stricture and a lesser risk of anal fistulization. However, the severity of the disease is unaffected.

Key words: Crohn's disease; Appendectomy; Surgery; Smoking

INTRODUCTION

Two common environmental factors, cigarette smoking and appendectomy, have been found to play a role in inflammatory bowel diseases (IBD). Smoking has a role both in disease onset and disease course, and it is well established that it has opposite effects on the two diseases, beneficial in ulcerative colitis (UC) and deleterious in Crohn's disease[1,2]. Previous appendectomy has a favourable effect on UC. Patients who have been appendectomized have a lesser risk of developing UC. Moreover, in the few appendectomized patients who develop UC, disease course is less severe, with a decreased need of colectomy compared to non-appendectomized patients[3,4]. Of note, the effects of smoking and appendectomy are additive[4]. In Crohn's disease, the effect of previous appendectomy remains debated. Some series reported an increased risk of Crohn's disease after appendectomy[5-7], and others did not[8-11]. These discrepancies may be partly linked to the inclusion or not of appendectomies performed close to the time of diagnosis. However, the largest study to date showed that the risk of Crohn's disease is increased up to 20 years after appendectomy[7]. Data concerning the effect of appendectomy on the clinical course of Crohn's disease are scarce and contradictory, one study reporting no effect[3] and another suggesting that previous appendectomy was associated with an increased risk of surgery[12]. In the study by Andersson *et al*[7], an increased risk of surgery for Crohn's disease was observed only in patients with perforated appendicitis.

The aim of the present study was to analyse the phenotype and clinical course of Crohn's disease in a large cohort of patients subjected to appendectomy compared with non-appendectomized patients.

MATERIALS AND METHODS

Patient population

From January 1995 to December 2004, all consecutive patients with Crohn's disease who came to our unit were

included in the study. The diagnostic criteria for Crohn's disease were those of Lennard-Jones[13]

Appendectomy status and smoking habits

Appendectomy and smoking status were specified during a direct interview of the patients. The date of appendectomy was noted and patients were classified according to the time span between appendectomy and diagnosis. Patients were classified as smokers if they had smoked more than 7 cigarettes per week for at least six months during the six months preceding diagnosis of Crohn's disease and/or thereafter[14].

Characteristics of Crohn's disease

The characteristics of Crohn's disease were completed according to the retrospective analysis of medical charts. The time of diagnosis was defined as the date of first detection of unequivocal inflammatory abnormalities of the intestine, as assessed from radiological, or endoscopic, or peroperative observations. The initial location of Crohn's disease lesions was determined by colonoscopy and small bowel X-ray. After diagnosis, patients were followed clinically with 3 to 4 visits per year, and only investigated again in case of flare-up or development of new symptoms. Morphological investigations included proctosigmoidoscopy, colonoscopy, and small bowel X-ray. Upper gastrointestinal endoscopy was only performed in case of gastroesophageal symptoms. Subjects were explored for ano-perineal disease at each visit.

Location and behavior of Crohn's disease were classified according to the Vienna classification[15]. First morphological demonstration of narrowing or penetrating complication was used to date the occurrence of the complication defining behavior[16].

Treatment of Crohn's disease

Our treatment policy has been exposed elsewhere[16]. Flare-up episodes were treated with mesalamine (3-4 g daily) or prednisolone (1 mg/kg per day, progressively tapered after four weeks), according to the clinical severity. When steroid therapy failed, patients seen before 1999 were given a 3-wk course of enteral or parenteral nutrition; those seen after June 1999, when Infliximab became available in France, received Infliximab 5 mg/kg.

As maintenance treatment, we used aminosalicylates (sulphasalazine, olsalazine or mesalamine, 2-3 g daily) for asymptomatic or moderately active forms of the disease, and immunosuppressive drugs for severe forms (steroid-dependent or poorly responsive to steroids). Azathioprine 2 mg/kg per day was used as first line immunosuppressive drug. In case of repeated flare-ups or chronic active disease in a patient receiving azathioprine, its dosage was increased to 2.5-3 mg/kg per day. Intramuscular methotrexate (20-25 mg weekly) was used in patients unresponsive or intolerant to azathioprine. Its dosage was tapered progressively to 10-15 mg, and re-augmented in case of clinical relapse.

Although the overall strategy remained mostly unchanged, there was a clear tendency over time to initiate immunosuppressants earlier in the disease course. Surgery was reserved for stenotic and extra-parietal complications, or intractable forms after a well-conducted medical management.

Phenotype and severity of Crohn's disease

Phenotyping Crohn's disease took into account disease location and the occurrence of a stricturing or penetrating complication. Overall severity of the disease was assessed in two ways: first retrospectively, taking into account the importance of the medical therapy, i.e. need for glucocorticoid, nutritional support, immunosuppressive drugs, and Infliximab, and finally incidence of excisional surgery. Second, patients who had not had ileo-cecal resection prior to inclusion were followed-up prospectively from the date of inclusion to December 2004, and activity of the disease was assessed prospectively by analyzing the occurrence of a flare-up each year. A patient-year was considered as active if a flare-up or a complication occurred during the year, and inactive otherwise.

Statistics

Continuous data are expressed as mean (standard deviation), and differences between the groups were tested for significance by Student's *t* test. Discrete data are given as percentages, and comparisons were made with Pearson's Chi-square test.

The retrospective study analyzed the effect of prior appendectomy on the long-term course of Crohn's disease. For this purpose, non-appendectomized patients were included in the control group until the time an ileo-cecal resection was eventually performed. To avoid biases related to the effect of ileo-cecal resection on the subsequent evolution of Crohn's disease, appendectomized patients were also censored at the time of ileo-cecal resection. For actuarial analysis, the Kaplan-Meier model was used, with the date of diagnosis as starting point. The curves were compared by the Log-rank test. Multivariate analyses were performed with Cox proportional hazards regression to adjust for confounding. All baseline variables suspected to be possible predictors of complication or intestinal surgery [young age (< 20 years), old age (equal to or above 40 years), gender, ethnicity (Caucasian or not), socioeconomic status (high or low-moderate), diagnosis after 1987, familial history, extra-intestinal manifestations, smoking status, initial disease location (esophago-gastro-duodenal, jejunal, ileal, colonic, and anoperineal lesions)], were entered into the model. Diagnosis after 1987 was retained as a variable because the use of immunosuppressive therapy became frequent since that year. Results of analysis are presented as hazard ratios (HRs) with 95% confidence intervals.

The prospective analysis included consecutive Crohn's disease patients seen between 1995 and 2004 who had had appendectomy but no ileo-cecal resection before 1995. Those patients were matched for sex, birth date (boxes of five years), date of diagnosis (boxes of five years), and Vienna classification disease location, with non-appendectomized patients without previous ileo-cecal resection. Patients of both groups were censored at the time of ileo-cecal resection. Calculations were performed using GB-stat statistical software.

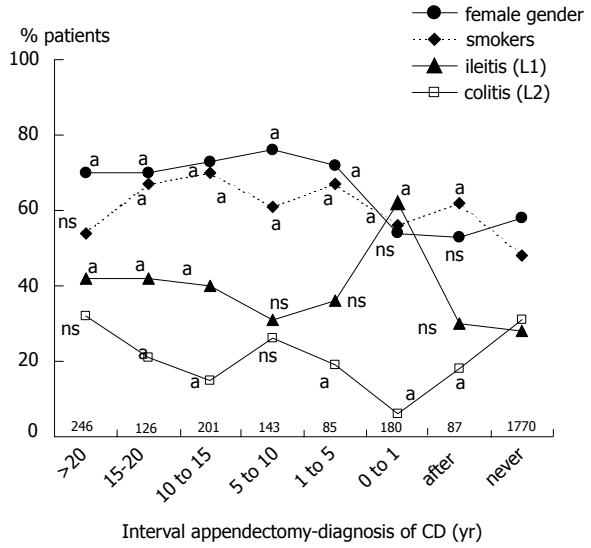

Figure 1 Characteristics of patients and Crohn's disease (given as percentages) according to appendectomy status and the time span between appendectomy and diagnosis of Crohn's disease. Numbers indicate the number of patients in each group. [a] and ns represent the *P* value of the comparison between the subgroup and the group of never appendectomized patients ([a]$P < 0.05$; ns: not significant).

Table 1 Main characteristics of Crohn's disease in non-appendectomized and appendectomized patients

	Non-appendectomized	Appendectomized	*P*
Number of patients	1770	716	
Female gender	1019 (58)	515 (72)	0.001
Age at diagnosis (yr)	27.5±12.8	32.1±13	0.001
Duration of disease (yr)[1]	9.6±8.6	9.1±8.1	NS
Caucasian ethnicity	1484 (84)	663 (93)	0.001
High socio-economic status	535 (30)	171 (24)	0.001
Disease onset after 1987	1374 (78)	554 (77)	NS
Familial history	272 (15)	79 (11)	0.004
Current smokers	856 (48)	444 (62)	0.001
Disease location			
Terminal ileum (L1)	489 (28)	277 (39)	
Colon (L2)	548 (31)	173 (24)	0.001
Ileocolon (L3)	514 (29)	187 (26)	
Upper gastrointestine (L4)	219 (12)	79 (11)	
Extra-intestinal manifestations	1136 (64)	422 (59)	0.01

[1] at last visit or when censored
Number in parentheses is percentages.

RESULTS

Appendectomy in relation to disease onset

Among 2926 patients with Crohn's disease, 1068 patients (38% of those with appendectomy status known) had been appendectomized, including 87 after Crohn's disease diagnosis. Compared to non-appendectomized patients, the 981 appendectomized patients before or at diagnosis were more often females (69 *vs* 58%, $P < 0.001$), Caucasians (90 *vs* 84%, $P < 0.001$), and active smokers (61 *vs* 48%, $P < 0.001$). They were also older at diagnosis (29.5±12.1 *vs* 27.4±12.7 years, $P < 0.001$), and the time span between first symptom attributable retrospectively to Crohn's disease and Crohn's disease diagnosis was longer (21.9±48.9 *vs*16.5±38.3 months in non-appendectomized patients, $P = 0.003$). Disease location differed significantly between the two groups, with a predominance of ileitis (L1 location) counterbalanced by a decrease of colitis (L2 location) in appendectomized patients (43 and 20%, *vs* 28 and 31%, respectively, in non-appendectomized patients, $P < 0.001$), without significant differences in the proportion of ileocolitis (L3) and upper digestive tract location (L4). Important differences were seen regarding gender distribution and disease location according to the time span between appendectomy and Crohn's disease diagnosis (Figure 1). A significant female predominance was observed only in patients appendectomized more than one year prior to Crohn's disease diagnosis. L1 was the main disease location whatever the interval between appendectomy and Crohn's disease diagnosis, however this increase was particularly marked in patients appendectomized at or within one year preceding the diagnosis. Compared to this latter subgroup, subgroups of appendectomized patients who had had appendectomy at various intervals prior to Crohn's disease differed significantly by a higher proportion of females, a lower prevalence of L1 location and a higher prevalence of L2 location. Finally, there was a higher proportion of smokers in appendectomized patients whatever the date of appendectomy. To avoid inclusion of patients for whom appendectomy could have been performed for a missed diagnosis of Crohn's disease, we excluded from further analysis, in addition to the 180 patients appendectomized within the year of diagnosis, the 85 patients in whom appendectomy had been performed within the five years before disease onset. Thus 716 patients were included in the retrospective analysis (Figure 2). Among those patients, appendectomy had been performed 5 to 76 years (median, 16 years) before diagnosis of Crohn's disease, at a median age of 11 years (range 0-67).

Retrospective analysis

The comparison of these 716 appendectomized patients with non-appendectomized patients is given in Table 1. The results were similar to the whole group of appendectomized patients, with a Crohn's disease diagnosis later in life and a predominance of both women and active smokers. In addition, appendectomized patients were more often Caucasian, of low-to-moderate socioeconomic status, without family history, and had less extra-intestinal manifestations. The most frequent disease location according to Vienna classification was ileum only in appendectomized patients, whereas it was colon only in non-appendectomized patients. More detailed comparison of initial and cumulative disease location revealed that prior appendectomy was associated with a higher prevalence of ileal involvement, while distal colon, rectum, and anus were more frequently spared (Figure 3). Cumulative disease behavior was inflammatory (B1) in 46%, stricturing (B2) in 15%, and penetrating (B3) in 39% of appendectomized patients, *vs* 43% (B1), 11% (B2), and 46% (B3), respectively, in non-appendectomized patients. Because disease behavior is highly dependent on disease location

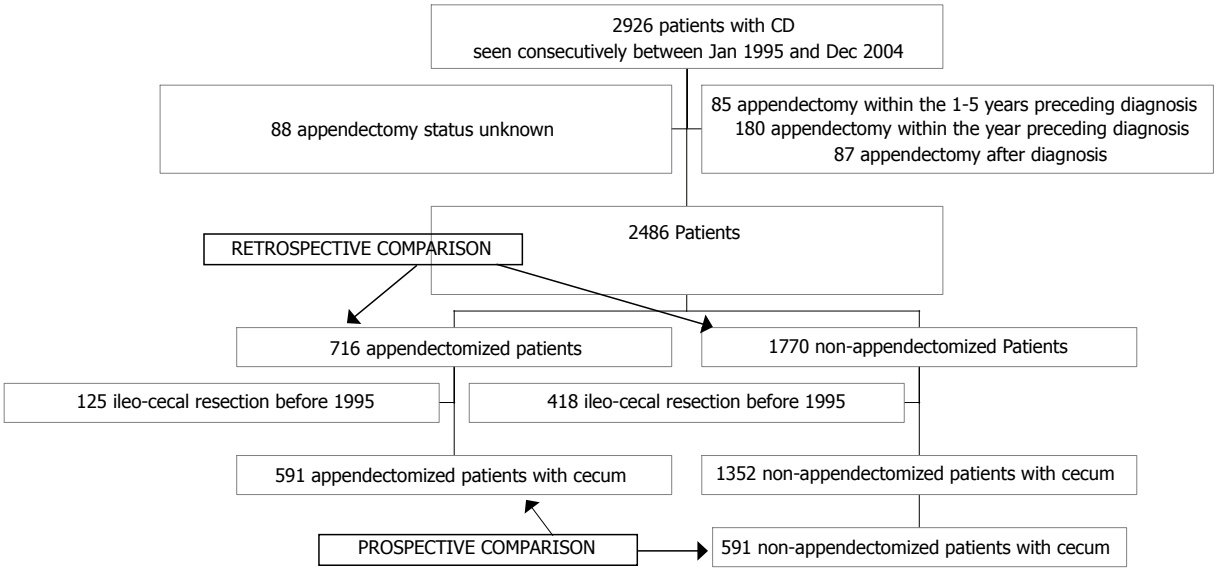

Figure 2 Flow chart of patients studied.

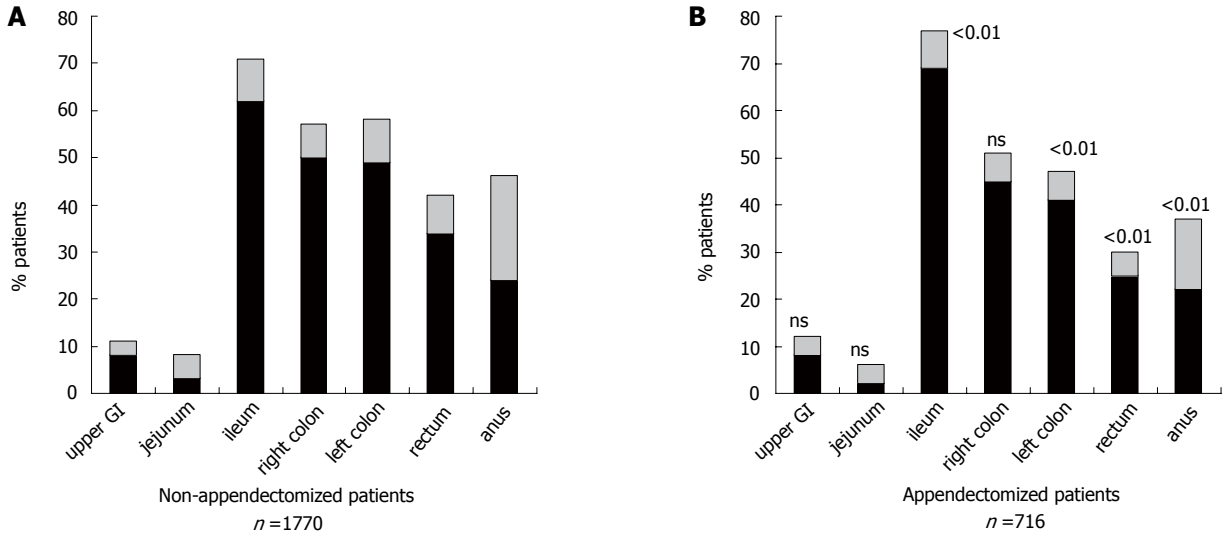

Figure 3 Initial and cumulative Crohn's disease involvement of various segments of the digestive tract in non-appendectomized and appendectomized patients. The numbers above the columns indicate the *P* values comparing the frequencies of cumulative involvement of each segment between the two groups.

and duration[16], the respective risks of stricture, intestinal perforation, and perianal perforation were calculated in each location group cumulatively (Table 2). These calculations demonstrated in appendectomized compared to non-appendectomized patients an increased risk of stricture in the L1 group, and a decreased risk of perianal perforating disease in the L2 group. Cox analysis in the whole population pooling the four location groups (2486 patients) confirmed that prior appendectomy was positively related to the risk of intestinal stricture (adjusted hazard ratio, 1.24; 95% confidence interval, 1.13 to 1.36; $P=0.02$) and inversely related to the risk of perianal perforation (adjusted hazard ratio, 0.75; 95% confidence interval, 0.68 to 0.83; $P=0.002$). The respective proportions of appendectomized and non-appendectomized patients who required oral steroids, enteral or parenteral nutrition, immunosuppressive therapy, infliximab, and surgery were not significantly different in the whole population nor in each location group (Table 3). In the whole population, the 10-yr cumulative need for first excisional surgery was significantly higher in appendectomized *vs* non-appendectomized patients (54±2% *vs*. 48±2%, respectively; $P=0.02$). However, multivariate analysis selected as predictive factors of surgery young age (adjusted hazard ratio, 0.80; 95 % confidence interval, 0.74 to 0.86; $P=0.002$), ileal involvement (adjusted hazard ratio, 1.73; 95% confidence interval, 1.60 to 1.87; $P<0.001$) and colonic involvement (adjusted hazard ratio, 0.66; 95 % confidence interval, 0.62 to 0.71; $P<0.001$) and did not confirm the specific role of appendectomy ($P=0.33$). Within each location group, the 10-yr cumulative need for first excisional surgery did not differ significantly (L1 74±4% *vs* 67±3%; L2 27±4% *vs* 38±3; L3 50±5% *vs* 39±3%; L4 51±8% *vs* 51±4%).

Table 2 5-yr and 10-yr cumulative risks of intestinal and perianal complications in non-appendectomized and appendectomized patients according to disease location

		Non-appendectomized				Appendectomized			
Location		**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**
Number of patients		**493**	**546**	**513**	**218**	**279**	**172**	**186**	**79**
Intestinal stricture	5-yr	23 ± 2	2 ± 1	9 ± 1	22 ± 3	30 ± 3 [b]	3 ± 2	12 ± 3	29 ± 6
	10-yr	34 ± 3	7 ± 1	17 ± 2	42 ± 5	46 ± 4 [b]	6 ± 2	24 ± 4	42 ± 7
Intestinal perforation	5-yr	30 ± 2	7 ± 1	12 ± 2	12 ± 2	27 ± 3	4 ± 2 [a]	13 ± 3	10 ± 4
	10-yr	42 ± 3	12 ± 2	25 ± 2	23 ± 4	37 ± 4	4 ± 2 [a]	23 ± 4	29 ± 7
Perianal fistulization	5-yr	14 ± 2	32 ± 2	31 ± 2	23 ± 3	11 ± 2	19 ± 3 [b]	26 ± 4	15 ± 4
	10-yr	18 ± 2	49 ± 3	41 ± 3	29 ± 4	17 ± 3	33 ± 5 [b]	40 ± 5	21 ± 6

[b]$P < 0.001$, [a]$P < 0.05$ *vs* appendectomized non-appendectomized patients (log rank).
Other curves were not significantly different.

Table 3 Cumulative therapeutic needs in non-appendectomized and appendectomized patients according to disease location

	Non-appendectomized				Appendectomized			
Location	**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**
Number of patients	**493**	**546**	**513**	**218**	**279**	**172**	**186**	**79**
Oral or IV steroids	396 (80)	469 (86)	462 (90)	195 (89)	228 (82)	150 (87)	173 (93)	71 (90)
Enteral or parenteral nutrition	53 (11)	1	94 (18)	48 (22)	23 (8)	0	35 (19)	13 (16)
Azathioprine or methotrexate	163 (33)	305 (56)	290 (57)	132 (61)	100 (36)	83 (48)	101 (54)	48 (61)
Infliximab	9 (2)	73 (13)	48 (9)	19 (9)	4 (1)	11 (6)	14 (8)	5 (6)
Intestinal resection	283 (57)	173 (32)	176 (34)	79 (36)	165 (59)	42 (24)	73 (39)	30 (38)

Numbers in parentheses are percentages.
Comparison between non-appendectomized and appendectomized patients revealed no significant difference

Table 4 Comparison between two groups of patients included in prospective follow-up study

	Non-appendectomized	Appendectomized
Number of patients	591	591
Number of females	427	427
Mean age at diagnosis (yr)	31.8±13.8	32.3±13.4
Mean age at inclusion (yr)	34.0±13.9	34.8±13.7
Diagnosis after 1987 (*n*)	539	524
Diagnosis after 1995 (*n*)	351	357
L1 location (*n*)	202	204
L2 location (*n*)	162	160
L3 location (*n*)	156	156
L4 location (*n*)	71	71

No difference between the two groups was significant

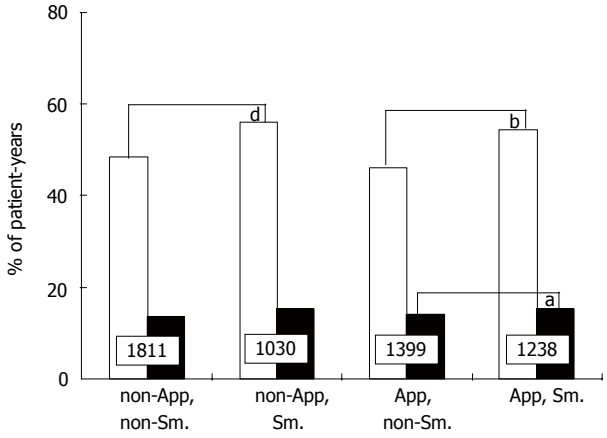

Figure 4 Percentages of patient-years with active disease (grey columns) and hospitalization (black columns), respectively, according to appendectomy and smoking status (App: appendectomized; Sm: smokers). Numbers indicate the number of patient-years in each group. [a]$P < 0.05$ *vs* App, non-Sm.; [b]$P < 0.001$ *vs* App, non-Sm.; [d]$P < 0.001$ *vs* non-App, non-Sm.

Prospective study

The prospective study included 591 appendectomized patients and 591 non-appendectomized matched controls, both selected on the basis of not having had ileo-cecal resection at inclusion into follow-up. The two groups were well matched (Table 4). One hundred and forty-five appendectomized (25%) and 143 non-appendectomized patients (24%), respectively, had an ileo-cecal resection after inclusion. After censoring, the median length of follow-up for the two cohorts was 4 years and encompassed a total of 5478 patient-years. The rate of years with active disease was 50% in appendectomized patients (1 318 out of 2 637 patient-years) *vs* 51% in non-appendectomized patients (1 455 out of 2 841 patient-years, NS). By contrast, in the same series of patients, non- or ex-smoking was associated with a decreased activity rate (1 521 out of 3 210 patient-years, 47%), compared to current smoking (1 252 out of 2 268, 55%; $P < 0.001$). Figure 4 gives the percentage of patient-years with active disease and hospitalization respectively, according to both smoking and appendectomy status. Previous appendectomy had no effect on year-by-year disease activity while smoking was significantly deleterious.

DISCUSSION

The present results show that previous appendectomy, while associated with some particularities in the location and behaviour of Crohn's disease, has no effect upon the course of the disease. Appendectomized patients are more prone to ileal disease and to formation of strictures, and less to distal colonic involvement and penetrating perianal disease. However, previous appendectomy does not change disease severity retrospectively assessed from therapeutic needs or prospectively assessed from year-by-year activity.

The role of appendectomy in Crohn's disease is difficult to assess because various biases may jeopardize the analysis. First, appendicitis may be the first manifestation of Crohn's disease. The appendix is frequently involved by Crohn's disease[17], granulomatous appendicitis may in some cases reveal Crohn's disease[18,19], and resection of a Crohn's disease appendix may be followed by a long period of remission[20,21]. However, most pathological studies concluded that idiopathic granulomatous appendicitis is nosologically distinct from Crohn's disease[22] and that most of these patients do not develop subsequent Crohn's disease. Moreover, when analyzing a cohort of Crohn's disease patients, Crohn's disease confined to the appendix was very unusual, observed in less than 1% of Crohn's disease patients[23]. Although the pathology reports of the removed appendix were not available in our series, the data suggest that the great majority of our patients had appendectomy performed not for Crohn's disease. Moreover, the frequency of appendectomy in our series is similar to other series[12] and not different from controls without Crohn's disease[24,25]. Another difficulty is to accurately define in time the patient population in which the effect of appendectomy may be assessed. On one hand, inclusion of all appendectomized patients whatever the time span between appendectomy and the onset of Crohn's disease may lead to an overrepresentation of ileal disease cases, as an acute abdominal pain in the lower right quadrant may reveal ileal Crohn's disease. On the other hand, prolonging the time span between appendectomy and Crohn's disease may select cases of Crohn's disease occurring later in life and thus susceptible to share a lesser activity. Actually, in the present study, the comparison of patients subgroups defined by the duration of the interval between appendectomy and onset of Crohn's disease showed that only patients appendectomized within one year prior to Crohn's disease diagnosis clearly differed from other subgroups through a higher prevalence of ileal disease, as it could be expected (Figure 1). The characteristics of the subgroup of appendectomized patients one to five years prior to Crohn's disease diagnosis were intermediate between the two adjacent subgroups. This led us to define the cutoff at five years prior to appendectomy. Such a cutoff is in agreement with the data of Frisch *et al*[6] who found that, five years after appendectomy, the relative risk of Crohn's disease was not further elevated. Finally, in the present study, selection bias were minimal, as we did not use a postal questionnaire, but included prospectively all consecutive patients seen over a 10-year period. The criteria used to assess the severity of the disease were objective, and were based on therapeutic needs. Both surgical and immunomodulator history are accepted as useful indicators of the over-all severity of Crohn's disease because these interventions are both easy to document retrospectively, and their use and the effects of their use may be complementary[14, 26]. Moreover, the prospective part of the study confirmed the results of the retrospective analysis.

Crohn's disease patients with prior appendectomy differed from non-appendectomized Crohn's disease patients in many aspects. There were more often females, smokers, of low-to-moderate socioeconomic status, and of Caucasian origin. In fact, these particularities might be more a consequence of appendectomy as such, than related to a particular Crohn's disease phenotype. Indeed, an epidemiological national survey in 1978-82 in France showed that appendectomy, but not appendicitis, was more frequent in females than in males[24], and an association has been shown between appendectomy and passive or active smoking[27]. Other epidemiological studies from UK have also reported an increased rate of incidental appendectomies in women [28]. Similarily, the contrast between a higher proportion of Caucasian patients and a lower proportion of patients of high socio-economic status fits better with what is known about epidemiology of appendicitis than with IBD[29,30]. In the Western world, appendectomy is performed more often in whites than in blacks[31], whereas data from UK suggest that the incidence of IBD in immigrants is similar to that of Europeans[32]. Changes in hygiene and water supply increased the risk of appendectomy[33] which in the last four decades has preferentially concerned populations of low-to-moderate socio-economic status, whereas patients with IBD seem to be better educated[34]. We believe that the overrepresentation of ileal disease in appendectomized Crohn's disease patients may be at least in part a consequence of the high prevalence of smokers in this group, smoking being associated with ileal Crohn's disease and not colonic Crohn's disease[35]. Alternative explanations of the more frequent ileal involvement in appendectomized patients would be a larger use of explorative procedures such as barium follow-through or colonoscopy because patients with previous appendectomy are more prone to small bowel obstruction[36,37] and non specific abdominal pain[38]. The occurrence of abdominal pain may give the opportunity to find out a paucisymptomatic ileitis which could have been ignored otherwise.

Comparisons of disease behaviour between appendectomized patients and controls revealed some subtle differences within each disease location group. The former patients were more exposed to stricture formation and less to penetrating anal disease. Actually, within the colon only group (L2 group), disease location tended to be more distal in non-appendectomized patients, perianal lesions were more frequent, and it could be expected that perianal perforating complications occurred more frequently. The increased prevalence of intestinal strictures in patients with prior appendectomy, although weak, seems more relevant. It may be hypothesized that the presence of intra-abdominal adherences and/or vasculature modifications secondary to prior laparotomy has a role in stricture formation.

The most important and unequivocal result of the pres-

ent study is the lack of effect of prior appendectomy on the severity of Crohn's disease. We observed an increased risk of surgery in appendectomized patients, but multivariate analysis revealed that this was related to confounding factors. Riegler *et al*[12] have reported an increased risk of surgery in 41 Crohn's disease patients with previous appendectomy when compared to 88 non-appendectomized patients, but patients appendectomized within one year prior to diagnosis of Crohn's disease were included in the analysis. In the study by Andersson, compared to Crohn's disease controls, Crohn's disease patients with a history of perforated appendicitis had a higher incidence of intestinal resections, those with appendectomy for other diagnoses had a lower incidence, but the incidence for the whole group with prior appendectomy was similar to controls[7]. Radford-Smith *et al*[3] have reported similar results in a retrospective analysis of 335 patients with Crohn's disease, of whom 36 had had prior appendectomy. Thus the effect of prior appendectomy on the risk of surgery seems nil or weak, or not related to appendectomy per se. Besides, therapeutic needs in terms of steroid and immunosuppressants requirements were very similar in appendectomized and non-appendectomized patients, whatever the disease location. Finally, the prospective follow-up of our appendectomized patients compared with controls matched for gender, age, and disease location, clearly demonstrated the lack of effect of prior appendectomy on the disease activity. This study was powered enough to support a firm conclusion, since concomitantly among the same patients, the deleterious effect of current smoking was highly significant.

In conclusion, this study shows that prior appendectomy is associated with a distinct phenotype of Crohn's disease. However these particularities may be attributable more to appendectomy than to Crohn's disease. Besides, in contrast to smoking, appendectomy has no effect on Crohn's disease severity. Taken together, these data indicate that appendectomy and Crohn's disease share common environmental or genetic characteristics whereas appendectomy per se does not exert any immune modulating effect. The response of IBD to the only environmental factors clearly documented so far, smoking and appendectomy, is quite different in UC and in Crohn's disease.

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CLINICAL RESEARCH

Short- and medium-term reproducibility of gastric emptying of a solid meal determined by a low dose of ^{13}C-octanoic acid and nondispersive isotope-selective infrared spectrometry

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Abstract

AIM: To evaluate the reproducibility of a modified ^{13}C breath test-based measurement of solid phase gastric emptying (GE) within the frames of a simple-repeated measure study protocol.

METHODS: Twelve healthy subjects (6 females and 6 males, mean age 24.9 ± 0.7 years) were recruited to undergo three identical GE examinations. In six subjects the first two examinations were performed 2 d apart, and the third session was carried out at a median interval of 19.5 d (range 18 - 20 d) from the second one. In another six subjects the first two measurements were taken 20 d apart (median, range: 17-23 d), whereas the third session took place 2 d after the second one. Probes of expiratory air collected before and during six hours after intake of a solid meal (378 kcal) labelled with 75 μL (68 mg) ^{13}C-octanoic acid, were measured for $^{13}CO_2$ enrichment with the nondispersive isotope-selective infrared spectrometry NDIRS apparatus.

RESULTS: Taking coefficients of variation for paired examinations into account, the short-term reproducibility of the GE measurement was slightly but not significantly better than the medium-term one: 7.7% and 11.2% for the lag phase (T-Lag), 7.3% and 10.9% for the gastric half emptying time (T½). The least differences in GE parameters detectable at $P = 0.05$ level in the 12 paired examinations were 9.6 and 15.6 min for T-Lag, 11.6 and 19.7 min for T½ by a two-day or two to three-week time gap, respectively.

CONCLUSION: The low-cost modification of the breath test involving a lower dose of ^{13}C-octanoic acid and NDIRS, renders good short- and medium-term reproducibility, as well as sensitivity of the measurement of gastric emptying of solids.

Key words: ^{13}C breath test; Gastric emptying; Nondispersive isotope-selective infrared spectrometry; ^{13}C-octanoic acid; Reproducibility

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INTRODUCTION

Since its first description and validation in 1993[1], ^{13}C-octanoic acid breath test (^{13}C-OABT) has dug its way towards the top of the list of the most widely used methods for the measurement of gastric emptying of solids. For example, recently published epidemiologically-oriented studies addressing the features of gastric emptying kinetics in large cohorts of patients involving hundreds of subjects suffering from functional dyspepsia, have been performed with ^{13}C-OABT[2-4]. Also the introduction of less expensive and technically demanding apparatus, based on the nondispersive isotope-selective infrared spectrometry (NDIRS), is an undeniable milestone which has opened the gate for a widespread use of ^{13}C breath tests[5-7], the ^{13}C-OABT making no exception in this respect[8-13].

Reproducibility is a vital feature of any measurement or diagnostic method used in medical practice. Few data are available on the repeatability of the ^{13}C-OABT in humans, and reports addressing this subject appear unsatisfactory[14,18]. Moreover, no previous study has reported reproducibility of the NDIRS variety of the ^{13}C-OABT. The aim of this study was to establish the short- and medium-term repro-

ducibility of the ^{13}C-OABT with NDIRS and a reduced dose of the ^{13}C substrate.

MATERIALS AND METHODS

Subjects

Twelve healthy subjects (6 females and 6 males) were recruited to participate in this study, their mean age was 24.9 ± 0.7 years, and the average body mass index amounted to 22.37 ± 1.06 kg/m^2.

During a screening interview, the participants declared themselves to be in full health according to the World Health Organisation criteria. Exclusion criteria comprised a history of surgery affecting the digestive tract anatomy with the exception of appendectomy, current use of any drugs which might affect gastrointestinal motility, and pregnancy. The recruitment procedure involved performance of a standard breath test with ^{13}C-urea, so that exclusively subjects with a negative test result for *Helicobacter pylori* infection were admitted. Except for three persons, all the participants were non-smokers. The study was conducted in accordance with the Helsinki Declaration, and each volunteer gave a written consent after getting information as to the aim, protocol and methodology of the study. During the introductory interview, the subjects were committed not to eat any food of naturally increased ^{13}C content, such as products made of maize, cane sugar, pineapple, kiwi fruit for 48 h preceding the examination.

Experimental protocol

Each subject underwent three sessions of gastric emptying measurement held on separate days. In six subjects the first two examinations were performed 2 d apart, and the third session was carried out at a median interval of 19.5 d (range 18-20 d) from the second one. In another six subjects, the first two gastric emptying measurements were taken 20 d apart (median, range 17-23 d), whereas the third session took place 2 d after the second one. The assignment of the order of intervals separating the sessions (short-long or long-short) was randomized. Ultimately the short-term reproducibility assessment involved performance of 12 pairs of gastric emptying examinations separated by a two-day break, whereas twelve pairs of examinations accomplished at a median interval of 21.5 d (range 17-23 d) were dedicated for the evaluation of the medium-term reproducibility.

The research was performed on volunteers who reported themselves to the laboratory in the morning, after a 12-h overnight fasting and abstaining from cigarette smoking (if applicable). At the beginning, a basal fasted probe of the exhaled air was put into an aluminium-covered plastic bag of about 1 L capacity (Fisher Analysen Instrumente GmbH, Germany). At the time point of "0", the subjects were given a solid test meal, a pancake made of two eggs, 30 g wheat flour and 0.1 g baking powder, which was additionally smeared with 50 g of strawberry jam before serving. The total energy content of the meal was 1574 kJ (378 kcal) and it contained 15.5 g proteins, 16.8 g fat, and 43.0 g carbohydrates. During the preparation procedure of the pancake, the two-egg yolks were temporarily separated from the egg whites and thoroughly mixed with 75 μL (68 mg) of ^{13}C-octanoic acid (INC610P, lot #T012A-L3241, Euriso-Top, France) which was instilled with a precision digital micropipette (Calibra 822-20/200, Socorex, Switzerland). Thereafter the yolks were added to and stirred with the remaining ingredients with an electric mixer. Finally the dough was transferred into a pan and fried to firm consistency with addition of 5 mL of sunflower oil. The time needed to consume the test meal did not exceed 10 min. No drink was served with the meal because the study intended to assess strictly the reproducibility of the solid phase gastric emptying.

Reckoning the passage of time from the defined "0" point, 26 probes of the expiratory air were collected postprandially every 10 min during the first hour, and then every 15 min for an additional 5 h. After ingestion of the meal, the subjects were asked not to take any additional food or drink for 6 h. They were enabled to rest sitting in a comfortably furnished room and allowed to watch video films.

Measurement of $^{13}CO_2$ and derivation of gastric emptying parameters

Concentrations of $^{13}CO_2$ in the probes of the exhaled air were measured with the NDIRS apparatus (*IRIS*, Wagner Analysen Technik Vertriebs GmbH, Germany; a model equipped with 16 ports for simultaneous mounting of bags with air samples was used). Repeatability of the $^{13}CO_2$ measurement with the NDIRS technique was checked by double measurements performed on 96 probes of the exhaled air originating from 3 randomly selected gastric emptying examinations. The procedure was performed in such a way that the second measurement was taken instantly without removing the bags containing the probes from the apparatus.

The determined $^{13}CO_2$ content within the total pool of the exhaled CO_2 was expressed in δ‰ PDB units, i.e. relative to the international standard - the calcium carbonate of the fossil Belemnitella of the cretaceous Pee Dee formation in South Carolina, USA (zero δ‰ PDB corresponds to 1.12372% ^{13}C atoms within $CaCO_3$). The obtained δ‰ PDB data were exported to an ASCII file for a subsequent analysis with a user-created Excel spreadsheet.

The changes in $^{13}CO_2$ concentration were recalculated to net increments expressed in ‰ DOB (*delta over baseline*) units according to the formula:

$$DOB_i = \delta‰\ PDB_i - \delta‰\ PDB_0$$

where **i** stands for the probe number, and the 0 index pertains to the basal probe of the expiratory air.

The momentary ^{13}C recovery at time i, D%$_i$_^{13}C, expressed in percent age of administered dose per hour (% dose per hour), was computed according to the equation:

$$D\%_i\_^{13}C = 100 \cdot (DOB_i/1000 \cdot 0.0112372 \cdot TCO_2) / dose$$

where TCO_2 = total expiratory CO_2 production in mmol/h and dose = amount in mmol of ^{13}C-octanoic acid given to the subjects. The TCO_2 was derived by multiplication by the rate of 300 mmol/m^2 per hour by the body surface area computed according to the formula by Haycock *et al*[19].

The ^{13}C recovery within a given period, C$_i$_^{13}C, ex-

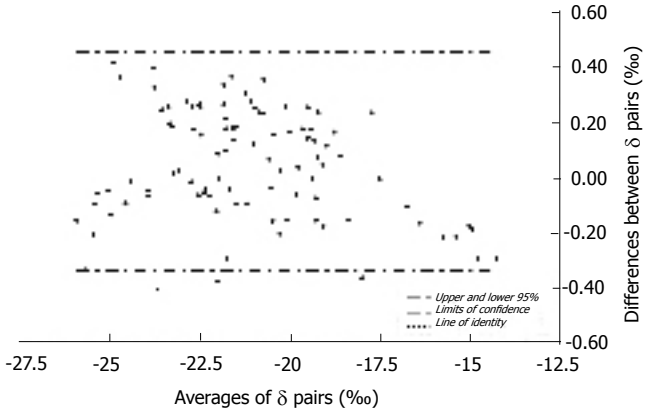

Figure 1 Repeatability of the measurement of $^{13}CO_2$ enrichment (δ) in expiratory air after intake of a solid meal labeled with 75 μL (68 mg) ^{13}C-octanoic acid. Ninety-six pairs of δ measurements taken from three randomly selected examinations were considered.

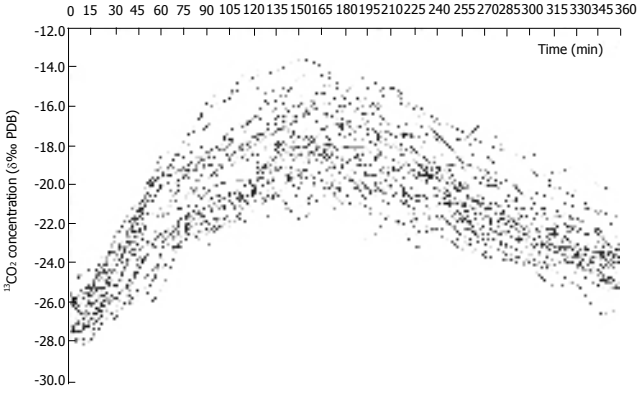

Figure 2 Individual curves reflecting the increment in $^{13}CO_2$ enrichment in exhaled air after intake of a 378 kcal solid meal containing 75 μL (68 mg) ^{13}C-octanoic acid (the 36 curves were obtained in 12 healthy subjects at three separate days).

pressed in percent age of dose, was computed following the trapezoid rule:

$C_i\_^{13}C = 0.5 \cdot (DOB_{i-1} + DOB_i)/1000 \cdot \triangle t \cdot 0.0112372 \cdot TCO_2 / dose$

where $\triangle t$ = the time span between consecutive DOB measurements

The cumulative ^{13}C recovery T_i -^{13}C in percent age of administered dose was then derived from a stepwise summation of $C_i\_^{13}C$ for i within the domain of 1 - 26.

Using algorithms of non-linear regression implemented in the Statistica 6.1 software[20], the curves of momentary ^{13}C recovery were fitted to the function:

$D\%_i\_^{13}C = at^be^{-ct}$

where t stands for time, and a, b, c are parameters of the function

which enables computation of three gastric emptying parameters[1, 21]: the lag phase, T-Lag = b/c; the gastric half emptying time, T½ = Gamma inv. (0.5; b+1; 1/c); and the gastric emptying coefficient, GEC = ln(a)

Statistical analysis

A null hypothesis, assuming a zero difference between repeated measurements, was checked with the paired Student's *t* test. The reproducibility of the gastric emptying parameters was expressed in terms of the coefficient of variation for paired examinations, CV_p[22,23]. Moreover Bland and Altman statistics was applied in order to calculate the repeatability coefficient, RC[24,25]. Finally, $\triangle_{0.05}$ - the least statistically significant difference detectable at P=0.05 level (two-tailed) was calculated for each gastric emptying parameter[26]. Results were presented as mean ± SE. All statistical analyses were performed with the Statistica 6.1 software, licence # adbp409a903816ar[20].

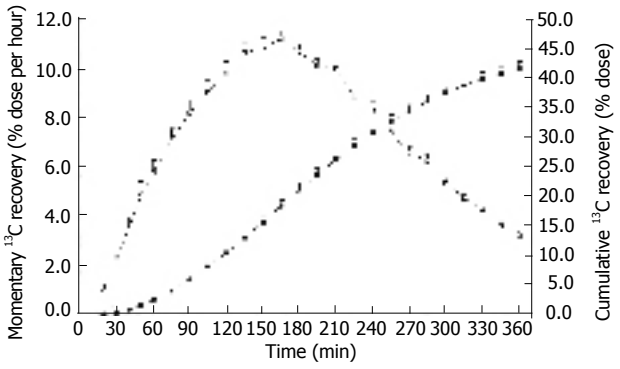

Figure 3 Momentary (empty squares) and cumulative (filled squares) ^{13}C recovery in exhaled air after ingestion of a 378 kcal solid meal containing 75 μL (68 mg) ^{13}C-octanoic acid. The data are grand means with standard errors (bars) calculated on average values obtained in 12 healthy volunteers during three examinations carried out on separate days.

RESULTS

Repeatability of NDIRS measurement of $^{13}CO_2$ enrichment in exhaled air

Bland and Altman statistics performed on the paired measurements of the $^{13}CO_2$ content within 96 samples of the expiratory air yielded a repeatability coefficient of 0.40‰, and the mean difference between the repeated measurements amounted to 0.07‰ (95% confidence interval: 0.34 - 0.46‰) (Figure 1). If the data pertaining to the $^{13}CO_2$ content within the expiratory air were expressed in terms of the delta over baseline values, the respective repeatability coefficient amounted to 0.62‰, with a mean difference of 0.28‰ between repeated measurements (95% confidence interval: 0.34 - 0.90‰).

Reproducibility of parameters characterizing gastric emptying kinetics

Ingestion of the solid test meal labeled with 75 μL of ^{13}C-octanoic acid brought about an expected increase of the $^{13}CO_2$ concentration in the exhaled air (Figure 2). The maximum net increment in the $^{13}CO_2$ concentration amounted to 9.1‰ (mean, 95% confidence intervals 5.3‰ to 12.9‰) during the 36 gastric emptying examinations. The maximum momentary ^{13}C recovery in the exhaled air was observed at 165 min (mean, 95% confidence intervals 112 min to 219 min) postprandially, and amounted on average to 11.50 ± 0.36 % dose per hour (Figure 3). During the whole 6 h observation period 41.80 ± 1.13 % of the administered tracer was eliminated in the form of $^{13}CO_2$ within the expiratory air (Figure 3).

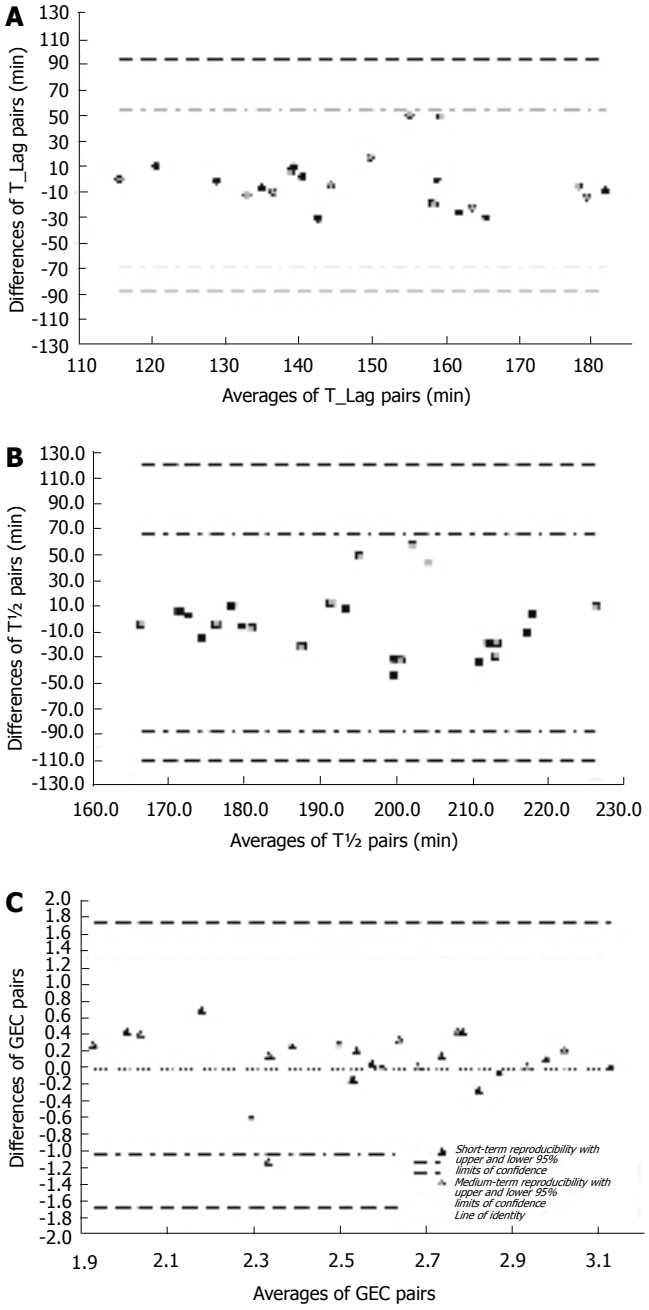

Figure 4 Bland and Altman statistics (plot of differences between pairs *vs* their names) of the short-(open symbols) and medium-term(filled symbols) reproducibility of the measurement of the lag phase (T-Lag, panel A), the gastric half emptying time (T½ , panel B), and the gastric emptying coefficient (GEC, panel C) of the solid phase gastric emptying with a breath test using 75 μL (68 mg) ^{13}C-octanoic acid and the isotope-selective nondispersive infrared spectrometry. On each panel the respective borders of the 95% confidence intervals are plotted - cf. the legend of the GEC graph, panel C.

A graphical depiction of the Bland and Altman statistics pertaining to the gastric emptying parameters is given in Figure 4, and a comprehensive statistical description of the reproducibility data is provided in Table 1. In no instance the null hypothesis of a zero difference between the repeated measurements had to be discarded. According to the data assembled in Table I, greater values of RC and CV_p characterized the medium-term compared to the short-term reproducibility. A comparison of the absolute values of the differences between the paired measurements implied rejection of a hypothesis that reproducibility of the gastric emptying was significantly worse when the examinations were separated by an extended time gap.

DISCUSSION

The novelty of the results furnished by the current study consists in: for the first time the reproducibility of the ^{13}C-OABT was evaluated with the NDIRS, a reduced dose of the ^{13}C-octanoic acid was applied for the test, the test meal was administered without allowance of any intake of liquids so that pure repeatability of the solid phase gastric emptying could be discerned, and a distinguishment was made between the short- and medium-term reproducibility.

Typically the ^{13}C-OABT is performed with 100 μL of ^{13}C-octanoic acid which is equivalent to 91 mg[2-5,8-12,14,16-18]. It was reported that a lower dose - 50 mg ^{13}C-octanoic acid can be used to examine the solid phase gastric emptying[21]. The NDIRS apparatus coped well with the 68 mg (75 μL) amount of ^{13}C-octanoic acid applied for labelling the test meal in the current study. This contention is supported by the finding of a low value of the repeatability coefficient (0.40‰) and a narrow 95% confidence interval of the differences between paired measurements of $^{13}CO_2$ enrichment (0.34 - 0.46‰) derived from a large number of breath samples covering a typical range of $^{13}CO_2$ concentration encountered throughout the 6 h of the ^{13}C-OABT. Since baseline fluctuation of $^{13}CO_2$ concentration in breath is expected to approach a standard deviation of 0.72‰[27], our results clearly indicate that the measurement error involved in the NDIRS technique is less than the natural baseline fluctuation of $^{13}CO_2$ concentration in breath. The finding quoted is in concordance with our previously published results[6,7,28].

While performing the ^{13}C-OABT, we generously allowed for a frequent sampling of the expiratory air (a total of 27 samples were collected throughout a single examination including the basal specimen) over a long observation period of 6 h. This approach was substantiated by findings of other authors clearly stating that too short a collection period and/or an infrequent sampling of the expiratory air may lead to erroneous estimation of the parameters of gastric emptying with the ^{13}C-OABT[16,17,29]. According to Choi *et al*[16,17], duration of the ^{13}C-OABT obligatory should be extended to six hours, since largely overestimated values of T_Lag and T½ would be derived from the data truncated to four hours.

Three parameters are originally proposed as quantitative descriptors of the gastric emptying kinetics examined with the ^{13}C-OABT, namely: T_Lag, T½, and GEC[1,21]. Nowadays researchers are increasingly inclined to use the T ½ only when reporting on their results with the ^{13}C-OABT. This apparently minimalistic approach has quite firm grounds. The shortcomings of GEC consist in the fact that its estimation is not independent of the endogenous CO_2 production, and in the case of fast gastric evacuation patterns this parameter may underestimate gastric emptying[1]. T_Lag of the ^{13}C-OABT is originally conceived to reflect the duration of the first phase of gastric evacuation, while the solid meal is ground to particles of 1-2 mm in diameter before the actual emptying can start. This assumption has not found any convincing corroboration in vali-

Table 1 Reproducibility of solid gastric emptying measurement with breath test at a 68 mg (75 μL) dose of ^{13}C-Octanoic acid and isotope-selective nondispersive infrared spectrometry

Observed values[1]	T-Lag, lag phase 149.7 ± 4.6 min		T½, gastric half emptying time 195.6 ± 4.2 min		GEC, gastric emptying coefficient 2.56 ± 0.09	
	Short-term	Medium-term	Short-term	Medium-term	Short-term	Medium-term
RC (min)	31.6	46.7	39.1	59.1	0.61	0.87
CVp (%)	7.7	11.2	7.3	10.9	8.3	12.5
△ 0.05 (min)	9.6	15.6	11.6	19.7	0.17	0.29

Short-term reproducibility involved twelve paired examinations performed 2 d apart, whereas medium-term reproducibility involved twelve paired examinations at a median interval of 21.5 d (range 17 - 23 d)

RC = repeatability coefficient, CVp = coefficient of variation for paired examinations, △ 0.05 = the least statistically significant difference detectable at $P = 0.05$ level, two-tailed

[1] These data are grand means calculated on average values obtained during three examinations carried out on separate days

dation studies of the ^{13}C-OABT against the scintigraphic determination of gastric emptying, which is the so called '*golden standard*' in the field of gastro-emptology[16,17]. Quite recently it was even demonstrated by Doppler ultrasonography that transpyloric flow starts already during ingestion of a ^{13}C-labelled solid meal, which results in a detectable excretion of $^{13}CO_2$[30]. It has been shown that trituration of the solid meal can lead to a pronounced shortening of this parameter[31]. Nevertheless, T_Lag will be more and more willingly replaced by the term '*time to reach the maximum of the momentary $^{13}CO_2$ excretion*' which is the revised representation of what the T-Lag derived mathematically from the function fitted to a $^{13}CO_2$ excretion curve actually is[1,8,9,21].

In the present study, we controlled rigorously and kept constant a number of factors which might affect reproducibility of the ^{13}C-OABT, namely meal composition, time of the day when the gastric emptying measurement was taken, posture of the subjects during the test. Ingestion of the test meal took place irrespective of actual phase of the duodenal migrating motor complex (MMC), because monitoring of the duodenal motor activity would require insertion of a manometric tube into the lumen of the duodenum - an invasive manoeuvre which might interfere with physiological conditions assumed for the ^{13}C-OABT performance; observation of a particular phase of the duodenal MMC could not ameliorate the reproducibility of the scintigraphic measurement of the gastric empting of the solid phase of a meal[32]. Homogeneity was the important feature of the test meal we applied, that is the pancake was uniformly labelled with ^{13}C-octanoic acid and eaten without addition of any liquid to assure the sole assessment of the reproducibility of the solid phase gastric emptying [33].

Because there are no other reports on the ^{13}C-OABT performed with the NDIRS, our data can be compared only to the results obtained by other authors applying the isotope ratio mass spectrometry (IRMS). Choi *et al*[17] reported that the reproducibility of T-Lag and T½ is 14% and 15% respectively. In the study by Delbende *et al*[18] the pertinent RC and CV_p amounted to 35.5 min and 14.3%, respectively. The reproducibility results of those two studies look very much alike indeed. The results of the present study indicated that the reproducibility of gastric emptying of solids determined by ^{13}C-OABT and the NDIRS was not worse than that by IRMS. Moreover, both the reproducibility and sensitivity (as assessed by the magnitude of the least detectable differences in the gastric emptying parameters) were preserved when the examinations were separated by a two- three-week time gap. Finally, it should be emphasized that the results obtained by others[17,18] and coming from our current study clearly demonstrate that the reproducibility of the measurement of gastric emptying of solids by the ^{13}C-OABT is equivalent to the intra-subject variability of the solid-phase gastric emptying observed with gamma scintigraphy[22,23]. This is an important finding due to the inherently indirect nature of ^{13}C-OABT. The reproducibility of ^{13}C-OABT would be affected by more factors than a scintigraphic GE measurement, namely the intestinal absorption of the ^{13}C-octanoic acid, its oxidation to $^{13}CO_2$ within the liver, as well as kinetics of the subsequent transfer of $^{13}CO_2$ to the expiratory air.

In conclusion, the low-cost modification of the breath test involving a lower dose of ^{13}C-octanoic acid and NDIRS, renders a good short- and medium-term reproducibility, as well as sensitivity of the measurement of gastric emptying of a solid meal.

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RAPID COMMUNICATION

Higher platelet P-selectin in male patients with inflammatory bowel disease compared to healthy males

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Abstract

AIM: To observe if the total amount of platelet P-selectin (tP-selectin) in patients with inflammatory bowel disease (IBD) was related to disease entity or activity, 5-aminosalicylic acid (5-ASA) medication or gender.

METHODS: tP-selectin was measured by immunoassay in seventeen IBD patients and twelve healthy controls.

RESULTS: Compared to controls, there was no difference of tP-selectin in patients related to disease entity or activity and 5-ASA medication. When the groups were split according to gender the male patient group showed higher levels of tP-selectin compared to male controls (153 ng/mL *vs* 94 ng/mL, $P < 0.05$).

CONCLUSION: Increased tP-selectin levels may alter the inflammatory response and susceptibility to thromboembolic disease. As previously shown with soluble P-selectin, tP-selectin shows gender dependent differences important to consider in future studies.

Key words: Platelet; P-selectin; Inflammatory bowel disease; 5-aminosalicylic acid; Gender

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INTRODUCTION

Upon platelet activation, intracellular P-selectin is expressed on the membrane surface and released from the platelets[1]. In addition to P-selectin, platelets release various inflammatory mediators that regulate both hemostasis and inflammation[2].

Ulcerative colitis (UC) and Crohn´s disease (CD) are disorders of unknown aetiology, jointly referred to as inflammatory bowel disease (IBD). Current research suggests platelet dysfunction as a contributor to the disease since increased risk of thromboembolic disease and abnormal platelet activity have been found. Increased surface expression of platelet P-selectin in patients with IBD did not appear to reflect disease activity[3,4], whereas soluble P-selectin in plasma recently was suggested to be parellel to the severity of UC[5]. Using a new technique to quantify the total amount of P-selectin in platelets, patients with hypertension have been reported to have higher levels of platelet P-selectin[6]. Our aim of this study was to observe if total amount of platelet P-selectin (tP-selectin) differs between IBD patients and healthy controls and if there is a difference regarding disease entity, disease activity, 5-aminosalicylic acid (5-ASA) medication and gender.

MATERIALS AND METHODS

Controls and patients with intake of anti-platelet drugs or steroids two weeks prior to sample collection were excluded. Seven female and five male healthy blood-donors served as a controls (mean age 44.9, range 25-65 years). The patient group consisted of six female and eleven male IBD patients (mean age 44.4, range 19-69 years). Diagnosis was verified with histological examination (biopsy and colonoscopy); twelve patients had UC and five patients had CD. Patients were sub-grouped according to disease activity, 5-ASA medication and gender. In UC eight patients were in relapse and in CD all patients were in remission. Ten patients had 5-ASA medication.

Isolated platelets (IP) were prepared as previously described[7]. Frozen IP samples were thawed and incubated with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 1 h. Thereafter, the permeabilised IP samples were gently mixed prior to measurement of tP-selectin levels according to standard procedures using an immunoassay from R&D Systems (Abingdon, Oxfordshire, UK).

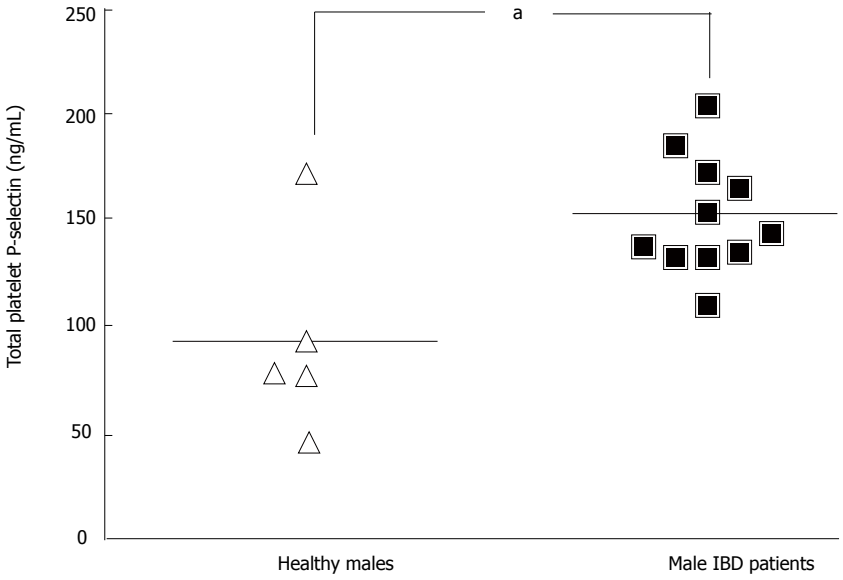

Figure 1 Total P-selectin in permeabilised platelets from 5 healthy male subjects and 11 male patients with inflammatory bowel disease. Horizontal lines represents the mean value. [a]$P < 0.05$ *vs* unpaired t test with Welch's correction.

Statistical analysis

All values are presented as mean ± standard error of the mean (SE). Statistical analyses were calculated by unpaired *t* test with Welch's correction for samples with different variances. Differences were considered significant when $P < 0.05$.

RESULTS

All values are in ng/mL. There was no significant difference of tP-selectin between controls, 133 ± 20.1 (n = 12), and patients, 154 ± 8.1 (n = 17). When samples were sub-grouped according to gender the male patients showed significant higher levels compared to the male controls; 153 ± 8.2 and 94.1 ± 21.1 (Figure 1). In the sub-grouped male group the mean and range of age were 46.8 and 32-55 years in the control group and 46.4 and 24-65 years in the male patient group, denoting that age is not the cause of the different levels. Both the female controls and the female patients tended to have higher levels compared to male controls, although insignificantly. There were no significant differences between the female controls and female patients; 161 ± 27.7 (n = 7) and 156 ± 18.7 (n = 6). The levels of tP-selectin in UC compared to CD were 151 ± 9.6 (n = 12) and 163 ± 16.3 (n = 5). Values regarding disease activity showed following results; remission, 157 ± 11.2 (n = 9) and relapse, 152 ± 11.7 (n = 8). None of the groups showed significant difference in comparison with the controls. The levels in male patients in remission compared to male patients in relapse were; 155 ± 13.7 (n = 6) and 152 ± 9.2 (n = 5). Patients with 5-ASA compared to patients without 5-ASA had tP-selectin levels of 152 ± 9.1 (n = 10) and 159 ± 15.6 (n = 7), respectively. There was no significant difference between the male patients with 5-ASA, 151 ± 8.4 (n = 6) compared to male patients without 5-ASA, 156 ± 16.0 (n = 5).

DISCUSSION

Plasma levels of soluble P-selectin has recently been suggested to relate to the severity of UC[5] and increased tP-selectin has been found in hypertensive patients[6]. Our previous study showed that patients with IBD in remission had higher basal platelet P-selectin surface expression[4]. To investigate if this was related to higher platelet P-selectin content we analysed tP-selectin in patients with IBD. There was no difference of tP-selectin in patients taken as one group compared to controls, irrespectively of disease entity or activity and 5-ASA medication. When the groups were split according to gender the male patient group showed significantly higher levels compared to male controls. Both healthy and patient females showed greater variance compared to the male groups. This may be caused by a hormonal influence on tP-selectin, since sP-selectin fluctuates during the menstrual cycle[8] and age (pre- and post-menopausal). Other sex-based differences that influence the disease activity in IBD have recently been reported[9]. In the present study, male patients had higher tP-selectin levels regardless of disease entity, disease activity and 5-ASA medication. Taken together with our previous findings[4], we suggest that male IBD patients have higher levels of tP-selectin and exhibit higher basal platelet P-selectin expression when in remission. This could have an exaggerated influence on the inflammatory response and susceptibility to thromboembolic disease.

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RAPID COMMUNICATION

Does endothelium agree with the concept of idiopathic hepatic vessel thrombosis?

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Abstract

AIM: To investigate the major steps of thrombogenesis and to identify the differences in these steps between idiopathic patient group and control group.

METHODS: Fibrinogenesis was studied by measuring the activated factor VII, total and free levels of tissue factor pathway inhibitor (TFPI). The fibrinolysis step was investigated by determining the global fibrinolytic capacity. The endothelial function was assessed by measuring the levels of soluble adhesion molecules, namely soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble E-selectin molecule. The exclusion criteria from "idiopathic" patient group were abdominal surgery, pregnancy, use of oral contraceptives, anti-phospholipid syndrome, Behçet's disease, cancer, myeloproliferative diseases. The congenital factors like mutations of factor-V Leiden and prothrombin, deficiencies of proteins C and S, antithrombin, hyperhomocysteinemia and hyperfibrinogenemia were ruled out. The total number of patients was reduced from 96 to 9 (7 with portal vein thrombosis, 2 Budd Chiari syndrome) by exclusion criteria.

RESULTS: The levels of adhesion molecules sICAM-1, sVCAM-1, free TFPI levels and global fibrinolytic capacity were significantly different ($P < 0.05$) in the patient group indicating an endothelial dysfunction and a lower fibrinolytic activity.

CONCLUSION: These results show that this patient group should be tested by means of endothelial dysfunction and managed differently.

Key words: Portal vein thrombosis; Budd-Chiari syndrome; Endothelial dysfunction; Soluble adhesion molecules; Fibrinolysis

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INTRODUCTION

Thrombosis in the hepatic vascular system (hepatic veins and portal veins) is an established risk factor for both morbidity and mortality due to inevitable consequences of disturbed liver vascular physiology and anatomy. The primary mechanism resulting in this condition may be clinically evident (ie, a mass-forming lesion adjacent to a major vessel resulting in compression and occlusion, which is called a "secondary" thrombosis) and by definition these patients are treated with therapeutic techniques to eliminate the cause. On the other hand, in some patients there may be a thrombophilic condition resulting in a "primary" thrombosis which usually is the case in this patient population. The clinical importance of presence or absence of a thrombophilic condition lies in the fact that the presence of a thrombophilic condition increases the mortality by a factor of 4[1].

Studies investigating the etiology of thrombosis in hepatic vasculature indicate that the most commonly-found predisposing thrombophilic conditions are deficiencies of proteins C and S as well as antithrombin (AT), mutations of factor V Leiden and prothrombin followed by myeloproliferative diseases and other less commonly-observed disorders including Behçet's disease[2-6]. On the other hand, there seems to be a patient population in whom there is not any obvious thrombophilic condition that can be found utilizing routine techniques but mortality risk increases still in the high risk group due to undiagnosed thrombophilic condition. This unique population is called "idiopathic" thrombosis group and their contribution to the main patient population is about

Table 1 Applied exclusion criteria

Exclusion criteria
Acute thrombosis of portal or hepatic veins
Any ultrasonographic (including Doppler studies) finding compatible with mass occupying lesion in liver
Positivity of viral hepatitis markers
Any finding in the liver biopsy suggesting parenchymal liver disease (including autoimmune liver diseases and metabolic liver diseases)
Acquired hematological abnormality leading to thrombophilia (myeloproliferative diseases, paroxysmal nocturnal hemoglobinuria, history of use of oral contraceptive or estrogen replacement treatment, anti-phospholipid syndrome, history of cancer, history of hepatobiliary surgery, history of pancreatitis)
Genetic hematological abnormality leading to thrombophilia (mutation of factor V Leiden, mutation of prothrombin, deficiencies in proteins C and S or antithrombin, hyperfibrinogenemia, hyperhomocysteinemia)
Severe and widespread atherosclerosis, uncontrolled or newly diagnosed malignancy elsewhere, systemic inflammatory diseases, history of recent operation

7%-22%[7,8]. In the current knowledge, hematological changes of this remaining "idiopathic" group is unclear, hence giving opportunity to investigate this patient population with advanced markers of thrombosis formation.

According to the current concept of pathologic thrombus formation, a thrombus is the result of convergence of multiple factors which may be either genetic or acquired. Therefore, it seems crucial to clarify the underlying possible pathogenetic factors or predisposing conditions in the "idiopathic" patient population in terms of tests not routinely used for the investigation of thrombogenesis.

In our study, we aimed to investigate the general characteristics of patients with idiopathic portal or hepatic vein thrombosis, to compare the "idiopathic" patient group with healthy adults in terms of major steps of thrombogenesis and fibrinolytic potentials, and to reconcile the "thrombophilia theory" in etiology of idiopathic portal hypertension with idiopathic portal vein thrombosis. The advanced tests that we used to investigate possible thrombophilia included activated factor VII (FVIIa) which is the most potent initiator of thrombin formation via tissue factor and its potent controller called tissue factor pathway inhibitor (TFPI; total and free fractions), adhesion molecules, namely soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin molecule as markers of endothelial dysfunction and global fibrinolytic capacity (GFC) to assess the steps of fibrinolysis.

This study was designed to investigate the endothelial dysfunction, steps of fibrinogenesis and fibrinolytic capacity in a patient group named as "idiopathic" after use of current techniques to investigate thrombophilia.

MATERIALS AND METHODS

Patient and control groups

All the patients diagnosed to have chronic portal vein or hepatic thrombosis with any appropriate diagnostic technique (Doppler-ultrasound, conventional angiography, computerized tomographic angiography or magnetic resonance angiography) between January 2000 and December 2003 were evaluated for possible inclusion to the study. One splenoportal angiography of our patient group showed portal vein cavernous transformation (Figure 1). The exclusion criteria (as shown in Table 1) were applied to eliminate chronic parenchymal liver diseases including cirrhosis, mass occupying lesion around great vessels and any genetic or acquired abnormality leading to thrombophilia.

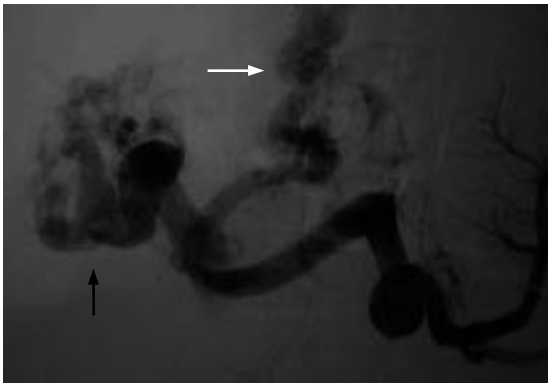

Figure 1 Splenoportal angiography showing cavernous transformation (bold arrow), eso phageal varices (white arrow) indicating severe portal hypertension.

All the patients underwent a series of evaluation comprised of viral markers of hepatitis, screening tests of metabolic liver diseases (Wilson's disease, hemochromatosis), liver biopsy, red blood cell count, full biochemical tests, reticulocyte counts and peripheral blood smear in order to rule out silent liver diseases or myeloproliferative blood diseases. Any increased hematocrit more than 50% indicated a scintigraphic total red cell mass count, whereas any abnormality in reticulocyte count or any suspicious finding in peripheral smear indicated a bone marrow aspiration. Biopsy studies were carried out to exclude silent or latent hematological diseases.

Genetic and other thrombophilic risks were evaluated by determining the levels of factor V Leiden and prothrombin mutations, proteins C and S, antithrombin, IgG and IgM classes of anti-cardiolipin and anti-phospholipid antibodies, homocysteine and fibrinogen. CD 55 and CD 59 clusters in peripheral blood were studied in all patients to rule out paroxysmal nocturnal hemoglobinuria after all hematological investigations.

In view of potential interference with the tests to be used in the study, patients with a history of uncontrolled diabetes and hypertension, severe and widespread atherosclerosis, oral contraceptive use or estrogen replacement treatment, use of anticoagulation drugs, uncontrolled or newly diagnosed malignancy elsewhere, systemic inflammatory diseases and recent (last 3 mo) major operation were excluded. Application of the exclusion criteria reduced the total number of 96 patients (portal vein involvement in 70, hepatic vein involvement in 26) to 9 patients comprised of 7 cases of portal vein thrombosis (PVT) including cavernous transformation of the portal vein, and 2 cases of hepatic vein thrombosis (HVT). After all these investigations, bone marrow biopsies were taken from patients with

Table 2 General characteristics of the patients (results were given as minimum, maximum and median)

Test	Result
Age	28-74 (48)
Female/Male	6/3
PVT (*n*)	7
HVT (*n*)	2
Hemoglobin (gr/L)	9.5-16.6 (13.5)
Platelet (count/mL)	62.000-358.000 (168.000)
ALT (U/L)	13-38 (23)
AST (U/L)	18-38 (27)
ALP (U/L)	87-355 (200)
GGT (U/L)	16-123 (34)
Total Bilirubin (mg/L)	0.24-2.08 (0.65)
INR	1.06-1.47 (1.14)
Protein C (%)	55-137 (87)
Protein S (%)	58-113 (85)
AT (%)	75-109 (90)
Homocysteine (umol/L)	9-19 (15)
Fibrinogen (mg/L)	181-420 (271)

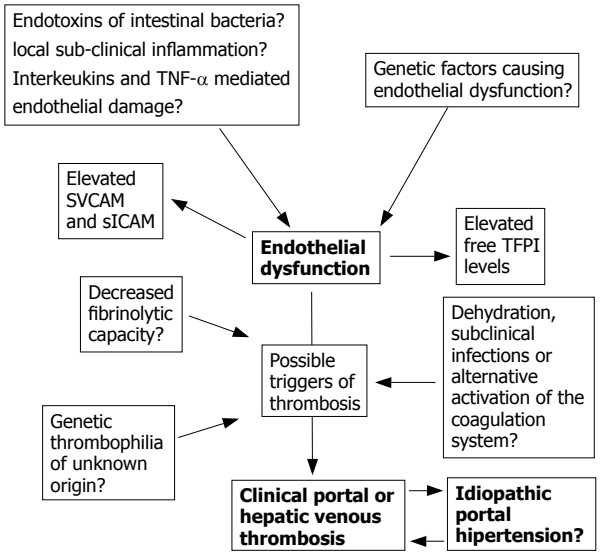

Figure 2 Results from our study and possible associations with other processes.

a diagnosis of idiopathic thrombosis to rule out any silent hematological diseases.

In excluded patients, the most common thrombophilic conditions were deficiency in proteins C and S and antithrombin III (28 patients, 29.1%), followed by myeloproliferative disorders (10 patients, 10.4%), factor V Leiden mutations (10 patients, 10.4%), prothrombin mutations (6 patients, 6.25%). The other conditions related with possible thrombophilia included cyst hydatid disease of the liver, Behcet's disease, metastasis of cancer, mastocytosis invasion of the liver, tuberculosis of the choleduct and abdominal surgery.

The control group comprised of 14 healthy adults (6 males, 8 females) with no history of cigarette smoking, hypertension, atherosclerotic vascular disease, systemic inflammatory condition and no use of medication (oral contraceptives and estrogen replacement treatment). An informed consent was obtained from each patient and control subject. The study protocol recieved approval of Ethical Committee of Hacettepe University.

Blood samples

Blood samples from patient and control groups were drawn from antecubital veins with a 20 G needle at 08:00 am after an overnight fasting. All samples were collected in 0.129 mol/L trisodium citrate tubes and centrifuged at 3000 r/min for 10 min, while plasma samples were stored at -30 ℃ for studies.

Evaluation of endothelial function

The endothelial function was tested by measuring serum concentrations of adhesion molecules, namely sVCAM-1, sICAM-1 and soluble E-selectin. The concentrations of these adhesion molecules were evaluated quantitatively by sandwich enzyme immunoassay (Parameter®, R&D systems, UK).

Evaluation of inital step of thrombogenesis and its control

The most potent initial step during formation of thrombin, tissue factor-(released from damaged endothelium) related activation of factor VII was evaluated by quantitative measurement of activated factor VII (Staclot® VIIa-TF, Diagnostica Stago, Asnieres, France). The major controlling step of this pathway, namely the TFPI (both total and free fractions) level, was tested quantitatively by one-step ELISA (Diagnostica Stago, Asnieres, France).

Fibrinolytic potential

Fibrinolytic capacity of each sample was evaluated by GFC, a semi-quantitative method designed to detect low potentials of given samples. This technique uses the measurement of generated D-dimer in the given sample when a standard amount of fibrin is added into it. The generated D-dimer was measured using macrolatex agglutination (Diagnostica Stago, Asnieres, France). Therefore, the results from this test were semi-quantitatively expressed as "normal GFC", "moderately low GFC" and "severely low GFC".

Statistical analysis

All the data were presented as minimum, maximum and median unless otherwise specified. Statistical tests used for evaluation of significant differences were Mann-Whitney U test and Fisher's exact test.

RESULTS

The general characteristics of the patients are shown in Table 2 and Figure 2. Four patients had portal vein cavernous transformation and 3 patients had pure portal vein thrombosis in the portal vein group, whereas HVT group had only large hepatic vein involvement. As the blood cell count was taken into account, patient group showed a general tendency towards thrombocytopenia due to presence of increased portal venous pressure leading to mild degree of hypersplenism. Liver enzymes were all normal or very slightly elevated owing to the strict exclusion criteria. One important fact was that the international normalized ratio (INR) of the patient group was normal, thus not affecting the hematological tests

Table 3 Comparison of patient and control groups by advanced tests (results were given as minimum, maximum and median)

Test (normal limits)	Patient group (*n*)	Control group (*n*)	Significance
FVIIa (25.5-96.9 mU/mL)	27-1232 (96)	39-325 (129)	NS[1]
Total TFPI (81.2±30.4 ng/mL)	22-114 (87.2)	43-112 (79)	NS
Free TFPI (10.0±4.8 ng/mL)	5-41 (19)	9-20 (13)	[a]$P<0.05$
sVCAM (395-714 ng/mL)	489-1890 (915)	410-890 (517)	[c]$P<0.05$
sICAM (115-306 ng/mL)	148-400 (252)	24-260 (164)	[b]$P<0.05$
E-Selectin (29.1-63.4 ng/mL)	14-68 (20)	11-64 (29)	NS

[1]NS: Not significant; [a]$P<0.05$, [c]$P<0.05$, [b]$P<0.05$ *vs* control group.

Table 4 Results and comparisons of GFC (global fibrinolytic capacity)

Group	Severely low GFC	Moderately low GFC	Normal GFC
Patient group	0	4	5
Control group	0	0	14
	Fisher's exact test		$P<0.05$

during the study. Proteins C and S showed that the values near the lower normal limits and even mildly low values were due to the decreased synthesis from liver as a result of abnormal blood flow dynamics of liver rather than a congenital deficiency.

The results of hematological evaluation of patient and control groups with advanced tests are shown in Table 3. The comparisons of FVIIa in both groups were similar, so were total TFPI levels. The results obtained from comparisons of free TFPI and adhesion molecules sVCAM-1 and sICAM-1 were striking. Levels of free TFPI, sVCAM-1 and sICAM-1 were significantly higher in patient group than in control group. sE-selectin levels did not differ between the two groups.

The results obtained from patient and control groups by GFC are shown in Table 4. The patient group showed a significant tendency towards moderately low fibrinolysis whereas fibriolysis was normal in control group.

DISCUSSION

The overall results and our proposals are shown in Figure 2. Firstly, the significant differences in vascular adhesion molecules indicated that there was an overall activation of endothelium comparable to normal subjects, suggesting that there is an unknown abnormality in endothelial functions. Adhesion molecules are synthesized from endothelium in response to bacterial endotoxins, interleukins and inflammatory mediators like tumor necrosis factor-α[9-11], indicating that this endothelial dysfunction is a pure result of endothelial dysfunction of splenoportal or hepatic venous systems. Since other external risk factors affecting endothelial function and our tests such as diabetes mellitus, hypertension, cigarette smoking or atherosclerosis were excluded in both patient and control groups, this finding may be of importance.

The comparable difference in free TFPI could be explained also by this endothelial dysfunction. TFPI is a protein molecule formed of three Kunitz particles synthesized and secreted from endothelium. Its main function is to inhibit the activated FVII-tissue factor complex before coagulation cascade becomes uncontrollable[12,13]. Severe deficiency in TFPI may lead to overt thrombosis in veins[14]. After synthesized from endothelium, the active component of this protein circulates in free form accounting for approximately 2.5 % of total TFPI pool and it is this free form that exhibits the main TFPI activity whereas the low density lipoprotein-bound TFPI shows no activity at all[12]. Our findings (a significant difference in free TFPI levels between two groups but no difference in total TFPI levels) were parallel to the findings of endothelial dysfunction confirmed by adhesion molecules, since the increased free TFPI levels indicated a stimulated synthesis from malfunctioning endothelium.

The results obtained from GFC studies indicated that in patient group there was a significant tendency towards moderately low GFC. This in a way is consistent with the recent thrombogenesis theory which accepts that thrombogenesis is not attributable to only one factor. In fact, thrombogenesis requires more than one risk factor for potent initiation of the pathway. From this point, we propose that either low GFC or endothelial dysfunction forms an appropriate milieu for development of this kind of "idiopathic" thrombosis.

The study presented can be questioned in some ways. Firstly, the number of patients studied was small to be conclusive about the hypothesis proposed before. But as discussed before, this patient population was a very rarely confronted group and similar studies are few. Secondly, the patient group had two different vessel involvements and could not be studied as they were homogenous. But if the liver functions and the patterns of thrombus formation were examined in detail, these two different vessel involvements would be similar in terms of idiopathic thrombus formation in milieu of normal liver function. Thirdly, the laboratory tests applied were not informative enough for our assumptions. Formation of thrombus can be evaluated in many ways including quantification of thrombin-antithrombin complexes or prothrombin fragments 1+2 or many other sophisticated tests. However, our hypothesis is not to test the presence of abnormal activation of prothrombin, but to find any possible factors triggering coagulation cascade, resulting in abnormal clot formation. Therefore we tested the most potent initiator of coagulation cascade. It can be argued to study the endothelial functions with other tests like plasminogen activator inhibitor-1 (PAI-1), von-Willebrand factor or thrombomodulin.

To our knowledge, there is no evidence that these tests with adhesion molecules can show endothelial dysfunction. These tests have no superiority over each other for estimation of the degree of endothelial dysfunction.

There is an ongoing debate over the presence of portal vein thrombosis in case of idiopathic portal hypertension (IPH). The pro-thrombogenesis theoreticians believe that there is a tendency towards thrombosis which manifests itself in any life time of a patient with IPH[15]. The anti-thrombogenesis theoreticians believe that although thrombophilia and repeated micro-thrombi over a long time may be one of the possible etiological factors, the presence of portal thrombosis is a finding against diagnosis of IPH[16]. We believe that thrombogenesis and silent or unknown risk factors may contribute to the development of IPH[17].

In conclusion, major idiopathic hepatic thrombosis (hepatic or portal veins) is not as rare as it was thought and our study may be an important clue to further studies in respect of endothelial dysfunction and other possible accompanying coagulation control defects like deficient fibrinolysis.

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RAPID COMMUNICATION

Pulse cyclophosphamide therapy for inflammatory bowel disease

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Abstract

AIM: To assess the efficacy of intravenous cyclophosphamide pulse therapy for refractory inflammatory bowel disease (IBD).

METHODS: We included in our cohort eight patients with (moderate/severe) steroid refractory IBD (4 with ulcerative colitis and 4 with Crohn's disease). They all received 6 cycles of intravenous cyclophosphamide (800 mg) per month.

RESULTS: Patients entered into remission after the second/third cyclophosphamide pulse. Disease activity decreased. There were no side effects and toxicity. All the patients went into long lasting remission. All Crohn's disease patients and 3 of 4 ulcerative colitis patients achieved complete remission. One patient with ulcerative colitis showed an impressive clinical response but did not enter into remission. For the maintenance, patients with Crohn's disease were treated with methotrexate (15 mg/wk) and patients with ulcerative colitis were treated with azathioprine (2.5 mg/kg body weight/d).

CONCLUSION: Remission was maintained in all patients for 6 mo on the average. The drug was well tolerated. These findings suggest that aggressive immunosuppressive therapy may be useful in some refractory patients and further controlled study should be considered in order to fully evaluate this type of treatment as a potential therapy for IBD.

Key words: Crohn's disease; Ulcerative colitis; Cyclophosphamide

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as inflammatory bowel disease (IBD), are relatively common inflammatory diseases of the gastrointestinal (GI) tract. Histopathologically and anatomically, these two conditions are distinct, with CD characterized by transmural inflammation that can occur throughout the GI tract, and UC characterized by more superficial inflammation confined to the colon and rectum. The etiology and pathogenesis of IBD are not yet completely understood. Although the details of the pathogenesis of IBD remain elusive, it is believed that exposure of genetically susceptible individuals to environmental factors (e.g., dietary antigens, luminal bacteria) seems to be an important factor for the development and chronicity.

Pharmacotherapy for IBD has essentially been unchanged for over twenty years, with therapy based on 5-aminosalicylic acid (5-ASA) preparations, corticosteroids, antibiotics and immunosuppressants. Many of the controversies about optimal use of these drugs in IBD arise as a consequence of methodological deficiencies in many of the trials combined with the difficulty in consistent patient selection due to the heterogeneous nature of both UC and CD. Current therapy for IBD is neither sufficient nor disease-modifying. The first-line therapy for IBD flares is typically medical in nature. Long-term treatment with non-specific anti-inflammatory drugs such as aminosalicylates, corticosteroids and immunosuppressants is often accompanied with undesirable and potentially serious side effects. Glucocorticoids are a mainstay for the treatment of severe inflammatory bowel disease. Aminosalicylates are efficacious in the treatment of active mild to moderate disease. Infliximab, a chimeric monoclonal anti-TNF alpha antibody can be used in treatment of refractory Crohn's disease[1]. Novel biologically-driven therapies are targeted to specific pathophysiological processes, offering the potential for better treatment outcomes.

The management of refractory, severe IBD is yet an unsolved problem. Accurate assessment of specific organ involvement and disease severity is vital if we are

going to tailor appropriate therapy for certain patient. In common with other disease, the aims of therapy for IBD fall into three categories, namely induction of remission, maintenance of remission, and prevention of relapse. All should be undertaken with minimal mortality and morbidity either from the disease itself or from the therapy

Cytotoxic drugs prevent cell division or cause cell death. They act predominantly on rapidly dividing cells such as T lymphocytes, and are therefore immunosuppressive and anti-inflammatory. When cytotoxic drugs are initially used in the treatment of cancer, they have profound effects on the immune system. This "unwanted" side effect has subsequently been exploited for the treatment of non-malignant disease where autoimmune mechanisms are considered important in the pathogenesis. Based on the previous observations of improvement in autoimmune diseases (i.e., vasculitides), cyclophosphamide can be the primary cytotoxic drug[2-4]. Pulse intravenous cyclophosphamide is probably equally effective as oral cyclophosphamide at inducing remission and this remission is usually maintained by continuing cyclophosphamide for 3-6 mo before changing to a combination of other per os therapy. Cyclophosphamide can be administered intravenously at 500-1000 mg/m^2 pulses monthly. The optimal duration of treatment with intravenous cyclophosphamide has not been determined for IBD, but treatment for six months with monthly pulses would be typical, followed by maintenance therapy with azathioprine or methotrexate. Therapy must be continued to prevent relapse and for maintenance.

Only sporadic but promising cases are reported and experiences with cyclophosphamide in severe IBD are very limited but most patients entering into remission have no major adverse events or side effects[5]. We here report some other cases to enrich the experiences. We designed a non-controlled prospective pilot study to investigate the effects of pulse cyclophosphamide therapy for IBD patients. The aim of this study was to examine the effect of this kind of therapy on UC and CD.

MATERIALS AND METHODS

Patient selection and treatment

All patients were recruited in this prospective uncontrolled pilot study from our out-patient clinic specializing in chronic inflammatory bowel diseases (at the 3rd Department of Medicine, University of Debrecen). Between September 2002 and December 2003 we included in our cohort eight patients with (moderate/severe) steroid refractory IBD (four patients with CD and four with UC).

All patients were diagnosed according to the standard criteria and underwent endoscopy and/or radiological studies, including ileo-colonoscopy, CT and/or double-contrast barium air enteroclysis during 6 mo of the study. Video endoscopy of the large intestine, including the terminal ileum, was performed before the first cyclophosphamide pulse and at wk 12. All the patients did not respond to conventional therapy (this was the only selection criteria for this study) and were treated with pulse cyclophosphamide therapy monthly (6 cycles). The bolus dose was applied independent of body weight. Cyclophosphamide (800 mg) was given during a 4 h hospital stay in 500 mL physiologic or normal saline (0.9%) in a period of 120 min. White blood cell (WBC) counts and urine tests for incidental microscopic haematuria were obtained on pre- and post-cyclophosphamide therapy day (0, 7, 14).

Dutcome measurement

All the patients were informed of the potential risks and benefits of cyclophosphamide therapy and a written informed consent was given in advance. We defined treatment failure as any of the followings: lack of an initial response, relapse after an initial response, and intolerance to cyclophosphamide necessitating discontinuation of the drug**.** We used the modified Truelove and Witts activity index for ulcerative colitis and best index (Crohn's disease activity index) for Crohn's disease as others[6,7]. We used working definitions for the assessment of the response both in UC and CD. Active disease was defined as symptomatic UC with a Truelove-Witts score greater than 6 points and symptomatic CD with a CDAI score greater than 150 points. Significant (moderate-severe) relapse was defined as symptomatic UC with a Truelove-Witts score greater than 10 points and symptomatic CD with a CDAI score greater than 150 points and/or the need for systemic steroid therapy (including oral budesonide), and treatment failure was defined as relapse, colectomy or any serious adverse event.

RESULTS

Patient histories are summarized in Table 1. CD patients were all females aged 36-65 years (mean 49 years) and UC patients were all males aged 29-59 years (mean 40.25 years). Each patient received six monthly treatment. All patients were continued on a daily regimen of methyl prednisolone and the daily dose was decreased according to individual clinical activity. Azathioprine (2.5 mg/kg body weight per day) and methotrexate (15 mg/wk) were initiated orally for UC and CD patients respectively to maintain remission following cyclophosphamide treatment.

The patients entered into remission after the second/third cyclophosphamide pulse. Disease activity decreased. There were no side effects and no toxicity. All the patients went into long lasting remission. Remission seemed stable (except for case 5: relapse after 6 mo). These findings suggested that aggressive immunosuppressive therapy might be useful in some refractory patients and further controlled study should be considered in order to fully evaluate this type of treatment as a potential therapy for IBD.

DISCUSSION

Current therapy for IBD is unsolved. Many drugs are prescribed for inflammatory bowel disease, either for treating active disease or for maintaining remission. However, no drug provides a completely satisfactory response in a large percentage of patients. Corticosteroids (steroids) at dosages equivalent to 40-60 mg/d prednisone are commonly prescribed for acute exacerbations of Crohn's disease and ulcerative colitis. Steroids are not

Table 1 Patient histories

Patient No	Age/sex	Disease	Duration of IBD (yr)	Localization	CDAI before/after 6 cycles	UC-DAI before/after 6 cycles	Maintenance therapy for remission
1	46/female	CD	26	colon-rectum	168/79		methotrexate
2	65/female	CD	18	ileum-colon-rectum	231/86		methotrexate
3	49/female	CD	22	ileum-colon	198/91		methotrexate
4	36/female	CD	5	ileum-colon	242/98		methotrexate
5	59/male	UC	5	rectum		14/6	azathioprine
6	44/male	UC	5	rectum		15/8	azathioprine
7	29/male	UC	4	recto-sigmoid		13/6	azathioprine
8	29/male	UC	8	recto-sigmoid		14/7	azathioprine

UC-DAI: Modified Truelove and Witts activity index for UC; CDAI: CD activity index; Methotrexate: 15 mg/wk; Azathioprine: 2.5 mg/kg body weight per day.

effective for maintenance of remission in either disease. However, some patients require long-term steroids for suppression of disease, particularly Crohn's disease. Taking steroids for a prolonged period can cause reversible or irreversible adverse effects[8-10]. These adverse effects are of sufficient concern to prompt consideration of alternative treatment strategies. Infliximab, human growth hormone, and other novel biotechnology treatments have been investigated as therapy for patients with inflammatory bowel disease. Although these biotechnology-derived treatments are promising, their cost is prohibitive for many patients. Because cyclophosphamide is effective for other inflammatory conditions and is relatively less expensive than some other agents, it is considered in the treatment of inflammatory bowel disease.

IBD continues to pose a challenge to clinicians. Over the past few years there have been significant advances in our understanding of its pathogenesis and treatment. These advances may lead to more specific and targeted treatments, with consequent improvements in clinical outcomes. Intravenous pulse cyclophosphamide therapy may be a safe and effective treatment for patients with severe IBD unresponsive to conventional treatment as a first-line adjunct to or replacement of systemic corticosteroids in the treatment of IBD. Last but not the least, costs of this kind of treatment are relatively low.

In conclusion, pulse cyclophosphamide therapy can be used in treatment of selected IBD patients. However, larger placebo-controlled studies in more diverse patient population are warranted.

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RAPID COMMUNICATION

Detection of PERV by polymerase chain reaction and its safety in bioartificial liver support system

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Abstract

AIM: To establish a method detecting porcine endogenous retrovirus (PERV) in China experimental minipigs and to evaluate the safety of PERV in three individuals treated with bioartificial liver support systems based on porcine hepatocytes.

METHODS: Porcine hepatocytes were isolated with two-stage perfusion method, then cultured in the bioreactor, which is separated by a semipermeable membrane (0.2 μm) from the lumen through which the patients' blood plasma was circulated. After post-hemoperfusion, patients' blood was obtained for screening. Additionally, samples of medium collected from both intraluminal and extraluminal compartments of the laboratory bioreactor and culture supernate *in vitro* was analyzed. The presence of viral sequences was estimated by polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, the infection of virus in the supernate of common culture was ascertained by exposure to the fetal liver cells.

RESULTS: PERV-specific gag sequences were found in the porcine hepatocytes using RT-PCR. and were detected in all samples from the intraluminal, extraluminal samples and culture supernate. However, culture supernatant from primary porcine hepatocytes (cleared of cellular debris) failed to infect human fetal liver cells. Finally, RT-PCR detected no PERV infection was found in the blood samples obtained from three patients at various times post-hemoperfusion.

CONCLUSION: The assays used are specific and sensitive, identified by second PCR. PERVs could be released from hepatocytes cultured in bioreactor without the stimulation of mitogen and could not be prevented by the hollow fiber semipermeable membrane, indicating the existence of PERV safety in extracorporeal bioartificial liver support system (EBLSS).

Key words: PERV; Bioartificial liver support systems; Polymerase chain reaction

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INTRODUCTION

Fulminant hepatic failure (FHF), which remains a disease with high mortality, cannot be reversed by conventional treatments[1-3]. Meanwhile, the complications of liver failure, such as, encephalopathy, cerebral edema and multi-organ failure made it more difficult to cure. Orthotopic liver transplantation has improved the survival rate to 70%~80% of patients with this almost deadly disease. However, many patients died while waiting for the organ because of the lack of donor liver. Therefore, more curiosity has been aroused to deal this with biological treatments, such as extracorporeal bioartificial liver support system (EBLSS)[2-6]. The shortage of human organs leads to the use of non-human donors as the main hepatic cell source. Porcine hepatocytes, including transgenic pig, have become an important cell source widely used in bioartificial liver support systems. Concerns have been raised about the safety of this potential therapy, especially the possibility of cross species transmission of porcine endogenous retroviruses (PERV) since vitro infection of human cell lines has been demonstrated in 1997[4,8-11]. PERVs are integrated into the genome of all pigs. They belong to the gammaretroviruses previously termed type C retroviruses. At least two subtypes of PERV, PERV-A and PERV-B, infect human cells *in vitro*. Viral particles have been shown to be released by mitogen stimulated porcine peripheral blood mononuclear cells (PBMC), *etc*[10, 12-13]. Clinical trials did not find provirus integration, indicating

that no virus infection had taken place. However, further study and more retrospective research should be done in this field.

PERV infection can be measured directly by PCR, RT-PCR[10,13]. Here, we present the methods for the detection of PERV and investigation of patients who had been treated with EBLSS based on porcine cells. All methods were specific and sensitive, and it was revealed that in all patients no PERV infection had occurred.

MATERIALS AND METHODS

Isolation of porcine hepatocytes

Liver cells were isolated by an adaptation of the two-step perfusion method[14,19-20]. Briefly, the animals were anesthetized with barbital (30 mg/kg, b.w, intraperitoneally) and their livers were removed intact. The liver was first perfused *in vitro* via the portal vein with warmed (37 ℃) Ca^{2+} and Mg^{2+} free Hanks balanced salt solution at a flow rate of 5-8 mL/min for 10-15 mL/min, and then perfused with 0.05% collagenase (Sigma, Type IV) in the same solution supplemented with 5 mmol/L $CaCl_2$ and 50 mmol/L HEPES. The reperfusion with collagenase soultion lasted 20 min at a rate of 5 mL/min at 37 ℃. After 10 min of incubation (37 ℃) with gentle shaking, the suspension was filtered and hepatocytes were sedimented at 50×g for 3 min. The viability of the isolated liver cells was determined by the trypan blue exclusion test.

PERV proviral PCR assays

DNA was extracted from porcine liver tissue using the preparation kits (Qiagen). For the detection of provirus, primers specific for the gag gene (forward primer 5' -GCGACCCACGCAGTTGCATA-3', and reverse primer 5'-CAGTTCCTTGCCCAGTGTCCTT-3') were used, and a second PCR was carried out to test the products using the primers 5'-TGATCTAGTGAGAGAGGCAGAC-3' and 5'-CGCACACTGGTCCTTGTCG-3'. For PCR amplification, the standard PCR program of one cycle of 95 ℃ for 10 min, 35 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min and one cycle of 72 ℃ for 7 min was applied. Liver tissues of human and experimental animals (rabbit and mice) were also gathered to analyze the specificity of PCR.

RT-PCR detection of PERV gag RNA sequences

For the detection of virus released from the cultured cells, viral RNA from supernatants of cultured cells was isolated. Cells were removed from supernate by centrifugation at 200×g for 10 min, thereafter cell debris was removed by centrifugation at 3 500×g for 10 min and an additional centrifugation step at 10 000×g for 30 min. Viral RNA was extracted using the RNA isolation kits (Qiagen)[15-18]. RNA was reverse transcribed using a one-step RT-PCR kit (promega) with no-RT PCR controlled.

Porcine hepatocytes cultured in fiber weaven bioreactor

Primary cells were harvested from 1 wk China Experimental minipigs by a modified two-step collagenase perfusion method[19]. After testing the viability of the cells by trypan blue exclusion, single porcine hepatocyte suspensions were cultivated in the extralumen of the fiber weaven bioreactor (developed by our institute (porous, 0.2 μm)). The bioreactor was filled with porcine hepatocyte about 1.0×10^8~1.4×10^9 in co-culture with non-parenchymal cells. DMEM containing EGF was circulated with an artificial capillary cell culture system from Cellmax. Both the biological function and enzyme release of the bioreactor were examined.

Patients

Patients included in the study were FHF, which was defined as occurring within 8 wk of onset of the precipitation illness (in the absence of pre-existing liver disease), and acute-on-chronic hepatic failure listed for liver transplantation with a progressive hepatic encephalopathy.

Extracorporeal liver support with the CellMax bioreactor

FHF patients were treated with hybrid liver support system, including a blood circuit with a continuous plasma separation unit and a second circuit for plasma perfusion of the CellMax bioreactor. Briefly, the bioreactor was filled with porcine hepatocyte about 6.0×10^8-1.4×10^9 in co-culture with non-parenchymal cells. Venous blood of FHF patients was elicited via a double-lumen dialysis catheter from the internal jugular vein and blood plasma were separated. Continuous hollow fibre plasma separation was performed at a rate of 100-150 mL/min and then stored in sterile chamber. For anticoagulation a continuous infusion of heparin was performed. After one or two days' circulatory culture, the bioreactor was then connected and plasma was continuously perfused at a rate of 150-200 mL/min from the chamber. Treated plasma was reunited with the blood cell and returned to the patients. A roller pump (Millipore ultrafiltration device) was used to circulate blood and a heater was used to maintain the temperature of patients and bioreactor at 37 ℃-39 ℃. After the perfusion, the patients' plasma samples were detected. Blood samples were obtained after the treatment.

In vitro infection experiments

For infection experiments, cell-free supernatants from cultured porcine hepatocytes were used to infect fetal liver cells in order to mimic the bioartificial liver support system *in vitro*. That is, cell-free supernatants from primary cultured porcine hepatocytes were collected and cell debris was removed by centrifugation at 10 000× for 30 min, then was used to infect fetal liver cells.

RESULTS

Cell yield and viability

The total isolated hepatocytes and non-parenchymal liver cells by the simplified two step perfusion method were $6.0\text{-}14.0\times10^8$ cell per liver. The estimated viability judged by the trypan blue test was 92%-96%.

Specificity of the PERV DNA assays

All these PCR assays gave negative results on tissue and serum samples from 2 HBV patients, as well as 2 HCV

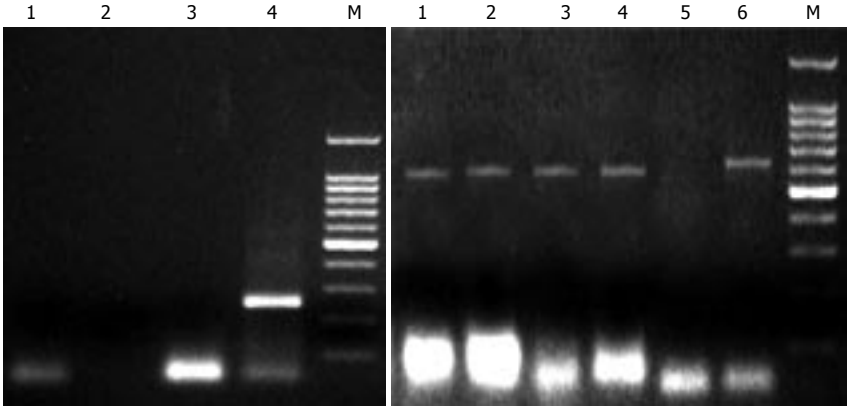

Figure1 PCR analysis of PERV proviral DNA in different liver tissues. Left: 1: normal person liver tissue, 2: HBV positive liver tissue, 3: HCV positive liver tissue, 4: second PCR of pig liver tissue, 5: Marker. Right: 1, 2, 3, 4, 6: pig liver cells, 5: nagative control.

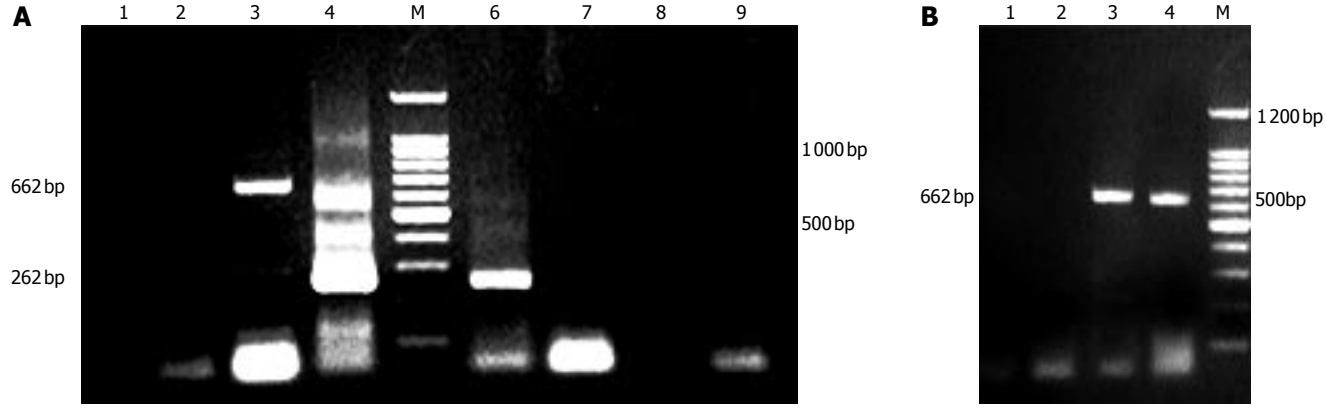

Figure 2 A: PERV RNA detection of supernate. 1:supernate of d 1, 2: supernate of d 3; 3:no RT PCR of d 3; 4:second PCR of d 3; 6: second PCR of d 5; 7: RT-PCR of d 7; 8: RT-PCR of d 9; 9: no RT PCR of d 9; **B:** PERV permeation of bioreactor. 1: intraluminal supernate of d 3 (no RT PCR), 2: extraluminal supernate of d 3 (no RT PCR), 3: intraluminal supernate of d 3 (RT-PCR); 4: extraluminal supernate of d 3 (RT-PCR).

patients and 1 rabbit, and positive results on tissue of China experimental swine, demonstrating 100% sensitivity, as shown in Figure 1. The detection of product with second PCR gave the same results, showing the usefulness and specificity of the method in observing PERV infection.

PERV release of cultured porcine hepatocytes in bioreactor

As depicted in Figure 2A, PERV could be released from porcine hepatocytes cultured not only in common flasks but also in fiber weaven bioreactor without the stimulation of mitogen. In the early stage, the quantity of virus so small that it couldnot be detected by PCR assays. From third day on, a large amount of PERVs could be released from the cells till their death. We also found that the semipermeable membrane (0.2 um) of bioreactor couldnot separate the virus, because in the both sides of the lumen we had evidence of the existence of PERV (Figure 2B).

PERV detection of patients using EBLSS

Continuous EBLSS treatment over a period of 4-6 h was safely performed and well tolerated by all the patients. No complications associated with therapy were observed during the treatment and the follow-up period of 1-2 years. After treatment, patients' blood was obtained for screening with the method mentioned above. No PERV RNA was detected in the serum of any of the 3 patients (Figure 3).

PERV infection experiments in vitro

PERV infection experiments with human fetal liver cells were performed. Supernatants from the cultured porcine hepatocytes were positive for PERV detection. Although it has been demonstrated that human kidney cells can be infected, it was also important to evaluate PERV infection of other human cells. In all fetal cells at one week post-infection, no evidence showed that PERV infection occurred with PCR and RT-PCR assays (Figure 4).

DISCUSSION

EBLSS based on porcine hepatocytes catches more interest as an effective temporary treatment to improve the chance of survival and a bridge to liver transplantation[5,6,20-24,38-41]. Because of the shortage of human cells, porcine endogenous retroviruse (PERV), found in 1970's, was focused for its safety in xenotransplantation and treatments based on porcine tissues or cells as vitro infection of human cell lines HEK 293 was demonstrated in 1997[4,34-37]. Laboratory surveillance of PERV infection in pig xenograft recipients and the treatments is critical for determining the safety of pig xenotransplantation. We used here highly specific PCR based assays for the molecular detection of PERV DNA and RNA sequences. In addition, we included a control PCR reaction without RT to confirm that the positive RT-

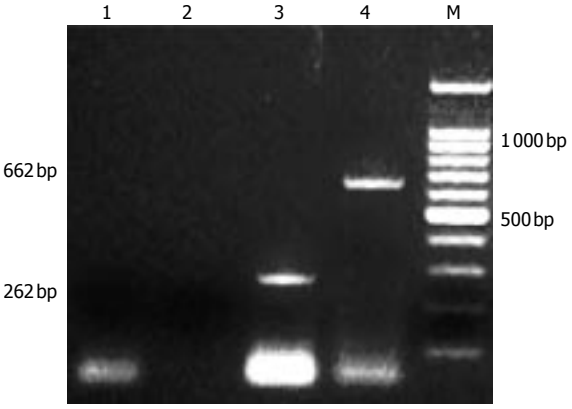

Figure3 infection of PERV. 1,2 RT-PCR and second PCR of cultured fetal hepatocytes, 3,4: positive control.

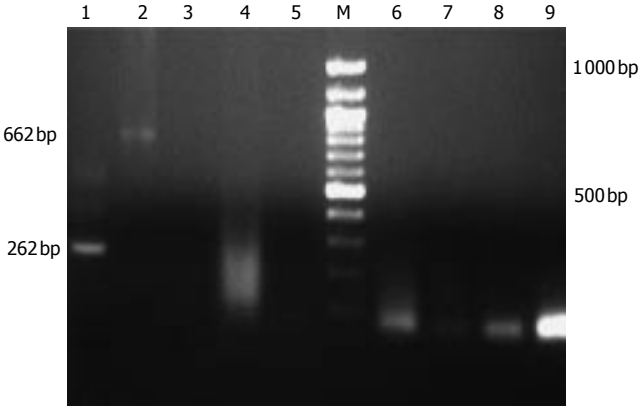

Figure4 PERV detection of patients treated with EBLSS. 1:second PCR of positive control, 2: positive control, 3:control; 4-9:RT-PCR and second PCR of patients.

PCR results were due to PERV RNA. we have developed polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) assays to detect proviral PERV gag sequences and PERV RNA sequences in serum samples by using specific primers. All these PCR assays gave positive results on tissue of China experimental swine, and the identification of product with second PCR gave the same results, showing the sensitivity and specificity of this method in observing PERV infection.

Viral particles have been shown to be released by pig PBMC, by cultured aorta endothelial cells stimulated with mitogen[10,12,13,25,26]. In several trials, human diabetic patients who were treated with pig pancreatic islet cells, with pig skin or with extracorporeal perfusion of pig liver did not show provirus integration[27,42], indicating that no virus infection had taken place. *In vitro* infection experiments revealed that PERVs infect not only human cell lines and primary cells, but also a variety of cells of other species including non-human primates. However, no transmission of PERVs *in vivo* was observed in non-human primates and several small animals gave high doses of PERV. Additional data showing the same results about the safety of PERV have been obtained from several experiments[16,29]. Nevertheless, considering the results of initial clinical xenotranplantations in humans, which found that persistent microchimerism was observed in 23 patients(totally 160) for up to 8.5 years, and the establishment of PERV infection model[27,28], it was worth paying more attention to its safety in xenotransplantation and EBLSS.

In this study, the retrospective investigation of the three patients with the treatment of EBLSS based on porcine hepatocytes was performed for possible PERV transmission. Our data are in agreement with other studies showing lack of PERV transmission. And in laboratory experiment we also found PERV could not be prevented by the hollow fibre membranes, indicating different opinion from the foregoing research that PERV particles could be prevented by using PES hollow fibre membranes with a molecular weight cut-off of 400 000 kD[31]. In fact, the pore size of bioreactor should be large enough to make material exchange possible, so, any efforts trying to block the virus with semipermeable membrane was probably in vain. The lack of PERV transmission in the patients investigated could be owing to short-term contacting with the EBLSS, lasting for 4-6 h during the treatments. And an effective inactivation of PERV released from pig cells may be another reason. Although cells of human could be infected productively with PERV as reported, no transmission was observed in patients treated with EBLSS in our study.

The latest data about the safety of xenotransplantation using porcine cells, tissues and organs has been obtained from experiments involving transplantation of encapsulated pig islet cells into diabetic rats and assessment of PERV infection in patients treated with a bioreactor based on porcine liver cells[29-33], demonstrating no PERV transmission occured. Although no data indicated that PERV infection had occurred in any of the patients treated with the BALSS containing porcine hepatocytes, longer and larger patient follow-up is required to supervise porcine retroviruses and EBLSS.

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RAPID COMMUNICATION

Clinical correlation of gallstone disease in a Chinese population in Taiwan: Experience at Cheng Hsin General Hospital

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Abstract

AIM: To explore the prevalence of gallstone disease (GSD) in Taiwan and condition-associated factors related to it.

METHODS: We studied a total of 2386 healthy adults (1235 males and 1151 females) voluntarily admitted to Cheng Hsin General Hospital for a paid physical check-up between January 2002 and December 2002. Blood samples and ultrasound sonography results were collected.

RESULTS: The overall prevalence of GSD among this study-population was 5.3%, including 1.7% ($n = 40$) having a single stone, 2.3% ($n = 55$) having multiple stones, and 1.3% ($n = 31$) having cholecystectomy. The prevalence revealed a statistically significant increase with increasing age ($P < 0.0001$). Females exhibited a greater prevalence of multiple stones than did males (3.0% *vs* 1.7%, $P = 0.04$). Using multiple logistic regression analysis, the following appeared to be significantly related to the prevalence of GSD: older age (40-49 years *vs* <40 years, OR = 1.63 [95% CI: 0.76-3.48], 50-59 years *vs* <40 years, OR = 4.93 [95% CI: 2.43-9.99], 60-69 years *vs* <40 years, OR = 6.82 [95% CI: 3.19-14.60], ≥70 years *vs* <40 years, OR = 10.65 [95% CI: 4.78-23.73]), higher BMI (≥27 kg/m^2 *vs* <24 kg/m^2, adjusted OR = 1.74 [95% CI: 1.04-2.88]), and higher FPG (≥126 mg/dL *vs* <110 mg/dL, OR = 1.71, 95%CI: 1.01-2.96).

CONCLUSION: Older age (≥50 years), obesity (BMI ≥ 27 kg/m^2), and type 2 diabetes (FPG ≥126 mg/dL) are associated with the prevalence of GSD.

Key words: Cross-sectional study; Gallstone disease; Prevalence; Risk factors

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INTRODUCTION

Gallstone disease (GSD) is one of the most common diseases in developed countries. Recent studies indicate varying prevalence of GSD with several predisposing factors in different study populations[1-9]. From a medical economic perspective, the direct and indirect costs of treating GSD patients were estimated at $16 billion and account for more than 800 000 hospitalizations yearly in the United States[9,10]. In Taiwan, the prevalence of GSD in the general population was 4.3% in 1989[8] while another voluntary screening revealed that the prevalence of GSD was 10.7% among healthy subjects in 1995[2]. For this reason, due to westernization of the diet and the environment, GSD is not rare in the Chinese population and has become one of the major health problems in Taiwan[7]. Without an appropriate and effective screening program for symptomatic GSD, the medical treatment of GSD and related complications contributes substantially to health care costs.

From the viewpoint of preventive medicine, it is

Table 1 Prevalence of each type of gallstone disease among 2386 Chinese subjects

Variable	Total screened number	Gallstone disease: Single stone prevalence number (%)	Multiple stones prevalence number (%)	Cholecystectomy prevalence number (%)
Gender				
Male	1235	21 (1.7)	21 (1.7)	17 (1.4)
Female	1151	19 (1.7)	34 (3.0)	14 (1.2)
P-value for χ^2-test		0.95	0.04	0.76
Age				
<40	745	4 (0.5)	5 (0.7)	2 (0.3)
40-49	723	6 (0.8)	7 (1.0)	6 (0.8)
50-59	504	11 (2.2)	21 (4.2)	8 (1.6)
60-69	252	7 (2.8)	17 (6.8)	6 (2.4)
70+	162	12 (7.4)	5 (3.1)	9 (5.6)
P-value for Cochran-Armitage trend test		<0.0001	<0.0001	<0.0001
Total	2386	40 (1.7)	55 (2.3)	31 (1.3)

not only important to be cognizant of the background prevalence of GSD regionally, but also to explore the complete spectrum of demographic and biological markers which may be related to development of GSD. Although most epidemiologic studies of GSD, demographic factors and biochemical markers have used case-control designs and cross-sectional data[1-9], to the best of our knowledge, some uncertainty still exists regarding the prevalence of the disease and the identity of the associated risk factors for the development of GSD. The present study was designed to explore potential associated risk factors and to improve understanding of the overall pathogenesis of GSD. The purpose of this study was to explore the context of associated risk factors for GSD prevalence amongst the general population aged 20 years or more, as determined by the application of a healthy volunteer subjects screening program at Cheng Hsin General Hospital, a fully certified regional hospital and teaching hospital in Taipei, Taiwan.

MATERIALS AND METHODS

Data resource and data collection

This cross-sectional study was conducted with a total of 2386 healthy adults (1235 males and 1151 females) voluntarily admitted to Cheng Hsin General Hospital for a paid physical check-up between January 2002 and December 2002. Blood samples and ultrasound sonography results were collected. Overnight-fasting blood samples were drawn via venipuncture from study participants by clinical nurses. Serum and plasma samples (from whole blood preserved with EDTA and NaF) were kept frozen (-20 ℃) until ready for analysis. The study-used definition of type-2 diabetes was from the 1997 ADA criteria[11]. Definitions of the following diseases / conditions were obesity: a body mass index (BMI) ≥27 Kg/m^2, high systolic blood pressure (SBP) ≥140 mmHg, high diastolic blood pressure (DBP) ≥90 mmHg, hypercholesterolemia (≥200 mg/dL), hypertriglyceridemia (≥200 mg/dL), low HDL (<35 mg/dL), high BUN (≥20 mg/dL), high creatinine (≥1.4 mg/dL), and hyperuricemia (≥7 mg/dL for males or ≥6 mg/dL for females). Serum ALT or AST levels ≥40 U/L were classified as elevated[12]. In addition, information on HBV and anti-HCV were collected using radioimmunoassay kits.

Diagnosis of gallstone disease

In the present study, GSD was diagnosed by a panel of specialists using real-time ultrasound sonography (TOSHIBA nemio SSA-550A, Japan) to examine the abdominal region after fasting for at least 8 h based on the presence of "movable hyper-echoic foci with acoustic shadow. Cases of GSD were classified as follows: single gallbladder stone, multiple gallbladder stones, and cholecystectomy, excluding gallbladder polyps. Cases were identified as any type of GSD study population.

Interobserver reliability in ultrasound sonography

In order to set up a consistent diagnosis of GSD between specialists, the Kappa statistic was used to assess the agreement of inter-observer reliability among study specialists. A pilot study was performed using 100 randomly selected healthy subjects other than the study participants. For inter-observer reliability, the Kappa value for diagnosis of GSD between specialists was 0.79 (95%CI: 0.61-0.95).

Statistical analysis

Statistical analysis was performed using SAS for Windows, (SAS version 9.0; SAS Institute Inc., Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant between two test populations. For univariate analysis, the two-sample, independent t-test method was adopted to assess differences in the mean value of continuous variables between subjects with and without GSD. Crude and adjusted odds ratios (adjustment for gender and age) were estimated and 95% confidence intervals were used. Multiple logistic regression was also performed in order to investigate the independence of risk factors associated with the prevalence of GSD.

RESULTS

The prevalence of each type of GSD amongst study subjects is presented in Table 1. The overall prevalence of GSD was 5.4%, including 1.7% ($n=40$) having a single stone, 2.3% ($n=55$) having multiple stones, and 1.3% ($n=31$) having cholecystectomy. Females (3.0%) had a significantly higher prevalence of multiple stones than males (1.7%) ($P=0.04$). There were no statistically significant differences in gender for other types of GSD. In addition, from the Cochran-Armitage trend test, the prevalence of each type of GSD showed an increase with age ($P<0.0001$). Subjects aged 50 years and over (96/918 = 10.5%) had a more than 5-fold risk for GSD compared with the subjects aged 50 years and less (30/1468 = 2.0%).

Figure 1 shows the gender- and age-specific prevalence of all types of GSD. Although there was no gender difference ($\chi^2=1.30$, $P=0.25$) for the overall prevalence

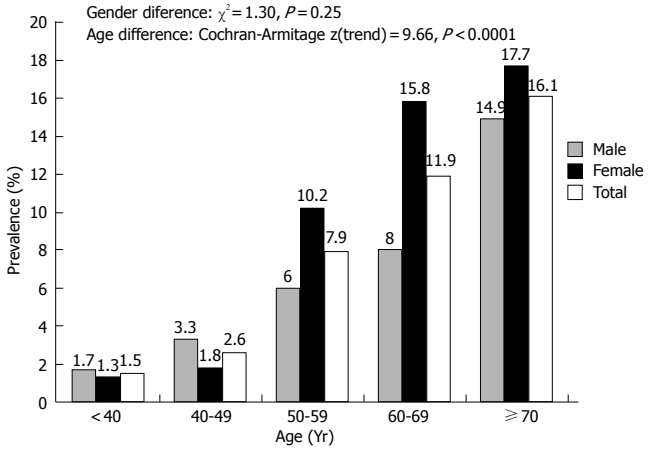

Figure 1 Gender- and age-specific prevalence of all types of gallstone disease among 2386 Chinese subjects.

Table 2 Comparison of characteristics in subjects with and without gallstone disease

Variable	Any type of gallstone disease			
	Yes (n = 126) Mean ± SD	No (n = 2260) Mean ± SD	Total (n = 2386) Mean ± SD	P-value for t test
Age (yr)	58.63±13.04	46.57±13.22	47.21±13.48	<0.0001
BMI (kg/m^2)	24.83±3.64	23.50±3.55	23.57±3.57	<0.0001
FPG (mg/dL)	109.22±33.21	96.98±25.21	97.63±25.84	<0.0001
SBP (mmHg)	130.06±20.07	122.55±18.40	122.94±18.56	<0.0001
DBP (mmHg)	81.27±12.22	79.56±12.56	79.65±12.55	0.14
Cholesterol (mg/dL)	216.93±41.06	210.51±37.72	210.87±37.94	0.07
Triglyceride (mg/dL)	143.39±98.63	130.12±108.06	130.86±107.58	0.18
HDL (mg/dL)	57.02±16.55	57.69±15.76	57.65±15.80	0.64
BUN (mg/dL)	14.94±6.28	13.55±5.56	13.63±5.61	0.02
Creatinine (mg/dL)	1.08±0.35	1.04±0.40	1.04±0.40	0.29
Uric acid (mg/dL)	6.74±1.67	6.59±1.72	6.60±1.72	0.35
AST (U/L)	32.79±30.13	27.59±17.59	27.88±18.54	0.06
ALT (U/L)	37.84±54.27	30.01±34.49	30.45±35.91	0.11

Table 3 Univariate analysis of associated clinical factors for gallstone disease among 2386 Chinese subjects

	Gallstone disease		Crude OR (95% CI)	Adjusted OR[1] (95% CI)
	Yes (n = 126)	No (n = 2260)		
Gender male	59	1176	1.00	-
female	67	1084	1.23 (0.86-1.76)	
Age (yr) <40	11	734	1.00	-
40-49	19	704	1.80 (0.85-3.81)	-
50-59	40	464	5.75 (2.92-11.32)	-
60-69	30	222	9.02 (4.45-18.29)	-
⩾70	26	136	12.76 (6.16-26.43)	-
BMI (Kg/m^2) <24	57	1343	1.00	1.00
24-27	38	585	1.53 (1.00-2.33)	1.36 (0.88-2.10)
⩾27	31	332	2.20 (1.40-3.46)	2.04 (1.28-3.26)
FPG (mg/dL) <110	93	2007	1.00	1.00
110-125	10	122	1.77 (0.90-3.49)	1.14 (0.57-2.28)
⩾126	23	126	3.95 (2.42-6.45)	1.95 (1.16-3.30)
High SBP No	93	1885	1.00	1.00
Yes	33	375	1.78 (1.18-2.69)	1.18 (0.54-1.33)
High DBP No	97	1801	1.00	1.00
Yes	29	459	1.17 (0.77-1.80)	0.95 (0.61-1.48)
Hypercholesterolemia No	93	1827	1.00	1.00
Yes	33	433	1.50 (1.00-2.26)	1.11 (0.72-1.69)
Hypertriglyceridemia No	100	1946	1.00	1.00
Yes	26	314	1.61 (1.03-2.52)	1.54 (0.97-2.46)
Low HDL No	122	2052	1.00	1.00
Yes	4	208	0.32 (0.12-0.89)	0.62 (0.22-1.73)
High BUN No	115	2118	1.00	1.00
Yes	11	142	1.43 (0.75-2.71)	0.67 (0.34-1.33)
High creatinine No	118	2214	1.00	1.00
Yes	8	46	3.26 (1.51-7.07)	1.22 (0.54-2.78)
Hyperuricemia No	43	963	1.00	1.00
Yes	83	1297	1.43 (0.98-2.09)	1.18 (0.80-1.75)
Higher AST No	106	2095	1.00	1.00
Yes	20	165	2.40 (1.45-3.97)	1.96 (1.16-3.32)
Higher ALT No	101	1883	1.00	1.00
Yes	25	377	1.24 (0.79-1.94)	1.48 (0.92-2.37)
HBV Negative	113	1975	1.00	1.00
Positive	13	285	0.80 (0.44-1.43)	0.98 (0.54-1.78)
Anti-HCV Negative	123	2204	1.00	1.00
Positive	3	56	0.96 (0.30-3.11)	0.63 (0.19-2.07)

[1]Adjustment for gender and age.

of GSD, there was a statistically significant increase with increasing study-subject age by means of the trend test ($z = 9.66$, $P < 0.0001$). The prevalence of GSD also showed an interaction effect between gender and age, that is, males proved to have a substantially greater overall prevalence of GSD than females among those aged less than 50 years, whereas there was a lower overall prevalence of GSD among those aged more than 50 years.

The results of the comparison of a variety of test characteristics and their potential association with the prevalent GSD for study subjects are illustrated in Table 2. Using a two-sample independent t-test, the associated factors that were significantly related to GSD included age (yes [58.63 ± 13.04 years] *vs* no [46.57 ± 13.22 years]), BMI (yes [24.83 ± 3.64 Kg/m^2] *vs* no [23.50 ± 3.55 Kg/m^2]), fasting plasma glucose (FPG) (yes [109.22 ± 33.21 mg/dL] *vs* no [96.98 ± 25.21mg/dL]), SBP (yes [130.06 ± 20.07 mmHg] *vs* no [122.55 ± 18.40 mmHg]), and BUN (yes [14.94 ± 6.28 mg/dL] *vs* no [13.55 ± 5.56 mg/dL]).

Table 3 presents the crude and adjusted odds ratios for the association between certain relevant associated risk factors and the prevalence of GSD. Compared to individuals without GSD, in addition to older age (40 - 49 years *vs* <40 years, OR = 1.80 [95% CI: 0.85 - 3.81], 50-59 years *vs* <40 years, OR = 5.75 [95% CI: 2.92-11.32], 60-69 years *vs* <40 years, OR = 9.02 [95% CI: 4.45-18.29], ⩾ 70 years *vs* <40 years, OR=12.76 [95% CI: 6.16-26.43]), subjects featuring GSD revealed a more-pronounced prevalence of: higher BMI (⩾27 kg/m^2 *vs* <24 kg/m^2, adjusted OR = 2.04 [95% CI: 1.28-3.26]), higher FPG (⩾ 126 mg/dL *vs* <110 mg/dL, OR = 1.95, 95% CI: 1.16-3.30), and higher AST (adjusted OR=1.96, 95%CI: 1.16-3.32) subsequent to adjustment for gender and age.

The effect of independent associated risk factors on GSD was examined using a multiple logistic regression model. As depicted in Table 4, subsequent to adjustment for confounding factors, the following appeared to be significantly related to GSD prevalence: age (40-49 years *vs* <40 years, OR = 1.63 [95% CI: 0.76-3.48], 50-59 years *vs*

Table 4 Multiple logistic regression of associated factors for gallstone disease among 2386 Chinese subjects

Variable	Gallstone disease (yes *vs* no) OR	95%CI
Gender (female *vs* male)	1.34	0.90-1.99
Age (40-49 *vs* <40 yr)	1.63	0.76-3.48
(50-59 *vs* <40 yr)	4.93	2.43-9.99
(60-69 *vs* <40 yr)	6.82	3.19-14.60
(≥70 *vs* <40 yr)	10.65	4.78-23.73
BMI (24-27 *vs* <24 kg/m^2)	1.25	0.79-1.97
(≥27 *vs* <24 kg/m^2)	1.74	1.04-2.88
FPG (110-125 *vs* <110 mg/dL)	0.96	0.47-1.97
(≥126 *vs* <110 mg/dL)	1.71	1.01-2.96
High SBP (yes *vs* no)	1.11	0.53-1.51
High DBP (yes *vs* no)	0.87	0.52-1.48
Hypercholesterolemia (yes *vs* no)	0.99	0.64-1.53
Hypertriglyceridemia (yes *vs* no)	1.25	0.75-2.08
Low HDL (yes *vs* no)	0.5	0.17-1.44
High BUN (yes *vs* no)	0.59	0.26-1.37
High creatinine (yes *vs* no)	2.02	0.71-5.75
Hyperuricemia (yes *vs* no)	0.97	0.64-1.46
Higher AST (yes *vs* no)	2.06	0.99-4.31
Higher ALT (yes *vs* no)	0.83	0.42-1.63
HBV (positive *vs* negative)	0.95	0.52-1.76
Anti-HCV (positive *vs* negative)	0.5	0.15-1.69

<40 years, OR = 4.93 [95%CI: 2.43 - 9.99], 60 - 69 years *vs* <40 years, OR = 6.82 [95% CI: 3.19 - 14.60], ≥70 years *vs* <40 years, OR = 10.65 [95% CI: 4.78 - 23.73]), the higher BMI (≥27 kg/m^2 *vs* <24 kg/m^2, adjusted OR = 1.74 [95% CI: 1.04 - 2.88]), and higher FPG (≥126 mg/dL *vs* <110 mg/dL, OR = 1.71, 95% CI: 1.01 - 2.96).

DISCUSSION

Prevalence of gallstone disease in the general population

One of the important benefits of early screening for GSD is that ultrasonography can lead to the discovery of an increasing proportion of asymptomatic cases for which one can use therapeutic nonsurgical approaches, such as litholitic bile acids, local solvents, and lithotripsy[13]. However, it appears that only a few published studies have attempted to determine the prevalence and possible etiology of GSD prevalence for the general population of Taiwan[2,8,14], which also face a GSD burden. In the present study, GSD appeared to be fairly common for the test population, affecting an estimated 5.3% of the general population in Taiwan. The prevalence of GSD amongst different test populations appears to vary, differing among different studies conducted in different countries[1,3-6]. In addition to different methods of GSD assessment, this disparity might be due to differences between different population stocks. The prevalence of GSD for our study population (5.3%) was lower than the corresponding figure presented in a previous hospital-based study conducted in the Veterans General Hospital (VGH), Taipei, Taiwan, which was reported to be 10.7%[2]. The apparent lower prevalence rate in our study may have been due to younger age of the participants. Another possible reason for such differences between the results of the VGH study and our results may simply have been related to the fact that although the kappa value for agreement of interobserver reliability seemed good [14], non-differential misclassification-bias identification could still be occurring and could lead to underestimation of GSD prevalence.

The implications of associated risk factors for gallstone disease

Our results reveal that older age represents a significant risk factor for GSD. Such a finding is consistent with results of other hospital-based and community-based studies conducted elsewhere[2-5,7,8]. GSD is very seldom found in children, and in that age group is mainly associated with haemolytic causes[15]. The long-term exposure to many other risk factors among elder persons may also account for the increased probability of developing GSD. Cholelithiasis is also an acquired disease contributed to by chronic environmental factors plus an aging effect[7]. In addition, it has been suggested that among the elderly, larger amounts of cholesterol are secreted by the liver, and the catabolism of cholesterol to bile acid is decreased[16,17].

Most previous epidemiologic studies have shown that females have a higher prevalence of GSD than males in the Western world. However, the male to female ratio seems to have changed from early reports, which showed figures of 1:4-6 to more recent studies where the ratio is 1:2 or less[3,4,18]. Pregnancy and sex hormones could be involved by altering biliary secretion or gallbladder motility, or both may play an important role in sex-related differences in the prevalence of GSD[13]. Another possible reason might be that estrogen replacement therapy or oral contraceptives are used[19]. Our findings showed that, except for multiple stones, females do not have a significantly higher prevalence of all types of GSD than males. This result is similar to that from other hospital-based or population-based studies conducted in Taiwan[2,7,8].

After adjustment for confounding factors, the present study also suggests that obesity is highly correlated with GSD prevalence. Supersaturated bile is the linkage between obesity and cholesterol GSD. Obesity can raise the saturation of bile by increasing biliary secretion of cholesterol - the latter probably depending on a higher synthesis of cholesterol in obese subjects[7,13,20]. While cholesterol GSD is common in Western countries, pigment GSD is still the principal component in Taiwan[2]. Our results also implied that obesity may be an independent associated risk factor leading to prevalent pigment GSD in the Chinese population.

It has been a matter of controversy whether diabetes mellitus is associated with GSD or not[7]. Consistent with previous studies[4,7], our results showed that diabetes mellitus is highly associated with GSD prevalence. From a clinical perspective, hyperglycemia inhibits bile secretion from the liver and disturbs gallbladder contraction [2]. Supersaturated bile in the gallbladder induces cholelithiasis in diabetic subjects[21]. The association of diabetics with GSD is stronger in subjects with a history of treated diabetes mellitus than in those with a simple history of diabetes and this could be an effect of hyperglycemia on gallbladder motility[22]. Furthermore, diabetes mellitus

combined with GSD may induce acute cholecystitis more frequently and have a higher possibility of progression to septicemia[7].

An increasing prevalence of cholecystolithiasis has been associated with the etiology and severity of chronic liver disease and cirrhosis[23]. Possible explanatory factors include overproduction and over-secretion of bilirubin due to increased extracorpuscular hemolysis, a reduction in the production and transportation of biliary salts and cholesterol secretion, stasis and changes in bile acidification, or even mucous hypersecretion and subtle alterations in gallbladder mucosal function[24,25]. However, although early liver dysfunction such as abnormal ALT, HBV, and HCV infection are endemic in Taiwan[12,26,27], the present study did not reveal liver function associated factors to be related to GSD prevalence. This finding is consistent with other hospital-based studies for the general population in Taiwan[2]. Further epidemiological and etiologic investigations are needed to clarify the pathophysiological mechanisms between early liver damage and pigment GSD among general Chinese populations.

Perceived limitations

A major limitation of the present study is the potential self-selection bias due to the hospital-based study design, that is, of it not being exactly representative of the whole general population. Second, it is well established that a large proportion of GSD patients remain asymptomatic and they are therefore often ignored for many years. Thus the present study may be representative of the clinical and not of the true prevalence. Thirdly, we do not detail to distinguish between cholesterol stones and pigment stones in this study, some measurement errors and different pathogenicity could occur. Fourthly, we did not consider how many individuals had progressive liver disease and explore the relationship between GSD and liver disease. Finally, our measurements were done only at a single time point and could not be able to be reflect long-term exposure to various demographic or biochemical aspects or factors, factors which might be important influencers of GSD. The solution to such a quandary is to conduct a number of prospective longitudinal analogous studies, the results of which would be expected to complement the cross-sectional findings of this study.

CONCLUSION

In conclusion, older age, obesity (BMI$\geqslant$27 kg/m^2), and type 2 diabetes (FPG$\geqslant$126 mg/dL) are associated with GSD prevalence. Further studies are needed not only to elucidate the temporal sequence of events that typically lead to GSD, but also to further explore the pathogenesis of GSD in the general Chinese population.

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RAPID COMMUNICATION

Correlation between ultrasonographic and pathologic diagnosis of liver fibrosis due to chronic virus hepatitis

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Abstract

AIM: To evaluate the validity of ultrasonographic and pathologic diagnosis of liver fibrosis in patients with chronic viral hepatitis.

METHODS: The liver fibrosis status in 324 patients was evaluated by both needle biopsy and ultrasonography. Liver fibrosis was divided into S0 -S4 stages. S4 stage was designated as definite cirrhosis. The ultrasonographic examination included qualitative variables, description of liver surface and parenchyma, and quantitative parameters, such as diameter of vessels, blood flow velocity and spleen size.

RESULTS: Ultrasonographic qualitative description of liver surface and parenchyma was related with the severity of fibrosis. Among the quantitative ultrasonographic parameters, cut-off value of spleen length (12.1 cm) had a sensitivity of 0.600 and a specificity of 0.753 for diagnosis of liver cirrhosis. The diameters of spleen (8 mm) and portal vein (12 mm) had a diagnostic sensitivity of 0.600 and 0.767, and a diagnostic specificity of 0.781 and 0.446, respectively. The diagnostic accuracy for liver cirrhosis was moderately satisfactory, and the negative predictive values of these parameters reached near 0.95.

CONCLUSION: Ultrasonography can predict the degree of liver fibrosis or cirrhosis. A single ultrasonographic parameter is limited in sensitivity and specificity for the diagnosis of early cirrhosis. The presence or absence of liver cirrhosis in patients with chronic virus hepatitis can be detected using 2 or 3 quantitative and qualitative parameters, especially the length of spleen, the diameter of spleen vein and echo pattern of liver surface.

INTRODUCTION

Chronic hepatitis virus B or C infection results in damage to hepatocytes and may eventually lead to liver fibrosis, cirrhosis and/or hepatocellular carcinoma[1-3]. The diagnosis of liver fibrosis and cirrhosis in patients with chronic virus hepatitis is of therapeutic and prognostic importance.

Although histologic examination of percutaneous biopsy specimens is the gold criterion for the severity of fibrosis and cirrhosis, biopsy is invasive and cannot be used repeatedly in follow-up. Moreover, liver biopsy can yield false negative results in nearly 20-30% of cases[4-7]. Therefore, it is important to use noninvasive methods in differentiation between liver fibrosis and cirrhosis.

Ultrasonography (US) is a noninvasive and inexpensive procedure for diagnosis of focal and diffuse parenchymal disease of liver. Although US cannot detect minute changes, it can show liver cirrhosis in patients with decompensated liver function[8-11]. However, correlation between US and histologic diagnosis has not been fully investigated in large series of patients. We conducted a prospective study to evaluate the validity of US for diagnosis of liver fibrosis in patients with chronic liver hepatitis without clinical or biochemical evidence of cirrhosis.

MATERIALS AND METHODS

Patients

From July 1999 to August 2002, 324 patients with chronic viral hepatitis undergoing US and histologic examination, were enrolled. Inclusion criteria included positive HBsAg and HBV-DNA or anti-HCV and HCV-RNA determined by PCR methods for at least 6 mo, and abnormal serum alanine transaminase level in recent 6 mo. Patients who had clinical or biochemical evidence of decompensated liver

Table 1 Relation between US quantitative parameters and fibrosis stages

Parameters	Stage of fibrosis	Mean±SD	*F*	*P*
Length of spleen (cm)				
	S0	10.811.32		
	S1	10.82±1.45		
	S2	11.35±2.02	5.1947	0.0005
	S3	13.31±2.68		
	S4	12.66±2.06		
Diameter of portal vein (cm)				
	S0	1.17±0.11		
	S1	1.18±0.17		
	S2	1.19±0.13	1.0369	0.3882
	S3	1.20±0.12		
	S4	1.27±0.13		
Diameter of spleen vein (cm)				
	S0	0.62±0.16		
	S1	0.67±0.15		
	S2	0.72±0.18	6.7896	0.0000
	S3	0.69±0.15		
	S4	0.81±0.20		

function or portal hypertension, positive HIV antibody, serum titer of antinuclear antibody >1:160, serum creatinine level over 1.5 upper limit of normal value, or known liver diseases of other etiologies, were excluded.

Histologic examination

Percutaneous liver biopsy specimens were obtained from the anterior segment of the right lobe in each patient under the guidance of US using the quick-cut or Menghini biopsy needle. The satisfactory size of specimens was longer than 1 cm. The liver tissue samples were stained with hematoxylin- eosin, Gordon- Sweet and van-Gieson methods. Three pathologists performed the histological examination. To evaluate the inter-observer variation, Kappa analysis was conducted to control the quality of pathological diagnosis. The average Kappa value was 0.8144, indicating the excellent consistency for the staging of liver fibrosis.

According to the Guidelines of Prevention and Treatment of Viral Hepatitis of Chinese Medical Association (2000), liver fibrosis was divided into S0-S4 stages: S0 stage-no fibrosis, S1 stage-enlarged portal tracts with fiber proliferation, S2 stage-fibrosis of portal tract with formation of fiber septa and intact architecture of liver lobule, S3 stage-fibrosis with distortion of lobule architecture but without cirrhosis, S4 stage-definite cirrhosis. Liver inflammation was divided into grades from G1 (mild) to G4 (severe)[12].

US examination

US examination was performed within 2 wk before or after liver biopsy. Acuson Asben system with a 3.5-5.0 MHz curved probe was utilized. The two US operators were unaware of the clinical details and the results of biopsy. The results were recorded on video- tapes and the final report was given based on the consensus of both operators. The standard protocol of US examination included variables describing the liver size, surface and parenchyma, the vessel structure, the blood flow velocity and spleen size.

Statistical analysis

Statistical analysis was performed using the SPSS 9.0 software. The significance of differences between subgroups was tested by the F test. $P<0.05$ was considered statistically significant. A receive-operating characteristic (ROC) curve was used to determine the best cut-off values of US parameters for diagnosis of liver cirrhosis.

RESULTS

A total of 324 patients were collected, 272 men (83.9%) and 52 women (16.1%). Their age ranged from 18 to 60 years with a mean ± SD of 35.56 ± 9.9 years. Based on virus markers, 306 patients had hepatitis B and 18 patients had hepatitis C. The mean duration of hepatitis, namely the duration from the date of clinical diagnosis to the date of liver biopsy, was 4.14 years. The histopathology showed S0 stage in 32 patients (9.9%), S1 stage in 116 patients (35.8%), S2 stage in 111 patients (34.3%), S3 stage in 35 patients (10.8%), S4 stage in 30 patients (9.3%), and inflammation G1 in 117 patients (36.1%), G2 in 110 patients (33.9%), G3 in 70 patients (21.6%) and G4 in 27 patients (8.3%).

The results of qualitative US were different in patients with various stages of liver fibrosis. The appearance of nodularities or irregular lines on liver surface and heterogenous distribution of nodularities in liver parenchyma, were related with advanced stages of liver fibrosis. But these descriptions could not definitely reflect the histopathologic diagnosis, because 97% patients in S0 subgroup and 66% patients in S4 subgroup showed smooth surface echo pattern. Only 13.7% patients in S4 stage subgroup showed moderate saw-teeth like liver surface echo pattern. No severe nodular surface was noted. The echo pattern of liver parenchyma and heterogenous distribution were significantly different in patients with various stages of liver fibrosis and were related to the severity of fibrosis. But coarse nodularity was frequently encountered: 25% in S0 subgroup patients, and 41% in S4 subgroup patients.

Table 1 summarizes the relation between liver fibrosis stages and quantitative US parameters, which showed the differences in subgroups with various stages of liver fibrosis. Among the quantitative US parameters, the spleen length and diameter of spleen vein were correlated with fibrosis stages ($P<0.05$). The spleen lengths were significantly different in S1/ S3, S2/S3, and S1/S4, but not significant in S3/S4 ($P=0.43$). The diameter of spleen vein was significantly different in S2/S4 and S3/S4 ($P=0.0068$ and $P=0.0036$). However, the diameter of portal vein only increased significantly in patients with S4 stage of liver fibrosis. These data suggested that the length of spleen began to increase at S3 of liver fibrosis, so that the difference in S3 / S4 was insignificant. The diameter of portal vein began to increase later than the length of spleen and diameter of spleen vein.

Based on receiver-operating characteristic (ROC) curve, the maximal sum of diagnostic sensitivity and specificity was considered as the best cut-off value of US parameters for prediction of the severity of liver fibrosis. The diag-

Table 2 Diagnostic values of three US parameters for early liver cirrhosis

Parameters and cut-off value	Sensitivity	Specificity	Accuracy	Positive predicative value	Negative predicative value
Length of spleen (12.1 cm)	0.600	0.753	0.737	0.198	0.948
Diameter of spleen vein (8 mm)	0.600	0.781	0.765	0.220	0.950
Diameter of portal vein (12 mm)	0.767	0.446	0.475	0.124	0.949

nostic values of three quantitative US parameters are listed in Table 2.

DISCUSSION

Liver cirrhosis can be detected by US in patients with portal hypertension. Schalm[13] reviewed the diagnostic methodology of liver cirrhosis and found that percutaneous liver biopsy has a sensitivity of below 85% in detection of liver cirrhosis. Liver biopsy could yield false negative results in nearly one third of cases, but it is currently considered the criterion for establishing a precise diagnosis and assessing the extent of fibrosis. The diagnostic sensitivity and specificity of US examination for liver cirrhosis vary widely with a diagnostic sensitivity of 0.125- 0.95 and a diagnostic specificity of 0.285- 1.0[14-16]. The diagnostic accuracy of US for early liver cirrhosis in patients with chronic virus hepatitis and compensated liver function has not been fully investigated.

At US scanning, liver surface nodularity reflects the presence of regenerative nodules and fibrous septa. Nodularity on liver surface and in parenchyma is independently associated with the diagnosis of cirrhosis and US is reliable for diagnosis of liver fibrosis. It was reported that the high frequency US transducer (7.5-12 MHz) can obtain satisfactory results for diagnosis of liver cirrhosis[17] while the low frequency US is not a reliable test for liver cirrhosis[18].

Gaiani *et al*[19] showed that 80.4% of cirrhosis can be detected in patients with compensated liver diseases of various etiologies using a US scoring system based on two US parameters. Colli *et al*[20] reported that US can detect severe fibrosis or cirrhosis with a specificity of 0.95 and a sensitivity of only 0.54. Moreover, surface nodularity could also be influenced by different factors, mainly local fatty infiltration. Hung *et al*[21] evaluated the validity of US in diagnosis of cirrhosis with a diagnostic sensitivity of liver cirrhosis of 0.775 and a specificity of 0.92 in patients with HBV infection. Zheng *et al*[22] studied the value of US in evaluation of liver fibrosis and compensated cirrhosis in comparison with serology and histology and found that hepatic parenchymal echo pattern, liver surface and thickness of gallbladder wall are three independent predictors of liver fibrosis. The diagnostic accuracy of US for compensated cirrhosis is 80.7%.

The accuracy of Doppler US measurement for diagnosis of early liver cirrhosis is still controversial. It was reported that decreased flow velocity in portal vein is sufficiently accurate in diagnosis of liver cirrhosis[23-25]. While other studies[26-28] showed that substantial variability exists in measurement of portal venous blood flow velocity and volume. Doppler US measurement does not represent the hepatic venous pressure gradient[29]. This controversy could be explained by the lack of standard technique of Doppler measurement. Furthermore, changes of hemodynamics in hepatic blood flow are influenced by multiple factors, such as extent of fibrosis, chronic inflammation, presence and size of esophageal varices, as well as porto-systemic shunts.

The results of this study showed that the maximal velocity of blood flow in portal vein was weakly related with liver cirrhosis, but the standard deviation of data was wide, suggesting that this Doppler US parameter is not important in evaluation of liver fibrosis.

Schalm[13] suggested that if histology shows no cirrhosis (nodules surrounded by fibrosis) but fibrosis and architectural distortion, diagnosis of cirrhosis should still be made when there is a US diagnosis of cirrhosis. Afdhal and Nunes[30] argued that a proper US examination can identify patients with cirrhosis when the biopsy findings are equivocal, or at variance with the clinical impression.

The results of this study showed that different stages of hepatic fibrosis could not be determined satisfactorily by US parameters. A single US parameter was limited in sensitivity and specificity for diagnosis of early cirrhosis. Early liver cirrhosis could be excluded using two or three quantitative and qualitative US parameters, especially the spleen length, diameter of spleen vein and echo pattern of liver surface, because the negative predictive values of these three quantitative US parameters were high. US can also be used in follow-up of patients with chronic virus hepatitis.

The limitations of our study are the relatively small number of patients with S0- S4 stages of liver fibrosis and no application of the high-frequency US probe in examination of liver surface.

In conclusion, US cannot be used as a specific diagnostic tool for chronic viral hepatitis, but US should be stressed in screening and follow-up of patients with chronic virus hepatitis. However, the results of this study do not decrease the value of liver biopsy because it has other indications in clinical practice of hepatology.

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RAPID COMMUNICATION

Clinicopathologic characteristics of esophagectomy for esophageal carcinoma in elderly patients

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Abstract

AIM: To evaluate the risk of esophagectomy for carcinoma of the esophagus in the elderly (70 years or more) compared with younger patients (<70 years) and to determine whether the short-term outcomes of esophagectomy in the elderly have improved in recent years.

METHODS: Preoperative risks, postoperative morbidity and mortality in 60 elderly patients (⩾70 years) with esophagectomy for carcinoma of the esophagus were compared with the findings in 1 782 younger patients (<70 years) with esophagectomy between January 1990 and December 2004. Changes in perioperative outcome and short-time survival in elderly patients between 1990 to 1997 and 1998 to 2004 were separately analyzed.

RESULTS: Preoperatively, there were significantly more patients with hypertension, pulmonary dysfunction, cardiac disease, and diabetes mellitus in the elderly patients as compared with the younger patients. No significant difference was found regarding the operation time, blood loss, organs in reconstruction and anastomotic site between the two groups, but elderly patients were more often to receive blood transfusion than younger patients. Significantly more transhiatal and fewer transthoracic esophagectomies were performed in the elderly patients as compared with the younger patients. Resection was considered curative in 71.66% (43/60) elderly and 64.92% (1 157/1 782) younger patients, which was not statistically significant ($P > 0.05$). There were no significant differences in the prevalence of surgical complications between the two groups. Postoperative cardiopulmonary medical complications were encountered more frequently in elderly patients. The hospital mortality rate was 3.3% (2/60) for elderly patients and 1.1% (19/1 782) for younger patients without a significant difference. When the study period was divided into a former (1990 to 1997) and a recent (1997 to 2004) period, operation time, blood loss, and percentage of patients receiving blood transfusion of the elderly patients significantly improved from the former period to the recent period. The hospital mortality rate of the elderly patients dropped from the former period (5.9%) to the recent period (2.3%), but it was not statistically significant.

CONCLUSION: Preoperative medical risk factors and postoperative cardiopulmonary complications after esophagectomy are more common in the elderly, but operative mortality is comparable to that of younger patients. These encouraging results and improvements in postoperative mortality and morbidity of the elderly patients in recent period are attributed to better surgical techniques and more intensive perioperative care in the elderly.

Key words: Esophagectomy; Carcinoma; Esophagus

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INTRODUCTION

Perioperative management of elderly patients after a major operation is an important issue because of the recent worldwide increase in the elderly population. Esophagectomy for esophageal carcinoma is a major procedure associated with a high mortality and morbidity, and advanced age is often considered a significant risk factor and even a relative contraindication to esophagectomy despite advances in modern surgical practice[1-3].

There have been a small number of studies on the relationship between the clinicopathologic characteristics and age of patients after esophagectomy for esophageal carcinoma. However, whether the prognosis of elderly patients after esophagectomy is more unfavorable than that in younger patients remains still controversial. Some reports emphasized the worse prognosis in elderly patients after esophagectomy[4,5], whereas others emphasized similar outcome irrespective of the age[6-8].

Table 1 Clinicopathologic characteristics *n* (%)

Variables	⩾70 years (*n* = 60)	< 70 years (*n* = 1782)	*P* value
Mean age (yr)	73.1±3.9	55.8±5.2	0.0007
Sex (male/female)	Sep-51	1453/329	0.61
Tumor location			0.74
Cervical	3 (5.0)	75 (4.2)	
Upper-third	7 (11.7)	186 (10.4)	
Middle-third	32 (53.3)	901 (50.6)	
Lower-third	16 (26.7)	591 (33.2)	
Double location	2 (3.3)	29 (1.6)	
Tumor size (cm)	5.2±2.1	6.5±2.7	0.85
Histological type			0.87
Squamous cell carcinoma	53 (88.3)	1 610 (90.3)	
Adenocarcinoma	4 (6.7)	97 (5.4)	
Other carcinomas	3 (5.0)	75 (4.2)	
Histological differentiation			0.76
Well	8 (13.3)	185 (10.4)	
Moderately	46 (76.7)	1 410 (79.1)	
Poorly	6 (10.0)	187 (10.5)	
TNM stage			0.89
0	1 (1.7)	15 (0.8)	
I	2 (3.3)	94 (5.3)	
II	9 (15.0)	251 (14.1)	
III	45 (75.0)	1 308 (73.4)	
IV	3 (5.0)	114 (6.4)	
Preoperative radiochemotherapy	19 (31.7)	624 (35.0)	0.68

Table 2 Preoperative risks *n* (%)

Risks	⩾70 years (*n* = 60)	< 70 years (*n* = 1 782)	*P* value
Weight loss	17 (28.3)	564 (31.6)	0.67
Anemia	13 (21.7)	344 (19.3)	0.62
Hypertension	26 (43.3)	512 (28.7)	0.02
Pulmonary dysfunction	43 (71.7)	479 (26.9)	0
Cardiac disease	23 (38.3)	324 (18.2)	0
Cirrhosis	8 (13.3)	155 (8.7)	0.24
Chronic renal disease	11 (18.3)	236 (13.2)	0.25
Diabetes mellitus	24 (40.0)	395 (22.2)	0.003

The purpose of this study was to evaluate the risk of esophagectomy for the esophageal carcinoma in the elderly (70 years or more) as compared with younger patients (<70 years) and to determine whether the short-term outcome of esophagectomy in the elderly have improved with increased experience of the surgical team and improved perioperative management in recent years.

MATERIALS AND METHODS

Patients

The subjects included 1 842 consecutive patients with primary carcinoma of the esophagus, who had been treated by esophageal resection and reconstruction between January 1990 and December 2004 in our institute. Our patients were unselected and consisted of all of the patients after esophagectomy for esophageal carcinoma during the study period. The patients comprised 1 563 men and 279 women. Sixty were elderly (⩾70 years) and 1 782 were younger patients (<70 years). Among the elderly, 17 patients were operated during the period 1990 to 1997, and data obtained were compared with those of 43 patients operated during the period 1998 to 2004.

All patients had detailed preoperative risk assessments based on history of chronic lung or heart disease, chest x-ray, electrocardiogram (ECG), arterial blood gas analysis, pulmonary function tests, and biochemical and hematological tests. The preoperative risk factors analyzed included weight loss more than 10%, anemia (hemoglobin less than 12 g/L), hypertension (prescribed history of hypertension, systolic blood pressure more than 140 mm Hg, and/or diastolic pressure more than 90 mm Hg), chronic pulmonary disease or abnormal lung dysfunction (forced expiratory volume at 1 second [FEV_1] < 70% of predicted normal), cardiac disease (history of ischemic heart disease, heart failure, or abnormal ECG), cirrhosis, chronic renal disease, and diabetes mellitus. Clinicopathologic characteristics, therapeutic methods, and the postoperative morbidity and mortality between elderly and younger patients were compared. Hospital mortality was defined as death within the same hospital admission after surgery, up to 6 mo after surgery. Resection was defined as curative when the tumor was confined to the esophagus with or without involvement of adjacent lymph nodes and all macroscopic tumors had been removed. Resection was palliative when there was infiltration of the tumor beyond the esophagus into mediastinal organs or when there was residual tumor after resection.

Statistical analysis

Comparisons between groups were performed using the Student's *t* test and χ^2 test or Fisher's exact test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Clinicopathologic characteristics and preoperative risks

The clinicopathologic characteristics between the elderly and younger groups are shown in Table 1. There were no significant differences in sex, tumor location, tumor size, histological type, histological differentiation, TNM stage and preoperative radiochemotherapy between the two groups. There were significantly more patients with hypertension, pulmonary dysfunction, cardiac disease, and diabetes mellitus in the elderly group, whereas weight loss, anemia, cirrhosis and chronic renal disease status were not statistically different between the two groups (Table 2).

Surgical treatment

Operative variables are shown in Table 3. No significant difference was found regarding the operation time, blood loss, organs in reconstruction and anastomotic site between the two groups, but elderly patients were more often to receive blood transfusion than younger patients. Significantly more transhiatal and fewer transthoracic esophagectomies were performed in the elderly patients. Resection was considered curative in 71.66% (43/60) elderly and 64.92% (1 157/1 782) younger patients, which difference was not significant ($P > 0.05$).

Table 3 Surgical treatment *n* (%)

Variables	⩾70 years (*n* = 60)	< 70 years (*n* = 1782)	*P* value
Operation time (min)	239±147	225±139	0.88
Blood loss (mL)	443±364	418±251	0.41
Blood transfusion	23 (38.3)	415 (23.3)	0.01
Types of operations			0
Transthoracic esophagectomy	41 (68.3)	1 563 (87.7)	
Transhiatal esophagectomy	17 (28.3)	190 (10.7)	
Thoracoscopic esophagectomy	2 (3.3)	29 (1.6)	
Organs in reconstruction			0.27
Stomach	53 (88.3)	1 435 (80.5)	
Colon	3 (5.0)	197 (11.1)	
Jejunum	4 (6.7)	150 (8.4)	
Anastomotic site			0.31
Cervical	14 (23.3)	326 (18.3)	
Intrathoracic	46 (76.7)	1 456 (81.7)	
Curative resection	43 (71.7)	1 157 (64.9)	0.34

Table 4 Postoperative morbidity and mortality *n* (%)

Variables	⩾70 years (*n* = 60)	< 70 years (n = 1782)	*P* value
Surgical complications			
Anastomotic leakage	2 (3.3)	35 (2.0)	0.34
Hemorrhage	1 (1.7)	19 (1.0)	0.49
Intra-abdominal abscess	0 (0.0)	8 (0.4)	1
Chylothorax	2 (3.3)	39 (2.2)	0.39
Thoracic empyema	0 (0.0)	12 (0.7)	1
Recurrent nerve paralysis	1 (1.7)	20 (1.1)	0.5
Wound dehiscence	1 (1.7)	23 (1.3)	0.55
Medical complications			
Pulmonary	26 (43.3)	501 (28.1)	0.01
Cardiac	23 (38.3)	352 (19.8)	0.001
Renal	1 (1.7)	46 (2.6)	1
Hepatic	0 (0.0)	15 (0.8)	1
Postoperative deaths	2 (3.3)	19 (1.1)	0.15
Anastomotic leakage	1 (1.7)	8 (0.4)	0.26
Pulmonary disease	1 (1.7)	9 (0.5)	0.28
Cerebrovascular accident	0 (0.0)	2 (0.1)	1

Postoperative morbidity and mortality

Postoperative morbidity and mortality in the elderly and younger groups are shown in Table 4. There were no significant differences in the prevalence of surgical complications between the two groups. Although the anastomotic leakage rate was low, it was still the most common surgical complication in each group. Postoperative medical complications were encountered more frequently in the elderly, mainly pulmonary and cardiac, whereas other medical complications were not statistically different between the two groups. No patient died on the operation table. The hospital mortality rate was 3.3% (2/60) for elderly patients and 1.1% (19/1 782) for younger patients without a significant difference. There was no significant difference in the types of complication as the causes of death between the two groups.

When the study period was divided into a former (1990 to 1997) and a recent (1997 to 2004) period, operative time, blood loss, and percentage of patients receiving blood transfusion of the elderly patients significantly improved from the former period to the recent period (Table 5). No significant differences in surgical complications were observed, but there was a significant decrease in postoperative cardiopulmonary complications from the former period to the recent period. The hospital mortality rate of the elderly patients dropped from the former period (5.9%) to the recent period (2.3%), but it was not statistically significant.

Table 5 Postoperative morbidity and mortality in elderly patients during the two periods *n* (%)

Variables	1990-1997 (*n* = 17)	1998-2004 (*n* = 43)	*P* value
Operation time (min)	255±157	212±104	0.008
Blood loss (mL)	520±375	402±249	0.003
Blood transfusion	13 (76.4)	19 (44.2)	0.04
Surgical complications			
Anastomotic leakage	1 (5.9)	1 (2.3)	0.49
Hemorrhage	0 (0.0)	1 (2.3)	1
Chylothorax	1 (5.9)	1 (2.3)	0.49
Recurrent nerve paralysis	1 (5.9)	0 (0.0)	1
Wound dehiscence	0 (0.0)	1 (2.3)	1
Medical complications			
Pulmonary	11 (64.7)	15 (34.9)	0.046
Cardiac	10 (58.8)	13 (30.2)	0.04
Renal	0 (0.0)	1 (2.3)	1
Hospital mortality rate	1 (5.9)	1 (2.3)	0.49

DISCUSSION

Esophageal carcinoma in the elderly has increased in part because of increasing life expectancy. Esophagectomy for carcinoma probably has the highest operative mortality of any elective surgical procedures[9]. Therefore, it is important to evaluate the risk of esophagectomy for carcinoma in elderly patients. Advanced age was once considered a relative contraindication to esophagectomy because of the high operative mortality rate[4,5]. At the same time, the malignant potential of neoplasms in elderly patients has occasionally been reported to be much less aggressive than that in younger patients[10]. Whether the prognosis of elderly patients with esophagectomy for esophageal carcinoma is more unfavorable than that in younger patients remains controversial.

Recently, more and more reports emphasized that esophagectomy could be performed in a high percentage of elderly patients and thus advanced age alone should not be considered as a contraindication for esophagectomy[6-8]. We shared this opinion and believed that resection should be offered whenever possible because it offered the only hope of cure and the best method of palliation[11]. The presence of risk factors has great impact on surgical outcome, hence thorough preoperative assessment should be carried out in all patients. Even in the presence of medical risk factors, resection is still preferred for the elderly unless the risk is prohibitively high. Most of our patients who did not have resection were unresectable because of extensive local or metastatic disease, and only a small portion (about 20%) of elderly patients were deemed unresectable because of poor physical conditions or cardiopulmonary status.

Preoperative risk assessment is an important aspect of patient selection for esophagectomy, as a significant number of these patients had cardiopulmonary or diabetes mellitus in the preoperative period and cardiopulmonary complications in the postoperative period. Pulmonary complication was one of the most common causes of surgical complication-related deaths in both groups. These results strongly suggest that greater preoperative precautions must be taken to manage cardiopulmonary complications in the elderly patients[12]. The anastomotic leakage and chylothorax rate were low in both elderly and younger patients, but these remained the main surgical complications. Pulmonary complication was the most common cause of postoperative death in both elderly and younger patients.

Although a significant number of patients had postoperative surgical or medical complications, only a few of them succumbed to death because of those complications. The mortality rate caused by surgical or medical complications in elderly patients was slightly higher but comparable to that of younger patients, despite higher cardiopulmonary risk and more cardiopulmonary complications in the elderly. The similar outcome was probably the result of significant improvement in surgical technique and more intensive perioperative patient care in our institution. For example, chest physiotherapy was instituted early before operation, and during the postoperative period, cricothyroidotomy was often performed to keep the airway clear of sputum. The more frequent use of transhiatal over transthoracic esophagectomy in elderly patients may also have contributed to the low cardiopulmonary–related mortality[13]. Moreover, shorter operative time, reduced blood loss and fewer perioperative blood transfusion in recent years may all have important impact on the reduced incidence of cardiopulmonary complications during this period[14-17]. Finally, there has been considerable improvement in postoperative pain control by epidural anesthesia block. Adequate analgesia decreases pulmonary complications by decreasing the disturbances of pulmonary mechanics after thoracotomy or laparotomy and enabling patients to generate effective cough[18]. The pulmonary complication rate decreased to only 34.9% in recent years in contrast to 64.7% in the previous era.

In conclusion, our study showed that preoperative medical risk factors and postoperative cardiopulmonary complications after esophagectomy are more common in the elderly, but operative mortality was comparable to that of younger patients. These encouraging results and improvements in postoperative mortality and morbidity in recent period are attributed to better surgical techniques and more intensive perioperative care in elderly patients. However, a careful patient selection procedure must be used to exclude the high-risk elderly patients from the operative list and thus will help to reduce the postoperative morbidity and mortality rate in this group of patients.

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RAPID COMMUNICATION

Value of mink vomit model in study of anti-emetic drugs

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Abstract

AIM: To establish a new, reliable vomit model of minks.

METHODS: Adult male minks were randomly divided into 8 groups ($n = 6$): cisplatin (7.5 mg/kg) intraperitoneal injection (ip) group, copper sulfate (40 mg/kg) intragastric injection (ig) group, apomorphine (1.6 mg/kg) subcutaneous injection (sc) group, and 18 Gy whole-body X-irradiation group, ondansetron injection group (2 mg/kg ip) 30 min later followed by cisplatin (7.5 mg/kg) ip, normal saline (NS) ip injection control group, metoclopramide injection group (4 mg/kg ip) 30 min later followed by apomorphine (1.6 mg/kg) sc, NS ig control group. The frequency of retching and vomiting was calculated. After behavioral experiment, distribution of 5-HT in the ileum was detected by immunohistologic method.

RESULTS: Cisplatin, apomorphine, copper sulfate and X-irradiation administered to minks evoked a profound emetic response in the animals. However, retching and vomiting were significantly inhibited by pretreatment with ondansetron and metoclopramide in cisplatin and copper sulfate groups ($P = 0.018$). Immunohistologic result showed that 5-HT released from enterochromaffin cells (EC cells) was involved in vomiting mechanism.

CONCLUSION: Mink vomit model has a great value in studying the vomiting mechanism and screening new antiemetic drugs.

Key words: Vomit; Mink; Cisplatin; Ondansetron; Apomorphine; X-irradiation

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INTRODUCTION

In the treatment of malignant disease, potentially curative chemotherapy and radiotherapy are commonly associated with a wide range of adverse events, including intractable nausea and vomiting, which can challenge patient compliance with a treatment regimen. Other forms of emesis, including those experienced post-operatively or in other diseases, can also present as serious problems. Studying the vomiting mechanism and screening for new anti-emetic drugs urge us to establish appropriate animal models with similar vomiting behavior to that of human beings. Ferret (*Mustela putorius furo*) is a relative ideal animal model in vomiting study worldwide[1]. However, its use is limited because of its high feeding cost and difficulties to survive. Fortunately minks (*Mustela vison*) and ferrets belonging to the same stoat genus are characterized by their inexpensiveness, wide availability and ease of feeding and raising. However, there are no reports on the use of minks in medical researches. This study was to establish a new and reliable vomit model of minks based on the effects of a variety of emetogens.

MATERIALS AND METHODS

Materials

Cisplatin powder was obtained from Qilu Pharmaceutical Factory, Jinan, China. Ondansetron hydrochloride injection was from Ningbo Tianheng Pharmaceutical Factory, Ningbo, China. Copper sulfate was from Shanghai Hengda Chemical Co., Shanghai, China. Apomorphine was from Sigma in St Louis, USA. Metoclopramide dihydrochloride injection was from Tianjin People's Pharmaceutical Factory, Tianjin, China. SABC immunohistology test kit was from Boster Biotechnology Co., Wuhan, China. Varian 6 MeV 2100-C X-irradiation Linear Accelerator was from Varian Corp., USA. Microscope was from Olympus, Japan. Adult male minks weighing 1.3-1.8 kg were provided by Qingdao Special Animal Center.

Animal experiments

Animals were used in the present study in accordance with the Qingdao University Guide for the Care and Use of Laboratory Animals.

To examine the effects of emetogens, the minks were divided into 4 groups ($n = 6$), and housed individually in an iron cage of 75 cm × 50 cm × 50 cm with free access to

Table 1 Effects of emetogens on minks (n = 6, mean±SD)

Emetogens	Dose (mg/kg)	Latency (min)	Retching (n)	Emesis (n)	Vomiting ratio
NS	-	-	0	0	0/6
Cisplatin	7.5	92.0±21	91.5±37	12.0±3	6/6
NS	-	-	0	0	0/6
Apomorphine	1.6	39.4±36	28.5±16	5.3±1.9	6/6
NS	-	-	0	0	0/6
Copper sulfate	40	7.0±2.0	29.0±9	8.7±2.3	6/6
Pseudo irradiation	0	-	0	0	0/6
X-ray	18 Gy	17.0±4	19.3±7	10.4±3	5/6

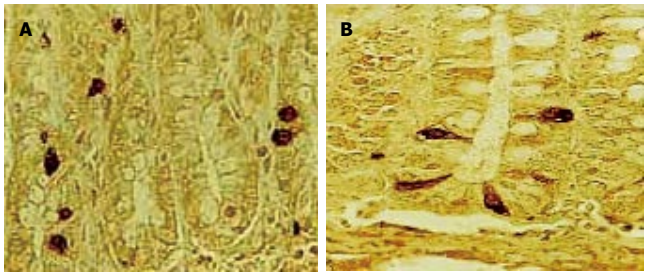

Figure 1 A: control group, 5-HT did not release from EC cells with a clear round rim; **B:** the cisplatin group, 5-HT released from EC cells with a tail.

Table 2 Effects of ondansetron or metoclopramide on emesis induced by cisplatin or apomorphine in minks (mean±SD, n = 6)

Emetogens	Dose (mg/kg)	Anti-emetics	Dose (mg/kg)	Latency (min)	Retching (n)	Emesis (n)
Cisplatin	7.5	NS		92.0±21	91.5±37	12±3
Cisplatin	7.5	Ondansetron	2	143.8±80	47.3±41	5.3±5[a]
Apomorphine	1.6	NS		39.4±36	28.5±16	5.3±1.9
Apomorphine	1.6	Metoclopramide	4	-a	1.3±3[b]	0.3±0.8[d]

[a]P = 0.018, t = 2.93 *vs* vehicle-pretreated cisplatin group; [b]P = 0.0022, t' = 4.08, [d]P = 0.003, t' = 5.94 *vs* vehicle-pretreated apomorphine group; -a: only 1 monk had retching and vomiting.

food and water

The minks in the first group were first given NS (5 mL/kg ip). After 24 h, the animals were administered cisplatin (7.5 mg/kg ip) in a volume of 5 mL/kg diluted with NS.

The minks in the second group were first given NS (15 mL/kg ig). After 24 h, the animals were given copper sulfate (40 mg/kg ig) in a volume of 15 mL/kg diluted with NS.

The minks in the third group were first given NS (0.4 mL/kg sc). After 24 h, the animals were given apomorphine (1.6 mg/kg sc) in a volume of 0.4 mL/kg diluted with NS.

The minks in the fourth group were first exposed to pseudo-irradiation. After 24 h, the animals received 18 Gy whole-body X-irradiation for 4.5 min.

To investigate the effects of anti-emetic agents on emesis induced by cisplatin or apomorphine, the minks in the first group were injected intraperitoneally with ondansetron injection (2 mg/kg) in a volume of 1 mL/kg 30 min before the intraperitoneal administration of cisplatin (7.5 mg/kg).

The minks in the second group were pretreated with NS (1 mL/kg ip) as control. After 30 min, the animals were administered cisplatin (7.5 mg/kg).

The minks in the third group were pretreated with metoclopramide injection (4 mg/kg ip) in a volume of 0.8 mL/kg, followed by apomorphine (1.6 mg/kg sc) after 30 min.

The minks in the fourth group were pretreated with NS (0.8 mL/kg ip) as control, followed by apomorphine (1.6 mg/kg sc) later 30 min.

All experiments were carried out starting at 9:00 am. After treatment, retching and vomiting were observed for 6 h, following the vomit criteria for ferrets described by Minami *et al*[2]. No observers were aware of the treatments. Salivating, flinching and other behaviors resembling the nausea of human beings were observed. During vomiting, the mink's head was protruding downwards ahead with open mouth, shrugging shoulder, contracting abdomen and occasional sound of vomiting. A vomiting cycle started the minute when vomiting began until smooth breaths recovered. A retching/vomiting referred to a vomiting action without or with stomach content spat out. The frequency of retching and vomiting was calculated.

Immunohistologic analysis

Minks in control group (n = 3) and cisplatin group (n = 3, 3 h after 7.5 mg/kg ip) were killed by cervical dislocation. A 3 cm long section was dissected 20 cm from the pylorus, and the distribution of 5-HT was detected under microscope by the immunohistologic method according to the instructions of the SABC test kit.

Statistical analysis

Data were expressed as mean±SD and analyzed by Student's t test. P < 0.05 was considered statistically significant.

RESULTS

Animal experiments

Cisplatin, apomorphine, copper sulfate and X-irradiation administered to the minks evoked a profound emetic response, and the manifestations of retching and vomiting in minks were similar to those in ferrets[2]. No vomiting occurred in control groups. The effects of emetogens over a 6 h period are shown in Table 1.

All minks in the NS + cisplatin group suffered from retching and vomiting. Vomiting started after 60 min and reached its peak after about 2 h. Five minks in the ondansetron pretreatment group suffered from retching and vomiting. The frequency of cisplatin-induced retching and vomiting after 6 h was significantly reduced by pretreatment with ondansetron.

All minks in the NS+apomorphine group and only one in the metoclopramide pretreatment group, suffered from retching and vomiting. The frequency of apomorphine-induced retching and vomiting after 6 h was significantly reduced by pretreatment with metoclopramide.

No significant difference was found between the pretreatment and control groups in terms of the latency and duration of emesis induced by cisplatin or apomorphine (Table 2).

Immunohistologic analysis

In the cisplatin group, 5-HT was released from EC cells which seemed to have a tail. In the control group, 5-HT was not released from EC cells which was round with a clear rim. Similar phenomena were also observed in other groups (Figure 1).

DISCUSSION

It is widely known that vomiting cannot be induced in rodents such as mice (except for the special strains), rats, and guinea pigs. Pigeons, cats and dogs are occasionally used in vomiting research and screening for new anti-emetics. Since pigeons often carry infectious viruses to human beings, no reports are available on vomiting induced by anticancer drugs in pigeons. Cats and dogs have a good learning ability. When vomiting is induced by emetogens, it often continues even anti-emetics[3] are administered. Ferret is a widely accepted animal model in vomiting study at present, mainly because its vomiting behavior resembles that of human beings[4] in terms of behavior, responses to emetogens and anti-emetic agents, and biochemistry change in vomit response. Ondansetron, a potential anti-emetic agent, was developed in ferret vomit model by Glaxo Company. However, since ferret is so expensive and difficult to raise, its wide use cannot be realized. In fact, no laboratory has carried out experiments in ferrets so far in China. Minks belong to the same stoat family of ferrets. As a vomit model, they not only share the advantages with ferrets, but also are inexpensive and can be raised quite easily and widely.

In the present study, the effects of a variety of emetogens, including cisplatin, copper sulfate, apomorphine and X-irradiation at the doses evoking a profound emetic response in ferrets, were identical in minks. The manifestations of retching and vomiting were similar to those in ferrets. As shown in our experiment, ondansetron and metodopramide can inhibit emesis induced by cisplatin and apomorphine in minks.

5-HT immunohistologic analysis showed that emetogens increased the release of 5-HT from EC cells in intestine, which may be an important mechanism involved in vomiting. This finding is consistent with the results reported previously[5]. However, it must be acknowledged that this study has its limitations because our experiments were mainly focused on the behavior of minks and the vomiting mechanism was studied in the initial stage. A further study is needed.

In conclusion, the mink vomit model is of great value in studying the vomiting mechanism and screening for new anti-emetic drugs.

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RAPID COMMUNICATION

Arg-gly-asp-mannose-6-phosphate inhibits activation and proliferation of hepatic stellate cells *in vitro*

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Abstract

AIM: To investigate the effect of arg-gly-asp-mannose-6 phosphate (RGD-M6P) on the activation and proliferation of primary hepatic stellate cells *in vitro*.

METHODS: Hepatic stellate cells (HSCs) were isolated from rats by *in situ* collagenase perfusion of liver and 18% Nycodenz gradient centrifugation and cultured on uncoated plastic plates for 24 h with DMEM containing 10% fetal bovine serum (FBS/DMEM) before the culture medium was substituted with 2% FBS/DMEM for another 24 h. Then, HSCs were cultured in 2% FBS/DMEM with transforming growth factor β1, M6P, RGD, or RGD-M6P, respectively. Cell morphology was observed under inverted microscope, smooth muscle α-actin (α-SMA) was detected by immunocytochemistry, type III procollagen (PCIII) in supernatant was determined by radioimmunoassay, and the proliferation rate of HSCs was assessed by flow cytometry.

RESULTS: RGD-M6P significantly inhibited the morphological transformation and the α-SMA and PC III expressions of HSCs *in vitro* and also dramatically prevented the proliferation of HSCs *in vitro*. Such effects were remarkably different from those of RGD or M6P.

CONCLUSION: The new compound, RGD-M6P, which has a dramatic effect on primary cultured HSCs *in vitro*, can inhibit the transformation of HSCs in culture caused by TGFβ1, suppresses the expression of PCIII and decreases proliferation rate of HSC. RGD-M6P can be applied as a selective drug carrier targeting at HSCs, which may be a new approach to the prevention and treatment of liver fibrosis.

Key words: RGD; Mannose-6-phosphate; Hepatic stellate cell; Liver fibrosis

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INTRODUCTION

Liver fibrosis is characterized by an excessive deposition of extracellular matrix constituents, resulting from an enhanced synthesis of matrix proteins (fibrogenesis) and decreased removal by matrix degrading enzymes (fibrolysis) in consequence of liver cell damage of various causes[1-3]. The major cell type responsible for hepatic fibrogenesis is the activated hepatic stellate cells (HSCs). Therefore, this cell type is an important target for antifibrotic therapy[4,5]. Pharmacotherapeutic intervention to alter HSC functions may act at different levels: inhibition of activation and transformation of HSCs, inactivation of profibrogenic cytokines, interference with matrix synthesis and stimulation of matrix degradation[6-11]. *In vivo*, however, antifibrotic drugs may not be efficiently taken up by HSCs or may produce undesirable side effects. Drug targeting may be explored to elicit cell-specific uptake of drugs, but in contrast to other cell types in the liver, a specific carrier agent to go into HSCs is not yet known[12]. Therefore, we focused on the development of a specific carrier for HSCs. The binding sites expressed on activated HSCs were considered for their ability to serve as potential targets for carrier molecules. Two of these are integrin and mannose 6-phosphate/insulin like growth factor II (M6P/IGF-II) receptors, which are over-expressed on activated rat HSCs, particularly during fibrosis[13,14]. Additionally, extracellular matrixes (ECM) regulate the morphology and biological activities of HSCs through integrins on the cell membrane[13]. It has been certified that the RGD sequence plays a vital role in the linkage between ECM and integrins[13]. This study was to synthesize a new compound, RGD-M6P targeting at integrins and/or M6P/IGF-II receptors over-expressed on activated HSCs and to study its effects on the activation and proliferation of primary cultured HSCs in order to find a new approach for regulating HSC activation and anti-hepato-fibrotic therapy. If successful, this may be of value in the design of other receptor-specific carriers.

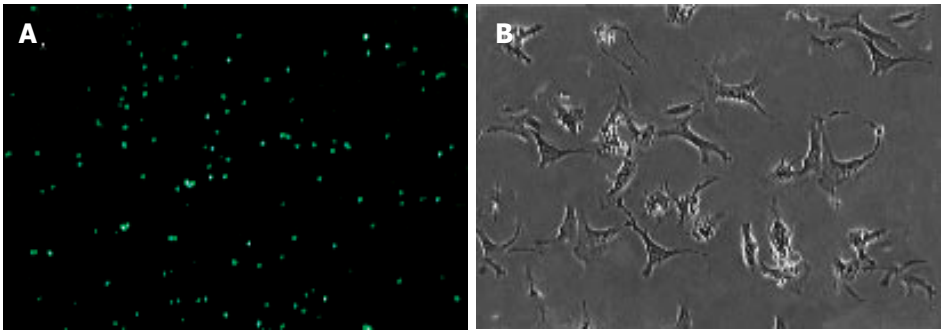

Figure 1 Small and round HSCs in the presence of vitamin-A droplets in cytoplasm (**A**) and spindle-like or asteroid membranous processes in HSCs (**B**).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 400-500 g were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences.

Main reagents

RGD, M6P and RGD-M6P were synthesized in Gastroenterological Laboratory of Xinhua Hospital, Shanghai Second Medical University. DMEM was purchased from Gibco Company. Procollagen III (PCIII) radioimmunoassay kits were purchased from Institute of Naval Medicine, Shanghai. α-SMA monoclonal antibody was provided from by Cruz Company.

Isolation of HSCs and grouping

HSCs were isolated and cultured. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital. After cannulation into the portal vein, the liver was perfused with calcium-free balanced NS containing 0.5 mg/mL collagenase and 1 mg/mL pronase E. Then the liver was removed, cut into small pieces and incubated in a solution containing 0.5 mg/mL collagenase. After washed, HSCs were purified by density gradient centrifugation with 18% Nycodenz, collected from the top layer, washed and suspended in DMEM supplemented with 10% FBS at the concentration of 1×10^6 cells/mL, and seeded on uncoated 24-well plastic plates at 1×10^5/well supplemented with 20% FBS/DMEM for 24 h. Then the HSCs were subjected to tetrandrine treatment after cultivation in 2% FBS/DMEM for another 24 h. Over 90% of isolated HSCs were viable as assessed by trypan blue exclusion with a purity of higher than 90% as determined by direct cell counting under a phase-contrast microscope and intrinsic vitamin A autofluorescence.

HSCs were subjected to different treatments for 5 d as follows: control group: without any treatment; TGFβ1 group: supplemented with 5 ng/mL TGFβ1; M6P group: supplemented with 100 μg/mL M6P and 5 ng/mL TGFβ1; RGD group: supplemented with 100 μg/mL RGD and 5 ng/mL TGFβ1; RGD-M6P group: supplemented with 200 μg/mL RGD-M6P and 5 ng/mL TGFβ1.

Immunocytochemical analysis of α-SMA

HSCs were cultured on 24-well plates with different treatments as described above. HSCs were fixed with ethanol/acetic acid after 5 d of treatment. α-SMA antibody, horse radish peroxidase-conjugated secondary antibody and diaminobenzidine were added sequentially according to the standard protocol. Semi-quantitative assessment of protein expression was performed using pathological image analysis system. The expression of α-SMA was estimated by gray value.

PCIII content

PCIII in supernatant was measured with a radioimmunoassay kit according to the manufacturer's instructions.

Flow cytometry

The cell cycle was analyzed by flow cytometry to assess the proliferation rate of HSCs. Adherent and floating cells were collected from a 6-well plate, centrifuged and washed twice with PBS at room temperature. Cell pellets were re-suspended in ice-cold 70% ethanol for overnight fixation at 4 ℃, centrifuged and re-suspended in 0.8 mL PBS. One microlitre 10 mg/μL RNase and 20 μL propidium iodide (1 mg/mL)were added and samples were incubated at 37 ℃ for 30 min before analysis by flow cytometry using a Beckman Coulter FC500. Proliferation was quantitatively measured as the percentage of cells in the G_2/M phase.

Statistical analysis

All data were expressed as mean±SD. Statistical analysis of values was performed with SPSS software (10.0 version). $P<0.05$ was considered statistically significant.

RESULTS

Morphological study of isolated HSCs in vitro

Over 90% of isolated HSCs were viable as assessed by trypan blue exclusion, which were small and round in appearance (Figure 1A). HSCs stimulated by 327 nm-length laser, showed a blue-green intrinsic autofluorescence due to vitamin-A rich droplets. After cultured for 72 h, HSCs presented a wall-adhesive growth pattern and many spindle-like or asteroid membranous processes and a reduction of vitamin-A droplets (Figure 1B). Overall, these findings indicated that HSCs freshly isolated from normal rats showed an activated form after 5 d of *in vitro* culture.

Expression of α-SMA in culture-activated HSCs

Immunocytochemical assay was used to evaluate the percentage of α-SMA-positive HSCs in different groups. The percentage of α-SMA-positive HSCs was significantly higher in TGFβ1 and M6P groups (Figures 2B and 2C)

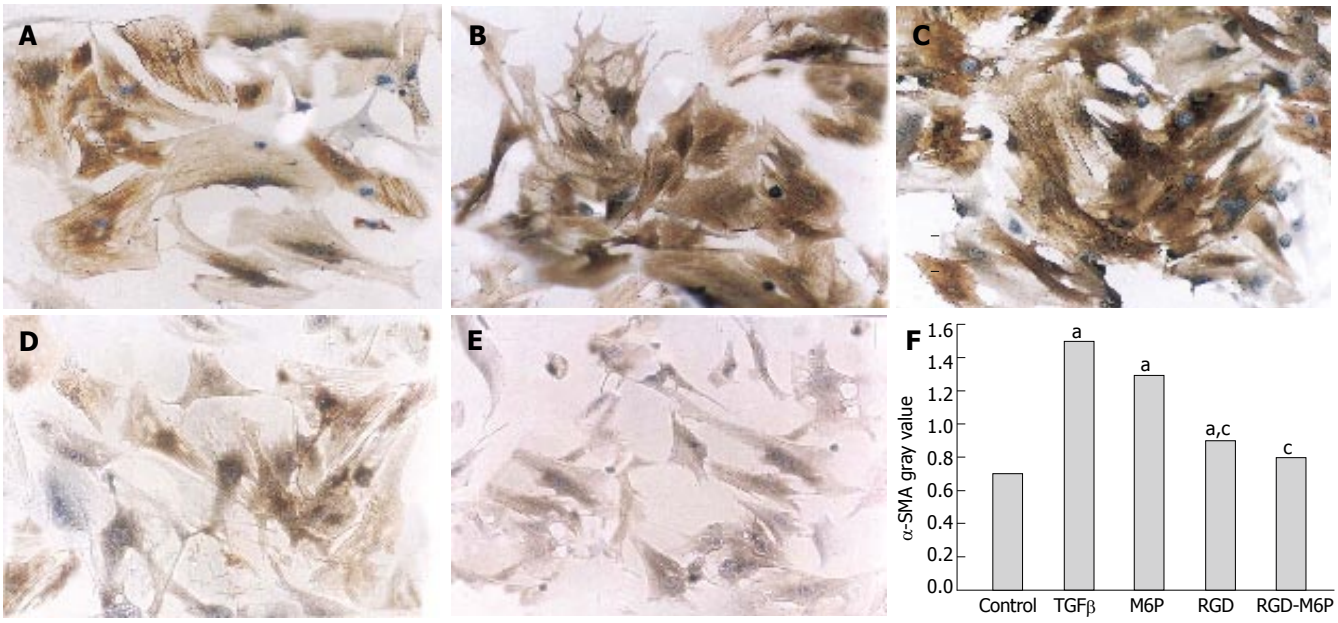

Figure 2 Immunocytochemical analysis of HSCs after cultured for 5 d (**A**), after treatment with TGFβ1 (**B**) and M6P (**C**), weak positivity of α-SMA RGD (**D**) and RGD-M6P (**E**), and elevated expression of α-SMA (**F**) in HSCs. [a]$P<0.05$ *vs* control groups; [c]$P<0.05$ *vs* TGFβ1.

Table 1 PCⅢ level in different groups (mean±SD)

Group	*n*	Concentration of PCⅢ (ng/mL)
Control group	5	323.59±16.14
TGFβ1 group	5	403.56±7.97[a]
M6P group	5	399.70±2.25[a]
RGD group	5	370.87±13.07[a,b]
RGD-M6P group	5	357.61±13.07[a,b]

[a]$P<0.01$ *vs* control groups; [b]$P<0.01$ *vs* TGFβ1 group.

than in control, RGD and RGD-M6P groups (Figures 2D and 2E). Even condensed α-SMA positive granules could be found (Figure 2B). No difference was found in the immunohistochemical study between RGD and RGD-M6P groups (Figure 2F). The cytomorphology study showed that the primary isolated HSCs were positive for α-SMA and the positivity was intensified through auto-activating characteristics of HSCs *in vitro*. Additionally, RGD and RGD-M6P could inhibit the activation of HSCs stimulated by TGFβ1.

PCⅢ excretion from culture-activated HSCs

PCIII is the most acceptable parameter of fibrogenesis as compared to other ECM contents, such as collagens I, III, IV, fibronectin, laminin and proteoglycans[15,16]. The level of PCIII in the control group was lower than that in the other groups ($P<0.01$, Table 1), and higher in TGFβ1 and M6P groups than in RGD and RGD-M6P groups. No difference was found in the level of supernatant PCIII between RGD and RGD-M6P groups. The results showed that the effect of RGD-M6P was better than that of M6P on inhibiting PCIII expression after stimulated by TGFβ1.

Proliferation of cultured HSCs

The activation of HSCs resulted in over-expression of α-SMA and increased proliferation of HSCs. Cell cycle analysis was used to evaluate the proliferation rate by flow cytometry. The primary isolated HSCs showed a characteristic proliferation (Figure 3A), which was further increased by TGFβ1 (Figure 3B). M6P showed no effect on the proliferation of HSCs (Figure 3C). On the contrary, RGD and RGD-M6P inhibited the proliferation of isolated HSCs significantly (Figures 3D and 3E).

DISCUSSION

In the past decade, significant advances have been made in anti-fibrosis therapy due to a better understanding of the cellular and molecular mechanism of hepatic fibrogenesis[1-3,8]. The key factors in the pathogenesis of liver fibrosis include the activation and proliferation of HSCs and their transformation into myofibroblasts[5]. Therefore, HSCs are the important target for antifibrotic therapies[4,12,17,18]. However, contrary to *in vitro* data, *in vivo* studies[13,14,19] demonstrated that most antifibrogenic agents cannot be efficiently taken up by HSCs and the binding sites expressed on (activated) HSCs can serve as potential targets for carrier molecules. Two of these are integrin and M6P/IGF-II receptors and their expressions are increased on activated rat HSCs, particularly during fibrosis. Some anti-fibrotic reagents targeting at HSCs have been developed[12,20], which allow cell-specific delivery of antifibrotic drugs to HSCs, thus enhancing their effectiveness *in vivo*.

Specific interactions between cells and ECM components are mediated by transmembrane proteins, especially heterodimeric integrins. Cell-matrix interactions guide or modulate cellular activities, such as adhesion, migration, differentiation, proliferation, and apoptosis[13]. Most integrins can recognize and bind to the amino acid sequence of RGD present in various matrix proteins. The expression of integrins is increased during liver fibrosis,

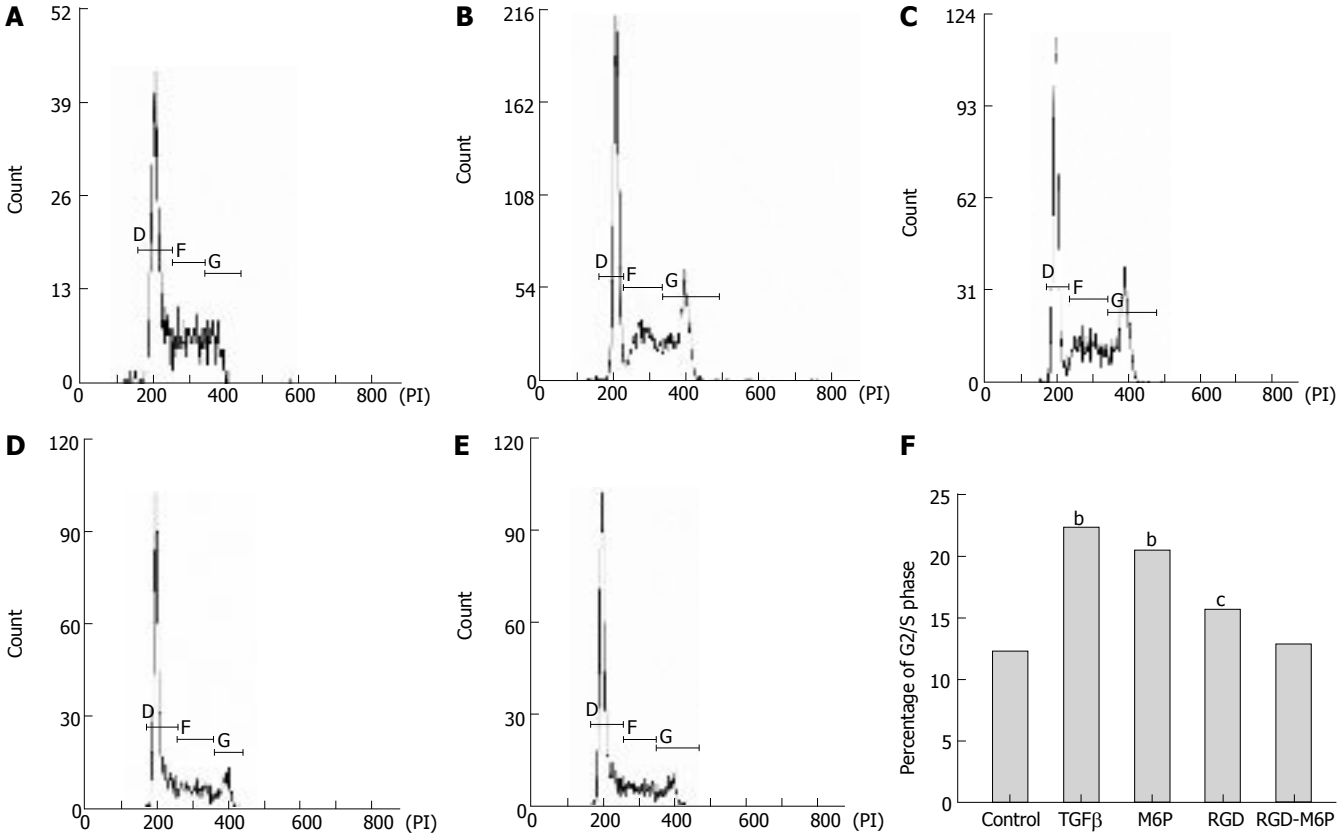

Figure 3 Flow cytometry analysis of cell cycle. PI histogram shows the details of cell cycle, in which the gates of D, F and G represent the G0/G1, S and G2/M phases, respectively, shown in corresponding figure.**A:** Proliferation of primary cultured HSCs; **B:** Increased proliferation of primary cultured HSCs in the presence of TGFβ1; **C:** Increased proliferation of primary cultured HSCs in the presence of M6P; **D** and **E:** Enlarged fraction of G2/M phase cells after treatment with RGD and RGD-M6P; **F:** Distribution of cell cycle phases was calculated using Multicycle I.O. $^{b}P < 0.01$ *vs* control, RGD and RGD-M6P groups; $^{c}P < 0.05$ *vs* control.

especially on the membrane of activated HSCs, suggesting that integrins can be used as a homing device to target at HSCs in fibrotic livers[19,21]. About 10%-20% of M6P/IGF-II receptors are expressed in cell membrane and over-expressed with the activation of HSCs[14]. It was reported that integrin antagonists can reduce the level of serum PCIII in fibrotic rats and inhibit the stimulating action of TGFβ1 on ECM expression[22-24]. Beliaars *et al*[25,26] showed that M6P-modified albumin accumulates in slices of normal and fibrotic liver and is taken up by HSCs. In the present study, we synthesized RGD-M6P and investigated its effects on the activation and proliferation of the primary HSCs *in vitro*.

The isolated primary HSCs in culture undergo auto-activation[27]. During this process, the expression of α-SMA is raised[28] and HSCs transform into fibroblast-like cells with increased proliferating activity[27], which might lack microenvironment *in vitro*. HSCs supplemented with TGFβ1 put forth more processes on their cell membrane, and the supernatant PCIII level is increased[29,30]. At the same time, the proliferation of HSCs is increased. Both RGD and RGD-M6P could inhibit the effect of TGFβ1 on the activation and proliferation of HSCs as well as PCIII expression. Though the M6P molecule participates in the activation of latent TGFβ1[31], it can be used as a candidate in the study of anti-fibrosis. In the present study, we could not find its anti-fibrosis activity because M6P acted on the activated TGFβ1 rather than the latent form. Moreover, RGD-M6P may have a more effective anti-fibrotic activity than RGD because RGD-M6P targets at the integrin and M6P/IGFII receptors on HSC membrane, thus more effectively modulating the HSC activity. But our present results failed to support the hypothesis. Further study is needed to explore the precise mechanism of this new complex.

In conclusion, integrins are related with TGFβ1 signal transduction and influence other signal transduction molecules such as JNK and NF-κB.

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RAPID COMMUNICATION

Detection of YMDD mutants using universal template real-time PCR

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Abstract

AIM: To establish a rapid and accurate method for the detection of lamivudine-resistant mutations in hepatitis B virus and monitor of lamivudine resistance during lamivudine treatment in patients with chronic hepatitis B virus infection.

METHODS: We established a real-time PCR method using a universal template and TaqMan probe to detect YMDD mutants. Variants of YVDD and YIDD were tested by individual reactions (reaction V and reaction I) and total hepatitis B viruses were detected in another reaction for control (reaction C). Results were determined by $\Delta Ct < 3.5$ (ΔCt = Ct of reaction V or I - Ct of reaction C). Clones of the HBV polymerase gene containing different YMDD mutations were tested. Serum samples from 163 lamivudine-treated patients with chronic hepatitis B virus infection were detected using this method and the results were confirmed by DNA sequencing.

RESULTS: As many as 1000 copies per milliliter of wide-type plasmid were detected and nonspecific priming was excluded. In the 163 samples from patients treated with lamivudine, lamivudine-resistant mutations were detected in 51 samples.

CONCLUSION: This universal real-time PCR is a rapid and accurate method for quantification of YMDD mutants of HBV virus in lamivudine-treated patients and can be used to monitor lamivudine-resistant mutations before and during lamivudine therapy.

Key words: HBV; YMDD; Mutation; UT-PCR

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INTRODUCTION

It is estimated that approximately 350 million persons worldwide are chronically infected with hepatitis B virus (HBV)[1,2]. HBV is the leading cause of liver cirrhosis and hepatocellular carcinima (HCC) and is responsible for 60 - 80% of all HCC cases[2,3]. Treatment of HBV infection has been revolutionized by the introduction of antiviral drugs such as lamivudine. However, long-term monotherapy commonly does not result in complete suppression of viral replication and is associated with the emergence of resistant mutants[4-7]. Specific mutations that result in replacement of methionine (M) in tyrosine-methionine-aspartate-aspartate (YMDD) motif of HBV reverse transcriptase (rt) by valine (V), isoleucine (I), or serine (S) confer resistance to lamivudine[8].

Mutations leading to lamivudine resistance can be detected by direct sequencing of HBV DNA after PCR amplification of a selected part of the viral polymerase gene. However, this is expensive, laborious and time-consuming with low level of sensitivity, usually detecting 20% the total virus population[9]. Other molecular techniques, such as restriction fragment length polymorphism (RFLP)[10], 5' nuclease assay[11], line probe assay[12], peptide nucleic acid-mediated PCR clamping[13,14], and oligonucleotide chips[15], overcome some of the limitations of DNA sequencing, but they are also time-consuming and expensive. Recent studies using real-time PCR have obtained quantitative results but cannot avoid nonspecific amplification[16,17].

In the present study, we established a rapid and accurate method for the detection of lamivudine-resistant mutations in HBV based on real-time PCR using a universal template. Positive and negative controls were included to evaluate the quality of the assay.

MATERIALS AND METHODS

Patients and extraction of HBV DNA

Serum samples were collected from 163 patients with chronic HBV infection who had received lamivudine monotherapy for one to two years. The ethical committee of our hospital approved the study and oral consent was obtained from the patients. HBV DNA was extracted from serum samples using the QIAamp blood kit (Qiagen,

Table 1 Primers and probe used in universal RT-PCR

Primers or probe	Sequence
Reverse primers	
Reaction V	5′-CCCCCAATACCACATCATCC-3′
Reaction I	5′-CCCCCAATACCACATCATCA-3′
Reaction C	5′-CCCCCAATACCACATCATC-3′
Forward primers	5′-tgaggagcacgagacggaagt*ATACA*A CACCTGTATTCCCATCCCAT-3′
Probe	5′-FAM ACTTCCGTCTCGTGCTCCTCA TAMRA-3′

Chatsworth, California) as described elsewhere[11].

Primers and probe

The primers and probe used in this study are summarized in Table 1. Two different reverse primers that would selectively amplify the YVDD (rtM204V) and YIDD (rtM204I) quasispecies and a common reverse primer to a highly conserved sequence within the polymerase open reading frame were used to discriminate different mutants. The amplicon was detected by real-time PCR with a TaqMan probe that annealed to the universal template linked to the primers. A universal forward primer was used in the control for amplification of all kinds of quasispecies. A universal template was linked to each primer as described previously[18]. In brief, the universal template (UT) sequence with a size of 21 bp was attached to the 5' end of the primers specific to YMDD variants[17]. The UT probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and DABCYL quencher at the 3' end. During the annealing phase, the UT probe specifically annealed to the 5' end of the UT-PCR primer and the 3' end of the UT-PCR primer specifically annealed to the target sequence and was extended. Due to the 5' exonuclease activity of DNA polymerase, the hybridized UT probe was hydrolyzed, leading to the separation of the reporter moiety from the quencher moiety and the generation of a fluorescent signal. The signal could be detected by ABI 7000 real-time PCR system (Applied Biosystems Inc. California, USA).

Real-time PCR

The amplification was performed on ABI 7000 with a final volume of 50 μl by incubating the reaction mixture at 50 ℃ for 2 min and at 95 ℃ for 5 min, followed by 40 cycles of PCR amplification at 94 ℃ for 20 s and at 55 ℃ for 30 s. The reaction mixture contained the following components: 1×PCR buffer, 100 nmol/L primers and probes, 400 μmol/L each of dATP, dGTP, dCTP and dUTP, 1.5 units of hot-start Taq DNA polymerase, 0.2 units of Amperase uracil N-glycosylase (UNG), 3 mmol/L $MgCl_2$, 20 mmol/L KCl. The amplification was optimized in Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). Variants of YVDD and YIDD and total HBV virus were amplified in individual reactions (reactions V, I and C for YVDD, YIDD and HBV virus respectively). The positive results were determined by $\Delta Ct < 3.5$. ΔCt = Ct of reaction V or I - Ct of reaction C.

DNA sequencing

The DNA sequence of the domain C of HBV polymerase gene was analyzed as described elsewhere[11]. In brief, HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified and sequenced by ABI 310 sequencer (Applied Biosystems Inc., California, USA)

RESULTS

Specificity of primers and probe

Mismatched templates and primers (mutant primers to wild-type templates and vice versa) were used to validate this assay. Dilution series of 10^8, 10^6, 10^4 copies per milliliter were used and PCR was performed with the mismatched primers. Nonspecific priming to the alternate template was observed at the template concentrations of 10^6 and 10^8 copies per milliliter but not at 10^4 copies per milliliter. Similar to previous study[17], the degree of cross-priming was at least 4 logs. For exclusion of nonspecific priming, the positive results were determined by $\Delta Ct < 3.5$. ΔCt = Ct of reaction V or I - Ct of reaction C.

Sensitivity and limit of detection

Mixing experiments were performed to determine the sensitivity of the assay. Mutant and wild-type plasmids were mixed with the ratio of 0:1, 1:1, 1:10, 1:100 and 1:1000 at end concentrations of 10^5, 10^6, 10^7 and 10^8 copies per milliliter respectively (five replicates for each mixture). In all the mixtures, the detection limit of the assay was 1000 copies per milliliter.

Comparison of real-time UT-PCR and sequencing

The assay was evaluated using serum from 163 lamivudine-treated patients with chronic HBV infection. All the patients treated with lamivudine alone for one to two years were HBV DNA positive by real-time PCR. Real-time UT-PCR was performed with all the samples together with positive and negative controls. Fifty-one (31%) of 163 samples were detected with YVDD (Figure 1) or YIDD mutants (YMDD in 112, YIDD in 21, YVDD in 27 and YIDD + YVDD in 3). YMDD mutations were detected in 19% (14 of 73) of the patients treated with lamivudine for one year and in 40% (37 of 90) of the patients treated for two years. The results of real-time UT-PCR and sequencing were concordant in 15 randomly selected samples (Table 2).

DISCUSSION

A more accurate and rapid method than DNA sequencing and RFLP is needed to detect and quantify the YMDD mutants in sera of chronic HBV infection patients before and during lamivudine therapy. A real-time UT-PCR assay has been developed for detection of YMDD mutations in a large number of patients. The detection limit of this assay is 1000 copies per milliliter of mutants and 1% within a mixed virus population at a concentration of 10^5 copies per milliliter of serum. This real-time UT-PCR assay is more sensitive than DNA sequencing. In addition, the result of the assay can be easily determined by the difference between Ct values of the mutant and the control and no quantitative standards are needed.

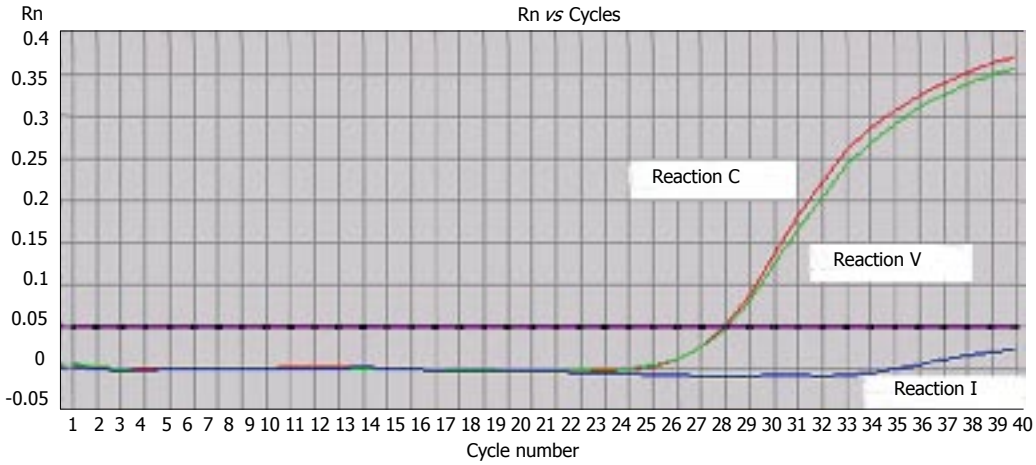

Figure 1 Detection of YMDD mutants using real-time UT-PCR. Three parallel reactions showed YVDD mutant.

Table 2 Comparison of results obtained by real-time PCR and sequencing

Samples	HBV DNA (Copies/mL)	Result of UT-PCR	Result of sequencing
1	2.49×10^5	YMDD	5′ -TATATGGATGAT-3′
2	4.73×10^7	YIDD	5′ -TATATTGATGAT-3′
3	6.10×10^3	YMDD	5′ -TATATGGATGAT-3′
4	6.24×10^6	YIDD	5′ -TATATTGATGAT-3′
5	2.98×10^9	YIDD	5′ -TATATTGATGAT-3′
6	8.13×10^6	YMDD	5′ -TATATGGATGAT-3′
7	1.35×10^4	YMDD	5′ -TATATGGATGAT-3′
8	3.57×10^3	YMDD	5′ -TATATGGATGAT-3′
9	2.74×10^6	YVDD	5′ -TATGTGGATGAT-3′
10	9.10×10^4	YVDD	5′ -TATGTGGATGAT-3′
11	7.02×10^4	YMDD	5′ -TATATGGATGAT-3′
12	5.18×10^5	YMDD	5′ -TATATGGATGAT-3′
13	3.51×10^3	YMDD	5′ -TATATGGATGAT-3′
14	8.04×10^4	YMDD	5′ -TATATGGATGAT-3′
15	4.29×10^6	YMDD	5′ -TATATGGATGAT-3′

Real-time UT-PCR has two major advantages, namely the universal probe can be used for many target sequences and it is less expensive compared with other sequence-specific fluorescent PCR techniques[18]. In the present study, different YMDD variants were detected using different primers specific to the target DNA and a probe specific to the universal template, showing that it is cost-effective and convenient to detect different sequences simultaneously.

The overall prevalence of the YMDD mutations in the patients treated with lamivudine for one and two years in this study was 19% (14 of 73) and 40% (37 of 90), respectively, which is consistent with prevalence reported by previous studies[4,5,19]. Mixture of YIDD and YVDD variants was observed in three samples.

In the present study, the primer used to detect rtM204I was specific to sequence ATT. Sequences of ATG and ATC could not be detected by this assay. However, this could be improved by adding two reverse primers specific to ATG and ATC into the reaction used to detect YIDD (data not shown).

Several real-time PCR methods are availabe for the detection of YMDD mutations[16,17,20,21]. Selective primers have been used[16,17]. One of the most important advantages of this study is that the result of mutants could be easily determined by the difference between Ct values of the mutant and the control without additional quantitative standards. Furthermore, nonspecific priming could be excluded by the cut-off of $\Delta Ct < 3.5$ because the degree of cross-priming was at least 4 logs in this study and elsewhere[17]. The impact of the intra-assay variability is reduced in this assay. However, this arbitrarily determined cut-off value reduces the sensitivity of the assay. The real-time UT-PCR is cost effective and convenient for large scale screening in clinical practices.

In conclusion, the real-time PCR assay established in this study is a rapid and accurate tool for detection of lamivudine-resistant mutations before and during lamivudine therapy.

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RAPID COMMUNICATION

Functional evaluation of a new bioartificial liver system *in vivo* and *in vivo*

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Abstract

AIM: To evaluate the functions of a new bioartificial liver (BAL) system *in vitro* and *in vitro*.

MEHTODS: The BAL system was configurated by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor. In the experiments of BAL *in vitro*, the levels of alanine aminotransferase (ALT), total bilirubin (TB), and albumin (ALB) in the circulating hepatocyte suspension and RPMI-1640 medium were determined during 6 h of circulation in the BAL device. In the experiments of BAL *in vitro*, acute liver failure (ALF) model in canine was induced by an end-side portocaval shunt combined with common bile duct ligation and transaction. Blood ALT, TB and ammonia levels of ALF in canines were determined before and after BAL treatment.

RESULTS: During 6 h of circulation *in vitro*, there was no significant change of ALT, whereas the TB and ALB levels gradually increased with time both in the hepatocyte suspension and in RPMI-1640 medium. In the BAL treatment group, blood ALT, TB and ammonia levels of ALF in canines decreased significantly.

CONCLUSION: The new BAL system has the ability to perform liver functions and can be used to treat ALF.

Key words: Bioartificial liver; Liver transplantation; Porcine hepatocyte

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INTRODUCTION

Acute hepatic failure (ALF) is a disease with a high mortality. Although significant improvements have been achieved in critical care therapy, the mortality rate of ALF patients is about 80%. In many patients liver failure is reversible and if short-term liver support is provided, the liver may regenerate. Survivors may recover full liver function and a normal life expectancy. For many years, liver transplantation has been the only curative treatment for this condition, subjecting many patients to replacement of a potentially self-regenerating organ, with the lifetime danger of immunosuppression and its attendant complications, such as malignancy. Additionally, because of the shortage of livers available for transplantation, many patients die before a transplant can be performed, or are too ill to undergo surgery at the time a liver becomes available. The survival of patients excluded from liver transplantation or those with potentially reversible ALF might be improved with temporary artificial liver support. Among a variety of liver support therapies, bioartificial liver (BAL) therapy is marked as the most promising solution. Some BAL devices are under trials in animals[1-4] and human beings[5-8]. The major problem is how to configure a BAL system. To attain enhanced efficacy of liver support, several BAL configurations have been proposed. The long-term maintenance of hepatocyte function is crucial for any BAL system. Some systems use hepatocytes attached to microcarrier beads or multicellular spheroid aggregates[9-11]. In regard to bioreactor design, various bioreactor configurations have been proposed that employ glass plates, hollow fiber membranes, encapsulation in biological matrices, and 3-dimensional carrier materials[12-15].

In this study, porcine hepatocytes were isolated by in situ recirculating collagenase perfusion method and cultured with spinner culture method to prepare hepatocyte spheroids. A new BAL system was configured by inoculating hepatocyte spheroids into cell circuit of a BIOLIV A3A hollow fiber bioreactor and the functions of the BAL system were evaluated *in vitro* as previously described[16,17]. At same time, the efficacy evaluation of the

BAL system was conducted in a model of ALF canines induced by an end-side portocaval shunt combined with common bile duct ligation and transaction.

MATERIALS AND METHODS

Materials

Chinese experimental male and female miniature pigs ($n=13$) weighing 2.5-4.0 kg, were provided by Beijing Agricultural University. The pigs were allowed free access to water and fasted for 12 h before the experiment. Hybrid male and female dogs ($n=16$) weighing 10-15 kg, were provided by the Experimental Animal Center of Drum Tower Hospital. Collagenase IV, RPMI-1640 medium, hepatocyte growth factor, nerve growth factor, and N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid (HEPES) were from Gibco BRL Life Technologies, Grand Island, NY, USA. Insulin, glucagon, transferrin, linoleic acid, glutamine, bovine serum albumin, Na_2SeO_3, $CuSO_4$•$5H_20$, $ZnSO_4$•$7H_2O$, poly (2-hydroxyethyl methacrylate) (poly-HEMA), penicillin, and streptomycin were from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. BIOLIV A3A hollow fiber cartridges with abnormal molecular weight cut-off of 70 Ka, a pore size of 200 nm, and a surface area of 1.06 m^2, were provided by Cell Biotech Ltd, Hong Kong, China.

Hepatocyte isolation and culture

Porcine hepatocytes were isolated by *in situ* recirculating collagenase perfusion method[18]. Hepatocytes were inoculated at a density of 5×10^7/mL in serum-free RPMI-1640 medium supplemented with 200 μg/L hydrocortisone, 1 mg/L hepatocyte growth factor, 10 μg/L nerve growth insulin, 4 μg/L glucagon, 6.25 mg/L transferrin, 10 mg/L linoleic acid, 2 mmol/L glutamine, 0.5 g/L bovine serum albumin, 3 nmol/L Na_2SeO_3, 0.1 μmol/L $CuSO_4$•$5H_2O$, 50 pmol/L $ZnSO_4$•$7H_2O$, 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 200 mg/L cefoperazone sodium, 100 000 U/L penicillin, and 100 mg/L streptomycin[19]. To inhibit cell attachment and induce the formation of cell spheroids in suspension, 250 mL bottles were coated with poly-HEMA. About 100 mL of hepatocyte suspension was placed in each bottle. The bottles were placed in an incubator (50 mL/L CO_2, 37 ℃) for 20 h and slowly rotated (12 revolutions/h) as previously described[20, 21].

Configuration of BAL system

The BAL system was configured by inoculating the hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor (BIOLIV A3A, Cell Biotech Limited, HK, China). The total cell circuit volume was 250 mL. Hepatocyte spheroids in serum-free RPMI-1640 medium containing 1.0×10^{10} primary porcine hepatocytes were infused into the outer space of the hollow fibers and the medium was circulated at 20 mL/min with continuous O_2 input (2 L/min) (Figure 1). The bioreactor was kept at 37.5 ℃ in an incubator[16].

Canine ALF model

Canine ALF model was induced by end-side portocaval shunt combined with common bile duct ligation and transaction as previously described[22]. All canines developed ALF 14 d after operation.

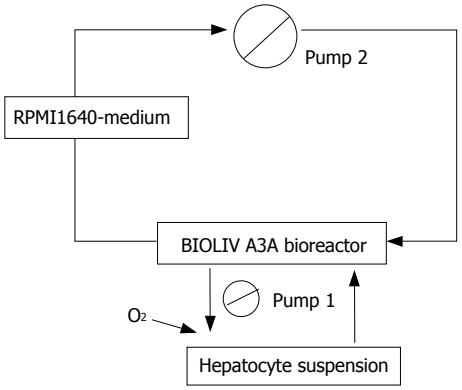

Figure 1 Schematic picture of the BAL system.

Determination of functions of BAL system in vitro

Independent experiments were performed 5 times with the BAL device. The RMPI-1640 medium flowed through the lumen of the hollow fibers at a rate of 30 mL/min. Samples of the hepatocyte suspension and RMPI-1640 medium were obtained at 2 h intervals during 6 h of circulation. Changes of alanine aminotransferase (ALT), total bilirubin (TB), and albumin (ALB) levels in the circulating hepatocytes and medium were determined with an automatic biochemical analyzer (Hitachi 7600, Japan).

Determination of functions of BAL system in vivo

Independent experiments were performed 8 times with the BAL device. Hemoperfusion was performed from femoral artery to femoral vein through the bioreactor at a rate of 30 mL/min. The ALF model canines were divided into two groups: the BAL treatment group ($n=8$) consisiting of canine ALF models was perfused using the above serum-free medium inoculated with porcine hepatocyte spheroids for 6 h, and the control group ($n=8$) consisting of the canine ALF model was perfused using the above serum-free medium without porcine hepatocytes for 6 h.

Blood samples were obtained before (pre-circulation) and after the treatment (post-circulation). Blood ALT and TB were determined with an automatic biochemical analyzer (Hitachi 7600, Japan). Blood ammonia was determined with a biochemical analyzer (DT60II, Johnson and Johnson Medical Ltd., USA).

Statistical analysis

Results were expressed as mean±SD. Statistical differences were evaluated by analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

ALT, TB and ALB levels in hepatocyte suspensions and RPMI-1640 media during circulation

The average viability of the isolated hepatocytes was 97% by trypan blue exclusion. The formation of hepatocyte spheroids was observed after a culture of 20 h. In the hepatocyte suspensions, there were no significant changes of ALT during 6 h of circulation, whereas the TB and

Table 1 Changes of ALT, TBi and ALB in hepatocyte suspensions and RPMI-1640 media during circulation (mean±SD)

Portion	Time (h)	Biochemical parameters		
		ALT(U/L)	TB(μmol/L)	ALB(g/L)
Hepatocyte suspensions (n=5)	0	13.7±3.5	3.5±2.4	2.8±1.5
	2	15.2±4.5	5.0±1.6[c]	3.1±1.6
	4	17.9±4.3	6.2±1.8[c]	3.6±0.9[c]
	6	18.4±3.2	8.4±2.6[c]	3.8±1.2[c]
RPMI1640 media (n=5)	0	0.8±0.2[a]	0.0±0.0[c]	0.5±0.1[a]
	2	1.5±0.5[a]	4.8±1.3[c]	1.8±0.3[ac]
	4	1.3±0.3[a]	5.7±1.6[c]	2.5±0.2[ac]
	6	1.4±1.0[a]	6.8±2.5[c]	2.8±0.3[ac]

[a]P<0.05 *vs* hepatocyte suspensions at the same circulation time, [c]P<0.05 *vs* 0 h.

Table 2 Changes of biochemical parameters before and after circulation in two groups (mean±SD)

Group	Biochemical parameters		
	ALT(U/L)	TB(μmol/L)	NH3(μmol/L)
BAL group (n=8)			
Pre-model	18.2±12.6	2.8±1.2	1.0±0.0
Pre-circulation	325.8±54.7[c]	72.6±23.5[c]	132.2±28.3[c]
Post-circulation	212.3±42.3[ac]	33.4±22.2[ac]	33.2±21.7[ac]
Control group (n=8)			
Pre-model	16.6±38.3	2.6±1.9	1.0±0.0
Pre-circulation	300.2±52.6[c]	74.3±23.7[c]	126.3±30.4[c]
Post-circulation	280.1±45.9[c]	65.5±35.6[c]	100.3±13.5[c]

[a]P<0.05 *vs* pre-circulation, [c]P<0.05 *vs* pre-model.

ALB levels increased gradually (P<0.05). The pre-circulation levels of ALT, TB and ALB were lower in the RPMI-1640 media than in hepatocyte suspensions (P<0.05). During 6 h of circulation, there was no significant change of ALT, whereas the TB and ALB levels gradually increased (P<0.05). At 2, 4, and 6 h of circulation, there were no significant differences of TB levels in RPMI-1640 media and hepatocyte suspensions, but the ALB concentration was lower in RPMI- 1640 media than in hepatocyte suspensions at all times (P<0.05, Table 1).

Changes of biochemical parameters in ALF canines after BAL circulation

The two groups were comparable in terms of body weight (12.6±2.2 kg in BAL group and 13.2±2.1 kg in controls) and biochemical parameters before circulation. The parameters after circulation are shown in Table 2. In the BAL treatment group, blood ALT, TB and ammonia levels significantly decreased from 455.1±225.2 U/L, 74.6±25.4 μmol/L and 131.2±28.3 μmol/L before circulation to 273.3±151.3 U/L, 33.1±11.7 μmol/L and 33.4±21.7 μmol/L after circulation. While in the control group, there were no significant differences in blood ALT, TB and ammonia levels between pre-circulation and post-circulation, though these indices were declined slightly (P>0.05). Blood ALT, TB and ammonia levels were lower in BAL group than in control group after circulation (P<0.05). The viability of hepatocytes was about 90% at the end of BAL treatment.

Survival rate of ALF canines

The survival rates of ALF canines 7 d after treatment were 100% (8/8) and 62.5% (5/8) in BAL group and control group respectively with no significant difference (P>0.05).

Adverse reaction related to BAL treatment

The ALF canines had no apparent signs of toxicity during and after BAL treatment.

DISCUSSION

Treatment of ALF is a formidable clinical challenge. Currently, liver transplantation is the most effective therapy. Although advances have been achieved in transplantation techniques, donor organ shortage remains a serious problem. The mortality of ALF is still high. However, many ALF patients can recover through liver regeneration after short-term liver support, which has prompted the design of an extracorporeal liver support device to "bridge" patients over until they either recover or receive a liver transplant. Among these devices, the BAL system is most promising. Various types of hepatocytes are inserted and different device designs have been proposed [23-25]. The BAL system differs from non-biologic artificial liver devices in the synthesis of essential metabolites and the selective removal of toxic substances, which are carried out by the cultured hepatocytes. An ideal BAL system can provide all the hepatic functions.

In this study, we configurated a new BAL system by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor and evaluated its functions *in vitro* and *in vivo*.

Nagaki *et al*[26] demonstrated that primary hepatocytes are superior to transformed hepatocytes as a source of biotransformation functions in the BAL system. The BAL system we developed uses viable porcine hepatocytes in the bioreactor to exert liver functions.

Since 10-30% of hepatocytes in the normal human liver are needed to maintain normal hepatic functions[27], Matsmura *et al*[28] proposed that a BAL requires 150-450 g of liver. Our BAL device contains 1.0×10^{10} hepatocytes to meet the need of patients with ALF. The liver of an adult canine weighs about 335 kg and contains 3.35×10^{9} hepatocytes. Therefore, the BAL device could be used in this experiment.

It has been reported that cell-cell interaction has an important role in maintaining the viability and the functions of hepatocytes[29]. In the present study, hepatocytes were incubated in serum-free medium and poly-HEMA-coated bottles by a continuous rotational method in order to restrict their attachment to the wall and promote the formation of hepatocyte spheroids. This method could not only facilitate cell-cell interaction and maintain cell functions, but it also meet the requirement of high-density culture in BA, thus reducing the possibility of immunoreaction by using serum-free medium with hormone and various growth factors[19].

The ideal bioreactor should provide a good environment for growth and metabolism of hepatocytes

as well as the efficient exchange of substances. At present, the most commonly used device is a hollow fiber bioreactor with many small hollow fibers made from semipermeable membranes. The device has two independent compartments that are separated by hollow fiber semipermeable membranes. The intratubular space is used to perfuse blood, while the extratubular space is used to culture hepatocytes. The blood or plasma of patients flows into the bioreactor, exchanging substances with hepatocytes through the semipermeable membranes. The membranes also provide immuno-isolation. In view of the molecular weight of albumin (68 ku), a hollow fiber membrane with 200 nm pore size and a MWCO of 70 ku was chosen for this study. The membrane allows passage of some relatively small molecules such as albumin, but restricts the passage of lymph cells and high molecular weight proteins. Blood-borne toxins and metabolic precursors are free to diffuse across the membrane to hepatocytes where they are metabolized. Metabolic products and detoxified toxins are free to diffuse back across the membrane to the flowing blood. Hepatocytes in the bioreactor also synthesize molecules (proteins, coagulation factors, enzymes, carrier molecules) that pass across the hollow fiber cartridge into blood. Continuous O_2 input could ensure hepatocytes to gain sufficient oxygen. Therefore, our BAL device has such a novelty as it uses hepatocyte spheroids and serum-free medium compared to previously reported BAL[13-16].

Experiments of BAL *in vitro* showed that TB and ALB levels in hepatocyte suspensions and in RPMI-1640 media increased during 6 h of circulation. There were no significant differences of TB levels in RPMI-1640 medium and hepatocytes suspensions after 2 h of circulation. ALB level was lower in RPMI-1640 samples than in hepatocyte suspensions at all periods of circulation. ALB levels in RPMI-1640 medium and hepatocyte suspension averaged 2.8 g/L and 3.8 g/L, respectively, after 6 h of circulation. During the 6 h of circulation, there were no significant changes of ALT levels in RPMI-1640 medium or hepatocyte suspensions. ALT level was lower in RPMI-1640 medium than in hepatocyte suspensions at all time points. Bilirubin and albumin could readily cross the semi-permeable membrane because of their low molecular weights. The results indicate that albumin synthesized by hepatocytes in the BAL system can cross into the circulating stream in the intratubular space of hollow fibers.

In the experiments of BAL *in vivo*, blood ALT, TBi and ammonia levels significantly decreased after 6 h of circulation in the BAL group. There were no significant differences in blood ALT, TB and ammonia between pre-circulation and post-circulation in the control group, though these indices were slightly decreased after circulation. These results suggest that our BAL system has the potential not only to protect against hepatocyte destruction but also to dilute excess ALT in the systemic serum, which is consistent with the previous report[30]. The survival rate was higher in BAL group than in control group, but there was no statistical significance, which may be related to the quantity of the samples. The viability of hepatocytes was about 90% at the end of BAL treatment, indicating that our BAL device has its advantages.

In conclusion, the new BAL system configurated in this research has a certain liver support effect and appears to have potential advantages for its clinical use in patients with ALF.

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CASE REPORT

Small bowel adenocarcinoma in Crohn's disease: A case report and review of literature

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Abstract

Small bowel adenocarcinomas are remarkable for their rarity, difficult diagnosis and poor prognosis. Here we report an unusual case of a 33-year-old patient in whom infiltrative adenocarcinoma of the small bowel was diagnosed after a 10-year history of Crohn's disease. In most previously reported cases, detection of Crohn's disease was subsequent to that of carcinoma of the small bowel or the patients involved had an even longer history of the disease. Our literature review suggests that the risk of small bowel adenocarcinoma is higher in patients with Crohn's disease than in the overall population. We present details on epidemiology as well as clinical and diagnostic aspects of this rare disease entity.

Key words: Crohn's disease, Small bowel adenocarcinoma, Case report

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INTRODUCTION

The incidence of inflammatory bowel disease (IBD) is increasing since World War II with levels around 6/100 000 for Crohn's disease (CD) and 15 to 20/100 000 for ulcerative colitis, a marked rise in the age group between twenty to forty years for both entities[1].

The ulcerations occur primarily in the small and large intestines, but may appear anywhere in the digestive tract from the mouth to the anus. Common symptoms of CD are abdominal pain, often in the lower right area, and diarrhoea, but rectal bleeding, weight loss and fever may also appear. Children with CD may suffer stunted growth and delayed development. The severity of the symptoms fluctuates erratically over time. Patients experience flare-ups between intervals of remission or reduced symptoms. The causes of this disease have not been identified yet; but both genetic factors that induce continued abnormal activation of the immune system[2,3] and environmental triggers, like Mycobacterium avium subspecies paratuberculosis[4], are likely to be involved.

Oral or topical preparations of 5-aminosalicylates represent first line therapy, and steroids and azathioprine are used in severe cases; metronidazole and TNF-alpha antibodies are used in fistulating disease. Fifty to seventy percent of Crohn's patients undergo surgery for progression of disease indicated by the presence of fistulas, tumor in the abdomen and development of ileus.

IBD is linked to large and small bowel carcinoma, especially to adenocarcinomas[5-7]. In the last twenty years, colorectal cancer has become the fourth most common cancer worldwide, and in Europe colorectal cancer represents the second most frequent cause of death from any cancer in men[8]. Even though only about 1% of all colorectal cancers is associated with ulcerative colitis or Crohn's colitis, the risk of colorectal cancer for any ulcerative colitis patient is found to be 2% at 10, 8% at 20 and 18% at 30 years, duration of disease, regardless of disease extent[9,10].

Small bowel carcinomas are uncommon representing only 1% to 5% of all gastrointestinal tract malignancies. The first observations suggested that particularly surgically bypassed bowel segments were exposed to high risk of small bowel adenocarcinoma[11,12]. However, the risk of small bowel carcinoma in patients with CD is much higher, being up to 60-fold of that in the general population[13, 14].

Neither clear risk factors nor methods for early diagnosis have been established by the few studies on this distinctly uncommon complication within this rare disease. Here we report on a patient in whom infiltrative adenocarcinoma of the ileum was diagnosed after a 10-year history of CD and also discuss possible risk factors, symptoms, feasible diagnostic approaches and treatment options on the basis of published reports.

CASE REPORT

A 33-year-old man presented in 1992 with recurrent pyrexia and abdominal pain but no diarrhoea. Enteroclysis

was performed and a diagnosis of ileal CD was made. His family history was negative for this disease. For persistent abdominal pain under therapy with 5-aminosalicylates, he was put on corticosteroids (prednisone, 12.5 mg daily). In the following four years the patient experienced repeated episodes of abdominal pain without diarrhoea.

Two years later, an abdominal ultrasound performed for reassessment of the disease, showed a thick intestinal loop from the left to the right upper abdomen. Unfortunately no further diagnostic or therapeutic steps were undertaken at that time.

In 2002, the patient, complaining of increasing abdominal pain, underwent ileocolonoscopy, which yielded no suspicious macroscopic or histopathological findings. Blood tests showed mild anaemia, signs of malabsorption (low proteins, phosphorous and iron deficiency) but none for inflammation. The recommended enteroclysis of the small bowel was not performed. In order to reduce corticosteroids, therapy with azathioprine was initiated (2 mg/kg weight/ per day).

In June 2003 he presented again with abdominal pain, vomiting and distended abdomen. Enteroclysis showed a dilated intestinal loop of the ileum, a pseudotumor in the right abdomen and two stenotic areas, one of which was high-grade and located in the right upper abdomen (Figure 1). Prednisone was increased to 50 mg/day, in addition to metronidazole and ciprofloxacine. Three weeks later, with a deterioration of obstructive symptoms, the patient underwent surgery.

The surgical specimens consisted of a 45 cm and a 7 cm long resected segments. Macroscopic examination showed high-grade inflammatory alteration of the ileum along a 20 cm segment and two upstream high-grade stenoses. The external surface of the diseased segment appeared brownish discoloured with adhering connective tissue. The internal surface of the ileum showed discontinuous mucous membrane, streaks of ulcers and whitened swelling of the bowel wall. Some foci had a 'cobblestone' appearance. The 7 cm long segment had a stenotic 'sandglass' formation.

Extensive histopathological examination in the first stricture revealed a poorly differentiated adenocarcinoma infiltrating the serosa, incipient infiltration of the mesenteric fat and lymphangiosis carcinomatosa as well as a metastatic peritoneal range. Tumor had seeded seven out of eleven lymph nodes examined. The resected specimen also showed adenoma's dysplasia adjacent to the carcinoma. Extended antral metaplastic lesions within agglutinated villi up to one margin were found.

The second specimen revealed proper margins of resection. In the centre of the macroscopic stricture was another focus of the adenocarcinoma. In addition, moderately florid inflammatory infiltrates with acute erosions were detected, compatible with Crohn's disease.

Immunohistochemistry was negative for NSE and chromogranin, only a few tumor cells were slightly positive for synaptophysin. CEA level determined only after surgery was 54.2 ng/ml (normal <50 ng/ml).

The patient recovered well from surgery and then underwent chemotherapy according to Folfox IV–scheme with oxaliplatin, 5-fluorouracil and leucovorin in six cycles.

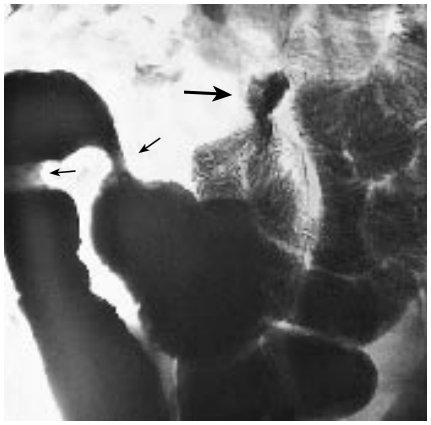

Figure 1 Enteroclysis: dilated intestinal loop of the ileum; altered Crohn's area (big arrow), one high grade and one low grade tumor stenosis in the right upper abdomen (two small arrows).

The patient tolerated the therapy quite well.

The computed tomography (CAT-scan) in October 2004 revealed up to two centimetres enlarged mesenteric and up to one centimetre enlarged retroperitoneal lymph nodes, without any further evidence for metastases. In November 2004 the patient presented with headache, vertigo and ambiopia and was diagnosed with meningeosis carcinomatosa. Intrathecal chemotherapy with methotrexate was started. He is now undergoing intrathecal chemotherapy with sustained-released cytarabine.

DISCUSSION

According to Parkin *et al* the age-standardized incidence of small intestine cancer (ICD-10 C17) ranges from 0.2 to 2.4 for males and from 0.2 to 1.8 for females worldwide[15]. The 'Statistik Austria' National Registry has 1384 documented cases of small intestine cancer between 1983-2000[16].

The association of CD with small bowel carcinoma is uncommon and to date only about 130 cases of small bowel carcinomas in patients with CD have been reported in the literature since the first description of this disease entity in 1956[17]. Cases and studies published in the last few decades, however, bear out from a 12-fold to an over 60-fold increased risk of small bowel cancer in CD[13, 14, 18]. This is in contrast to publications that still emphasize the popular position that CD is primarily associated with carcinoma of the colon. Adenocarcinoma is the most common forms of all small bowel malignancy and there appears to be an increased risk for developing ileal carcinoma in CD patients[19-23].

Most of ileal carcinoma in CD are located in strictures[11,24,25] and are often incidentally diagnosed postoperatively as in our case report. The occult carcinomas in strictures pose a challenge to diagnostic investigations using conventional modalities such as small bowel series and upper and lower gastrointestinal endoscopy. CT is now considered the imaging modality of choice[26-28], and a fat density target sign in CT[29] is also getting greater attention as reliable marker for diagnosing CD or even small bowel carcinoma. Abdominal MRI[30], double-contrast enteroclysis[31] and endoscopy[32], especially video wireless capsule endoscopy[33,34], are promising new diagnostic tools.

Other interesting characteristics are adjacent metapla-

sia, adenoma and epithelial dysplasia[35-38], which underline the importance of further research with respect to sequence-dysplasia in ileal adenocarcinomas in relation to Crohn's disease.

Risk factors for small intestine carcinoma in CD are chronic active course with stricture, fistulas and onset of disease before the age of 30 years[25,39,40]. Further reported risk factors are: early onset, age between 30 and 50 years, male sex and smoking[13,14,41-45]. Therapy of CD with corticosteroids, azathioprine and TNF-alpha antibodies are also considered as potential risk factors. It has been suggested in previous studies that azathioprine, administered mostly combined with steroids to patients with a long history of Crohn's disease, frequent recurrence or those allergic to 5-aminosalicylates has a carcinogenic potential[14,46-49]. In the light of these reports, it is interesting to raise the question whether azathioprine therapy initiated in our patient after normal ileocolonoscopy 18 mo before diagnosis of carcinoma might have contributed to the acceleration of the malignant disease. In contrast to azathioprine, 5-aminosalicylates are considered preventive against the development of large and small bowel adenocarcinoma in inflammatory bowel disease[13,23,50-53]. Mesalazine is now used for treating light to moderate Crohn's colitis and ileitis postoperatively to maintain remissions, but its potential to prevent malignancy needs to be evaluated. TNF-alpha antibodies, also mostly combined with immunosuppression, are used in patients with refractory, steroid-dependent and fistulating CD. There is a theoretical risk of increased rate of malignancies due to antagonism of TNF-alpha, but to date there is no clear proof of such an effect[54-56].

Prognosis of small bowel adenocarcinoma is poor, and the mortality at 1 and 2 years ranges from 30-60% dependent on the stage of the cancer[13,21,57-59].

Further prognostic factors are based on histologic findings such as positive surgical margins, poor differentiation, depth of tumor invasion, positive lymph nodes and extramural venous spread in small bowel adenocarcinoma[60].

CONCLUSION

Small bowel adenocarcinoma in Crohn's disease is rare and preoperative diagnosis continues to present challenges. Long-term prognosis is poor - all the more it is important to be vigilant. Patients with increased risk are those with longstanding complicated CD presenting with a 'de novo' clinical picture of obstruction. Male patients, in particular smokers, are considered to be at increased risk. Since the diagnosis is difficult to make, attending physicians must exercise a high level of clinical suspicion for operative cure. The preventive potential of 5-ASA in adenocarcinoma of the colon suggests that this drug should be preferred to azathioprine in patients to maintain remission.

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CASE REPORT

Bile duct hamartomas (von Mayenburg complexes) mimicking liver metastases from bile duct cancer: MRC findings

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Abstract

We present a case of a 72-year-old man with a common bile duct cancer, who was initially believed to have multiple liver metastases based on computed tomography findings, and in whom magnetic resonance cholangiography (MRC) revealed a diagnosis of bile duct hamartomas. At exploration for pancreaticoduodenectomy, liver palpation revealed disseminated nodules at the surface of the liver. These nodules showed gray-white nodular lesions of about 0.5 cm in diameter scattered on the surface of both liver lobes, which were looked like multiple liver metastases from bile duct cancer. Frozen section of the liver biopsy disclosed multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma characteristics of multiple bile duct hamartomas (BDHs). Only two reports have described the MRC features of bile duct hamartomas. Of all imaging procedures, MRC provides the most relevant features for the imaging diagnosis of bile duct hamartomas.

Key words: Bile duct hamartoma; Magnetic resonance cholangiography; Multiple liver metastases

Nagano Y, Matsuo K, Gorai K, Sugimori K, Kunisaki C, Ike H, Tanaka K, Imada T, Shimada H. Bile duct hamartomas (von Mayenburg complexes) mimicking liver metastases from bile duct cancer: MRC findings. *World J Gastroenterol* 2006; 12(8): 1321-1323

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INTRODUCTION

Multiple bile duct hamartomas (BDHs) are small benign neoplasms of the liver, pathologically containing cystic dilated bile ducts embedded in a fibrous stroma[1]. In almost all cases, these patients are believed to have multiple liver metastases following initial imaging[2], and diagnosis of BDHs is frequently not considered until after liver biopsy[3]. It is difficult to be certain that all of the lesions identified radiologically, such as ultrasonography (US), computed tomography (CT), are definitely BDHs, necessitating referral for open biopsy to exclude liver metastases. Although the magnetic resonance imaging (MRI) appearance has been reported in 14 cases previously, only two reports have been published for BDHs with illustration of magnetic resonance cholangiography (MRC) features[3,4]. In this report, MRC may be useful to allow accurate characterization of the liver lesions, thereby permitting the diagnosis of von Mayenburg complexes to be made with careful analysis of the MRI, and MRC images.

CASE REPORT

A 72-year-old man was admitted to our hospital in September 2003 with a history of epigastralgia. The physical examination was unremarkable. His serum levels of hepatobiliary enzymes were increased, however, that of total bilirubin (T Bil, 0.7 μmol/L) and direct bilirubin (D Bil, 0.3 μmol/L) were normal. Tumor markers, carbohydrate antigen19-9 (CA19-9) and carcinoembryonic antigen (CEA) were almost within normal range. Ultrasonography showed a dilatation of the common bile duct (CBD) and intrahepatic bile duct. Endoscopic retrograde cholangiography (ERC) showed the irregular stenosis of the lower CBD (1.5 cm in length) and upper part of CBD was dilated. Biopsied specimens from this tumor histologically revealed adenocarcinomas. On the basis of these findings, we diagnosed this tumor as a carcinoma of the CBD. Additionally, multiple, very small and hypodense nodules with ring-like enhancement in both hepatic lobes were found on early phase of enhanced CT (Figure 1).

The lesions were too small to characterize accurately by either modality, but were consistent with disseminated liver metastases. On MRC images (TR 16 000 ms TE 107.8 ms), multiple irregularly delineated hyperintense nodules were seen, not communicating with the biliary tree (Figure 2). These findings suggested the bile duct hamartoma (BDH) (von Mayenburg complexes).

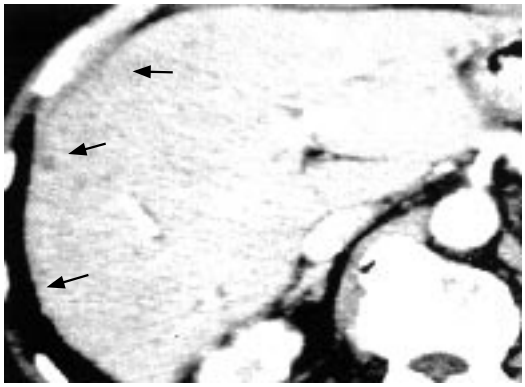

Figure 1 Contrast-enhanced computed tomography showing multiple, very small and hypodense nodules with ring-like enhancement (0.5 cm in diameter) in both hepatic lobes.

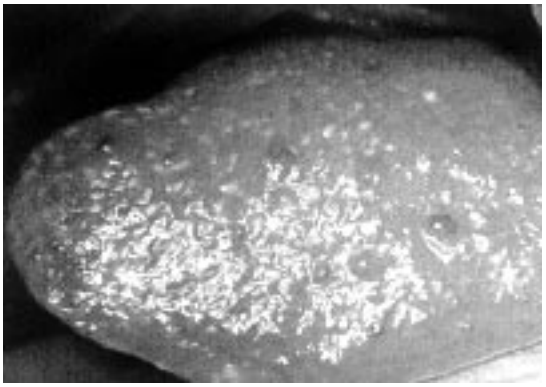

Figure 3 Intraoperative macroscopic view showing gray-white nodular lesions (about 0.5 cm in diameter) scattered on the surface of the both liver lobes.

Figure 2 MR cholangiography (TR 16 000 ms TE 107.8 ms) showing multiple irregularly delineated hyperintense nodules, not communicating with the biliary tree.

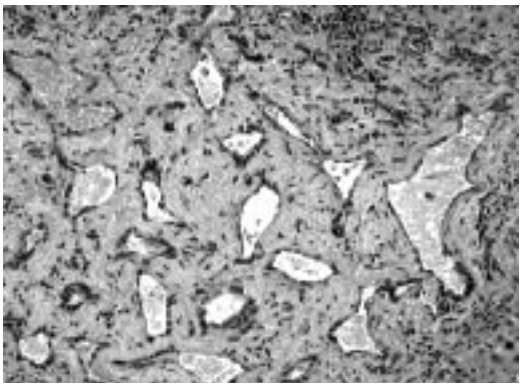

Figure 4 Microscopic view of liver biopsy showing multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma (Hematoxylin and eosin, x100).

At exploration for pancreatoduodenectomy, liver palpation revealed disseminated nodules at the surface of the liver. These nodules showed gray-white nodular lesions (about 0.5 cm in diameter) scattered on the surface of the both liver lobes, which were looked like multiple liver metastases from the bile duct cancer (Figure 3). Frozen section of the liver biopsy disclosed multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma characteristics of BDHs with no evidence of malignancy (Figure 4).

DISCUSSION

On gross pathology, Von Meyenburg complexes of the liver present as gray-white nodular lesions scattered throughout the liver parenchyma, usually measuring between 0.1 and 1.0 cm in diameter[3]. In this case, enhanced CT showed low density small nodules with ring-like enhancement, therefore multiple liver metastases were suspected. The major differential diagnoses, especially on frozen section, are intrahepatic cholangiocarcinoma and metastatic adenocarcinoma. The clinical importance of bile duct hamartomas is its ability to mimic metastatic disease of the liver. Chamlou *et al*[5] reported that, in the pancreatic cancer patient, the biliary duct hamartoma was erroneously confused intraoperatively with hepatic metastasis on frozen sections, and the pancreatoduodenectomy was therefore not performed at the first operation. Because of prevalence of biliary hamartomas, these entities should be considered as possibilities when liver lesions are noted intraoperatively.

MRI can aid in the differentiation of liver cysts and liver metastases by identifying the hyperintense signal from liver cysts on heavily T2-weighted sequences. To our best of knowledge, only two reports have described the MRC features of BDHs[3,4]. MRC can be extremely important in evaluating anomalies of bile ducts. Additionally, it allows differentiation between dilated biliary ducts, saccular dilatation of biliary duct system (Caroli's diseases), and periductal non-communicating cystic lesion, including polycystic disease, bile duct hamartomas and multiple abscess. In previous reports, MRC images showed multiple hyperintense cystic lesions of small diameter scattered in both liver lobes with normal appearance of the intrahepatic and extrahepatic bile ducts. No communication between the hamartomas and the intrahepatic bile ducts was observed. In this case, MRC findings are similar to the previous reports and compatible with BDHs. MRC offers the ability to characterize small liver lesions characteristics of BDH.

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Meetings

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Adelaide
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April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
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February 24-26, 2006
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2	4 days	four days	In text narration
3	day	d	After Arabic numerals
4	Four d	Four days	At the beginning of a sentence
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6	2 hs	2 h	After Arabic numerals
7	hr, hrs,	h	After Arabic numerals
8	10 seconds	10 s	After Arabic numerals
9	10 year	10 years	In text narration
10	Ten yr	Ten years	At the beginning of a sentence
11	0,1,2 years	0,1,2 yr	In figures and tables
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13	4 weeks	4 wk	
14	Four wk	Four weeks	At the beginning of a sentence
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21	1 M	1 mol/L	
22	10 μM	10 μmol/L	
23	1N HCl	1 mol/L HCl	
24	1N H_2SO_4	0.5 mol/L H_2SO_4	
25	4rd edition	4^{th} edition	
26	15 year experience	15- year experience	
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28	25 $g.kg^{-1}/d^{-1}$	25 g/(kg·d) or 25 g/kg per day	
29	6900	6 900	
30	1000 rpm	1 000 r/min	
31	sec	s	After Arabic numerals
32	1 pg $\cdot L^{-1}$	1 pg/L	
33	10 kilograms	10 kg	
34	13 000 rpm	13 000 *g*	High speed; *g* should be in italic and suitable conversion.
35	1000 *g*	1 000 r/min	Low speed. *g* cannot be used.
36	Gene bank	GenBank	International classified genetic materials collection bank
37	Ten L	Ten liters	At the beginning of a sentence
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39	umol	μmol	
40	30 sec	30 s	
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45	$^{*}F=9.87$, $^{\S}F=25.9$, $^{\#}F=67.4$	$^{1}F=9.87$,$^{2}F=25.9$, $^{3}F=67.4$	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	$10\times10\times10cm^3$	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$x\pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	*im*	im	intramuscular injection
70	*iv*	iv	intravenous injection
71	Wang et al	Wang *et al.*	
72	EcoRI	*Eco*RI	*Eco* in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
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RAPID COMMUNICATION

Sensitivity and inter-observer variability for capsule endoscopy image analysis in a cohort of novice readers

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Abstract

AIM: To determine the performance of novice readers (4th year medical students) for detecting capsule endoscopy findings.

METHODS: Ten capsule endoscopy cases of small bowel lesions were administered to the readers. Gold standard findings were pre-defined by gastroenterologists. Ten gold standard "targets" were identified among the 10 cases. Readers were given a 30-min overview of Rapid Reader software and instructed to mark any potential areas of abnormalities. A software program was developed using SAS to analyze the thumbnailed findings.

RESULTS: The overall sensitivity for detecting the gold standard findings was 80%. As a group, at least 5 out of 10 readers detected each gold standard finding per recording. All the gold standard targets were identified when the readers' results were combined. Incidental finding/false positive rate ranged between 8.2-59.8 per reader.

CONCLUSION: A panel of medical students with minimal endoscopic experience can achieve high sensitivity in detecting lesions on capsule endoscopy. A group of novice readers can pre-screen recordings to thumbnail potential areas of small bowel lesions for further review. These thumbnails must be reviewed to determine the clinical relevance. Further studies are ongoing to assess other cohorts.

Key words: Capsule endoscopy analysis; Small bowel lesions; Novice readers; Sensitivity

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INTRODUCTION

Capsule endoscopy is a new diagnostic procedure developed for the complete examination of the small intestine through video images transmitted from an ingestible camera[1-3]. Briefly, the PillCam™ capsule endoscopy and diagnostic imaging system (GIVEN Imaging, Yoqneam, Israel) is a commercially available system consisting of three major components: PillCam™ capsule which captures images and transmits digital pictures (at 2 frames/s) over an 8-h period, sensor array and data recorder, which receives and records the data transmitted from the PillCam™ capsule and RAPID™ Workstation, which is used to initialize the data recorder and to download and process the raw data from the data recorder[4,5]. The processed information, composed of approximately 50 000 still images collected over an 8-h period, can be reviewed as a continuous video stream. The reported time range typically needed for a complete review of a single capsule endoscopy recording case is anywhere from 50[6,7] to 120 min[8].

Numerous studies have now demonstrated that the sensitivity and specificity of capsule endoscopy are advantageous over the traditional diagnostic methods of small bowel lesions[5-7,9-11]. Capsule endoscopy may also reduce total medical utilization and costs as well as improve patient's quality of life in certain circumstances[12].

One feature that can affect the diagnostic yield of capsule endoscopy is the image analysis process, i.e. the ability of the person reviewing the images (reader) to accurately detect significant lesions and interpret the findings. This process is time consuming and requires individuals to focus their undivided attention viewing the large number of images.

Currently, the process of capsule endoscopy image analysis has not been standardized with respect to the selection and training of individual readers, determination of the gold standard to which findings are compared to assess sensitivity and false positive rates or reporting of findings and diagnoses. Unfortunately, these important issues have not been well studied previously. Studies of inter-observer variability have been limited to anecdotal reports between 1 and 4 different readers[13-20,23,24]. Furthermore, since capsule endoscopy image analysis is a time consuming process, the arduous process of image recognition and analysis is often delegated to individuals having received minimal pre-training with little consideration of their ability to achieve competency in reading capsule endoscopy recordings. A survey was conducted at the 2003 Given International Capsule Endoscopy Conference and found that 82% of gastroenterologists reported that they are the first readers to interpret the capsule endoscopy recordings, while 18% use a resident physician assistant and/or nurse to interpret the capsule endoscopy recordings first[21].

In this clinical study, our aim was to determine the sensitivity, incidental finding and/or false positive rate, and intraclass correlation of novice capsule endoscopy readers who were 4th year US medical students with minimal endoscopic background for detecting pre-specified capsule endoscopy findings. Previous studies have shown that it is not a simple task to achieve 100% sensitivity on capsule endoscopy recording[13-20]. In addition, it was reported that since the pathology is visualized in more than a small percentage of images from each capsule endoscopy recording, a fatigue gastroenterologist may analyze the capsule endoscopy recording at a very rapid speed, being likely to miss lesions[14]. Hence, we propose that analysis of the same capsule endoscopy recording by multiple readers might be an effective method to achieve 100% sensitivity and decrease medical errors. If the combined results of the novice readers show a high sensitivity, then perhaps novice readers can be considered as physician extenders in analyzing capsule endoscopy recordings. In our study, instead of manually analyzing the capsule endoscopy readers' results, we used statistical software to perform the analysis in an attempt to decrease the time required for this process. The reason is that manual comparison of the readers' findings can be labor intensive and time consuming, if a large number of readers are evaluated. Finally, the method of using medical students as novice readers to analyze the images of a diagnostic modality has been described in the literature[25].

MATERIALS AND METHODS

Capsule endoscopy recordings

Ten recordings with definitive sites of small bowel lesions were administered to the readers in a pre-set order (lesions - AVM-3, small bowel tumor - 1, radiation enteritis - 1, ulcers/aphthous lesions - 3, and foreign body with ulceration - 2). Two gastroenterologists (attending physicians at the tertiary medical center) selected these 10 recordings.

Capsule endoscopy readers

The novice capsule endoscopy readers consisted of a group of ten 4th year medical students with minimal endoscopic background from David Geffen School of Medicine at UCLA (Los Angeles, CA, USA). All the participating medical students signed an informed consent agreement as approved by the local institutional review board.

The readers were blinded to the patients' clinical history because this study was to assess the readers' abilities to detect small bowel lesions on capsule endoscopy recordings rather than to test their medical knowledge. Furthermore, the readers were blinded to each other's capsule endoscopy findings.

Gold standard

The gold standard for the true positive findings was predefined by the two gastroenterologists (over 150 capsule endoscopy cases each at the time when this study was started) who independently reviewed all the available data for each of the 10 recordings, including pertinent medical history; previous endoscopic, radiologic and surgical examinations; the complete 8-h capsule endoscopy recording. The experts' consensus of positive findings was used to calculate the sensitivity and false positive measures for each individual reader as defined below. Ten gold standard "targets" were identified among the 10 cases. We did not include any negative findings in this study because our aim was to evaluate the readers' ability to detect positive findings. However, the readers did not know that there was at least one gold standard finding per case.

Capsule endoscopy image analysis

Each reader reviewed the entire 8-h recordings for all 10 cases to localize the thumbnailed significant lesions within the small intestine. Significant upper and lower gastrointestinal lesions could be detected by capsule endoscopy, but the lesions of esophagus, stomach, and colon were not analyzed. Presumably, lesions in these areas were detected during routine endoscopic evaluation.

The readers analyzed the 10 recordings in a consecutive order over a 30-90 d period. They were also asked to record how long it took for them to interpret each case and were told to use a cautious and highly inclusive approach, while interpreting the capsule endoscopy recordings in order to minimize the chance of missing any clinically significant lesions. All findings identified by each reader were marked, thumbnailed and annotated using the Rapid Reader software program (GIVEN Imaging, Yoqneam, Israel). The readers were given a 30-min overview of the Rapid Reader software and instructions for thumbnailing. Active gastrointestinal bleeding was often detected by the Suspected Blood Indicator program (two capsule endoscopy cases we used had active gastrointestinal bleeding lesions); however, we did not allow the readers to

Table 1 Gold standard findings and time intervals

Location	Case	Time intervals	Findings
1	1	22 500–32 900	Aphthous ulceration (from NSAIDS use)
2	2	4 542–6 842	Aphthous ulceration (from NSAIDS use)
3	3	16 144–16 157	Duodenal bleeding (AVM)
4	4	25 302–25 372	Staples and ulcerations (from prior Surgery)
5	5	24 244–24 700	Small bowel tumor
6	6	23 596–23 692	Aphthous ulceration (from Crohn's disease)
7	7	32 657–50 000	Radiation enteritis
8	8	20 000–26 000	Chicken bone
9	9	1350–2434	Bleeding angiodysplasia
10	10	12 600–12 800	Bleeding angiodysplasia

use the Suspected Blood Indicator program on the Rapid Reader, since we wanted to assess the readers' true abilities to detect the lesions on capsule endoscopy recordings. Furthermore, we felt that active bleeding lesions on the capsule endoscopy recordings should be easily and consistently detectable by the readers.

Outcome accuracy measures and analysis

The percentage of cases where a reader had at least one finding in the gold standard areas of a case was expressed as the reader's sensitivity. If a reader had a finding outside the gold standard time interval, it was considered an incidental finding/false positive rate.

The time series for each case was divided into time intervals using the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC, USA). Within each time interval, it was noted whether or not each reader had at least one finding. For a given time interval size, a time interval could be designated as being in a true problem area (part of the time interval was in the "gold standard" area for that case) or not. Each time interval could also be designated as having a "finding" or not, where the "finding" was yes if $X/10$ readers had a finding in that time interval. X was the reader's threshold. Reader's threshold was defined as the minimum number of readers out of all the readers who had to have a finding in a given time interval in order to consider it as a true positive finding (namely a true problem area). The clinical implication of the optimal time interval size and reader's threshold analyses was that this method could inform the capsule endoscopy readers, where the time series and the thumbnailed findings occurred the most. Therefore, the readers would know to which parts of the capsule endoscopy recording they needed to pay extra attention during the image analysis and review process. This is especially important and perhaps shortens the time needed for the gastroenterologists reviewing the thumbnailed findings of the screeners (in this case, the novice readers).

There were a total of 128 time interval size/reader's threshold combinations per case. In each of these time interval size/reader's threshold combinations, sensitivity and specificity were estimated for each case. Sensitivity was estimated as the probability of a finding, given the finding being in a region with a true finding (in the "gold standard" region), while specificity was estimated as the probability of no finding, given that the finding was not in an incidental/false positive region. Among the time interval size/reader's threshold combinations with 100% sensitivity, we calculated the incidental finding/false positive rate (the number of time intervals with a finding outside the gold standard area divided by the total number of time intervals outside the gold standard area) and the standardized number of minutes viewing incidental finding/false positive time intervals (the incidental finding/false positive rate multiplied by the average number of true negative time intervals for that reader's threshold/time interval size, multiplied by the time interval size in minutes). For each of these quantities, we calculated the average and the maximum value across all 10 capsule endoscopy cases. Separate analyses were conducted using different time interval sizes, which ranged in length from 20 to 25 000 s, in order to determine the impact of time interval size on the results.

Intraclass correlation was assessed separately for each case. The intraclass correlation among all 10 readers measured the agreement among the readers in their evaluation of capsule endoscopy recordings, above the agreement was expected by chance. The intraclass correlation coefficient was estimated for each time interval size and for each capsule endoscopy recording.

RESULTS

Based on the gold standard findings, 10 targets were specified in the 10 recordings used in this study (Table 1). The average time taken by the readers to interpret each case was 118 min. The overall sensitivity among the 10 readers was 0.80 (80%) for time interval size of 20 s. All findings were detected in 6 out of 10 readers. On a case level, the gold standard finding was identified by all the 10 readers in case #2 but only 5 readers for case #6. The individual reader sensitivity ranged between 60-100%, with reader #8 achieving 100% sensitivity, while reader #5 achieving only 60% sensitivity (Table 2). The readers were able to identify all the gastric, duodenal, and cecal images accurately. The number of incidental false positive finding ranged from a minimum of 8.2 in reader #1 to a maximum of 59.8 in reader #10 per recording (Table 3). By case, the number of incidental false positive findings ranged from 12 in case #9 to 40.1 in case #5. Intraclass correlation varied with case, but seemed to increase with increased time interval size. The overall intraclass correlation was <0.40 but cases #2 and #9, being low compared to fair agreement (Figure 1).

In the time interval size and reader's threshold analyses, the minimum time interval size for which sensitivity in all 10 recordings achieved 100% was 3 000 s. All possible reader thresholds achieved 100% sensitivity for all 10 recordings in at least one of the time interval sizes examined. The average percentage of incidental false positive findings in the 10 cases ranged from 28% with a reader's threshold of 7 and time interval of 5 000 s to 66% with a reader's threshold of 3 and a time interval of 10 000 s (Figure 2A). The maximum percentage of the incidental false positive findings in the 10 recordings ranged from 56% for time interval of 5 000 s and reader's threshold of 7-100% for several combinations (Figure 2B).

Overall, in the optimal time interval size and reader's threshold

Table 2 Findings and sensitivity

Reader number	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Total findings	Sensitivity
1	1	1	0	1	1	1	1	0	1	1	8	0.8
2	1	1	1	0	0	1	1	1	1	1	8	0.8
3	1	1	1	0	0	1	1	1	1	1	8	0.8
4	1	1	1	1	1	0	0	1	1	1	8	0.8
5	1	1	0	0	1	0	0	1	1	1	6	0.6
6	0	1	1	1	0	1	1	1	0	1	7	0.7
7	1	1	1	1	1	0	1	1	1	1	9	0.9
8	1	1	1	1	1	1	1	1	1	1	10	1
9	1	1	1	1	0	0	1	1	1	1	8	0.8
10	1	1	1	1	1	0	1	1	1	0	8	0.8
Overall	9	10	8	7	6	5	8	9	9	9	80	0.8

Average sensitivity = 80%.

Table 3 False positive/incidental findings

Reader number	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Min	Mean	Max	SD
1	16	14	3	14	10	4	5	5	6	5	3	8.2	16	4.849
2	17	39	6	7	64	6	15	6	19	10	6	18.9	64	18.788
3	34	39	27	11	51	9	17	16	12	14	9	23	51	14.158
4	32	31	10	7	13	62	8	12	5	10	5	19	62	17.858
5	25	7	11	4	2	1	2	27	6	9	1	9.4	27	9.324
6	6	18	9	8	27	8	17	6	4	9	4	11.2	27	7.193
7	23	19	29	53	22	25	33	33	21	42	19	30	53	10.708
8	29	20	11	28	22	7	10	2	8	13	2	15	29	9.226
9	26	62	34	68	29	11	19	14	7	5	5	27.5	68	21.936
10	10	28	17	107	161	34	12	187	32	10	10	59.8	187	66.813
Mean	21.8	27.7	15.7	30.7	40.1	16.7	13.8	30.8	12	12.7				
SD	9.31	15.94	10.64	35.54	46.37	18.87	8.73	55.77	9.17	10.69				

SD = standard deviation.
Note: The number in each case is the number of thumbnailed findings for that reader falling outside the gold standard target time range.

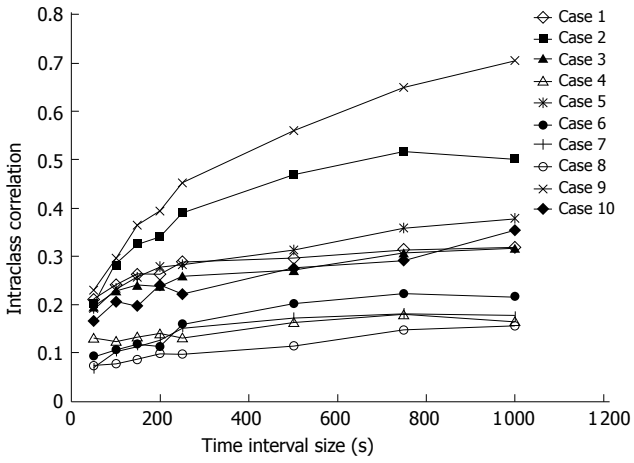

Figure 1 Inter-reader concordance analyzed separately by case.

analyses, the combination of 5 000 s and reader's threshold of 7/10 was the most optimal. This combination resulted in a reader's sensitivity of 100%, a low average incidental finding/false positive percentage and a low number of minutes viewing incidental finding/false positive time interval video images. However, while this time interval was still relatively large (83 min), smaller time interval sizes had higher rates of errors and/or failed to yield 100% sensitivity.

DISCUSSION

Capsule endoscopy is a newly developed diagnostic modality that allows visualization of the entire small intestine. However, the process of selecting, training, and validating an individual's ability to accurately perform capsule endoscopy image analysis has not been well studied. Therefore, we performed a systemic study to compare and validate the capsule endoscopy readers' performance on capsule endoscopy image analysis. The goal of our study was to determine the sensitivity and incidental finding/false positive rate as well as the intraclass correlation of novice readers and to determine if the concept of analyzing the same capsule endoscopy recording by multiple novice readers was an effective and accurate approach for capsule endoscopy image interpretation.

We hypothesized that novice readers could reliably

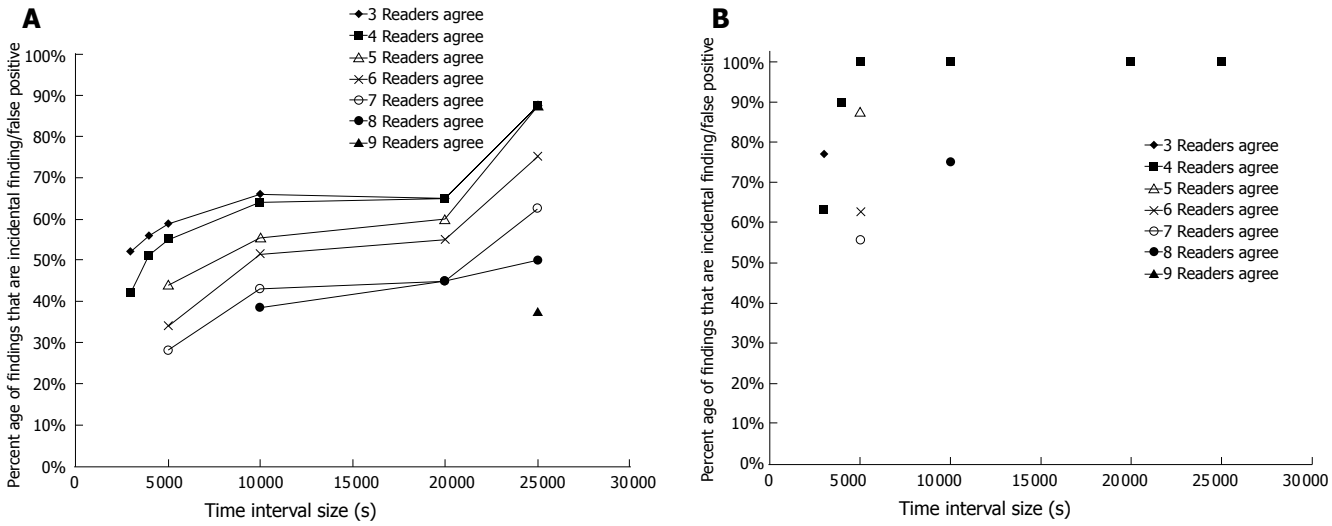

Figure 2 Average (**A**) and maximum (**B**) incidental finding/false positive rate across 10 cases by reader's threshold and time interval size.

detect small bowel lesions with a high sensitivity and a large number of incidental/false positive findings. In our study, each reader made a moderate number of incidental or false positive findings per recording. The following factors can help explain the moderate number of incidental or false positive findings: 1) the novice readers were asked to perform the capsule endoscopy analysis in a detailed, thorough and highly cautious fashion in an attempt to minimize the possibility of missing small bowel lesions; 2) the readers were untrained in interpreting capsule endoscopy images; 3) the "lodging" of capsule around the same spot in the small bowel caused some of the readers to thumbnail the same lesion several times; and 4) some of the incidental or false positive findings were small lesions, such as focal petechiae, areas of erythema or "mucosal breaks" with their clinical significance being still debatable.

We found that novice readers with minimal endoscopic experience were able to detect lesions on capsule endoscopy with a moderate-to-high sensitivity. Though the majority of the readers were unable to achieve 100% individual sensitivity, if we view the results by this panel of novice readers as a whole, every single gold standard target was detected. Therefore, perhaps the concept of analyzing the same capsule endoscopy recording by multiple novice readers may be an alternative yet effective and accurate method to interpret the capsule endoscopy images. This alternative approach might decrease the risk of having any lesions undetected by a single reader.

Inter-observer variability in analyzing capsule endoscopy recordings has been studied by Levinthal *et al*[14]. The combined sensitivity of the group of novice readers from our study is comparable with the result achieved by Levinthal *et al*[14]. In another published series, inter-observer variability was evaluated by comparing the interpretation results on 20 capsule endoscopy cases of an attending gastroenterologist and a 4th year therapeutic endoscopy student who has reviewed 15 capsule endoscopy cases prior to the participation of this study[15]. The authors found that there is a complete agreement between the two readers in 18/20 cases. Nonetheless, this study only compared the clinically significant findings and did not report the number of incidental/false positive findings.

However, studies on inter-observer variability on capsule endoscopy interpretation are mostly documented in abstract forms. Hoffman *et al*[16] showed that physician extenders could save gastroenterologists' time in capsule endoscopy interpretation.

Analyzing capsule endoscopy recordings requires a significant time commitment from the gastroenterologist. As a result, a few studies have investigated the potential of using physician extenders to serve as screeners for interpreting capsule endoscopy images. The results from our study showed that novice readers could achieve a high sensitivity in capsule endoscopy analysis when their results were combined as a group. Therefore, to analyze the same capsule endoscopy recordings by multiple novice readers may be the most effective and accurate method for detecting all significant lesions on capsule endoscopy. This is especially important because some lesions may appear in a single frame and could be easily missed by a single reader. An analogy to this method is the airport luggage screening process, in which the luggage is screened through the X-ray/CT scanners, whereby the television screen is monitored by "highly trained" individuals who detect the "high risk items" (analogous to lesions). Suspicious bags are subsequently re-X-rayed and screened by several individuals and then high-risk items are manually inspected (analogous to endoscopy, push enteroscopy or surgical investigation).

Our study is the first systematic study to date addressing the issues of inter-observer variability in capsule endoscopy image analysis by a large group (>4 individuals) of readers. The most important clinical conclusion of our study is that a panel of novice readers with minimal endoscopic experience can detect small bowel lesions on capsule endoscopy recordings and pre-screen recordings to thumbnail potential abnormalities with a high sensitivity, allowing the gastroenterologists to review only the thumbnailed potential abnormalities. This concept serves as an alternative method to those proposed

in the previous studies (i.e. using gastroenterology students or endoscopy nurses). Furthermore, perhaps the most effective way to accurately detect all abnormalities on capsule endoscopy recordings is to analyze the same capsule endoscopy case by a number of readers. This approach to capsule endoscopy image analysis may decrease the number of medical errors. Our results suggest once again that physician extenders can serve as screeners for interpreting capsule endoscopy images and save a significant amount of time of the gastroenterologists and make capsule endoscopy more cost-effective and attractive to practising gastroenterologists. However, due to the moderate number of incidental/false positive findings, gastroenterologists must review these thumbnails to determine the clinical relevance of each finding. Future studies should also estimate the amount of time that gastroenterologists have to spend on the assessment of all the incidental and false positive findings by the physician extenders. Additional studies are ongoing to assess other reader cohorts' (endoscopy nurses, gastroenterology students, medical residents, non-medical personnel) abilities to detect abnormalities on capsule endoscopy before physician extenders begin to screen capsule endoscopy in everyday clinical practise.

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RAPID COMMUNICATION

Serological pattern "anti-HBc alone": Characterization of 552 individuals and clinical significance

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Abstract

AIM: To investigate the prevalence and clinical significance of "anti-HBc alone" in an unselected population of patients and employees of a university hospital in southern Germany.

METHODS: All individuals with the pattern "anti-HBc alone" were registered over a time span of 82 mo. HBV-DNA was measured in serum and liver samples, and clinical charts were reviewed.

RESULTS: Five hundred and fifty two individuals were "anti-HBc alone" (of 3004 anti-HBc positive individuals; 18.4%), and this pattern affected males (20.5%) more often than females (15.3%; $P < 0.001$). HBV-DNA was detected in serum of 44 of 545 "anti-HBc alone" individuals (8.1%), and in paraffin embedded liver tissue in 16 of 39 patients tested (41.0%). There was no association between the detection of HBV genomes and the presence of biochemical, ultrasonic or histological signs of liver damage. Thirty-eight "anti-HBc alone" patients with cirrhosis or primary liver carcinoma had at least one additional risk factor. HCV-coinfection was present in 20.4% of all individuals with "anti-HBc alone" and was the only factor associated with a worse clinical outcome.

CONCLUSION: In an HBV low prevalence area, no evidence is found that HBV alone causes severe liver damage in individuals with "anti-HBc alone". Recommendations for the management of these individuals are given.

Key words: HBV markers; HBV serology; Hepatitis B virus; Hepatocellular carcinoma; Occult HBV infection

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INTRODUCTION

Antibodies to hepatitis B virus (HBV) core antigen (anti-HBc) are the most important marker of HBV infection. They are present when symptoms of hepatitis first appear and usually persist for life, irrespective if the infection resolves or remains chronic. Complete recovery from acute and chronic hepatitis B is associated with the loss of HBV surface antigen (HBsAg) and appearance of HBsAg-specific antibodies (anti-HBs) in serum. Thus, anti-HBc is usually accompanied by HBsAg or anti-HBs. However, the detection of "anti-HBc alone" (as the only marker of HBV infection) is not an infrequent serological pattern. In areas with low HBV prevalence (most parts of Europe and the United States) "anti-HBc alone" is found in 10-20% of all individuals with HBV markers, according to 1-4% of the general population[1,2]. This pattern poses problems because it provides no exact diagnosis. It is seen in acute infections, in the interval between the loss of HBsAg and the appearance of anti-HBs, as well as in chronic and past infections. Some individuals with "anti-HBc alone" carry HBV in their serum, their proportion varies greatly between 0.2% in blood donors and 47% in iv drug abusers[1].

The clinical significance of "anti-HBc alone" is greatly unknown. Longitudinal studies to explore the long-term clinical outcome are hardly practicable. We therefore investigated all individuals found positive for "anti-HBc alone" in our diagnostic laboratory over a period of 82 mo in regard to their virological and clinical findings.

MATERIALS AND METHODS

Study design

From July 1996 through April 2003, all individuals were registered whose sera tested reactive for anti-HBc and negative for HBsAg and anti-HBs for the first time. Our laboratory routinely received samples from patients and employees of a University hospital in Southern Germany.

Table 1 Distribution of HBV, HCV and HIV markers (tested with AxSYM™) in all individuals referred to the laboratory

	HBsAg neg anti-HBs neg	Anti-HBc pos HBsAg pos Anti-HBs neg	HBsAg neg anti-HBs pos	Anti-HBc neg
Study period		82 mo (6/96-4/03)		12 mo (1/03-12/03)
n (% of anti-HBc pos)	552 (18.4 %)	412 (13.7 %)	2040 (67.9 %)	4398
Male/female (ratio)	370/180 (2.06 : 1)	251/145 (1.73 : 1)	1185/851 (1.39 : 1)	2631/1766 (1.49 : 1)
Age (yr; mean±SD)	58.5±16.5	42.8±17.4	57.4±16.7	50.4±19.6
Anti-HCV pos/*n* tested (%)	112/550 (20.36 %)	20/323 (6.19 %)	144/1983 (7.26 %)	95/4285 (2.22 %)
Anti-HIV pos/*n* tested (%)	17/540 (3.15 %)	8/206 (3.88 %)	23/1571 (1.46 %)	7/3768 (0.19 %)

Totally 552 individuals (≥12 mo of age) with reactivity to "anti-HBc alone" were included in a database, and results of previous or follow-up testing for HBV markers were added when available for the study period. Clinical charts were reviewed to collect data about the medical history and clinical status of each individual.

Serological test

HBsAg, anti-HBs, anti-HBc, and anti-HBe were tested with microparticle enzyme immunoassays (AxSYM HBsAg™, AxSYM AUSAB™, AxSYM CORE™, AxSYM Anti-HBe™; Abbott Laboratories, Abbott Park, IL). Reactivity to "anti-HBc alone" (HBsAg and anti-HBs negative) was confirmed with a second microparticle enzyme immunoassay (IMx Core, Abbott, Delkenheim, Germany). When the new chemiluminescent microparticle immunoassay ARCHITECT anti-HBc™ (Abbott Laboratories, Abbott Park, IL) became available, all newly identified "anti-HBc alone" patients were additionally tested with this assay if there was enough serum available ($n=113$).

Antibodies against HCV and HIV were measured with microparticle enzyme immunoassays (AXSYM HCV™, AXSYM HIV-1/ HIV-2™; Abbott Laboratories, Abbott Park, IL). Positive test results were confirmed with Western blot assays Abbott MATRIX HCV 2.0 (Abbott Diagnostics, Wiesbaden-Dielkenhein, Germany) and NEW LAV BLOT I and II (BIORAD Laboratories, Munich, Germany), respectively.

PCR assays

HBV-DNA was isolated from serum with the QIAmp™ blood kit (Qiagen, Hilden, Germany). Paraffin embedded liver tissue was available for 39 patients from liver biopsies (hepatitis C, $n=16$; cirrhosis of the liver, $n=5$; liver abscess, $n=2$; deteriorating liver function after gastric perforation, $n=1$) or from liver surgery (liver transplantation for HCV associated cirrhosis, $n=4$; liver transplantation for alcohol associated cirrhosis, $n=3$; hepatocellular carcinoma, $n=4$; liver metastases, $n=2$; thrombosis of the portal vein, $n=1$; planned organ donation after brain death due to cerebral hemorrhage, $n=1$). Five to ten 5-μm sections were deparaffined as described before[3] and DNA was prepared using the QIAmp™ blood and tissue kit (Qiagen, Hilden, Germany). HBV-DNA was quantitatively measured using a kinetic fluorescence detection system (TaqMan PCR)[4]. The lower detection limit of this assay was 50-100 genomic copies per mL.

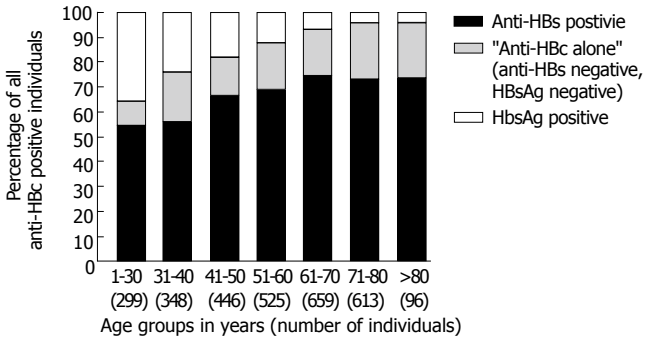

Figure 1 Distribution of HBV markers in 2986 anti-HBc positive individuals of different age groups, all referred to one virological laboratory over a period of 82 mo.

HCV-RNA in serum was detected using the Cobas Amplicor 2, 0 (Roche Diagnostics, GmbH, Mannheim, Germany).

Statistical analysis

The SPSS program package version 10.0 (SPSS Inc., Chicago, IL) was utilized to perform Fisher's exact test, χ^2 test and *t*-test. The significance level was set at 5%, all *P*s resulted from two-sided tests.

RESULTS

HBV serology

From July 1996 through April 2003, 3004 individuals tested positive for anti-HBc (Table 1) and 552 showed reactivity to "anti-HBc alone" (18.4%). "Anti-HBc alone" was seen more often in males (370/1806; 20.5%) than in females (180/1176; 15.3%; $P<0.001$). The percentage of individuals positive for "anti-HBc alone" was highest in the age group 71-80 years (139/613; 22.7%) and lowest in the youngest group 1-30 years of age (28/299; 9.4%; Figure 1).

In 113 "anti-HBc alone" individuals, apart from the confirmatory test, an additional assay for anti-HBc was performed (ARCHITECT™): anti-HBc was positive in 111 individuals, negative in one individual, and alternating negative and positive in two consecutive samples from another individual.

HCV and HIV coinfection

One hundred and twelve of 550 "anti-HBc alone" individuals (20.4%) were positive for anti-HCV. HCV-RNA was detected in 98 of 108 anti-HCV positive patients tested (90.7%). Five hundred and forty "anti-HBc alone" individuals were tested for anti-HIV and 17 (3.1%) were positive.

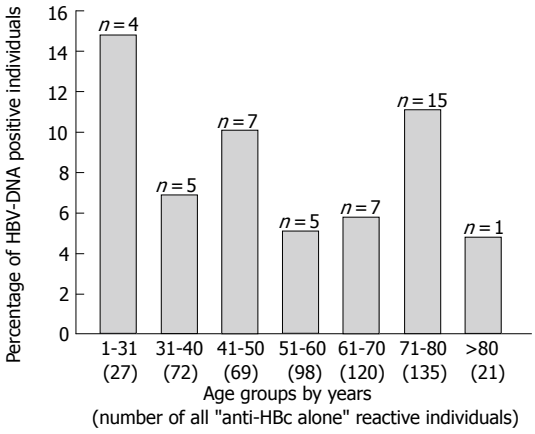

Figure 2 Percentage of HBV-DNA positive individuals (in serum) of all "anti-HBc alone" reactive individuals of different age groups.

Detection of HBV-DNA in serum and liver

Sera from 545 "anti-HBc alone" individuals were tested for the presence of HBV-DNA by PCR and 44 were positive (8.1%). The viral load was between 50 (lower limit of detection) and 1000 copies/ml serum. The percentage of HBV-DNA positive individuals was highest in the youngest group 1-30 years of age (4/27; 14.8%) and lowest in the age group > 80 years (1/21; 4.8%; Figure 2).

Paraffin embedded liver tissue was available from 39 patients. HBV-DNA was detected in 16 (41.0%): in 12 of 32 liver samples (37.5%) which had originally been taken because a chronic liver damage was suspected, and in 4 of 7 liver samples (57.1%) taken for other reasons. Considering the results of the histological examination, HBV-DNA was detected in 2 of 17 livers with cirrhosis, in 3 of 9 primary liver cancers and in 11 of 13 livers without cirrhosis or primary carcinoma.

Only 2 of the 39 patients with available liver samples were HBV-DNA positive in serum: one patient with chronic hepatitis C (no cirrhosis or carcinoma) was HBV-DNA positive both in serum and liver, and one patient with hepatocellular carcinoma was HBV-DNA positive only in serum but not in liver. Thus, liver HBV-DNA was positive in 1 of 2 patients with serum HBV-DNA and in 15 of 37 patients without serum HBV-DNA.

Clinical presentation

The 552 "anti-HBc alone" individuals were initially tested for HBV markers due to the following reasons: routine screening prior to invasive procedures ($n = 416$); diagnostic evaluation of cirrhosis of the liver ($n = 20$), hepatocellular carcinoma ($n = 11$), or elevation of transaminases ($n = 10$); diagnostic evaluation of a known HCV ($n = 28$) or HIV ($n = 16$) infection; routine screening of health care workers ($n = 6$); follow up evaluation of a known hepatitis B infection ($n = 4$); and evaluation of a needle stick injury recipient ($n = 3$). No diagnosis or cause of testing was given in 38 cases. In 90 patients (16.3%), the positive anti-HBc status was known prior to the actual investigation. Thirteen patients (2.4%) had suffered from jaundice of unknown origin earlier in their life. Information about suspected modes of infection or risk factors was available for 141 patients (25.5%): origin from an HBV hyperendemic area ($n = 95$), intravenous drug abuse ($n = 21$), HIV-infection ($n = 17$), previous blood transfusion or transplantation ($n = 17$), health care worker ($n = 10$), and HBV-positive sex partner ($n = 4$). Twenty individuals had more than one of these risk factors.

Alanine transaminase (ALT) activity was measured in 445 individuals and was normal in 279, up to twofold elevated in 89 and more than twofold elevated in 77 patients. Ultrasonic investigation of the liver was performed in 237 patients and revealed enlargement/steatosis of the liver in 69, signs of cirrhosis in 20, liver carcinoma in 10, and was non-distinctive in 20 patients. Histological examination of the liver was performed in 43 patients and showed cirrhosis in 16, hepatocellular carcinoma with cirrhosis in 10, active viral hepatitis in 8 (all with chronic HCV infection, HBV-DNA in serum positive in 1/8), steatosis of the liver in 3, liver abscess in 2, metastases of colon carcinoma in 2, primary biliary cirrhosis in one, and combined hepatocellular-cholangiocarcinoma without cirrhosis in one patient.

Combining all results of clinical, ultrasonic and histological examinations, we identified 38 "anti-HBc alone" patients with severe chronic liver damage (cirrhosis of the liver, $n = 27$; hepatocellular carcinoma, $n = 10$; combined hepatocellular-cholangiocarcinoma, $n = 1$). All 38 patients had at least one additional risk factor capable of causing the severe liver damage: chronic HCV infection ($n = 18$), alcohol abuse ($n = 14$), combined alcohol abuse and HCV infection ($n = 5$), and non-alcoholic steatohepatitis ($n = 1$).

"Anti-HBc alone" reactive individuals with positive and negative HBV-DNA in serum were compared regarding sex, age, ALT level, liver pathology, anti-HBs-titer, anti-HBe-status, HCV- and HIV coinfection. Statistically significant differences were not found (Table 2).

In those patients investigated, the detection of anti-HCV antibodies was associated with an elevation of ALT activity ($P < 0.001$) and with the presence of cirrhosis or primary carcinoma of the liver (Table 3).

Serological and clinical course

In 151 of the 552 "anti-HBc alone" patients (27.4%), more than one serum was investigated during the study period. In 111 individuals, reactivity to "anti-HBc alone" was confirmed by subsequent testing after a mean follow up period of 17.1 mo (minimum-maximum, 0-77 mo). Eleven individuals were anti-HBs positive (16.8 ± 8.4 IU/L) when retested after a mean of 11.5 months (1-38 mo), and 10 individuals had anti-HBs titers fluctuating slightly above and below 10 IU/L over a period of 44.9 mo (10-82 mo).

One "anti-HBc alone" patient (male, 45 years, HBV-DNA in serum positive) showed HBV reactivation with reappearance of HBsAg, HBeAg, increase of HBV-DNA and loss of anti-HBs - but normal ALT activity - after treatment with rituximab (humanized monoclonal antibody to CD 20) and chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisone) for non-Hodgkin lymphoma.

For 18 patients, a previous serum sample was available not showing the "anti-HBc alone" pattern. Twelve patients were anti-HBs positive (16.6 ± 4.7 IU/L) at an average of

Table 2 Clinical data in individuals with reactivity to "anti-HBc alone"

		HBV-DNA-PCR in serum[1]	
		Positive	Negative
n		44	501
Males/females[2]		27/17	336/163
Age (mean±SD)		57.2±18.6	58.5±16.2
ALT[2]	Normal	23/35 (65.7%)	251/402 (62.4%)
	≤Twofold increase	7/35 (20.0%)	81/402 (20.1%)
	> Twofold increase	5/35 (14.3%)	70/402 (17.4%)
Liver ultrasound[2]	no pathological findings	5/11 (45.5%)	112/204 (54.9%)
	Steatosis	5/11 (45.5%)	63/204 (30.9%)
	Cirrhosis	0/11	20/204 (9.8%)
	Carcinoma	1/11 (9.1%)	9/204 (4.4%)
Liver histology[2]	No pathological findings	0/2	5/42 (11.9%)
	Viral inflammation	1/2 (50%)	7/42 (16.7%)
	Steatosis	0 /2	3/42 (7.1%)
	Cirrhosis	0 /2	17/42 (40.5%)
	Carcinoma	1/2 (50%)	10/42 (23.8%)
Anti-HBs titer (IU/L)		1.9±2.5	2.6±3.0
Anti-HBe pos/*n* tested		6/9 (66.7%)	53/138 (38.4%)
Anti-HCV pos/*n* tested		7/44 (15.9%)	103/500 (20.6%)
Anti-HIV pos/*n* tested		0/43 (0 %)	17/491 (3.5%)

[1] There were no statistically significant differences between "anti-HBc alone" individuals with positive and negative HBV-DNA in serum regarding all parameters listed in Table 2.
[2] Missing numbers - not available/ not tested.

Table 3 Clinical data in "anti-HBc alone" individuals and anti-HCV status

		Anti-HCV		*P*-value
		Positive	Negative	
Sex	Males	76	290	NS[2]
	Females	36	141	
ALT[1]	Normal	31	244	<0.001
	≤Twofold increase	20	67	
	>Twofold increase	48	29	
Liver ultrasound[1]	No pathological findings	27	89	0.03
	Steatosis	25	43	
	Cirrhosis	11	9	
	Carcinoma	6	4	
Liver histology[1]	No pathological findings	0	0	<0.001
	Viral inflammation	8	0	
	Steatosis	2	1	
	Cirrhosis	12	4	
	Carcinoma	6	4	

[1] Missing numbers: not available/ not tested.
[2] NS: not significant.

31.3 mo (1-59 mo) before being detected as "anti-HBc alone".

Six patients were previously HBsAg positive. Three of them had an acute HBV infection recently: (1) one female patient (14 years old) lost HBsAg after 9 mo, but remained HBV-DNA positive and anti-HBs negative during an additional 34 mo; (2) one male patient (45 years old) was "anti-HBc alone" (HBV-DNA positive) 4 mo after the acute infection and tested anti-HBs-positive after additional 4 mo; (3) one male patient (17 years old) was "anti-HBc alone" (HBV-DNA negative) 4 mo after the acute infection and was anti-HBs positive when retested after an additional 10 mo. Two patients just cleared a chronic HBV infection: one male patient (37 years old) had a chronic HBV and HCV infection for at least 6 years and cleared HBsAg (but not HBV-DNA or HCV-RNA) after treatment with PEG-interferon and ribavirin; the other male patient (59 years old) was discovered to be HBsAg positive by routine screening (ALT normal, HBV-DNA negative), and was "anti-HBc alone" when retested after 5 mo. The sixth patient (male, 41 years old) showed reverse seroconversion (reappearance of HBsAg and loss of anti-HBs) and moderate ALT elevations 14 mo after allogeneic hematopoietic stem cell transplantation[5]. He lost HBsAg again after 4 mo and had alternating anti-HBs positive and negative ("anti-HBc alone") but HBV-DNA negative during follow up (53 mo).

DISCUSSION

The present study has primarily been undertaken to assess the clinical significance of the serological pattern "anti-HBc alone". Over a period of 6 years and 8 mo, 552 individuals with this pattern were identified in our laboratory, accounting for 18.4% of all individuals with positive anti-HBC. Forty-four of 545 "anti-HBc alone" patients had circulating HBV-DNA with less than 1 000 copies/ml. Depending on the sensitivity of the PCR assay, the proportion of HBV-DNA carriers in this study (8.1%; detection limit 50 HBV-DNA-copies/ml) complies with other studies reporting carrier rates of 3.3% and 7.7% (detection limits of 5 000 and 100 HBV-DNA copies/mL, respectively) in unselected German individuals or blood donors[2,6]. Even more "anti-HBc alone" individuals with very low viremia would probably be found if the sensitivity of the assay were increased. In paraffin embedded liver tissue, we detected HBV-DNA in 16/39 "anti-HBc an alone" patients (41.0%), only one of them was also PCR positive in serum. Thus, a significant fraction of "anti-HBc alone" individuals carry HBV genomes. However, HBV-DNA has also been detected in individuals with anti-HBs antibodies. Two German studies have reported a total of six HBV-DNA-positive blood donors with anti-HBs levels greater than 100 IU/L[7,8]. After the resolution of an HBV infection, a periodical release of virions from hepatocytes, as first described by Reherman *et al*[9], can occur in HBsAg-negative, anti-HBc positive individuals, although more often in persons who lack anti-HBs compared to those with detectable anti-HBs[10].

The essential question about these "occult" infections applies to their clinical impact. Since HBV-DNA has been found in serum and liver samples of HBsAg-negative patients with cirrhosis or primary liver cancer[11-13], it has been suggested that "anti-HBc alone" individuals may suffer from chronic liver injury with the associated oncogenic potential. Herein, we identified 38 "anti-HBc alone" patients with severe chronic liver damage. All 38 patients had at least one other risk factor which could cause the liver damage by itself. In addition, HBV-DNA was less often detected in the liver of these patients (2/17 with cirrhosis, 3/9 with primary carcinoma) than in liver samples from patients without chronic liver damage

(11/13). When all "anti-HBc alone" individuals of this study were analyzed, there was no association between the detection of HBV-DNA in serum and ultrasonic, histological or biochemical evidence of liver damage. Therefore, the present study provides no evidence that HBV alone causes liver damage in "anti-HBc alone" individuals.

Although an occult HBV infection in "anti-HBc alone" individuals seems to be inoffensive, it might become injurious in the case of immunosuppression leading to viral reactivation. In patients with past infection (anti-HBs and anti-HBc positive), HBV reactivation has been reported during chemotherapy, HIV infection, and after kidney and bone marrow transplantation[14-17]. In the present study, HBV reactivation with reappearance of HBsAg was observed in one "anti-HBc alone" patient after treatment with rituximab (humanized monoclonal antibody to CD 20) and chemotherapy for non-Hodgkin lymphoma. This patient had no clinical hepatitis, but there are at least two reported cases of HBV reactivation with liver inflammation after rituximab and chemotherapy: one patient died from liver failure[18], and the other reactivation occurred in a patient with preexisting anti-HBs and anti-HBc antibodies[19]. Our findings justify the cautious use of rituximab in "anti-HBc alone" patients and emphasize that these patients carry the risk for HBV reactivation during immunosuppression in general.

When a patient is diagnosed to be reactive for "anti-HBc alone", several explanations might apply to this phenomenon. First, a certain proportion of "anti-HBc alone" individuals will be false positive, depending both on the anti-HBc test used and on the HBV prevalence of the population investigated. We aimed to minimize false positives by including only individuals who were anti-HBc positive in a second confirmatory assay. When a subset of 113 individuals was investigated with a third anti-HBc test, reactivity was confirmed in 111 (98%). Although all three assays were from the same manufacturer, we estimated that the population was truly anti-HBc reactive. An alternative assay from a different manufacturer might provide a better confirmation, but there was no serum for additional assays available.

Second, "anti-HBc alone" individuals might be in the window phase of an acute HBV infection when HBsAg disappears followed by anti-HBs a few weeks later. Herein, only 6 of 552 "anti-HBc alone" individuals were known to be HBsAg positive before: three were in the window period after an acute infection, two probably just cleared a chronic infection, and one lost HBsAg again after HBV reactivation following bone marrow transplantation. In a low HBV prevalence area as in Germany, the proportion of "anti-HBc alone" individuals in the window period is very low, but individuals in this period are probably infectious.

Third, "anti-HBc" alone can also reflect an HBV infection which has resolved many years or decades earlier. In the majority of our patients, reactivity for anti-HBc was an incidental finding, most were healthy and not aware of a previous liver inflammation. In 151 of the 552 "anti-HBc alone" individuals, more than one sample was investigated during the study period: 12 individuals were anti-HBs positive before being detected as "anti-HBc alone", 10 individuals had anti-HBs titers fluctuating around 10 IU/L during follow up, and 11 individuals were weakly anti-HBs positive when retested. These findings suggest that a significant proportion of "anti-HBc alone" individuals are comparable to those still showing anti-HBs.

Fourth, a large fraction of "anti-HBc alone" patients is assumed to have an unresolved chronic infection with low grade, possibly intermittent virus production. These individuals have detectable HBV-DNA in serum (8.1% in this study) and are potentially infectious. The negative HBsAg assay is unclear in these cases, but can be caused by very low concentrations of HBsAg, fixation of HBsAg in immune complexes, or by HBsAg mutations[20,21]. From a practical point of view, there seems to be no clear-cut distinction between "late immunity" and "unresolved infection": HBV-DNA has also been detected in anti-HBs positive individuals, and HBV reactivation can occur both in anti-HBs positive and in "anti-HBc alone" patients. In the present study, there was no difference in the clinical picture between HBV-DNA negative and positive individuals with "anti-HBc alone".

One final explanation for the phenomenon "anti-HBc alone" is the suppression of HBV replication by an HCV coinfection. In the present study, 112/550 "anti-HBc alone" individuals (20.4%) were coinfected with HCV, and patients with HCV coinfection more often had an elevated ALT activity or chronic liver damage. The detrimental effect of HCV coinfection has previously been reported in HBsAg positive patients with chronic HBV infection[22,23].

In conclusion, we performed an extensive study including 552 "anti-HBc alone" individuals of all age groups and found no single patient who had severe liver damage and no additional risk factor other than the "anti-HBc alone" pattern. There was also no association between the detection of HBV genomes in serum and/or liver and damage of the liver. Therefore, the probability that HBV alone causes severe liver damage in "anti-HBc alone" patients seems to be low.

This study supports suggestions for the practical management of "anti-HBc alone" individuals which have previously been made by a group of experts[1]: When this pattern is observed for the first time, a false positive anti-HBc reactivity should be ruled out by a second anti-HBc test, preferably an assay with a different format. In some cases, anti-HBe will also be present and confirm the authenticity of the hepatitis B markers. All "anti-HBc alone" individuals must be screened for an HCV coinfection. To search for a chronic HBV infection (or an infection in the window phase), viral DNA should be measured with the most sensitive DNA amplification method (capable of detecting $\leqslant$100 genomes per mL), and the ALT activity should be determined. It appears sufficient to re-evaluate individuals without HBV-DNA and with normal ALT level after a longer period of time, e.g. every five years. In case of a relevant ALT elevation, a liver biopsy seems appropriate to reach a conclusion concerning therapy. Antiviral treatment should be considered if HBV-DNA and biochemical and histological signs of active hepatitis are present. Since "anti-HBc alone" patients with this constellation are rare, they should be monitored by an

experienced hepatologist. Individuals with positive HBV-DNA PCR and normal ALT values should be assessed in yearly intervals. When "anti-HBc alone" individuals are subjected to severe immunosuppression (e.g. during anti-tumor chemotherapy or transplantation), HBV-DNA assays should be applied to recognize viral reactivation. Individuals with "anti-HBc alone" must be advised not to donate blood.

Further research is needed to answer open questions about the pattern "anti-HBc alone", e.g. concerning the reason for the male predominance and the infectivity of affected individuals.

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RAPID COMMUNICATION

Hypertriglyceridemia is positively correlated with the development of colorectal tubular adenoma in Japanese men

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Abstract

AIM: To determine the real association between serum lipid levels and colonic polyp formation.

METHODS: We performed a large scale retrospective study to analyze the correlation between the incidence of colorectal adenoma or carcinoma and the fasting serum levels of total cholesterol (TC) and triglycerides (TG) in patients who underwent total colonoscopy for screening for colon cancer.

RESULTS: Both levels were significantly elevated in patients with adenomas as compared with patients without any neoplastic lesion (TC 207.6±29.5 *vs* 199.5 ±34.3, $n=4883$, $P<0.001$; TG 135.0±82.2 *vs* 108.7± 71.5, $n=4874$, $P<0.001$). The difference was significant in patients with tubular adenoma but not in those with villous or serrated adenoma. Multiple logistic regression analysis including age and sex revealed that TG was an independent correlation factor in male ($P<0.01$), but not in female patients. The level of TG in patients with invasive carcinoma did not show a significant elevation from that in patients with adenoma. These findings suggest that hypertriglyceridemia is an independent risk factor for colonic adenoma in men.

CONCLUSION: Although a high level of serum triglyceride does not appear to be mechanically involved in the development of carcinoma, reduction of serum TG and intensive surveillance with total colonoscopy may have benefit in men with hypertriglyceridemia.

Key words: Triglyceride; Hyperlipidemi; Colorectal; Adenoma; Colonoscopy

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INTRODUCTION

Many studies have suggested that a high intake of dietary fat tends to promote colon carcinogenesis[1-4]. Regarding serum lipid levels, early studies showed an inverse association between cholesterol levels and colon cancer, whereas many contradictory results have also been reported later[5-8]. These findings suggest that the association of low serum cholesterol with malignancies is a consequence of the impaired nutritional status rather than a cause of cancer.

As compared with total cholesterol, less information is available on the relationship between serum triglyceride and colorectal neoplasm. The striking similarity of lifestyle risk factors for colorectal cancer and insulin resistance suggests that hypertriglyceridemia as well as hyperinsulinemia and hyperglycemia associated with insulin resistance support the development of colon cancer[9-12]. Moreover, recent animal studies showed direct evidence suggesting a positive effect of triglyceride on the development of aberrant crypt foci[13] and adenomatous polyps[14,15]. This suggests overlapping mechanisms that increase the risk of colon cancer and hyperlipidemia. On the contrary, other reports failed to detect a significant association between serum levels of triglyceride as well as cholesterol and risk of colorectal cancer in human[16,17] and animal studies[18].

Thus, the true association between circulating lipid levels and colonic neoplasm development has not been satisfactorily determined in humans. In our clinic, we have performed a large number of total colonoscopies for screening for colon cancer, and intensively attempted to find colonic adenomatous polyps, since it is generally accepted that most colorectal cancers arise from adenomatous polyps[19]. From our experience, we retrospectively analyzed the relationship between serum lipid levels and the presence of colonic adenoma as well as carcinoma.

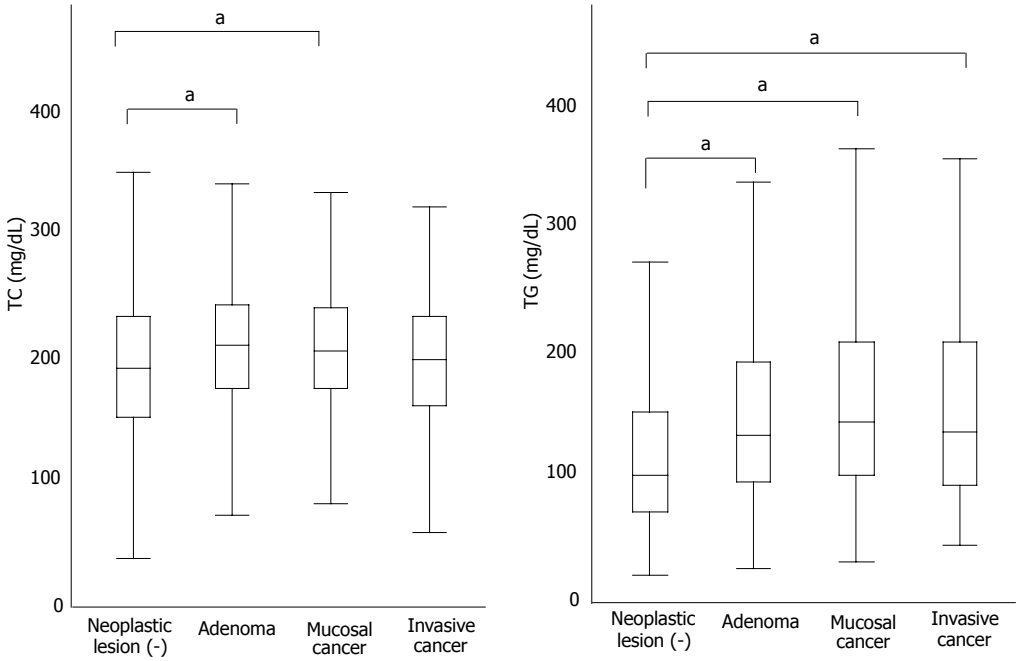

Figure 1 The average mean± SD of total cholesterol (TC) and triglyceride (TG) of patients with adenoma without cancer (n = 3821), with adenoma as well as mucosal cancer (n = 386), with invasive cancer as well as adenoma (110), and without any neoplastic lesions (n = 956); a means P value less than 0.05 by ANOVA.

MATERIALS AND METHODS

Patients

A total of 4887 patients underwent total colonoscopy to screen for colon cancer from 1995 to 2003 in our clinic. The entire colon was carefully examined with a high resolution magnifying chromo-endoscope. More than 30 min was spent performing each colonoscopy, in order to avoid overlooking small lesions. All the adenomatous polyps including mucosal cancers were simultaneously resected by endoscopic polypectomy technique and examined histopathologically. Invasive cancer was found in 110 (2.3%) of 4887 patients even without subjective complaints. All the patients also had 1 to 77 adenomatous polyps in other parts of the colon. In the remaining 4777 patients, no adenomas were found in 956 (19.6%) patients, whereas at least one adenoma could be detected in the other 3821 (78.2%) patients, and all of them were endoscopically removed simultaneously. In 386 cases, 1 to 3 adenomas were histologically diagnosed to contain mucosal carcinoma. In these 3931 patients with adenomas (including patients with invasive cancer), only one adenoma was detected in 660 (13.5%) patients, 2 in 549 (11.2%) and 3 in 458 (9.4%) patients. In the other patients, 1934 (39.6%) had 4 to 9 adenomas, while the other 788 (16.1%) patients had 10 or more adenomas in the entire colon. Histological examination revealed mucosal cancer in 386 (9.9%) patients and that most of the resected adenomas were diagnosed as tubular adenoma, and that tubular adenoma was detected in 3928 (80%) of the total patients. Villous adenoma and serrated adenoma were observed in 53 (1.4%) and 36 (0.9%) cases, patients.

Determination of serum cholesterol and triglycerides

The fasting serum levels of two major lipids, total cholesterol (TC) and triglycerides (TG), were measured just before colonoscopy in 4883 and 4874 cases, respectively. Then, the relationship between the incidence of colonic neoplasm and fasting serum levels of these lipids was retrospectively evaluated.

Statistical analysis

Statistical analysis was performed with JUMP software. The serum lipid levels in patients were analyzed by ANOVA and Fisher's exact test. The odds ratios were evaluated by multiple logistic regression tests. Values of P less than 0.05 were considered statistically significant.

RESULTS

Serum levels of TC and TG in patients with tubular, villous and serrated adenomas

The serum levels of TC and TG in patients with adenoma versus those with mucosal or invasive cancer showed a significant difference by ANOVA (Figure 1). Both values were significantly elevated in patients with adenomas as compared with patients without any neoplastic lesions (TC 207.6 ± 29.5 *vs* 199.5 ± 34.3, $P < 0.001$; TG 135.0 ± 82.2 *vs* 108.7 ± 71.5 $P < 0.001$). Patients with invasive or mucosal cancer showed very similar levels of TG (142.0 ± 75.1, 139.3 ± 91.6, respectively) to those with adenomas, which were significantly higher than that in patients without adenomas ($P < 0.001$). For TC, however, patients with mucosal cancer also showed a very similar level to those with adenomas (206.2 ± 28.7), while patients with invasive cancer showed a relatively reduced level (200.8 ± 28.3), which was similar to that in patients without neoplastic lesions. LDL cholesterol was examined in 1483 patients and showed a similar profile to TC (data not shown).

Next, we examined the difference in these lipid levels in patients with adenomas of different histology. As shown in Table 1, the serum levels of these lipids were significantly different in patients with tubular adenomas, but not in patients with villous or serrated adenomas. This indicates that hyperlipidemia has a specific association

Table1 Serum levels of Total cholesterol (TC) and Triglyceride (TG) in patients with tubular, villous and serrated adenomas

Pathological finding	TC	*n*	*P* value	TG	*n*	*P* value
tubular adenoma						
abesnt	199.6±34.4	958		108.9±71.0	954	
present	207.3 ±29.3	3925	$P<0.0001$	135.8±81.8	3920	$P<0.0001$
villous adenoma						
abesnt	205.8±30.6	4831		130.5±80.7	4821	
present	211.5±24.6	52	NS	132.0±60.8	53	NS
serrated adenoma						
abesnt	205.8±30.6	4848		130.4±80.3	4838	
present	208.8±24.1	35	NS	149.3±102.3	36	NS

NS: not significant.

Table 2 Univariate analysis of risk factors for tubular adenoma

Tubular adenoma	Total	Negative (%)	Positive (%)	*P* value
Age (yr)				
10-56	2475	765 (30.9)	1710 (69.1)	
57-94	2412	194 (8.0)	2218 (92.0)	<0.0001
Gender				
Male	2997	402 (13.4)	2595 (86.6)	
Female	1890	557 (29.5)	1333 (70.5)	<0.0001
Hypercholesterolemia				
Negative	3318	695 (21.0)	2623 (79.0)	
Positive	1565	263 (16.8)	1302 (83.2)	<0.0001
Unknown	4			
Hypertriglyceridemia				
Negative	3488	784 (22.5)	2704 (77.5)	
Positive	1386	170 (12.3)	1216 (87.7)	<0.0001
Unknown	13			

Hypercholesterolemia and hypertriglyceridemia were defined as total cholesterol>220mg/dL and TG>150 mg/dL, respectively.

Table 3 Adjusted odds ratios and 95% CIs for tubular adenoma for a change of 1SD in male or female patients

	TC			TG		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Total (3787)						
Gross OR	1.3	1.21-1.39	<0.0001	1.64	1.48-1.82	<0.0001
Age adjusted OR	1.01	0.93-1.09	0.79	1.34	1.21-1.48	<0.0001
Age, gender and TC or TG adjusted OR	1.04	0.96-1.13	0.334	1.13	1.01-1.09	0.013
Male (2997)						
Gross OR	1.32	1.19-1.47	<0.0001	1.35	1.19-1.56	<0.0001
Age adjusted OR	1.09	0.98-1.23	0.13	1.24	1.09-1.43	0.0015
Age and TC or TG adjusted OR	1.04	0.91-1.07	0.555	1.23	1.07-1.42	0.0041
Female (1890)						
Gross OR	1.49	1.34-1.65	<0.0001	1.42	1.25-1.64	<0.0001
Age adjusted OR	1.08	0.96-1.21	0.21	1.04	0.92-1.17	0.56
Age and TC or TG adjusted OR	1.06	0.95-1.02	0.29	1.02	0.91-1.16	0.7

with the development of tubular adenoma, but not the development of carcinomas or other types of adenomas.

Analysis of risk factors for tubular adenomas

We then performed multivariate analysis to determine the real association between serum lipid levels and tubular adenomas. Since univariate analysis showed that male and aged patients had colonic adenomas more frequently than their counterparts, we separately calculated the odds ratios for a change of one standard deviation (SD) adjusted for age in male and female patients (Tables 2 and 3). Multiple logistic regression analysis showed a clear contrast between TC and TG. When age and sex were considered, TC lost the independent relationship with adenoma, possibly because of the strong association with age. In contrast, TG was determined to be an independent correlation factor with tubular adenoma in total ($P<0.05$) and male ($P<0.01$) patients, but not in female patients.

DISCUSSION

Epidemiologic studies have suggested that a high fat intake is strongly related to the development of colorectal cancer, although it is unclear whether serum lipid levels can predict the high risk population for colon cancer. Our results clearly indicate that hyperlipidemia is associated with the development of adenomatous polyps with tubular histology in the colon. Although a positive association between hyperlipidemia and colonic neoplasm has already been reported, this large scale study revealed two additional findings.

The first is that the serum levels of cholesterol and triglycerides are increased in patients with adenoma compared to those without adenoma, while serum levels in patients with cancer are not elevated compared to those with adenoma. This indicates that a high fat intake that increases serum lipid levels may play a role in the development of adenoma but not in the development of carcinoma from adenoma.

The other important finding is that TG, but not TC, was independently correlated with the development of tubular adenoma, and the association was observed only in male but not in female patients. Recent studies have demonstrated that APC-deficient FAP model mice showed a hyperlipidemic state, possibly due to reduced activity of lipoprotein lipase, and that systemic administration of pioglitazone, a PPAR ligand, to the mice markedly reduced the serum level of triglycerides as well as intestinal polyp

formation[14,15]. These results are in agreement with our findings, and suggest the possibility that pioglitazone may be clinically effective to prevent polyp formation in humans.

The reason that the association was evident in male, but not in female patients, is yet unknown. The triglyceride level was significantly lower in women than in men in our series (106.17 ± 63.00 *vs* 145.90 ± 86.39, $P < 0.001$), and tubular adenoma was observed in 70.5% of female patients versus 86.6% of male patients ($P < 0.001$). Since it was reported that the usage of estrogen reduced the incidence of colon adenoma as well as carcinoma by about 30%, resulting in reduced colon cancer-related mortality [20], hormonal effects may be mechanically involved in the suppression of adenoma formation in the colon.

In summary, hypertriglyceridemia was shown to be an independent risk factor for colonic adenoma in men, although a high level of serum triglyceride did not appear to be directly involved in the development of colon carcinoma. Since all the adenomas were exclusively polypectomied in this clinic, no invasive cancers were detected at annual follow-up of those patients. Reduction of serum TG and intensive surveillance with total colonoscopy may be effective to prevent colon cancer in men with hypertriglyceridemia.

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RAPID COMMUNICATION

Triple therapy of interferon and ribavirin with zinc supplementation for patients with chronic hepatitis C: A randomized controlled clinical trial

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Abstract

AIM: To study the therapeutic effect of interferon (IFN) and ribavirin with zinc supplement on patients with chronic hepatitis C viral (HCV) infection.

METHODS: A total of 102 patients confirmed histologically to have chronic HCV infection with genotype 1b and more than 100 KIU/mL of HCV were randomly assigned to each arm of the study and each received 10 million units of pegylated interferon (IFN-alpha-2b) daily for 4 wk followed by the same dose every other day for 20 wk plus ribavirin (600 or 800 mg/d depending on body weight), with or without polaprezinc (150 mg/d) orally for 24 wk. The primary endpoint was sustained virological response (SVR) defined as negative HCV-RNA in the serum 6 mo after treatment.

RESULTS: There were no differences in the clinical background between the two groups except for more females in the dual therapy group than in the other group ($P < 0.05$). SVR was observed in 33.3% of the triple therapy group and 33.3% of the dual therapy group. The side effects were almost the same in both groups except for gastrointestinal symptoms, which were less in the triple therapy group ($P = 0.019$).

CONCLUSION: Considered together, triple therapy of zinc plus IFN and ribavirin for HCV infection patients with genotype 1b and high viral load is not better than dual therapy except for lower incidence of gastrointestinal side effects.

Key words: HCV; Zinc; Interferon; Ribavirin

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INTRODUCTION

Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma. It has become the most common indication for liver transplantation in many centers[1,2]. Improvement of antiviral therapy for patients with chronic HCV infection has been recently achieved by the introduction of pegylated interferon (IFN-alpha-2b) combined with ribavirin[3-5]. Because the sustained virological response rate in patients with HCV genotype 1b and high viral load is still insufficient, the search continues for new treatment strategies.

On the other hand, patients with chronic liver disease exhibit metabolic imbalances of trace elements such as high levels of iron and copper, and low levels of zinc, selenium, phosphorus, calcium and magnesium[6]. Of these trace elements, zinc is a constituent of a number of enzymes involved in a large number of metabolic processes. Mild-to-severe zinc deficiency disturbs several biological functions such as gene expression, protein synthesis, immunity, skeletal growth and maturation, gonad development, pregnancy outcomes, taste perception and appetite[7,8]. Zinc has been studied for its antiviral effects on HIV [9], rhinovirus[10] and herpes virus[11]. In addition to its anti-inflammatory effects, zinc has an antioxidant effect[12] and induces metallothionein (MT) which has radical scavenging and immunomodulatory effects[13]. Because zinc has many beneficial effects on viral eradication, we performed a preliminary study which showed the beneficial effect of IFN combined with zinc supplementation on chronic hepatitis C [14,15]. Here we present the results of a randomized controlled study of IFN with or without zinc supplementation for the treatment of chronic hepatitis C.

MATERIALS AND METHODS

Patients

Adult patients of both genders aged 24 - 70 years with

compensated chronic HCV infection were chosen. Each of the recruited patients fulfilled the following inclusion criteria: genotype 1b, more than 10^5 copies/mL of HCV in the serum, elevated serum alanine aminotransferase (ALT) concentration for at least 6 mo before initiation of treatment, and a liver biopsy specimen taken in the preceding 6 mo of study entry showing chronic hepatitis. Patients were excluded when they suffered from any of the following conditions: decompensated liver disease, other causes of liver disease such as hepatitis B infection, autoimmune disorders, hemoglobin value < 11 g/dL, white blood cell count < 3/nL, thrombocytopenia < 70/nL, neoplastic disease, severe cardiac disease, other severe concurrent diseases such as preexisting psychiatric conditions, and pregnancy or lactation. Informed consent was obtained from all patients enrolled in the study after a thorough explanation of the aims, risks and benefits of this therapy.

Study design

This randomized trial was conducted between January 2001 and December 2003 at 13 hospitals in Gunma, Japan. Eligible patients were randomly assigned to two study treatment groups with a ratio of 1:1 without stratification. Patients who met the entry criteria were randomly allocated into one of the two groups. The first group (triple therapy group) received oral triple therapy of 75 mg polaprezinc twice daily (Plomac; Zeria Pharmaceutical Co., Tokyo, Japan), IFN-alpha-2b (Intron A; Schering-Plough, Tokyo), and 600 mg or 800 mg ribavirin per day adjusted according to body weight (600 mg for weight below 60 kg and 800 mg for weight ⩾60 kg) for 24 wk. The second group (dual therapy group) received combination therapy of IFN-alpha-2b and ribavirin at doses similar to those listed above, but no zinc supplementation. Since 75 milligrams of polaprezinc contained 17 mg of zinc, the patients received 34 mg of zinc per day. All patients were intramuscularly injected with 10 million units of IFN every day for 4 wk followed by three times a week for 20 wk. Patients were followed-up for another 24 wk after the treatment, i.e., until week 48.

The response to treatment was assessed as follows. Sustained virological response (SVR) was defined as undetectable HCV RNA in serum at the end of follow-up by analysis with Ampliform HCV version 2 with a lower limit of detection of 100 copies/mL. Analyses were performed in all patients who were randomized to one of the treatment groups. All the other patients whose HCV-RNA was positive 24 wk after the treatment were classified as non-responders.

Histopathological examination of liver

In all patients, a liver biopsy was performed in the preceding 6 mo before therapy. Hepatic inflammation (grade) and fibrosis (stage) were assessed by the semiquantitative histological score proposed by Scheuer[16] and Desmet *et al*[17]. Portal/periportal inflammatory activity, lobular inflammatory activity and degenerative liver cell changes were scored using a scale of 0 to 3 for the criterion of inflammatory activity (1: mild, 2: moderate, 3: severe). The degree of fibrosis was scored using a scale of 0 to 4 (0: absent, 1: mild without septa, 2: moderate with a few septa, 3: numerous septa without cirrhosis, 4: cirrhosis).

Table 1 Comparison of clinical data of patients with chronic hepatitis C who received dual or triple therapy

	Triple therapy group	Dual therapy group	*P* value
Demography			
Male/Female	24/26	38/14	0.015[1]
Age[a] (yr)	57±23 (27-69)	53.8±9.8 (23-70)	NS[2]
Body weight[a] (kg)	60.5±9.8 (35-93)	63.5±12.4 (35-93)	NS[2]
BMI[a] (kg/m2)	23.5±3.1	23.4±2.1	NS[2]
Naïve/Retreatment	34/15	40/11	NS[1]
Biochemistry			
HCV RNA (kcopy/mL)	700 (100-1490)	720 (110-2310)	NS[2]
ALT[a] (U/L)	95.6±61.1	97.4±59.8	NS[2]
Ferritin[a]	216.8±244.4	214±139.6	NS[2]
Serum zinc[a] (μg/dL)	73.3±20.3	69.8±17.2	NS[2]
Histopathology			
F 0/1/2/3/4	3/12/23/10/1	1/20/15/13/1	NS[1]
A 1/2/3	26/20/2	20/24/5	NS[1]

[a]Data are mean ± SE and (range).
[1] Fisher's exact test. [2] Wilcoxon
NS, not significant.

Statistical analysis

Chi-square test and Fisher's exact test (two-tailed) for frequency tables were used for statistical analysis. A logistic multiple regression model was used to examine relationships between baseline clinical characteristics and the binary outcome of response to therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for the logistic multiple regression model. Variable selection was an important step in characterizing prognostic relations and identifying variables most strongly related to the outcome. The final model was then obtained by fitting only the main effect terms. $P<0.05$ (two-sided) was considered statistically significant. Statistical analyses were conducted by the StatView 5.0 and SAS version 8.2 statistical analysis packages.

RESULTS

Response to treatment

We recruited 102 patients with genotype 1b and more than 100 KIU/mL of HCV-RNA who met the predefined inclusion and exclusion criteria. The clinical background of the two treatment groups was not different except for gender (Table 1).

Twenty-four patients dropped out because of side effects and inability of follow-up due to unknown reasons. Thus, 78 patients including 39 patients on triple therapy and 39 who did not receive zinc supplementation were the subjects of this study (Figure 1). At the end of the study (48 wk), the SVR rate was 33.3% (13/39) in both the dual therapy and triple therapy groups (Figure 2). One week after the beginning of treatment, the viral disappearance rate in the triple therapy group (20.0% [5/25]) was not significantly different from that in the dual therapy group (10.3% [3/29]). Four weeks after the beginning of treatment, the viral disappearance rate in the triple therapy group was 69.7% (23/33), which was not significantly different from that in the dual therapy group (57.1% [16/28]).

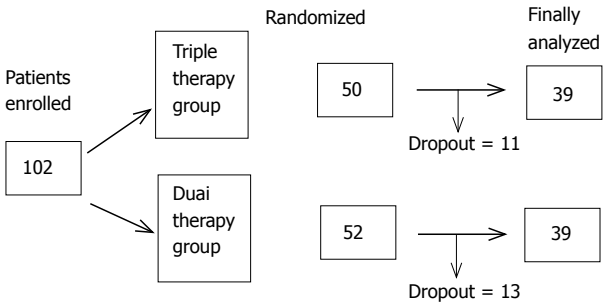

Figure 1 Profile of enrolled patients.

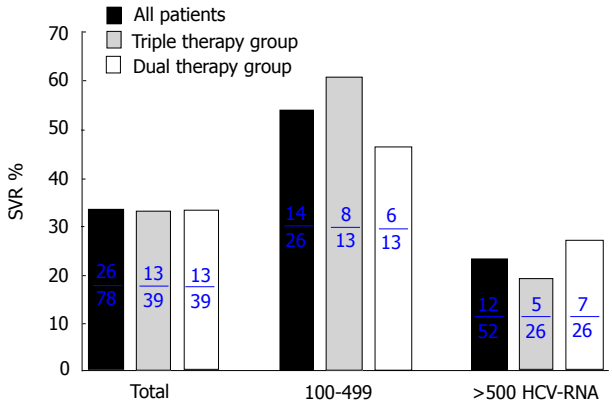

Figure 2 Rates of sustained virological response (SVR) to dual and triple therapies. Zinc supplementation had no significant effect on SVR.

Table 2 Virological response rates for dual and triple therapy groups at different weeks (w) after commencement of each treatment

HCV RNA (kcopy/mL)	Triple therapy group				Dual therapy group			
	1wk	4wk	24wk	48wk	1wk	4wk	24wk	48wk
>500	1/17	13/21	21/26	5/26	1/20	10/20	25/26	7/26
	5.9%	61.9%	69.2%	19.2%	5.0%	50.0%	96.2%	26.9%
					NS	NS	NS	NS
100-499	4/8	10/12	13/13	8/13	2/9	6/8	13/13	6/13
	50.0%	83.3%	100%	61.5%	22.2%	75.0%	100%	46.2%
					NS	NS	NS	NS
All patients	5/25	23/33	34/39	13/39	3/29	16/28	38/39	13/39
	20.0%	69.7%	87.2%	33.3%	10.3%	57.1%	97.4%	33.3%
					NS	NS	NS	NS

Fisher's exact test. NS, not significant.

While there were no significant differences between the two groups, patients of the triple therapy group tended to show an earlier decrease of the viral load compared to the dual therapy group (Table 2).

With regard to HCV-RNA load in the serum, the SVR rate of patients with HCV-RNA between 100 and 499 KIU/mL was 64.3% (8/13) in the triple therapy group, which was not different from that in the dual therapy group (46.2% [6/13]). Similarly, the SVR rate of patients with HCV-RNA >500 KIU/mL was not significantly different between the triple therapy and dual therapy groups (5/26 [19.2%] and 7/26 [26.9%], respectively).

Table 3 The logistic multiple regression model

Variable	Odds ratio	95%CI	*P*-value
HCV RNA (kcopy/mL)			
<500	1.000	0.014-0.690	0.0197
⩾500	0.097		
Body weight (kg)			
<64	1.000	0.931-63.301	0.0583
⩾64	7.677		
Pre-treatment platelet count ($\times 10^4/\mu L$)			
<16	1.000	0.032-1.909	0.1798
⩾16	0.246		

CI: confidence interval

Table 4 Summary of adverse events *n* (%)

	Triple therapy group	Dual therapy group	
Fever	35 (89.7)	38 (97.4)	NS
Anorexia	26 (66.7)	25 (64.1)	NS
Fatigue	24 (61.5)	22 (59.0)	NS
Arthralgia	20 (51.3)	18 (46.1)	NS
Eczema	16 (41.0)	13 (33.3)	NS
Nausea	14 (35.9)	13 (33.3)	NS
Abdominal discomfort	8 (20.5)	18 (46.2)	$P<0.019$
Stomatitis	13 (33.3)	12 (30.8)	NS
Headache	11 (28.2)	14 (35.9)	NS
Hair loss	12 (30.8)	7 (17.9)	NS
Psychosomatic	3 (7.7)	1 (2.6)	NS

Fisher's exact test. NS, not significant.

Factors associated with sustained virological response

A logistic multiple regression model was used for statistical analysis including HCV-RNA, body weight and pre-treatment platelet count. The analysis identified HCV-RNA level as a significant determinant of SVR when data of both groups were pooled together (Table 3).

Adverse effects

The side effects of the treatment were almost the same in both groups, including flu-like symptoms, mild reduction in hemoglobin, mild thrombocytopenia and leukopenia (Tables 4 and 5). The number of dropouts due to adverse effects was similar in the two groups (11 of 50 in the zinc group and 13 of 52 in the control group). The main reasons for dropout were anemia, fatigue, appetite loss and psychosomatic complaints (Table 6). The treatment protocol did not correlate significantly with the number of cases with adverse effects listed in Tables 4 - 6 except for abdominal discomfort. Fewer patients of the triple therapy group developed this side effect than those of the other group ($P=0.019$, Table 4).

DISCUSSION

The metabolism of trace elements is distorted in chronic

Table 5 Laboratory abnormalities

	Triple therapy group	Dual therapy group	
Decrease in WBC (<2 000 μL)	5	5	NS
(<1 500 μL)	1	0	NS
Decrease in hemoglobin (> 10 g/dL)	13	14	NS
(>8.5 g/dL)	3	4	NS
Decrease in platelet count (<50 000 μL)	1	3	NS
IFN dose reduction	9	10	NS
Ribavirin dose reduction	15	17	NS

Fisher's exact test. NS, not significant.

Table 6 Reasons for withdrawal from the study

Causes	Triple therapy group	Dual therapy group	
Anemia	2	3	NS
Psychosomatic	3	4	NS
Anorexia & Fatigue	2	3	NS
HCC[1]	1	0	NS
Dizziness	0	1	NS
Lost to follow-up	3	2	NS
Total	11	13	NS

Fisher's exact test. NS, not significant.
[1]Hepatocellular carcinoma was detected during the treatment.

liver disease[6]. We have previously reported that zinc deficiency in patients with chronic HCV can be improved by treatment with IFN[18]. In our previous pilot study, the beneficial effects of combined treatment of zinc and IFN have been confirmed with regard to both eradication of the virus and lowering or normalization of ALT[15].

In view of the clinical background of patients before treatment, the number of female patients in our study was greater than that of male patients in the triple therapy group, but univariate analysis did not reveal a significant sex difference in the effect of treatment.

Although patients on triple therapy showed early disappearance of HCV-RNA, the results 24 wk after the treatment showed no overall beneficial effect of zinc supplementation and are similar to those of previous reports[15]. Furthermore, the triple therapy tended to have the same beneficial effects of IFN + ribavirin dual therapy in patients with moderate viral load (between 100 and 499 KIU/mL). It is possible that longer treatment (more than 24 wk) and perhaps higher dosages of IFN and ribavirin may produce different results. Based on the adverse effects of the treatment, the only advantage of the triple therapy is the significantly lower incidence of abdominal discomfort compared to the dual therapy.

Polaprezinc, a synthetic agent, N- (3-aminopropionyl)-L-histidinate zinc, is a chelating compound for ulcer healing and has been used experimentally to prevent gastric ulceration in rats as well as clinically as an antipeptic ulcer drug[19,20]. Zinc present in the orally administered polaprezinc is absorbed, leading to a rise in serum concentration of zinc[21]. In this respect, absorption of polaprezinc is more favorable than other zinc-containing derivatives such as zinc sulfate[21] . While the dose of polaprezinc could increase serum zinc concentration and possibly improve the SVR, caution should be exercised with regard to the side effects of higher doses of polaprezinc, as it is known to induce abdominal discomfort in some patients (personal communication).

In conclusion, 24-wk zinc supplementation has no additive effect on the anti-HCV dual therapy of IFN-alpha-2 and ribavirin, apart from reducing the incidence of abdominal discomfort.

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