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MINIREVIEWS

Portal vein thrombosis in liver cirrhosis

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Abstract

Portal vein thrombosis (PVT) is considered to be a frequent complication of liver cirrhosis. However, unlike PVT in patients without cirrhosis, very few data are available on the natural history and management of PVT in cirrhosis, despite its association with potentially life-threatening conditions, such as gastroesophageal bleeding and acute intestinal ischemia. Moreover, no consensus regarding PVT in cirrhosis exists. Suggested causes of PVT in cirrhosis include reduced portal blood flow velocity, multiple congenital or acquired thrombophilic factors, inherited or acquired conditions, and derangement of liver architecture. However, the understanding of PVT in cirrhosis is incomplete. In addition, information on the management of PVT in cirrhosis is inadequate. The aims of this review are to: (1) assemble data on the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrhosis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the recent knowledge concerning diagnostic and therapeutic procedures.

Key words: Portal vein thrombosis; Liver cirrhosis; Thrombophilic factors; Anticoagulation; Splenectomy

Core tip: Portal vein thrombosis (PVT) is considered to be a frequent complication of liver cirrhosis; however, very few data are available on the natural history and management of PVT in cirrhosis, despite its association with potentially life-threatening conditions. The understanding and information on the management of PVT in cirrhosis are incomplete. The aims of this review are to: (1) assemble data on the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrhosis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the recent knowledge concerning diagnostic and therapeutic procedures.

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INTRODUCTION

Portal vein thrombosis (PVT), an obstruction of the portal vein or its branches by a blood clot, is encountered in a variety of clinical settings, such as myeloproliferative disease, cirrhosis, cancer and infection. More patients with cirrhosis are being diagnosed with PVT be-

cause current imaging techniques allow for the detection of asymptomatic PVT during routine ultrasonographic examination. PVT has a variety of clinical presentations, from asymptomatic to life-threatening conditions such as gastroesophageal bleeding and acute intestinal ischemia[1-3]. Although liver transplantation has altered the prognosis of patients with cirrhosis, the presence of PVT can exclude a patient from a transplant listing or negatively impact post-transplantation survival[4].

It remains unclear whether PVT is a consequence of severe liver disease, a factor aggravating underlying liver disease, or both. PVT is considered to be a frequent complication of liver cirrhosis; however, unlike PVT in patients without cirrhosis, very few data are available on its natural course and management despite its association with potentially life-threatening conditions. In addition, no consensus regarding PVT with cirrhosis exists. There is a growing need for optimal, evidence-based management of PVT in cirrhosis.

The aims of this review are to: (1) assemble evidence regarding the physiopathological mechanism, clinical findings, diagnosis and management of PVT in cirrhosis; (2) describe the principal factors most frequently involved in PVT development; and (3) summarize the most recent knowledge concerning diagnostic and therapeutic procedures.

DEFINITION OF ACUTE AND CHRONIC PVT

From a clinical point of view, PVT comprises two different entities: acute PVT and chronic PVT. Each represents a successive stage of the same disease. Although they share similar causes, they differ with respect to their management[5].

Acute PVT is characterized by the sudden formation of a thrombus within the portal vein[6]. The thrombus can involve variable portions of the mesenteric veins and/or splenic vein. Occlusion can be complete or partial, leaving a peripheral circulating channel.

In patients with chronic PVT, also known as portal cavernoma, the obstructed portal vein is replaced by a network of hepatopetal collateral veins connecting the patent portion of the vein upstream of the thrombus to the patent portion downstream. The number, size and location of these collaterals are extremely variable from patient to patient[6].

However, in patients with cirrhosis, it may be difficult to establish the "age" of the thrombosis because the criteria commonly used in patients without cirrhosis to define acute or chronic PVT (presence of collateral circulation and signs of portal hypertension) are already features of liver disease[7,8].

PREVALENCE

The prevalence of PVT in patients with cirrhosis has been reported more frequently in recent years. The reported prevalence of PVT is in the range of 0.6%-15.8% in patients with liver cirrhosis or portal hypertension[9-15]. The presence of PVT is reportedly 0.6% when evaluated by angiographic studies[9], 4.4% when evaluated by ultrasound[10], and 10%-12% when evaluated by computed tomography and magnetic resonance imaging[1,11]. Moreover, the prevalence of PVT increases with patient age and liver disease severity, reaching 15% in patients awaiting liver transplantation[12-15].

The etiology of liver disease influenced the prevalence of PVT in a study of 885 patients who underwent liver transplantation. The prevalence of PVT was 3.6% in primary sclerosing cholangitis, 8% in primary biliary cirrhosis, 16% in alcoholic and hepatitis B virus-related cirrhosis, and 35% in hepatocellular carcinoma (HCC)[12].

ETIOLOGY OF PVT IN CIRRHOSIS

Inherited and acquired thrombophilic disorders, bacterial infection[16,17] and sluggish portal flow[2,18,19] may all play a role in the high prevalence of PVT in patients with cirrhosis.

Cirrhosis was recently considered to be a hypercoagulable state, not a hypocoagulable state. The levels of both pro- and anti-coagulation proteins are reduced under conditions of hepatic synthetic impairment in patients with liver cirrhosis. Coagulation and anticoagulation mechanisms remain balanced but are carried out at a lower level[20-22]. The net result is a hemostatic balance that is compensated under normal circumstances, with no tendency for bleeding or thrombosis[22]. In cirrhosis, however, this equilibrium can easily tilt towards either bleeding or thrombosis[19,23-26]. Some authors have demonstrated that elevated levels of factor Ⅷ (a procoagulant driver) in combination with decreased levels of protein C (an anticoagulant driver), both of which are typically found in patients with cirrhosis (*i.e.*, procoagulant imbalance), are probably related to partial resistance to the *in vitro* anticoagulant action of thrombomodulin[27-30]. However, in patients with impaired synthetic function and low plasma levels of natural coagulation inhibitors, there is currently no simple way to ascribe such a low level to a pre-existing deficiency[31].

A thrombophilic genotype, including factor V Leiden G1691A mutation[32,33], methylenetetrahydrofolate reductase (TT677) mutation[34,35] and prothrombin (G20210A)[11,36], is associated with the formation of PVT. However, they may play a minor pathogenic role in the formation of PVT.

Reduced portal flow velocity seems to be the most important predictive variable for PVT development in patients with cirrhosis[37-39]. Amitrano *et al*[38] suggested that portal blood stasis in patients with cirrhosis is the main change favoring thrombosis, even in the presence of other local, systemic, congenital and acquired factors. Kinjo *et al*[39] performed Doppler ultrasonographic examinations after splenectomy in patients with cirrhosis and showed that portal venous flow was dramatically decreased by 49.2% in the PVT group but only by 6.6% in

the non-PVT group.

Splenectomy has recently been reported to play a role in the surgical strategy for HCC and interferon-based therapy for hepatitis C[40-45]. In addition, it can improve the prognosis for patients with cirrhosis by allowing them to receive interferon therapy or undergo treatment for HCC[44,45]. Despite the good results demonstrated in these studies, the high prevalence of PVT after splenectomy in patients with cirrhosis remains problematic[39,46]. It has been suggested that blood turbulence or stasis in the stump of the splenic vein after splenectomy might result in increased coagulability, leading to the propagation of splenic venous thrombus formation in the portal system after splenectomy[47]. Splenomegaly and a large splenic vein diameter are independent risk factors for PVT after splenectomy in patients with concomitant cirrhosis and portal hypertension[39,46,47].

The role of sclerotherapy as a potential trigger for PVT is controversial[48,49]. Some recent reports showed that thrombopoietin receptor agonists might be associated with an increased incidence of PVT in patients with cirrhosis[50,51].

CLINICAL FINDINGS

Clinical findings of PVT in cirrhosis vary from asymptomatic to life-threatening conditions. Partial PVT, which is now often detected by routine ultrasonography or computed tomography, might be associated with few symptoms. However, complete PVT may present as abdominal or lumbar pain with sudden onset or progression over a few days. Rapid, complete obstruction of the portal vein or mesenteric veins without involvement of the mesenteric venous arches induces intestinal congestion, which manifests as severe, continuous, colicky abdominal pain and occasionally as nonbloody diarrhea[1-3]. The bleeding risk appears to be higher in patients with PVT and cirrhosis than in patients with cirrhosis alone (39% *vs* 27%, respectively)[52]. In many patients, however, the thrombus is partial and its aspects and location change in follow-up images. Laboratory findings, including the levels of aminotransaminase, fibrin and fibrinogen degradation products, and d-dimers, are often normal in many cases of developing PVT.

Chronic PVT is commonly diagnosed after a fortuitous finding of hypersplenism or portal hypertension. In the majority of patients it is asymptomatic. Gastrointestinal bleeding is better tolerated by patients with chronic PVT than in those with other forms of portal hypertension, probably because patients with PVT are usually younger and have no liver dysfunction. The occurrence of ascites or encephalopathy in patients with chronic PVT is uncommon and is usually encountered only transiently following gastrointestinal bleeding or when unrelated renal failure or marked sepsis is present in older patients[3]. Liver test results are typically normal in patients with portal cavernoma in the absence of underlying liver disease. Biliary symptoms related to portal cholangiopathy (jaundice, biliary pain, cholangitis, cholecystitis or pancreatitis) rarely reveal the presence of a cavernoma[53,54]. Hepatopulmonary syndrome is present in about 10% of patients.

DIAGNOSIS

More of these patients are being diagnosed with PVT because current imaging techniques allow for the detection of asymptomatic PVT during routine ultrasonography in patients with cirrhosis.

Ultrasound and Doppler ultrasound are almost always sufficient for a diagnosis of PVT[55,56]. In most patients, the diagnosis of acute PVT can be rapidly established using noninvasive imaging. Ultrasound sonography can show hyperechoic material in the vessel lumen with distension of the portal vein and its tributaries. Doppler imaging shows the absence of flow in part or all of the lumen.

Enhanced computed tomography (CT) can show a lack of luminal enhancement in the portal vein, increased hepatic enhancement in the arterial phase, and decreased hepatic enhancement in the portal phase[57]. CT and magnetic resonance (MR) angiography are more sensitive techniques than Doppler imaging with respect to assessment of the extent of the thrombus within the portal venous system[56-58]. Definitive diagnosis of PVT can be obtained by MR imaging (MRI) and CT; the former provides a better evaluation of the extent of the thrombosis, particularly in the mesenteric vein, reaching a sensitivity and specificity of 98%-100%. CT provides information not only about the extent of the thrombosis and the development of collateral circulation, but also about the state of the abdominal organs. It is the procedure of choice when intestinal ischemia or hepatocellular carcinoma is suspected[59,60]. A diagnosis of cavernoma is readily achieved by abdominal imaging with ultrasound, CT or MRI, which shows serpiginous structures while the main portal vein and/or its main branches are not visible.

A recent study showed that positive intrathrombus enhancement on contrast-enhanced sonograms is an accurate predictor of recanalization in patients with recent portal thrombosis[61].

TREATMENT

Optimal management of PVT in cirrhosis is not addressed in any current consensus publication. There are a few reports about the factors that influence recanalization or the extent of thrombosis; however, the actual impact of PVT treatment on the natural course of cirrhosis has not been investigated. No randomized controlled trials have been performed and most existing evidence concerning PVT treatment is based on case series and is of low quality.

PVT increases the risk of variceal bleeding and is reportedly an independent risk factor for the inability to

control variceal bleeding[62]. In addition, PVT can be a life-threatening emergency when it extends to the superior mesenteric vein, leading to intestinal infarction. Anticoagulated patients with cirrhosis have better recanalization rates and PVT extension than non-anticoagulated patients[63]. Therefore, in patients with concomitant cirrhosis and PVT, a treatment algorithm that includes anticoagulation and transjugular intrahepatic portosystemic shunting (TIPS) provides a good chance of complete repermeation, reduces portal hypertensive complications and decreases the rate of thrombosis progression[63]. Francoz *et al*[4] evaluated patients with cirrhosis awaiting liver transplantation and found that survival was significantly lower in those with complete PVT at the time of surgery ($P = 0.04$). Furthermore, the rate of partial or complete recanalization was significantly higher among patients receiving anticoagulation therapy than among those not receiving anticoagulation therapy ($P = 0.002$).

Conversely, some reports have shown that PVT has little influence on prognosis in patients with cirrhosis. Maruyama *et al*[64] evaluated 150 patients with virus-related cirrhosis but without PVT at baseline; PVT developed in 28% of patients (42/150), with a cumulative incidence of 12.8%, 20% and 38.7% at 1, 5 and 8-10 years, respectively. The natural course of thrombosis was improvement in 47.6% of patients, unchanged in 45.2%, and worsened in 7.2%. Spontaneous resolution or an unchanged appearance was the most common outcome of PVT; therefore, cirrhotic PVT had little influence on prognosis. In their multivariate analysis, Luca *et al*[65] noted that there was no clear association between progression or regression of partial PVT and clinical outcome and that the Child-Pugh score at the time of diagnosis was the only independent predictor of survival.

In the field of liver transplantation, there is accumulating evidence that PVT, especially thrombus extension to the superior mesenteric vein, may adversely affect the outcome of transplantation. Thus, patients with concomitant cirrhosis and PVT who are on the waiting list for liver transplantation should be treated with anticoagulation therapy[66,67]. PVT prior to liver transplantation is an independent prognostic factor for post-transplant survival[68,69] and complete or partial PV recanalization has been associated with a better survival rate after liver transplantation[4]. It has also been shown that individuals with PVT at the time of liver transplantation are at higher risk of recurrent PVT after transplantation and of requiring retransplantation[30,70]. The increased mortality and morbidity rates associated with PVT are mostly restricted to the first year after liver transplantation[4,62] and actuarial survival after 1 year is good. Therefore, PVT cannot be considered to be a contraindication to liver transplantation[71].

Anticoagulation therapy is of proven benefit in patients with acute deep vein thrombosis[72]. The optimal anticoagulation regimen for the treatment and monitoring of PVT has not yet been fully explored and no clear recommendations exist regarding this issue in recent guidelines or consensus publications[6,28]. Treatment strategies most often include the use of anticoagulation, while thrombectomy and TIPS are considered second-line options.

The goal of anticoagulation therapy for acute PVT is to recanalize the obstructed veins, which will prevent intestinal infarction and portal hypertension. Correction of the causal factors should be achieved as soon as possible.

Vitamin K antagonists (VKA) have been used in some studies to treat PVT in patients with cirrhosis. The rate of PV recanalization in patients with cirrhosis treated with VKA is about 40%[4,73]. Orally administered VKA is more acceptable to patients; however, treatment with VKA is particularly difficult in patients with cirrhosis, mostly because anticoagulation monitoring is complex in this particular situation. Notably, international normalized ratio (INR) monitoring in patients with liver disease probably overestimates the bleeding risk because this international sensitivity index is determined using plasma from patients taking VKA[74]. The INR has only been validated in individuals with normal liver function on stable anticoagulation. A 29% variation in the mean INR was reported in patients with cirrhosis in a study in which three different thromboplastin reagents were used[75]. It is also unclear whether a target INR between 2 and 3 is adequate in individuals with an abnormal INR before anticoagulation therapy[30].

No consensus exists regarding the optimal duration of anticoagulation therapy in these settings. Complete recanalization can be delayed until the sixth month of anticoagulation therapy[5,76]. However, whether this is also true for patients with cirrhosis who develop acute PVT remains to be determined.

Randomized controlled trials of anticoagulation therapy for the prevention of recurrent thrombosis are lacking in cirrhotic PVT. In patients with deep vein thrombosis, a lack of complete recanalization indicates a high risk of recurrence after cessation of anticoagulation therapy[77]. The frequent association with permanent prothrombotic disorders and the risk of intestinal infarction support the use of anticoagulation. However, an increased risk of bleeding secondary to portal hypertension raises concerns. Delgado *et al*[73] reported that re-thrombosis after complete recanalization occurred in 38.5% of patients with cirrhosis after anticoagulation therapy was stopped. Thatipelli *et al*[78] stated that prolonged anticoagulant therapy does not appear to be justified based on the low rate of recurrence and substantial rate of major hemorrhage. To avoid the extension of thrombosis to the splanchnic vessels, prophylactic anticoagulation should be continued in patients with underling thrombophilic conditions or in patients who are likely candidates for future liver transplantation[4,63].

The choice of the anticoagulation regimen must also account for the potential need to reverse the effect of anticoagulation. There is no current consensus or guideline on whether nonselective beta blockers, endoscopic

variceal ligation or combination therapy is better for variceal bleeding prophylaxis[7,79].

An attractive alternative to oral anticoagulants could be the use of low-molecular-weight heparin (LMWH), the dosing of which is weight based and thus does not necessitate screening. A 50%-80% portal vein recanalization rate was recently reported with the use of LMWH in 38 patients with cirrhosis, with only a few episodes of non-severe variceal bleeding[62,80]. Villa *et al*[81] performed a randomized controlled trial to evaluate the safety and efficacy of enoxaparin, a LMWH, in preventing PVT in patients with advanced cirrhosis. They demonstrated that the actuarial probability of PVT was lower in the enoxaparin group ($P = 0.006$). The actuarial probability of survival was higher in the enoxaparin group ($P = 0.020$). No relevant side effects or hemorrhagic events were observed in their study. Enoxaparin appeared to delay the occurrence of hepatic decompensation and improve survival. However, an increased volume of distribution, such as that produced by ascites and edema, in patients with cirrhosis makes it difficult to determine the optimal dose of LMWH[82].

The administration of antithrombin Ⅲ (AT-Ⅲ) could be an attractive alternative to PVT in cirrhosis. Kawanaka *et al*[83] demonstrated that the low AT-Ⅲ activity and further decreases in this activity are associated with PVT after splenectomy in patients with cirrhosis and that treatment with AT-Ⅲ concentrates is likely to prevent the development of PVT in these patients.

TIPS and anticoagulation therapy are considered to be optimal treatment choices for PVT in cirrhosis[84]. TIPS completely recanalized the portal venous system in 57% of patients with cirrhosis and resulted in a marked decrease in 30% without major procedure-related complications. Despite problems associated with patency (bare stents, 38% in 12 mo and 85% in 24 mo; covered stents, 21% in 12 mo and 29% in 24 mo) and encephalopathy (27% at 12 mo, 32% at 24 mo), the long-term outcome of TIPS placement for cirrhotic PVT is excellent[85]. In addition, PVT prior to liver transplantation is an independent prognostic factor for post-transplant survival and TIPS prevents total portal vein occlusion in liver transplantation candidates with partial PVT[86].

In patients with both cirrhosis and chronic PVT, there is no consensus on the indication for anticoagulant therapy. As described in the consensus for PVT in patients without cirrhosis, therapy for chronic PVT can be separated into prevention and treatment of gastrointestinal bleeding, prevention of recurrent thrombosis and treatment of portal cholangiopathy. When PVT is longstanding and cavernous transformation has occurred in the portal vein, prophylactic anticoagulation is reversed only in patients with thrombophilic conditions and/or a high risk of thrombus extension into the superior mesenteric vein. There is still sufficient evidence in favor of interventional therapy such as TIPS[87]. Data on endoscopic ligation are lacking in adult patients with chronic PVT.

CONCLUSION

PVT is a common problem in patients with cirrhosis, mostly in individuals with advanced liver disease. However, many unknown pathophysiological aspects of PVT and unresolved issues encountered in everyday practice remain to be addressed. The most optimal, most efficient and safest modalities for treatment, screening and monitoring must be established in future controlled trials.

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ORIGINAL ARTICLE

Oxidative stress and extracellular matrices after hepatectomy and liver transplantation in rats

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Abstract

AIM: To investigate oxidative stress (OS)-mediated damage and the behavior of extracellular matrices in various rat models because shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade after surgery. These injuries also cause fatal liver damage.

METHODS: Rats were divided into four groups according to the surgery performed: control; hepatectomy with 40% liver remnant (60% hepatectomy); orthotopic liver transplantation (OLT) with whole liver graft (100% OLT); and split OLT (SOLT) with 40% graft (40% SOLT). Survival was evaluated. Blood and liver samples were collected at 6 h after surgery. Biochemical and histopathological examinations were performed. OS-induced damage, 4-hydroxynonenal, ataxia-telangiectasia mutated kinase, histone H2AX, phosphatidylinositol 3-kinase (PI3K) and Akt were evaluated by western blotting. Behavior of extracellular matrices, matrix metalloproteinase (MMP)-9, MMP-2, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were also evaluated by western blotting and zymography.

RESULTS: Although 100% OLT survived, 60% hepatectomy and 40% SOLT showed poor survival. Histopathological, immunohistological, biochemical and protein assays revealed that 60% hepatectomy, 100% OLT and 40% SOLT showed liver damage. PI3K and Akt were decreased in 60% hepatectomy and 40% SOLT. For protein expression, 40% SOLT showed differences in MMP-9, MMP-2 and TIMP-2. TIMP-1 showed differences in 60% hepatectomy and 40% SOLT. For protein activity, MMP-9 demonstrated significant differences in 60% hepatectomy, 100% OLT and 40% SOLT.

CONCLUSION: Under conditions with an insufficient liver remnant, prevention of OS-induced damage *via* the Akt/PI3K pathway may be key to improve the postoperative course. MMP-9 may be also a therapeutic target after surgery.

Key words: Free radicals; Akt; Phosphatidylinositol 3-kinase; Matrix metalloproteinase; Tissue inhibitors of metalloproteinase

Core tip: Although shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade after surgery, these injuries also cause fatal liver damage. Postoperative liver damage is still a critical matter in the field of liver surgery. Oxidative stress and extracellular matrices are important for liver regeneration after surgery and these may be important keys to overcome current problems in the field of liver surgery. Here, we investigated oxidative stress-mediated damage and the behavior of extracellular matrices in various rat models with liver surgery.

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INTRODUCTION

Liver resection is considered the standard treatment for primary malignant tumors and liver metastases. Advanced surgical techniques for hepatectomy, development of preoperative evaluation, and improvements in intensive postoperative care have resulted in a decline in perioperative morbidity and mortality. However, postoperative liver failure still occurs despite these developments. Extended hepatectomy has the advantage of high curability but increases morbidity and mortality[1]. Insufficient volume of the remnant liver is correlated with perioperative morbidity and mortality[1]. Prognosis of postoperative liver failure due to insufficient liver remnant is poor[1,2].

Orthotopic liver transplantation (OLT) is an accepted therapy for end-stage liver disease and currently provides long-term survival and good quality of life. However, cold ischemia/warm reperfusion (CIWR) injury is still a major cause of morbidity and mortality after OLT[3]. Currently, strategic procedures are needed to improve the liver tolerance against CIWR injury. A small-for-size graft (SFSG) is used for deceased donor liver transplantation (DDLT) and living donor liver transplantation (LDLT)[4,5]. The SFSG is defined as a ratio of graft weight against standard liver volume < 40%[6,7]. An inevitable insufficiency of graft size cannot be avoided in the LDLT or split orthotopic liver transplantation (SOLT) for DDLT. The SFSG in LDLT or SOLT is accompanied with CIWR injury and shear stress with portal hypertension. Hence, the SFSG results in high mortality and morbidity. The choice of a left-side graft is preferred from the viewpoint of greater donor safety and expanded donor candidates in LDLT[7,8]. Guaranteed SOLT with successful outcomes resolves a donor shortage in DDLT[4,5]. Currently, the 40% SFSG is a critical matter to overcome the donor shortage in DDLT and ensure donor safety in LDLT[4].

Oxygen is required for cell survival. However, it also poses a potential hazard *via* reactive oxygen species (ROS) and reactive nitrogen species (RNS), with biological and functional alterations of lipids, proteins and DNA[9-11]. Control of ROS/RNS production plays physiological roles, especially in regulating cell signaling, cell proliferation, differentiation and apoptosis[9-11]. Oxidative stress (OS) mediated by free radicals is defined as an imbalance between the production of ROS/RNS and the antioxidant capacity of the cell[9-11].

The extracellular matrix has important effects on inflammation, carcinogenesis and regeneration[12-14]. There are diverse types of proteases that control remodeling of the extracellular matrix, trigger liver regeneration and drive tumor progression[12-14]. Matrix metalloproteinases (MMPs) are a family of enzymes that degrade constituents of extracellular matrices and basement membranes. Currently, a total of 28 MMPs have been identified[14]. MMPs have been intensively studied and shown to play key roles in inflammation, carcinogenesis and regeneration[12-15]. MMP-2 and MMP-9 are implicated in liver injury and remodeling. In particular, previous researchers reported that MMP-9 and MMP-2 contribute to liver failure after liver surgery[12-21]. Tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous inhibitors of MMPs. Alteration in the MMP-TIMP balance is linked to pathophysiological conditions[22,23]. Currently, four members have been identified in the TIMP family which can inhibit various MMPs[24]. In particular, many researchers have focused on TIMP-1 and TIMP-2 during liver regeneration[25-28].

Although shear stress with portal hypertension and CIWR injury trigger the liver regeneration cascade after liver surgery, these injuries also cause fatal liver damage[29-31]. Initial damage is confirmed at the early postoperative period after liver surgery[3,12,13,18,29-31]. Therapeutic strategies to reduce this damage have the advantage of improving clinical results after liver surgery and overcoming the current issue of insufficient liver volume in the field of liver surgery. In the present preliminary study, we investigated OS-mediated damage and the behavior of extracellular matrices in various rat models with shear stress and portal hypertension and/or CIWR injury.

MATERIALS AND METHODS

Animals

Lewis rats (RT-1^l) were purchased from Harlan Laboratories (Indianapolis, IN, United States). Male rats were 8-12 wk old and weighed 250 g. The experimental protocols were approved by the Ethical Committee of our institution (Mayo Clinic, Institutional Animal Care and Use Committee, No. A19609). Rats were cared for in accordance with the Institutional Guidelines for Animal Welfare based on The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical procedures and postoperative care

Comprehensive details of the surgical procedures for rat

Table 1 Study design

Group	Hepatic remnant volume	Cold ischemia warm reperfusion	Shear stress portal hypertension
Control	100%, native liver	-	-
60%-hepatectomy	40%, native liver	-	+
100%-OLT	100%, syngeneic graft	+	-
40%-SOLT	40%, syngeneic graft	+	+

OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

and postoperative care in our institution have been previously described[32-34]. In the hepatectomy model, 40% of liver remnant consisted of the left median and lateral segments[32,33]. In the transplantation model, the syngeneic graft had a cold ischemic time of 3-4 h at 4 ℃ in normal Ringer's solution[33]. The 40% SFSG was also formed by the left median and lateral segments at the back table[34]. To avoid any irrelevant signaling, the hepatic artery was reconstructed by ultramicrosurgery[33]. Each rat was kept separately after surgery and body temperature was maintained by a heating pad. Postoperative observation was performed every 30 min until 6 h after surgery and 1.0 mL of warm lactate Ringer's solution was routinely administered every 1 h until 6 h after surgery. In the transplantation model, we previously demonstrated the importance of a shortened anhepatic phase and exclusion of unreliable samples based on autopsy findings[33,34]. In this study, the anhepatic phase was kept within 20 min in the transplantation model. No surgical complications were observed in each case at sampling autopsy.

Study design

Rats were divided into four groups according to the surgery performed: (1) laparotomy only (control); (2) hepatectomy with 40% liver remnant (60% hepatectomy); (3) OLT with whole liver graft (100% OLT); and (4) SOLT with 40% SFSG (40% SOLT) (Table 1). The survival study was performed on 10 rats in each group. Cell signaling involved in proliferation, differentiation and apoptosis was confirmed at the early postoperative period after liver surgery and subsequently progressive necrosis was observed, as described previously[3,12,13,18,29-31]. Serum and plasma were collected at 6 h after surgery (n = 5, in each group). Liver samples were also collected at 6 h after surgery for histopathological/immunohistological assessments, western blotting and gelatin zymography (n = 5, in each group).

Biochemical assays and coagulation profile

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), the international normalized ratio of prothrombin time (PT-INR) and hyaluronic acid (HA) were measured. Serum AST, ALT and T-Bil were assessed by commercial kits (SGOT, SGPT and total bilirubin reagent, respectively; Biotron, Hemet, CA, United States). The PT-INR in the plasma was measured by the i-STAT System (Abbott, Princeton, NJ, United States). Serum HA was measured using a commercial kit (Quantikine Hyaluronan ELISA Kit; R and D Systems, Minneapolis, MN, United States).

Histopathological and immunohistological assessments

Liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sliced into 4-μm sections. The morphological characteristics and graft injury scores were assessed after hematoxylin-eosin (HE) staining. The graft damage score has been described previously[34]. Scores were counted in 10 fields (× 100 magnification) in each slide and these scores were averaged in each HE slide.

Induction of apoptosis was assessed by immunostaining of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, S7100; Chemicon International, Billerica, MA, United States) and cysteine aspartic acid protease (caspase) 3 [Cleaved Caspase-3 (Asp175) Antibody, 9661S; Cell Signaling Technology, Danvers, MA, United States]. A TUNEL-positive nucleus was stained brown and a negative nucleus was counterstained light blue. A caspase-3-positive nucleus was stained brown and a negative nucleus was counterstained blue. Slides were scanned with an automated high-throughput scanning system (Scanscope XT, Aperio Technologies, Vista, CA, United States). To quantify the immunohistological findings, positive-stained nuclei were counted by Aperio Imagescope software (Aperio Technologies). All nuclei were classified into four color intensity levels and the higher two levels were considered as positive. The ratio of positive-stained nuclei to all nuclei was calculated and the mean ratio/mm^2 was determined.

Western blotting and gelatin zymography

The primary antibodies for malondialdehyde (MDA) (Anti-Malondialdehyde antibody, ab6463; Abcam, Cambridge, MA, United States); 4-hydroxynonenal (4-HNE) (4 Hydroxynonenal antibody, ab46545; Abcam); ataxia telangiectasia mutated kinase (ATM) (Phospho-ATM/ATR Substrate Rabbit mAb, 2909; Cell Signaling Technology); phosphorylated histone H2AX (γH2AX) (Phospho-Histone H2A.X Antibody, 2577; Cell Signaling Technology); phosphatidylinositol 3-kinase (PI3K) (Phospho-PI3K p85/p55 Antibody, 4228; Cell Signaling Technology); Akt (Phospho-Akt Rabbit mAb, 4058; Cell Signaling Technology); superoxide dismutase (SOD) (Cu/Zn Superoxide Dismutase, LS-B2907; LifeSpan BioSciences, Seattle, WA, United States); catalase (Catalase, LS-B2554; LifeSpan BioSciences); MMP-9 (Anti-MMP-9, Catalytic domain, AB19016; Millipore, Temecula, CA,

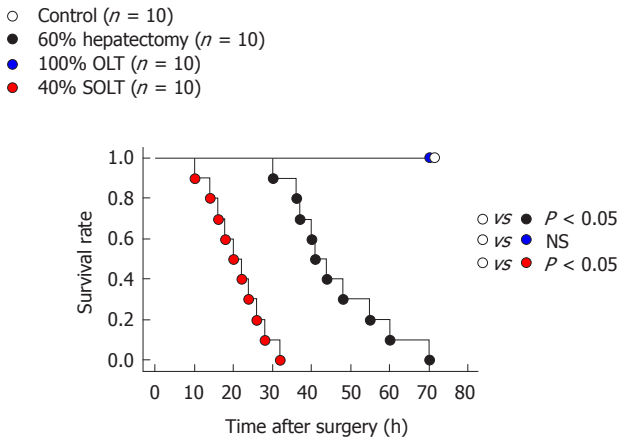


Figure 1 Survival curves. OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

United States); MMP-2 [MMP-2 antibody (MMP2/8B4), ab7032; Abcam]; TIMP-1 [Anti-TIMP-1 Mouse mAb (102D1), IM63; Calbiochem, San Diego, CA, United States]; and TIMP-2 [Anti-TIMP2 antibody (3A4), ab1828; Abcam] were used. Glyceraldehyde-3-phosphate dehydrogenase served as a control. Signals were quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA, United States). Gelatinase activity was visualized by fluorescence microscopy (Olympus BX50; Olympus Optical, Tokyo, Japan).

Statistical analysis

The results were presented as mean ± SD. Student's *t* test was used for the comparison of unpaired continuous variables between groups. Survival curves were constructed by the Kaplan-Meier method (Log-rank test). Statistical calculations were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant.

RESULTS

Survival curves

Survival curves for each group are shown in Figure 1. All rats that underwent a laparotomy or 100% OLT survived. The 60% hepatectomy and 40% SOLT groups clearly showed poorer survival than the controls ($P < 0.0001$). Insufficient liver remnant resulted in poor survivals after 60% hepatectomy. Especially, 40% SOLT showed very poor survivals.

Liver parenchymal damage

In comparison with the controls (0.1 ± 0.1 points), there were significant differences in the graft damage score for 60% hepatectomy (3.7 ± 0.7 points, $P < 0.0001$), 100% OLT (4.0 ± 0.6 points, $P < 0.0001$) and 40% SOLT (5.8 ± 1.1 points, $P < 0.0001$) (Figure 2A).

Immunohistological assessment of apoptosis induction

In comparison with the controls (0.003 ± 0.004), the rates of TUNEL-positive nuclei showed significant differences in 60% hepatectomy (0.017 ± 0.009, $P = 0.0278$), 100% OLT (0.107 ± 0.012, $P = 0.0001$) and 40% SOLT (0.166 ± 0.052, $P < 0.0001$) (Figure 2B). In comparison with the controls (0.002 ± 0.002), the rates of caspase-3-positive nuclei revealed significant differences in 60% hepatectomy (0.044 ± 0.023, $P = 0.0033$), 100% OLT (0.063 ± 0.014, $P < 0.0001$) and 40% SOLT (0.115 ± 0.019, $P < 0.0001$) (Figure 2C).

Conventional liver function tests, coagulation profile and endothelial damage

In comparison with the controls (42.5 ± 8.6 U/L), AST levels showed significant differences in 60% hepatectomy (202.4 ± 41.9 U/L, $P < 0.0001$), 100% OLT (290.5 ± 31.9 U/L, $P < 0.0001$) and 40% SOLT (387.4 ± 36.8 U/L, $P < 0.0001$) (Figure 3A). In comparison with the controls (59.8 ± 9.6 U/L), ALT levels showed significant differences in 60% hepatectomy (213.8 ± 57.0 U/L, $P < 0.0001$), 100% OLT (309.4 ± 38.3 U/L, $P < 0.0001$) and 40% SOLT (392.2 ± 76.7 U/L, $P < 0.0001$) (Figure 3B). In comparison with the controls (0.41 ± 0.13 mg/dL), there were no significant differences in T-Bil levels in 60% hepatectomy (0.50 ± 0.26 mg/dL, $P = 0.4798$) and 100% OLT (0.58 ± 0.15 mg/dL, $P = 0.0801$), but there was in 40% SOLT (1.37 ± 0.29 mg/dL, $P = 0.0001$) (Figure 3C).

In comparison with the controls (0.99 ± 0.04), PT-INR values revealed significant differences in 60% hepatectomy (1.16 ± 0.09, $P = 0.0052$), 100% OLT (1.12 ± 0.04, $P = 0.0008$) and 40% SOLT (1.22 ± 0.06, $P < 0.0001$) (Figure 3D).

In comparison with the controls (76.6 ± 14.9 ng/mL), HA levels demonstrated significant differences in 60% hepatectomy (264.0 ± 58.8 mg/dL, $P = 0.0001$), 100% OLT (188.0 ± 29.0 mg/dL, $P < 0.0001$) and 40% SOLT (350.2 ± 136.6 mg/dL, $P = 0.0021$) (Figure 3E).

Oxidative stress

The western blotting intensities of MDA in each group are shown in Figure 4A. In comparison with the controls (1.00 ± 0.10), normalized MDA showed significant differences in 60% hepatectomy (1.64 ± 0.39, $P = 0.0074$), 100% OLT (2.12 ± 0.78, $P = 0.0133$) and 40% SOLT (2.30 ± 0.26, $P < 0.0001$) (Figure 4B).

Lipid peroxidation

In comparison with the controls (1.00 ± 0.09), normalized 4-HNE showed significant differences in 60% hepatectomy (1.30 ± 0.20, $P = 0.0152$), 100% OLT (1.41 ± 0.20, $P = 0.0028$) and 40% SOLT (1.40 ± 0.19, $P = 0.0032$) (Figure 4C).

Responses and repairs to DNA damage

In comparison with the controls (1.00 ± 0.098), normalized ATM showed significant differences in 60% hepatectomy (1.15 ± 0.09, $P = 0.0336$), 100% OLT (1.28 ± 0.10, $P = 0.0015$) and 40% SOLT (1.21 ± 0.09, $P = 0.0053$) (Figure 4D). In comparison with the controls (1.00 ± 0.17), normalized γH2AX showed significant differences

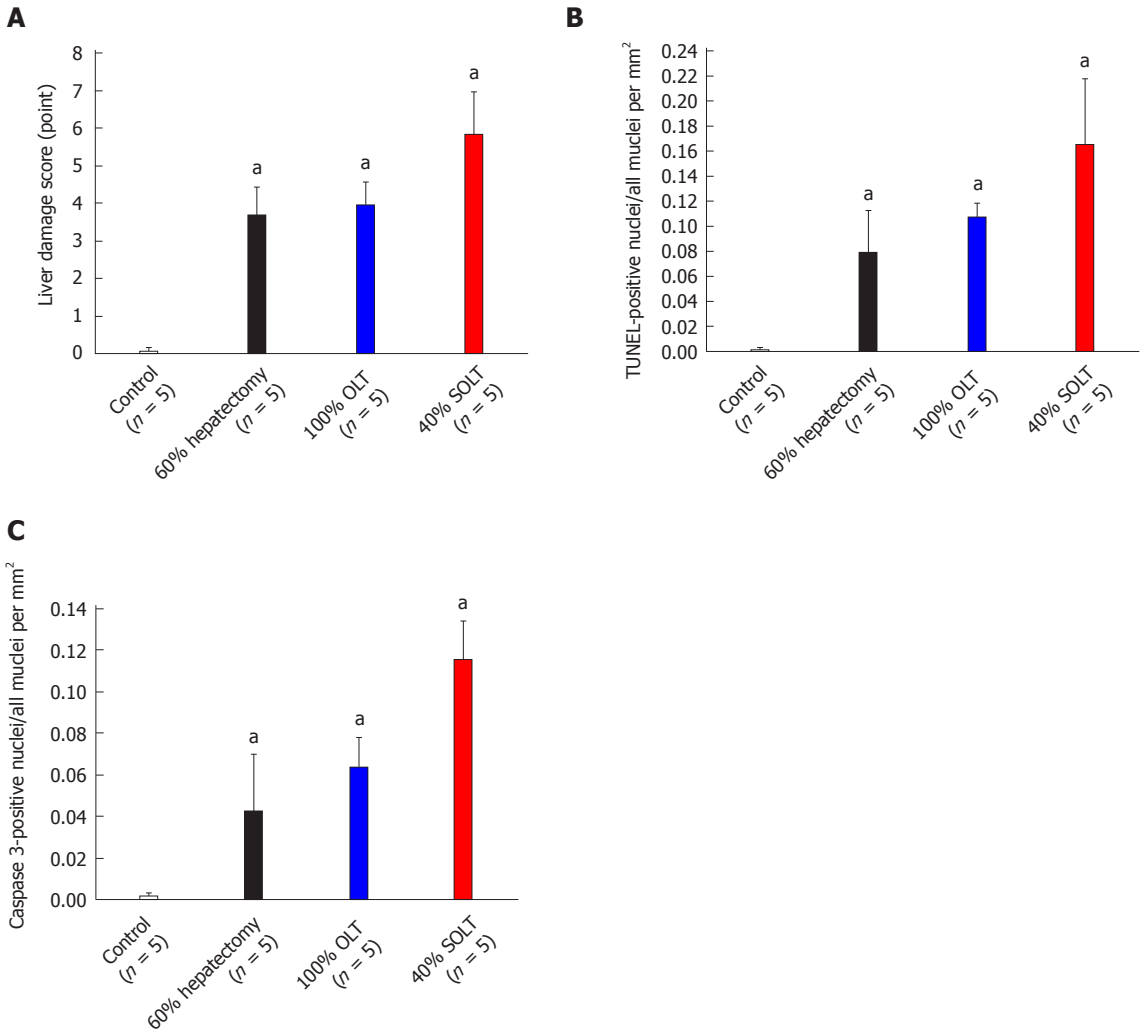


Figure 2 Histopathological and immunohistological assessments. A: Liver damage score in HE staining; B: TUNEL-positive rate; C: Caspase-3-positive rate. [a]*P* < 0.05 *vs* control. OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

in 60% hepatectomy (1.39 ± 0.29, *P* = 0.0071), 100% OLT (1.67 ± 0.38, *P* = 0.0303) and 40% SOLT (2.59 ± 0.66, *P* = 0.0008) (Figure 4E).

Promotion of cell survival

The western blotting intensities of PI3K and Akt in each group are shown in Figure 4F.

In comparison with the controls (1.00 ± 0.08), there was no significant difference in normalized PI3K in 100% OLT (0.92 ± 0.09, *P* = 0.1726), but there were significant differences in 60% hepatectomy (00.36 ± 0.11, *P* < 0.0001) and 40% SOLT (0.42 ± 0.19, *P* = 0.0002) (Figure 4G). In comparison with the controls (1.00 ± 0.12), there was no significant difference in normalized Akt in 100% OLT (0.92 ± 0.37, *P* = 0.6486), but there were significant differences in 60% hepatectomy (0.37 ± 0.23, *P* = 0.0007) and 40% SOLT (0.34 ± 0.24, *P* = 0.0006) (Figure 4H).

Activities of antioxidant enzymes

In comparison with the controls (1.00 ± 0.09), normalized SOD did not show significant differences in 60% hepatectomy (0.97 ± 0.09, *P* = 0.6503), 100% OLT (0.96 ± 0.11, *P* = 0.5461) and 40% SOLT (0.87 ± 0.09, *P* = 0.0595) (Figure 4I). In comparison with the controls (1.00 ± 0.17), normalized catalase also revealed no significant differences in 60% hepatectomy (0.91 ± 0.11, *P* = 0.3665), 100% OLT (0.90 ± 0.15, *P* = 0.3365) and 40% SOLT (0.95 ± 0.14, *P* = 0.6454) (Figure 4J).

Behavior of MMP-9, MMP-2, TIMP-1 and TIMP-2

Protein expression and activity of MMP-9 are shown in Figure 5A. Protein expression was evaluated by western blot densitometry (Figure 5B-D). In comparison with the controls (1.00 ± 0.34), there were no significant differences in normalized MMP-9 in 60% hepatectomy (1.14 ± 0.43, *P* = 0.5811) and 100% OLT (1.18 ± 0.35, *P* = 0.4254), but there was a significant difference in 40% SOLT (2.16 ± 0.26, *P* = 0.0003) (Figure 5B). In comparison with the controls (1.00 ± 0.16), there were no significant differences in normalized MMP-2 in 60% hepatectomy (0.78 ± 0.17, *P* = 0.0716) and 100% OLT (0.80 ± 0.23, *P* = 0.1437), but there was a significant difference in 40% SOLT (0.78 ± 0.12, *P* = 0.0385) (Figure

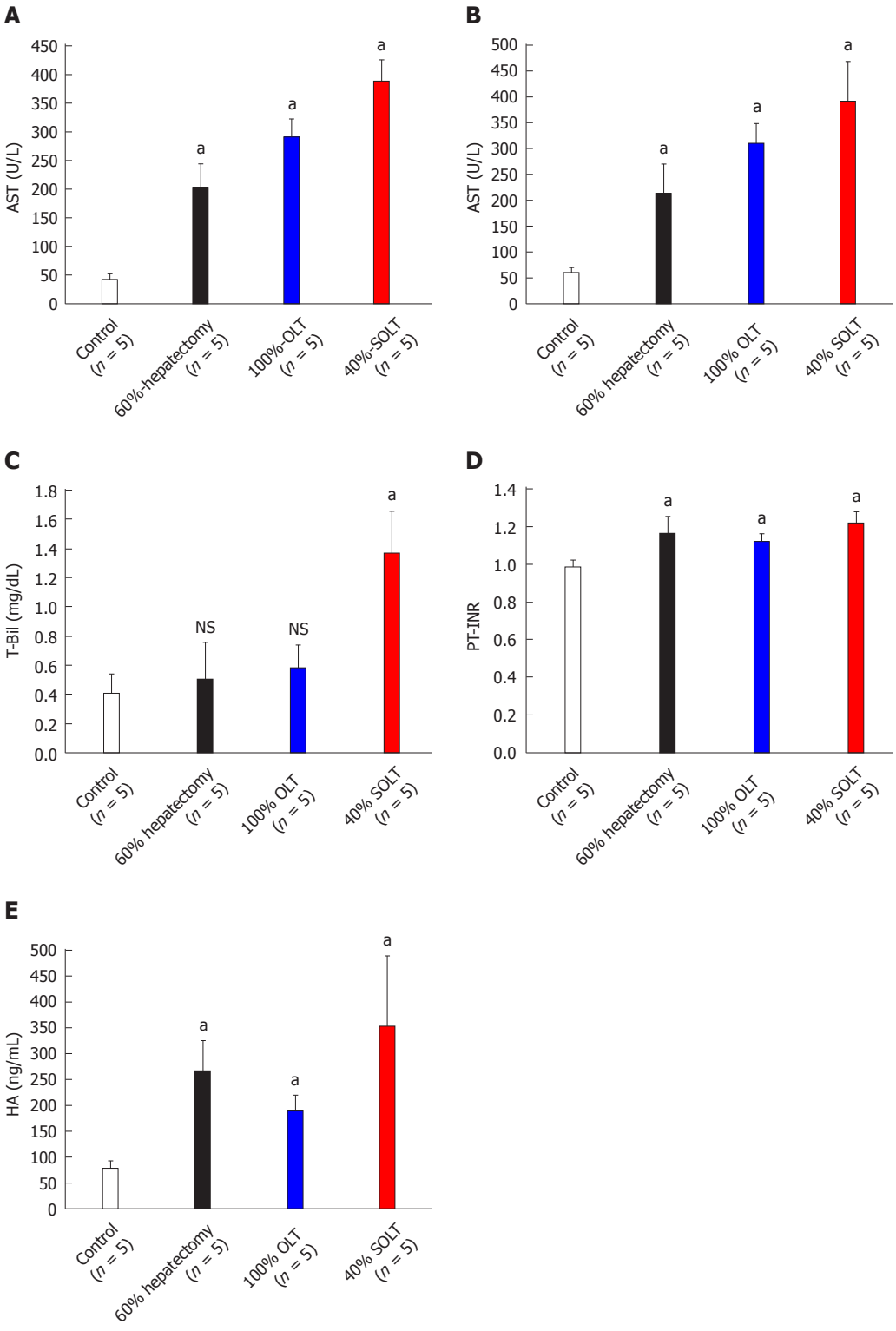
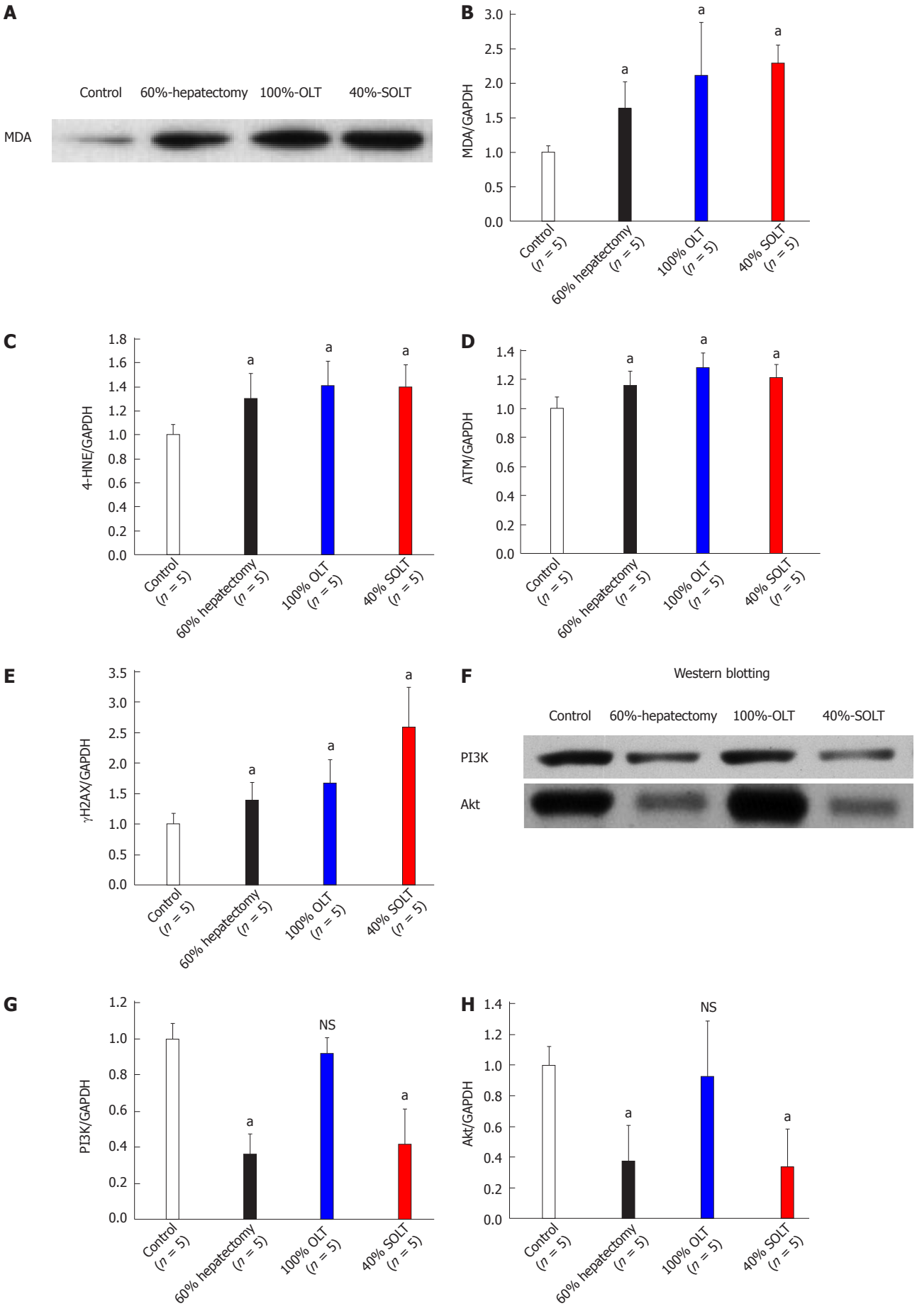


Figure 3 Biochemical and coagulation profiles. A: Serum aspartate aminotransferase (AST); B: Serum alanine aminotransferase (ALT); C: Serum total bilirubin (T-Bil); D: Plasma international normalized ratio of prothrombin time (PT-INR); E: Serum hyaluronic acid (HA). $^{a}P < 0.05$ *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

5C). In comparison with the controls (1.00 ± 0.30), there was no significant difference in normalized TIMP-1 in 100% OLT (0.82 ± 0.43, $P = 0.4654$), but there were significant differences in 60% hepatectomy (1.41 ± 0.26, $P = 0.0491$) and 40% SOLT (1.46 ± 0.32, $P = 0.0486$) (Figure 5D). In comparison with the controls (1.00 ± 0.24), there were no significant differences in normalized TIMP-2 in 60% hepatectomy (1.23 ± 0.24, $P = 0.1605$) and 100% OLT (0.95 ± 0.17, $P = 0.6846$), but there was a significant difference in 40% SOLT (1.28 ± 0.12, $P = 0.0471$) (Figure 5E).

Protein activities were evaluated by intensity in zy-


A
Control
60%-hepatectomy
100%-OLT
40%-SOLT
MDA
B
MDA/GAPDH
C
4-HNE/GAPDH
D
ATM/GAPDH
E
γH2AX/GAPDH
F
Western blotting
Control
60%-hepatectomy
100%-OLT
40%-SOLT
PI3K
Akt
G
PI3K/GAPDH
NS
H
Akt/GAPDH
NS
Control (n = 5)
60% hepatectomy (n = 5)
100% OLT (n = 5)
40% SOLT (n = 5)

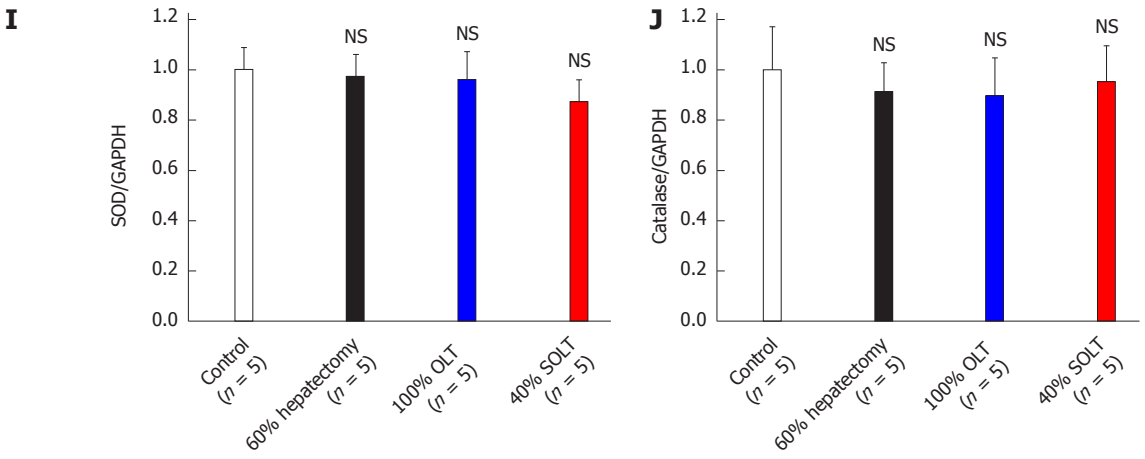


Figure 4 Protein expression of malondialdehyde, 4-hydroxynonenal, ataxia-telangiectasia mutated kinas/H2AX, phosphatidylinositol 3-kinase/Akt and antioxidant enzymes. A: Actual intensities of malondialdehyde (MDA) in western blotting; B: Normalized MDA; C: Normalized 4-hydroxynonenal (4-HNE); D: Normalized ataxia-telangiectasia mutated kinase (ATM); E: Normalized γH2AX; F: Actual intensities of phosphatidylinositol 3-kinase (PI3K) and Akt in western blotting; G: Normalized PI3K; H: Normalized Akt; I: Normalized superoxide dismutase (SOD); J: Normalized catalase. $^{a}P < 0.05$ *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

mography (Figure 5F-I). In comparison with the controls (1.00 ± 0.15), relative MMP-9 clearly demonstrated significant differences in 60% hepatectomy (1.37 ± 0.23, $P = 0.0156$), 100% OLT (1.47 ± 0.33, $P = 0.0211$) and 40% SOLT (2.10 ± 0.75, $P = 0.0125$) (Figure 5F). In comparison with the controls (1.00 ± 0.17), relative MMP-2 did not reveal significant differences in 60% hepatectomy (1.03 ± 0.12, $P = 0.7444$), 100% OLT (0.98 ± 0.15, $P = 0.8821$) and 40% SOLT (1.04 ± 0.13, $P = 0.6847$) (Figure 5G). In comparison with the controls (1.00 ± 0.15), relative TIMP-1 did not reveal significant differences in 60% hepatectomy (0.96 ± 0.29, $P = 0.7926$), 100% OLT (0.98 ± 0.09, $P = 0.8217$) and 40% SOLT (0.91 ± 0.26, $P = 0.5347$) (Figure 5H). In comparison with the controls (1.00 ± 0.12), relative TIMP-2 did not show significant differences in 60% hepatectomy (1.04 ± 0.09, $P = 0.5974$), 100% OLT (1.03 ± 0.11, $P = 0.6845$) and 40% SOLT (1.03 ± 0.16, $P = 0.7495$) (Figure 5I).

Statistical differences between groups

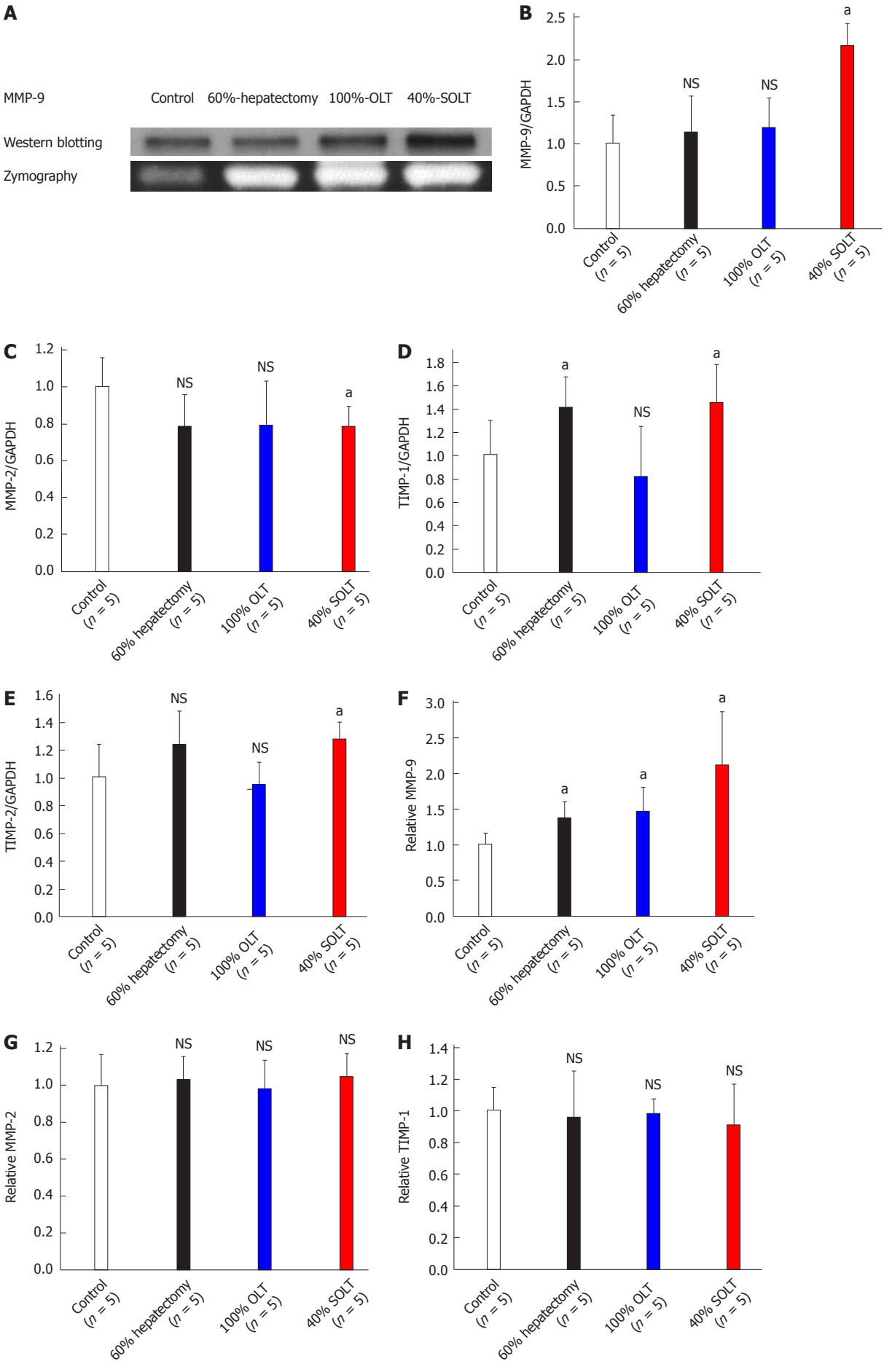
As described above, the data in comparisons with the controls are shown. Statistical differences between groups are summarized in Table 2.

DISCUSSION

In survival and histopathological studies, 40% SOLT involved dual damage (*i.e.*, shear stress with portal hypertension and CIWR injury) and showed the poorest survival and most severe liver damage. Although 100% OLT showed good survival, CIWR injury was observed by histopathological and biochemical findings. Here, we used plasma PT-INR and serum HA levels as markers of sinusoidal endothelial damage and all groups showed significant differences. Survival in the 60% hepatectomy and 40% SOLT groups seemed to be higher than in the 100% OLT group and this may reflect the damage induced by shear stress and portal hypertension. Our histopathological, immunohistological and biochemical findings revealed that liver damage and apoptotic induction were observed in the early postoperative period after liver surgery, as in previous studies[3,12,13,18,29-31]. Paradoxically, the early postoperative period may have a therapeutic potential for a subsequent course after liver surgery.

OS causes DNA damage and subsequent apoptosis and is an imbalance between production of free radicals and antioxidant defenses[9-11]. From the viewpoint of production of free radicals, ROS/RNS can attack and damage a variety of critical biological molecules, including lipids, essential cellular proteins and DNA[9-11]. Products of lipid peroxidation can be easily detected in biological fluids and tissues and can reliably and rapidly reflect the sensitive and specific signals of lipid peroxidation that occur *in vivo*[35,36]. The compound 4-HNE is an end product of lipoperoxidation with antiproliferative and proapoptotic properties[35,36]. Our results with MDA and 4-HNE confirmed that OS occurred even in the early postoperative period.

With regard to DNA damage responses, the protein kinase ATM can be initiated through rapid intermolecular autophosphorylation induced by DNA damage, phosphorylate various proteins and subsequently amplify the responses to DNA damage[36,37]. This DNA damage-inducible kinase activates H2AX[38]. H2AX is required for cell cycle arrest and DNA repair following double-stranded DNA breaks[38,39]. DNA damage results in the rapid phosphorylation of H2AX by ATM[38,40]. Within minutes of DNA damage, H2AX is phosphorylated at the sites of the DNA damage[38]. This early event in the DNA-damage response is required for the recruitment of many DNA-damage response proteins. Therefore, histone H2AX is activated by ATM after DNA dam-


A
MMP-9
Control
60%-hepatectomy
100%-OLT
40%-SOLT
Western blotting
Zymography
B
MMP-9/GAPDH
C
MMP-2/GAPDH
D
TIMP-1/GAPDH
E
TIMP-2/GAPDH
F
Relative MMP-9
G
Relative MMP-2
H
Relative TIMP-1
NS
a
Control (n = 5)
60% hepatectomy (n = 5)
100% OLT (n = 5)
40% SOLT (n = 5)

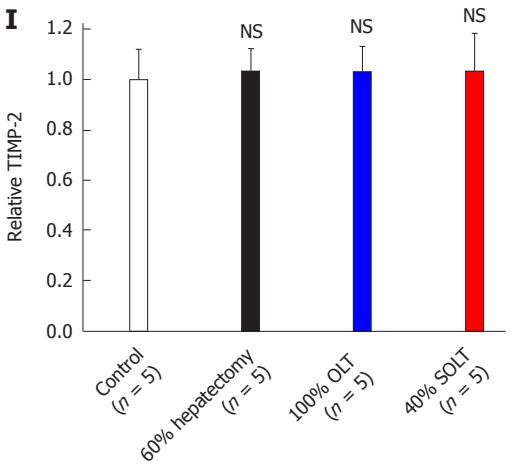


Figure 5 Protein expression and activities of matrix metalloproteinases and tissue inhibitor of metalloproteinases. A: Actual protein expression and activities of matrix metalloproteinase (MMP)-9; B: Normalized MMP-9; C: Normalized MMP-2; D: Normalized tissue inhibitor of metalloproteinase (TIMP)-1; E: Normalized TIMP-2; F: Relative MMP-9; G: Relative MMP-2; H: Relative TIMP-1; I: Relative TIMP-2. [a]$P < 0.05$ *vs* control. NS: Not significant ($P \geqslant 0.05$); OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation.

Table 2 Statistical differences between groups

	Control *vs* 60%-hepatectomy	Control *vs* 100%-OLT	Control *vs* 40%-SOLT	60%-hepatectomy *vs* 100%-OLT	60%-hepatectomy *vs* 40%-SOLT	100%-OLT *vs* 40%-SOLT
Survival rate	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Liver damage score	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
TUNEL positive ratio	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Caspase-3 positive ratio	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
AST	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
ALT	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS
T-Bil	NS	NS	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
PT-INR	$P < 0.05$	p <0.05	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
HA	$P < 0.05$	p <0.05	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Western blotting						
MDA	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	NS
4-HNE	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	NS	NS
ATM	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	NS	NS
γH2AX	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
PI3K	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Akt	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
SOD	NS	NS	NS	NS	NS	NS
Catalase	NS	NS	NS	NS	NS	NS
MMP-9	NS	NS	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$
MMP-2	NS	NS	$P < 0.05$	NS	NS	NS
TIMP-1	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
TIMP-2	NS	NS	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Zymography						
MMP-9	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	p <0.05
MMP-2	NS	NS	NS	NS	NS	NS
TIMP-1	NS	NS	NS	NS	NS	NS
TIMP-2	NS	NS	NS	NS	NS	NS

OLT: Orthotopic liver transplantation; SOLT: Split orthotopic liver transplantation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; T-Bil: Total bilirubin; PT-INR: International normalized ratio of prothrombin time; HA: Hyaluronic acid; MDA: Malondialdehyde; 4-HNE: 4-hydroxynonenal; ATM: Ataxia-telangiectasia mutated kinase; PI3K: Phosphatidylinositol 3-kinase; SOD: Superoxide dismutase; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase.

age[38]. Thus, the ATM/H2AX signaling pathway is important in the response to and repair of DNA damage induced by OS[38,41]. Our results with ATM and H2AX clearly showed that OS after liver surgery caused DNA damage signaling and triggered subsequent DNA repair. In this study, groups with only CIWR injury (*i.e.*, 100% OLT) caused OS-induced damage and subsequent apoptotic process. However, this group showed differences not in PI3K/Akt, but in ATM/H2AX. These results suggested that CIWR injury induce apoptosis due to OS *via* the ATM/H2AX pathway.

Akt also plays a critical role in controlling apoptosis

and promotes cell survival to prohibit apoptosis[42-44]. Apoptotic machinery is inhibited by the activation of Akt[42-44]. Akt is an integral component of the antiapoptotic process related to the activation of PI3K[42-45]. Our results clearly showed that groups with accompanying shear stress and portal hypertension (*i.e.*, 60% hepatectomy and 40% SOLT) had decreased PI3K and Akt. This suggested that a subsequent apoptotic process was triggered in these groups. Shear stress and portal hypertension due to insufficient liver volume induce apoptosis due to OS *via* the Akt/PI3K pathway.

With regard to antioxidant defense, scavenging enzymes of free radicals, such as SOD and catalase, also play an important role in reducing DNA damage and subsequent apoptosis[10,11]. Cells are normally able to defend themselves against OS-induced damage through this scavenging system[10,11]. Our results revealed that this scavenging system did not appear to be triggered, although these scavenging enzymes can cope with large amounts of ROS[46]. Shear stress with portal hypertension and/or CIWR injury after liver surgeries in this study caused considerable liver damage. A possible explanation is that this scavenging system failed to stimulate some reactive molecules because of considerable damage after liver surgery.

MMPs have been intensively studied and shown to play key roles in inflammation, carcinogenesis and regeneration and many researchers have already focused on MMP-2 and MMP-9 after liver surgery[12-21]. In the present study, 40% SOLT increased protein expression of MMP-2 in western blotting, although zymography did not show any differences. Contrary to MMP-2, postoperative MMP-9 clearly showed differences in protein expression and function. Additionally, MMP-9 showed high reproducibility in our previous studies[20,47,48]. The present results for MMP-9 suggested that MMP-9 clearly increased even in the early postoperative period after liver surgery and MMP-9 is a major therapeutic target after liver surgery.

TIMPs are also important after liver surgery. Many researchers have focused on TIMP-1 and TIMP-2 during liver regeneration[25-28]. Some researchers have focused on postoperative behavior of TIMP-1[28]. In particular, TIMP-1 has extrahepatic effects during liver failure[23,49-52] and therefore we initially expected that TIMP-1 would show differences in the liver samples. However, zymography for TIMP-1 did not show any differences, although groups with shear stress and portal hypertension (*i.e.*, 60% hepatectomy and 40% SOLT) showed increased protein expression of TIMP-1 in western blotting. TIMP-1 is an endogenous inhibitor of MMP-9 and a balance of MMP-9/TIMP-1 is linked[22,23]. However, the behavior of TIMP-1 in the postoperative liver is still unclear and further studies are required.

Liver damage and apoptotic induction are confirmed even in the early postoperative period after liver surgery but liver injury triggers the liver regeneration cascade after surgery. Once hepatic failure occurs after liver surgery, this damage is usually intractable and fatal. Therefore, the early postoperative period may be a suitable time for treatment to achieve a good postoperative course after liver surgery and our lab focused on OS-mediated damage and the behavior of extracellular matrices after liver surgery[20,48,51,53-56]. The inhibition of apoptotic induction due to OS *via* the ATM/H2AX pathway may be important for a strategy against CIWR injury, even in the condition of sufficient liver volume. Under conditions with insufficient liver remnant, the prevention of apoptotic induction due to OS *via* the Akt/PI3K pathway may be key to improving postoperative course. Also, MMP-9 may be a reliable therapeutic target, especially in the condition of CIWR injury with insufficient liver volume. We hope that our results will be informative for researchers in the hepatology field.

ACKNOWLEDGMENTS

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COMMENTS

Background

After liver surgery, shear stress with portal hypertension and cold ischemia/warm reperfusion injury trigger the liver regeneration cascade and also cause fatal liver damage.

Research frontiers

Changes and behaviors of oxidative stress and extracellular matrices are still unknown.

Innovations and breakthroughs

Here, the authors investigate the oxidative stress-mediated damage and the behavior of extracellular matrices after liver surgery in various rat models.

Applications

Under conditions with insufficient liver remnant, prevention of oxidative stress-induced damage *via* the Akt/PI3K pathway may be key to improve postoperative course. MMP-9 may be also a therapeutic target after liver surgery.

Terminology

Regulations for oxidative stress and MMP-9 may have a therapeutic potential, in order to resolve the current problems after liver surgery.

Peer review

This is a very interesting paper about the pathophysiology of hepatic failure after hepatectomy and liver transplantation.

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BRIEF ARTICLE

Disease dependent qualitative and quantitative differences in the inflammatory response to ascites occurring in cirrhotics

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Abstract

AIM: To assess differing patterns and levels of ascitic fluid cyctokine and growth factors exist between those with a high risk and low risk of spontaneous bacterial peritonitis (SBP).

METHODS: A total of 57 consecutive patients with ascites requiring a large volume paracentesis were studied. Their age, gender, specific underlying disease conditions were recorded after a review of their clinical records. Each underwent a routine assessment prior to their paracentesis consisting of a complete blood count, complete metabolic profile and prothrombin time/international normalized ratio (INR) determination. The ascitic fluid was cultured and a complete cell count and albumin determination was obtained on the fluid. In addition, blood and ascitic fluid was assessed for the levels of interleukin interleukin (IL)-1A, IL-1B, IL-2, IL-4, IL-8, IL-10, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) utilizing the Randox Biochip platforms (Boston, MA). A serum-ascites gradient, for each cytokine and growth factor was calculated. The results are reported as mean ± SEM between disease groups with statistical analysis consisting of the student *t*-test (two tailed) with a *P* value of 0.05 defining significance.

RESULTS: No clinically important demographic or biochemical differences between the 4 groups studied were evident. In contrast, marked difference in the cytokine and growth factors levels and pattern were evident between the 4 disease groups. Individuals with alcoholic cirrhosis had the highest levels of IL-1A, IL-1B, IL-4, IFNγ. Those with malignant disease had the highest levels of IL-2. Those with hepatitis C virus (HCV) associated cirrhosis had the highest value for IL-6, IL-8, IL-10, MCP-1 and VEGF. Those with cardiac disease had the highest level of TNF-α and EGF. The calculated serum- ascites gradients for the cardiac and malignant disease groups had a greater frequency of negative values signifying greater levels of IL-8, IL-10 and MCP-1 in ascites than did those with alcohol or HCV disease.

CONCLUSION: These data document important differences in the cytokine and growth factor levels in plasma, ascitic fluid and the calculated plasma - ascites fluid gradients in cirrhotics requiring a large volume paracentesis. These differences may be important in determining the risk for bacterial peritonitis.

Key words: Ascites; Cirrhosis; Growth factors; Inflammation; Procalcitonin

Core tip: Previous studies have examined factors relative to the pathogenesis of spontaneous bacterial peritonitis (SBP) in patients with cirrhosis of the liver. This study was designed to examine the role of cytokines in decompensated cirrhotics requiring a large-volume paracentesis for ascites management and to compare the biomarker responses present in both the plasma and ascitic fluid of cirrhotics of differing etiologies. Factors likely to represent protective cytokines associated with a reduced risk for SBP include epidermal growth factor, tumor necrosis factor-α, interleukin (IL)-1A, IL-8, and IL-10. Those are more likely to be associated with potential for SBP include: IL-1B, IL-4, monocyte chemotactic protein -1, and interfero-γ.

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INTRODUCTION

Large-volume ascites occurring in cirrhotic patients has been shown to manifest an inflammatory response characterized by increased levels of cytokines, interleukins and several growth factors[1]. The pathophysiologic mechanisms responsible for the development of cirrhosis and ultimately decompensated cirrhosis vary as a function of the underlying hepatic disease[2,3]. These differences in pathophysiology may be reflected in the cytokine, interleukin and growth factor induced response that occurs. Moreover, depending upon the site of inflammatory cell activation, differences in plasma and ascitic fluid levels of inducible cytokines and growth factors may exist. These differences may explain in part an increase rate of bacterial translocation and subsequent spontaneous bacterial peritonitis (SBP) development.

Previous studies have reported that cytokine characteristics of the Th1 response are increased in decompensated cirrhosis especially with infection[4]. Interleukin (IL)-4 which is a major cytokine of the Th2 response was not significantly different between decompensated cirrhotic patients with infected or non-infected ascites[4]. The current study was designed to confirm these findings and expanding it to study the role of growth factors in cirrhotics with non-infected ascites.

The aim of this investigation was: (1) to identify and quantitate the plasma and ascitic fluid biomarkers of inflammation in decompensated cirrhotics requiring a large-volume paracentesis for ascites management and (2) to compare and contrast the biomarker responses present in both the plasma and ascitic fluid of cirrhotics of differing etiologies.

MATERIALS AND METHODS

Subjects

A total of 57 consecutive cirrhotics requiring a large-volume paracentesis for clinical reasons were studied. Their age, gender and the specific disease etiology for their cirrhosis was determined by a review of their clinical records and, when necessary additional clinical testing procedures. Four distinct etiologic groups of cirrhotics were identified and the cytokine levels were compared between groups in an effort to examine the role of the etiologic factor responsible for cirrhosis in each subgroup.

Inclusion and exclusion criteria

Inclusion criteria: (1) cirrhosis documented by imaging (either an abnormal CT or US) or liver biopsy; (2) ascites requiring a large volume paracentesis because of tense ascites and failure to control the ascites with diuretics (furosemide and spironolactone); and (3) willingness to undergo a large volume paracentesis and sign an informed written consent documenting their participation and allowing for the additional studies required as a result of their participation.

Exclusion criteria: (1) no evidence for cirrhosis; (2) no ascites or adequate ascites control with diuretics; and (3) unwillingness to participate and sign an informed written consent.

Investigations

Each subject had the following routine laboratory studies determined: complete blood count, complete metabolic profile consisting of blood urea nitrogen, creatinine, glucose, total bilirubin, alkaline phosphatase, aspartate and alanine aminotransferases, total protein, albumin, and prothrombin time/INR. Each patient had a calculated Child-Turcotte-Pugh (CTP) score and the following studies were obtained on their ascitic fluid: cell counts for red blood cells, white blood cells and differential, albumin and ascitic fluid cultures. In addition to these routine measures, the plasma and ascitic fluid of each subject was assayed for a panel of biomarkers of inflammation to include the following: procalcitonin, IL-1A, IL-1B, IL-2, IL-4, IL-8, IL-10, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Procalcitonin was assayed utilizing the BioMerieux Vidas assay (Lombard, Illinois). The interleukins, inflammatory cytokines and growth factors were assayed utilizing Randox Biochip assay Platforms (Boston, Massachusetts). All results were compared to that of a normal human plasma panel utilized as a control sample which was obtained commercially from Bioreclamation, LLC (Liverpool, NY, United States). In addition, the levels of the analytes

Table 1 Characteristics of the 57 subjects' studies and of procalcitonin cytokines, and growth factors in plasma (mean ± SEM)

Parameter	ETOH	HCV	Malignancy	Cardiac
n	25	20	8	4
Male/female	18/7	14/6	5/3	2/2
CTP score	9.1 ± 0.2	8.2 ± 0.1	8.1 ± 0.1	8.1 ± 0.1
Laboratory tests				
Creatinine (mg/dL)	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.3	1.3 ± 0.2
Prothrombin time (s)	14.0 ± 0.4	13.8 ± 1.0	13.6 ± 0.2	12.5 ± 0.2
Total bilirubin (mg/dL)	1.4 ± 0.1	1.2 ± 0.2	1.4 ± 0.2	1.9 ± 0.3
Albumin (g/dL)	3.1 ± 0.2	3.2 ± 0.2	3.0 ± 0.4	3.2 ± 0.3
PCT	0.375 ± 0.215	0.440 ± 0.230	0.954 ± 0.242	0.092 ± 0.70
IL-1A	0.26 ± 0.146	0.160 ± 0.070	0.182 ± 0.106	0.135 ± 0.065
IL-1B	4.710 ± 2.252	1.747 ± 0.800	1.982 ± 0.106	1.610 ± 0.990
IL-2	2.498 ± 1.333	1.203 ± 0.548	2.690 ± 1.905	1.025 ± 0.375
IL-4	3.157 ± 1.429	2.580 ± 1.005	1.508 ± 0.422	1.430 ± 0.090
IL-6	83.791 ± 47.204	164.430 ± 70.891	105.392 ± 60.511	129.700 ± 112.500
IL-8	92.790 ± 44.935	334.513 ± 184.222	104.165 ± 61.670	16.415 ± 6.815
IL-10	1.335 ± 0.454	1.620 ± 0.779	1.117 ± 0.297	1.415 ± 0.615
MCP-1	111.139 ± 17.746	326.407 ± 137.768	116.052 ± 32.101	88.350 ± 35.150
IFNγ	1.148 ± 0.650	0.303 ± 0.058	0.712 ± 0.201	0.725 ± 0.525
TNF-α	3.887 ± 1.218	5.843 ± 2.248	4.805 ± 1.304	13.600 ± 11.80
EGF	37.451 ±11.642	92.453 ± 42.231	70.690 ± 36.431	126.00 ± 35.150
VEGF	11.658 ± 4.419	194.347 ± 130.788	20.523 ± 7.739	41.470 ± 32.870

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; PCT: Procalcitonin cytokines; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

present in plasma were compared against those in the patients' ascitic fluid. Plasma- Ascites gradients were calculated for each analyte and the mean for each disease group was calculated.

Human research approval

The IRB of Cook County Health and Hospital System approved this study prior to its initiation. Each subject signed an informed written consent before their participation in the study. Moreover, the Cook County Health and Hospital System funded the study in its entirety.

Statistical analysis

The mean and standard error of the mean for each parameter was determined and the differences between the means of the various disease groups studies were calculated utilizing the students *t*-test. A P value < 0.05 was considered to be significant.

RESULTS

The characteristics of the 57 subjects studied are shown in Table 1. No clinically important differences between the 4 disease groups studied were evident. In contrast, the procalcitonin levels in plasma varied substantially between groups with the greatest values being present in the group with malignancy (Table 1). The lowest procalcitonin values were seen in those with cardiac cirrhosis. The alcoholic and hepatitis C positive groups had plasma procalcitonin levels that were midway between these two extremes (Table 1).

The ascitic fluid procalcitonin levels mirrored the plasma levels with the greatest values being found in the group with malignancy and the lowest levels being present in those with cardiac disease. Again the other two groups had values midway between these two extremes (Table 2). Interestingly, however the alcoholic subgroup had an ascitic fluid procalcitonin value that was greater than that of the hepatitis C positive subgroup such that the relative position of the procalcitonin level in the two subgroups was reversed as compared to that found in plasma (Tables 1 and 2).

The cytokine and growth factor values varied markedly between groups for each parameter studied (Tables 3 and 4). The mean levels of the various factors measured in plasma aligned from the highest to the lowest for each disease group is reported in Table 3. Individuals with alcoholic liver disease had the highest IL-1a, IL-1b, IL-4 and interferon gamma levels. Individuals with malignant the liver disease had the highest values for IL-2. Those with hepatitis C had the highest levels IL-6, IL-8, IL-10, MCP-1 and VEGF.

The mean values for the ascitic fluid levels of the same 12 factors aligned from the highest to the lowest is presented in Table 4. The cardiac group had the greatest values for 5 of the 12 factors measured followed by the alcoholic group with 3 and the other 2 groups with 2 each. The malignancy group at the lowest value for 5 factors followed by the cardiac group with 3 and the other 2 groups with 2 each. Because of the variability in the measured values, the groups did not differ statistically, but when one examines the mean values per se considerable differences are seen between the various groups with mean plasma values ranging from 1.5-20 times the values of the lowest value for each parameter (Table 4). Similarly, when one examines the mean values in the ascitic fluid, the range of values for a given factor between groups ranged from 1.3-10 times the value

Table 2 Procalcitonin, cytokines and growth factors in the ascitic fluid (mean ± SEM)

	ETOH	HCV	Malignancy	Cardiac
n	25	20	8	4
PCT	0.221 ± 0.129	0.125 ± 0.115	0.647 ± 0.497	0.043 ± 0.033
IL-1A	0.168 ± 0.042	0.200 ± 0.058	0.153 ± 0.052	0.700 ± 0.400
IL-1B	7.479 ± 4.813	4.3 ± 1.193	4.900 ± 1.021	5.000 ± 0.300
IL-2	1.368 ± 1.628	0.667 ± 0.067	1.990 ± 1.044	0.650 ± 0.050
IL-4	9.268 ± 1.628	15.833 ± 4.932	6.533 ± 0.984	10.555 ± 4.85
IL-6	687.177 ± 30.115	807.764 ± 0.867	707.525 ± 54.339	790.05 ± 39.750
IL-8	338.015 ± 91.838	329.567 ± 91.926	229.200 ± 105.057	718.25 ± 80.150
IL-10	74.686 ± 39.663	15.200 ± 3.5	9.043 ± 3.876	76.500 ± 44.700
MCP-1	919.608 ± 41.636	537.600 ± 88.357	576.728 ± 206.393	421.900 ± 82.6
IFNγ	2.114 ± 0.671	0.467 ± 0.267	1.488 ± 0.838	0.2 ± 0.1
TNF-α	22.818 ± 8.882	30.433 ± 20.055	9.672 ± 1.786	70.950 ± 61.750
EGF	1.318 ± 0.345	0.967 ± 0.167	2.407 ± 0.465	4.9 ± 3.4
VEGF	54.383 ± 12.143	129.733 ± 63.589	689.12 ± 499.836	119.450 ± 117.950

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; PCT: Procalcitonin cytokines; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

Table 3 Mean levels of the plasma factors aligned from the highest to the lowest for each disease group

	Highest values → Lowest values			
IL-1A	ETOH	Malignancy	HCV	Cardiac
	0.260	0.182	0.160	0.135
IL-1B	ETOH	Malignancy	HCV	Cardiac
	4.710	1.082	1.747	1.61
IL-2	Malignancy	ETOH	HCV	Cardiac
	2.690	2.498	1.203	1.025
IL-4	ETOH	HCV	Malignancy	Cardiac
	3.157	2.580	1.508	1.43
IL-6	HCV	Cardiac	Malignancy	ETOH
	164.430	129.700	109.392	83.791
IL-8	HCV	Malignancy	ETOH	Cardiac
	334.513	104.265	92.790	16.415
IL-10	HCV	Cardiac	ETOH	Malignancy
	1.620	1.415	1.335	1.117
MCP-1	HCV	Malignancy	ETOH	Cardiac
	326.407	116.052	111.130	88.35
IFN-γ	ETOH	Cardiac	Malignancy	HCV
	1.148	0.725	0.712	0.303
TNF-α	Cardiac	HCV	Malignancy	ETOH
	13.600	5.843	4.805	3.887
EGF	Cardiac	HCV	Malignancy	ETOH
	126.000	92.453	70.690	37.451
VEGF	HCV	Cardiac	Malignancy	ETOH
	194.347	41.470	20.523	11.658

HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

of the lowest value (Table 5). The plasma-ascitic fluid gradients for each parameter were determined and are reported in (Table 5). A positive value for the plasma-ascitic fluid gradient identifies those factors wherein the plasma level was greater than the ascitic fluid level. In contrast, a negative value for the plasma-ascitic fluid gradient identifies those factors wherein the greater value was present in the ascitic fluid. A positive value suggests that the cytokine assayed arose from a systemic response while a negative value suggests that the response arose primarily in the abdominal cavity and that either a peritoneal or mesenteric origin for the cytokine.

DISCUSSION

This study extends the finding of an earlier study evaluating cytokine, and growth factor levels in the plasma and ascitic fluid of cirrhotics[1]. In both studies, the inflammatory cytokines IL-4, IL-6, IL-8, IL-10, TNF-α and MCP-1 have been shown to be increased in both the ascitic fluid and plasma of cirrhotics with large volume ascites. The present study performed in a completely different and slightly larger patient population extends the earlier study by documenting differences in the cytokine profiles based on the individuals underlying disease etiol-

Table 4 Mean values of the various factors in the ascitic fluid aligned from the highest to the lowest

	Highest values → Lowest values			
IL-1A	Cardiac	HCV	ETOH	Malignancy
	0.700	0.200	0.168	0.153
IL-1B	ETOH	Cardiac	Malignancy	HCV
	7.479	5.000	4.900	4.300
IL-2	Malignancy	ETOH	HCV	Cardiac
	1.990	1.368	0.667	0.650
IL-4	HCV	Cardiac	ETOH	Malignancy
	15.833	10.555	9.268	6.533
IL-6	HCV	Cardiac	Malignancy	ETOH
	807.764	790.050	707.525	687.177
IL-8	Cardiac	ETOH	HCV	Malignancy
	718.250	338.015	329.567	229.200
IL-10	Cardiac	ETOH	HCV	Malignancy
	76.500	74.686	15.200	9.043
MCP-1	ETOH	Malignancy	HCV	Cardiac
	919.608	576.728	537.600	421.900
IFN-γ	ETOH	Malignancy	HCV	Cardiac
	2.114	1.488	0.467	0.200
TNF-α	Cardiac	HCV	ETOH	Malignancy
	70.950	30.433	22.818	9.672
EGF	Cardiac	Malignancy	ETOH	HCV
	4.900	2.407	1.318	0.967
VEGF	Malignancy	HCV	Cardiac	ETOH
	689.120	129.733	119.456	54.383

HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

Table 5 Mean plasma- ascitic cytokine gradients segregated by disease etiology

	HCV	ETOH	Malignancy	Cardiac
IL-1A	-0.040	0.092	0.029	-0.565
IL-1B	-2.553	-2.769	1977.100	-3.390
IL-2	0.540	1.530	0.700	0.875
IL-4	-13.253	-6.111	-5.025	-9.125
IL-6	-643.337	-603.386	-602.133	-639.35
IL-8	-5.054	-245.225	-125.035	-701.835
IL-10	-13.580	-73.331	-7.926	-75.085
MCP-1	-201.193	-303.469	-460.673	-333.35
IFN-γ	-0.164	-0.966	-0.776	0.525
TNF-α	-24.590	-18.931	-4.777	57.050
EGF	96.486	36.176	-68.283	121.100
VEGF	64.614	-42.725	-668.649	-77.980

No significant difference between any of these groups $P > 0.05$. HCV: Hepatitis C virus; IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor.

ogy for the cirrhosis[1]. The current data suggests therefore that the pathophysiologic responses to the various hepatic disease etiologies in some way may determine, at least in part, the innate immune responses that occur and account for the differences in the cytokine and growth factor levels in the ascitic fluid and plasma[2,3]. The IL-6 and MCP-1 levels were universally increased in all four cirrhotic groups. VEGF levels were increased most markedly in those with malignancy and to a lesser degree in those with cardiac and alcohol induced disease. Individuals with cirrhosis due to hepatitis C had the lowest VEGF levels. In contrast, the hepatitis C positive group had the greatest levels of IL-4 present in both plasma and ascitic fluid.

The finding of an increase in VEGF levels in cirrhotics with malignancy is interesting but not particularly surprising as malignant disorders are known to be associated with increased VEGF levels[5,6]. The increase of VEGF levels in cardiac and alcohol induced liver disease is surprising and differs markedly from that seen in those with hepatitis C. This observation is consistent with the data reported in other studies wherein increased organ remodeling has been observed in individuals with cardiac and alcohol related disease but not so in those with hepatitis C virus[2,3].

These data also support the role of the peritoneal based immune response in the pathogenesis of both bacterial translocations spontaneous bacterial peritoni-

tis[1,7-14]. More specifically, they are consistent with the clinical observations that spontaneous bacterial peritonitis occurs less frequently in patients with cardiac and malignant ascites as contrasted to those with alcoholic liver disease and chronic viral induced liver disease.

As shown in Table 4, cardiac disease associated ascites has the highest ascetic fluid levels of IL-1A, IL-8, IL-10, TNF-α, and EGF. Conversely, the cardiac disease associated ascites has the lowest levels of IL-2 and MCP-1. Those with malignancy associated ascites have the highest levels of IL-2 and VEGF and the lowest levels of IL-1A, IL-4, IL-8, IL-10, and TNF-α.

The present findings for these two distinct etiologic groups suggest that the ascitic fluid immune response manifested in the ascitic fluid may account in some way for the lower rate of spontaneous bacterial peritonitis in individuals with ascites due to these two unique causes of cirrhosis.

Factors likely to represent protective cytokines associated with a reduced risk for SBP include EGF, TNF-α, IL-1A, IL-8, and IL-10. Those are more likely to be associated with potential for SBP include: IL-1B, IL-4, MCP-1, and IFN-γ (Table 4).

The data shown in Table 5 consisting of the serum-ascites gradient enables one to determine whether the primary source of the measured factor arose from the vascular space or the peritoneal cavity. Specifically, those with the positive value identify a primary vascular source of the measured factor while a negative value identifies these factors having their origin in the peritoneal cavity.

In summary, the present data suggest the well-recognized factors that include a reduced plasma oncotic pressure, increased splanchnic venous congestion and pressure, increased vascular permeability and an overwhelmed lymphatic mechanism for removing ascitic fluid account substantially for the development of clinical ascites. They suggest that unique immune related responses that differ between various hepatic disease states may also contribute to the development of ascites and the likelihood of developing spontaneous bacterial peritonitis. Further, these data suggest further that a better understanding of the different immune response characteristics present in cirrhotics of different etiologies may enable disease specific modulation of the immune response in each and thereby contribute to the development of improved therapies that control not only to the development of ascites but also overall disease progression.

COMMENTS

Background

Large-volume ascites occurring in cirrhotic patients has been shown to manifest an inflammatory response characterized by increased levels of cytokines, interleukins and several growth factors. The pathophysiologic mechanisms responsible for the development of cirrhosis and ultimately decompensated cirrhosis vary as a function of the underlying hepatic disease.

Research frontiers

This study extends the finding of an earlier study evaluating cytokine, and growth factor levels in the plasma and ascitic fluid of cirrhotics.

Innovations and breakthroughs

The current data suggests therefore that the pathophysiologic responses to the various hepatic disease etiologies in some way may determine, at least in part, the innate immune responses that occur and account for the differences in the cytokine and growth factor levels in the ascitic fluid and plasma.

Applications

The authors suggest that unique immune related responses that differ between various hepatic disease states may also contribute to the development of ascites and the likelihood of developing spontaneous bacterial peritonitis.

Peer review

The authors performed plasma and ascitic cytokines among various etiologies of cirrhosis.

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BRIEF ARTICLE

Association between inherited monogenic liver disorders and chronic hepatitis C

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Abstract

AIM: To determine the frequencies of mutations that cause inherited monogenic liver disorders in patients with chronic hepatitis C.

METHODS: This study included 86 patients with chronic hepatitis C (55 men, 31 women; mean age at diagnosis, 38.36 ± 14.52 years) who had undergone antiviral therapy comprising pegylated interferon and ribavirin. Viral load, biochemical parameter changes, and liver biopsy morphological data were evaluated in all patients. The control group comprised 271 unrelated individuals representing the general population of Latvia for mutation frequency calculations. The most frequent mutations that cause inherited liver disorders [gene (mutation): *ATP7B* (H1069Q), *HFE* (C282Y, H63D), *UGT1A1* (TA)7, and *SERPINA1* (PiZ)] were detected by polymerase chain reaction (PCR), bidirectional PCR allele-specific amplification, restriction fragment length polymorphism analysis, and sequencing.

RESULTS: The viral genotype was detected in 80 of the 86 patients. Viral genotypes 1, 2, and 3 were present in 61 (76%), 7 (9%), and 12 (15%) patients, respectively. Among all 86 patients, 50 (58%) reached an early viral response and 70 (81%) reached a sustained viral response. All 16 patients who did not reach a sustained viral response had viral genotype 1. Case-control analysis revealed a statistically significant difference in only the H1069Q mutation between patients and controls (patients, 0.057; controls, 0.012; odds ratio, 5.514; 95%CI: 1.119-29.827, P = 0.022). However, the H1069Q mutation was not associated with antiviral treatment outcomes or biochemical indices. The (TA) 7 mutation of the *UGT1A1* gene was associated with decreased ferritin levels (beta regression coefficient = -295.7, P = 0.0087).

CONCLUSION: Genetic mutations that cause inherited liver diseases in patients with hepatitis C should be studied in detail.

Key words: Hepatitis C; Hepatolenticular degeneration (Wilson's disease); *ATP7B*; *SERPINA1*; *UGT1A1*; *HFE*

Core tip: This is the first study to evaluate the association between hepatitis C and the most frequently inherited monogenic liver diseases (hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease) and their causative mutations. This case-control study revealed an association between hepatitis C and the mutation that causes Wilson's disease. In addition, biochemical data analysis

revealed an association between hepatitis C and the mutation that causes Gilbert's syndrome.

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INTRODUCTION

Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV), which primarily affects the liver. An estimated 130 to 200 million people worldwide are infected with HCV[1]. The most common monogenic inborn errors of metabolism associated with liver disease are hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Wilson's disease, and Gilbert's syndrome. These diseases have a particularly high frequency in Northern Europe and Latvia[2-4]. Hereditary hemochromatosis is characterized by excessive iron overload and is most commonly caused by *HFE* gene mutations[2]. The frequency of the most common *HFE* mutation, C282Y, is 0.035 in Latvia and 0.026 Lithuania; however, the frequency of hereditary hemochromatosis is lower at 0.013[5]. Alpha-1 antitrypsin deficiency is caused by the absence of the proteinase inhibitor alpha-1 antitrypsin, and affected patients develop liver disease and emphysema in the third or fourth decade of life[3]. Wilson's disease is a progressive autosomal recessive disorder of copper metabolism. The carrier frequency of the causative mutation is 1:80 in Latvia and 1:90 in Europe[4]. Finally, Gilbert's syndrome is characterized by benign unconjugated hyperbilirubinemia with a frequency of 5.0% to 14.8% in Europe[6]. Most reports on the coexistence of monogenic liver diseases and HCV infection have focused primarily on hereditary hemochromatosis[7] because elevated iron levels are necessary for viral replication[8,9]. Although the associations of HCV infection with alpha-1 antitrypsin deficiency[10,11] and Gilbert's syndrome[12-14] have been investigated, the association of HCV infection with Wilson's disease remains unclear. Copper reportedly plays a potential role in the development of HCV infection[15,16].

The aim of the present study was to determine the frequency of mutations that cause inherited monogenic liver disorders in patients with chronic HCV infection who have undergone antiviral therapy and in whom the viral response status is known.

MATERIALS AND METHODS

Ethics

This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the *Central Medical Ethics Committee* of *Latvia*. All study participants signed an informed consent form that was issued according to the regulations of the *Central Medical Ethics Committee* of *Latvia*.

Subjects

Eighty-six patients with HCV infection who had undergone antiviral treatment with ribavirin and pegylated interferon were included in this study. These patients comprised 55 men and 31 women with a mean age at diagnosis of 38.36 ± 14.52 years (men, 37.27 ± 15.69 years; women, 40.25 ± 12.26 years). All patients were of European descent. The pretherapeutic alanine transaminase level, iron level, ferritin level, viral load, and HCV genotype were evaluated in all patients. The Knodell histology activity index was used for morphological examination.

The control group comprised 271 unrelated individuals chosen to represent the general population of Latvia. Participants in the control group underwent polymorphism frequency determination only. Biochemical association analysis, clinical examination, and exclusion of HCV infection were not performed in this group.

Genotyping methods

Peripheral blood genomic DNA was purified by standard phenol: chloroform extraction and ethanol precipitation with slight modification as described elsewhere[17] using reagents from Sigma Aldrich, Inc. (St. Louis, MO, United States). A summary of the methods used in this study is presented in Table 1[4,18-20]. Reagents used for polymerase chain reaction (PCR) (buffers, dNTP mix, Taq polymerase, and agarose) were obtained from Thermo Fisher Scientific (Waltham, MA, United States). Synthetic oligonucleotides, the sequences of which have been previously published[4,18-20], were obtained from Metabion GmbH (Martinsried, Germany). Fluorescent PCR products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, United States) using the reagents described in the manufacturer's protocol.

Statistical analysis

PLINK software[21] was used for genotyping data analysis and quality control. Analysis adhered to a call rate of < 98% and Hardy-Weinberg equilibrium P value of $\leqslant$ 0.05. The chi-square test was used to compare the patient and control groups with a significance threshold of $P < 0.05$. SPSS software v.16.0 (SPSS Inc., Chicago, IL, United States) was used to compare mean biochemical marker values between the patient and control groups and between the two patient groups [with and without a sustained viral response (SVR), defined as the inability to detect viral RNA six months after therapy[22]. Parametric values were compared using ANOVA, and nonparametric data were evaluated with the Mann-Whitney test. Genotype association analysis with biochemical markers was conducted using a full linear model comprising

Table 1 Genotyping methods used in the present study

Disease	Gene	Mutation	rs[1]	Analysis method
Hereditary hemochromatosis	*HFE*	C282Y	rs1800562	PCR-RFLP with restrictase *RsaI*[18]
		H63D	rs1799945	PCR-RFLP with restrictase *MboI*[18]
Gilbert's syndrome	*UGT1A1*	(TA)7, UGT1A1*28	rs8175347	Fluorescent PCR[20]
Alpha-1 antitrypsin deficiency	*SERPINA1*	PIZ	rs28929474	Bi-PASA[19]
Wilson's disease	*ATP7B*	H1069Q	rs76151636	Bi-PASA[4]

[1]Single nucleotide polymorphism database number (http://www.ncbi.nlm.nih.gov/snp/). PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism analysis; Bi-PASA: Bidirectional PCR allele-specific amplification.

Table 2 Allelic frequencies in patient and control groups

Gene	rs[1]	Mutation	Patients (n = 86)	Controls (n = 271)	OR	95%CI	P value
UGT1A1	rs8175347	(TA)7	0.371	0.350	1.098	0.643-1.871	0.796
HFE	rs1799945	C282Y	0.048	0.035	1.420	0.358-5.218	0.522
	rs1800562	H63D	0.096	0.121	0.740	0.301-1.760	0.563
ATP7B	rs76151636	H1069Q	0.057	0.012	5.514	1.119-29.827	0.022
SERPINA1	rs28929474	PIZ	0.012	0.016	0.363	0.013-5.158	0.576

[1]Single nucleotide polymorphism database number (http://www.ncbi.nlm.nih.gov/snp/). OR: Odds ratio.

three genetic effects: additive effects of allele dosage, dominance deviation from additivity (a negative value indicates a recessive allele), and the 2-df joint test of both additive and dominance. Beta was evaluated as the regression coefficient. Data were accepted as statistically significant at a P value of < 0.05. Sex and age (with confirmed HCV infection) were used separately as covariates. The adjusted beta coefficient and P value were applied for each covariate. The association of the HCV genotype with the therapy response was assessed using the χ^2 test.

RESULTS

Viral RNA was not detectable in 50 (58%) of the 86 patients (30 men, 20 women) in the third month of antiviral therapy. Sixteen (19%) patients (13 men, 3 women) did not reach an SVR. The HCV genotype was determined in 80 patients; genotypes 1, 2, and 3 were present in 61, 7, and 12 patients, respectively. All patients who did not reach an SVR had viral genotype 1; for this reason, the odds ratio (OR) and 95%CI were not calculated ($P = 0.015$).

Genetic marker analysis revealed a significantly higher frequency of the *ATP7B* H1069Q mutation in patients than in controls (0.057 *vs* 0.012, respectively; OR = 5.514; 95%CI: 1.119-29.827, $P = 0.022$). Further results of the genetic marker analysis are shown in Table 2.

The presence of inherited liver disease was confirmed in nine patients (eight had Gilbert's syndrome with genotype (TA)7/(TA)7, and one had hereditary hemochromatosis with genotype C282Y/H63D). The presence of inherited liver disease was confirmed in 30 (11%) controls; all had Gilbert's syndrome.

In the comparison of patients who had reached an SVR with those who had viral persistence (*i.e.*, negative response to treatment), a significant association was found between the iron level and the presence of viral persistence (Table 3). Neither the other biochemical markers nor the histology activity index showed statistically significant differences between the patient and control groups.

In the patient group, association analysis was performed between genetic markers and the biochemical markers alanine transaminase level, ferritin level, iron level, and viral load. A statistically significant association was found only between the ferritin level and the (TA)7 allele of the *UGT1A1* gene. The strongest model for the association of the *UGT1A1* gene with the ferritin level was dominance deviation from additivity (beta = -295.7, $P = 0.0087$), and the statistical significance remained after adjusting for age ($\text{beta}_{\text{adjusted}} = -264.4$, $P_{\text{adjusted}} = 0.0219$) and sex ($\text{beta}_{\text{adjusted}} = -249.3$, $P_{\text{adjusted}} = 0.0305$). The associations between the other biochemical indices and genetic markers were not statistically significant for any of the analyzed models.

DISCUSSION

Numerous studies have been conducted to identify host and viral factors that influence antiviral therapy efficiency in patients with HCV infection. Approximately 40% to 50% of individuals with viral genotype 1 and 80% with genotypes 2 and 3 reach an SVR[1]. Compared with these previously reported rates, a higher number of patients with genotype 1 in the present study reached SVR. In addition, all patients with genotypes 2 and 3 reached an SVR. These differences between our study results and those in the literature are likely due to our small patient group and relatively young patient age (38.36 ± 14.52 years) because increasing age is a risk factor for ineffective therapy[22]. Various risk factors are reportedly

Table 3 Characterization of the patient group

	Result of antiviral therapy	Mean	95%CI of mean		*P* value
			Lower bound	Upper bound	
Age in year at diagnosis	Sustained viral response	38.76	34.88	42.64	0.788
	Viral persistence	37.87	32.07	43.66	
	Total	38.43	35.24	41.61	
Alanine transaminase level	Sustained viral response	106.45	81.73	131.17	0.056
	Viral persistence	153.75	104.27	203.23	
	Total	123.65	99.86	147.44	
Iron level	Sustained viral response	20.84	18.19	23.49	0.015
	Viral persistence	29.92	22.05	37.80	
	Total	24.71	20.98	28.45	
Ferritin level	Sustained viral response	298.67	185.52	411.82	0.354
	Viral persistence	397.79	197.96	597.62	
	Total	336.09	231.98	440.20	
Viral load	Sustained viral response	1.91E + 06	1.12E + 06	2.70E + 06	0.115
	Viral persistence	4.21E + 06	5.15E + 05	7.90E + 06	
	Total	2.75E + 06	1.35E + 06	4.14E + 06	

associated with an individual patient's response to antiviral treatment, including the homocysteine level, vitamin D level, and many other parameters[23,24]. However, only sex, age, liver disease progression, viral genotype, and insulin resistance are included in the clinical guidelines as possible risk factors[22]. In the present study, the only markers that significantly influenced the efficacy of antiviral therapy were the alanine transaminase level (P = 0.056) and the iron level (P = 0.015). Iron is necessary for the replication of HCV; however, iron depletion therapy before antiviral therapy has not been proven to be effective[25]. The small size of our patient group is the main reason why the other data did not show a statistically significant impact on the efficacy of antiviral therapy.

Of all mutations that cause inherited liver diseases, the most extensively studied are those that cause hereditary hemochromatosis[26]. Although we did not detect a statistically significant association between HCV infection and the C282Y or H63D mutation in our study, the C282Y mutation was found to be more common in the patient group than in the control group (frequency of 0.048 *vs* 0.035, respectively) (Table 2). We also failed to detect an association between the iron or ferritin level with either the C282Y or H63D mutation. This result may have been due to our small patient group and/or the ages of our patients (men, 37.27 ± 15.69 years; women, 40.25 ± 12.26 years). Our patients may have been too young to manifest the symptoms characteristic of hereditary hemochromatosis because symptoms related to iron overload usually appear between the ages of 40 and 60 years in men and after menopause in women[27]. In addition, the higher serum iron levels seen in our patients with HCV infection may have been caused by various factors other than *HFE* gene mutations; *e.g.*, hepatocyte necrosis or increased intestinal iron uptake[28].

In contrast to previous reports[10,11], we did not detect an association between HCV infection and alpha-1 antitrypsin deficiency. Again, this may have been due to the small number of patients in our study and/or the fact that liver symptoms in patients with alpha-1 antitrypsin deficiency more commonly manifest in childhood or late adulthood. Advanced liver disease generally occurs around the age of 66 years in individuals heterozygous for the PIZ mutation[29]. The mean age of our patients was 38.43 years at the completion of analysis.

Gilbert's syndrome, also termed benign hyperbilirubinemia[30], was included in our study because an estimated 10% to 15% of European descent individuals are affected by this syndrome and because previous data have demonstrated anti-inflammatory and antioxidant functions of bilirubin[31,32]. The (TA)7 polymorphism of the *UGT1A1* gene was shown to be significantly associated with the ferritin level (beta = -295.7, P = 0.0087). Some studies have proposed that ferritin, being an acute-phase reactant, behaves as a marker of more active and advanced liver disease. Patients with chronic HCV infection and high serum ferritin levels reportedly have significantly more severe liver inflammation and fibrosis than do patients with normal serum ferritin levels[7,33]. In our study, patients with viral persistence had slightly elevated ferritin levels. Based on the analysis of the association of the ferritin level with genetic markers, the (TA)7 polymorphism could be associated with less prominent liver inflammation and lower ferritin levels in patients with HCV infection. This may in turn lead to a better antiviral treatment response, and future studies should address this notion. Our results also support the idea that more extensive liver inflammation can lead to viral persistence as evidenced by the fact that alanine transaminase levels were higher in patients with viral persistence.

Interestingly, the H1069Q mutation of the *ATP7B* gene was found to be associated with chronic HCV infection. We included this mutation in our analysis because a high rate of Latvians reportedly carry this mutation[4]. The *ATP7B* gene is involved in copper metabolism. Copper is well known to be critical for the proper functioning of both the humoral and innate immune systems; however, its precise mechanisms of action are unknown[34]. The spontaneous elimination or

persistence of HCV infection depends on the host's immune status[35]. Previous reports have stated that the host response to HCV infection may be primarily dependent on the human leukocyte antigen system. However, other factors, such as copper, may also influence the host response because changes in copper levels in patients with HCV infection have been reported[15,36,37]. We propose that the H1069Q mutation of the *ATP7B* gene may be an important modifier in patients with HCV infection. Future studies should investigate this in detail, especially considering the fact that Wilson's disease is treatable.

The main limitation of our study was the small number of patients and controls. No analysis was performed to exclude HCV infection in the control group. However, this was a pilot study. Research involving larger numbers of patients and controls in whom HCV infection has been excluded is warranted.

COMMENTS

Background

Inherited monogenic liver diseases and their causative mutations may represent genetic factors responsible for changing the host response to hepatitis C virus (HCV) infection. Although the association between HCV infection and hereditary hemochromatosis has been extensively studied, only a few studies on the associations between HCV and the mutations causing alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease have been performed.

Research frontiers

Mutations that cause inherited liver diseases are highly distributed and associated with chronic inflammation and liver damage. This is one of the critical points in HCV infection.

Innovations and breakthroughs

Many studies have been performed in an attempt to identify host genetic factors that can influence the efficacy of antiviral therapy in patients with chronic HCV infection. This is first study to analyze all of the most common genetic disorders in one patient group. The results of this pilot study show that this research should be continued with a larger group of patients.

Applications

Therapy for inherited liver disorders is either already available or is currently under investigation. If the importance of such therapies with respect to alleviating liver damage in HCV infection is proven, the efficacy of antiviral therapy may be improved by establishing treatment that is more specifically targeted not only to the viral life cycle, but also to factors directly associated with the development of liver damage. Alpha-1 antitrypsin deficiency is a liver disease caused by the absence of the proteinase inhibitor alpha-1 antitrypsin. Wilson's disease is a progressive autosomal recessive disorder of copper metabolism. Gilbert's syndrome is characterized by benign unconjugated hyperbilirubinemia.

Terminology

Hepatitis C is an infectious liver disease caused by the HCV, that affects an estimated 130 to 200 million people worldwide. Inherited monogenic liver disorders are inherited diseases, caused by mutations in one gene (autosomal recessive inheritance), in which the primary manifestation is liver damage.

Peer review

This case-control study is the first to examine the association between HCV and frequently inherited monogenic liver diseases (hereditary hemochromatosis, alpha-1 antitrypsin deficiency, Gilbert's syndrome, and Wilson's disease) and their causative mutations. This study revealed an association between HCV and the mutation responsible for Wilson's disease. Biochemical experiments revealed an association between HCV and the mutation that causes Gilbert's syndrome. This is a well-designed study that brings new insight into the association between inherited liver diseases and HCV.

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BRIEF ARTICLE

Methylsulfonylmethane suppresses hepatic tumor development through activation of apoptosis

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Abstract

AIM: To investigate the effect of methylsulfonylmethane (MSM), recently reported to have anti-cancer effects, in liver cancer cells and transgenic mice.

METHODS: Three liver cancer cell lines, HepG2, Huh7-Mock and Huh7-H-*ras*^{G12V}, were used. Cell growth was measured by Cell Counting Kit-8 and soft agar assay. Western blot analysis was used to detect caspases, poly (ADP-ribose) polymerase (PARP), and B-cell lymphoma 2 (Bcl-2) expressions. For *in vivo* study, we administered MSM to H-*ras*^{12V} transgenic mice for 3 mo.

RESULTS: MSM decreased the growth of HepG2, Huh7-Mock and Huh7-H-*ras*^{G12V} cells in a dose-dependent manner. That was correlated with significantly increased apoptosis and reduced cell numbers in MSM treated cells. Cleaved caspase-8, cleaved caspase-3 and cleaved PARP were remarkably increased in the liver cancer cells treated with 500 mmol/L of MSM; however, Bcl-2 was slightly decreased in 500 mmol/L. Liver tumor development was greatly inhibited in the H-*ras*^{12V} transgenic mice treated with MSM, compared to control, by showing reduced tumor size and number. Cleaved PARP was significantly increased in non-tumor treated with MSM compared to control.

CONCLUSION: Liver injury was also significantly attenuated in the mice treated with MSM. Taken together, all the results suggest that MSM has anti-cancer effects through inducing apoptosis in liver cancer.

Key words: Methylsulfonylmethane; Anti-cancer effects; Liver cancer cells; Transgenic mice; Hepatic tumorigenesis

Core tip: Methylsulfonylmethane (MSM) is an organic sulfur-containing compound. MSM suppressed hepatic tumor growth through activation of apoptosis. MSM could be a potential candidate as an anti-liver cancer agent.

Kim JH, Shin HJ, Ha HL, Park YH, Kwon TH, Jung MR, Moon HB, Cho ES, Son HY, Yu DY. Methylsulfonylmethane suppresses hepatic tumor development through activation of apoptosis. *World J Hepatol* 2014; 6(2): 98-106 Available from:

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INTRODUCTION

Liver cancer is the sixth most common malignancy and the third most common cause of cancer-related mortality worldwide[1]. Approximately 560000 cases are diagnosed each year and 550000 deaths are due to liver cancer. In most countries, 75%-90% of liver cancers are hepatocellular carcinomas[2]. The main risk factors of liver cancer include infection with hepatitis B virus (HBV) or hepatitis C virus (HCV)[3]. Other risk factors include excessive alcohol consumption, nonalcoholic steatohepatitis, autoimmune hepatitis, primary biliary cirrhosis, particularly aflatoxin B and various genetic metabolic diseases[4]. However, mortality is diminishing with the development of vaccine and therapy methods. In particular, therapy using natural extracts with no side effects has been reported. It was recently reported that tetrandrine induces apoptosis in human hepatocellular carcinoma[5] and berbamine induces apoptosis and tumor growth inhibition[6].

Apoptosis is a physiological process for involution and atrophy of various tissues and organs during development and maintenance of tissue homeostasis[7]. The apoptosis pathway is mediated by death receptors that include tumor necrosis factor receptor (TNFR), Fas and TNF-related apoptosis-inducing ligand (TRAIL). These ligands lead to the recruitment and activation of initiator cysteine aspartic proteases (caspases) such as caspases-8 and 10. These lead to the activation of caspase-3. The active caspase-3 involves DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and biochemical changes[8]. Otherwise, apoptosis is initiated by the stress-mediated release of cytochrome-c. The cytochrome-c activates initiator caspase, typically caspase-9, which leads to the activation of the executioner caspase-3. In response to apoptotic stimuli, pro-apoptotic members of the B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) become activated and act on the mitochondria to induce the release of cytochrome-c[8].

Methylsulfonylmethane (MSM), an organic sulfur-containing compound, inhibits LPS-induced release of pro-inflammatory mediators in murine macrophages through downregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling[9]. Moreover, the effect of MSM has been reported in cancer. MSM suppresses breast cancer growth by down-regulating signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 5b (STAT5b) pathways[10]. Apoptotic effects in other cancer cells, such as esophageal, gastric and liver cancer cells, were reported[11].

To further understand the effect of MSM in prevention of hepatic tumorigenesis, we have examined it in liver cancer cell lines and liver cancer mouse model.

MATERIALS AND METHODS

Cell culture and stable cell lines

HepG2 and Huh7 cell lines were maintained in a DMEM (HyClone, United States), supplemented with a 10% FBS (HyClone, United States), penicillin/streptomycin (HyClone, United States) in a CO_2 incubator at 37 ℃. Huh7-H-ras^{G12V} cell lines were generated by stably transfecting H-*ras*^{G12V} in Huh7 cells. The pCAG-HA-H-*ras*^{G12V}-neo was constructed as follows. The coding sequences for mutated H-*ras*^{G12V} were inserted by PCR cloning into the EcoR I site of the pCAG-HA-neo vector and confirmed by restriction mapping and DNA sequencing. Huh7 cells were plated in 6-well culture plates for 24 h prior to transfection. Cells were transfected with 3 μg of pCAG-HA-H-*ras*^{G12V}-neo construct using a Lipofectamine 2000 reagent (Invitrogen, United States), according to the manufacturer's instructions. After 48 h, cells were trypsinized and plated in a medium containing 400 μg/mL neomycin (G418). Following selection for 2 wk, total populations of neomycin-resistant cells were pooled and single-cells sorted into 96-well plates with a growth medium containing 400 μg/mL neomycin. Sorted single cells were grown under selection for an additional 2 wk and expanded into stable cell lines. The candidate clones were analyzed by Western blot analysis using a HA (Roche, Germany) antibody.

Animals

The generation of H-*ras*^{12V} transgenic liver cancer mouse model was previously described[12]. We used 3 mo old H-*ras*^{12V} transgenic male mice. H-*ras*^{12V} transgenic mice were divided into two groups and administered with PBS (control group, $n = 5$) and methylsulfonylmethane (MSM, 100 μg/g) (treated group, $n = 6$) every day for 3 mo. The genotyping of PCR primers for the H-*ras*^{12V} were 5'-CTAGCGCTGCAGGAATTC-3' and 5'-GTAGTTTAACACATTATACACT-3'. The mice were housed in a pathogen-free animal facility under standard 12 h light/dark cycle. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Reagents

MSM and crystal violet were purchased from Sigma-Aldrich Co. (United States).

Cell growth assay (anchorage-independent)

The cell growth after treatment with MSM was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). HepG2 and Huh7 (Mock, H-*ras*^{G12V}) cells were suspended at a concentration of 5×10^3 cells/well and cultured in 96-well flat bottomed microplate. After exposure to MSM at different time points (0, 24, 48, 72 and 96 h),

CCK-8 (10 μL) was added to each well of a 96-well flat bottomed microplate containing 100 μL of culture medium and MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) and the plate was incubated for 2 h at 37 ℃. Viable cells were counted by absorbance measurements at 450 nm using auto microplate reader (VERSAmax™, United States).

Soft agar assay (Anchorage-dependent)

HepG2, Huh7 - Mock, and Huh7-H-*ras*^{G12V} (5×10^3 cells) were suspended in 1 mL of DMEM containing 0.3% agar in cell-growth medium and plated in triplicates over a first layer of 0.6% agar in cell-growth medium. The cells were grown at 37 ℃ and 5% CO_2. Then the viable colonies were stained with 0.01% crystal violet (Sigma, United States) for 2 h. We treated with MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) on the top agar on day 0. The MSM contained medium was changed every day.

Flow cytometry analysis

Apoptosis was also evaluated by flow cytometry after Annexin V-FITC/PI (BD Bioscience, United States) staining. The cells were digested with trypsin and resuspended in 100 μL of binding buffer, 5 μL of Annexin V-FITC and added with 5 μL of PI, and the mixture was incubated at room temperature for 15 min in the dark. The cells were analyzed using a BD FACSCalibur (BD Bioscience, United States) and divided into four groups: normal cells (Annexin V negative and PI positive), early apoptotic cells (Annexin V positive and PI negative), late apoptotic cells (Annexin V positive and PI positive) and necrotic cells (Annexin V negative and PI positive). The percentages of the different cell groups were determined by a scatter plot analysis.

Western blot analysis

We homogenized liver cancer cell lysates in lysis buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L glycerol phosphate, 1% Triton X-100 and 10% glycerol) with protease (Sigma, United States) and a phosphatase-inhibitor cocktail (Roche, Germany). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. For Western blot analysis, 30 μg protein lysates were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, United States). The membranes were primarily blotted with primary antibodies against GAPDH (Lab Frontier, South Korea), HA (Roche, Germany), cleaved caspase-3, cleaved caspase-8, cleaved PARP [Poly (ADP-ribose) polymerase] or Bcl-2 (Cell Signaling Technology Inc., United States) at 4 ℃ overnight. They were washed five times with 10 mmol/L Tris-HCl (pH 7.5) containing 150 mmol/L NaCl and 0.2% Tween-20 (TBST) and incubated with horseradish peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG (Pierce, United States) for 1 h at room temperature. After the removal of excess antibodies by washing with TBST, specific binding was detected using a SuperSignal chemiluminescent substrate (Pierce, United States) according to the manufacturer's instructions.

Live cell counting by trypan blue stain

Huh7-H-*ras*^{G12V} cells were suspended at a concentration of 0.3×10^6 cells/well and cultured in 6-well plate. After exposure to MSM for 24 h, cells were calculated by trypan blue stain through an electron microscope (Nikon, Japan).

Blood plasma analysis

Once a mo during the experimental period, blood samples were taken from orbital venous congestion. Plasma was prepared by centrifugation of the blood at 10000 rpm for 5 min at 4 ℃ and stored at -70 ℃ until analysis. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured with an automatic chemistry analyzer (Hitachi 7150, Japan).

Liver histology

The liver was removed from the mice and immediately fixed in a buffer solution of 10% formalin for pathological analysis. Fixed tissues were processed routinely for paraffin embedding and 5 μm sections were prepared and stained with hematoxylin and eosin (H and E). Stained areas were viewed using an optical microscope.

Statistical analysis

Data were analyzed using SigmaStat 3.1 software. All data are presented as the mean ± the standard error of the mean (SEM) from at least three independent experiments. Comparisons between groups were analyzed by Student's *t*-test for paired and unpaired measure. P value < 0.001 was considered statistically significant.

RESULTS

MSM inhibits cell growth in liver cancer cell lines

To investigate the effect of MSM in cell growth, cell lines such as HepG2, Huh7-Mock, and Huh7-H-*ras*^{G12V} were exposed to MSM in a dose-dependent manner. Cell growth was analyzed at 0, 24, 48, 72 and 96 h using CCK-8. HepG2 cell growth was significantly inhibited with treatment of 500 mmol/L but the growth of Huh7-Mock and Huh7-H-*ras*^{G12V} cells was significantly reduced with treatment of 200 mmol/L and 500 mmol/L of MSM (Figure 1A). To assess the effect of MSM in colony formation, we conducted a soft agar assay. As shown in Figure 1B, colony size and number of HepG2 cell were decreased in a dose-dependent manner. However, Huh7-Mock and Huh7-H-*ras*^{G12V} cells were remarkably inhibited in 200 mmol/L and 500 mmol/L (Figure 1B). These results suggest that MSM treatment inhibits cell growth significantly in liver cancer cell lines.

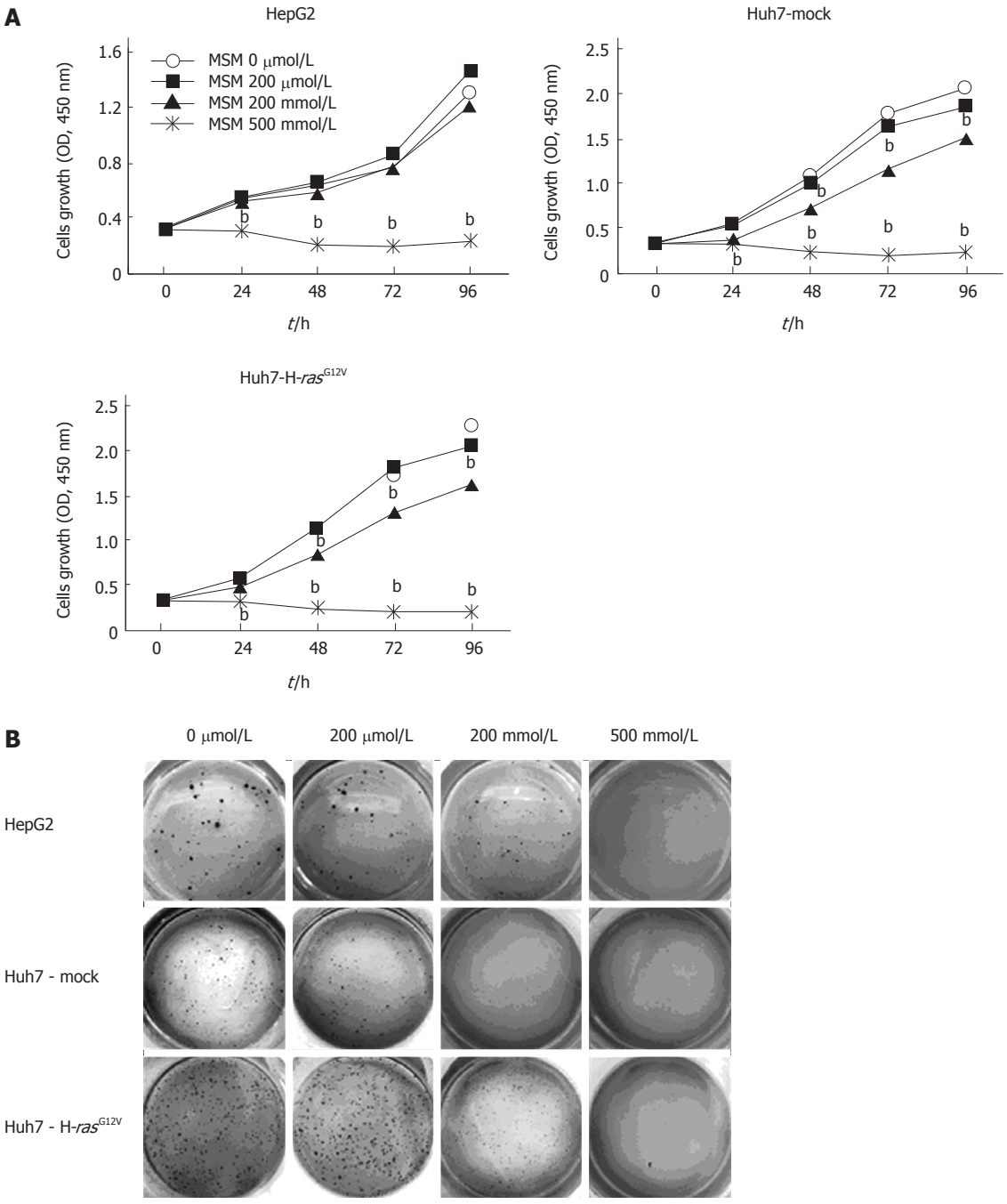

Figure 1 Inhibitory effect of methylsulfonylmethane in liver cancer cell lines. A: Cellular proliferation effects of MSM were measured by CCK-8 assay. Cells were treated with MSM (0, 200 μmol/L, 200 mmol/L, and 500 mmol/L) at different time points (0, 24, 48, 72 and 96 h); B: Anchorage-independent growth assay was performed in liver cancer cells treated with MSM in a dose-dependent manner. $^{b}P < 0.001$ *vs* control. MSM: Methylsulfonylmethane.

MSM induces apoptosis in liver cancer cell lines

To determine whether MSM induces apoptosis in liver cancer cell lines, we utilized Annexin V/PI staining, observed morphology and counted live cells. The Annexin V/PI staining was performed to examine the reversion of phosphatidylserine, a marker for apoptosis. Our results showed that the proportion of apoptotic cells was induced by treatment of 200 mmol/L and 500 mmol/L in liver cancer cell lines. Particularly, the apoptosis rate was increased 6-fold in all liver cancer cell lines treated with 500 mmol/L compared to control (Figure 2A). We examined the morphology and counted live cells of Huh7-H-*ras*^{G12V}. Morphological changes were observed in 200 mmol/L and 500 mmol/L. Also, adherent cells were decreased in 200 mmol/L and 500 mmol/L treated with MSM. In addition, live cell number was significantly reduced in 500 mmol/L of MSM compared to control (Figure 2B). The data indicate that MSM treatment induces apoptosis in liver cancer cell lines.

MSM activates caspase-3, -8 and PARP in liver cancer cell lines

The expression of HA-H-*ras*^{G12V} in Huh7-H-*ras*^{G12V} was confirmed by Western blot. HA-H-*ras*^{G12V} was overex-

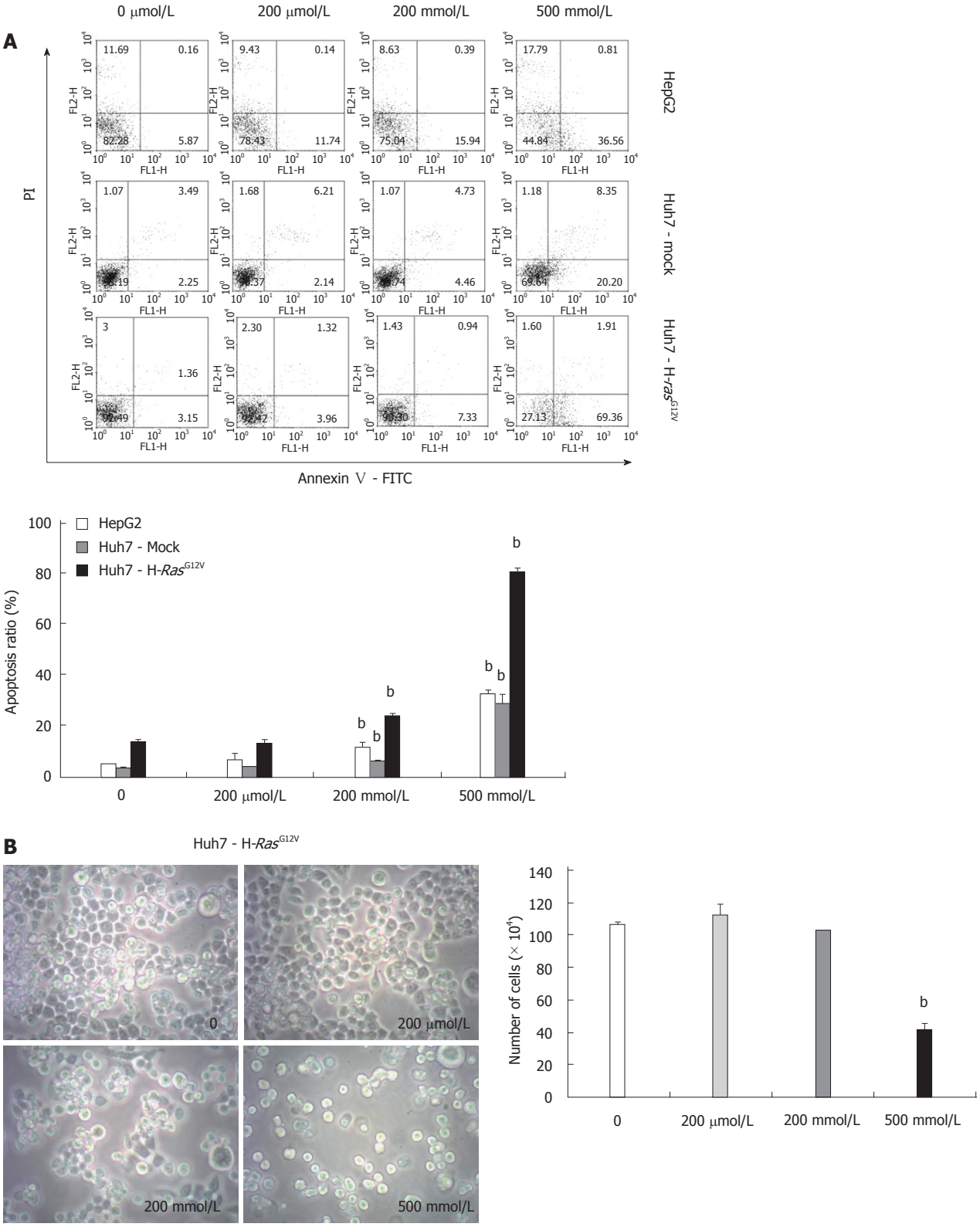

Figure 2 Methylsulfonylmethane induces apoptosis in liver cancer cell lines. A: Detection of apoptotic cells by Annexin V. Cells were treated with MSM in a dose-dependent manner for 24 h; B: Dose-dependent effects of MSM on the morphology and live cell counting of Huh7-H-*ras*^{G12V} cell line for 24 h. $^{b}P < 0.001$ *vs* control. MSM: Methylsulfonylmethane.

pressed in Huh7-H-*ras*^{G12V} cell (Figure 3A). To understand the mechanisms involved in MSM-induced apoptosis in liver cancer cell lines, we first determined caspase activity. The protein levels of cleaved caspase-3, cleaved caspase-8 and cleaved PARP were significantly increased in liver cancer cell lines treated with 500 mmol/L (Figure 3B). To investigate whether mitochondrial anti-apoptosis proteins are involved in regulating MSM-induced apop-

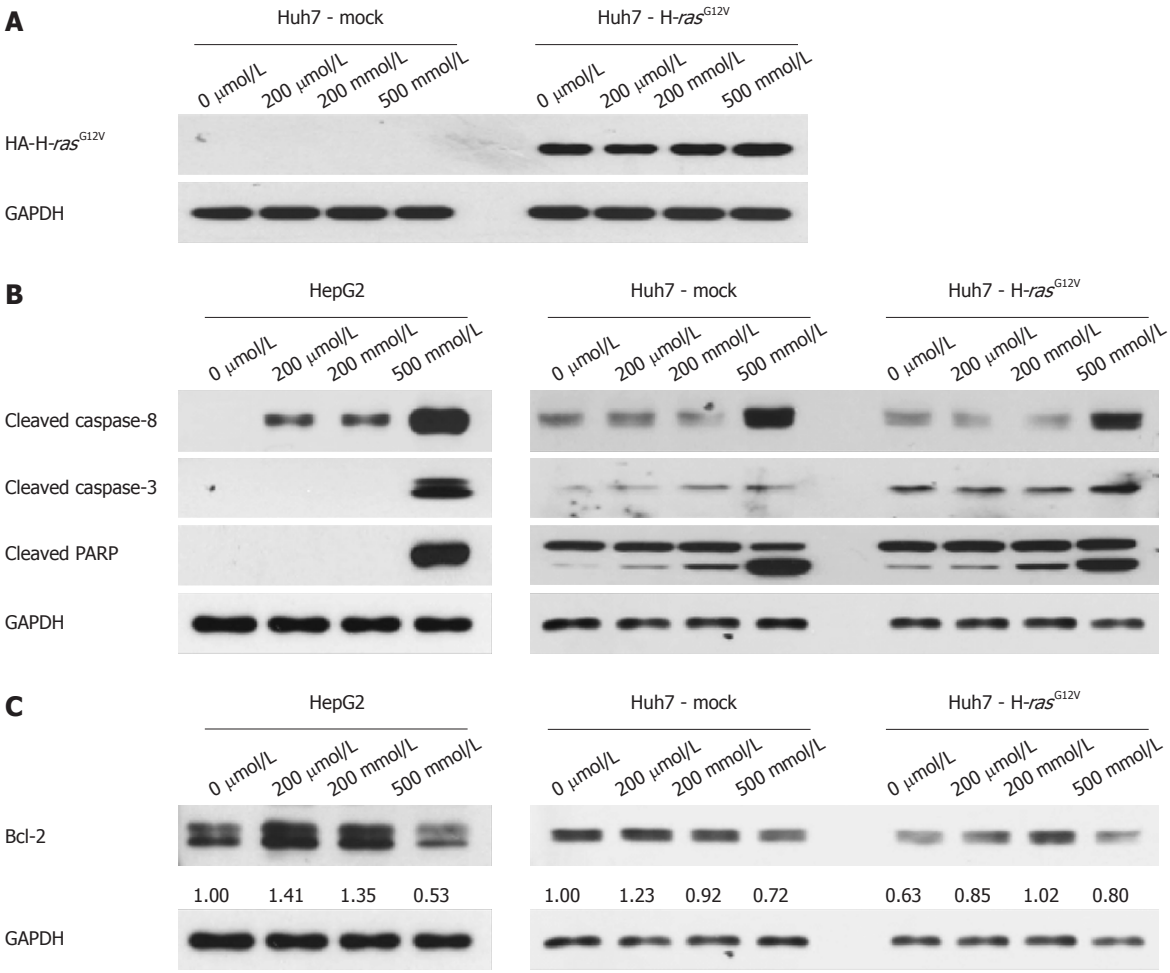

Figure 3 Methylsulfonylmethane increases caspase-3, -8 and PARP activation in liver cancer cell lines. A: Expression levels of HA-H-*ras*^{G12V} in Huh7-H-*ras*^{G12V}; B: Expression levels of cleaved caspase-3, cleaved caspase-8 and cleaved PARP in liver cancer cells were determined by Western blotting analysis. Cells were treated with MSM in a dose-dependent manner for 24 h; C: Expression of Bcl-2 in liver cancer cells treated with MSM in a dose-dependent manner for 24 h. Numbers under each result indicate a fold increase of band density as compared to control, GAPDH. MSM: Methylsulfonylmethane; PARP: Poly (ADP-ribose) polymerase.

tosis of liver cancer cell lines, we examined the protein level of Bcl-2. The result indicated that Bcl-2 was decreased in liver cancer cell lines treated with 500 mmol/L (Figure 3C). It suggests that MSM induced apoptosis by regulating the expression of cleaved caspase-3, cleaved caspase-8 and cleaved PARP.

MSM inhibits hepatic tumorigenesis in H-ras^{12V} *transgenic mice*

To investigate the suppression effects of MSM in hepatic tumorigenesis, H-*ras*^{12V} transgenic mice were orally administered with MSM (100 μg/g) for 3 mo. Tumor volume and number were significantly reduced in the MSM treated group compared to the control group (Figure 4A). To determine histological changes, we examined the H and E staining in mouse liver. As shown in representative photomicrographs of liver histology, the tumor size in the MSM treated group was dramatically decreased compared to the control group. Moreover, necrosis was observed in the MSM treated group (Figure 4B). To confirm in vitro data on apoptosis, we examined the protein levels of cleaved PARP. Cleaved PARP was increased in MSM treated non-tumors compared to PBS treated control; however, not in tumor (Figure 4C). To examine the effect of MSM in liver function of H-*ras*^{12V} transgenic mice, we checked AST and ALT levels. AST levels were significantly lower in the MSM treated group for 3 mo than the control group. ALT levels were also lower in the MSM treated group for 1 mo than the control group (Figure 4D). These data suggest that MSM suppresses liver damage in H-*ras*^{12V} transgenic mice.

DISCUSSION

MSM is naturally obtained from various species of fruits, vegetables, grains, animals and animal products. The chemical structure of MSM is a combination of oxygen in dimethyl sulfoxide (DMSO), so also called dimethyl sulfone ($DMSO_2$). The anti-inflammatory effects of MSM on lipopolysaccharide-induced inflammatory responses in murine macrophages[9] and effects of MSM in breast cancer by down regulating STAT3 and STAT5b pathways[10] were reported. However, the effect of MSM has not been studied in liver cancer. In the present study,

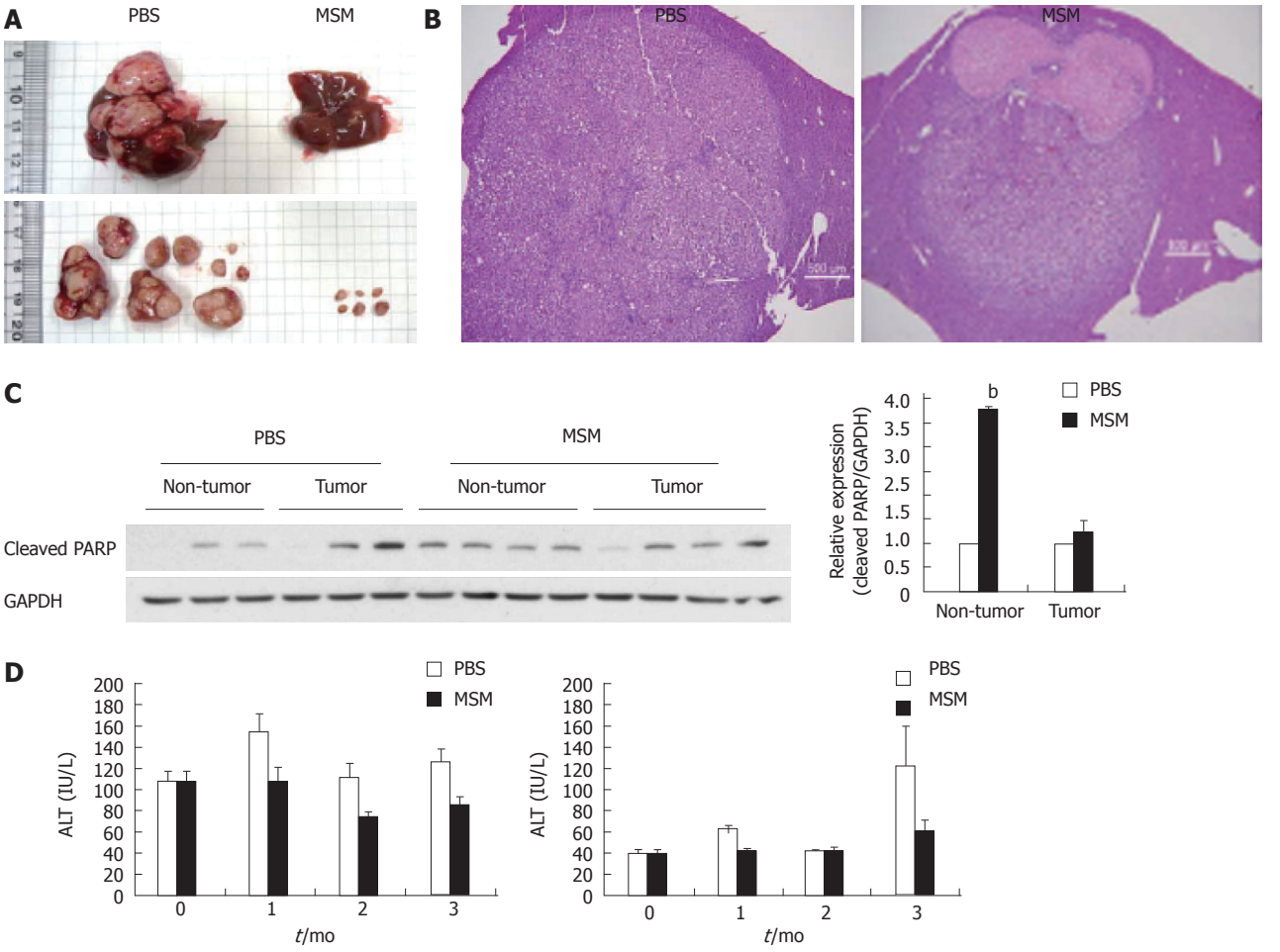

Figure 4 Methylsulfonylmethane inhibits hepatic tumorigenesis in H-*ras*^{12V} transgenic mice. H-*ras*^{12V} transgenic mice were administered with PBS and MSM (100 μg/g) every day for 3 mo. A: Liver morphologies of PBS (control) group (left) and MSM group (right) after administration for 3 mo; B: Hematoxylin and eosin stained section in livers of PBS and MSM treated H-*ras*^{12V} transgenic mice (Scale bars, 500 μm); C: Expression levels of cleaved PARP in H-*ras*^{12V} livers; D: The levels of AST and ALT in plasma of H-*ras*^{12V} transgenic mice treated with MSM. MSM: Methylsulfonylmethane; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

we found that MSM dramatically inhibits hepatic tumor cell growth. We performed CCK-8 and soft agar assay. Anchorage dependent tumor cell growth inhibition was found in liver cancer cells, such as HepG2, Huh7-Mock and Huh7-H-*ras*^{G12V} treated with 500 mmol/L of MSM (Figure 1A). Anchorage independent cell growth was also inhibited in the liver cancer cells treated with both 200 mmol/L and 500 mmol/L of MSM (Figure 1B). The results indicate that MSM is effective in inhibiting liver cancer cell growth.

To further investigate the apoptosis in liver cancer cells treated with MSM, we examined apoptotic cells by Annexin V/PI staining. Apoptotic cells were significantly increased by treatment of 500 mmol/L in liver cancer cell lines (Figure 2A) and the morphology of Huh7-H-*ras*^{G12V} cells was changed in 200 mmol/L and 500 mmol/L. The numbers of live cells were reduced in 500 mmol/L (Figure 2B). The result indicated that MSM caused apoptosis in liver cancer cell lines.

Cancer chemotherapy is known to induce tumor cell death in a variety of cell types in part by promoting the intracellular ROS. Recently, salinomycin-induced apoptosis of human prostate cancer cells was due to accumulated ROS[13]. In our study, ROS levels were significantly increased in 500 mmol/L of MSM in Huh7 cell lines (Supplemental Figure 1), suggesting that MSM treatment regulated ROS levels in liver cancer cell lines.

To clarify the apoptotic mechanism stimulated by MSM, we studied both the death receptor pathway and the mitochondrial pathways[14]. Cell surface death receptors, such as Fas which bind their ligands, initiate signaling to activate caspase-8, caspase-3 to induce apoptosis, and signaling involved in mitochondrial release of cytochrome-c, which activates caspase-9 and caspase-3[15]. We performed Western blot. Bcl-2 was decreased in all of the cell lines treated with 500 mmol/L (Figure 3C) and MSM treatment led to an increased apoptotic response involving caspase-3, caspase-8 and PARP activation in liver cancer cell lines (Figure 3B). The results demonstrate that MSM induces apoptosis through activation of the caspase pathway.

We performed *in vivo* studies to investigate the liver tumor growth suppressive function of MSM. We orally administered MSM (100 μg/g) to H-*ras*^{12V} transgenic

mice for 3 mo. During the administration, body weight ratio was not changed between MSM treated group and control group (Supplemental Figure 2). However, the AST and ALT levels of MSM treated group were lower than the control group (Figure 4D). In addition, tumor volume and number were noticeably reduced in the MSM treated group (Figure 4A). The expression of cleaved PARP was increased in MSM treated non-tumors compared to PBS treated control; however, similar in tumors between the PBS and MSM treated group (Figure 4C). As shown in photomicrographs of liver histology, tumor size of the MSM treated group was decreased compared to the control group (Figure 4B). All the data suggest that MSM improves liver function and suppresses hepatic tumorigenesis through activation of apoptosis.

MSM was efficacious with treatment of 500 mmol/L in inhibition of hepatic tumor cell growth. In addition, the apoptosis rate was increased 6-fold in all of liver cancer cell lines treated with 500 mmol/L compared to control. These results indicate that MSM is efficacious with treatment of the highest dose in liver cancer cells, consistent with the result that MSM suppresses breast cancer cell growth at 300 mmol/L[10]. MSM is an edible natural organic compound present in many food items and is not associated with any toxic effects, even at higher concentrations[16,17]. MSM administration with high dose (100 μg/g) to H-*ras*^{12V} transgenic mice for 3 mo did not affect body weight ratio but improved liver function by showing lowered AST and ALT levels and remarkably retarded hepatic tumor growth in the MSM treated group. All the results suggest that MSM could be available for inhibition of hepatic tumor growth. Further research is needed to be feasible in humans.

In summary, we showed that MSM induced growth inhibition and apoptosis in hepatic tumorigenesis. Therefore, MSM could be a potential candidate as an anticancer agent.

COMMENTS

Background

Liver cancer is the third most common cause of cancer-related mortality worldwide. However, there are only a few effective ways to prevent or treat liver cancer. Therefore, studies are going on in the area of liver cancer. Methylsulfonylmethane (MSM), an organic sulfur-containing compound, is naturally obtained from various species of vegetables, grains, animals and animal products. Recently, a study has reported that MSM can be used to inhibit breast cancer growth.

Research frontiers

MSM is an edible natural organic compound present in many food items and is not associated with any toxic effects, even at higher concentrations. Research is focused on finding the efficacy of higher doses of MSM treatment in cells and mice with H-ras activated liver cancer.

Innovations and breakthroughs

MSM decreased the growth of Huh7-H-rasG12V cells in a dose-dependent manner. That was correlated with significantly increased apoptosis in MSM treated cells. Cleaved caspase-8, cleaved caspase-3 and cleaved PARP were remarkably increased in the liver cancer cells treated with 500 mmol/L of MSM. Liver tumor development was greatly inhibited in the H-ras12V transgenic mice treated with MSM compared to control, by showing reduced tumor size and number.

Applications

The results suggest that MSM could be a potential candidate for prevention of liver cancer.

Terminology

MSM is a very simple organic sulfur-containing compound with a molar mass of 94.13 g/mol. MSM contains only eleven atoms and is found in foods, including fruits, vegetables, grains and beverages.

Peer review

This study described the efficacy of MSM treatment in cells and mice with H-ras activated liver cancer well. The results are interesting and indicate that MSM could be used for preventing hepatic tumor growth.

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