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Research Article

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Rho inhibitor prevents ischemia–reperfusion injury in rat steatotic liver

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Background & Aims: Hepatic stellate cells are thought to play a role in modulating intrahepatic vascular resistance based on their capacity to contract via Rho signaling. We investigated the effect of a Rho-kinase inhibitor on ischemia–reperfusion injury in the steatotic liver.

Methods: Steatotic livers, induced by a choline-deficient diet in rats, were subjected to ischemia–reperfusion injury. Hepatic stellate cells isolated from steatotic livers were analyzed for contractility and Rho signaling activity. The portal pressure of the perfused rat liver and the survival rate after ischemia–reperfusion were also investigated.

Results: Hepatic stellate cells from steatotic livers showed increased contractility and upregulation of Rho-kinase 2 compared with those from normal livers. Furthermore, endothelin-1 significantly enhanced the contractility and phosphorylation level of myosin light chain and cofilin in hepatic stellate cells isolated from steatotic livers. A specific Rho-kinase inhibitor, fasudil, significantly suppressed the contractility and decreased the phosphorylation levels of myosin light chain and cofilin. Serum levels of endothelin-1 were markedly increased after IR in rats with steatotic livers, whereas fasudil significantly decreased endothelin-1 serum levels. Rats with steatotic livers showed a significant increase in portal perfusion pressure after ischemia–reperfusion and a significant decrease in survival rate; fasudil treatment significantly reduced these effects.

Conclusions: Activation of Rho/Rho-kinase signaling in hepatic stellate cells isolated from steatotic livers is associated with an increased susceptibility to ischemia–reperfusion injury. A Rho-kinase inhibitor attenuated the activation of hepatic stellate cells isolated from steatotic livers and improved ischemia–reperfusion injury in steatotic rats.

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Introduction

Liver steatosis increases the risk of postoperative morbidity and mortality after liver surgery including liver transplantation [1–3]. Ischemia–reperfusion (IR) injury is one of the most critical complications commonly associated with liver surgery [4–6]. Although it is known that steatotic liver (SL) is particularly vulnerable to IR injury, the mechanisms underlying this increased susceptibility have not yet been clarified.

Experimental studies have indicated that the degree of steatosis is correlated with hepatic microcirculatory disturbances [4,5]. Fat droplet accumulation in the cytoplasm of hepatocytes is associated with an increase in cell volume, which may result in the partial or complete obstruction of the hepatic sinusoidal space and the reduction of sinusoidal blood flow. A continuous state of chronic cellular hypoxia persists in fatty hepatocytes, predisposing the SL to IR injury [7]. The sinusoidal lumens are narrowed by fibrin microthrombi and cellular debris during reperfusion, further decreasing sinusoidal perfusion.

Hepatic stellate cells (HSCs) play an important role in the regulation of hepatic microcirculation. HSCs undergo contraction or relaxation in response to certain stimuli and, as a result, regulate microcirculation by increasing or decreasing the diameter of the sinusoidal lumen [8]. HSCs also play an important role in IR injury [9]. Because HSCs are oxygen-sensing cells [10], they are likely to be activated by exposure to IR-induced oxidative stress, resulting in the disruption of hepatic microcirculation.

The Rho family of small GTPases is known to regulate cell shape and motility through reorganization of the actin cytoskeleton [11]. One of the putative Rho target proteins, the serine/threonine kinase ROCK, mediates cytoskeleton-dependent cell functions by enhancing the phosphorylation of myosin light

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Abbreviations: IR, ischemia–reperfusion; SL, steatotic liver; HSCs, hepatic stellate cells; ROCK, Rho-kinase; MLC, myosin light chain; P-MLC, phosphorylated myosin light chain; NL, normal liver; fasudil, fasudil hydrochloride hydrate; NO, nitric oxide; L-NAME, *N*-nitro-L-arginine methyl ester; ET-1, endothelin-1; P-Cofilin, phosphorylated cofilin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; H&E, hematoxylin and eosin; TUNEL, TdT-mediated dUTP-digoxigenin nick-end labeling; HSCs-SL, hepatic stellate cells isolated from rat steatotic liver; HSCs-NL, hepatic stellate cells isolated from rat normal liver; SECs, sinusoidal endothelial cells.

chain (MLC) [12]. An increase in phosphorylated MLC (P-MLC) increases the contractility of actomyosin and causes smooth muscle contraction [13]. In addition, P-MLC facilitates the clustering of integrins and the bundling of actin fibers [14], resulting in stimulus-induced cell adhesion and motility.

The contraction of HSCs narrows the sinusoidal lumen and reduces hepatic microcirculatory flow via Rho signaling. We reported previously that the Rho/ROCK signaling pathway played an important role in the activation of HSCs and that a ROCK inhibitor attenuated hepatic injury after warm IR and orthotopic liver transplantation in a rat model [9]. Recently, HSC activation has been shown to be correlated with the severity of steatosis in the liver [15–17]. However, little is known about the connection between activated HSCs and IR injury in SL. Furthermore, there are few studies on the involvement of Rho signaling in the activation of HSCs in SL.

The aim of the present study was to investigate the association between Rho signaling and the activation of HSCs in SL. We also examined whether inhibition of the Rho/ROCK pathway could ameliorate IR injury in the steatotic rat liver.

Materials and methods

Animals

Four-week-old male Wistar rats were purchased from Charles River Breeding Laboratories (Osaka, Japan). Rats were fed either a choline-deficient diet (Hiroshima Institute for Experimental Animals, Hiroshima, Japan) to encourage the development of SL, or a normal diet, which resulted in the development of a normal liver (NL). All animal experiments were performed according to the guidelines set by the US National Institutes of Health (1996).

Liver IR

Under anesthesia, whole rat livers were subjected to warm ischemia by clamping the hepatic artery and portal vein with microvascular clips. The specific ROCK inhibitor fasudil hydrochloride hydrate (fasudil; kindly donated by Asahi Kasei Co., Tokyo, Japan) was used to investigate the effect of ROCK inhibition on liver IR injury. Selected rats were pretreated with 10 mg/kg fasudil (intraperitoneal injection) 30 min before the induction of ischemia.

Isolation of HSCs

HSCs were isolated from rat livers according to previously described procedures [9,18]. Purity was estimated by ordinal light and fluorescence microscopic examination and by indirect enzyme immunoreactivity with an antidesmin antibody (Dako, Versailles, France). HSCs were grown in standard tissue culture plastic flasks in Dulbecco's minimum essential medium with 10% fetal bovine serum and antibiotics.

Collagen gel contraction assay

The contractility of the HSCs was evaluated using hydrated collagen gel lattices on 24-well culture plates as described previously with some modifications [9,19]. To investigate the influence of nitric oxide (NO) on HSCs, the NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; Cayman Chemical, Ann Arbor, MI) was used.

Western blot analysis

Primary rat HSCs were left untreated or were treated with 10 μM fasudil and/or 5 nM endothelin-1 (ET-1; Sigma-Aldrich Inc., Tokyo, Japan) for 30 min before homogenization in lysis buffer (Cell Lysis Buffer; Cell Signaling Technology, Danvers, MA). Western blot analysis was performed according to previously described procedures with some modifications [20]. Specific antibodies against β-actin were from Abcam (Tokyo, Japan); those against MLC were from Sigma-Aldrich Inc., and those against P-MLC, cofilin, phosphorylated cofilin (P-Cofilin), and Rho-kinase 2(ROCK2) were from Cell Signaling Technology. The protein expression of ROCK2 was normalized to the level of β-actin. The phosphorylation levels were normalized to the levels of total MLC or cofilin protein expression.

Biochemical assessment

Blood samples were collected from the inferior vena cava. Serum ET-1 concentrations were measured using an Endothelin-1 EIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) according to the manufacturer's instructions. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were assayed by standard enzymatic methods.

Measurement of portal perfusion pressure in the isolated rat liver

Portal pressure in isolated perfused rat livers was measured according to previously described procedures, with some modifications [21]. The perfusion with Krebs–Henseleit buffer (Sigma-Aldrich Inc.) was continued until the monitored inlet pressure value became stable at a constant flow rate of 0.3 ml min^{-1} liver $volume^{-1}$ (ml).

Confocal immunofluorescence and histological study

Phalloidin staining of isolated HSCs and liver sections was performed according to previously described procedures [22]. Samples were observed under a conventional fluorescence microscope or a laser confocal microscope. For the histological study, liver specimens were collected from the middle hepatic lobe after IR. Formalin-fixed liver tissue sections were stained with hematoxylin and eosin (H&E) and examined microscopically. To assess the activity of HSCs in liver sections, phalloidin staining was performed. To assess the grade of the steatosis, sections were stained for oil red O. Furthermore, the detection of apoptosis in liver tissue sections was achieved by TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining as previously reported [23].

Statistical analysis

Survival rates were compared using the Kaplan-Meier method and analyzed by the log-rank test. Other data are expressed as average values (SD). Statistical analysis among experimental groups was performed using the *t*-test. *p* values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS software, version 16 (SPSS Japan Inc., Tokyo, Japan).

Results

Changes in the morphology of HSCs isolated from steatotic rat livers

At 10 weeks, rats fed a choline-deficient diet developed liver steatosis, characterized by more than 60% of fatty filtration in the hepatocytes with few inflammatory cells and slight fibrosis (Fig. 1A). HSCs isolated from rat SLs (HSCs-SL) showed increased stress fiber formation and F-actin expression compared to HSCs isolated from normal rat livers (HSCs-NL), which were suppressed by fasudil treatment (Fig. 1B). Phalloidin staining of liver sections showed stress fiber formation and F-actin expression around sinusoidal spaces in SL after IR, as well as the suppression of these changes by fasudil (Supplementary Fig. 1).

Contractility of HSCs isolated from normal and steatotic rat livers

To evaluate differences in contractility, HSCs-NL and HSCs-SL were cultured on hydrated collagen gels. Contraction was measured as the reduction in the initial area of the gel. In the absence of vasoactive agents, the areas of the gels with HSCs-SL were

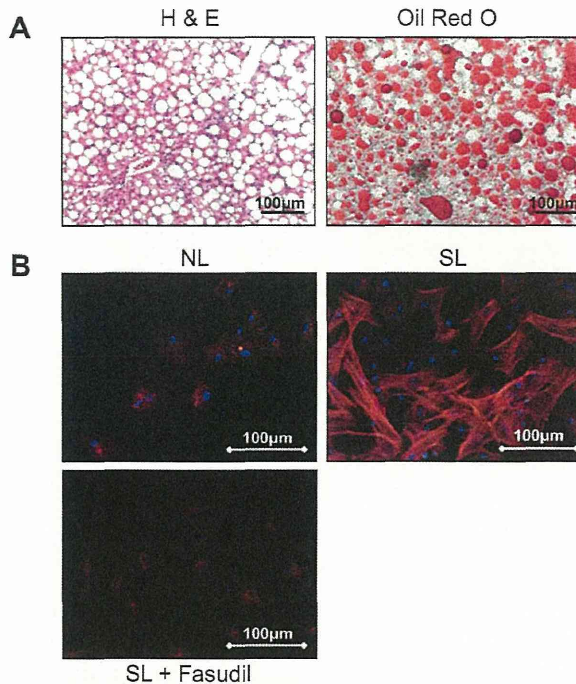

Fig. 1. Oil red O staining and morphology changes of HSCs isolated from rat SL. (A) Representative H&E- and oil red O-stained sections. Rats fed a choline-deficient diet for 6 weeks had more than 60% of macrovesicular steatosis. (B) Images show differences in F-actin expression in isolated HSCs from NL, SL, and SL treated with fasudil (10 μM, 24 h). Cells were stained to show F-actin (red) and nuclei (blue). HSCs isolated from NL showed slight stress fiber formation and F-actin expression. By contrast, HSCs isolated from SL had an elongated, fusiform morphology with prominent dendritic processes. Fasudil suppressed both stress fiber formation and F-actin expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

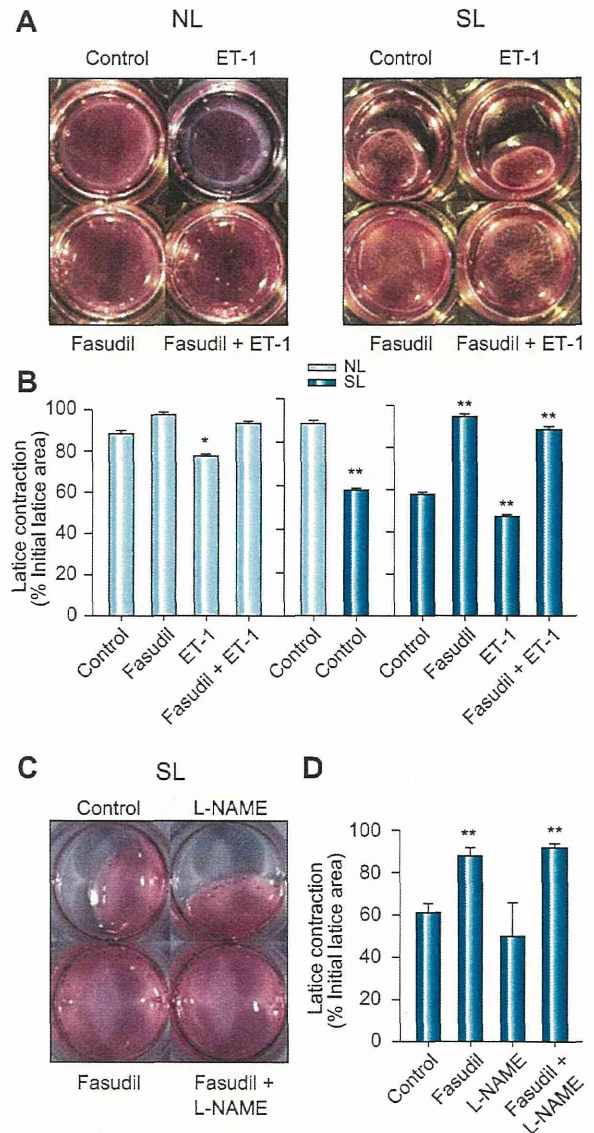

Fig. 2. Collagen gel contraction assay. (A) Contraction of collagen gels induced by the activation of isolated HSCs in untreated NL and SL, and in NL and SL treated with ET-1, fasudil or a combination of the two. Without the addition of HSCs, the collagen gels did not contract during the observation period (not shown). Control, medium alone; ET-1, 5 nM ET-1; Fasudil, 10 μM fasudil; Fasudil + ET-1, 10 μM fasudil and 5 nM ET-1. (C) Contraction of collagen gels in SL and SL treated with L-NAME, fasudil or a combination of the two. Control, medium alone; L-NAME, 100 μM L-NAME; Fasudil, 10 μM fasudil; Fasudil + L-NAME, 10 μM fasudil, and 100 μM L-NAME. (B and D) Changes in the collagen gel area induced by contraction of HSCs. HSCs isolated from rat NL, closed bars; HSCs isolated from rat SL, open bars. Average values (SD) of three independent experiments are shown. *p <0.05 compared to each control; **p <0.01 compared to each control.

significantly smaller than those with HSCs-NL (p <0.01). In the presence of fasudil (10 μM), this reduction in the areas of gels with HSCs-SL was not observed. The gel areas containing HSCs-SL were significantly smaller in the presence of ET-1 (5 nM) than those with HSCs-SL in the absence of ET-1 (p <0.01). In sharp contrast, however, when fasudil was added to the culture medium in the presence of ET-1, the shrinkage of the gels with HSCs-SL was suppressed (p <0.01; Fig. 2A and B). Furthermore, the NO synthase inhibitor L-NAME (100 μM) was used to investigate the influence of NO on HSCs. Fasudil suppressed the contraction of HSCs-SL even in the presence of L-NAME (p <0.01; Fig. 2C and D).

Expression of ROCK2 and phosphorylation of MLC and cofilin

To examine the possible involvement of the Rho/ROCK pathway in the activation of HSCs, the expression of ROCK2 and the phosphorylation state of MLC and cofilin, a downstream effector of Rho/ROCK signaling, were assessed by Western blot analysis with monoclonal antibodies to ROCK2 and the phosphorylated form of MLC and cofilin. Quantitative analysis using a scanning densitometer confirmed that ROCK2 was significantly overexpressed in HSCs-SL compared with HSCs-NL (p <0.01) (Fig. 3A). The phosphorylation level of MLC in HSCs-SL was significantly increased compared with that in HSCs-NL. The phosphorylation levels of MLC and cofilin were significantly enhanced by ET-1 in HSCs-SL

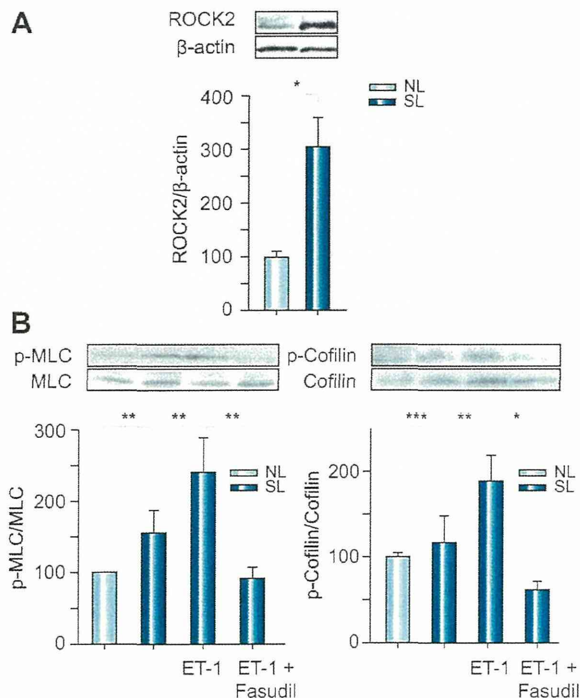

Fig. 3. Western blot analyses in rat HSCs isolated from rat NL or SL. (A) Expression level of ROCK2. (B) Total and phosphorylated MLC and cofilin. HSCs isolated from rat SL were either left untreated or cultured with 10 μM fasudil and/or 5 nM ET-1 for 30 min. The protein expression of ROCK2 was normalized to the level of β-actin. The phosphorylation levels of MLC and cofilin were normalized to total MLC and cofilin protein expression, respectively. Each figure is representative of three independent experiments. Average values (SD) for individual groups are shown. *$p<0.01$, **$p<0.05$, ***N.S.

($p<0.05$, in both), but the effects were suppressed by fasudil ($p<0.05$, 0.01, respectively; Fig. 3B).

Influence of IR on the secretion of ET-1

Serum ET-1 concentrations were measured after 30 min of ischemia followed by 3 h of reperfusion using ELISA. Serum ET-1 concentrations significantly increased after IR in rats with NL ($p<0.01$). Serum ET-1 concentrations after IR were significantly higher in rats with SL than in rats with NL ($p<0.01$). Furthermore, fasudil significantly suppressed the serum ET-1 concentrations after IR ($p<0.01$; Fig. 4).

Influence of IR on portal perfusion pressure

To determine the influence of IR on the microvascular blood flow in the hepatic lobule, the portal perfusion pressure was assessed in isolated rat livers. Perfusion pressures were measured after 45 min of ischemia followed by 15 min of reperfusion. The portal perfusion pressures in rats with SL were significantly higher than those in rats with NL ($p<0.05$). The portal perfusion pressures in rats with SL after IR were significantly higher than those in rats with SL that did not undergo IR ($p<0.01$), and the effect was suppressed by fasudil ($p<0.01$; Fig. 5).

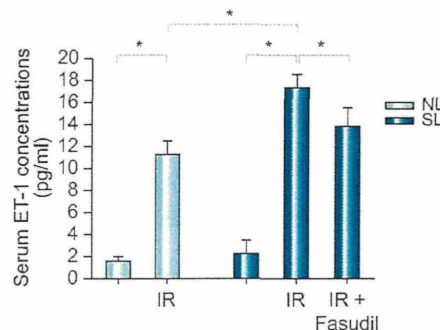

Fig. 4. Serum ET-1 concentrations, reflecting secretion from SECs and HSCs. Blood samples were collected from rats with NL or SL after 30 min of ischemia followed by 3 h of reperfusion. A group of the SL rats received fasudil (10 mg/kg) 30 min before ischemia. Average values (SD) for individual groups are shown; for all groups, n = 6. *$p<0.01$.

Biochemical assessment, histological study, and survival rates after IR

AST and ALT are well-established markers of hepatocellular injury after IR. Serum AST and ALT levels were measured after 30 min of ischemia followed by 3 or 24 h of reperfusion. The increase in AST at 3 and 24 h and in ALT at 3 h after IR of the untreated rats with SL was significantly higher than in fasudil-treated rats with SL (Fig. 6A). For histological analysis, liver specimens were obtained after 45 min of ischemia followed by 24 h of reperfusion. Liver specimens from untreated rats with SL after IR showed distortion of architecture, sinusoidal congestion, microthrombus, and extensive areas of coagulative necrosis. In contrast, specimens from the SL group treated with 10 mg/kg fasudil showed almost normal hepatic structure (Fig. 6B). TUNEL staining of liver tissue sections after IR showed that most hepatocytes in NL were TUNEL positive, whereas only minimal TUNEL staining was found in SL (Supplementary Fig. 2). The survival rate of rats with SL that underwent 45 min of ischemia was significantly lower than that of rats with NL ($p<0.01$). However, treat-

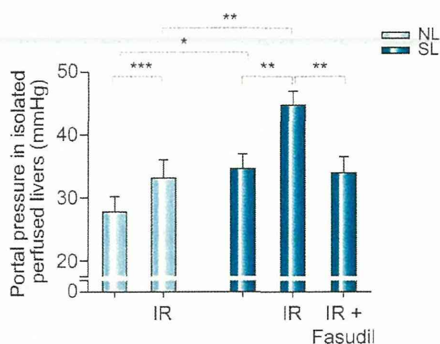

Fig. 5. Portal pressures in isolated perfused livers from rats with NL and SL. Livers were untreated, treated with 45 min of ischemia followed by 15 min of reperfusion, or preinjected with 10 mg/kg fasudil intraperitoneally 30 min before IR. Average values (SD) for individual groups are shown; for all groups, n = 5. *$p<0.05$; **$p<0.01$, ***N.S.

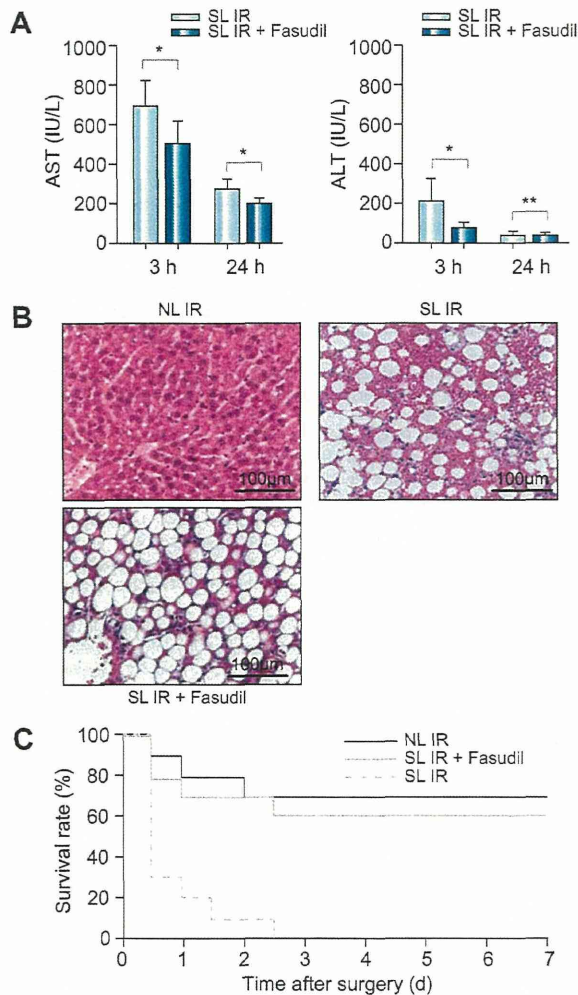

Fig. 6. Influence of IR in rats with SL. Rats with NL or SL were treated with IR. A fraction of the SL group received fasudil (10 mg/kg) 30 min before IR. (A) Serum levels of AST and ALT in rats with SL after 30 min of ischemia followed by 3 and 24 h of reperfusion. Average values (SD) for individual groups are shown; for all groups, n = 5. *p <0.05, **N.S. (B) Histological examination of NL and SL after 45 min of ischemia followed by 24 h of reperfusion. Liver specimens from untreated rats with SL showed severe fat accumulation, distortion of architecture, sinusoidal congestion, and extensive areas of coagulative necrosis. Liver specimens from the group that received fasudil similarly showed severe fat accumulation but had a nearly normal hepatic structure and minimal sinusoidal enlargement in the hepatic lobule center compared with the untreated SL. Representative H&E-stained liver sections. (C) The survival rates of rats with NL or SL that underwent 45 min of ischemia. Although the survival rate of the SL group was significantly lower than that of the NL group (p <0.01), fasudil treatment significantly improved the survival rate of the SL group (p <0.01), n = 10.

ment with fasudil significantly improved the survival rate of rats with SL after IR (p <0.01; Fig. 6C).

Discussion

The SL is known to be vulnerable to IR compared with the NL. The present study is the first report to provide evidence of the effect of Rho/ROCK signaling activation in HSCs on the increased susceptibility of SL to IR injury in rats. The present results showed that the activation of HSCs in SL was associated with the upregulation of ROCK2 and that the enhanced activation of ROCK2 was involved in the induction of IR injury in SL. The intra-abdominal infusion of fasudil, a specific inhibitor of ROCK, significantly alleviated IR injury in SL.

ROCK is a downstream effector of the small GTPase Rho involved in the regulation of cytoskeletal rearrangements and cell migration. ROCK is involved in the contraction of activated HSCs, which play an important role in regulating hepatic microcirculation. Intrahepatic upregulation of ROCK contributes to increased intrahepatic resistance in cirrhotic rats and to an increased sensitivity of cirrhotic livers to vasoconstrictors [24].

In the current study, HSCs-SL showed greater stress fiber formation and overexpression of ROCK compared with NL. The contractility of the HSCs and the phosphorylation of MLC in the HSCs were significantly enhanced in SL compared with NL. These results indicated a significant activation of the HSCs-SL compared with the HSCs-NL. There have been few studies using HSCs-SL. However, liver biopsy specimens from SL and nonalcoholic steatohepatitis showed the presence of activated HSCs, identified immunohistologically using a specific monoclonal antibody to detect cytoplasmic α-smooth muscle actin, which is not present in quiescent cells. These findings revealed a correlation between the degree of HSC activation and hepatic fibrosis, and the study of these specimens suggested a trend toward increased HSC activation with increasing fat accumulation, although this lacked statistical significance [15–17]. The hepatic expression of ROCK has been shown to be elevated in livers from cirrhotic rats and patients with alcohol-induced cirrhosis, and intrahepatic upregulation of ROCK contributes to portal hypertension via an increase in hepatic vascular resistance [24]. These results indicate that the activation of HSCs may be related to the overexpression of ROCK.

In our previous report using normal rat livers, the IR-induced impairment of sinusoidal microcirculation resulted, in part, from the contraction of HSCs, and Y-27632, a specific ROCK inhibitor, suppressed the IR-induced microcirculatory disturbance by promoting the relaxation of HSCs [9].

In the present study, the portal perfusion pressure was significantly increased in SL compared with NL. The perfusion pressure in SL was further increased after IR, and fasudil significantly suppressed this pressure increase. Serum ET-1 concentration was also significantly elevated after IR, and the increase in ET-1 concentration was suppressed by the administration of fasudil. These findings indicate that fasudil attenuates microvascular injury following ischemia reperfusion in SL. ET-1, which activates the Rho/ROCK pathway and elevates portal pressure via contraction of HSCs, was used as an alternative marker for IR injury in our *in vitro* studies (including a collagen gel contraction assay and measurement of MLC and cofilin phosphorylation in HSCs). This was done because it was extremely difficult to isolate HSCs from rats with SL undergoing IR owing to insufficient perfusion of pronase and collagenase, and even when successful, the isolated HSCs showed very low viability for use in further *in vitro* studies.

The contractility of the HSCs and the phosphorylation of MLC and cofilin were significantly enhanced by ET-1 in the HSCs-SL, and fasudil attenuated these effects. Furthermore, fasudil prolonged the survival of rats with SL undergoing IR and attenuated

sinusoidal congestion and hepatocyte necrosis. These results indicate that fasudil, a specific ROCK inhibitor, suppresses IR-induced liver injury by ameliorating the hemodynamic disturbance through the modulation of Rho signaling in SL, which is more vulnerable to IR than NL.

Fasudil was used as a ROCK inhibitor in the present study based on the clinical application of the inhibitor for the release of cerebral vasospasm after subarachnoid hemorrhage [25]. Fasudil was administered at a dose of 10 mg/kg by intraperitoneal injection before IR because the area under the serum fasudil concentration curve for rats after the intraperitoneal injection of the inhibitor (10 mg/kg) was 4490 ng h/ml, which was almost compatible or slightly higher than that of fasudil in humans [26]. The use of fasudil may be a new therapeutic strategy to prevent hepatic IR injury.

The results of the current study indicated that the effect of fasudil on the contractility of HSCs-SL was mediated by the direct inhibition of ROCK, and independent of the NO effect in HSCs. Anegawa *et al.* reported that in a rat model of secondary biliary cirrhosis bile duct ligation, ROCK activation with resultant eNOS activation was substantially involved in the pathogenesis of portal hypertension. Moreover, fasudil significantly suppressed ROCK activity and increased eNOS phosphorylation through a reduction of the binding of serine/threonine Akt to ROCK and an increase of the binding of Akt to eNOS [27]. The improvement of hepatic hemodynamics by fasudil has been shown to be mediated by an enhancement of NO production by sinusoidal endothelial cells (SECs), rather than by direct inhibition of Rho-kinase in HSCs. Our result was not compatible with Anegawa's report. This may be due to differences in the experimental model used. We used a collagen gel contraction assay to show that the effect of fasudil on the contractility of HSCs-SL was mediated by direct inhibition of ROCK, while in the study by Anegawa *et al.*, a bile duct ligation-induced secondary biliary cirrhosis model revealed that the hemodynamic effects of the *in vivo* administration of fasudil were associated with the production of NO by SECs, and not by direct inhibition of ROCK. The relationship between SL and NO synthesis remains to be elucidated, and further investigation is necessary. In addition, ROCK inhibitors have been reported to improve the VLDL transport functions of hepatocytes in SL, which might be one of the mechanisms underlying their protective effect against IR injury [28].

In summary, activation of Rho/ROCK signaling in HSCs-SL is associated with an increased susceptibility to IR injury. Inhibition of ROCK attenuates the activation of the HSCs-SL and improves IR injury in rats with liver steatosis.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.04.029.

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Brief Communication

Human CD47 Expression Permits Survival of Porcine Cells in Immunodeficient Mice That Express SIRPα Capable of Binding to Human CD47

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Signal regulatory protein α (SIRPα) is a critical immune inhibitory receptor on macrophages, and its interaction with CD47 prevents autologous phagocytosis. We have previously shown that pig CD47 does not interact with human SIRPα, and that human CD47 expression inhibits phagocytosis of porcine cells by human macrophages in vitro. In this study, we have investigated the potential of human CD47 expression to promote porcine cell survival in vivo. Human CD47-expressing and control porcine B-lymphoma cells were transplanted into T- and B-cell-deficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice that express SIRPα capable of interacting with human CD47. Only the human CD47-expressing porcine lymphoma cells survived and were able to form tumors in NOD/SCID mice; however, both the control and human CD47-expressing porcine cells survived in macrophage-depleted NOD/SCID mice. These results indicate that transgenic expression of human CD47 may provide an effective approach to inhibiting macrophage-mediated xenograft rejection in clinical xenotransplantation.

Key words: CD47; Macrophage; Pig; Signal regulatory protein α (SIRPα); Xenotransplantation

INTRODUCTION

Xenotransplantation from pigs may provide a solution to the scarcity of human donors, but this type of clinical translation is primarily hampered by strong xenoimmune responses (7,16,20,28). Because of the extensive molecular incompatibilities between the donor and host, innate immune responses, including those mediated by natural antibodies, complement, macrophages, and natural killer (NK) cells, play a much greater role in the rejection of xenografts than in allograft rejection. Recipient macrophages are activated and rapidly recruited after xenotransplantation, and their responses to xenoantigens occur before T-cell activation (10). Macrophages cause almost immediate rejection of xenogeneic bone marrow cells, even in the absence of adaptive immunity (1,3), which poses a formidable obstacle to the application of mixed chimerism for induction of xenotransplantation tolerance. Macrophages have also been found to mediate the rejection of porcine islet xenografts in both rodents (9,11,17,26) and primates (19).

Macrophage activation is regulated by the balance between stimulatory and inhibitory signals. CD47 (also known as the integrin-associated protein) is a member

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of the Ig superfamily and it is expressed ubiquitously in all tissues (5). Signaling regulatory protein α (SIRPα) (also known as CD172a or SHPS-1) is an inhibitory receptor expressed on macrophages and dendritic cells (DCs) that recognizes CD47 as a "marker of self" (2,5). The CD47–SIRPα interaction provides a "don't eat me" signal to macrophages, which is required for preventing phagocytosis of normal self-hematopoietic cells. We have recently shown that the lack of interaction between pig CD47 and mouse SIRPα is critically associated with macrophage-mediated xenograft rejection in mice (24). We also observed that pig CD47 does not functionally interact with human SIRPα, and that human CD47 expression reduces phagocytosis of porcine cells by human macrophages in vitro (12). A recent study demonstrated that, due to polymorphisms in the nonobese diabetic (NOD) SIRPα allele, NOD mouse SIRPα is capable of cross-reacting with human CD47, and such cross-reactivity prevents human hematopoietic cells from rejection by macrophages in the mouse model (21). In the present study, we used NOD/SCID (severe combined immunodeficient) mice to assess the potential of human CD47 expression to inhibit macrophage-mediated rejection of porcine cells in vivo.

MATERIALS AND METHODS

Mice and Cell Lines

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed in a specific pathogen-free microisolator environment. Protocols involving animals used in this study were approved by the Massachusetts General Hospital Subcommittee of Research Animal Care, and all of the experiments were performed accordingly. Human CD47-expressing (hCD47-LCL) and control (pKS-LCL) porcine cell lines were generated by transfecting porcine B lymphoma cell line (LCL) cells with pKS336-hCD47 or empty pKS336 vector, respectively, as described previously (12).

In Vitro Cytotoxicity Assay

hCD47-LCL cells and pKS-LCL cells were mixed (at 1:1 ratio) and cocultured (4×10^4/well) with or without human macrophages in 24-well plates, and the ratios of hCD47-LCL cells to pKS-LCL cells in the cultures were determined every day for 8 days by flow cytometry using anti-human CD47 mAb (B6H12; Pharmingen, San Diego, CA) and anti-pig major histocompatibility complex class I (pMHC-I; clone 2.27.3). Human macrophages were differentiated from human monocytic leukemia cell line THP-1 cells (ATCC, Manassas, VA) by stimulation with phorbol myristate acetate (100 ng/ml) for 2 days, and were used after washing out the nonadherent cells.

Porcine Cell Transplantation in NOD/SCID Mice

Porcine cells were injected into the peritoneal cavity or renal subcapsular space of NOD/SCID mice. Some NOD/SCID mice were treated with clodronate liposomes to deplete macrophages. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany), and liposome encapsulation was performed as described previously (22). NOD/SCID mice were intravenously injected with clodronate liposomes every 5 days until analysis. Porcine cell survival was determined by flow cytometric analysis (FACScalibur; BD Biosciences, San Jose, CA) using fluorescence-conjugated anti-pMHC-I (clone 2.27.3) and anti-human CD47 (B6H12). Each experimental group contained between 3 and 12 mice.

Statistical Analysis

Significant differences between groups were determined by Student's *t*-test using Prism 4 (GraphPad Software, San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Human CD47 Expression Enables Porcine LCL Cells to Survive in NOD/SCID Mice

We first compared the survival of hCD47-LCL and pKS-LCL) porcine LCL cells in NOD/SCID mice after IP injection. In vitro assay confirmed that hCD47-LCL cells are significantly more resistant than pKS-LCL cells to destruction by human macrophages (Fig. 1), which is consistent with our previous observations (12). NOD/SCID mice were injected IP with the 1:1 mixed hCD47-LCL and pKS-LCL cells (5×10^7/mouse in total) (Fig. 2A), and sacrificed either when they first showed signs consistent with tumor development (lethargy, hunched posture, weight loss, and palpable abdominal swelling and/or mass) or at day 45 postinjection. In the 12 mice examined, five developed visible tumors (Fig. 2B). Tumor cell suspensions were subsequently prepared and stained with anti-pig class I and anti-human CD47 in order to detect the survival of hCD47-LCL versus pKS-LCL cells. Flow cytometric analysis of the tumor cell suspensions revealed that all tumor cells from these mice expressed human CD47, indicating that hCD47-LCL, but not pKS-LCL, cells were capable of surviving in NOD/SCID mice (Table 1, Fig. 2B).

Similar results were obtained when a mixture (1:1) of hCD47-LCL and pKS-LCL cells was injected into the renal subcapsular space of NOD/SCID mice. These mice were sacrificed between 2 and 5 weeks after LCL cell injection, and tumors were found in four of the five mice analyzed (Table 1). Again, all surviving tumor cells detected in these mice were determined to be human $CD47^+$ hCD47-LCL cells by flow cytometric analysis