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ORIGINAL ARTICLE

Protective effects of simultaneous splenectomy on small-for-size liver graft injury in rat liver transplantation

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Summary

Splenectomy is an effective technique in living donor liver transplantation (LDLT) with small-for-size (SFS) liver grafts for overcoming SFS liver graft injury. However, the protective mechanism of splenectomy is still unclear. The aim of this study was to investigate how splenectomy could attenuate SFS graft injury through the measurement of biochemical factors, particularly the expression of endothelin (ET)-1, which is a key molecule of microcirculatory disorders by mediating sinusoidal vasoconstriction. We performed rat orthotopic liver transplantation using SFS liver grafts with or without splenectomy. We investigated intragraft expression of ET-1 mRNA and hepatic protein levels of ET-1. In addition, portal pressure, hepatic injury and morphological changes, and survival rate were evaluated. In result, intragraft ET-1 mRNA expression after SFS liver transplantation was significantly downregulated by splenectomy, and hepatic expression of ET-1 in SFS grafts was rarely observed. Splenectomy inhibited the increase in portal pressure, ameliorated SFS liver graft injury and improved the graft survival rate after SFS liver transplantation. In conclusion, splenectomy improved the SFS liver injury and decreased the expression of ET-1 by attenuating portal hypertension on SFS liver transplantation. Downregulation of intragraft ET-1 expression plays important roles in the protective mechanism of splenectomy in SFS liver transplantation.

Introduction

Living donor liver transplantation (LDLT) has become an important treatment for the patients with end-stage liver disease and hepatic malignancies because of a shortage of deceased donors [1]. However, widespread application of LDLT has revealed a problem of inadequate graft size so-called small-for-size (SFS) liver grafts, particularly in adult-to-adult transplantation [2,3]. An SFS liver graft is defined as a graft involving <40–50% of the standard liver volume or a graft to recipient weight ratio (GRWR) of 0.8–1.0% [3]. An SFS liver graft that does not meet the functional demands of the recipient results in serious liver failure including poor bile production, delayed synthetic function, prolonged cholestasis and intractable ascites, and often leads to death in the absence of retransplantation. In addition to such serious complications, recent studies reported that SFS liver grafts are associated with acute rejection and metastasis [4,5]. To minimize risk for living donors, transplant surgeons aim at procuring the minimal liver volume necessary, and this potentially leads to an SFS liver graft. Therefore, understanding the pathogenesis and improving the therapeutic strategies are crucial for overcoming serious liver failure caused by SFS liver graft injury in LDLT.

Although several pathogenic mechanisms, such as insufficient graft volume, poor graft quality, insufficient outflow drainage and severity of the original liver disease, have been reported for SFS liver graft injury, one of the major causes is excessive portal venous inflow into a relatively small graft [2,6]. Excessive portal inflow induces mechanical injury to

an SFS liver graft and aggravates hepatic ischaemia-reperfusion injury (IRI) [7], particularly hepatic microcirculatory disturbances via irreversible sinusoidal endothelial cell injury after SFS liver transplantation [8]. Previous studies have reported that endothelin (ET)-1, a powerful vasoactive peptide, is a key molecule in the pathogenesis of microcirculatory disorders that follow IRI. Therefore, ET-1, which results from shear stress induced by portal hypertension, is also considered a cause of microcirculatory disturbance because it mediates sinusoidal vasoconstriction after SFS liver transplantation [9,10].

Several surgical interventions that modify portal hypertension after SFS liver transplantation have already been clinically applied; these interventions include portosystemic shunt, splenic arterial ligation and splenectomy [11]. Among these surgical interventions, splenectomy has been reported as a simple, powerful and effective technique in LDLT patients with SFS liver grafts, not only for modifying portal hypertension but also for overcoming SFS graft dysfunction [12,13]. However, the exact details regarding the protective mechanism of splenectomy in SFS liver transplantation, particularly the intragraft molecular response, are still unclear.

In this study, we hypothesized that simultaneous splenectomy in SFS grafts downregulates intragraft expression of ET-1 by decreasing portal hypertension in a rat liver transplantation model. The aim of this study was to investigate how splenectomy could attenuate SFS graft injury through the measurement of biochemical factors, particularly the expression of ET-1 in SFS liver grafts. Elucidating the protective mechanism of splenectomy in SFS liver transplantation in rats is important for the development of new clinical therapeutic strategies to prevent SFS liver graft injury in LDLT patients.

Materials and methods

Animals

Male Lewis rats (LEW; Japan SLC, Hamamatsu, Japan), weighting 200–280 g, were used as both donors and recipients. Animals were housed in standard animal laboratories with a 12 h light/dark cycle and free access to water and standard laboratory chow *ad libitum*. Animals were fasted for 24 h before the operation. All experiments followed our institution's criteria for the care and use of laboratory animals in research, which conform to the National Institutes of Health Guidelines.

Surgical procedure and experimental design

All surgical procedures were performed under isoflurane inhalation anaesthesia. To reduce graft size, lobe ligation and the piercing suture technique were used. Right lobe liver graft was selected as the SFS liver graft and the median ratio of graft weight to recipient liver weight was 31.8% (range 22.5–34.8%). Liver grafts were preserved in ice-cold saline solution before implantation into recipients and the mean time of cold preservation was 45 min. Orthotopic liver transplantation was performed with the two-cuff technique, which is the modified technique described by Kamada *et al.* [14]. The suprahepatic inferior vena cava was anastomosed using 8-0 nylon continuous sutures. The portal vein and infrahepatic inferior vena cava were anastomosed with the cuff technique. The bile duct was anastomosed with an intraluminal stent. After implantation of SFS liver grafts, animals in the SFS liver transplantation with splenectomy (SLT + SP) group underwent splenectomy immediately. Postoperatively, animals were allowed to recover from surgery with free access to food and water *ad libitum*.

Animals were divided into four groups; the simple laparotomy group (Sham group), the whole liver transplantation group (WLT group), the SFS liver transplantation group (SLT group) and the SLT + SP group. Animals were sacrificed 12 or 24 h after surgery to collect graft liver tissue specimens. Blood samples were collected 24 h after surgery.

Portal pressure measurement

After anaesthesia induction, a 24-G catheter was inserted into the ileocolic vein and connected to a pressure transducer (MU-881R, Nihonkouden, Tokyo, Japan) for continuous pressure monitoring. Portal pressure was measured immediately after liver transplantation in the SLT and SLT + SP groups. As a control, the portal pressure before liver transplantation was also measured (for each group, $n = 5$).

Evaluation of liver injury by blood analysis and histological study

For blood analysis, all blood samples were centrifuged at 1400 *g* for 10 min at 4 °C, and the serum was frozen at −20 °C for further measurement. Serum alanine aminotransferase (ALT) and bilirubin levels were measured as an indicator of hepatic graft injury using a diagnostic kit (WAKO Pure Chemical Industries, Osaka, Japan) and Quantichrom™ bilirubin assay kit (for each group, $n = 6$). For histological studies, liver tissue specimens from each group were fixed in 10% buffered formalin and embedded in paraffin for light microscopy. Sections were cut and stained with haematoxylin and eosin.

Hepatic gene expression profiles by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)

In this study, we aimed to demonstrate the relationship between excessive portal flow and ET-1 expression level.

Therefore, we performed real-time quantitative RT-PCR to quantify the ET-1 expression. Total RNA was extracted from liver tissues using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA). First-strand cDNA was synthesized using a Ready To Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ, USA). ET-1 mRNA was quantified using a Light Cycler (Roche Diagnostics, Mannheim, Germany) with the double-strand-specific dye SYBR Green I for each group, ($n = 5$). ET-1 expression was adjusted using the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and was reported as the ratio to GAPDH mRNA. The following primers were used:

ET-1 Sense: (5′-CTGGTGGAGGGAAGAAAACT-3′)
ET-1 Antisense: (5′-GGGCTCGCTCTATGTAAGTC-3′)
GAPDH Sense: (5′-TGAACGGGAAGCTCACTGG-3′)
GAPDH Antisense: (5′-TCCACCACCCTGTTGCTG TA-3′)

Hepatic ET-1 expressions by immunohistochemical staining

We performed immunohistochemical staining to determine the localization of the intragraft expression of ET-1. Liver tissue specimens were fixed in 4% paraformaldehyde (Wako Chemical Co., Osaka, Japan) in phosphate-buffered saline (PBS), washed with PBS, dehydrated with 70%, 90% and 100% ethanol and xylene, then embedded in paraffin. From each paraffin block containing liver tissue specimens, 2.0-μm sections were cut and mounted onto a superfrosted slide glass. For antigen retrieval, the sections were overheated in a microwave (500 W) for 5 min, 3 times in citrate buffer (pH 6.0) and blocked with nonspecific staining blocking regent (DAKO, Glostrup, Denmark). After overnight incubation at 4 °C with monoclonal anti-ET-1 antibody (no. 18201; IBL Corp., Gunma, Japan) at a dilution of 1:100, the sections were processed according to the standard immunoperoxidase method using an EnVision Kits (K0675; DAKO, Glostrup, Denmark). The sections were counterstained with haematoxylin.

Survival study

Ten animals in the WLT, SLT and SLT+SP groups were allowed to survive until death, and survival rates were determined. Rats that had lived for more than 7 days after liver transplantation were considered long-time survivors.

Statistical analysis

All data are expressed as the mean ± SD. The Mann–Whitney *U*-test was used for statistical comparison. Survival rates were assessed using the Kaplan–Meier test, and the statistical significance between experimental groups was determined by the log-rank test. *P* value of < 0.05 was considered statistically significant.

Results

Splenectomy inhibited the increase in portal pressure after SFS liver transplantation

The portal pressure in the SLT group was significantly increased by SFS liver transplantation compared with that in the control group ($P < 0.01$). In contrast, the portal pressure after liver transplantation in the SLT + SP group was significantly lower than that in the SLT group ($P < 0.05$). No significant difference was observed between the portal pressures in the SLT + SP and control groups (Fig. 1).

Splenectomy ameliorated SFS liver graft injury in rat liver transplantation

We determined the extent of liver graft injury by measuring serum levels of ALT and bilirubin. At 24 h after liver transplantation, serum ALT levels in the SLT group were significantly higher than the WLT group ($P < 0.01$). In contrast, serum ALT levels in the SLT + SP group were not increased, and there was no significant difference between the SLT + SP group and the WLT group (Fig. 2). Serum bilirubin levels of the SLT group, at 24 h after liver transplantation, were also significantly higher than the WLT group ($P < 0.01$) and the SLT + SP group ($P < 0.05$), and there was no significant difference in serum bilirubin levels between the SLT + SP group and the WLT group (Fig. 3).

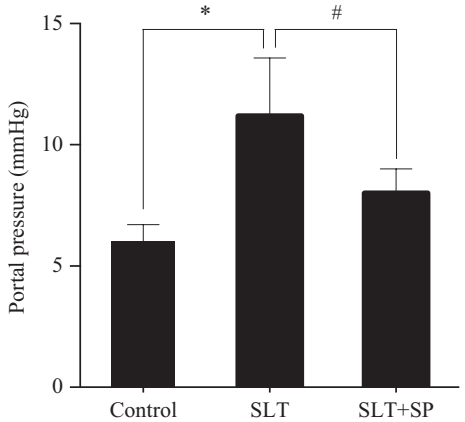

Figure 1 In the SLT group, portal pressure was significantly increased by SFS liver transplantation compared with that of control group (11.2 ± 2.39 vs. 6.0 ± 0.71 mmHg; *$P < 0.01$). The portal pressure after liver transplantation in the SLT + SP group (8.0 ± 1.00 mmHg) was significantly lower than that in the SLT group (#$P < 0.05$). Data were mean ± SD. $n = 5$ per group.

Hepatic architecture in SFS graft, particularly the integrity of sinusoids, was well protected by splenectomy

Normal liver architecture was observed in the Sham group as a control (Fig. 4a). In the WLT group, morphological changes were also not observed after liver transplantation (Fig. 4b). In the SLT group, swollen liver cells compressed the sinusoidal space, especially around the central vein, and vacuolar changes in the cytoplasm were observed (Fig. 4c). In contrast, hepatic lobular architecture was well preserved, and the portal tracts showed normal morphology in the SLT + SP group (Fig. 4d).

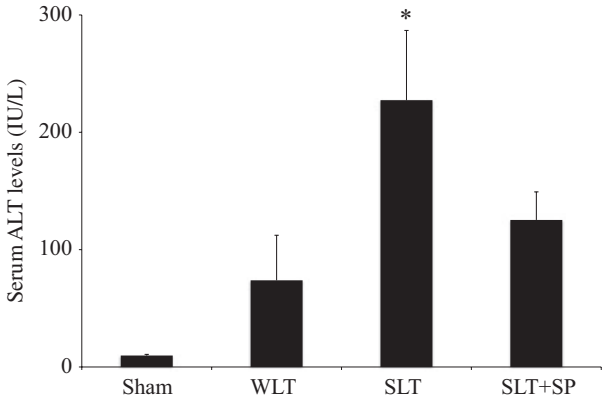

Figure 2 Serum ALT levels in the SLT group were significantly higher than the WLT group and the SLT + SP group (*$P < 0.01$). There was no significant difference in ALT levels between the SLT + SP group and the WLT group. Data were mean ± SD. $n = 6$ per group.

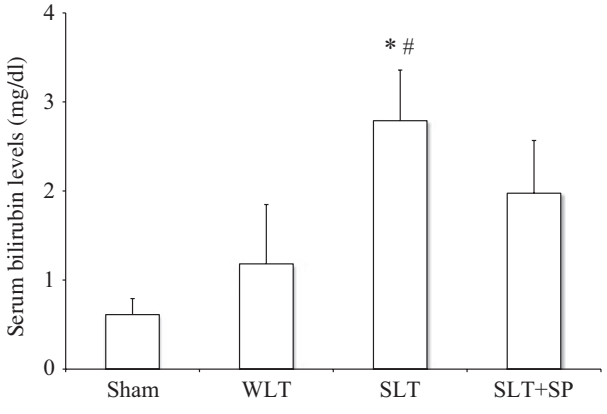

Figure 3 Serum bilirubin levels in the SLT group were significantly higher than the WLT group (*$P < 0.01$) and the SLT + SP group (#$P < 0.05$). There was no significant difference in ALT levels between the SLT + SP group and the WLT group. Data were mean ± SD. $n = 6$ per group.

Intragraft ET-1 mRNA expression after SFS liver transplantation was significantly downregulated by simultaneous splenectomy

Intragraft expression of ET-1 mRNA was detected by quantitative real-time RT-PCR. There was no significant difference in intragraft expression of ET-1 mRNA at 12 h after liver transplantation in every group. At 24 h after liver transplantation, intragraft expression of ET-1 mRNA in the SLT group was significantly higher than the other groups ($P < 0.05$). In contrast, expression in the SLT + SP group was not increased, and there was no significant difference between the SLT + SP group and the WLT groups (Fig. 5).

Hepatic expression of ET-1 in SFS grafts was rarely observed with simultaneous splenectomy

To examine the actual distribution and localization of hepatic ET-1 expression in SFS liver grafts, we determined hepatic protein levels of ET-1 by immunohistochemical staining at 24 h after transplantation. No hepatic ET-1 expression was observed in the Sham group (Fig. 6a). In the SLT group, ET-1 positive cells were seen in sinusoids, which are around the central veins (Fig. 6c). In contrast, ET-1 positive cells were clearly decreased in hepatic tissue of the SLT + SP group than in the SLT group (Fig. 6d). The number of ET-1 positive cells in the WLT group was approximately the same as that in the SLT + SP group (Fig. 6b).

Simultaneous splenectomy significantly improved the graft survival rate after SFS liver transplantation

The WLT group survived indefinitely and was healthy in appearance. In the SLT group, 40% achieved long-time survival and the others died within 3 days after liver transplantation. On the other hand, all animals in the SLT + SP group survived indefinitely and showed significant survival rate improvement compared with the SLT group ($P < 0.01$) (Fig. 7).

Discussion

According to previous studies, the major complications associated with SFS liver graft injury result from hepatic sinusoidal damage caused by portal hypertension, severe inflammatory response triggered by shear stress and hepatic microcirculatory disturbances because of vascular mediators [2,8]. However, the detailed mechanisms of SFS liver injury and subsequent liver failure are still unclear. In clinical studies, it is apparent that modulation of excessive portal inflow for shear stress improvement is effective in reducing SFS liver graft injury [2,15,16]. Although splenectomy is one of the major clinical strategies for

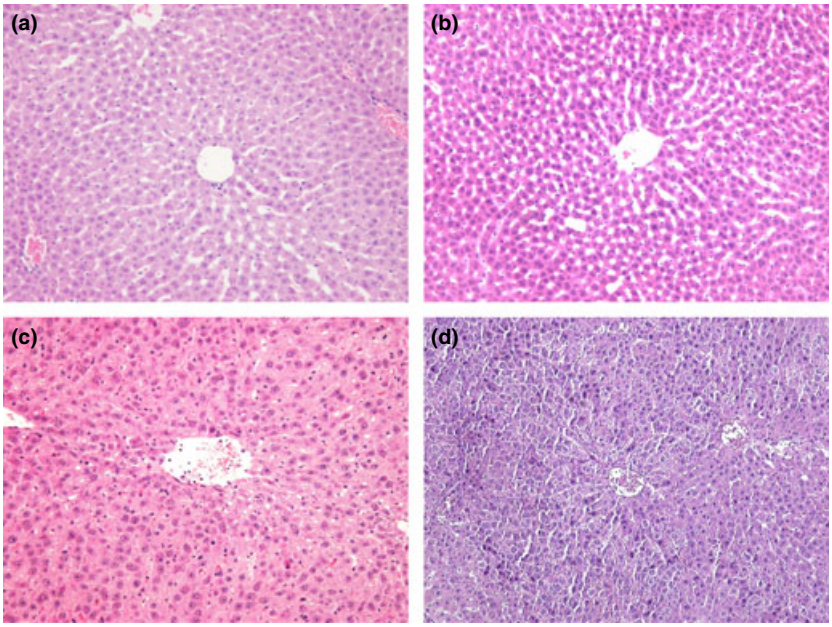

Figure 4 Normal liver architecture was observed in the Sham group as a control (a). In the WLT group, morphological changes were also not observed after liver transplantation (b). In the SLT group, swollen liver cells compressed the sinusoidal space, especially around the central vein, and vacuolar changes in the cytoplasm were observed (c). In contrast, hepatic lobular architecture was well preserved and the portal tracts showed normal morphology in the SLT + SP group (d) (Original magnification ×100).

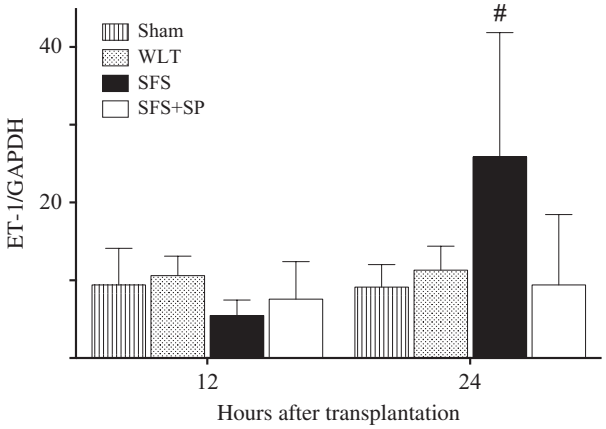

Figure 5 There was no significant difference in intragraft expression of ET-1 mRNA at 12 h after liver transplantation. However, 24 h after liver transplantation, intragraft expression of ET-1 mRNA in the SLT group was significantly higher than in the other groups (#$P < 0.05$). In contrast, intragraft expression of ET-1 mRNA of the SLT + SP group was not increased, and there was no significant difference between the SLT + SP group and the WLT group. Data were mean ± SD. $n = 5$ per group.

modulation of excessive portal inflow, few reports have revealed the relationship between splenectomy and the intragraft molecular response. In this study, we showed that simultaneous splenectomy reduced liver injury 24 h after SFS liver transplantation in an experimental rat model. Our results showed that simultaneous splenectomy in SFS liver transplantation significantly decreased intragraft ET-1 expression, not immediately after reperfusion but 24 h after transplantation. These findings indicated that portal decompression secondary to splenectomy led to downregulation of ET-1 and precluded SFS liver graft injury by improving sinusoidal microcirculatory disturbance.

ET-1, a 21 amino acid peptide, plays a crucial role in many biological processes. The major function of ET-1 is local control of vascular tone by binding to endothelin receptors, such as endothelin receptor A (ETA), on sinusoidal hepatic stellate cells (HSCs) [17]. Increased ET-1 levels have various adverse effects on liver, including vasoconstriction of the sinusoids, diminution of perfusion rate, intrahepatic vascular resistance, promotion of leucocyte–endothelium interaction and portal hypertension [18]. Therefore, many experimental studies have implicated ET-1 in the hepatic injury caused by microcirculatory disturbances [19]. A previous study conducted at our institution reported that hepatic ET-1 production and activation of HSCs were increased after hepatic IRI during obstructive cholestasis [20].

Recently, many studies have demonstrated the contribution of ET-1 in the molecular mechanism of portal hypertension caused by SFS liver transplantation. Chen *et al.* [21] reported that SFS liver graft injury was related to high levels of ET-1 binding to upregulation of ETA on hepatic stellate cells. Man *et al.* [22] demonstrated an imbalance of vasoregulatory genes, such as ET-1 and ETA, by cDNA microarray and that early overexpression of several

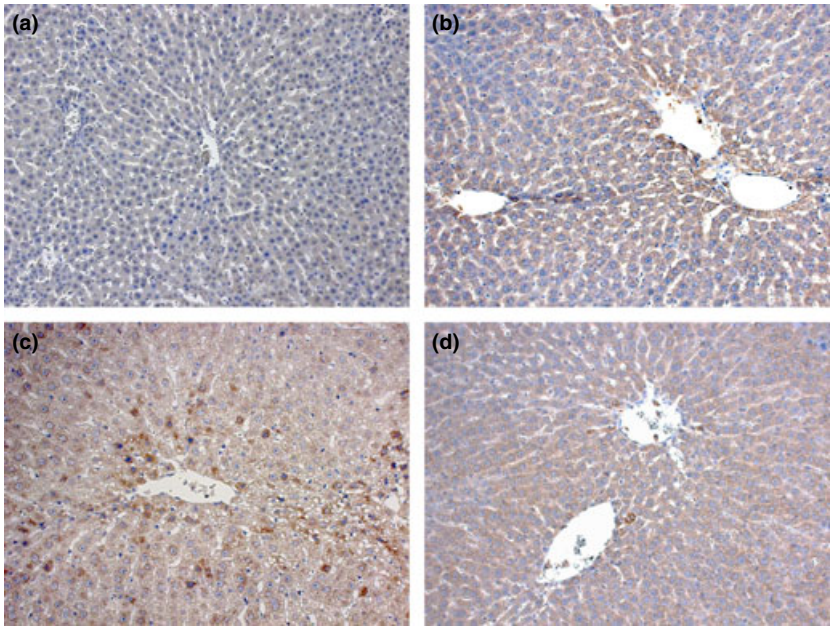

Figure 6 To examine the ET-1 expression in SFS liver grafts, we determined hepatic protein levels of ET-1 by immunohistochemical staining. No hepatic ET-1 expression was observed in the Sham group (a). In the SLT group at 24 h after transplantation, ET-1 positive cells were seen in sinusoids, which are around the central veins and Glisson's capsule (c). In contrast, ET-1 positive cells were clearly decreased in hepatic tissue of the SLT + SP group than in the SLT group (d). The number of ET-1 positive cells in the WLT group was approximately the same as that in the SLT + SP group (b) (Original magnification ×200).

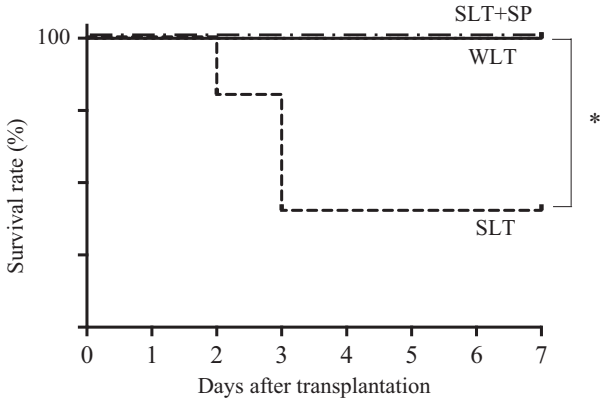

Figure 7 All animals in the WLT group (solid line) survived indefinitely. In the SLT group (broken line), 40% achieved long-time survival and the others died within 3 days after liver transplantation. On the other hand, SLT + SP group (dot-dash line) showed significant survival rate improvement compared with the SLT group (*$P < 0.01$). $n = 10$ per group.

adhesion molecules also plays an important role in disturbance of hepatic microcirculation in the early phase after SFS liver transplantation. Palmes *et al.* [23] reported that hepatic microcirculatory disorders were related to ET-1/nitric oxide (NO) imbalance in SFS liver injury. On the basis of these data, ET-1 has been focused on as a biochemical therapeutic target for SFS liver graft injuries [24]. Suppression of ET-1 by administration of a selective ETA antagonist reduced sinusoidal microcirculatory disturbance and SFS liver graft dysfunction [25]. Treatment with FK409, a NO releaser, improved SFS liver graft injury via downregulation of the early growth response-1 pathway [26]. Moreover, administration of somatostatin, which is known to have an effect on portal decompression, led to downregulation of ET-1 and improved SFS graft injury [27]. In addition to these drug administration methods, the use of a preservation solution containing activated protein C also improved SFS graft injury through portal decompression [28]. However, few of these strategies have been clinically applied.

Most clinical studies have focused on interventional strategies to modify excessive portal venous inflow into SFS liver graft. Among those surgical strategies, portocaval shunting, splenic artery ligation and splenectomy have been clinically applied to patients with SFS grafts in LDLT [2]. Troisi *et al.* [29] reported that using portocaval shunts for grafts with GRWR <0.8% improved 1-year graft survival from 20% to 75%. However, a major concern with portocaval shunt is the portal-flow stealing phenomenon, and a logical conclusion is that the presence of a shunt negatively affects outcome if portal flow decreases excessively [30]. Humar *et al.* [31] reported splenic artery ligation performed in seven patients with SFS graft dysfunction, and six patients successfully recovered from liver failure. Shimada *et al.* [32] performed splenectomy or splenic artery ligation in 8 patients with SFS grafts (GRWR; 0.7%) and

demonstrated that these surgical procedures could decrease both the portal pressure after transplantation and the mortality rate. Yoshizumi *et al.* [33] investigated 50 recipients with a graft weight to standard liver weight ratio of <40% and showed that SFS liver transplantation without splenectomy was a risk factor for the occurrence of liver failure. In addition, Yoshizumi *et al.* also showed that splenectomy is more beneficial than splenic artery ligation in SFS liver transplantation because splenectomy was more effective in treating pancytopenia.

The most noted adverse effects of splenectomy are septic complications. Splenectomy in liver transplantation is strongly associated with septic complications and poor diagnosis [34]. Moreover, because of portal hypertension, simultaneous splenectomy can be difficult in patients with huge collaterals. In such cases, simultaneous splenectomy affects both the extent operation time and blood loss during the operation. However, recent reports have revealed no such septic complications in LDLT [12,32,35], and recent advances in surgical devices have made surgical dissection with numerous collateral vessels easier [36].

In this study, we demonstrated that the inhibition of excessive portal inflow into the liver graft decreased the intragraft ET-1 expression level in the splenectomy group. However, these data may not be only indicative of the underlying mechanism of the protective effects of splenectomy. Recent studies have also demonstrated the mechanisms underlying the improvement in liver injury by simultaneous splenectomy, except for portal decompression. Kuriyama *et al.* [37] reported that the elimination of splenic inflammatory cell recruitment into the liver was the cytoprotective effect of splenectomy in SFS liver transplantation. The other studies have shown that platelets, which increase after splenectomy, can promote liver regeneration [38,39]. In patients with hepatitis C virus, splenectomy results in significantly improved platelet counts after LDLT [35,40]. Transforming growth factor beta 1, which is a major antiproliferative factor for hepatocytes, was produced and secreted by the spleen during the early phase of liver regeneration in rats [41]. In addition, Uehara *et al.* [42] reported that ET-1 derived from the spleen increased intrahepatic resistance by downregulating the Rho-kinase pathway and that splenectomy improved liver function by enhancing intrahepatic microcirculation in a liver cirrhosis model. These data suggest that splenectomy has various protective effects in SFS liver transplantation by modulation of excessive portal inflow and other molecular mechanisms.

In conclusion, we clearly demonstrated that simultaneous splenectomy improved the SFS liver injury and decreased the expression of ET-1 by attenuating the effect of portal hypertension on SFS liver transplantation in rats. These results suggested that downregulation of intragraft ET-1 expression plays important roles in the protective mechanism of simultaneous splenectomy in SFS liver transplantation. To clarify the effect of splenectomy on LDLT, further clinical investigations are required, such as multicenter prospective studies.

Authorship

TY and TT: involved in preforming experiments, analysing data and manuscript writing. HS, HY, HO, and AK: involved in analysing data and designing study. HY, KF, SK, DO, DS, and HN: involved in data collection, literature search and preparation of the manuscript. MM: involved in designing study and interpretation of results.

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