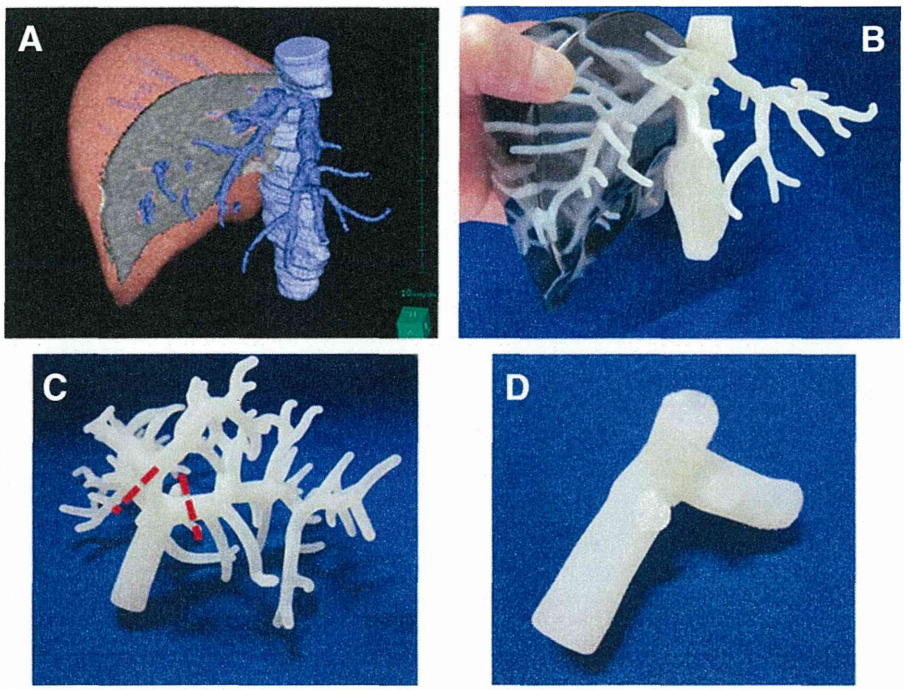

Figure. 3. (A,B) A 3D solid model of the graft obtained from a 3D printer was constructed from the data obtained with the Synapse Vincent program. A gumlike material was used to make (C) the recipient's PV tree with (D) a PV Y-graft, and these models were effective for allowing the reconstructed shape of the anastomosis to be understood with appropriate spatial perception.

estimate each vessel's circulating and draining areas. On the basis of this estimate, we are able to evaluate whether to reconstruct the MHV tributaries or anomalous hepatic veins in LDLT.

With the recent development of 3D printers, solid 3D models can be made from images obtained with a 3D volume analyzer. In this case, we made a 3D model from the data generated by the Synapse Vincent program (Fig. 3A,B). The solid 3D model was effective as a preoperative simulation, and the plastic liver and gum-like material made to represent the vessels made it easy to imagine the reconstructed shape and angle of the anastomosis with appropriate spatial perception (Fig. 3C,D), which helped us to choose an appropriate surgical strategy. It should also be noted that the use of a 3D printed model during surgical planning was helpful not only for the operating surgeons, but also for young surgeons as training, so that they could understand the detailed representation of the future complex anastomosis in LDLT. Although making a 3D solid model for every case is currently expensive, it is considered to be worthwhile for select cases such as ours because of its efficacy in facilitating operation planning. We highly recommend using a 3D printed model in cases with complex vascular or biliary anatomy, which require multiple or complicated anastomoses with or without the use of graft interposition.

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Hepatology Research

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Original Article

Prospective study of the safety and efficacy of intermittent inflow occlusion (Pringle maneuver) in living donor left hepatectomy

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Aim: The impact of intermittent inflow occlusion (Pringle maneuver) in living donor hepatectomy on the outcome of both the donor and the recipient is unknown. The aim of this study is to elucidate the safety and efficacy of Pringle maneuver in living donor hepatectomy.

Methods: Twenty consecutive cases of living donors who underwent left hepatectomy were prospectively divided into two groups, with (Group A, $n = 10$) or without (Group B, $n = 10$) the Pringle maneuver during hepatectomy. Intraoperative blood loss, postoperative liver functions in the donors and recipient outcome were reviewed.

Results: Median blood loss was significantly less in group A than in group B. Median alanine aminotransferase was significantly higher on postoperative day 1 in group A than in group B, but the difference was not significant at 7 days after surgery. Eight of 10 recipients in each group survived with good graft function with a median follow-up period of 20 months in group A and 19 months in group B.

Conclusion: The Pringle maneuver was safely applied in living donor hepatectomy, but the only benefit was the reduction of blood loss during the donor surgery, and no positive impact on the recipient outcome.

Key words: hepatectomy, ischemic reperfusion injury, liver transplantation, living donor, Pringle maneuver

INTRODUCTION

REDUCING BLOOD LOSS is essential to achieving good outcomes in hepatectomy, and intermittent inflow occlusion (the Pringle maneuver) has been widely adopted to control intraoperative bleeding during liver transection.[1–3] Although it has been proven that the Pringle maneuver does not cause remnant liver injury, the possibility of ischemia–reperfusion injury is of great concern in living donor hepatectomy, both in the graft and in the remnant liver. On the other hand, the Pringle maneuver is reported to protect the hepatocyte against ischemia–reperfusion injury by means of a mechanism known as ischemic preconditioning, via heme oxygenase-1 (HO-1) upregulation in the liver.[4,5] Accordingly, Imamura *et al.* proposed that transplant physicians should not hesitate to use the Pringle maneuver in living donor hepatectomy.[6] However, the safety and feasibility of the Pringle maneuver in living donor liver transplantation (LDLT) have not been fully elucidated. The aim of this study is to elucidate the impact of the Pringle maneuver on the outcome of living donor hepatectomy and on graft function in the recipient.

METHODS

THIS PROSPECTIVE STUDY was approved by the ethics committee of Nagasaki University Hospital (no. 09082890), and all patients were enrolled in this study after providing fully informed consent. Surgical procedures were the same in the groups as has been described in detail elsewhere.[7–9] Liver parenchymal transection was performed utilizing saline-linked electric

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cautery and an ultrasonic surgical aspirator.[7] The Pringle maneuver was performed in group A, with intermittent inflow occlusion, repeated 15-min occlusion and 5 min of reperfusion. Intraoperative blood loss, serum lactate level just after hepatectomy and postoperative liver functions in the donors, and recipient outcomes were reviewed.

Immunohistochemistry of HO-1 and TLR4

Liver tissues were obtained from segment 4 according to Couinaud's nomenclature using a 16-G needle just before removal of the liver during donor surgery. Formalin-fixed paraffin-embedded (4-μm) sections were used for hematoxylin–eosin staining, and immunohistochemistry staining with HO-1 and Toll-like receptor 4 (TLR4) was performed using a high-temperature antigen-unmasking technique. The sections were boiled in pH 6.0 ethylenediaminetetraacetic acid buffer solution at 95 C for 40 min. After 10 min of blocking with 0.1% H_2O_2/methanol, the sections were incubated for 30 min at room temperature in a humidified chamber with primary antibodies. As the primary antibodies, anti-HO-1 rabbit antihuman polyclonal antibody (dilution 1:100; Lifespan Biosciences, Seattle, WA, USA) and anti-TLR4 rabbit antihuman polyclonal antibody (dilution 1:70; Bioworld Technology, Newmarket Suffolk, UK) were used. After a 30-min reaction with the primary antibodies, the slides were reacted with Histofine Simple Stain RAT MAX PO (MULTI) (Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 min. The slides were visualized with H_2O_2 and 3,3′-diaminobenzidine-tetrachloride solution at room temperature for 5 min and stained in hematoxylin for 1.5 min. The coverslips were mounted with 90% glycerol containing 1 mg/mL *P*-phenylenediamine.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. We considered $P < 0.05$ to be statistically significant.

RESULTS

Characteristics of the patients

TWENTY CONSECUTIVE LIVING donors who underwent left hepatectomy including the middle hepatic vein (MHV) without the caudate lobe from September 2009 to January 2011 were prospectively divided into two groups, those who underwent (group A, $n = 10$) and those who did not undergo (group B, $n = 10$) the Pringle maneuver during hepatectomy. These donors were divided into these two groups alternatively, so that this study is not randomized, but a controlled clinical trial. The characteristics of both donors and recipients are listed in Table 1. Only the sex of the recipient was significantly different between the groups, possibly due to the small sample size.

Outcomes of the donor surgery

As shown in Table 2, the median blood loss was significantly less in group A than in group B, both in total (303 g [range, 170–480] in group A vs 720 g [range, 360–2200] in group B, $P < 0.05$) and during hepatectomy (85 g [range, 10–132] in group A vs 420 g [range, 170–1200] in group B, $P < 0.01$). Intraoperative serum lactate level after hepatectomy was not significantly different between the groups (2.2 mmol/L [range, 1.0–4.2] in group A vs 2.2 mmol/L [range, 1.0–3.4] in group B). The median alanine aminotransferase (ALT) level was significantly higher on postoperative day 1 in group A than in group B (689 IU/L [range, 329–1279] in group A vs 321 IU/L [range, 281–644] in group B, $P < 0.05$), but the difference was not significant at 7 days after surgery and was normalized in both groups A and B at the last follow up (Fig. 1). The median levels of total bilirubin and prothrombin time international normalized ratio (PT-INR) were not significantly different throughout the postoperative course between the groups. With regard to the postoperative complications, one minor bile leakage which was defined as grade I in Clavien's classifications was seen in one patient from group A. All donors are currently doing well with normal liver functions at a median follow-up period of 26 months (range, 17–32) in group A and 23 months (range, 16–31) in group B, respectively (Table 2).

Outcomes of the recipients

The graft functions in the recipients are shown in Figure 2. The median ALT level was significantly higher on postoperative day 1 in group A than in group B (408 IU/L [range, 396–419] in group A vs 219 IU/L [range, 159–917] in group B, $P < 0.05$), but this difference had disappeared at 7 and 28 days after transplantation. Although there was no significant difference

Table 1 Characteristics of donors and recipients

	Group A Pringle(+)	Group B Pringle(−)	
n	10	10	
Donors			
Age	31 (20–56)	36 (31–55)	NS
Sex, M/F	8/2	7/3	NS
Recipients			
Age	58 (27–70)	61 (46–69)	NS
Sex, M/F	8/2	2/8	$P < 0.05$
Original diagnoses			
C-LC	7	5	
PBC	0	2	
B-LC	1	0	
Alcoholic-LC	1	1	
FHF	1	0	
Caroli disease	0	1	
MELD	15 (7–27)	14 (7–31)	NS
Blood type combination			
Identical/compatible	8	10	NS
Incompatible	2	0	
Graft weight (g)	373 (306–459)	387 (310–467)	NS
Estimated residual liver volume in the donor (%)	32 (25–38)	31 (27–34)	NS
GV/RSLV (%)	40.4 (31.1–53)	41.8 (32–54.4)	NS

Data are presented as median (range). B-LC, hepatitis B-related liver cirrhosis; C-LC, hepatitis C-related liver cirrhosis; FHF, fulminant hepatic failure; GV, graft volume; MELD, Model for End-Stage Liver Disease; NS, no significant change; PBC, primary biliary cirrhosis; RSLV, recipient standard liver volume.

in median total bilirubin at 1 day and 28 days after transplantation, this parameter was significantly lower on postoperative day 7 in group A than in group B (3.2 mg/dL [range, 1.6–17.0] in group A vs 9.1 mg/dL [range, 4.6–18.4] in group B, $P < 0.05$). The median levels of PT-INR were not significantly different throughout the postoperative course between the groups. As for the outcomes for the recipients associated

Table 2 Outcome of the donors

	Group A Pringle(+)	Group B Pringle(−)	
Duration of surgery (min)	407 (325–454)	388 (343–515)	NS
Duration of liver resection (min)	72 (51–83)	75 (64–102)	NS
Blood loss (g)	303 (170–480)	720 (360–2200)	$P < 0.01$
Blood loss during liver resection (g)	85 (10–132)	420 (170–1200)	$P < 0.01$
Serum lactate after hepatectomy (mmol/L)	2.2 (1.0–4.2)	2.2 (1.0–3.4)	NS
Complications			
Clavien classification			
I	1	0	
II	0	0	
III	0	0	NS
IV	0	0	
V	0	0	

NS, no significant difference.

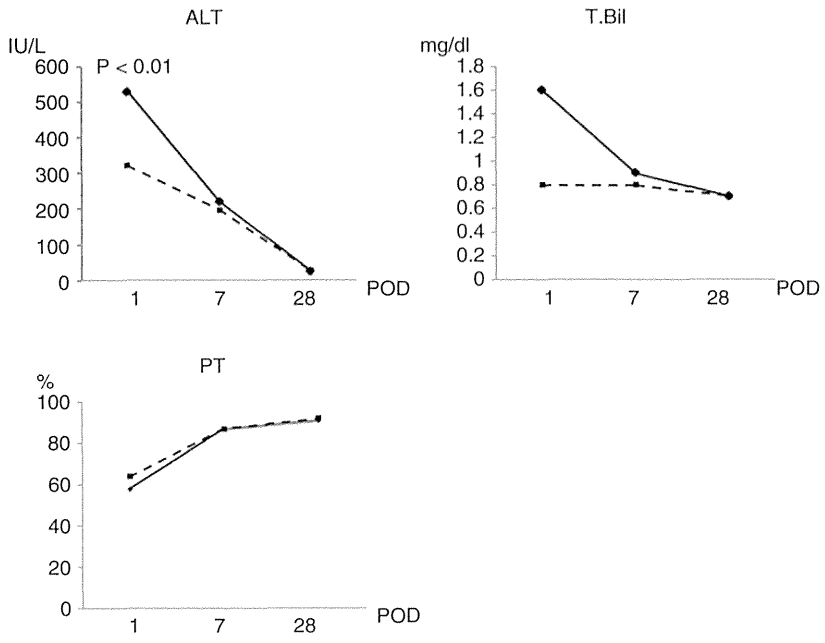

Figure 1 Serial changes of liver function tests after surgery in the donors, with (solid line) or without (dotted line) Pringle maneuver during donor surgery. ALT, alanine aminotransferase; POD, postoperative day; PT, prothrombin time; T.Bil, total bilirubin.

with these donors, the recipients for eight of 10 of the donors in each group survived with good graft function with a median follow-up period of 20 months (range, 11–26) in group A and 19 months (range, 10–25) in group B.

Immunohistochemistry of HO-1 and TLR4 in the graft

In the immunohistochemistry of the graft just after hepatectomy, the expressions of both HO-1 and TLR4 in

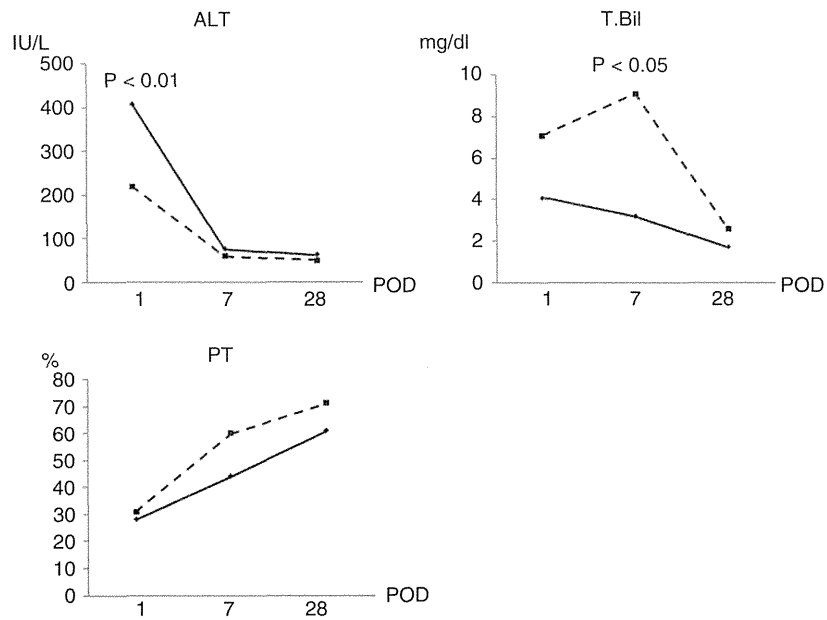

Figure 2 Serial changes of liver function tests after transplantation in the recipients, who received the graft from the donors with (solid line) or without (dotted line) Pringle maneuver during donor surgery. ALT, alanine aminotransferase; POD, postoperative day; PT, prothrombin time; T.Bil, total bilirubin.

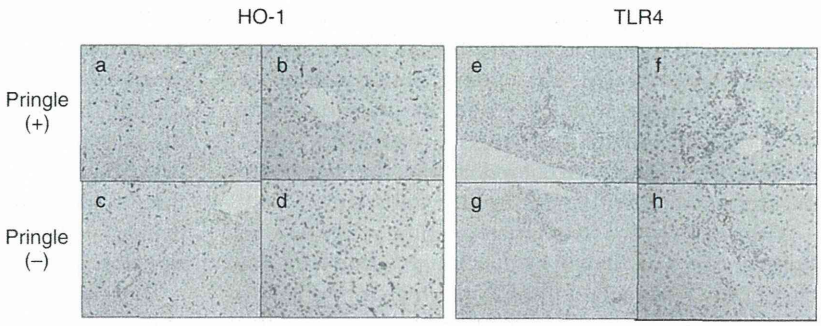

Figure 3 Immunohistochemistry of (a–d) heme oxygenase-1 (HO-1) and (e–h) Toll-like receptor 4 in the graft just before the removal of the graft in donor surgery. The expressions of HO-1 and TLR4 were not different between the Pringle group and the non-Pringle group. HO-1 was stained in the sinusoid, while TLR4 was stained in the portal triad in both groups (original magnification: [a,c,e,g] ×50; [b,d,f,h] ×100).

group A were similar to those in group B. HO-1 was stained in sinusoids, while TLR4 was stained in the portal triad in both groups (Fig. 3).

DISCUSSION

THIS STUDY SHOWED that the Pringle maneuver was safely applied in living donor hepatectomy, without causing severe injury in either the graft or the remnant liver in the donors. Although there has been no official tracking of whether the Pringle maneuver is generally adopted in living donor hepatectomy, most transplant centers do not utilize this technique, probably due to the concern that ischemic injury of the graft may occur, despite the fact that the safety of the Pringle maneuver has been proven in hepatectomy, even for cirrhotic livers.[1–3] Several single-arm retrospective studies showed that the Pringle maneuver could be safely applied in living donor hepatectomy,[10,11] and Imamura *et al.* mentioned that the graft function may be even better with ischemic preconditioning.[6] Recently, Park and colleagues also reported in their prospective randomized study that the Pringle maneuver was safely applied in living donor transplantation using a right lobe graft, with a graft-to-recipient bodyweight ratio of more than 0.9% and less than 30% steatosis.[12] They evaluated several cytokines that should be related to ischemic liver injury or liver regeneration, and none of them were significantly different between the groups with or without the Pringle maneuver. In our center, left lobe graft with MHV is the first line in adult-to-adult LDLT, so we evaluated cases of left lobe graft only. According to our study, even though these left lobe grafts were generally smaller in volume (median, 40–41% of recipient standard liver volume) than right lobe grafts, the graft function seemed to be as good as that of right lobe grafts.

However, although the final outcomes of the liver function in both the donors and recipients were not significantly different between the groups with or without the Pringle maneuver, hepatocyte injury during donor surgery seemed more severe in the Pringle group than in the group that did not undergo the Pringle maneuver because the ALT level was significantly higher in the Pringle group immediately after surgery in the donors. The ALT level result in our study was generally higher than in the previous reports.[6,10–12] This difference may be related to the fact that our procedure in liver resection was a two-surgeon technique using saline-linked electric cautery. We previously showed that our technique can be safely applied in living donor hepatectomy and with less blood loss and bile leakage than in the cases using ultrasonic surgical aspirator alone.[7] We believe that the high ALT level in the current study is related to the hepatocyte damage due to transient heat injury, which might have been enhanced by ischemic injury. In regard to the serum lactate level just after hepatectomy, there was no significant difference between the groups. Pietsch *et al.* showed in their study that serum lactate level during liver resection was significantly higher in the cases with Pringle maneuver than that in those without Pringle maneuver.[13] The result is not similar to that in our study, possibly because of absolutely normal liver of living donor in this study. Also, the mean level of lactate was 2.6 mmol/L in the Pringle group in their study, not so different from that in our study (median level of 2.2 mmol/L). However, we propose that as long as the possibility of more severe hepatocyte damage exists, the decision to use the Pringle maneuver should be made with caution in living donor hepatectomy, because donor safety is the top priority in LDLT. The indication of the Pringle maneuver should be based on the balance between the benefit (reduction of blood loss) and the

risk (possible severe hepatocyte damage) in the donor, because there seemed to be no obvious benefit for the recipient. Accordingly, our current policy is that we adopt the Pringle maneuver only in cases involving slight anemia before the surgery, emergency LDLT without time for saving blood before the surgery or uncomfortable bleeding during liver transection.

With regard to the liver function in the recipient after surgery, ALT was higher in the Pringle group the day after LDLT, while total bilirubin was higher in the non-Pringle group at day 7 after LDLT. In principle, this result may be related to the small sample size, and liver function after LDLT is easily affected by many factors, including blood flow, rejection, biliary stricture, infection, and so on. The only significant difference in patient characteristics was sex in the recipient. The number of female recipients was higher in the group with Pringle maneuver, possibly because of small sample size. Some studies have showed that the sex mismatch (female to male) is one of the significant factors related to poor outcome in LDLT.[14,15] In our study, the majority of donors were male in both groups, but sex mismatch may be related to the higher bilirubin level at day 7 after LDLT. In any case, we found that the Pringle maneuver seemed to have no beneficial impact on recipient liver function.

Heme oxygenase is the rate-limiting enzyme involved in heme metabolism. The inducible form, HO-1, is expressed in response to various stimuli, including peroxide, heat, hyperoxide, endotoxin and inflammatory cytokines. HO-1 is well recognized as one of the factors protecting hepatocyte against ischemic–reperfusion injury with its antioxidant, maintaining microcirculation and modulating the cell cycle and anti-inflammatory functions.[4,5] Several animal studies showed that HO-1 was expressed significantly more in the liver with ischemic preconditioning, but in our study, HO-1 was expressed in the livers that did not undergo the Pringle maneuver to the same degree as in those that underwent the Pringle maneuver. Because HO-1 is expressed by various stimuli as mentioned above, surgical intervention itself may induce HO-1 in a clinical situation, including heat injury with saline-linked cautery.

Toll-like receptor 4 belongs to the interleukin-1 receptor family, and triggers host inflammatory responses that are mediated by macrophages, neutrophils and complement.[16,17] Shen *et al.* reported that TLR4 may function as a putative HO-1 repressor in hepatic ischemic–reperfusion injury in their study using TLR4 knockout mice with 90 min of warm ischemia and 6 h of reperfusion.[18] In the current study, the expression of TLR4 in the Pringle group was found to be similar to that in the non-Pringle group in liver tissue on the basis of immunohistochemistry. Like HO-1, TLR4 is expressed by various stimuli, so that at least in the clinical setting in the current study, there was no significant difference in the expression of both HO-1 and TLR4 with or without the Pringle maneuver. Finally, we could not show any beneficial impact of the Pringle maneuver on the graft function in relation to ischemic preconditioning.

There are several limitations in this study, with the main one being that the number of cases was too small. A randomized control trial with sufficient number of cases should be performed to show the actual efficacy of the Pringle maneuver in LDLT, both in the donor and the recipient.

In conclusion, the Pringle maneuver was safely applied in LDLT, but the only obvious benefit was the reduction of blood loss in the donor surgery. Because the hepatocyte injury may be severe after the Pringle maneuver, this technique should be adopted in selected cases in which it is a priority to reduce the blood loss during donor surgery.

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CASE REPORT

Expression of alpha smooth muscle actin in living donor liver transplant recipients

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Abstract

Recently, there have been reports from liver biopsies that showed the progression of liver fibrosis in liver transplant patients after the cessation of immunosuppression. Herein, we focused on activated hepatic stellate cells expressing alpha smooth muscle actin (α-SMA) to understand the correlation between immunosuppressant medication and liver fibrosis. The study enrolled two pediatric patients who underwent living donor liver transplantation and ceased immunosuppressant therapy. The number of α-SMA-positive cells in the specimens obtained by liver biopsy from these two patients showed a three-fold increase compared with the number from four transplanted pediatric patients who were continuing immunosuppressant therapy. In addition, the α-SMA-positive area evaluated using the WinRooF image processing software program continued to increase over time in three adult transplanted patients with liver fibrosis, and the α-SMA-positive area was increasing even during the pre-fibrotic stage in these adult cases, according to a retrospective review. Therefore, α-SMA could be a useful marker for the detection of early stage fibrosis.

Key words: Living donor liver transplantation; Liver fibrosis; Immunosuppressant therapy; Alpha smooth muscle actin; Hepatic stellate cells

Core tip: The primary finding presented in this case report is that there is that the cessation of immunosuppressant therapy may promote liver fibrosis in patients after liver transplantation, even though normal liver function is maintained. In addition, the alpha smooth muscle actin (α-SMA)-positive area increased during the pre-fibrotic stage. Therefore, α-SMA may serve as a useful marker to detect early stage fibrosis.

Hirabaru M, Mochizuki K, Takatsuki M, Soyama A, Kosaka T, Kuroki T, Shimokawa I, Eguchi S. Expression of alpha smooth muscle actin in living donor liver transplant recipients. *World J Gastroenterol* 2014; 20(22): 7067-7074 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i22/7067.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i22.7067

INTRODUCTION

Liver transplantation is an established treatment for hepatic failure. Recent developments in surgical techniques, anesthesia and perioperative management have contributed to a decrease in early mortality after liver transplantation. However, the mortality in patients with chronic hepatic failure has remained unchanged[1]. Some of the causes of

the poor prognosis for these patients include renal disorders, vascular disorders, malignant tumors, and the use of immunosuppressant medication[2-4]. Therefore, a reduction in such medication may reduce the mortality rate[5].

Despite many reports describing patients who have acquired immune tolerance[6,7], the characteristics of patients with immune tolerance are still unknown[8]. Clinical immune tolerance refers to the state of maintaining normal organ graft function even after the cessation of immunosuppressant medication[9,10]. In practice, the cessation of immunosuppressant medication varies depending on each patient's condition and must be individualized; although some patients have a favorable postoperative course and can successfully achieve a reduction of immunosuppressant medication, other patients have no choice but to stop the treatment, such as in the case of infection with the Epstein-Barr virus (EBV). The probability of adult patients acquiring immune tolerance has been reported to be 8%-33%[11-18], and this rate has been suggested to be much higher in pediatric patients[6,19,20].

However, liver transplant recipients with no abnormalities in hepatic function after the cessation of immunosuppressant medication have recently been reported to developed hepatic fibrosis, with the hepatic fibrosis improving after resumption of the medication[21]. Therefore, there is a need to understand the mechanism(s) of hepatic fibrosis induced by withdrawal of immunosuppression. We have herein focused on hepatic stellate cells (HSCs), which may be involved in hepatic fibrosis. HSCs constitute a large portion of the hepatic interstitium, representing 5%-8% of the total number of liver cells[22]. In the healthy liver, HSCs are quiescent, but can be activated by factors, including TGFβ1 and IFNγ, that are released by Kupffer cells (KC) and T cells after injury or stimulation[22,23]. The appearance of alpha smooth muscle actin (α-SMA) in the activated HSCs can be detected using α-SMA immunostaining[24]. Activated HSCs undergo apoptosis at sites of acute inflammation but induce sinusoidal sclerosis, leading to the development of sinusoidal portal hypertension at sites of chronic inflammation. The activated HSCs have also been suggested to be responsible for the expression of type I collagen and the progression of fibrosis[25]. We therefore predict that an immune response may cause fibrosis in patients who have discontinued immunosuppressant medication, however the mechanism underlying this response remains to be determined.

We performed immunohistological analysis to determine the mechanism underlying the fibrosis associated with immunosuppressant medication in two pediatric patients who were doing well with good graft function without immunosuppression for several years after receiving living donor liver transplantation (LDLT).

CASE REPORT

Patients

A total of 163 patients underwent LDLT in our department from August 1997 to May 2012. Among them, 12 were pediatric patients who were less than 18 years of age, and 2 of these pediatric patients had ceased immunosuppressant medication for a long period. One patient was an 18-year-old male who underwent LDLT for biliary atresia (BA) at 5-years of age. In this case, immunosuppression (IS) was stopped according to the weaning protocol because of his good condition 68 mo after the LDLT. Another patient was an 11-year-old female who underwent LDLT for BA at 11-mo of age. Her IS was stopped non-electively because of EBV infection 3 mo after the LDLT. A total of eight liver biopsies were performed in these two patients. As a control, this study also included four pediatric patients who did not have hepatic function abnormalities or fibrosis and continued their immunosuppressant medication (no-tolerance cases). To examine whether the findings in these pediatric cases were also relevant to adult patients with fibrosis, three randomly selected patients with liver fibrosis not due to hepatitis C were evaluated.

Specimens were collected by ultrasound-guided core needle biopsy. Each specimen was stained with hematoxylin eosin, and the severity of fibrosis was determined using Ishak's modified staging system[26]. The evaluation of each specimen was conducted blindly by two pathologists.

Immunohistochemistry

Four-micrometer-thick sections, cut from formalin-fixed, paraffin-embedded tissues, were immunohistochemically stained for SMA, CD68, and CD79α. The following primary antibodies and a staining kit [MAX-PO (MULTI), Nichirei Corporation, Tokyo, Japan] containing peroxidase-labeled -secondary antibodies were used: anti-alpha-SMA (Nichirei; Code 412021), anti-CD68 (Dako, Tokyo, Japan; Code M0814), and anti-CD79α (Dako; Code N162830). The immunostaining was performed according to the manufacturer's instructions.

Histology score-based semiquantitative analysis

A semiquantitative analysis was performed by light microscopy at × 100 magnification, and the number of positively immunostained cells was calculated in five arbitrarily selected fields of view.

Computer-assisted semiquantitative analysis

The tissues in the α-SMA-stained area were subjected to objective semiquantitative analysis using the WinROOF image processing software program (MITANI Corporation, Tokyo, Japan). The ratio of the positive area in the specimen to the total area was calculated.

Immunohistochemistry in pediatric patients with immune tolerance

The number of cells in five randomly selected fields of view (Table 1) that were α-SMA-positive was 250.5 ± 102.8 (mean ± SD) in the two pediatric patients with tolerance, whereas the count was 69.6 ± 67.7 in the four pediatric cases without tolerance. The numbers of