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Attenuation of Portal Hypertension by Continuous Portal Infusion of PGE1 and Immunologic Impact in Adult-to-Adult Living-Donor Liver Transplantation

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Background. Small-for-size syndrome remains the greatest limiting factor of expanding segmental liver transplantation from living donors. Portal hyperperfusion is considered to substantially contribute to small-for-size syndrome. We investigated the impact of continuous portal infusion of prostaglandin E1 (PGE1) on small-for-size grafts (SFSGs) in adult-to-adult living-donor liver transplantation (LDLT).

Methods. From July 2003 to December 2009, LDLT was performed in 122 patients. We introduced continuous portal infusion of PGE1 to five SFSG patients (PG group) from November 2007 to December 2009 and retrospectively compared them with a historical control group of eight relevant SFSG patients without PGE1 infusion (non-PG group) from July 2003 to October 2007 to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Splenectomy cases were excluded from analysis.

Results. The PG group demonstrated significantly lower postoperative portal pressure than the non-PG group. Moreover, the PG group demonstrated significantly improved liver function in the early posttransplantation period and significantly better recovery from hyperammonemia at 1 week after transplantation and from hyperbilirubinemia in the late posttransplantation period. Overall survival was significantly better in the PG group than in the non-PG group. Three patients in the non-PG group died of rejection-related reasons. Interestingly, immunomonitoring assay revealed that antidonor immune responses were significantly accelerated in the non-PG group compared with the PG group after LDLT. In contrast, the PG group showed well-suppressed antidonor immune responses.

Conclusion. Continuous portal infusion of PGE1 for SFSG attenuated portal hypertension, improved graft function, and suppressed antidonor immune responses, resulting in better survival.

Keywords: Living-donor liver transplantation, Small-for-size graft, Portal hypertension, Alloimmune response, Prostaglandin E1.

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Segmental liver transplantation based on cadaveric splitting or living-donor liver transplantation (LDLT) has been developed for treating patients with end-stage liver disease. It is also a means of overcoming organ shortage and wait-list mortality. However, small-for-size syndrome (SFSS) remains the greatest limiting factor for the expansion of segmental liver transplantation from either cadaveric or living donors (*1, 2*). If the volume of the engrafted liver is considerably less than the standard liver weight in patients with end-stage liver disease who are undergoing partial liver transplantation, excessive portal venous inflow might cause early portal hypertension (*3, 4*) and increased morbidity and mortality due to SFSS (*5*). Previous data have suggested that, in recipients of adult-to-adult LDLT, one of the most challenging tasks is to match a good size graft. Emphasis has more recently been placed not only on the evaluation of the ratio between donor and recipient liver volume but also on the degree of portal hypertension and the stage of liver disease in the recipient, consistent with the result in a pig model (*6–8*). Therefore, the importance of portal pressure during LDLT is now recognized.

We have demonstrated that continuous portal infusion of prostaglandin E1 (PGE1) considerably improved the congestion

of the residual liver after extended hepatectomy in a rat model (9). Based on this result, we applied a continuous portal infusion of PGE1 for small-for-size grafts (SFSGs) in LDLT in the clinical setting.

We here investigated the clinical significance of controlling portal pressure by continuous portal infusion of PGE1 after surgery in LDLT with SFSGs, focusing on portal decompression, postoperative liver function, survival, and the antidonor immune status of the recipient retrospectively.

RESULTS

Patients' Demographic and Clinical Characteristics

Thirteen patients receiving SFSGs were retrospectively analyzed in this study. The patients' demographic and clinical characteristics are shown in Table 1. Of these patients, five received a continuous portal injection of PGE1 after transplantation (PG group) from November 2007 to December 2009 (era 2), whereas eight were historical controls from July 2003 to October 2007 (era 1) without PGE1 infusion (non-PG group). There was no significant difference in age or underlying disease between the two groups. Preoperative examination of the hepatic reserve showed similar Child-Pugh scores (PG group, 10.0±0.71; non-PG group, 9.00±0.83). Patients' model for end-stage liver disease scores, which were used as recipient severity indices, was similar between groups (mean [range], 16.8 [8–30] and 15.1 [9–28], respectively). Portal vein pressure (PVP) at laparotomy was also similar between the two groups (25.2 [17–34] and 20.3 [17–24] mm Hg, respectively). Concerning the graft, one patient in each group showed minimal fatty metamorphosis (<0.1%) on histology and there was no significant difference in graft-to-recipient body weight ratio (GRWR) between the two groups (0.680 [0.63–0.71] and 0.655 [0.51–0.72], respectively).

Furthermore, factors related to surgical invasiveness in those two groups, such as hemorrhage level, operation time, and graft ischemia duration, were similar. No donor had donor-specific antigens, and there was no difference in the number of human leukocyte antigen (HLA) mismatch (Table 1). Three donor candidates in each group underwent liver biopsy. Among them, one in each group showed minimal fatty metamorphosis (<0.1%) on histology. Of note, three of five patients in the PGE1 group and three of eight patients in the non-PGE1 group received right-lobe grafts. All patients receiving right lobes in both groups had grafts with middle hepatic vein (MHV) tributaries more than 5 mm in diameter, and all draining tributaries were reconstructed with the

TABLE 1. Patients' demographic and clinical characteristics

Variables	PG group (n=5)	Non-PG group (n=8)	*P*
Recipient factors			
Age, years	56.4±3.4	57.9±4.4	0.510[a]
Gender, male/female	5/0	3/5	0.075[b]
Child-Pugh score	10.0±1.6	9.0±1.9	0.325[a]
MELD score	16.8±8.2	15.1±5.8	0.702[a]
PVP, mm Hg, at laparotomy	25.2±6.1	20.9±3.0	0.199[a]
Disease background			
Viral hepatitis (B/C)	1/2	1/5	>0.999[b]
Alcoholic	1	1	>0.999[b]
Acute hepatic failure	1	0	0.385[b]
Cholestatic disease	0	1	>0.999[b]
Donor factors			
Age, years	26.2±3.3	33.3±10.5	0.113[a]
Gender, male/female	0/5	5/3	0.075[b]
Graft factors			
Graft type, right/left	3/2	3/5	0.592[b]
GRWR, %	0.68±0.03	0.66±0.09	0.510[a]
Reconstruction of hepatic vein	3	3	0.592[b]
HLA class I mismatch	1.20±0.49	1.63±0.23	0.453[a]
HLA class II mismatch	0.60±0.24	1.00±0.00	—
DSA	0	0	—
Surgical factors			
Operation time, min	781.0±153.6	755.9±106.0	0.758[a]
Bleeding, mL	5322.0±2295.3	5751.4±6371.2	0.866[a]
Total ischemia time, min	117.0±35.5	118.9±31.4	0.925[a]

[a] Unpaired *t* test with Welch's correction.
[b] Fisher's exact test.
DSA, donor-specific antibody; HLA, human leukocyte antigen; GRWR, graft-to-recipient body weight ratio; MELD, model for end-stage liver disease; PVP, portal vein pressure.

recipients' native MHV trunk as reported previously (*10*). There was no thrombosis in those reconstructed tributaries after surgery. One patient of each group had grafts with inferior right hepatic vein, which were reconstructed using direct anastomosis to inferior vena cava in each case.

Continuous PGE1 Infusion Attenuated Portal Hypertension After Reperfusion in SFSGs

After laparotomy, we inserted a catheter from the mesenteric vein to the distal side of the portal vein and measured the PVP during the operation. All patients exhibited portal hypertension during laparotomy. In the PG group, after reflow of the portal and hepatic veins was confirmed, we started PGE1 infusion into the portal vein through a catheter. Continuous infusion of PGE1 resulted in a significant reduction of PVP at the time of abdominal closure in the PG group compared with the non-PG group ($P<0.005$; Fig. 1A). The mean PVP at the time of abdominal closure was 15.4±1.17 mm Hg in the PG group and 20.5±1.47 mm Hg in the non-PG group (Fig. 1A). Furthermore, the PVP ratio at the end of the operation, compared with that at laparotomy, showed effective portal decompression in the PG group and non-PG group, respectively (0.62±0.04 vs. 0.99±0.06; $P<0.001$; Fig. 1B). Importantly, none of the patients in the PG group developed hypoperfusion after PGE1 portal infusion.

Clinical Course of Graft Liver Function

Graft liver function markers, including serum transaminases, arterial ketone body ratio (AKBR), ammonia, and total bilirubin, after surgery were compared between the PG group and the non-PG group.

Elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly attenuated in the PG group compared with the non-PG group on days 1 and 2 (Fig. 2). Similarly, the AKBR, which reflects the hepatic mitochondrial redox state and is considered an accurate index of the functional reserve of the graft liver after transplantation, was significantly higher in the PG group. However, these values became comparable between the two groups after day 3. Strikingly, significantly better recovery from hyperammonemia was seen in the PG group for 1 week after surgery. The serum total bilirubin level was comparable between the two groups by day 28 after LDLT. Nonetheless, hyperbilirubinemia was significantly improved in the PG group after day 28 but remained prolonged in the non-PG group. These results indicate that continuous infusion of PGE1 significantly improved the liver function after LDLT with SFSGs.

Complications and Prognosis

In the PG group, no complications associated with the portal vein catheter were observed after surgery (e.g., post-removal bleeding, catheter infection, or portal thrombosis). One patient in the non-PG group and none in the PG group developed SFSS. Postoperative death occurred in 5 patients of the non-PG group and in none in the PG group. In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In contrast, in the PG group, the 1-and 2-year survival rates were both 100%, a difference that was statistically significant ($P<0.05$; Fig. 3). The main causes of death in the non-PG group were graft dysfunction, rejection, and subsequent infection as well as bacterial sepsis after biliary stenosis. No patients in the PG group had a

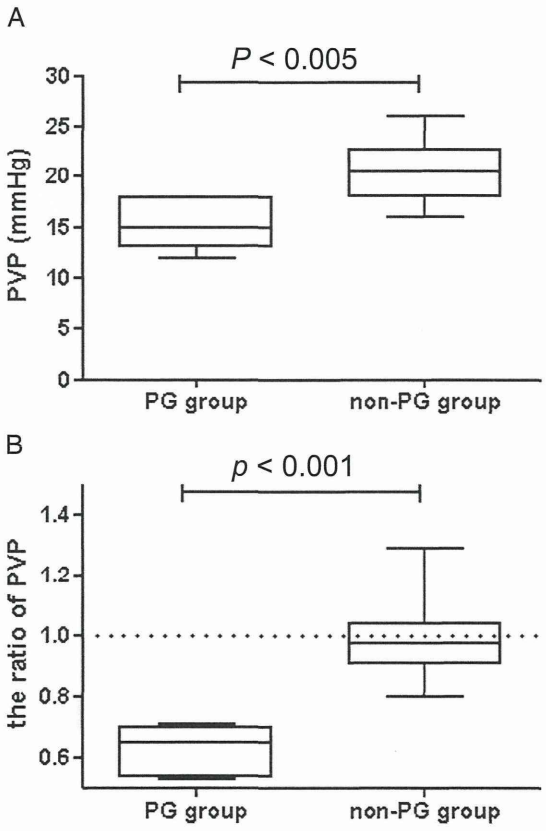

FIGURE 1. PVP value at the end of the operation (A) and ratio of PVP at the end of the operation to that at laparotomy (B) in the PG group and the non-PG group. An unpaired *t* test with Welch's correction was used to compare PVP and the ratio of PVP between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. *$P<0.05$; ***$P<0.001$. PVP, portal vein pressure.

rejection episode. Rejection was diagnosed by liver biopsy and histologic findings showed features of SFSG and/or portal hypertension with rejection (see **Figure S1, SDC**, http://links.lww.com/TP/A807). The 2-year survival of SFSG patients (non-PG group) in era 1 (July 2003 to October 2007) was significantly worse than that of the non-SFSG patients in the same period (37.5% vs. 77.8%; $P<0.05$), whereas the 2-year survival of SFSG patients (PG group) in era 2 (November 2007 to December 2009) was not statistically different from that of the non-SFSG patients in the same period (100% vs. 77.1%). Of note, the 2-year survival of non-SFSG patients was similar between eras 1 and 2 (Fig. 4).

Estimation of Immunosuppressive Status After Surgery by Using the Carboxyfluorescein Diacetate Succinimidyl Ester-Mixed Lymphocyte Reaction Assay

Because the main cause of death in 3 patients in the non-PG group was related to rejection, we retrospectively analyzed the immunosuppressive postoperative status of both groups. All patients and their donors consented to be

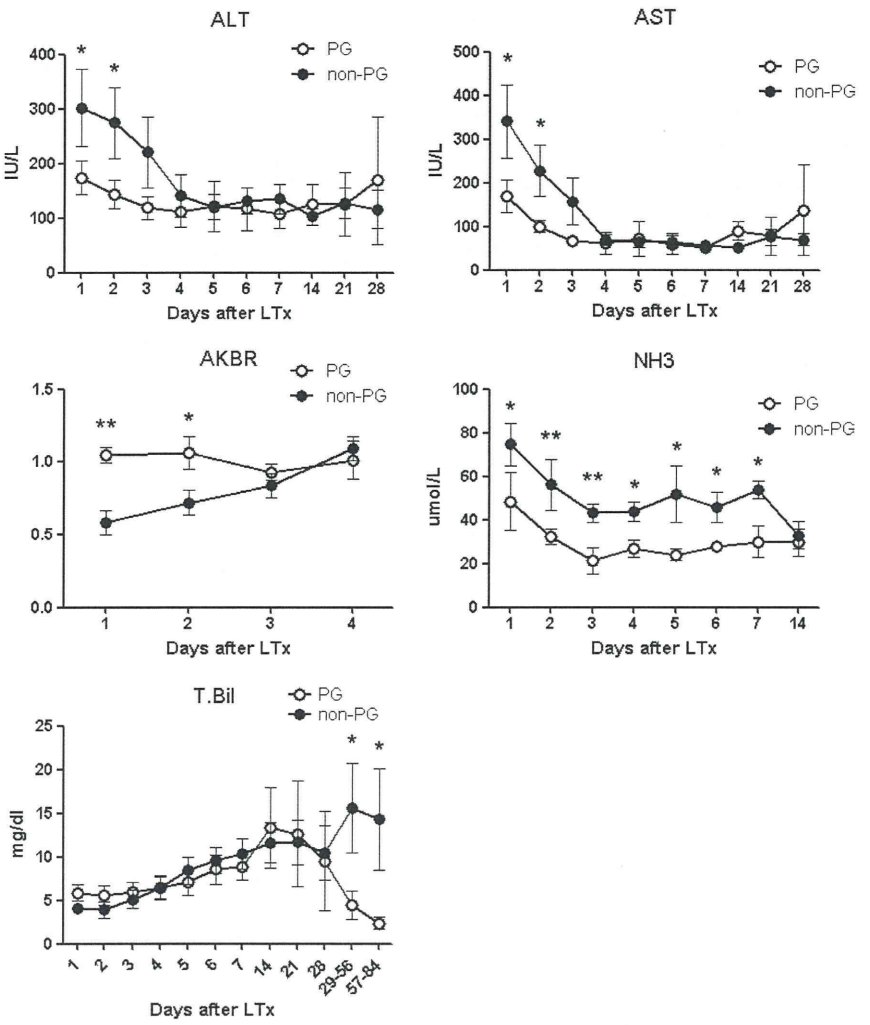

FIGURE 2. Liver function tests (ALT, AST, AKBR, NH_3, and T.Bil) of patients with (PG group; open circle) or without PGE1 portal infusion (non-PG group; closed circle) after LDLT. Data are mean±SEM for individual groups. An unpaired *t* test with Welch's correction was used to compare each of the indicated parameters between the PG group and the non-PG group. **P*<0.05; ****P*<0.01. AKBR, arterial ketone body ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDLT, living-donor liver transplantation; LTx, liver transplantation; NH_3, ammonia; T.Bil, total bilirubin.

subjected to a mixed lymphocyte reaction (MLR) assay with the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. In all five patients of the PG group, suppressed $CD8^+$ T-cell proliferation, which is defined as a stimulation index (SI)<2, was observed in the antidonor MLR assay (i.e., a hyporesponse to donor; mean SI, 1.10±0.13; Fig. 4A). The mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells, which are activated cytotoxic T cells, was 9.24±5.93 (Fig. 4B). In contrast, in five of the eight patients in the non-PG group, accelerated $CD8^+$ T-cell proliferation was observed in the antidonor MLR assay (i.e., a hyperresponse to donor; mean SI, 2.85±0.50; Fig. 4A). Furthermore, the mean percentage of $CD25^+$ cells among the proliferating $CD8^+$ T cells was 63.82±8.63 (Fig. 4B). These differences between the two groups were significant. Of note, three patients in the non-PG group who showed high antidonor response (i.e., SI of $CD8^+$ T cells>3) required steroid pulse treatment and died of graft dysfunction or infection after rejection. Two patients who showed a relatively high antidonor response (i.e., SI of $CD8^+$ T cells>2) required an increase in immunosuppressant doses. These results indicated that patients with SFSGs show accelerated antidonor immune responses and that continuous portal infusion of PGE1 suppressed this type of antidonor immune response.

DISCUSSION

Various approaches to controlling excessive portal flow and pressure have been proposed, such as dual grafting to increase graft volume (*11*, *12*). Although this concept is simple, it requires two healthy living donors and involves increased risk to donors. Another approach is portal decompression with a portosystemic shunt (*13*, *14*) or splenic artery manipulation, including splenectomy, embolization, and ligation (*15*–*17*). This method is more favored in terms of availability and donor risk. Nonetheless, there is

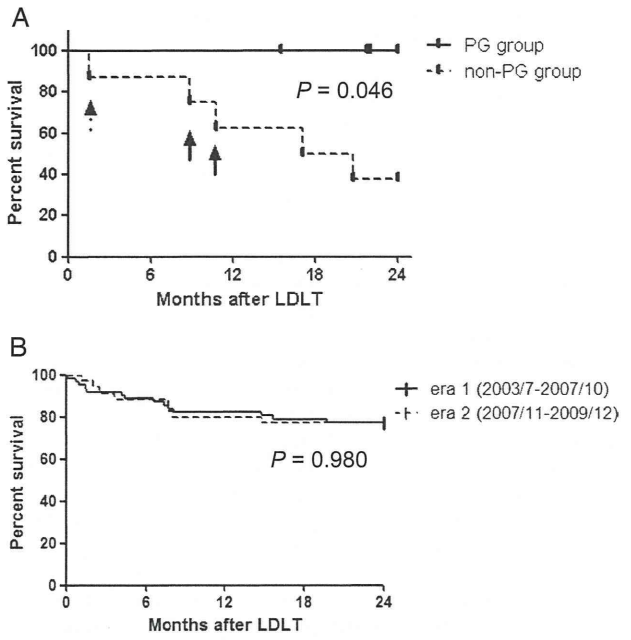

FIGURE 3. A, Kaplan–Meier patient survival curves of patients with (PG group; n=5; solid line) or without PGE1 portal infusion after LDLT (non-PG group; n=8; dotted line). In the non-PG group, the 1- and 2-year survival rates were 62.5% and 37.5%, respectively. In the PG group, the 1- and 2-year survival rates were both 100%, a difference that was statistically significant. *P<0.05. Dashed arrow represents a patient's death due to SFSS and rejection followed by infection, and solid arrows represent patients' death due to rejection-related reasons. B, Kaplan–Meier patient survival curves of non-SFSG patients in era 1 (from July 2003 to October 2007; n=62; solid line) or era 2 (from November 2007 to December 2009; n=35; dotted line). In the era 1 and era 2 groups, the 2-year survival rate was 77.4% and 77.1%, respectively, with no statistical difference (P=0.980). ABO-incompatible cases and splenectomy cases were excluded from analysis. LDLT, living-donor liver transplantation; PGE1, prostaglandin E1; SFSG, small-for-size graft; SFSS, small-for-size syndrome.

a considerable risk of infection in splenectomy or splenic artery ligation (*18*). Moreover, significantly higher mortality was observed in patients who had splenectomy mainly due to septic complications in liver transplantation (*19, 20*). In fact, we experienced one SFSG case in which the patient died of sudden sepsis without any primary focus 4 years after transplantation with splenectomy. Therefore, another method to control portal pressure and preserve the spleen is likely more preferable.

We have reported that portal administration of PGE1, a vasodilator of vessels containing smooth muscle (*21, 22*), prevented congestion of residual liver tissues in a rat extended hepatectomy model. In this study, we tried various vasodilators; however, residual liver congestion after hepatectomy was improved only by continuous portal infusion of PGE1. We also tried systemic continuous venous infusion of PGE1 at the same dose, but this was not effective. This suggests the therapeutic potential of portal PGE1 injection to prevent portal hypertension after LDLT with SFSGs.

We translated this method to adult LDLT with SFSGs, and portal infusion of PGE1 successfully reduced PVP, resulting in improved liver graft function in both early and late posttransplantation periods. This result was unexpected because the portal infusion of PGE1 was given for only the first week yet improved the long-term survival of recipients.

We used a CFSE-MLR assay to objectively evaluate the antidonor responses of the recipients (*23, 24*). The lack of $CD8^+$ and $CD25^+$ T-cell proliferation in antidonor MLR reflects the suppression of the antidonor response. In this immunologic investigation, all patients given the continuous portal infusion of PGE1 showed a well-suppressed response of the antidonor $CD8^+$ T cells (Fig. 4). In contrast, surprisingly, patients without the PGE1 treatment showed

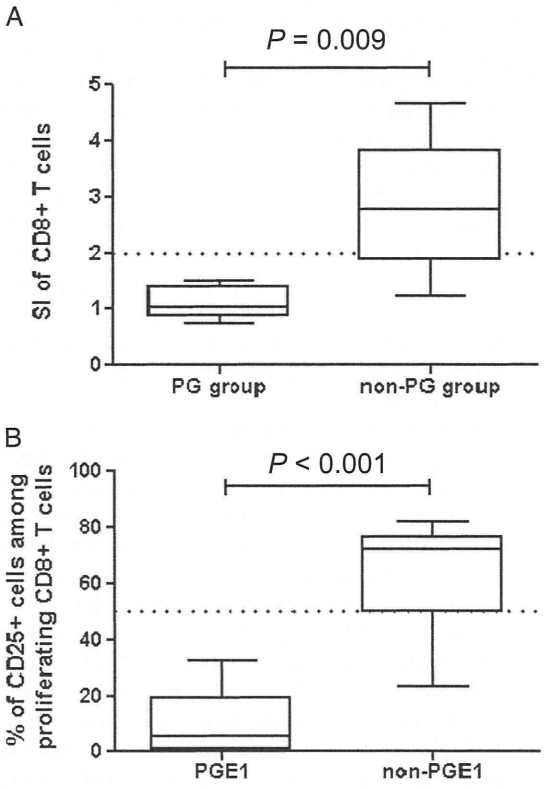

FIGURE 4. SIs of $CD8^+$ T-cell subsets in the antidonor MLR assay of patients in the PG group (n=5) and the non-PG group (n=8) on the third to fourth weeks after transplantation (A) and percentage of $CD25^+$ cells among proliferating $CD8^+$ T cells in patients of the PG group and the non-PG group (B). $CD8^+$ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the $CD4^+$ and $CD8^+$ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of the allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic (self to donor) combination by that of the self-control. An unpaired t test with Welch's correction was used to compare the SI and percentage of $CD25^+$ cells between the PG group and the non-PG group. The box plot represents the 25th to 75th percentiles, the dark line is the median, and the extended bars represent the 10th to the 90th percentiles. **P<0.01; ***P<0.001. MLR, mixed lymphocyte reaction; SI, stimulation index.

an accelerated response of antidonor $CD8^+$ T cells despite the use of the same immunosuppressive protocol. Therefore, SFSG likely accelerated the antidonor response, enhanced rejection, and might result in a worse survival rate, although SFSS is multifactorial in nature. This finding is consistent with the results in an animal model (*25*) and our preliminary data using mouse model (data not shown). To our knowledge, this is the first clinical report to state that SFSG may accelerate antidonor immune responses in LDLT.

We previously reported that liver sinusoidal endothelial cells (LSECs) of grafts induce allospecific immunotolerance by suppressing reactive T cells through Fas ligand and/or PD-L1 signaling (*26–28*). Based on this concept, one possible mechanism is that the sinusoidal structure and LSECs could be damaged by portal hypertension and lose their tolerogenicity, resulting in accelerated antidonor immunoresponse and rejection. This interpretation is consistent with that of previous reports stating that portal hypertension disrupts sinusoids and LSECs in the liver (*8*). Another possibility is antirejection and/or the anti-inflammatory effect of PGE1 itself. It has been reported that PGE1 could prevent ischemia-reperfusion injury by inducing heat shock protein (*29*) or by inhibition of neutrophil adherence (*30*). It has been also reported that administration of PGE1 could prevent and suppress the rejection process in heart transplantation (*31*) and in renal transplantation (*32*). Furthermore, it has been reported that PGE1 protects human LSECs from apoptosis, which is consistent with our findings (*33*).

The main limitation of our study is its retrospective nature. Another limitation is the relatively small number of patients in each subgroup analyzed, although the background characteristics of each group and the survival rates of non-SFSG patients were similar. A randomized study is ideal; however, performing a prospective randomized study for this approach is difficult because of the high mortality of SFSG patients without PGE1 perfusion, as shown in Results.

In conclusion, continuous infusion of PGE1 is suggested to be useful in improving SFSG function and survival after LDLT. Improved understanding of underlying mechanisms may have important implications for clinical managements such as antirejection therapy or preventing ischemia-reperfusion injury in liver transplantation with SFSGs.

MATERIALS AND METHODS

Patients

From July 2003 to December 2009, LDLT was performed on 122 adult patients with end-stage liver disease. We introduced continuous portal infusion of PGE1 to five patients with SFSGs that exhibited a GRWR less than 0.72% from November 2007 to December 2009 (era 2). These patients (PG group) were the subjects of this retrospective case–control study. We compared them with a historical group of eight relevant patients who received SFSG without PGE1 infusion (non-PG group) from July 2003 to October 2007 (era 1) to determine the safety and efficacy of continuous PGE1 portal infusion for SFSGs. Because we introduced the portal infusion of PGE1 in November 2007, all patients with SFSGs in era 2 received PGE1 infusion. One SFSG case with splenectomy in era 1 was excluded from this study. The study protocol was approved by the ethics committee of Hiroshima University, and all patients provided informed consent before surgery. None of the patients receiving PGE1 portal infusion showed clinical evidence of insertion site infection or bleeding after catheter removal throughout the follow-up period.

Operation, PVP Measurement, and Continuous Portal Infusion of PGE1

The graft-harvesting technique, recipient surgery, perioperative recipient management, and immunosuppression regimens were conducted as described previously with minor modifications (*34, 35*). In brief, the right lobe without the MHV or the left lobe with the MHV was harvested from the donor as follows. Before parenchymal transection, the right or left lobe was mobilized and the short hepatic veins were transected. For the right lobe, during parenchymal transection, the major right tributaries of the MHV were clamped using a vascular clip and then transected. After hepatectomy, ex vivo perfusion of the graft was performed through the portal vein. The initial perfusate was saline solution (500 mL); then, the University of Wisconsin solution (1000 mL) was used as the perfusate.

To measure the PVP in the recipient during the operation, an 18G catheter was inserted from the mesenteric vein to the portal vein after laparotomy (*36*). The implantation was performed after total hepatectomy. The graft vein was anastomosed to the equivalent vein of the recipient in an end-to-end fashion. Thereafter, the graft was reperfused before microsurgical reconstruction of the hepatic artery (end-to-end anastomosis of the graft hepatic artery to the recipient hepatic artery). The bile duct of the graft liver was anastomosed in an end-to-end fashion to the recipient's common hepatic bile duct. In the non-PG group, the portal catheter was removed at the time of abdominal closure. In the PG group, PGE1 was administered through a portal catheter at the graft portal reflow. PGE1 was continuously administered for 1 week ($0.01\ g\ kg^{-1}\ min^{-1}$) and then the catheter was noninvasively removed. AST, ALT, serum bilirubin, serum ammonia, and AKBR levels were measured as liver function indices. The initial immunosuppressive regimen consisted of tacrolimus and steroids. Doppler ultrasonography and computed tomographic scans were routinely performed daily and biweekly, respectively.

Immunosuppression was initiated with a protocol based on tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan) and methylprednisolone. Methylprednisolone was withdrawn gradually according to the protocol. The dose of tacrolimus was controlled according to blood concentration and adjusted daily. The target trough level was set at 15 ng/mL for 2 weeks and 10 ng/mL for another 2 weeks. Continuous venous infusion of heparin for therapeutic heparinization was routinely done to prevent thrombosis, which was monitored using coagulation tests. Rejection was diagnosed and proven by biopsy histologically and MLR assay. Patients were followed for 2 years after LDLT, and survival was defined as the period between LDLT and death.

Immune Monitoring by In Vitro CFSE-MLR Assay

CFSE-MLR was routinely performed to evaluate the recipient's antidonor immune response 2 to 4 weeks after surgery.

For CFSE-MLR, peripheral blood mononuclear cells prepared from the blood of the recipients (autologous control), donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy. Those obtained from the recipients for use as the responder cells were labeled with 5 mM CFSE (Molecular Probes, Eugene, OR), as described previously (*24*). The stimulator and responder cells (2×10^6 each) were incubated in 24-well flat-bottomed plates in a total volume of 2 mL culture medium at 37°C under 5% CO_2 for 5 days. After culture for MLR, $CD4^+$ and $CD8^+$ T-cell proliferation, CD25 expression of proliferating T-cell subsets and SI were quantified by flow cytometry as described previously (*37, 38*) and described in the SDC Materials and Methods in detail (see http://links.lww.com/TP/A807).

Statistical Analysis

Statistical analysis and comparisons were performed using PRISM version 4.0 (GraphPad, San Diego, CA). Data are expressed as mean±SEM. An unpaired *t* test with Welch's correction was used to compare groups. *P* values <0.05 were considered statistically significant.

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Expression of Recipient CD47 on Rat Insulinoma Cell Xenografts Prevents Macrophage-Mediated Rejection through SIRPα Inhibitory Signaling in Mice

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Abstract

We have previously proven that the interspecies incompatibility of CD47 is responsible for *in vitro* phagocytosis of xenogeneic cells by host macrophages. Utilizing an *in vivo* model in the present study, we investigated whether genetically engineered expression of mouse CD47 in rat insulinoma cells (INS-1E) could inhibit macrophage-mediated xenograft rejection. INS-1E cells transfected with the pRc/CMV-mouse CD47 vector (mCD47-INS-1E) induced SIRPα-tyrosine phosphorylation in mouse macrophages in vitro, whereas cells transfected with the control vector (cont-INS-1E) did not. When these cells were injected into the peritoneal cavity of streptozotocin-induced diabetic Rag2$^{-/-}$γ chain $^{-/-}$ mice, which lack T, B, and NK cells, the expression of mouse CD47 on the INS-1E cells markedly reduced the susceptibility of these cells to phagocytosis by macrophages. Moreover, these mice became normoglycemic after receiving mCD47-INS-1E, whereas the mice that received cont-INS-1E failed to achieve normoglycemia. Furthermore, injection of an anti-mouse SIRPα blocking monoclonal antibody into the mouse recipients of mCD47-INS-1E cells prevented achievement of normoglycemia. These results demonstrate that interspecies incompatibility of CD47 significantly contributes to *in vivo* rejection of xenogeneic cells by macrophages. Thus, genetic induction of the expression of recipient CD47 on xenogeneic donor cells could provide inhibitory signals to recipient macrophages via SIPRα; this constitutes a novel approach for preventing macrophage-mediated xenograft rejection.

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Introduction

Xenotransplantation, using organs, tissues, and cells from other species as the transplant source, has the potential to resolve the severe shortage of human donors; however, robust immune responses to xenografts remain a major obstacle to the clinical application of this approach [1,2]. Extensive genetic disparities between the donor and recipient are thought to contribute significantly to the more vigorous rejection of xenografts than allografts; these disparities, however, remain ill-defined. Vigorous innate immune cell activation can be accounted for by both recognition of xenoantigens by activating receptors, and an incompatibility in inhibitory receptor–ligand interactions [3].

In human xenograft recipients, innate humoral and cellular xenoimmune responses are both predominantly elicited by preformed and induced xenoreactive antibodies (Abs). Among the components of innate immunity, macrophages, which can be activated by prophagocytic signaling pathways through Fcγ receptors, play a significant role in targeting xenogeneic cells that have been opsonized with these Abs. Genetically engineered α-1,3-galactosyltransferase (GalT)-knockout pigs, which no longer express the major xenoantigens Galα1,3Galβ1,4GlcNAc (Gal) carbohydrate residues held promise for conferring protection against such xenoreactive Ab-mediated rejection [4–6].

However, we have previously demonstrated that human reticuloendothelial macrophages can phagocytose porcine cells even in the absence of Ab or complement opsonization, and that removing Gal epitopes from porcine cells failed to prevent this phagocytosis [7]. Similarly, other groups have also reported that non-human primate macrophages mediate rapid rejection of porcine pancreatic islets [8], which express little or no Gal antigens [9]. These results suggest that regulation of macrophages in human recipients may be required to achieve successful engraftment of porcine xenografts.

We have recently proven that the interspecies incompatibility of CD47 is responsible for *in vitro* phagocytosis of xenogeneic porcine cells by human macrophages [10]. CD47 is an ubiquitously expressed cell surface protein of the immunoglobulin superfamily that serves as a ligand for signal regulatory protein (SIRP) α, an immune inhibitory receptor on macrophages. CD47 and SIRPα constitute a cell–cell communication system (the CD47-SIRPα system); such interactions play important roles in both hematopoietic and immunological regulation [11–14]. In addition, the CD47-SIRPα system has been implicated in negative regulation of

phagocytosis by macrophages [15]; specifically, when expressed on the surface of several cell types (i.e., erythrocytes, platelets, or leukocytes), CD47 can protect against phagocytosis by macrophages by binding to SIRPα [15]. Moreover, CD47 inhibits both Fcγ and complement receptor-mediated phagocytosis through its SIRPα receptors [16].

We have previously verified that porcine CD47 does not induce tyrosine phosphorylation of SIRPα in human macrophages, and that manipulation of porcine cells for expression of human CD47 markedly reduces the susceptibility of these cells to phagocytosis by human macrophages *in vitro* [10]. These results indicated that genetic induction of the expression of recipient-type CD47 on xenogeneic donor cells could provide inhibitory signaling to SIPRα on host macrophages, suggesting a novel approach for preventing macrophage-mediated xenograft rejection.

Here, we have now investigated this postulate in a rat-to-mouse *in vivo* model, in which the interspecies incompatibility of CD47 (85% amino acid sequence homology between these species [17]) would normally cause active phagocytosis of rat cells by mouse macrophages.

Materials and Methods

Antibodies

An anti-SIRPα Ab (P84) was used to block the macrophage inhibitory receptor, SIRPα [18]. Biotin-conjugated rat anti-mouse CD11b (M1/70; BD Pharmingen, San Diego, CA, USA), purified anti-mouse CD47 Ab (miap301; BD Pharmingen), and APC Streptavidin (BD Pharmingen) were used for FACS analysis via immunofluorescence using a FACSCalibur® (BD Biosciences, Franklin Lakes, NJ, USA). In FACS analyses, nonspecific binding of labeled mAbs was blocked with rat anti–mouse FCγR mAb, 2.4G2. Rabbit polyclonal Ab against SIRPα (Abcam, La Jolla, CA, USA), purified rat anti-mouse CD172a (P84) Ab (BD Pharmingen), purified rabbit polyclonal anti-phosphotyrosine Ab (BD Transduction Laboratories, Lexington, KY, USA), and horse radish peroxidase (HRP)-conjugated rabbit secondary Ab (Amersham Biosciences Co., Piscataway, NJ, USA) were used for immunoprecipitation and western blot analysis.

Cell Cultures

All cells were maintained at 37°C under a humidified atmosphere of 5% CO2 in air. A rat insulinoma cell line (INS-1E) [19] was kindly provided by Dr. Claes B. Wollheim (University of Geneva, Switzerland). Cells were cultured in RPMI1640 containing 10% FCS with 5 μM 2-mercaptoethanol (2-ME; Katayama, Osaka, Japan), 10% HEPES buffer (Gibco, NY, USA), and 100 IU/mL penicillin-100 μg/mL streptomycin (Gibco). P84 hybridoma cells producing an anti-SIRPα Ab were kindly provided by T. Matozaki (Gunma University, Gunma, Japan). The mouse macrophages were cultured in DMEM containing 10% FCS with 5 μM 2-ME, 10% HEPES buffer, and 100 IU/mL penicillin-100 μg/mL streptomycin.

Animals

Rag2$^{-/-}$ γ chain $^{-/-}$ mice were purchased from Taconic (One Hudson City Centre Hudson, NY, USA). All animal protocols described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the local committee for animal experiments, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Mouse Macrophage Preparation

To prepare peritoneal macrophages, peritoneal cells were harvested from B6 mice after intraperitoneal injection of PBS, plated in a Gelatin Cellware 75-cm2 Vented Flask (BD Biosciences) and cultured at 37°C for 2 h. Macrophages were used after non-adherent cells were washed off.

T Cell Isolation

T cells were negatively isolated from wild type B6 splenocytes using a cocktail of biotin-conjugated monoclonal antibodies against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, anti-MHC class II, and Ter-119 and anti-biotin antibody-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of T cells was ≥95% (data not shown), as determined by CD3e surface staining using FACS analysis.

Immunoprecipitation and Immunoblotting

Peritoneal macrophages (2×106) were incubated for 16 h before experiments and rinsed once with PBS. Then, 2×107 mouse or rat RBCs or INS-1E cells were added to the macrophage cultures, and incubated at 37°C for 30 min. The cells were lysed in 0.3 mL of lysis buffer [1% NP-40, 1 mM PMSF, 50 mM β-glycophosphate, 20 mM NaF, 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin, and 2 mM sodium pervanadate] by rotation on ice for 15 min.

For immunoprecipitation, the lysates were mixed with rat anti-mouse SIRPα Ab (P84) and 50% slurry of protein G–sepharose beads (Sigma-Aldrich, St. Louis, MO, USA) by rotation at 4°C for 8 h. Precipitated proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. Thereafter, polyclonal rabbit anti-phosphotyrosine Ab (BD Pharmingen) and HRP-linked anti-rabbit IgG Ab (Amersham Biosciences Co.) were used as primary and secondary Abs, respectively, in western blot analysis. Alternatively, the membrane was stained with rabbit polyclonal Ab against SIRPα (Abcam), followed by HRP-linked anti-rabbit IgG Ab (Amersham Biosciences Co.).

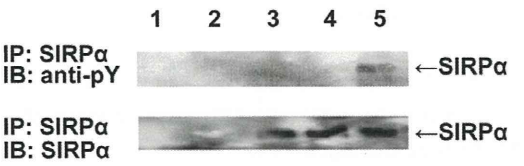

Figure 1. Tyrosine phosphorylation of SIRPα in mouse macrophages was induced by incubation with mouse red blood cells (RBCs), but not with rat RBCs. Differentiated mouse macrophages were incubated with mouse or rat RBCs at 37°C for 30 min. The cells were lysed, and the lysates were mixed with mouse anti-mouse SIRPα antibodies and 50% slurry of protein G-sepharose beads by rotation at 4°C for 8 hrs. Precipitated proteins were separated by 8% SDS-PAGE, followed by blotting to a nitrocellulose membrane. Rabbit immunoaffinity-purified anti-phosphotyrosine IgG and goat anti-rabbit HRP-conjugated IgG were used as primary and secondary antibodies, respectively. Rat RBCs alone (lane 1), mouse RBCs alone (lane 2), mouse macrophages incubated in medium alone (lane 3), or mouse macrophages incubated with rat (lane 4) or mouse (lane 5) RBCs are shown. Immunoblotting with anti-mouse SIRPα was used as loading control. IP, immunoprecipitation; IB, immunoblotting; anti-pY, anti-phosphotyrosine.
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