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SYSTEMIC THROMBOLYTIC THERAPY FOR LATE-ONSET PORTAL VEIN THROMBOSIS AFTER LIVING-DONOR LIVER TRANSPLANTATION

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Background. **There have been few reports of noninvasive treatment for portal vein thrombosis (PVT) after liver transplantation.**

Methods. **One adult patient and four pediatric patients developed PVT 5, 14, 14, 16, and 43 months after living-donor liver transplantation, respectively. All five of the patients received a 10-day course of systemic thrombolytic therapy with recombinant tissue plasminogen activator.**

Results. **After the treatment was completed, successful portal vein recanalization was demonstrated on ultrasonography or multidetector three-dimensional computed tomography angiography in all five cases.**

Conclusion. **Noninvasive treatment with systemic recombinant tissue plasminogen activator should be considered for selected patients with late-onset PVT after liver transplantation.**

Portal vein thrombosis (PVT) is one of the life-threatening complications of liver transplantation, especially it when occurs in the immediate posttransplant period (*1, 2*). Acute PVT may lead to portal hypertension or hepatic ischemia with catastrophic sequelae. Late-onset PVT, on the other hand, is generally well tolerated, although it may eventually lead to graft compromise requiring aggressive intervention (*3*). Although there have been several reports on the use of surgery and interventional radiology in the management of

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both early (*4, 5*) and late (*6*) PVT, there have been only a few cases, thus far, of late-onset PVT that have been successfully treated with medical management alone using systemic thrombolytic therapy (*7*). We report five cases (four pediatric, one adult) of successful recanalization of late-onset PVT after living-donor liver transplantation (LDLT) with systemic thrombolytic therapy using intravenous recombinant tissue plasminogen activator (rtPA).

PATIENTS AND METHODS

Of 130 LDLT cases between June 1994 and February 2003, five patients (3.8%) developed PVT after transplantation. All of them were identified more than 3 months after transplantation. Patient characteristics are listed in Table 1. In all cases, PVT was first detected by Doppler ultrasonography, which was performed as a component of the routine patient follow-up or as the initial step in the workup of new-onset liver dysfunction, with or without symptomatology. PVT was subsequently verified by multidetector three-dimensional computed tomography (3D CT) angiography. As the first-line therapy, all patients received the same protocol of systemic thrombolytic therapy using rtPA (Actilyse, Boehringer Ingelheim, Ingelhiem/Rhein, Germany) by continuous intravenous infusion at a dose of 0.25 mg/kg/d for 10 days. Heparin was administered with a loading dose of 200 IU/kg/d and gradually increased to a maintenance dose of 350 IU/kg/d for 10 days. This 10-day course was repeated if the patient revealed insufficient improvement with only partial regression of the PVT. No additional anticoagulants were administered thereafter. After resolution of the PVT, patients were followed every 1 or 2 months by Doppler ultrasonography or multidetector 3D CT angiography.

RESULTS

The clinical course and outcome of the patients are summarized in Table 2.

Case 1

A 2-year-old boy weighing 9.4 kg underwent LDLT for biliary atresia. He received a left lateral segment graft from his mother, who was ABO blood group identical. During surgery, the recipient's portal vein trunk was found to be hypoplastic (diameter=4.5 mm); therefore, a branch patch of its bifurcation was anastomosed to the graft portal vein using 7–0 monofilament polyglyconate suture with a 7-mm growth factor. A successful reconstruction was accomplished without any complications. The immunosuppression regimen consisted of microemulsified cyclosporine, azathioprine, and steroids. The posttransplant course was uneventful, and the patient did well until 14 months post-LDLT when a PVT was detected on a routine Doppler ultrasonography study. 3D CT angiography revealed portal vein occlusion with PVT. There was no ascites, and PVT was localized along the anastomotic site with no extension deep into the intrahepatic portal vein. Because the patient was clinically well with acceptable liver function (aspartate aminotransferase [AST], 63 U/L; alanine aminotransferase [ALT], 21 U/L; total bilirubin, 1.0 mg/dL), a course of rtPA was administered without any complications. After the course of treatment was completed, multidetector 3D CT angiography showed complete regression of the PVT. The patient is currently doing well with normal liver function 2 months after rtPA treatment.

Case 2

A 7-month-old boy weighing 6.9 kg underwent LDLT for biliary atresia. The patient received a left lateral segment graft from his mother, who was ABO blood group compatible. During surgery, the recipient's portal vein trunk was found to be hypoplastic (diameter=4 mm); therefore, a branch patch of its bifurcation was anastomosed to the graft portal vein using 7–0 monofilament polyglyconate suture with a 10-mm growth factor. Immediately after the reconstruction, Doppler ultrasonography revealed a low mean portal flow velocity of 5 cm/sec, which was attributed to rotation of the graft into the right subphrenic space. The graft was therefore elevated by placing a balloon catheter containing 75 mL of water as a tissue expander posterior to it and fixating the falciform ligament to the anterior abdominal wall. This maneuver resulted in a sufficient mean portal flow velocity of 47 cm/sec. The balloon catheter was removed 56 days later, after staged deflation, without any complications. The immunosuppression regimen consisted of microemulsified cyclosporine, azathioprine, and steroids. The posttransplant course was uneventful, and portal vein flow, as assessed on Doppler ultrasonography, continued to be satisfactory. Fifteen months post-LDLT, the patient presented with sudden onset melena accompanied by mild liver dysfunction (AST, 59 U/L; ALT, 23 U/L; total bilirubin, 1.2 mg/dL). Doppler ultrasonography and 3D CT angiography at that time revealed portal vein occlusion at the anastomotic site without visualization of the intrahepatic portal system. There was no ascites. A PVT was suspected, and because the patient was generally well with only mild anemia and no evidence of active bleeding, a course of rtPA was administered. The patient tolerated the treatment well, and there were no complications. After the treatment course was completed, multidetector 3D CT angiography revealed recanalization of the portal vein with partial visualization of the intrahepatic portal system. The patient received another course of rtPA 6 months later, with no significant improvement compared with the initial result after a single course of thrombolysis. The patient is currently doing well with normal liver function and no further bleeding 4 months after the second treatment.

TABLE 1. Patient characteristics

No.	Sex	Age	BW (kg)	Diagnosis	Donor	GRWR (%)	ABO matching	PV before Tx
19	M	2 yr	9.4	BA	Father	3.9	Identical	MPV 4 mm, patent, hepatofugal flow, 4 cm/sec
57	M	7 mo	6.9	BA	Mother	2.1	Compatible	MPV 4.5 mm, patent, hepatopetal flow
69	F	6 mo	6.5	BA	Mother	4.7	Identical	MPV 4.2 mm, patent, hepatopetal flow, low flow
76	M	34 yr	65.6	HBV-LC	Wife	1	Identical	MPV 13 mm, patent, hepatopetal flow, 6 cm/sec
82	M	11 mo	7.8	BA	Mother	3	Identical	MPV 4.3 mm, patent, hepatopetal flow, 10 cm/sec

BW, body weight; GRWR, graft-to-recipient weight ratio; PV, portal vein; Tx, transplantation; MPV, main portal vein; HBV, hepatitis B virus; LC, liver cirrhosis; BA, biliary atresia.

TABLE 2. Patient outcome

No.	Tx-PVT	F/U after rtPA	Doppler at PVT	Doppler current	AST (IU/L)/ALT (IU/L)/Bil (mg/dL) at PVT	AST (IU/L)/ALT (IU/L)/Bil (mg/dL) current
19	43 mo	2 mo	PVT(+), no flow	CR, 10 cm/sec	63/21/1	37/19/1.2
57	16 mo	4 mo	PVT(+), low flow, 6 cm/sec	PR, 8 cm/sec	59/23/1.2	40/17/1.4
69	21 mo	2 mo	No flow	PR, 10 cm/sec	29/11/1.9	34/17/1.4
76	5 mo	10 mo	PVT(+), anastomotic stricture	CR	216/122/5.1	22/15/0.8
82	14 mo	2 mo	PVT(+), 8 cm/sec	PR	49/26/1	52/25/0.8

Tx, transplantation; PVT, portal vein thrombosis; rtPA, recombinant tissue plasminogen activator; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Bil, total bilirubin; CR, complete regression of PVT; PR, partial regression of PVT; F/U, follow-up.

Case 3

A 6-month-old girl weighing 6.5 kg underwent LDLT for biliary atresia. She received a left lateral segment graft from her mother, who was ABO blood group identical. During surgery, the recipient's portal vein trunk was found to be hypoplastic with low portal flow, and she required reanastomosis twice to achieve sufficient flow. The successful reconstruction was accomplished with anastomosis between the recipient's branch patch bifurcation of the main portal vein and graft left portal vein using 7–0 monofilament polyglyconate sutures. During the final period of operation, the patient required a polytetrafluoroethylene sheet to close the abdominal fascia to avoid the graft compression because the graft was too big to close directly (graft-to-recipient weight ratio, 4.7%). The immunosuppression regimen consisted of microemulsified cyclosporine, azathioprine, and steroids. The posttransplant course was uneventful, and she did well until 21 months post-LDLT when PVT was detected by ultrasonography study. PVT seemed to be localized along the anastomotic site, and there was no blood flow in the intrahepatic portal vein (Fig. 1); 3D CT angiography subsequently revealed portal vein occlusion with PVT. There was no ascites. The patient was apparently doing well with acceptable liver function (AST, 29 U/L; ALT, 11 U/L; total bilirubin 1.9 mg/dL), so a course of rtPA was administered. The patient tolerated the treatment without any complications. After the treatment course was completed, Doppler ultrasonography showed partial regression of PVT with portal flow velocity of 10 cm/sec (Fig. 1). The patient is currently doing well with normal liver function 2 months after the treatment.

Case 4

A 34-year-old man underwent LDLT for hepatitis B-related liver cirrhosis. He received a right lobe graft from his wife, who was ABO blood group identical. Although the portal vein appeared grossly normal, transection revealed subintimal thrombosis in the portal vein trunk. After thrombectomy, portal vein reconstruction was performed with the recipient's left portal vein because thrombosis was observed to have been more extensive in the right branch. A 6–0 monofilament polypropylene suture material was used for anastomosis, leaving a 10-mm growth factor. The immunosuppression regimen consisted of tacrolimus and steroids. One month after transplantation, the patient developed a

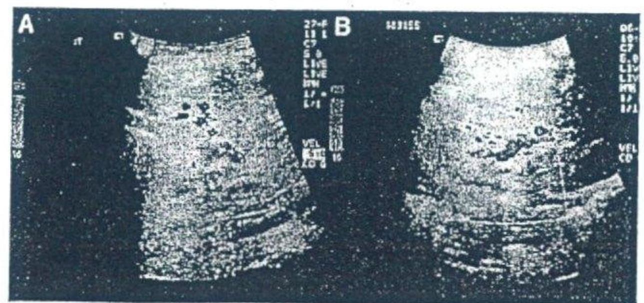

FIGURE 1. Doppler ultrasonography study before (A) and after (B) the systemic thrombolytic therapy in case 3. Before the treatment, no portal flow was detected at umbilical portion of the graft, whereas it reversed with flow velocity of 10 cm/sec after the therapy.

bile leak, which was appropriately treated by percutaneous drainage. Because T-tube cholangiography revealed no abnormality in the biliary anastomosis, we believed that the bile leak originated from the cut surface of the liver. The posttransplant course was otherwise uneventful, and portal vein flow remained acceptable. Five months after surgery, the patient was seen in the clinic and found to have mild liver dysfunction (AST, 216 U/L; ALT, 122 U/L; total bilirubin 5.1 mg/dL), although he continued to be asymptomatic. Multidetector 3D CT angiography revealed portal vein stenosis with thrombus formation extending from the splenic vein confluence to the anastomotic site. There was no ascites. Despite the finding of suspected anastomotic stenosis, we decided to try systemic thrombolytic therapy first (the less-invasive procedure) because the patient was generally doing well. Follow-up CT studies showed recanalization of the portal vein after completion of treatment, although the anastomotic stricture remained (Fig. 2). The patient is currently doing well with normal liver function 10 months after the treatment.

Case 5

An 11-month-old boy weighing 7.8-kg underwent LDLT for biliary atresia. The patient received a left lateral segment graft from his mother, who was ABO blood group identical. During surgery, the recipient's portal vein trunk was found to be only 6 mm in diameter, so a branch patch of its bifurcation was anastomosed to the graft portal vein using 7–0 monofilament polyglyconate sutures with a 10-mm growth factor. Although there was no pretransplant PVT identified, the patient required reanastomosis of the portal vein four times intraoperatively because of insufficient portal flow with repeated fresh PVT. A successful reconstruction was finally accomplished with an acceptable mean portal flow velocity of 29 cm/sec. The immunosuppression regimen consisted of microemulsified cyclosporine, azathioprine, and steroids. The posttransplant course was uneventful with no deterioration of portal flow. The patient did well until 14 months post-LDLT when PVT was detected on a routine Doppler ultrasonography study. 3D CT angiography revealed a portal vein occlusion without visualization of the intrahepatic portal system. There was no ascites, and PVT was localized along the anastomosis with no extension deep into the intrahepatic portal vein. A course of rtPA was opted for because the patient was clinically well with good liver function (AST, 49 U/L; ALT, 26 U/L; total bilirubin 1.0 mg/dL). The patient tolerated the treatment well, and there were no complications. Follow-up Doppler ultrasonography after completing the treatment course showed only partial regression of the PVT. Therefore, the patient received another course of rtPA 2 months later, but there was no remarkable improvement compared with the result after the first course. The patient is currently doing well with normal liver function 2 months after the second treatment.

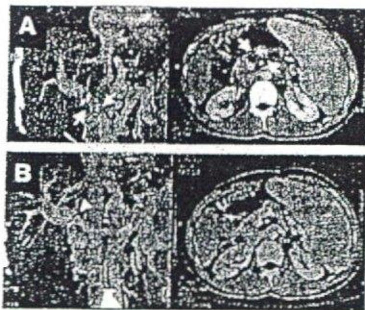

FIGURE 2. Multidetector three-dimensional computed tomography (3D CT) angiography before (A) and after (B) the systemic thrombolytic therapy in case 4. Before the treatment, the thrombus extended from the splenic vein confluence to the portal vein anastomosis (*arrow*). Although the entire thrombus disappeared after the treatment, an anastomotic stricture remained (*arrowhead*).

DISCUSSION

Medical management of late-onset posttransplant PVT using systemic administration of rtPA is an attractive alternative to surgical intervention because it is noninvasive, generally safe, and effective as demonstrated in the five cases reported. Several authors have reported successful reestablishment of portal vein flow through mechanical repair by interventional radiology or surgery (*3–6*). In our literature search, only one group has attempted using medical thrombolysis for late-onset posttransplant PVT (*7*). Invasive approaches have traditionally been favored, because the majority of portal vein thromboses are attributed to blood flow alterations secondary to kinking or stenosis at the anastomotic site. In these cases, the underlying structural abnormality should be corrected by balloon dilatation (*8*), stenting (*4*), or bypass surgery (*6*). However, Guckelberger and colleagues (*7*) reported that after successful pharmacologic thrombolytic therapy, there may be no abnormality evident at the anastomotic site, and the exact cause of the PVT may remain undetermined. Because the cause of posttransplant PVT varies from patient to patient, the ideal treatment modality needs to be determined on a case-by-case basis. Factors predisposing to posttransplant PVT include the presence of PVT before transplant (as in case 4) and portal vein hypoplasia, which was generally observed in biliary atresia (as in cases 1–3 and 5) (*9, 10*). The adult case was further complicated by a posttransplant stenosis of the portal vein anastomosis. Despite these findings, a noninvasive approach was chosen because all of the patients were clinically stable with only mild symptomatology and liver dysfunction, if any. Following the protocol outlined by Guckelberger et al. (*7*), systemic administration of a short, low-dose course of rtPA was instituted as the first-line therapy. However, the most favorable indication of this procedure should be no structural abnormality and no severe extent of thrombosis. Cherukuri et al. (*11*) reported long-term patency after the treatment of PVT with interventional thrombolysis and stent placement in such cases. Accordingly, the adult case in our series may be considered for an interventional approach later during follow-up.

The systemic thrombolytic therapy was originally introduced as treatment for hepatic veno-occlusive disease after bone marrow transplantation in pediatric patients (*12*) and was only later adapted for PVT after liver transplantation. We have shown that rtPA can be used safely, even in patients with recent bleeding, as long as there is no evidence of active bleeding during the treatment period. However, during treatment, we have to pay special attention not to miss the bleeding complication, which sometimes can be life threatening,

such as central nervous system, gastrointestinal, or hepatic hemorrhage, as reported in the case of use in myocardial infarction (*13–15*). Accordingly, patients receiving rtPA treatment should be monitored closely for signs of bleeding to promptly institute intervention when necessary.

In terms of imaging studies for characterizing suspected posttransplant vascular complications, interventional radiologic procedures have the advantage of being not only therapeutic but also diagnostic (*4, 8*). These procedures are less invasive than surgical intervention, but they are highly dependent on the expertise of the interventional radiologist and should be performed with a surgeon on standby (*16*). An even less invasive diagnostic tool, however, is multidetector 3D CT angiography, which can accurately detect vascular abnormalities and is more reliable than conventional angiography (*17*). The combination of this innovative diagnostic modality, multidetector 3D CT angiography, with systemic thrombolytic therapy, is a feasible option for minimally invasive management of late-onset posttransplant PVT.

PVT may recur after medical thrombolysis, especially in patients with pathologic native vessels, as demonstrated in the cases described in this report. Therefore, regular and careful long-term follow-up is necessary. Prophylaxis with oral anticoagulant medication may improve outcomes; we, however, chose not to administer long-term oral anticoagulation because its efficacy in this particular clinical situation is still not clear. Although systemic thrombolytic therapy may resolve a PVT occurring in a structurally normal vessel, it may also serve as a bridge to a more definitive surgical solution in cases in which the PVT is secondary to a structural defect such as an anastomotic stricture. In our series, the adult case with anastomotic stricture may be considered for interventional treatment such as stent placement later in the follow-up.

Three patients revealed partial regression of PVT after the treatment, two of whom received another course of rtPA thereafter. No remarkable improvement was observed in both patients after the second treatment, but Dakik and Nasrallah (*18*) reported that repeated doses of rtPA could recanalize the coronary artery with failed thrombolysis after a first dose of rtPA. Accordingly, our policy is to try a repeated course of systemic thrombolytic therapy when complete regression of PVT is not achieved after the first dose, especially in the case with no suspected structural abnormality and as long as the patient is doing well with no significant liver dysfunction.

On the basis of our experience, this systemic therapy may be a possible option for acute and late-onset hepatic artery thrombosis after transplantation or pretransplant PVT, both of which have never been reported.

CONCLUSION

Although longer follow-up is necessary, systemic thrombolytic therapy using low-dose rtPA seems effective for late-onset PVT after liver transplantation. This noninvasive approach should be considered in selected cases with stable clinical status and no active bleeding.

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IMMUNODYNAMICS OF BASILIXIMAB IN LIVER ALLOGRAFT RECIPIENT UNDER CONTINUOUS HEMODIAFILTRATION

Basiliximab is a high-affinity human-murine chimerized monoclonal antibody that blocks binding of interleukin-2 to the IL-2R α-chain (CD25) on activated T lymphocytes. Blockade of these receptors decreases the incidence of acute cellular rejection of liver and renal allografts (*1*).

However, no clinical data is available regarding the serum levels of basiliximab under continuous hemodiafiltration (CHDF), which is increasingly used for patients with acute renal failure (ARF) with borderline hemodynamic stability (*2*). We report herein the immunodynamics of basiliximab in a living donor liver transplantation (LDLT) recipient who was kept on CHDF for ARF during the perioperative period.

A 57-year-old Japanese man with postnecrotic liver cirrhosis due to hepatitis C virus infection was admitted to a referral hospital with recent onset of fever and jaundice in July 2002. Despite undergoing conservative therapy for 3 days, his general status worsened to a grade III hepatic coma and he was transferred to our hospital for possible liver transplantation.

Emergency LDLT was performed successfully on September 9, 2002, using the 450 g right lobe of his ABO-identical brother (0.99% of the his overall body weight, 54.6% of the standard liver volume). Immunosuppression was achieved by intravenous solumedrol tapered from 500 mg/day to 20 mg/day, plus mycophenolate mofetil 2 g/day and 20 mg of intravenous basiliximab (Simulect, Novartis, Basel, Switzerland) on days 0 and 4. On postoperative day 2, the patient regained consciousness and was extubated. For preexisting renal failure (creatinine 4.2 mg/dL; urea nitrogen 50 mg/dL), the patient was kept on CHDF throughout the perioperative period until he expired on day 30 due to MRSA pneumonia, which developed on day 21 after LDLT. The rates of each component of CHDF were as follows: blood flow, 80 ml/min; hemofiltration, 1000 ml/h; dialysate, 500 ml/h; and fluid supplement, 500 ml/h.

Throughout the postoperative period on CHDF, the serum levels of basiliximab were within the therapeutic range (>200 ng/ml) (Fig. 1) (*4*). The percentage of CD25 positive lymphocytes was as low as 0.1% on posttransplant day 4 and 0.2% on posttransplant day 18.

In our patient, the effects of basiliximab were apparently unaffected by CHDF. Thus far, the removal of basiliximab has been reported with 64.4% reduction after 2.5 L of plasma pheresis (*4*) and also through ascites in patients after liver transplantation (*5*). Although our patient expired due to MRSA pneumonia, there was no evidence of a relationship between basiliximab and the MRSA pneumonia, as only CD25 positive lymphocytes seemed to be affected by basiliximab.

A membrane made of polymethylmethacrylate (PMMA) was used for CHDF because of its lesser affinity to anti-hepatitis B surface antigen antibody, compared to polyacrylonitrile (PAN) membrane (*3*). CHDF with other membranes such as PAN could attach human antibodies and decrease serum levels accordingly. The relationship between the CHDF membrane and human antibodies should be further elucidated, thus providing important information regarding the kinetics of drugs such as basiliximab, since long half-life time stands behind its popularity.

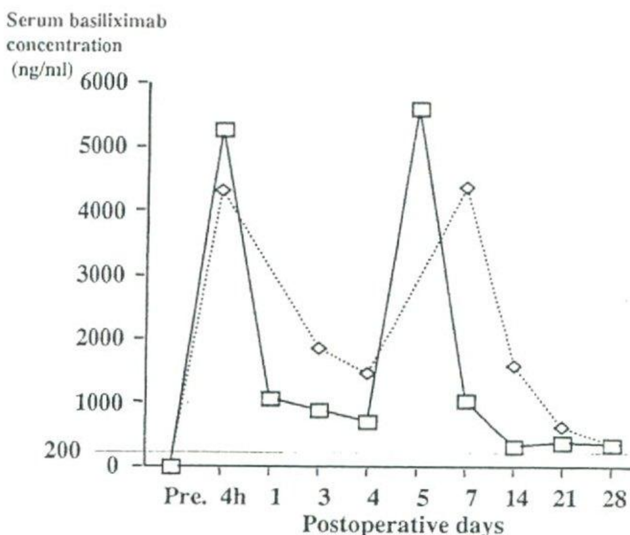

FIGURE 1. The serum levels of basiliximab were maintained above 200 ng/ml (*solid line*) throughout the observation period for the liver transplant patient who underwent continuous hemodiafiltration (CHDF), as compared to the liver transplant patient (24-year-old female, biliary atrasia) who did not undergo CHDF (*broken line*).

We reported the maintenance of the serum levels of basiliximab under CHDF in a patient after LDLT. Although our report involves only one case, supplemental basiliximab does not seem necessary under such a circumstance.

We thank Dr. Marizel Rouilly (Novartis Pharma AG, Basel, Switzerland) for measuring the serum levels of basiliximab.

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INTESTINAL CRYPTOSPORIDIOSIS MIMICKING ACUTE GRAFT-VERSUS-HOST DISEASE FOLLOWING MATCHED UNRELATED HEMATOPOIETIC STEM CELL TRANSPLANTATION

In this report, we discuss two female patients (Patient 1: 64 years, Patient 2: 48 years) with acute myeloid leukemia and myelodysplastic syndrome, respectively. Both patients presented with massive diarrhea after HLA-matched, unrelated peripheral blood stem cell transplantation (PBSCT). The conditioning regimen for Patient 1 consisted of 30 mg/m^2/d fludarabine for five days, 150 mg/m^2/d carmustine for two days, and 110 mg/m^2/d melphalan for one day. Patient 2 received 4 mg/kg/d busulfan for four days and 60 mg/kg/d cyclophosphamide for two days (*1*). Graft versus host disease (GVHD)-prophylaxis consisted of 20 mg/kg/d anti-thymocyte-globulin (ATG-S, Fresenius, Graefeling, Germany) for two days and Cyclosporine A (CsA) in both patients. Patient 1 also received mycophenolate mofetil, whereas Patient 2 received a short course of methotrexate on days +1, +3 and +6 (*1*). On day +13, Patient 1 developed severe watery diarrhea without any additional signs of acute GVHD. In contrast, Patient 2 had extensive chronic GVHD (skin, liver, bile ducts, lung, eyes) and was therefore still on immunosuppression with mycophenolate mofetil (500 mg three times a day) and prednisolone (5 mg twice a day) when she developed watery diarrhea 25 months after transplantation.

In both patients, coloscopy was suggestive for acute intestinal GVHD grade III-IV (Fig. 1A). Although initial histomorphological studies were concordant with the macroscopic findings, further investigations revealed intestinal cryptosporidiosis (Fig. 1B). Other potential bacterial or viral etiologies were excluded. Besides GVHD as the most common cause, side effects of chemotherapy or other medications and infectious agents (e.g. bacteria, viruses or fungi) must be considered and should be tested regularly (*2*). Protozoal infections are rare (0.5%) but life-threatening diseases in immunocompromised patients (*2*). *Cryptosporidium* is transmitted by person-to-person contact via the fecal-oral route or indirectly by sputum and vomitus (*3*). *Cryptosporidium* species have been reported to be resistant to a wide range of disinfectants and antiseptics, so timely and accurate diagnosis is of clinical importance.

Currently, no antibiotic treatment of cryptosporidiosis has been reported to reliably eradicate the protozoon (*3*). Paromomycin, azithromycin, spiramycin, nitazoxanide and albendazole are the commonly used antibiotics, but in most cases treatment failed to eradicate *Cryptosporidia* successfully (*3*). Numeric T cell reconstitution and reduction of immunosuppressive therapy were reported to be relevant for the healing of opportunistic HIV-1 associated cryptosporidiosis (*4*). Our report confirms that this seems also to be valid for cryptosporidiosis in the allogeneic hematopoietic stem cell transplantation (HSCT) setting. Patient 1 suffered from severe T lymphocytopenia, whereas Patient 2 revealed a normal CD4 count at time of diagnosis, but was still on immunosuppression rendering her susceptible for the opportunistic protozoon. Moreover, the importance of functional intact T cell response was recently demonstrated in two patients with CD40L deficiency, who cleared *Cryptosporidium parvum* after successful bone marrow transplantation (*5*).

The few reported cases of post-HSCT cryptosporidiosis were observed after either allogeneic T-cell-depleted bone marrow transplantation, CD34-positive selected autologous PBSCT, or intensified immunosuppression due to GVHD (*6*). Besides reduction of immunosuppression, Nachbaur et al. (*6*) administered low dose recombinant human interleukin-2 (rhIL-2) subcutaneously, to influence T lymphocyte subset reconstitution in patients having undergone autologous PBSCT and suffering from intestinal cryptosporidiosis. Since this treatment was approved only in the autologous setting and might induce GVHD in the allogeneic setting, it was not applied in our patients. In contrast to these cases, cryptosporidiosis resolved in our cases, solely by administering anti-infectious treatments (Patient 1: azithromycin plus paromomycin, Patient 2: albendazole) and carefully reducing the immunosuppression (Figs. 1C and 1D).

We conclude that, in HSCT recipients with diarrhea, it is mandatory to include specific techniques for the identification of *Cryptosporidium* in the stool to the diagnostic panel. Due to the high diagnostic yield, gastrointestinal endoscopy should be performed and the biopsies should be specifically stained and examined by an experienced pathologist aware of intestinal parasitoses. Intestinal cryptosporidiosis in patients after allogeneic HSCT can mimic intestinal GVHD. Since acute GVHD requires intensive immunosuppression, whereas therapy of intestinal cryptosporidiosis consists of

Cytotoxic T-Cell Elimination During Anti-CD4–Induced Rat Liver Acceptance and Rapid Replacement of Functional Graft Antigen–Presenting Cells

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In previous studies, we showed that primed T cells were eliminated in long-term survival Wistar Furth (WF) recipient rats with spontaneously accepted Lewis (LEW) liver graft and that the grafted liver lost the ability to elicit rejection reaction early after liver transplantation. We hypothesized that the same phenomenon may be observed in tolerant animals after immunosuppression in a rejector rat strain combination (WF→LEW). Furthermore, we proposed the repopulation of liver allograft with host antigen–presenting cells rapidly after transplantation. Recipient LEW rats that underwent anti-CD4 therapy accepted the WF liver allografts after a transient rejection reaction. In tolerant animals, alloreactive CD8 T cell precursors were present, but primed T cells were absent. Intraperitoneal challenge with grafted WF liver homogenates obtained from recipient LEW rats on day 4 after transplantation did not induce transient rejection responses in long-term survival recipient LEW rats, a finding that differed from the results of experiments using normal WF liver homogenates. However, challenge with grafted WF liver homogenates, similar to those of normal LEW liver homogenates, induced rejection responses in long-term survival recipient WF rats with LEW liver allograft. Flow cytometric analysis confirmed that most of nonparenchymal cells in the grafted WF liver were recipient (LEW) genotype. These observations showed that the deletional mechanism of effector T cells also is observed in this setting, and professional donor antigen–presenting cells are replaced by those of recipient genotype within the graft during the early phase of transplantation. (*Liver Transpl 2004;10:734–742.*)

Abbreviations: ALT, alanine aminotransferase; APCs, antigen-presenting cells; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; LEW, Lewis; mAb, monoclonal antibody; PVG, Piebald virol Glaxo pigmented; MMC, mitomycin C; PFA, paraformaldehyde; NPC, nonparenchymal cells; TB, total bilirubin; WF, Wistar Furth; IP, intraperitoneal challenge.

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Deletional mechanisms of effector T cells may play a role in transplantation tolerance.[1,2] We previously have shown that treatment with anti-CD4 monoclonal antibody (mAb) induced heart allograft tolerance but not skin allograft acceptance in the Wistar Furth (WF) to Lewis (LEW) rat strain combination. We also examined the differences in cellular responses between heart-bearing and skin-rejected hosts.[3] Our results showed that primed T cells were present in anti-CD4-treated WF skin-rejected LEW rats and that the secondary donor heart and skin grafts resulted in accelerated rejection in the hosts, in sharp contrast to anti-CD4-treated WF heart-bearing LEW rats. That is, no primed T cells were present, and the survival of secondary donor grafts was prolonged in the heart-bearing hosts.

In rat liver transplantation, the grafted liver itself has been reported to be involved in immune tolerance using a retransplantation model, and the Kupffer cell population in the graft at the time of retransplantation has been shown to be almost completely of recipient origin.[4] Because it is conceivable that the chimeric retransplanted liver is associated with reduced immunogenicity,[5] the establishment of tolerance may be caused by the pathway of indirect allorecognition. In tissue and organ transplantation, the importance of the direct pathway in graft rejection has been stressed,[6–8] and the indirect pathway has been suggested to be more important in graft rejection.[9,10] In liver transplantation, however, hepatocytes exert an immunologic effect on graft acceptance[11] and appear to regulate both the direct and indirect pathways.[12] Recent investigations have shown the role of the passenger leukocyte genotype in the rejection and acceptance of rat liver allografts.[13] They noted that recipient-type passenger leukocytes in the hepatic allograft were involved in graft acceptance in the rejector rat strain combination.

In this study, we used an anti-CD4 induced liver acceptance model. We observed that primed T cells were absent in tolerant animals overcoming acute rejection responses and that functional donor antigen-presenting cells (APCs) were almost completely replaced by those of the recipient genotype within the graft by

day 4 after grafting in the rejector rat strain combination.

Materials and Methods

Animals

Male WF ($RT1^u$; A^u, B^u, D^u) rats were obtained from a colony raised in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine (Nagasaki, Japan). Male LEW ($RT1^l$; A^l, B^l, D^l) and PVG ($RT1^c$; A^c, B^c, D^c) rats were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine.

Monoclonal Antibodies

Antirat cluster designation (CD3) mAb, a mouse antibody of the IgM immunoglobulin class, was produced using hybridoma 1F4. Antirat CD4 mAb, a mouse antibody of the IgG2a immunoglobulin class, was produced using hybridoma Oxford 38. Antirat CD8 mAb, a mouse antibody of the IgG2b immunoglobulin class, was produced using hybridoma 10B5. Dr. Y. Hashimoto (Tohoku University, Sendai, Japan) kindly provided 1F4, Dr. S. Miyagawa (Osaka University Medical School, Osaka, Japan) kindly provided OX38, and Dr. N. Sato (Sapporo Medical College, Sapporo, Japan) kindly provided 10B5.

Mixed Lymphocyte Culture

Responder spleen cells (2×10^5) were cultured with 4×10^5 mitomycin C (MMC) –treated stimulator cells (Kyowa Hakko Co, Tokyo, Japan) in round-bottomed, 96-well tissue culture plates (Corning 25860, Corning, NY) for 4–5 days at 37°C under 5% CO_2 in air. The culture medium was Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin and 5×10^{-5} M 2-mercaptoethanol (2-ME). MMC-treated stimulator cells were prepared by incubating spleen cells (2×10^7/mL) with 50 μg/mL MMC for 30 minutes at 37°C. Assays were performed in triplicate. Sixteen hours before harvesting, the culture was pulsed with 3.7 KBq 3H-thymidine, and radioactivity was measured using a liquid scintillation counter.

Cell-Mediated Cytotoxicity Assays

Responder spleen cells (2×10^7) were cultured with 4×10^7 MMC or paraformaldehyde (PFA) (Wako, Osaka, Japan) –treated stimulator cells at 37°C under 5% CO_2 in air. PFA treatment was performed by incubating spleen cells (2×10^8/mL) with PFA at a concentration of 0.5% for 20 minutes at 4°C. Effector cells were produced by culture for 5 days. Target cells were labeled with ^{51}Cr by incubating spleen concanavalin A (Con A) blasts (5×10^6) with 2 Megabecquerel (MBq) of $Na_2{}^{51}CrO_4$ (NEN, Boston, MA) in 0.5 mL of culture medium for 40 minutes at 37°C. The labeled cells were washed twice with culture medium before use. Samples of 2×10^4 labeled target cells (in a volume of 100 μl) were incubated with serial dilutions of the effector cell suspension (100 μl). The plates were incubated for 4 hr at 37°C under 5% CO2 in air. In antibody blocking assays, an equal volume (50 μl) of serially diluted antibody, effector cell suspension, and 2×10^4 labeled target cells were incubated together. The supernatant was then removed from each well, and the radioactivity was measured with an ALOKA ARC-300 gamma counter (Tokyo, Japan). Percent specific lysis was calculated using the following equation: $(a - b/c - b) \times 100$, where a = counts per minute in the supernatant of target cells mixed with effector cells, b = counts per minute in the supernatant of target cells incubated alone, and c = counts per minute after lysis of target cells with Nonidet P-40. Assays were performed in duplicate in round-bottomed, 96-well plates.

Isolation of Liver Nonparenchymal Cells (NPC)

Grafted WF livers obtained from recipient LEW rats 100 days after transplantation were used as the source of cells in this study. The grafted liver was perfused with 200 mL Hanks balanced salt solution, then perfused for 8 minutes with 37 mg collagenase (Wako, Osaka, Japan) in 75 mL Hanks buffer supplemented with CaC_{12} (0.56 g/L). The liver was surgically removed and cut into small pieces. After removal of undigested tissues, the cells were resuspended in Hanks buffer. Resuspended cells were centrifuged at 50 g for 1 minute and the NPC-rich supernatant was recovered. After repeating this procedure 3 times to eliminate hepatocytes, the supernatant was centrifuged at 180 g for 5 minutes to pellet a mixed NPC fraction. After the cells were resuspended in Hanks buffer, the NPC were further enriched by centrifugation at 200 g for 6 minutes in a 2-step Percoll gradient (25 and 50%).

Pretreatment of Cells With Anti-CD3 mAb (1F4) and Complement

The method was described previously by Yamaguchi, Kanematsu, Shiku, and Nakayama.[5]

Preparation of Alloantibody

Female WF and LEW rats aged 5–8 weeks were inoculated intraperitoneally with LEW and WF spleen cells (1–2 × 108) once a week, respectively. Sera obtained from live rats 10 days after the fifth inoculation were used as an antibody against major histocompatibility complex alloantigens. Specificity of sera (WF anti-LEW antiserum and LEW anti-WF antiserum) was confirmed by flow cytometry using spleen cells as targets.[14]

Flow Cytometric Analysis

The cells were incubated with antiserum, diluted (1:100) with Eagle's minimum essential medium, for 30 minutes at 4°C. After 2 washes with phosphate buffered saline, the cells were incubated with fluorescein isothiocyanate–labeled goat anti-rat IgG-F(ab')2 (MP Biomedicals Inc.-Cappel Products, Irvine, CA; diluted 1:100) for 30 minutes at 4°C. After 2 washes with PBS, the cells were suspended in PBS and examined in an fluorescence-activated cell sorter can (Becton Dick-

inson Co., Mountain View, CA). Cells (1×10^4) were sorted after live gating on the leukocyte population to concentrate the analysis, and single histograms were obtained.

Measurement of Serum Levels of Alanine Aminotransferase (ALT) and Total Bilirubin (TB)

Blood samples were obtained from the tail vein. The serum concentrations of alanine ALT and TB were measured with an autoanalyzer (UVIDEC-77, Jasco, Tokyo). The normal values for ALT and TB were less than 35 U/L and 0.4 mg/dL, respectively.

Inoculation of Liver Homogenates

The liver was perfused through the portal vein with 20 mL of ice-cold Haltman's solution, then removed and homogenized in a manual homogenizer. Cells were then pelleted by centrifugation and inoculated intraperitoneally into recipient rats that survived for more than 100 days after transplantation. We used recipient rats with no serologic evidence of liver dysfunction in blood samples obtained before experiments; we excluded animals that suffered technical death as a result of the experiment. Liver homogenates consisted microscopically of parenchymal and NPCs. Cell viability was more than 90% as determined by Trypan blue exclusion, and the volume equivalent to 5% of a normal liver homogenates (donor) efficiently sensitized nondonor type animals for donor skin grafts.

In Vivo Administration of mAb

Anti-CD4 mAb (OX38, purified antibody, 1 mg) was injected on days 0 and 2 into recipient LEW rats through the penile vein after WF liver transplantation.

Operative Procedure

Orthotopic liver transplantation was performed by the cuff and nonarterialized method, as previously described.[5] Rejection of the liver graft was judged by death of the animals.

Results

Transient Rejection Responses in Anti-CD4-Treated Recipient LEW Rats After WF Liver Transplantation

We performed liver transplantations in the WF to LEW rat strain combination (WF→LEW). All grafts were rejected within 38 days (n = 8). However, administration of depletional anti-CD4 mAb (OX38) prolonged the survival of recipient LEW rats, except in cases of technical failure (mean graft survival > 200 days, n = 10). As shown in Fig. 1, WF liver transplantation into transiently CD4-depleted LEW rats was associated with a rise in serum TB concentration at 2 weeks; however, the serum TB reverted to normal at 8 weeks. In contrast, serum TB concentrations increased in recipient LEW rats without immunosuppression 2 weeks after transplantation and further increased until the death of the animals. In the LEW-to-LEW rat strain combination, no rise in serum TB levels was observed.

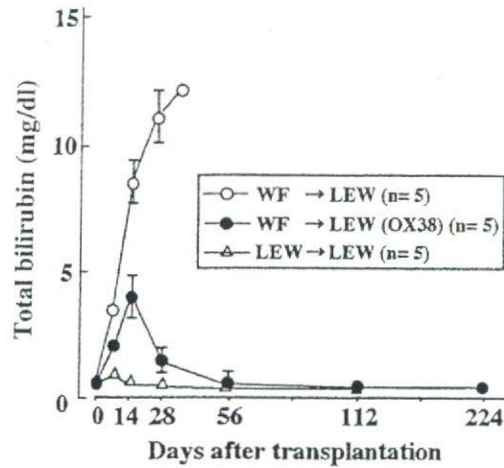

Figure 1. Serum TB concentrations after liver transplantation in WF to LEW rat strain combination. Closed circles are serum levels of TB in recipient LEW rat (n = 5) that were transiently depleted of CD4 T cells after WF liver transplantation. Open circles (n = 5) are recipient LEW rats without anti-CD4 therapy. Open triangles (n = 5) are syngeneic rat strain combination.

Serum levels of aminotransferase and the loss of body weight in anti-CD4-treated recipients were almost identical to those in recipients without anti-CD4 therapy at 1 or 2 weeks after transplantation (data not shown). The percentage of CD4 T cells at 3, 7, and 14 days after OX38 treatment was 0.2%, 3.4%, and 14.1%, respectively.[3]

In Vitro Cellular Responses to Donor Antigens in Long-Term Survival Recipient LEW Rats Bearing WF Liver Transplant

The mixed lymphocyte culture response of spleen cells from transiently CD4-depleted LEW rats bearing WF liver graft for more than 100 days was similar to that of spleen cells from untreated LEW rats after in vitro stimulation with MMC-treated WF spleen cells (Fig. 2A). The generation of cytotoxic T lymphocytes (CTLs) in spleen cells from recipient LEW rats was also similar to that in spleen cells from naïve LEW rats (Fig. 2B). Each experiment (A and B) was carried out twice, and essentially the same results were obtained.

Characterization of CTL Generated in Spleen Cells From Anti-CD4-Treated WF Liver-Bearing LEW Rats

Cytotoxicity was observed in Con A blasts from WF but not PVG strains (Fig. 3A), and it was blocked by the

addition of anti-CD3 mAb (1F4) and anti-CD8 mAb (10B5) but not anti-CD4 mAb (OX38) to the culture (Fig. 3B). Furthermore, we investigated the presence of primed T cells in the recipient LEW rats. As shown in Fig. 3C, no generation of CTL was observed in the spleens from recipient LEW rats 100 days after WF liver grafting after in vitro stimulation with PFA-treated WF spleen cells, a finding similar to that in naïve LEW rats. However, spleen cells from anti-CD4–treated WF skin rejected LEW rats 100 days after skin grafting generated CTL after in vitro stimulation with PFA-treated WF spleen cells (ref-3). These results demonstrated the presence of CD8 CTL precursors (A and B) and the absence of primed T cells in anti-CD4-treated WF liver–bearing LEW rats (C). Each experiment was carried out twice (A and B) or more than 3 times (C), and essentially the same results were obtained.

Loss of Immunostimulatory (Costimulatory) Capacity of WF Liver Transplants to Elicit Immune Attack on the Host Immune System

The response of recipient rats to grafted liver was investigated by intraperitoneal challenge of liver homogenate.[5] The experimental design is shown in Fig. 4. As given in Table 1, intraperitoneal challenge with a volume equivalent to 5–50% of a normal WF liver homogenate into recipient LEW rats surviving for more

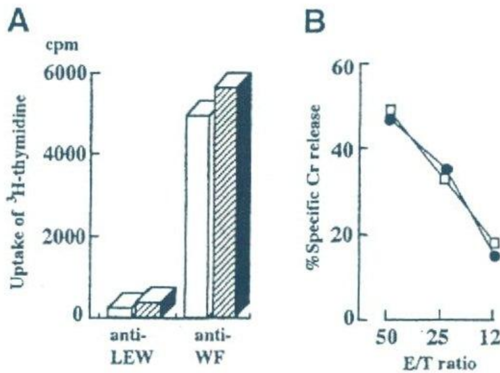

Figure 2. In vitro cellular responses. Mixed lymphocyte reaction (MLR) response (A) and cell cytotoxicity (B) in spleen cells from LEW rats that were transiently depleted of CD4 T cells and bore WF liver grafts for more than 100 days. In A, responder spleen cells from normal LEW rats (open bars) and recipient LEW rats with WF liver transplant (hatched bars) were cultured with MMC-treated LEW or WF stimulator cells. In B, responder spleen cells were obtained from naïve LEW rats (open squares) and LEW rats with WF liver transplant (closed circles). Target cells were WF splenic blast cells. Abbreviation: E/T, effector to target cell.

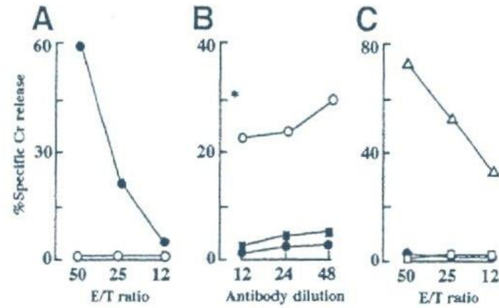

Figure 3. Characterization of CTL. (A) Specificity analysis of CTL generated in anti-CD4-treated WF liver–bearing LEW rats on in vitro stimulation with MMC-treated WF stimulator spleen cells. Splenic Con A blasts from WF (closed circles) and PVG (open circles) rats were used as targets. (B) Antibody blocking assays. Anti-CD3 mAb (closed circles), anti-CD8 mAb (closed squares), or anti-CD4 mAb (open circles) was added to the culture. Asterisk indicates cytotoxicity without antibodies. The E/T ratio in this experiment was 25:1. (C) Generation of CTL on in vitro stimulation with PFA-treated WF stimulator spleen cells. Responder spleen cells were obtained from anti-CD4-treated WF liver-bearing LEW rats (closed circles), naïve LEW rats (open squares) or anti-CD4-treated WF skin rejected LEW rats 100 days after skin grafting (open triangles). Abbreviation: E/T, effector to target cell.

than 100 days after WF liver grafting induced a transient elevation of serum levels of ALT that reached a peak level on days 5–6 (Fig. 4A). In subsequent experiments, we used more than 50% volume of the total liver homogenate for intraperitoneal challenge. No elevation of ALT was observed after administration of normal LEW (Fig. 4B) and PVG (Fig. 4C) liver homogenates. We found that intraperitoneal challenge

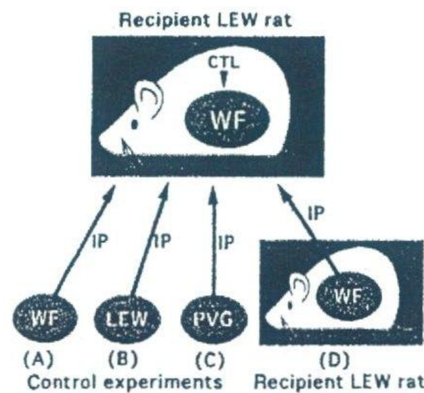

Figure 4. Experimental design. Normal WF (A), LEW (B), PVG (C) (control) and grafted WF (D) liver homogenates were intraperitoneally administered into recipient LEW rats that survived for more than 100 days after WF liver grafting.

Table 1. Serum Levels of ALT in the Long-term Surviving LEW Rats with WF Liver Transplant After Intraperitoneal Injection with Liver Homogenates

Injected Liver	ALT (U/L)*			
	0	5d	6d	14d#
(A) Naïve WF liver (5% volume, control)	28 (24–31)	203 (178–222)	250 (210–264)	32 (25–40)
Naïve WF liver (50% volume, control)	26 (21–27)	212 (197–246)	202 (177–219)	31 (23–37)
(B) Naïve LEW liver (control)	24 (20–26)	23 (22–30)	21 (18–27)	19 (16–25)
(C) Naïve PVG liver (control)	27 (19–27)	27 (26–34)	28 (20–32)	25 (24–30)
(D) Grafted WF liver (POD 1)	24 (20–29)	109 (96–132)	134 (113–153)	30 (23–35)
Grafted WF liver (POD 3)	27 (23–31)	38 (37–79)	140 (130–176)	27 (23–37)
Grafted WF liver (POD 4)	21 (20–25)	26 (22–31)	25 (22–30)	20 (17–23)
Grafted WF liver (POD 5)	25 (21–31)	22 (18–24)	19 (15–24)	18 (16–23)
Grafted WF liver (POD 100)	26 (19–27)	21 (17–28)	23 (18–29)	25 (24–30)

Abbreviations: ALT, alanine aminotransferase; POD, postoperative day (The point at which grafted WF liver homogenates were obtained after grafting).
NOTE. Each group consisted of 3 rats.
*Data are given as median (range) value. #Day after i.p. injection.

with normal WF spleen cells ($1-10 \times 10^7$), but not LEW spleen cells, induced a transient elevation of serum levels of ALT and that administration of normal WF liver homogenate into naïve WF and LEW rats did not increase AST levels (data not shown). That is, the elevation of ALT is caused by the generation of effector cells in WF liver–grafted LEW recipient by intraperitoneal challenge of donor (WF) APCs, and the effector cells destroy donor (WF) hepatocyte in the grafted WF liver. In the next step, we investigated the kinetics of costimulatory properties of the grafted WF liver in transiently CD4-depleted recipient LEW rats (Fig. 4D). Challenge with grafted WF liver homogenate obtained from recipient LEW rats on days 1 and 3—but not on days 4, 5, and 100 after transplantation—induced a transient elevation of serum ALT levels. These results indicate that the costimulatory capacity of the grafted WF liver to elicit immune attack disappeared almost completely by day 4 after grafting. Each experimental group shown in Table 1 consisted of 3 animals.

Replacement of the Alloimmunostimulatory Ability of WF Liver Graft With That of Recipient Genotype

We examined the change in alloimmunostimulatory ability of the grafted WF liver in transiently CD4-depleted recipient LEW rats (WF→LEW). Liver homogenates were intraperitoneally injected into LEW liver-transplanted WF rats (LEW→WF) that survived for more than 100 days after grafting without immunosuppression. The experimental design is presented in Fig. 5. We reported that intraperitoneal challenge with normal LEW spleen cells (1×10^8) induced a transient elevation of serum levels of ALT and that the liver damage was blocked by in vivo administration of anti-CD8 mAb (10B5).[15] Challenge with normal LEW liver homogenate (Fig. 5A), but not normal WF liver homogenate (Fig. 5B), into LEW liver-transplanted WF rats induced a transient elevation of serum ALT concentrations (Table 2). This liver damage was blocked by treatment of recipient WF rats with anti-CD8 mAb.[5] However, grafted WF liver homogenate obtained from transiently CD4-depleted LEW recipients at 1, 3, 4, 10, and 100 days after transplantation induced a transient elevation of ALT (Fig. 5C). No

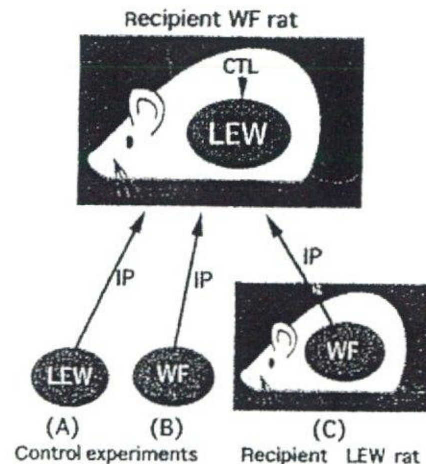

Figure 5. Experimental design. Normal LEW (A), WF (B) (control) and grafted WF (C) liver homogenates were intraperitoneally administered into recipient WF rats that survived for more than 100 days after LEW liver grafting.

Table 2. Serum Levels of ALT in the Long-term Surviving WF Rats with LEW Liver Transplant After Intraperitoneal Injection with Liver Homogenates

Injected Materials	ALT (U/L)*			
	0	5d	6d	14d#
(A) Naïve LEW liver (control)	31 (30–35)	312 (253–337)	253 (222–277)	23 (21–28)
(B) Naïve WF liver (control)	25 (24–31)	31 (25–35)	22 (20–26)	26 (25–30)
(C) Grafted WF liver (POD 1)	28 (25–34)	98 (94–129)	169 (139–206)	18 (15–22)
Grafted WF liver (POD 3)	25 (21–33)	232 (193–260)	237 (208–279)	30 (23–35)
Grafted WF liver (POD 4)	21 (20–26)	191 (163–210)	186 (154–210)	24 (23–32)
Grafted WF liver (POD 10)	30 (27–33)	277 (226–305)	214 (163–218)	28 (25–33)
Grafted WF liver (POD 100)	29 (25–34)	207 (193–245)	200 (183–239)	29 (27–37)
LEW blood (0.3 ml)	28 (25–32)	26 (25–30)	35 (30–39)	27 (25–33)

Abbreviations: ALT, alanine aminotransferase; POD, postoperative day (The point at which grafted WF liver homogenates were obtained after grafting).
NOTE. Each group consisted of 3 rats.
*Data are given as median (range) value. #Day after i.p. injection.

elevation of ALT was noted after administration of recipient LEW blood (0.3 mL). In view of the change of color after infusion of blood into the normal liver that had been perfused with lactated Ringer's solution through the portal vein, we confirmed that LEW recipient blood volume residing in the perfused WF liver transplant was less than 0.1 mL. Each experimental group shown in Table 2 consisted of 3 animals. Histologically, cellular infiltrates around the portal tract and in sinusoids were observed in the grafted LEW liver obtained from recipient WF rats 7 days after intraperitoneal challenge with the grafted WF liver homogenate (Fig. 6). These results (Table 1 and 2) indicated that reciprocal migration of functional APCs between graft and host occurred 1 day after transplantation, and alloimmunostimulatory ability of the grafted WF liver was

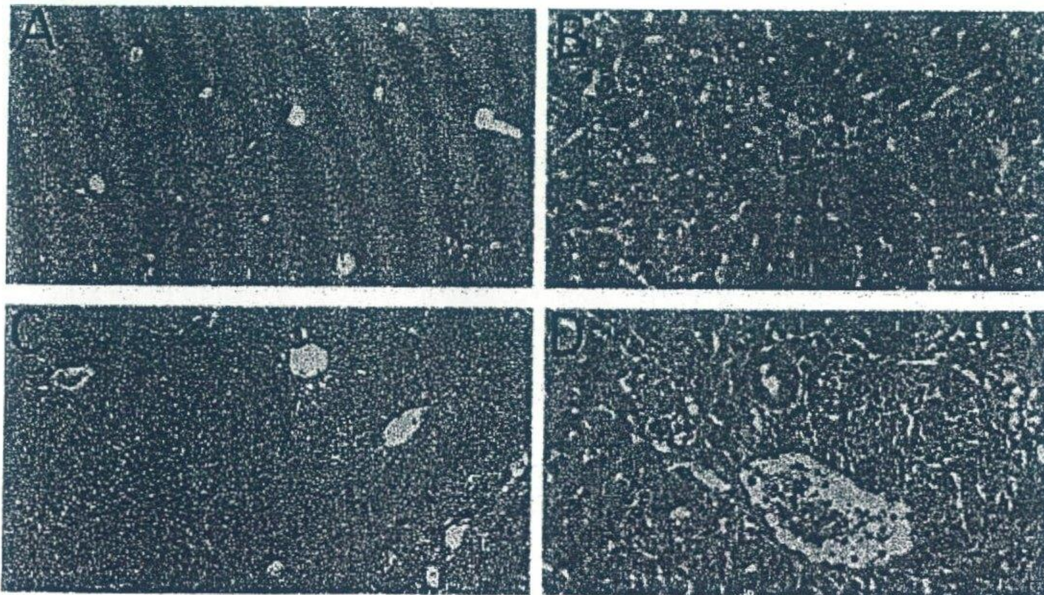

Figure 6. Histologic examination (hematoxylin and eosin stain) of grafted LEW liver. Grafted LEW livers from long-term surviving recipient WF rats were obtained before (A and B) and on day 7 after (C and D) intraperitoneal administration of grafted WF liver homogenates parked for 4 days in recipient LEW rats. The same animal was used for this kinetic study. This experiment was carried out twice and essentially the same results were obtained. (Magnification, A and C; ×25, B and D; ×100).

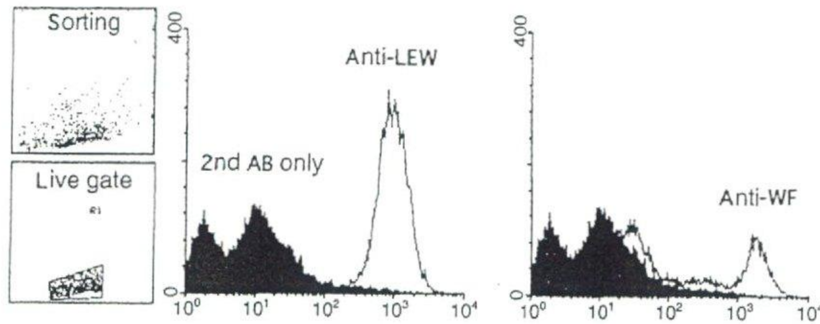

Figure 7. Flow cytometric analysis (genotype of NPC). Dot plot at cell sorting (left upper), live-gating (left lower), and the response of WF anti-LEW antiserum (middle) and LEW anti-WF antiserum (right) against NPC, respectively.

almost completely replaced by that of LEW genotype at day 4 after transplantation.

Genotype of NPC Within the Grafted WF Liver

We next examined the genotype of NPC within the graft by flow cytometry. We noted that NPC in the grafted WF liver included many CD3 positive cells (more than 30% of the NPC), different from those in the normal WF liver (data not shown). Then, the NPC treated with anti-CD3 mAb and complement were used as samples for flow cytometric analysis. Elimination of CD3 positive cells was confirmed by flow cytometry (data not shown). As shown in Fig. 7, the majority of the NPC within the grafted WF liver was recipient (LEW) genotype. This experiment was carried out 3 times, and essentially the same results were obtained.

Discussion

Researchers, including the current authors, have shown that donor APCs migrating from the liver graft were reduced in the periphery early after transplantation[14,16] and that primed CTLs were eliminated in the recipient animal or within the spontaneously accepted liver graft.[5,17,18] Similarly, in this study, using an anti-CD4-induced liver acceptance model, alloreactive CD8 T-cell precursors persisted in tolerized animals even though no primed CTLs were present. Because transient rejection reaction early after liver transplantation was observed in this model, effector T cells were probably eliminated in a manner similar to that in the spontaneously accepted liver transplantation model. We previously reported that primed T cells were present in WF rats that had been immunized with homogenized LEW liver for at least 1 year after the immunization, a finding that contrasts sharply with the LEW liver–bearing WF rats (nonrejector rat strain combination).[5] These results indicate that the continuous existence of liver parenchymal components produced by liver regeneration or the liver graft itself is essential for the elimination of primed T cells. Other investigators have shown that the presence of donor parenchymal tissue plays an important role in the induction of stable unresponsiveness.[11] They have suggested that the role of hepatic parenchyma is related to the continuous release of soluble antigens.

We previously have shown the presence of suppressive mechanisms for cytotoxic T cells in anti-CD4-induced rat heart tolerance by adoptive transfer experiments.[3] The same combination (WF→LEW) as that in the current study was used in the heart transplantation model, but no effector T cells were generated in the recipients even when a challenge test was performed in the tolerant animals with heart grafts. This finding was quite different from that in the liver transplantation model in the current study. We speculated that some other regulatory mechanisms are associated closely with CTL elimination in liver transplantation.

The indirect pathway probably remains permanently active because of the traffic of recipient APCs through the graft[19] and may be a prerequisite for the establishment of tolerance in the host.[20] As described previously, the maintenance of indirect recognition pathway by the persistent presence of donor hepatocytes appears to play some part in liver transplantation tolerance. The results of previous studies[5,14] and the current study indicate that, because the transplanted liver tissue itself does not possess the costimulatory capacity to stimulate naïve T cells, the immune response through the direct pathway disappears immediately after hepatic implantation, followed by an immune response through the indirect pathway. Gassel et al.[4] reported that when DA liver from primary LEW recipient was retransplanted into naïve LEW rat without immunosuppression, the retransplanted liver was

accepted without signs of chronic rejection. They surmised that mechanisms in the liver graft itself were involved in tolerance induction. Yoo-Ott et al.[12] reported that donor-derived hepatocytes protected cardiac allografts from acute and chronic rejection in rat. A chimeric liver probably has the ability to stimulate T cells via the indirect pathway.[10] Indeed, we observed in a LEW to WF rat strain combination that the long-term surviving grafted LEW liver from recipient WF rats had the ability to stimulate naïve CD4 T cells in vivo (data not shown).

In this study, we investigated the kinetic change of alloimmunogenicity of the liver graft in rejector rat strain combination (WF→LEW) and found that the professional APCs within the graft were replaced by those of recipient genotype by day 4. In kidney transplantation, by contrast, the depletion of donor passenger leukocytes from the graft was achieved by parking the graft for more than 14–50 days, depending on the strain combination.[21] The phenomenon of kidney graft was thus deemed to be a slow process.[22] Donor T cells within the liver also are replaced with those of the recipient in mice within 2–4 days after transplantation.[17] Interestingly, Kreisel et al.[13] showed that chimeric August Copenhagen Irish (ACI) livers created by recipient-type (LEW) or third-party (Brown Norway [BN]) passenger leukocytes were rejected at a significantly slower rate in rejector rat strain combination (ACI→LEW). The authors surmised that the MHC framework on the surface of passenger leukocytes plays an important role as a critical regulator of the immune response and that recipient T cells may receive a tolerogenic signal when they encounter donor allopeptide presented by non–donor-type APCs.

It would be of value to know which subsets of APCs within the graft were replaced. Hove et al.[23] recently reported that nonlymphoid cells (vascular endothelial cells, biliary epithelial cells, hepatocytes) of recipient origin were found within human liver allografts. The evidence of chimerism was apparent 3 months after transplantation, although it had not been observed 1 week after transplantation. APCs of recipient origin in the liver graft observed in the current study appeared to be highly immunogenic passenger leukocytes. We previously examined the alloimmunostimulatory capacity of the recipient WF spleen after LEW liver transplantation (LEW→WF), and we found that the alloimmunostimulatory ability of LEW cells in the WF spleen reached a peak 12 hours after grafting and rapidly disappeared thereafter.[14] That is, immunogenic donor passenger leukocytes within the graft migrated into the recipient spleen immediately after liver transplantation. Furthermore, we showed that the immunogenic passenger leukocytes of donor genotype were nylon–wool adherent cells. On in vitro proliferation assay, the allostimulatory ability of donor (LEW) passenger leukocytes is more potent than that of normal LEW spleen cells (stimulation indexes are 28 and 9, respectively) (unpublished data by Yamaguchi and Hashimoto, 2000). Although no direct evidence is available, the highly immunogenic APCs replaced by recipient genotypes are likely to be dendritic cells. It was reported that dendritic cells obtained from the liver showed potent allostimulatory ability in vitro.[24] Meanwhile, the vascular endothelium and biliary epithelium, including hepatocytes, may not be potent APCs because the grafted livers parked for several days in recipients had no immunostimulatory capacity in our study.

Lu et al.[25,26] have shown the correlation between donor liver–derived leukocytes, in particular populations of migratory dendritic cells, and apoptosis in activated allogeneic T cells. Our previous results indicated that a period of 2 months is required for primed T-cell elimination in rat liver transplantation.[5] Continuous regulatory mechanisms may be essential for this T-cell manipulation. As described previously, because donor APCs were almost eliminated in the periphery early after liver transplantation, recipient APCs that present donor-specific peptide may be involved in effector T-cell elimination. The professional and the high number of recipient APCs in the liver allograft may lead to have the capacity to modulate the CTL function.

In summary, we noted the rapid repopulation of functional graft APCs with recipient genotype after liver transplantation, and we surmised that this may be associated with manipulating host immune responses. In addition, we observed CTL elimination in the process of graft acceptance in the rejector rat strain combination. We concluded that the deletional mechanism of primed T cells caused by the continuous presence of parenchymal cells is critical for tolerance induction in liver transplantation. In clinical liver transplantation, if efforts are made to achieve the effector T-cell elimination, great benefit will be brought in the survival. However, it is also important to know that the liver graft itself participates intensely in immunologic unresponsiveness different from other organ transplantation.

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Clinical Transplantation

Risk factors for intraoperative portal vein thrombosis in pediatric living donor liver transplantation

Cheng YF, Chen CL, Huang TL, Chen TY, Chen YS, Takatsuki M, Wang CC, Chiu KW, Tsang LL, Sun PL, Jawan B. Risk factors for intraoperative portal vein thrombosis in pediatric living donor liver transplantation.
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Abstract: Pathologic changes of the recipient native portal venous system may cause thrombosis of the portal vein, especially in pediatric living donor liver transplantation (LDLT). This study assessed the utility of Doppler ultrasound (US) for the detection of intraoperative portal vein occlusion and identification of predisposing risk factors in the recipients. Seventy-three pediatric recipients who underwent LDLT at Chang Gung Memorial Hospital, Taiwan, from 1994 to 2002 were included. Preoperative and intraoperative Doppler US evaluation of the portal vein was performed. Age, body weight, native liver disease, type of graft, graft recipient weight ratio (GRWR), type of portal anastomosis, portal velocity, portal venous size and presence of portosystemic shunt were analyzed for statistical significance of predisposing risk factors. Eight episodes of intraoperative portal vein thrombosis, with typical findings of absent Doppler flow in portal vein and prominent hepatic artery with a resistant index lower than 0.5 ($p < 0.001$), were detected during transplantation, which was then corrected by thrombectomy and re-anastomosis. Children age ≤ 1 yr ($p = 0.025$), weight ≤ 10 kg ($p = 0.024$), low portal flow ≤ 7 cm/s ($p = 0.021$), portal venous size ≤ 4 mm ($p = 0.001$), and GRWR > 3 ($p < 0.017$) were all risk factors for intraoperative portal vein thrombosis. Doppler US is essential in the preoperative evaluation, early detection and monitoring of outcome of the portal vein in liver transplant.

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Key words: Doppler ultrasound – intra-operation – living donor liver transplantation – portal vein thrombosis – pediatric-risk factor

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Liver transplantation has become an important treatment option in the management of end-stage liver disease (1). The combination of recent improvements in operative technique, immunosuppression therapy, and organ utilization has contributed to better post-transplant outcomes (2). However, vascular complications are still significant causes of graft failure in liver transplantation, especially in pediatric cases. The incidence of portal vein thrombosis is not uncommon in pediatric transplant recipients ranging from 4 to 16% (3, 4). Children with pathological portal veins, most commonly seen in biliary atresia, remain a challenge to the surgeon although various technical skills has been employed for portal vein reconstruction to attain optimal flow (5, 6). Graft loss of up to 70% has been reported (7). In our experience, portal vein thrombosis occur early right after portal vein anastomosis during transplantation. The goal of this study was to identify characteristic Doppler pattern and the risk factors of perioperative portal vein thrombosis in children undergoing living donor liver transplantation (LDLT).

Methods and materials

Seventy-three pediatric patients underwent living LDLT in our center from 1994 to 2002, among whom one re-transplanted patient was excluded.

All grafts were harvested from healthy adult living donors who expressed a fully informed voluntary offer. The donors were 76 parents (19 fathers and 47 mothers), four grandparents (three grandmothers and one grandfather), and one aunt. The required volume for liver resection of the donors, which was 1–3% of the recipient's body weight, was calculated on preoperative computed tomography. Range of resection for donation was selected from among left lateral segment, extended lateral segment with a part of the medial segment, left lobe with middle hepatic vein.

Preoperative study of the vascular system were Doppler ultrasound (US) for portal vein to record flow direction, caliber size and velocity, and angiography, magnetic resonance venography or computer tomography angiography to document the presence of collateral circulation and portosystemic shunt.

Intraoperative Doppler US studies of the hepatic veins, portal vein and hepatic artery was performed sequentially after reperfusion of the portal vein and hepatic artery and immediately after abdominal closure. Re-examination was also performed after re-reconstructive management during operation. An Acuson 128 scanner (Acuson, Mountain View, CA, USA) with 7.0 or 4.0 MHz scanner in the imaging and Doppler mode was used in all recipients to measure the angle with corrected flow velocity and the cross-sectional area of the horizontal portion of the left portal vein. Operations for the donors and recipients were performed according to the principles we reported earlier (8). Two methods of portal vein reconstruction, branch patch and vein graft interposition were employed.

Analysis of risk factors for intraoperative portal vein thrombosis

Patients with and without intraoperative portal vein thrombosis were compared using the ninel clinico-pathological variables related to potential risk of occlusion, including six host-related factors: age, body weight, native liver disease, type of the graft, GRWR and type of portal anastomosis. Anatomical factors documented on imaging studies included for analysis were flow rate and caliber of the pre-transplant native portal vein, and presence of portosystemic shunt (>5 mm).

Results

There were 36 males and 37 females patients with age of 2.98 ± 3.08 yr (mean $\pm$ SD range, 0.5–17) and body weight of 12.44 ± 7.88 kg (mean $\pm$ SD range, 1–63). Pre-transplant diseases included biliary atresia (60 cases), glycogen storage disease (five cases), Wilson's disease (one case), Alagille syndrome (one case) and neonatal hepatitis (six cases). The 1-yr actuarial survival rate after LDLT of the 73 cases was 97.26%.

Condition of the native portal vein at preoperative survey

Portal vein blood flow was hepatopedal in 62 cases and hepatofugal in eight cases. Absent portal flow was noted in three cases. The caliber of the portal vein was 5.49 ± 2.12 mm (mean $\pm$ SD range, 0–16), and the velocity of the portal flow was 7.64 ± 6.38 cm/s (mean $\pm$ SD range, −12–19). Twenty-nine cases had prominent portal-systemic shunt (>5 mm).

Type of grafts and operation

The grafts transplanted included 40 left lateral segments, 27 extended left lateral segments, four left lobe with middle hepatic vein and two right liver lobes. The GRWR was 2.64 ± 0.93 (mean $\pm$ SD range, 1.27–5.12) for maintenance of adequate graft weight. Seventy-two cases had patch anastomosis for portal vein reconstruction, and one patient had ovarian vein graft due to total occlusion of the native portal vein before surgery.

Complications after portal vein reconstruction

The portal vein velocity was 24.46 ± 9.68 with range of 12–51 cm/s in non-complicated cases. Portal vein occlusion was noted in eight cases right after re-perfusion. Absent portal blood flow (velocity = 0), readily detectable strong pulsative hepatic artery, and increased velocity at the end diastolic phase were observed in all cases on intraoperative Doppler US. Marked decrease of resistance index below 0.5 (0.46 ± 0.02 with range of 0.45–0.49) was noted in all eight cases with portal vein thrombosis. All these values subjected to statistical evaluation showed significant differences between the group with intraoperative portal vein thrombosis (0.46 ± 0.02 with range of 0.45–0.49) and the uncomplicated group (0.78 ± 0.09 with range of 0.60–0.92) ($p < 0.001$).

All eight cases with intraoperative portal vein thrombosis had blood clot at both side of the anastomosis on subsequent re-operation for thrombectomy and reanastomosis of the portal vein. Closure of splenorenal shunt (n = 2) and prominent coronary vein (n = 4) was also per-

formed in six cases. No recurrent portal vein thrombosis was noted after liver transplantation among these eight cases.

One case had late portal vein occlusion at 6 months after transplantation due to intestinal perforation with repeated peritonitis. No other related risk factor was found in this patient. Among the eight cases with pre-transplant hepatofugal flow, intraoperative portal vein thrombosis was found in two patients. Among the three cases with preoperative absent portal flow, interposition graft with the ovarian vein was used in one patient, while patent portal vein was noted in the explanted native liver of the other two patients.

The size and velocity of the native portal vein, the clinical characteristics of the recipient's age, body weight, type of graft, native liver disease, and the graft weight and anastomotic method used, were subjected to statistical analysis to evaluate the risk factors for intraoperative portal vein occlusion. Age equal or younger than 1 yr ($p = 0.025$), body weight ≤10 kg ($p = 0.024$), portal flow ≤7 cm/s ($p = 0.021$), portal vein caliber ≤4 mm ($p = 0.001$), and graft recipient weight ratio (GRWR) >3 ($p < 0.017$) were found to be associated with higher risk for intraoperative portal vein occlusion (Tables 1 and 2).

Discussion

Technically satisfactory vascular anastomosis to allow adequate blood supply to the graft is essential for successful liver transplantation and long-term graft survival. Direct visualization of congestive liver with decreased blood pressure or palpation of a pulsatile vessel is suggestive of intraoperative hepatic vein or hepatic artery occlusion, respectively, but intraoperative portal vein thrombosis is clinically silent (9, 10). Our study demonstrate the critical role of intraoperative Doppler US in early detection of intraoperative portal vein thrombosis that allow early intervention to avoid prolonged warm ischemia time leading to suboptimal liver graft or even primary non-functioning graft.

Advance surgical techniques such as direct venous graft, jump graft, or even hemiportcaval anastomosis has been developed to manage occlusive portal vein anomalies but high morbidity and mortality rate is still noted due to the complexities of the procedures (11, 12). Thus attempts to identify risk factors for intraoperative portal vein thrombosis is imperative to provide better preoperative planning or intraoperative management to secure optimal graft survival.

Table 1. Host-factors related to intraoperative portal vein occlusion

	No. of cases	Mean ± SD	Intraoperative portal vein thrombosis (n)	No intraoperative portal vein thrombosis (n)	p-values Fisher's exact test)
Age (yr)					
≤ 1	19	0.79 ± 0.18	5	14	0.025
>1	54	3.7 ± 3.09	3	51	
Body weight (kg)					
≤ 10	35	7.95 ± 1.42	7	28	0.024
>10	38	16.57 ± 9.16	1	371	
Native liver disease					NS
Biliary atresia	60		6	54	
Glycogen storage disease	5		0	5	
Wilson disease	1		0	1	
Alagille syndrome	1		0	1	
Neonatal hepatitis	6		2	4	
Type of graft					NS
LLS	40		7	33	
ELLS	27		1	28	
LL	4		0	4	
RL	2		0	4	
GRWR					
≤ 3	48	2.07 ± .49	2	496	
>3	25	3.72 ± 0.55	6	19	0.017
Type of anastomosis					NS
Branch patch	72		8	64	
Venous graft	1		0	1	

LLS, left lateral segment; ELLS, extended left lateral segment; LL, left lobe; RL, right lobe; GRWR, graft recipient weight ratio.