Extravasated Platelet Aggregation in Liver Zone 3 Is Associated With Thrombocytopenia and Deterioration of Graft Function After Living-Donor Liver Transplant

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Extravasated Platelet Aggregation in Liver Zone 3 Is Associated With Thrombocytopenia and Deterioration of Graft Function After Living-Donor Liver Transplant

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Abstract

Objectives: **Continuous thrombocytopenia after liver transplant is associated with a less favorable prognosis, but this pathogenesis remains unclear. We focused on the consumption of platelets in the allograft. We assessed platelet consumption in allografts, and evaluated the pathology of platelet aggregation in an allograft tissue and its involvement in clinical outcomes.**

Materials and Methods: **We took biopsy specimens from 20 patients. To examine the localization of platelet aggregation, CD42b was assayed immunohistochemically, and its level of expression correlated with clinical data and outcomes.**

Results: **Platelet aggregation in zone 3 was 70%, compared with 30% in zone 1 and 50% in zone 2. Platelets were found mainly as extravasated platelet aggregates in local microenvironments. Patients were stratified according to the extent of extravasated platelet aggregates in zone 3 into extravasated platelet aggregate-negative and -positive groups. Graft weight/recipient body weight ratio with the extravasated platelet aggregate-positive group was significantly lower than that of the extravasated platelet aggregate-negative group. Platelet count after surgery was lower, while total bilirubin and prothrombin time/international normalized ratio were higher in the extravasated platelet aggregate-positive than they were in the extravasated platelet aggregate-negative group.**

Conclusions: **Extravasated platelet aggregates in the zone 3 of allograft tissue cause the consumption of platelets and continuous thrombocytopenia after transplant, and may be the clinical marker for deterioration of graft function. Platelet activation and degranulation following the release by platelets of some negative regulators may be involved partially in liver damage.**

Key words: *CD42b, Liver biopsy, Allograft tissue*

Introduction

There is increasing evidence that continuous thrombocytopenia soon after liver transplant (LT) is associated with a less favorable prognosis.[1,2] Few pathophysiological studies have assessed post-LT thrombocytopenia, because of difficulties in obtaining tissue samples.[3] Evaluation of allograft tissue from a living-donor LT (LDLT) recipient with thrombocytopenia plus sinusoidal obstruction syndrome (SOS) shows platelet aggregation in the space of Disse along the sinusoidal vessels and platelet phagocytosis by hepatocytes, a morphology called extravasated platelet aggregation (EPA).[4]

In our case, the progression of SOS resulted in graft dysfunction and the patient died. These findings indicate that platelets have the potential to aggregate and be consumed in allograft tissue after transplant. Moreover, platelet aggregation in the allograft may be partly involved in graft dysfunction. We immunohistochemically evaluated the presence and extent of platelet aggregation in allograft tissue obtained after transplant, using antibodies to the platelet marker

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CD42b (platelet glycoprotein Ib), and assessed the clinical effects of platelet aggregation on graft function.

Materials and Methods

Patients and biopsy material

Of the 45 consecutive patients who underwent LT at our institution from August 2005 to February 2014, we selected those who underwent liver biopsy within 60 days of LDLT (median, 28 d). Liver biopsies were obtained from only LDLT recipients. Pediatric recipients and recipients of deceased-donor liver transplants were excluded. Thus, a total of 20 patients were included.

After transplant, all patients received immunosuppressive therapy, consisting of tacrolimus, prednisolone, and mycophenolate mofetil. All LDLTs were performed after obtaining full informed consent from the patients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethical Review Committee of the Institute.

Evaluation of platelet aggregation in allograft tissues

The avidin-biotin peroxidase complex method was used for immunohistochemistry. The primary antibody was mouse monoclonal anti-CD42b antibody (1:100, EPR6995; Abcam, Tokyo, Japan). CD42b (platelet glycoprotein Ib) is a specific marker for platelets, so a CD42b-positive reaction was taken to indicate the presence of platelets. We previously reported that normal spleens were positive and normal livers negative for CD42b.[4] The distribution of CD42b-immunoreactivity in allograft tissues was categorized using the Rappaport classification.[5] The extent of CD42b-immunoreactivity in each zone was evaluated by light microscopy at ×200 magnification, and was categorized by 2 independent observers as − (no staining); + (< 33% of each zone); ++ (33% to 66%; and +++ (> 66%). The relations between platelet aggregation in allograft tissues and clinical data, including preoperative status, graft variables, operative data, and clinical outcomes, were evaluated.

Statistical analyses

Continuous values are presented as means ± standard deviation (SD), and are compared by the Mann-Whitney *U* test. Differences in frequency were analyzed by the chi-square test. All statistical analyses were performed using StatMate IV software (Release 8.0.1, SAS Institute Japan), with $P < .05$ considered statistically significant.

Results

Expression of CD42b in allograft tissues

The results are shown in Table 1. CD42b-immunoreactivity was observed in liver zone one of 6 of the 20 patients (30.0%), with the intensity of staining being grade one in all 6. CD42b-immunoreactivity was observed in zone two of 10 patients (50.0%), being grade one in 8 and grade two in 2. CD42b-immunoreactivity was present in zone three in 14 patients (70.0%), being grade one in 6, grade two in 5, and grade three in 3. These results indicated that platelet aggregation was more likely present in liver zone 3 than in zones 1 and 2. Representative results from one of the LDLT recipients with grade 3 CD42b intensity in zone 3 are shown in Figure 1. At low magnification (×100), CD42b-immunoreactivity was mainly observed in zone 3 (Figure 1A). Higher magnification (×200) showed that > 66% of zone 3 was immunopositive for CD42b (Figure 1B).

Table 1. Evaluation of Platelet Aggregation in Allograft Tissues

	CD42b-immunoreactivity				
	-	+	++	+++	Expression Rate
Zone 1	14	6	0	0	6/20 (30.0%)
Zone 2	10	8	2	0	10/20 (50.0%)
Zone 3	6	6	5	3	14/20 (70.0%)

Histologic localization of CD42b-immunoreactivity in local microenvironments

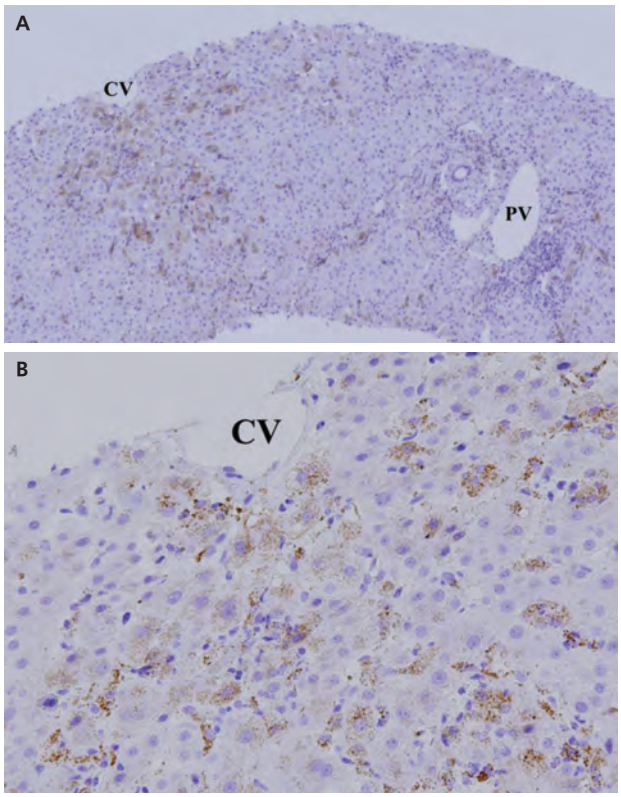
High magnification after incubation of tissue samples with antibody to CD42b showed that platelet aggregation could be classified into 2 patterns: aggregates attached to hepatocytes along the sinusoids (Figure 2A), and dots in hepatocyte cytoplasm (Figure 2B). These platelet aggregate morphologies are identical to those previously reported.[4]

Relation of platelet aggregation in zone 3 and clinical features during the perioperative period

To determine the associations between EPA in zone 3 and the clinical features of LDLT patients, patients were stratified according to the extent of CD42b-immunoreactivity. Patients with grade 0/1 staining were classified as the EPA-negative group, and those with grade 2/3 staining as the EPA-positive group.

The groups were comparable in preoperative recipient factors, including age, sex, underlying diseases, Model for End-Stage Liver Disease score, and Child-Pugh score; and in donor and graft characteristics including donor age, donor sex, percentage with ABO incompatibility, and graft type (Table 2). The graft volume/recipient standard liver volume ratio was lower in the EPA-positive than it was in the EPA-negative group, although the difference was not statistically significant. The graft weight/recipient body weight ratio, however, was significantly lower in the EPA-positive than it was in the EPA-negative group. There were no significant between-group differences in intraoperative factors including blood loss, operative time, or the duration of graft ischemia.

Figure 1. Immunohistochemical Staining of Liver Allograft Tissues With Antibody to CD42b



Abbreviations: CV, central vein; PV, portal vein

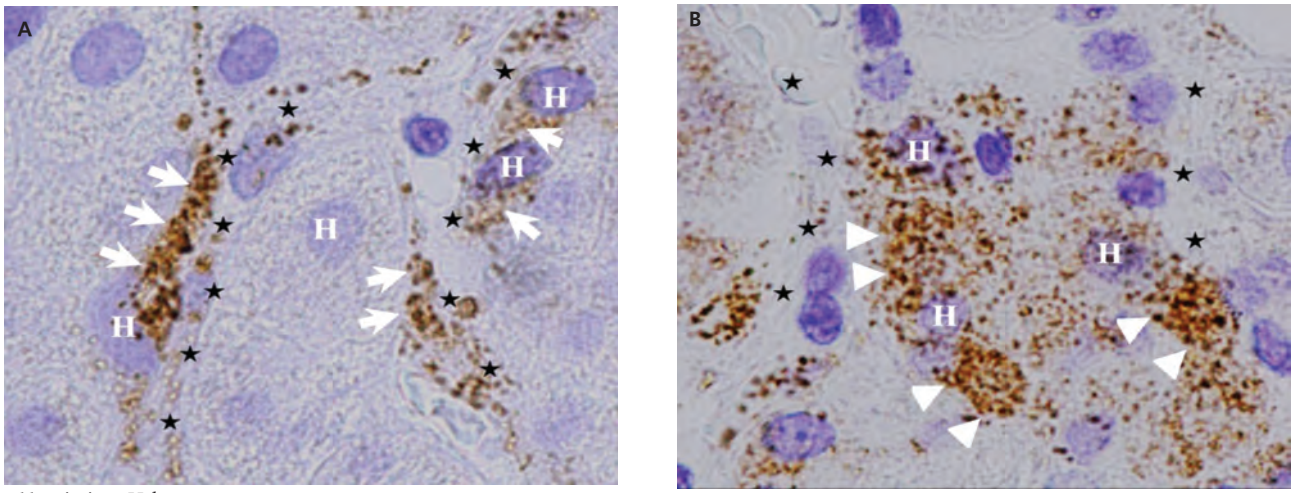
Representative results from one LDLT recipient with grade 3 CD42b expression in zone 3. At low magnification, CD42b was mainly observed in zone 3 (A, × 40). Moreover, CD42b expression constituted more than 66 % of zone 3 (B, × 200).

Table 2. Evaluation of Platelet Aggregation in Allograft Tissues

Clinical Parameters	EPA-Negative Group (n = 12)	EPA-Positive Group (n = 8)	*P* Value
Preoperative recipient factors			
Recipient age (years)	51.5 ± 9.7	53.8 ± 13.4	.21
Recipient sex (male/female)	7/5	7/1	.37
Indication			.47
Hepatitis (B/C/alcoholic/NASH)	9 (2/5/1/1)	5 (1/4/0/0)	
Cholestatic disease	2	3	
Other	1 (FAP)	0	
MELD score	10.3 ± 5.6	15.1 ± 6.9	.14
Child-Pugh score	8.4 ± 2.5	9.7 ± 2.6	.28
Donor and graft characteristics			
Donor age (years)	39.3 ± 14.0	43.8 ± 13.3	.46
Donor sex (male/female)	8/4	4/4	.64
ABO incompatible (n)	1	1	.76
Graft type (right/left/post)	10/2/0	4/3/1	.27
GRWR (%)	0.985 ± 0.21	0.776 ± 0.11	.04
GV/SLV (%)	50.8 ± 8.1	43.1 ± 9.4	.06
Intra-operative factors			
Blood loss (mL)	3984 ± 4728	4785 ± 4056	.41
Operative time (min)	995 ± 203	1028 ± 99	.33
Cold ischemic time (min)	76.4 ± 58.9	64.5 ± 55.2	.9
Warm ischemic time (min)	47.0 ± 19.6	68.7 ± 34.4	.11

Abbreviations: EPA, extravasated platelet aggregation; FAP, familial amyloid polyneuropathy; GRWR, graft weight/recipient body weight ratio; GV/SLV, graft volume/recipient standard liver volume ratio; MELD, model for end-stage liver disease; NASH, non-alcoholic steatohepatitis

Figure 2. Histologic Localization of CD42b-Immunoreactivity in Local Liver Microenvironments



Abbreviations: H, hepatocyte

At higher magnification, CD42b was present as aggregates attached to hepatocytes (arrows) along the sinusoid (★) (A, × 1000), and in hepatocyte cytoplasm (arrowheads) (B, × 1000).

Table 3. Perioperative Laboratory Data of the Living-Donor Liver Transplant Recipients

	Pre	POD 14	POD 28	POD 42	POD 56
Platelet ($\times 10^3/\mu L$)					
EPA-negative group	81.5 ± 54.7	160.5 ± 85.3	174.0 ± 72.2	176.0 ± 75.4	163.9 ± 81.5
EPA-positive group	61.3 ± 22.5	80.3 ± 30.6*	127.6 ± 82.2	109.8 ± 58.1	130.6 ± 56.6
Total bilirubin (mg/dL)					
EPA-negative group	4.51 ± 6.06	2.71 ± 2.47	2.44 ± 2.94	2.88 ± 3.46	2.16 ± 2.72
EPA-positive group	5.77 ± 5.80	6.81 ± 2.83**	7.05 ± 7.35*	8.02 ± 10.54	5.76 ± 8.01*
AST (IU/L)					
EPA-negative group	63.3 ± 40.2	49.5 ± 26.1	86.3 ± 81.9	62.4 ± 47.9	53.1 ± 29.5
EPA-positive group	53.6 ± 25.1	43.1 ± 34.4	77.8 ± 59.7	50.6 ± 49.0	38.0 ± 21.6
ALP (IU/L)					
EPA-negative group	475 ± 504	379 ± 193	575 ± 379	509 ± 228	468 ± 224
EPA-positive group	382 ± 80	383 ± 168	655 ± 422	616 ± 197	655 ± 251
PT-INR					
EPA-negative group	1.28 ± 0.14	1.07 ± 0.15	1.10 ± 0.19	1.12 ± 0.21	1.07 ± 0.16
EPA-positive group	1.44 ± 0.25	1.26 ± 0.20*	1.25 ± 0.25	1.28 ± 0.35	1.09 ± 0.13

Abbreviations: ALP, alkaline phosphatase; AST, aspartate transaminase; EPA, extravasated platelet aggregation; POD, postoperative day; PT-INR, prothrombin time-international normalized ratio

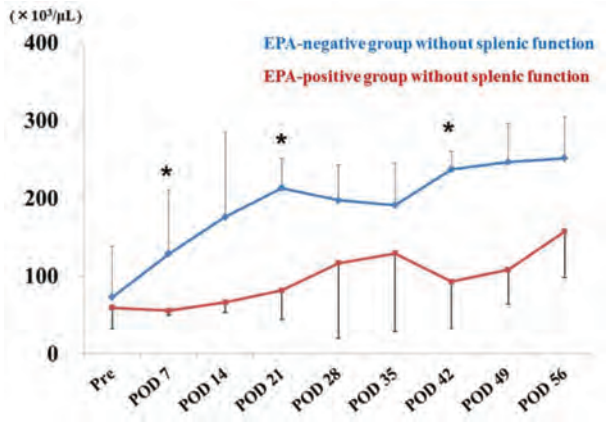
*$P < .05$.

**$P < .01$ compared with the EPA-negative group.

Laboratory data of the recipients after LDLT are shown in the Table 3. Platelet counts on postoperative day (POD) 14 were significantly lower in the EPA-positive than they were in the EPA-negative group. Platelets counts in the EPA-negative group were higher than $15 \times 10^4/mm^3$ on POD 14, and remained so until POD 56. In contrast, similar platelet counts were not observed in the EPA-positive group until POD 56. Total bilirubin was significantly higher on PODs 14, 28, and 56 in the EPA-positive group, as was prothrombin time/international normalized ratio on POD 14. There were no significant differences in aspartate aminotransferase and alkaline phosphatase concentrations in these 2 groups.

Inasmuch as platelet counts after LDLT are affected by splenic function, we compared postoperative platelet counts only in LDLT recipients without splenic function. Four patients in the EPA-negative group had undergone splenectomy, whereas 5 patients in the EPA-positive group had undergone splenectomy, and 1 had undergone preoperative partial splenic embolization. Platelet counts on PODs 7, 21, and 42 were significantly lower in the EPA-positive patients than they were in the EPA-negative patients without splenic function (Figure 3). Platelet counts in all 4 of these EPA-negative patients were higher than $20 \times 10^4/mm^3$ by POD 21. By contrast, platelet counts in these 6 EPA-positive patients were not over $20 \times 10^4/mm^3$ until POD 56.

Figure 3. Perioperative Platelet Counts of the Living Donor Liver Transplant Recipients Without Splenic Function



Platelet counts were significantly lower in EPA-positive than in EPA-negative patients without splenic function on PODs 7, 21 and 42.

*$P < .05$ compared with EPA-positive patients.

Postoperative complications and clinical outcomes

Table 4 shows the complications and clinical outcomes for patients in both groups. Five patients had intra-abdominal hemorrhage, requiring hemostatic therapy, either interventional radiology or laparotomy. Bacterial infections, including pneumonia (n = 1), cholangitis (n = 2), sepsis (n = 4), and fungal infections (n = 1) were diagnosed based on their clinical manifestations and the isolation of organisms. Ten patients experienced *cytomegalovirus* infection, diagnosed as the presence of *cytomegalovirus* antigenemia. Two patients experienced acute renal failure, requiring renal replacement therapy. Patients with biopsy-proven acute (n = 7) and chronic (n = 1) rejection were treated with augmented immunosuppressive therapy. There were no signi-ficant differences between the EPA-negative patients and the EPA-positive patients in the rates of each postoperative complication. In-hospital mortality and 1-year mortality rates were higher in the EPA-positive group than they were in the EPA-negative group, although these differences did not reach statistical significance. One patient in the EPA-negative group died within 12 months

of hospital release from hepatocellular carcinoma recurrence. In contrast, 4 patients in the EPA-positive group died within 12 months, 1 each from sepsis with overwhelming postsplenectomy infection, graft dysfunction caused by hepatic artery thrombosis, small-for-size graft syndrome, and chronic rejection with SOS.

Table 4. Postoperative Complications and Clinical Outcomes

	EPA-Negative Group (n = 12)	EPA-Positive Group (n = 8)	*P* Value
Intra-abdominal hemorrhage	3	2	.59
HAT	0	1	.83
Bile leakage	1	2	.70
Biliary stricture	1	1	.64
Perforation of small intestine	0	1	.83
Gastrointestinal bleeding	1	1	.64
Pneumonia	0	1	.83
Cholangitis	1	1	.64
Sepsis	1	3	.30
Fungal infection	0	1	.83
CMV infection	6	4	.64
Acute renal failure	0	2	.28
Acute rejection	4	3	.77
Chronic rejection	0	1	.83
In-hospital mortality	0	3	.09
1-year mortality	1	4	.11

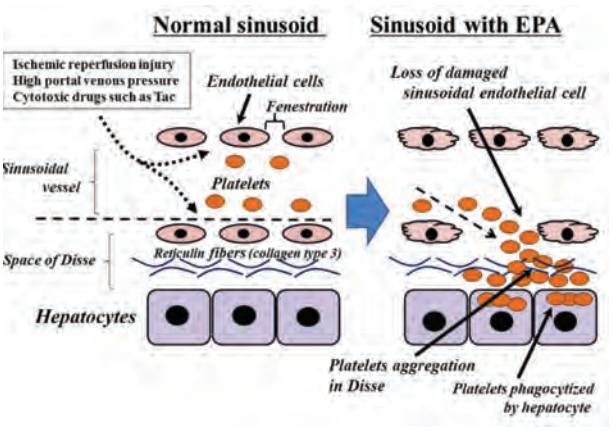
Abbreviations: CMV, *cytomegalovirus*; EPA, extravasated platelet aggregation; HAT, hepatic artery thrombosis

Discussion

Few clinical studies to date have assessed the pathogenesis of continuous thrombocytopenia after LDLT. Platelet consumption because of splenomegaly and hypersplenism was caused by elevated portal venous pressure in a small sized graft,[6] and prolonged thrombocytopenia after LDLT was associated with a decrease in ADAMTS13 (a disintegrinlike and metalloproteinase with thrombospondin type-1 motifs 13), which cleaves multimers of von Willebrand factor into smaller sizes and prevents platelet aggregation and/or thrombus formation.[2] Pathophysiological examination has shown a relation between platelet aggregation in allograft tissue obtained soon after reperfusion and clinical features.[7,8] However, the regional specificity of platelet aggregation in allografts has not been fully assessed. This study showed that platelet aggregation in allografts was mainly present in zone 3 as EPA. Platelet counts were lower in the EPA-positive patients than they were in EPA-negative patients, with similar platelet counts over time in patients with and without splenic function. These findings suggest that the consumption of platelets in allografts, especially in zone 3, can reduce platelet counts after transplant.

We have hypothesized that the pathogenesis of EPA in allograft tissue involves several steps (Figure 4).[4] Damage to sinusoidal endothelial cells may be caused by ischemic reperfusion injury,[9] the shear stress of high portal venous pressure,[10] and/or cytotoxic drugs such as tacrolimus,[11] resulting in the denuding of the sinusoidal endothelium or loss of fenestrations, and allowing platelets to enter the space of Disse. This space contains reticulin fibers, most of which contain collagen type three.[12] Platelets have been found to bind to, and form aggregates, with collagen type three,[13] resulting in platelet aggregation in the space of Disse. Subsequently, platelets may bind to hepatocytes through asialoglycoprotein receptor, resulting in phagocytosis.[14,15] We found that graft weight/recipient body weight ratio was significantly lower in the EPA-positive group than it was in the EPA-negative group. Graft weight/recipient body weight ratio is related to portal venous pressure, with recipients of small-sized grafts having significantly higher portal venous pressure recipients of larger grafts.[16] Living-donor liver transplant patients implanted with small-sized grafts experience transient portal hypertension, accompanied by sinusoidal endothelial cell damage because of the shear stress of portal hypertension.[10] Thus, sinusoidal endothelial cells may be more damaged in EPA-positive than in EPA-negative patients.

Figure 4. Schematic Model of the Pathogenic Mechanism of EPA-Induced Sinusoid Damage



The normal sinusoid consists of a blood vessel with endothelial fenestrations. Sinusoidal endothelial cell damage because of ischemic reperfusion injury, shear stress of high portal venous pressure, and cytotoxic drugs (eg, tacrolimus) may result in the denuding of the sinusoidal endothelium or loss of fenestrations, allowing platelets to enter the space of Disse. This space contains reticulin fibers, which consist primarily of collagen type 3. Platelets can easily attach to collagen type 3, forming aggregates. Additionally, the extravasated platelets in the space of Disse can be phagocytized by hepatocytes through asialoglycoprotein receptor.

Interestingly, EPA appeared mainly in zone 3. Generally, zone 3 hepatocytes contain lower levels of glutathione than those in other zones.[17] Glutathione mediates the detoxification of drugs, their metabolites, and reactive oxygen species.[18,19] Sinusoidal endothelial cells in LDLT recipients are exposed to the cytotoxic agent tacrolimus,[11] as well as to reactive oxygen species induced by liver ischemia/reperfusion injury.[20] The relative deficiency of glutathione in zone 3 hepatocytes might result in greater damage to sinusoidal endothelial cells in this zone.[21] Additionally, the shear stress of portal hypertension because of small-sized grafts likely exacerbated sinusoidal endothelial cell damage in EPA-positive patients, resulting in a greater likelihood of EPA in zone 3. Sinusoidal obstruction syndrome, previously called *veno-occlusive disease*, is a liver disease characterized by damage to liver tissue in zone 3 and is clinically diagnosed by the triad of jaundice, painful hepatomegaly, and ascites/weight gain.[22] Sinusoidal obstruction syndrome is confirmed histologically, including by fibrous obliteration of small hepatic veins by connective tissue and centrilobular hemorrhagic necrosis.[22] Previously, we reported that an allograft parenchyma with EPA in zone 3 progressed from central perivenulitis to centrilobular fibrosis with a finding of SOS.[4] Extravasated platelet aggregation in zone 3 soon after transplant may be a sign of SOS.

Postoperative laboratory data showed that total bilirubin and prothrombin time/international normalized ratio were higher in the EPA-positive group than they were in the EPA-negative group. These findings suggested a greater degree of deterioration of graft function, including bile secretion and hepatic synthetic activity in the EPA-positive group. In addition, clinical outcomes, including 1-year mortality rate, tended to be poorer in the EPA-positive group. Similarly, 1-year survival rates were found to be significantly lower in LDLT recipients with platelet counts less than $100 \times 10^3/\mu L$ on POD 14, similar to our EPA-positive group, than in patients with higher platelet counts not (58.3% vs 90.3%).[2] Increased platelet aggregation in allograft tissue obtained before and 2 hours after reperfusion of deceased-donor liver transplants was associated with significantly higher serum levels of aspartate aminotransferase and lactate, and longer time in the intensive care unit after transplant.[8] These results indicate that platelet aggregation or platelet consumption in the allograft may partially contribute to the deterioration in graft function.

Platelet activation and degranulation of EPA, followed by the release from platelets of negative regulators, may be partially involved in liver damage.[4] In particular, plasminogen activator inhibitor-1, which is abundant in platelets, suppresses fibrinolysis, and the progression to fibrosis in the tissue microenvironment. Additionally, plasminogen activator inhibitor-1 acts as a negative regulator of hepatocyte proliferation by inhibiting urokinase-type plasminogen activator, which activates hepatocyte growth factor.[23,24] Further experimental investigation is necessary to confirm whether negative regulators released by activated platelets contribute to liver damage.

In conclusion, EPA in zone 3 of LDLT allograft tissue causes platelet consumption and continuous thrombocytopenia after transplant, and may be a clinical marker for deterioration in graft function and allograft parenchyma injury with SOS. Measures to reduce damage to the sinusoidal endothelium and platelet aggregation may prevent the occurrence of EPA. Portal venous pressure control because of prophylactic splenic artery modulation, including splenic arterial ligation or splenectomy for small-size graft,[25,26] or ischemic preconditioning,[8,27] may be effective for endothelial protection. Moreover, prophylactic administration of endothelial protective and antiplatelet agents prior to the development of irreversible damage may be effective. The phosphodiesterase 3 inhibitors cilostazol and milrinone, may be appropriate, owing to their antiplatelet properties, their ability to increase tolerance to ischemia/reperfusion injury,[28] and their induction of immune tolerance by enhancing regulatory T-cell responses.[29] In rats, phosphodiesterase 3 inhibitors have been found to protect against SOS,[30] and to attenuate graft injury in an orthotopic LT model.[31] In clinical practice, we have locally infused a phosphodiesterase 3 inhibitor into LDLT recipients and patients with small remnant liver volume after hepatectomy such as trisegmentectomy, with favorable outcomes (unpublished data).

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