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Impact of post-transplant flow cytometric panel-reactive antibodies on late-onset hepatic venous outflow obstruction following pediatric living donor liver transplantation

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Summary

The development of late-onset hepatic venous outflow obstruction (LOHVOO) following pediatric living donor liver transplantation (LDLT) can lead to uncontrollable fibrotic damage in liver grafts, even long-term patency of the graft outflow is achieved with appropriate therapeutic modalities. The aim of this study was to verify our hypothesis that some immunological responses, particularly cellular and/or antibody-mediated rejection (AMR), are associated with LOHVOO, which occurs following damage to liver sinusoidal endothelial cells in zone 3 of liver grafts. One hundred and eighty-nine patients underwent LDLT between May 2001 and December 2010 at our institute. Nine patients (4.8%) were identified as having LOHVOO. The preoperative factors, operative factors, and mortality, morbidity, and survival rates were examined and compared between the groups with and without LOHVOO. No statistical differences were observed between the groups with regard to preoperative factors, technical factors, or postoperative complications. However, FlowPRA reactivity was found to be a statistically significant risk factor for LOHVOO ($P = 0.006$). The patients with both class I- and class II-reactive antibodies also had a significant risk of developing LOHVOO ($P = 0.03$) and exhibited significantly higher retransplant rates. In conclusion, although further studies are needed to clarify this phenomenon, the pathophysiological mechanism underlying the development of LOHVOO after LDLT may be explained by immune-mediated responses that facilitate damage in zone 3 of liver grafts.

Introduction

Surgical refinement of technical-variant liver allografts in pediatric liver transplantation (LT), including reduced-size, split, and living donor liver transplantation (LDLT), has been used to overcome the shortage of donor organs and size discrepancies between recipients and donors [1]. However, the incidence of vascular complications following pediatric LT must be addressed with a view to decreasing postoperative morbidity [1–4]. Hepatic venous outflow obstruction (HVOO) is a rare vascular complication of LT, with an incidence of only 0.8% in all cases [1,5]; however, it can result in graft loss without appropriate management [2,6,7]. Hepatic vein reconstruction is one of the most crucial factors, and the creation of a wide outflow orifice is an important factor for avoiding the development of HVOO. The causes of early-onset HVOO are often related to technical issues, while late-onset HVOO (LOHVOO) is caused by subsequent fibrosis associated with inflammatory processes, such as bile leakage, abscess formation and compression, or twisting of the site of anastomosis due to graft growth [2,6,8,9]. LOHVOO can cause insidious hepatic deterioration, and achieving its complete correction is sometimes very difficult because irreversible fibrotic changes can occur around the anastomotic site [2,6,9]. Interventional radiological treatment (IVRT) [3,5,7,10,11] has been reported to be an effective therapeutic option; however, some patients experience recurrence with the pro-

gression of fibrosis in zone 3 area of liver graft, requiring the administration of multiple rounds of IVRT [11] and placement of an expandable metallic stent (EMS) [10]. Such patients should be considered for retransplantation [12] if they continue to exhibit uncontrollable fibrotic damage in their allografts.

Veno-occlusive disease (VOD)/sinusoidal obstruction syndrome (SOS) is a rare cause of liver graft dysfunction that involves the fibrous obliteration of small hepatic veins by connective tissues [13,14]. The cause of VOD/SOS remains unknown; however, previous reports have shown that the disease is strongly associated with a particular form of acute rejection (AR) with prominent endothelial involvement that occurs via endothelialitis-induced damage to the centrilobular wall in the zone 3 area [15]. The advanced stage of VOD/SOS can result in the same conditions as LOHVOO, ultimately inducing progressive liver failure without appropriate therapy. Based on these pathophysiological findings, we hypothesize that various immunological responses, particularly antibody-mediated rejection (AMR), are associated with LOHVOO.

Antibody-mediated rejection occurs as the result of antibody deposition on the graft endothelium with subsequent complement activation and is triggered by antibody-mediated immunity, including the production of anti-human leukocyte antigen (HLA) antibodies by plasma cells in sensitized patients [16]. With regard to renal transplantation, HLA-specific alloantibodies found in post-transplant patients have been shown to be strongly associated with a poor graft function and graft loss; however, little evidence for these findings in LT patients is available [17]. The flow cytometric panel-reactive antibody assay (FlowPRA) is a screening test used to assess the presence or absence of anti-HLA antibodies against class I and class II HLA antigens using beads coated on the surface of HLA antigens [18,19]. We conducted a retrospective analysis to evaluate antibody-mediated immunocompatibility in patients with LOHVOO. The aim of this study was to assess the incidence and outcomes of LOHVOO in our series in order to determine the impact of the FlowPRA screening test in the management of LOHVOO.

Patients and methods

One hundred and eighty-nine children underwent primary LDLT at Jichi Medical University Hospital (JMUH) between May 2001 and December 2010. The records of these patients were retrospectively reviewed for patient demographics, including age, gender, primary liver disease, type of graft, and graft blood-type combination. The median follow-up period was 8.2 years (range: 67 days–10.2 years). The age at LDLT ranged from 13 days to 16.6 years (median: 1.5 years). The recipient body weight at LDLT ranged from 2.6 to 59.0 kg (median: 9.9 kg). The original diseases included cholestatic liver diseases (n = 154), metabolic liver diseases (n = 17), fulminant hepatic failure (n = 8), liver cirrhosis (n = 4), congenital absence of the portal vein (n = 4), primary sclerosing cholangitis (n = 2), and hepatic malignancy (n = 1). In each case, the recipient operation was performed in a piggyback fashion without the use of venovenous bypass, as described elsewhere [20]. The anastomotic orifice was prepared according to the number of graft hepatic veins, the shape of the graft, and the anatomy of the recipient IVC. The standard types of recipient orifices on the IVC are shown in Fig. 1a: (i) a new wide orifice on the recipient IVC connecting all three hepatic veins; (ii) one orifice connecting the left and middle hepatic veins via an incision in the IVC on the lower caudal side; and (iii) other types. The diameter of the new orifice

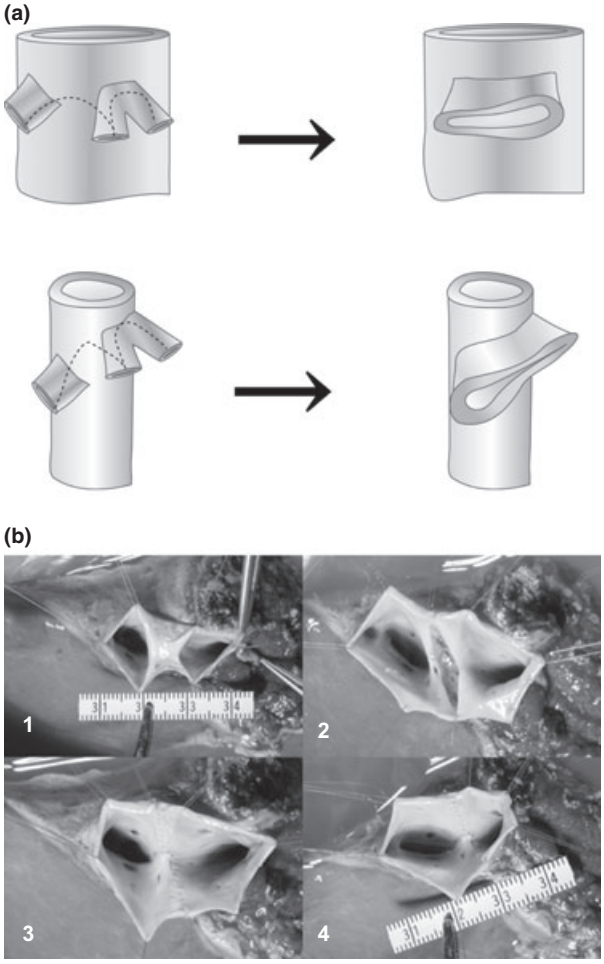

Figure 1 (a) A new wide orifice on the recipient inferior vena cava connecting all three hepatic veins (type A). (b) Left lobe graft outflow venoplasty. One septovenoplasty between the left and middle hepatic veins was required to ensure an adequately wide outflow orifice.

was adjusted to that of the graft hepatic vein using an incision in the IVC wall or sutures from the left corner of the hepatic vein as needed. The graft hepatic veins were made into one orifice using graft outflow venoplasty with a back-table procedure if the distance between the separated hepatic veins was short [21] (Fig. 1b). The graft hepatic veins were anastomosed separately if they were located far apart. Anastomosis was performed in an end-to-side fashion with running sutures of 5-0 or 6-0 polypropylene monofilament. No conduits or patch grafts were used for hepatic vein reconstruction. The patients received a left lateral segment (LLS; n = 153, 80.5%), left lobe (LL; n = 30, 15.8%), or r-LLS that consisted of monosegments and reduced monosegments (n = 7, 3.7%). The graft-to-recipient weight ratio (GRWR) ranged from 0.71% to 4.32%, with a mean of 2.43%. The hepatic venous flow after LDLT was assessed using Doppler ultrasound every day for the first week, once to twice a week during the rest of the period of hospitalization and at least once every 3 months after discharge. We defined LOHVOO as HVOO occurring more than 1 month post-transplant. The diagnosis of HVOO was made based on the presence of intractable ascites, an abnormal hepatic venous flow pattern, histological findings suggestive of HVOO, or liver dysfunction. Doppler ultrasound findings suggestive of HVOO included the disappearance of the pulsatile hepatic venous flow and flattening of the hepatic venous waves. The computed tomography findings included a narrow segment of the venous outflow (Fig. 2a). Liver biopsy findings suggestive of HVOO included congestion, hemorrhage, necrosis, and fibrosis around the central veins (Fig. 2c). Common abnormal laboratory findings included hypo-albuminemia and hyperbilirubinemia. Hepatic vein venograms were obtained when a diagnosis of HVOO was suspected (Fig. 2b). If patients with a pressure gradient across the area of stenosis of more than 3 mmHg were considered to have significant HVOO requiring treatment, interventional balloon venoplasty was initiated. Patients with HVOO were followed as outpatients, and their clinical manifestations, laboratory data, and Doppler ultrasound findings were closely observed every 1–2 months with the administration of anticoagulation therapy. Hepatic vein venography was repeated if recurrence of HVOO was suspected. When recurrence was confirmed, balloon venoplasty was additionally performed.

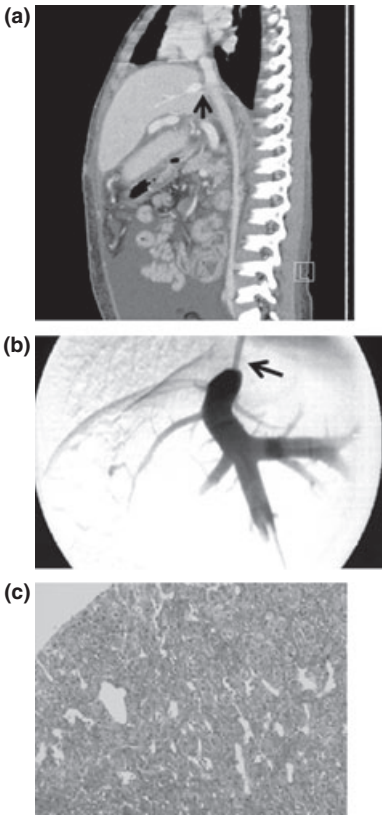

Figure 2 (a) Sagittal view of contrast-enhanced computed tomography of the hepatic venous outflow obstruction (arrow). (b) Hepatic venogram showing stenosis of the venous outflow (arrow). (c) Well-developed perivenular and sinusoidal fibrosis in an allograft biopsy (Azan-Mallory stain).

Determination of anti-HLA antibodies (FlowPRA Screening)

All patients were tested with flow cytometric panel-reactive antibody assays, as described below. Fresh serum samples were used in this study for patients who underwent LDLT within the past 5 years. Frozen serum samples, which were kept in a refrigerator until thawing for the analysis, were used in patients who had undergone LDLT more than 5 years previously. Beads coated with the HLA antigens were added to the patients' serum using the FlowPRA Screening Kit (One Lambda, Inc., Canoga Park, CA, USA) and left at room temperature for 30 min. Fluorescein isothiocyanate-labeled anti-human IgG antibodies (BD Biosciences Pharmigen, San Diego, CA, USA) were then added as a secondary antibody, and the reaction mixture was allowed to stand for another 30 min. After washing the samples twice, determination was started using the fluorescence-activated cell sorter (FACS). The reaction was considered to be positive when 10% or more of the beads were stained in comparison with that observed in the negative control, and multimodal staining was detected.

Data analysis

All data are presented as the medians or means and standard deviations (SDs). The statistical analyses were performed using Student's t-test, the chi-square test, Fisher's

exact test, a Kaplan–Meier analysis, or the log-rank test as appropriate, and differences at $P < 0.05$ were considered to be significant.

Results

Late-onset hepatic venous outflow obstruction occurred in nine cases (4.8% (9/189); five females and four males). The perioperative factors, postoperative complications, and outcomes of the cases were evaluated. There were no significant differences between the groups in age, gender, body weight, total bilirubin, albumin, prothrombin international normalized ratio (INR), serum creatinine, pediatric model for end-stage liver disease (PELD) score, blood compatibility (incompatible versus identical and compatible), or graft type (reduced left lateral segmental graft versus others). Surgical factors included the operative time, amount of blood loss and transfusion per weight, cold and warm ischemia time, weight of the native liver and graft, percentage of real graft volume/standard liver volume (%SLV), graft-to-recipient body weight ratio (GRWR), and recipient-to-donor body weight ratio (RDWR). The combination of the recipient orifice on the IVC and graft HV was not significantly different from those observed in the patients without LOHVOO (Table 1). In addition, there were no significant differences in the incidence of other vascular complications, such as hepatic arterial and portal venous thrombosis, or biliary complications, such as bile leakage and bile duct stenosis ($P > 0.05$). Other factors of the postoperative status, such as acute rejection, overall relaparotomy rate, and duration of hospital stay did not differ significantly between the two groups, with the exception of the retransplant rate, which was significantly higher in the group with LOHVOO ($P < 0.001$) (Table 2).

Of all patients in this study with available serum for FlowPRA screening tests, twenty were positive for class I antibodies only, two were positive for class II antibodies only, and six were positive for both class I and class II antibodies. The remaining patients were negative for both class I and class II antibodies. Of the nine LOHVOO patients, four were positive for class I antibodies only, none were positive for class II antibodies only, and three were positive for both class I and class II antibodies. The remaining two patients were negative for both class I and II antibodies. FlowPRA reactivity was found to be a statistically significant risk factor for LOHVOO ($P < 0.001$) (Table 2). The patients with both class I- and class II-reactive antibodies also had a significant risk of developing LOHVOO ($P < 0.001$). A multivariate analysis showed that positive FlowPRA findings and a double-positive status for class I- and class II-reactive antibodies were also risk factors for LOHVOO. Seven of the nine LOHVOO patients successfully underwent balloon venoplasty following hepatic vein venography. Four patients (44.4%) developed recurrence after undergoing initial balloon venoplasty. There was no difference concerning the immunosuppressive drug combinations between the groups. All patients with LOHVOO were compliant with drug treatment. At the time of LOHVOO, the CNI level with respect to the post-LT period was within the target range in all patients. Seven patients

Table 1. The type of hepatic vein reconstruction in recipients with and without LOHVOO.

	LOHVOO (n = 9)	No LOHVOO (n = 180)	P value
Type of recipient hepatic venous orifice			
(A) One orifice; all three HVs	8	136	0.421
(B) One orifice; LHV+MHV	0	18	
(C) Others	1	26	
Type of graft hepatic vein			
(a) Without venoplasty	1	25	0.795
(b) With venoplasty	8	155	
Combination of the recipient orifice			
(A) & (a)	0	14	0.557
(A) & (b)	8	122	
(B) & (a)	0	7	
(B) & (b)	0	11	
(C) & (a)	1	12	
(C) & (b)	0	14	

LOHVOO, late-onset hepatic venous outflow obstruction.
Parametric variables are expressed as the mean ± SD.

Table 2. The postoperative characteristics of recipients with and without LOHVOO

	LOHVOO (n = 9)	No LOHVOO (n = 180)	P value
Vascular complications			
Hepatic arterial thrombosis	2	13	0.320
Portal vein thrombosis/stenosis	2	27	0.557
Biliary complications			
Bile leakage	0	6	0.578
Bile duct stenosis	0	35	0.143
Relaparotomy	1	15	0.770
Retransplantation	2	5	<0.001*
Acute cellular rejection	5	65	0.409
Duration of hospital stay (day)	59.5 ± 38.8	54.1 ± 27.4	0.253
FlowPRA reactivity			
FlowPRA(+/−)	7	21	<0.001*
Class I (+)/Class II (−)	4	16	<0.001*
Class I (−)/Class II (+)	0	2	0.180
Class I (+)/Class II (+)	3	3	<0.001*
Class I (−)/Class II (−)	2	159	<0.001*

LOHVOO, late-onset hepatic venous outflow obstruction.
Parametric variables are expressed as the mean ± SD.
*P value <0.05 was considered to be statistically significant.

required other therapeutic options (Fig. 3): two patients underwent multiple sessions (more than five times) of balloon venoplasty, one patient underwent stent placement, and two patients underwent retransplantation. All of these patients had positive FlowPRA findings and a double-positive status for class I- and class II-reactive antibodies. One of the patients remained alive without recurrence after undergoing multiple sessions of balloon venoplasty with moderate perivenular fibrosis. One of the patients developed restenosis inside the stent and required living donor retransplantation. One patient experienced prolonged LOHVOO with progressive graft failure despite the use of all therapeutic options and finally underwent living donor retransplantation. One patient required multiple sessions of balloon venoplasty (more than 10 times) and was considered a candidate for stent placement. In two patients, LOHVOO was successfully controlled with initial cellular and/or AMR therapy without the need for interventional balloon venoplasty.

There were no significant differences in the patient survival rate between the two groups: 94.5% in the group with LOHVOO and 93.3% in the group without LOHVOO ($P = 0.442$).

Discussion

Vascular complications in LT patients include major morbidities; however, overall venous outflow complications are relatively uncommon. Stenosis of the inferior vena cava (IVC) is reported to occur in less than 2% of cases [22]. HVOO is slightly more common; however, the incidence varies with the type of transplant. In a review of 600 pediatric LTs, the rate of the HVOO was 1% in patients receiving whole liver grafts and 2% in patients undergoing LDLT, while a rate of 4% was observed in patients with reduced-size or split grafts [1]. In one review of 264 piggyback transplants, only two cases (0.8%) of late-onset HVOO were detected [1,5]. On the other hand, HVOO is more likely to occur after LDLT because considerable graft growth is seen after implantation, and the grafts have a tendency to tilt into the large abdominal cavity, which may lead to functional stenosis via twisting in the outflow tract [2,6]. Additionally, this type of anastomotic obstruction is more likely to be secondary to perivenular fibrosis or intimal hyperplasia [3,6]. One report noted that HVOO is predominantly present in patients of younger ages and lower weighs among pediatric cases [2]. In contrast, another article [6] reported that factors related to donor–recipient size mismatching, the RDWR (<0.1%), and the use of technical-variant small grafts (reduced left lateral grafts) are significant risk factors. However, the present study did not show any significant increases in the risks associated with these factors. The size of the anastomotic orifice, orientation of the vessels, and position of the graft are important determinants in maintaining the patency of reconstructed hepatic veins. However, a good outflow is not guaranteed postoperatively because the graft position changes during accommodation of the graft in the abdominal cavity [2]. Balloon angioplasty is the first choice treatment for LOHVOO, and the long-term efficacy of this procedure has been

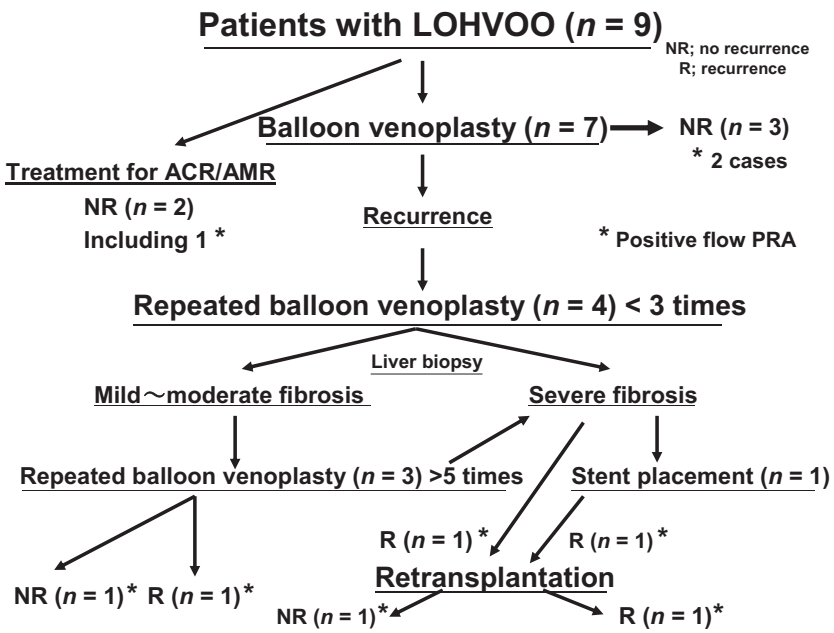

Figure 3 Clinical outcomes of the recipients with late-onset hepatic venous outflow obstruction (LOHVOO). *indicates a positive FlowPRA case.

previously reported [3,5,7,10,11]. However, some patients experience recurrence requiring multiple rounds of IVRT and should be considered for metallic expandable stent placement, as 20–45% of patients with LOHVOO develop recurrent HVOO after undergoing initial IVRT [6,10,11]. Following stent implantation, some patients exhibit the complete reversal of fibrosis in the zone 3 area, while others do not achieve histological recovery from fibrotic changes, even if they demonstrate long-term patency of the hepatic veins hemodynamically after EMS placement. Although various technical innovations have been introduced in the field of pediatric LDLT, the incidences of HVOO and recurrent HVOO after intervention have not been sufficiently reduced [4,6,8,9]. The incidence of HVOO following pediatric retransplantation has been reported to be 15%, although, in such cases, the patient has theoretically already recovered to widen the open stricture portion [12]. Regarding different approaches to solving these issues, we would like to discuss the pathophysiological aspects of HVOO in order to describe the existence of an immune-mediated phenomenon.

The principal target for acute rejection is presumably the major histocompatibility complex (MHC) antigen system, as is the case with other types of organ transplants, although to date, there has been no confirmation of a consistent correlation between acute rejection and the degree of HLA matching in human liver transplants. One reason for this finding is that the expression of MHC antigens by hepatocytes is limited to low levels of MHC class I and no class II. Biliary epithelial cells, which constitutively make up 3% of the nonparenchymal cells of the liver, express MHC class I, but not class II, antigens. In contrast, liver sinusoidal endothelial cells (LSECs), which comprise 60% of the nonparenchymal cells of the liver, express both HLA class I and class II antigens [17]. As hepatocytes are surrounded by LSECs on the vascular side, it may be that LSECs protect hepatocytes from immune attack via a combination of barrier and tolerogenic effects [23]. The presence of any antibodies that attack LSECs facilitates the acute cellular and/or AMR of LTs by downregulating the expression of cytokine transforming growth factor and upregulating the expressions of all reactive T cells [24]. In patients with cellular rejection, most inflammatory responses are localized to the portal tracts and perivenular areas, which are rich in HLA class II antigens [25]. The sinusoidal endothelium is generally not a primary target of the cellular immune response; rather, LSECs are associated with resistance of the liver to cellular-mediated rejection. In patients with AMR, on the other hand, target cells with alloantigens are directly recognized by alloantibodies, and the anatomic pattern of liver injury is expected to parallel the distribution of antigens. ABO blood-type antigens are relatively restricted to portal areas, whereas HLA class I antigens are expressed on all endothelial cells [26], and antibodies against sinusoidal endothelial cells are associated with rejection episodes [24].

The other important point is the significance of central perivenulitis (CP) and VOD/SOS in LT patients. First, CP encompasses dropout of zone 3 hepatocytes, red blood cell extravasation, and perivenular mononuclear inflammation. In a previous report [27] of pediatric LT patients, CP associated with zone 3-based fibrosis was detected in 27% of all allograft biopsies and 66% of allografts overall. The authors were unable to conclude that CP leads to ductopenic chronic rejection and advanced fibrosis; however, affected patients may have a risk of potential graft loss if they lack appropriate baseline immunosuppression. Second, VOD/SOS is a rare cause of liver graft dysfunction with fibrous obliteration of small hepatic veins by connective tissues [13,14]. The cause of VOD/SOS in LT patients remains unknown; however, previous reports have shown that the disease is strongly associated with a particular form of AR with prominent LSEC involvement via endothelialitis-induced damage to the centrilobular wall of the zone 3 area [15]. Following damage to LSECs, histological changes occur, such as narrowing of the sublobular and central veins due to subendothelial edema and congestion of the hepatic sinusoids caused by pale necrotic hepatocytes. Fragmented red blood cells and other coagulation factors are deposited and clotted in the subendothelial space of the central veins and perivenular zones. Finally, the sinusoidal and venous lumens are obliterated by type I, III, and IV collagen accompanied by increases in the number of hepatic stellate cells (HSCs) that line the sinusoids and fibrous bridges between the central venules [13,14]. It is reasonable that graft fibrosis is caused by an imbalance in the homeostasis of the extracellular matrix in the liver resulting from increases in collagen production, reductions in extracellular matrix degradation, or both. Activated HSCs are currently considered to be the major collagen-producing cells in injured livers and acquire a myofibroblast phenotype that can be recognized by the cell expression of alpha-smooth muscle actin [28]. Although multiple factors regulate HSC activation, transforming growth factor beta-1 is considered to be the primary fibrogenic cytokine in patients with chronic liver diseases, including chronic hepatitis C, and acts through the activation of resident quiescent HSCs into myofibroblast-like cells. The advanced stage of VOD/SOS involves the same conditions as LOHVOO, and both diseases result in progressive liver failure without appropriate therapy. Based on these findings, we showed that CP, VOD after LT, and LOHVOO involve the same pathophysiological aspects in each different histological stage with regard to damage of LSECs, which are rich in HLA class I and class II antigens, in the zone 3 area. Uncontrollable graft fibrosis may progress due to activated HSCs in sensitized patients

with anti-HLA class I and/or class II antigens, despite achieving long-term patency of the graft outflow following EMS placement and/or surgical repair; however, further investigation is needed (Fig. 4).

Given the low concern regarding antidonor alloantibodies, pre-and post-transplant measurements of sensitization in LT patients have been largely ignored, and there are little data regarding the effects of these antibodies [17]. Biological evidence of AMR includes the presence of circulating donor-specific antibodies (DSA) and subsequent deposition of complement component C4d on the graft endothelium as a consequence of complement activation secondary to antigen-antibody binding. FlowPRA [18,19] has numerous advantages over the conventional lymphocytotoxic cross-match test (LCT); however, it is better to conduct FlowPRA single antigen I and II beads tests and LABScreen single antigen multiplex solid-phase immunoassays, which make it possible to better identify DSA and non-DSA qualitatively in order to rule out patients at high risk of developing AMR. Complement degradation product C4d has also become an important marker of AMR in patients undergoing other types of solid organ allograft biopsies. A previous report [29] demonstrated that extensive C4d staining in cross-match-positive patients is associated with AMR and a poor graft survival; however, the meaning of C4d staining in liver grafts remains unclear. One recent article showed that humoral alloreactivity mediated by antibodies against donor HLA molecules appears to be frequently intertwined with the cellular mechanisms of rejection and plays a role in the development of ductopenia [30]. Another article noted that the high prevalence of graft fibrosis and anticlass II DSA suggests that humoral alloreactivity may contribute to the process of unexplained late graft fibrosis following liver transplantation [31]. In this study, we tested only "post-transplant," not preformed FlowPRA. We also only tested for DSA in positive PRA patients, not all transplanted patients. This means that we cannot report specific immunological data showing donor–recipient specificity, which may be an epiphenomenon, as it is unlikely that this type of immunological attack would cause large vessel stenosis amenable to dilatation and stenting. We now try to examine DSA and C4d stains in all patients and acknowledge that more immunological investigations of donor–recipient specificity are required.

In conclusion, the FlowPRA reactivity was found to be a statistically significant risk factor for LOHVOO, and patients with both class I- and class II-reactive antibodies had a significant risk of developing LOHVOO, with a significantly higher retransplant rate. The pathophysiological mechanisms of LOHVOO may be explained by immune-mediated responses that facilitate damage in zone 3 of liver grafts. At this time, further studies are needed to confirm our findings of an association with preformed class I and II antibodies and DSA.

Authorship

TU: designed the study, performed the acquisition of data, conducted the analysis and interpretation of data, and drafted the manuscript; KM, YI, YS, TW, NY, NO: performed the acquisition of data. KM: critically revised the manuscript for important intellectual content.

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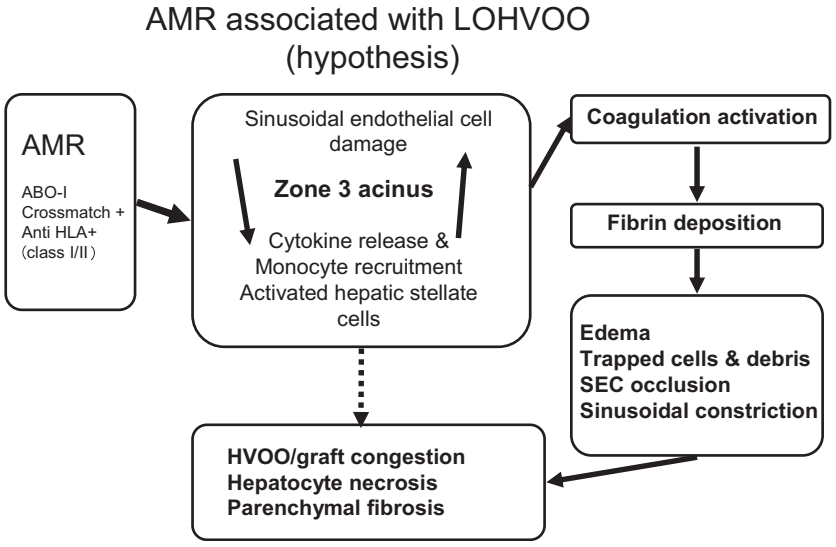

Figure 4 A hypothetical mechanism of antibody-mediated rejection associated with late-onset hepatic venous outflow obstruction (LOHVOO).

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