5–90) and 5 (2–30) days in groups 1 and 2 respectively ($P = 0{\cdot}028$).

With hyperbilirubinaemia defined as a peak total bilirubin level above 85 μmol/l, several risk factors, including mild macrovesicular steatosis, RLVR, age, estimated blood loss during surgery and operating time, were evaluated. Five of the ten donors in group 1 had hyperbilirubinaemia. Only mild macrovesicular steatosis was significantly associated with hyperbilirubinaemia in univariable analysis ($P = 0{\cdot}030$). Multivariable analysis also showed mild macrovesicular steatosis to be independently associated with hyperbilirubinaemia (odds ratio 7·94 (95 per cent confidence interval 1·17 to 54·03); $P = 0{\cdot}034$) (*Table 3*). Liver enzyme levels 1 day after surgery tended to be higher in group 1, but not significantly so (*Fig. 2b,c*). GGT levels immediately after surgery and on postoperative days 1, 2 and 3 were significantly higher in group 1 ($P = 0{\cdot}007$, $P = 0{\cdot}033$, $P = 0{\cdot}002$ and $P < 0{\cdot}001$ respectively) (*Fig. 2d*).

Remnant liver regeneration

From January 2005, 21 donors (five in group 1 and 16 in group 2) had CT to determine remnant liver regeneration; four of these patients had macrovesicular steatosis of less than 5 per cent and one patient had 10–15 per cent steatosis. RLVR immediately after surgery was not significantly different between the two groups

Table 3 Logistic regression model of risk factors for patients with hyperbilirubinaemia

	No. of patients	Odds ratio	*P*
Histology			
Normal	5 of 31		
Mild macrovesicular steatosis	5 of 10	7·94 (1·17, 54·03)	0·034
Remnant liver volume ratio (%)			
< 40	7 of 23		
≥ 40	3 of 18	3·47 (0·28, 43·44)	0·335
Age (years)			
< 40	6 of 27		
≥ 40	4 of 14	0·72 (0·11, 4·75)	0·735
Estimated blood loss (ml)			
< 1000	9 of 34		
≥ 1000	1 of 7	0·24 (0·01, 4·51)	0·338
Operating time (h)			
< 10	8 of 31		
≥ 10	2 of 10	1·02 (0·15, 8·06)	0·940
Sex			
M	9 of 29		
F	1 of 12	5·01 (0·46, 55·05)	0·187

Values in parentheses are 95 per cent confidence intervals.

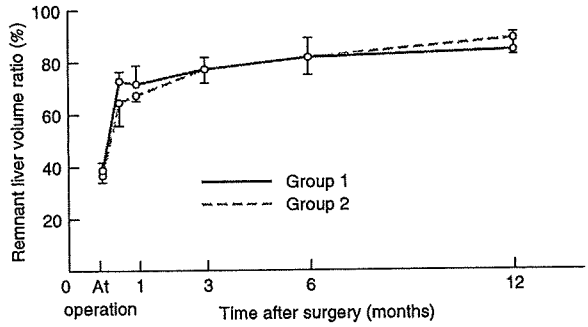

Fig. 3 Remnant liver volume, determined by computed tomographic volumetry and expressed as a percentage of preoperative liver volume, in five donors with macrovesicular steatosis (group 1) and 16 donors with normal liver histology (group 2). Values are median with 75th and 25th percentiles. There were no significant differences at any time point (Mann–Whitney *U* test)

(37·1 and 39·0 per cent in groups 1 and 2 respectively; $P = 0{\cdot}482$). In general, liver regeneration was prominent during the first 2 weeks after the operation and continued more slowly thereafter in both groups (*Fig. 3*). Some 3 months after surgery, the volume ratio ((current liver volume/original liver volume) × 100) was 77·5 and 77·6 per cent in groups 1 and 2 respectively ($P = 0{\cdot}823$), and median RLVR at 12 months after surgery was 84·8 and 89·1 per cent ($P = 0{\cdot}571$). There were no significant differences between the groups at any time point.

Complications

Eight donors in group 1 and 13 in group 2 had at least one complication. In group 1, three patients had a grade I complication and four a grade II complication. One donor in group 1 had a bile duct stricture (grade IIIa complication) at 2 months after surgery that was managed endoscopically. In group 2, four donors had a grade I and eight had a grade II complication. One patient in group 2 had a biloma and pleural effusion (grade IIIa complication), both treated by percutaneous drainage. No donor in either group had a grade IIIb, IV or V complication, and none had a vascular complication. None of the complications led to serious sequelae and there were no remnant disabilities. All donors subsequently returned to their daily life activities.

Discussion

This study of the effects of mild macrovesicular steatosis on donor outcome after right hepatectomy, including a comparison of liver function test results, showed that

donors with mild macrovesicular steatosis had higher total bilirubin and GGT levels than donors with normal liver histology. It has been reported that intrahepatocyte fat deposition occludes sinusoidal blood flow, resulting in hypoxic injury to hepatocytes and bile duct epithelial cells[10,24]. This mechanism may be related to higher levels of total bilirubin and GGT, even in donors with only mild macrovesicular steatosis. Moreover, mild macrovesicular steatosis was found to be an independent risk factor for hyperbilirubinaemia after right hepatectomy.

The patient group studied here was of only limited size and therefore it is difficult to draw firm conclusions with respect to the negative effects of mild macrovesicular steatosis. A few reports[4,7,22] have suggested that there is a risk associated with mild macrovesicular steatosis after right hepatectomy in living liver donors, although this has not been investigated fully. Nevertheless, the present results suggest that this pathology may be related to an adverse outcome for living liver donors. Consequently, from the point of view of donor safety, the presence of mild macrovesicular steatosis following right hepatectomy may be a reason for concern in living liver donor selection.

For preoperative evaluation of macrovesicular steatosis in living donor candidates in the present study, BMI, L/S ratio and liver function tests were used for screening. The results show that ALT and GGT may predict mild macrovesicular steatosis more reliably than BMI or L/S ratio. However, the levels of these enzymes in donors with mild macrovesicular steatosis were nearly within the normal range, casting doubt on their reliability in preoperative screening for mild steatosis in living liver donors. Rinella and colleagues[25] found a significant correlation between BMI and overall grade of macrovesicular steatosis, and that no patient with a BMI below 25 kg/m^2 had macrovesicular steatosis. Iwasaki and co-workers[18] reported that the L/S ratio may be a useful tool for determining macrovesicular steatosis, and considered the likely optimal cut-off L/S ratio for excluding macrovesicular steatosis greater than 30 per cent to be 1·1. These authors concluded that macrovesicular steatosis in the donor of 30 per cent was acceptable for the liver graft, and could avoid the serious complications associated with moderate or severe macrovesicular steatosis. As even mild macrovesicular steatosis can negatively affect the postoperative course of the donor, prediction remains an important issue in regard to donor safety. Unfortunately, it remains difficult to detect this pathology conclusively with non-invasive methods, such as liver function tests, CT, BMI or other imaging modalities[25].

At present, preoperative liver biopsy is recommended for the detection of macrovesicular steatosis as well as other liver pathologies, such as fibrosis and hepatitis[19,26–28]. The degree of macrovesicular steatosis can be reduced by treatment, including exercise, diet and medication[29,30]. It is important to establish indications for preoperative liver biopsy in order to avoid a poor outcome in both donor and recipient due to overlooked graft lesions. Thus, indications for preoperative liver biopsy may need to be extended, especially when right hepatectomy is undertaken for donor candidates. The authors' current policy is to exclude candidates from right hepatectomy if they have macrovesicular steatosis greater than 5 per cent. When mild macrovesicular steatosis is found during evaluation of a donor candidate, diet and exercise are recommended initially. When selecting candidates as living liver donors, careful attention must be paid not only to macrovesicular steatosis but also to other factors, such as remnant liver volume and donor age, as these may also affect donor outcome[31].

The relationship between macrovesicular steatosis and remnant liver regeneration following hepatectomy remains unclear[6,17,32–34]. The present results indicate that mild macrovesicular steatosis does not impair liver regeneration after right hepatectomy. However, the incidence of postoperative complications tended to be higher in donors with mild steatosis. Most of the complications in donors were minor and there was no permanent disability. It is difficult to draw firm conclusions with respect to the association between mild steatosis and complications, and additional investigations are needed to confirm such putative negative effects of mild macrovesicular steatosis. In normal liver resections for hepatic lesions, the incidence of complications was reported to be 23 per cent[35], compared with 16·1 per cent in a review of living liver donor outcomes[1]. For right liver graft donors, the reported complication rate ranges from 18·9 to 37 per cent[1,2,4,36–38]. To assess living liver donor outcome accurately, a universal system for evaluating donor complications should be established, and both short- and long-term observations of donors are needed[3,39–42].

In conclusion, mild macrovesicular steatosis appears to be an independent risk factor for hyperbilirubinaemia in living liver donors undergoing right hepatectomy. Mild macrovesicular steatosis may be related to a negative outcome in living liver donors, but larger studies are needed to confirm the present results. LDLT is an important procedure for patients with end-stage liver disease, in which one of the main issues is donor safety.

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ORIGINAL ARTICLE

Serial Measurement of Doppler Hepatic Hemodynamic Parameters for the Diagnosis of Acute Rejection After Live Donor Liver Transplantation

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To elucidate the role of Doppler hepatic hemodynamic parameters as surrogate markers of acute rejection (AR) after live donor liver transplantation (LDLT), serial Doppler measurements were prospectively performed during the first 2 weeks after LDLT to compare the longitudinal hepatic hemodynamic changes between patients with histologically proven AR and patients without histologically proven AR. Forty-six patients that had undergone adult-to-adult LDLT using a right lobe graft were enrolled in this study. The portal venous maximum velocity (PVV; cm/second), portal venous flow volume, hepatic arterial peak systolic velocity, hepatic arterial pulsatility index, hepatic venous maximum velocity, hepatic venous pulsatility index, and splenic arterial pulsatility index were measured. Fourteen patients were diagnosed by biopsy to have clinically relevant AR. Markedly increased PVV was seen soon after surgery and gradually decreased in both patients with clinically relevant AR and patients without clinically relevant AR. This serial change of decreasing PVV was significantly greater in patients with clinically relevant AR ($P < 0.0001$). After postoperative day 6, the PVV in patients with clinically relevant AR was significantly lower than that in patients without clinically relevant AR (PVV on postoperative day 6: 35.6 ± 21.3 versus 58.3 ± 27.1 cm/second, respectively, $P = 0.0080$). A PVV cutoff value of 20.2 cm/second demonstrated the best accuracy for predicting clinically relevant AR. The sensitivity and specificity for predicting clinically relevant AR were 92.9% and 87.1%, respectively. The area under the curve was 0.94. In conclusion, serial Doppler measurement of hepatic parameters in LDLT is useful for the diagnosis of clinically relevant AR. Clinically relevant AR should therefore be suspected when a marked unexpected decrease in the PVV is observed. *Liver Transpl 15:1119-1125, 2009.* © 2009 AASLD.

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Acute rejection (AR) after live donor liver transplantation (LDLT) is one of the most common clinical problems and often happens 5 to 10 days postoperatively.[1-3] AR is first suspected upon the presentation of clinical symptoms such as high fever, a rise in the serum total bilirubin value, and an increase in the transaminase value.[3-5] Next, liver biopsy is performed because the diagnosis of AR is dependent on liver biopsy. Although liver biopsy is safe, it is invasive and is associated with complications such as bleeding and sepsis.[6,7] Moreover, perihepatic space in patients with small grafts is thought to lessen the tamponade effect afforded by the abdominal wall soft tissues after biopsy, thereby rendering the biopsy site more prone to uncontrolled bleeding. It is desirable to avoid unnecessary liver biopsy whenever possible.[3]

Doppler ultrasonography is noninvasive and is widely used after LDLT to predict vascular complications.[8,9]

Abbreviations: AR, acute rejection; GRWR, graft-to-recipient weight ratio; HAPSV, hepatic arterial peak systolic velocity; HCC, hepatocellular carcinoma; HVPI, hepatic venous pulsatility index; HVV, hepatic venous maximum velocity; IRHV, inferior right hepatic vein; LC/non-LC, liver cirrhosis/non-liver cirrhosis; LDLT, live donor liver transplantation; M/F, male/female; POD, postoperative day; PVFV, portal venous flow volume; PVV, portal venous maximum velocity; RAI, rejection activity index; ROC, receiver operating characteristic; SAPI, splenic arterial pulsatility index; V5, drainage vein of segment 5; V8, drainage vein of segment 8.

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Moreover, there have been many reports that Doppler ultrasonography is useful for the diagnosis of AR.[5,8-12] Mohr et al.[10] reported that a sharp decrease in portal venous flow can be observed in patients with AR and that the hyperkinetic velocity returns to normal within a few days after successful antirejection therapy. Harms et al.[11] found a decrease in the damping index of the portal vein and an increase in the damping index of the hepatic veins in patients with AR. Recently, Bolognesi et al.[5] reported that rejection is associated with a marked reduction in the portal venous velocity and a slight increase in the splenic arterial index. Furthermore, hemodynamic measurements by Doppler ultrasonography are useful for the diagnosis of AR in LDLT. We previously reported that a decreased portal venous maximum velocity with an increased hepatic arterial peak systolic velocity (HAPSV) preceded changes in the laboratory findings of patients with AR.[12] However, in most studies, Doppler measurements were performed only when AR was suspected on the basis of the clinical signs and were reported for orthotopic liver transplantation.

In this study, serial quantitative Doppler measurements of hepatic hemodynamics were prospectively performed during the first 2 weeks after LDLT, and the hepatic hemodynamic changes of patients with histologically proved and clinically relevant AR and patients without it were compared. In addition, the accuracy and role of Doppler hepatic hemodynamic parameters as surrogate markers of clinically relevant AR after LDLT were evaluated in patients after LDLT.

PATIENTS AND METHODS

From May 2002 to March 2007, over 100 LDLT procedures were performed at Nagoya University Hospital with the approval of the Nagoya University Ethics Committee. Among them, adult-to-adult LDLT using a right lobe graft was performed in 46 patients. These 46 patients were enrolled in this study. A right lobe graft without the middle hepatic vein was used in 44 patients, and a right lobe graft with the middle hepatic vein was used in 2 patients. Reconstruction of the drainage veins, except for the right hepatic vein, was performed by means of an inferior right hepatic vein (IRHV) reconstruction in 4 patients, a drainage vein of segment 5 (V5)/IRHV reconstruction in 2 patients, a drainage vein of segment 8 (V8)/IRHV reconstruction in 1 patient, a V5/V8/IRHV reconstruction in 1 patient, and a V5/V8 reconstruction in 2 patients. LDLT procedures were performed for cirrhosis caused by hepatitis B virus [14: 11 patients with hepatocellular carcinoma (HCC) and 3 patients without HCC], cirrhosis caused by hepatitis C virus (14: 10 patients with HCC and 4 patients without HCC), alcoholic cirrhosis with HCC (1), primary biliary cirrhosis (8), primary sclerosis cholangitis (2), biliary atresia (1), familial amyloidotic polyneuropathy (1), fulminant hepatitis (4), and cryptogenic cirrhosis (1). The mean graft-to-recipient body weight ratio was 1.13% ± 0.30%. The mean age was 52.7 ± 9.0 years. Liver biopsy was performed for clinically suspected AR whenever there was clinical and biochemical deterioration suggestive of liver dysfunction. The indications for performing liver biopsy were determined by transplant surgeons who did not perform Doppler ultrasonography. In principle, transplant surgeons performed liver biopsy when both aspartate aminotransferase and alanine aminotransferase levels continuously exceeded 100 IU/L, regardless of the Doppler hemodynamic results.

Doppler Ultrasonography

Postoperative Doppler ultrasonography was performed on fasting patients during each of the first 14 days after LDLT. The parameters were measured in patients during suspended normal respiration. All the Doppler studies were performed with color and pulsed Doppler units using a 2- to 5-MHz convex probe (HDI5000, Philips, Bothell, WA). Doppler ultrasonography was used to measure the portal venous maximum velocity (PVV; cm/second) and portal venous flow volume (PVFV; mL/minute) in the right branch of the portal vein and the HAPSV (cm/second) and hepatic arterial pulsatility index in the intrahepatic right hepatic artery. The hepatic venous maximum velocity (HVV; cm/second) and hepatic venous pulsatility index (HVPI) were measured in the right hepatic vein about 2.0 cm inside the organ and analyzed by its waveform. Hepatic venous waveforms are classified as continuous, biphasic, and triphasic. Hepatic venous waveforms have been reported to show dumping in AR.[10] Although the pulsatility index is used to assess arterial pulsatility, we used it for the hepatic vein to assess the pulsatility of the hepatic venous waveforms. The splenic arterial pulsatility index (SAPI) was based on the flow in the splenic artery at the splenic hilum, with identification of the branch of the splenic artery by color Doppler ultrasonography. The pulsatility index was automatically calculated as (peak systolic velocity − peak end diastolic velocity)/mean velocity on the hepatic artery, hepatic vein, and splenic artery. The axial size of the sample volume was maintained in the 2- to 3-mm range. The angle between the Doppler beam and long axis of the vessel was held at less than 60 degrees. PVV, HAPSV, and HVV were automatically determined for samples of the Doppler signal during 2 cardiac cycles. PVFV was calculated as the product of the right portal venous mean velocity and the cross-sectional area. The Doppler ultrasound operators (H.S., M.H., and K.K.) were blinded to the results of the biochemical tests and biopsy procedures for the patients. The images were interpreted prospectively by one of the operators at the time of evaluation. To decrease interobserver variability in the Doppler ultrasound results, all 3 ultrasound operators participated in a cooperative training program according to Sabbà et al.[13] and Sacerdoti et al.[14] before the beginning of the study.

Histological Assessment of AR

The diagnosis of AR was established by an analysis of a core-needle biopsy specimen that was obtained with a

TABLE 1. Histologic Findings at Biopsy

Patient	Portal Inflammation	Bile Duct Inflammation	Endothelitis	RAI Score	Day at Biopsy
1	2	2	3	7	10
2	3	3	3	9	6
3	3	1	1	5	8
4	2	1	1	4	9
5	2	1	2	5	9
6	2	2	3	7	7
7	2	2	1	5	8
8	3	2	3	8	6
9	2	2	2	6	7
10	2	2	1	5	9
11	2	1	1	4	10
12	2	1	1	4	7
13	1	1	2	4	10
14	2	2	1	5	8

Abbreviation: RAI, rejection activity index.

16G biopsy needle (Monopty disposable biopsy system, Bard). All biopsy specimens were examined by multiple pathologists who were unaware of the Doppler ultrasound results. Biopsy specimens were assigned scores according to the Banff method, and the rejection activity index (RAI) was calculated. The RAI was calculated by the determination of separate scores (RAI scores of 1-3) for portal inflammation, bile duct inflammation damage, and venous endothelial inflammation according to the severity of the involvement. The total score was the sum of the scores for those single components.[3] On the basis of the histological results, the patients were grouped according to the presence or absence of clinically relevant AR, which was defined as an RAI score of 4 or greater.

Statistical Analysis

The results are expressed as the mean ± standard deviation. Differences between 2 groups of continuous variables were evaluated by an analysis of variance with the Scheff test. Differences between 2 groups concerning longitudinal changes were evaluated by a repeated analysis of variance with the Scheff test. The sensitivity and specificity of the Doppler parameters for the presence of clinically relevant AR were determined according to the standard formulas, and receiver operating characteristic (ROC) curves were constructed. An ROC curve is a plot of the sensitivity versus 1 − the specificity for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve, with values close to 1.0 indicating a high diagnostic accuracy. The diagnostic value of the Doppler parameters was assessed with the area under the ROC curve. The cutoff point was defined as the greatest sum of the sensitivity and specificity estimates.

The results were considered significant when P was less than 0.05. The statistical analysis was performed with a commercially available software program (JMP statistical software, version 6.0, SAS Institute, Cary, NC).

RESULTS

Seventeen patients who were suspected of having AR on the basis of their clinical data underwent needle biopsy. Fourteen patients were diagnosed with clinically relevant AR. Table 1 shows the histological findings. In these 14 patients, the time between LDLT and biopsy was 6 to 10 days.

There were no significant differences in the backgrounds of the patients with clinically relevant AR and those without clinically relevant AR, except for sex (Table 2).

Vascular complications occurred in 3 patients, including portal venous thrombosis in 1 patient, arterial thrombosis in 1 patient, and an intrahepatic arterial aneurysm in 1 patient. No other patients demonstrated any vascular complications that required intervention. Acute renal failure occurred in 1 patient. Infectious complications, such as pneumonia, cholangitis, peritonitis, or sepsis, occurred in 8 patients.

One patient developed portal thrombosis, and the PVV was 0 cm/second. This patient was excluded from the analysis performed to predict clinically relevant AR.

Differences in the Perioperative Hepatic Hemodynamic Parameters Between Patients With Clinically Relevant AR and Patients Without Clinically Relevant AR

Portal Venous Flow

A markedly increased PVV in comparison with the normal PVV values in healthy subjects[15-18] (we previously reported 20.86 ± 5.18 cm/second[17]) was seen soon after surgery and gradually decreased in both the patients with clinically relevant AR and the patients with-

TABLE 2. Backgrounds of Patients With AR and Those Without AR

	Patients With AR (n = 14)	Patients Without AR (n = 32)	*P* Value
Age (years)	49.1 ± 10.7	54.2 ± 7.9	0.080
Sex (M/F)	4/10	22/9	0.011
GRWR	1.21 ± 0.26	1.10 ± 0.31	0.260
LC/non-LC	13/1	29/3	0.782

Abbreviations: AR, acute rejection; GRWR, graft-to-recipient weight ratio; LC/non-LC, liver cirrhosis/non-liver cirrhosis; M/F, male/female.

out clinically relevant AR. This serial decrease in the PVV was significantly greater in patients with clinically relevant AR ($P < 0.0001$; Fig. 1A).

There were no differences in the PVV between patients with clinically relevant AR and patients without clinically relevant AR from postoperative day 1 (POD1) to POD5 (PVV on POD1: 88.7 ± 21.9 versus 99.3 ± 31.2 cm/second, respectively, P = 0.2554). However, after POD6, the PVV in patients with clinically relevant AR was significantly lower than that in patients without clinically relevant AR (PVV on POD6: 35.6 ± 21.3 versus 58.3 ± 27.1 cm/second, respectively, P = 0.0080). The maximum percentage decrease of PVV in patients with clinically relevant AR was significantly higher than that in patients without clinically relevant AR (59.7% ± 21.3% versus 29.9% ± 10.6%, respectively, $P < 0.0001$).

To evaluate the sensitivity and specificity, an ROC curve was drawn with the PVV and the percentage decrease of PVV. PVV was the most valuable parameter for predicting clinically relevant AR. The best accuracy for predicting clinically relevant AR was found with a PVV of 20.2 cm/second. The sensitivity and specificity for predicting clinically relevant AR were 92.9% and 87.1%, respectively. The area under the curve was 0.94 (Fig. 2) in PVV. The area under the curves was 0.92 a percentage decrease of PVV (cutoff, 47%).

There were no findings of AR in 3 patients who underwent liver biopsy, and we diagnosed the cause of liver dysfunction in these patients to be endotoxemia, cholangitis, and prolonged hepatitis, respectively. The PVV in these patients was stable, and the minimum PVVs during the first 2 weeks were 47.6, 33.0, and 31.6 cm/second, respectively.

The serial change of the PVFV was similar to that of the PVV (Fig. 1B).

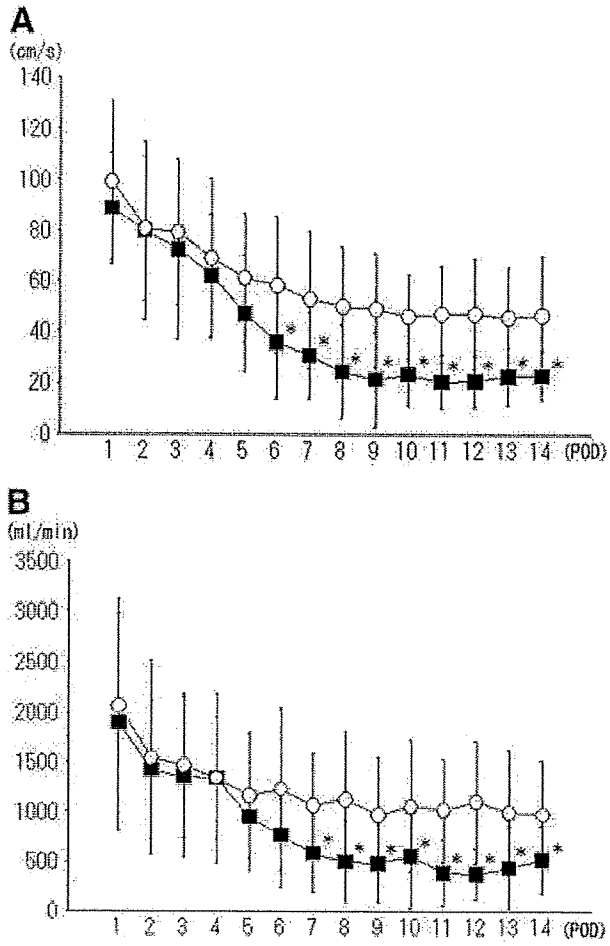

Figure 1. (A) Differences in the portal venous maximum velocity between the patients with acute rejection and the patients without acute rejection. (B) Differences in the portal venous flow volume between the patients with acute rejection and the patients without acute rejection. Open circles indicate patients without acute rejection; closed squares indicate patients with acute rejection. *P < 0.05. Abbreviation: POD, postoperative day.

Hepatic Arterial Flow

The HAPSV on POD1 in patients with clinically relevant AR was significantly lower than that in patients without clinically relevant AR (51.3 ± 23.8 versus 72.7 ± 30.0 cm/second, respectively, P = 0.0228). However, there were no significant serial changes in the HAPSV. The HAPSV reciprocally increased to the decreased PVV in each patient with clinically relevant AR at the time of onset of clinically relevant AR. The maximum percentage increase of HAPSV in patients with clinically relevant AR was significantly higher than that in patients without clinically relevant AR (107.9% ± 77.5% versus 65.6% ± 35.3%, respectively, P = 0.0162).

To evaluate the sensitivity and specificity, an ROC curve was drawn with the percentage increase of

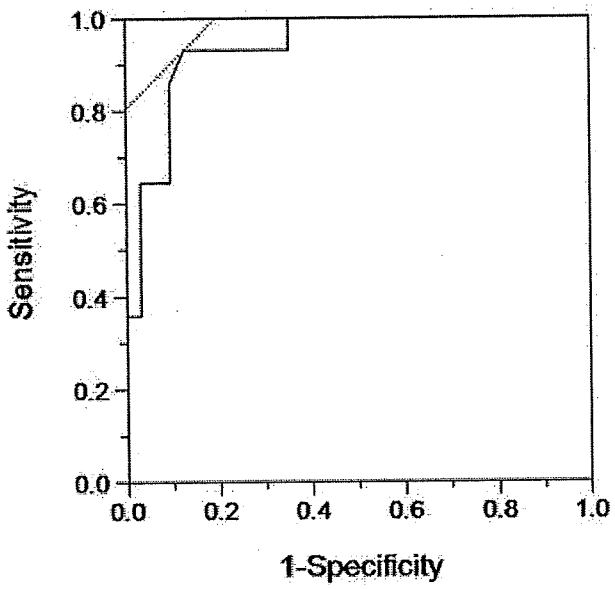

Figure 2. Receiver operating characteristic curve for the prediction of acute rejection according to the portal venous velocity. A portal venous maximum velocity of 20.2 cm/second demonstrated the best accuracy for the prediction of acute rejection. The sensitivity and specificity for predicting acute rejection were 92.9% and 87.1%, respectively (P < 0.0001). The area under the curve was 0.94.

HAPSV. The area under the curves demonstrated 0.71 a percentage increase of HAPSV (cutoff, 91.6%).

There were no differences in the hepatic arterial pulsatility index between patients with clinically relevant AR and patients without clinically relevant AR.

Hepatic Venous Flow

There were no significant differences in the HVV between patients with clinically relevant AR and patients without clinically relevant AR except on POD5. The HVV on POD5 in patients with clinically relevant AR was higher than that in patients without clinically relevant AR (65.4 ± 26.7 versus 49.5 ± 22.4 cm/second, respectively, P = 0.0436).

There were significant differences in the serial change of HVPI between patients with clinically relevant AR and those without clinically relevant AR (P < 0.0001). The HVPI was significantly lower in patients with clinically relevant AR than that in patients without clinically relevant AR on POD3, POD4, POD5, and POD14 (Fig. 3).

Splenic Arterial Doppler Parameter

The SAPI was measured in 10 patients with clinically relevant AR and in 28 patients without clinically relevant AR. The SAPI could not be measured in the remaining patients because they had undergone either splenectomy or splenic arterial ligation.

There were no significant differences during the postoperative period. However, the SAPI increased when clinically relevant AR occurred. The maximum percentage increase of SAPI in patients with clinically relevant AR was significantly higher than that in patients without clinically relevant AR (36.2% ± 23.9% versus 21.7% ± 13.1%, respectively, P = 0.0241).

To evaluate the sensitivity and specificity, an ROC curve was drawn with the percentage decrease of SAPI. The area under the curves demonstrated 0.71 a percentage decrease of SAPI (cutoff, 25.4%).

DISCUSSION

Many authors have reported the predictive value of Doppler ultrasonography for AR after liver transplantation.[5,10-12,19,20] However, most of them used transient Doppler measurements after AR or evaluated only a single parameter. In the current study, the serial changes of hepatic Doppler hemodynamic parameters, including the portal venous flow, hepatic arterial flow, and hepatic venous flow, were measured and analyzed before and after clinically relevant AR in patients with LDLT using a right lobe graft. Even in normal subjects, the hepatic hemodynamics differed between the right lobe and left lobe. To perform a quantitative analysis, we analyzed only patients with a right lobe graft in this study.

The typical hepatic hemodynamic changes were markedly decreased PVV and PVFV, reciprocally increased HAPSV due to the hepatic arterial buffer response, decreased HVPI (which indicated a flat hepatic venous waveform), and increased SAPI. Among those parameters, PVV was the most accurate parameter for the diagnosis of clinically relevant AR.

Mohr et al.[10] first reported that patients with AR show a sharp decrease in the portal venous flow and that the hyperkinetic portal velocity returns within a few days after successful antirejection therapy. However, the use of Doppler ultrasound in the assessment of liver graft hemodynamics during AR has been controversial. Kok et al.[19] reported that serial Doppler ul-

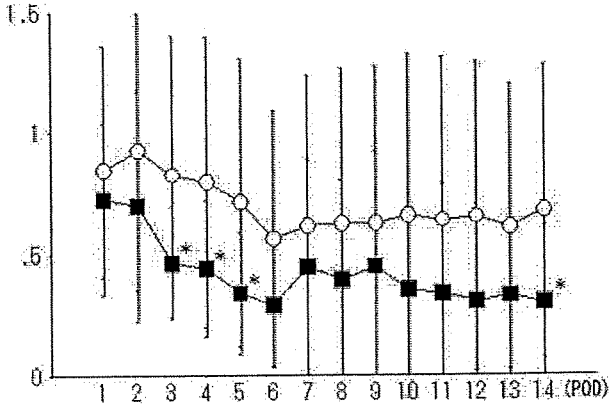

Figure 3. Differences in the hepatic venous pulsatility index between the patients with acute rejection and the patients without acute rejection. Open circles indicate patients without acute rejection; closed squares indicate patients with acute rejection. *P < 0.05. Abbreviation: POD, postoperative day.

trasound examinations were not helpful in predicting AR. Recently, Bolognesi et al.[5] reported that rejection was associated with a marked reduction in the portal venous velocity and a slight increase in the splenic arterial index. They speculated that the difference between their results and those of Kok et al. could be attributed to the selection of patients who underwent liver biopsy. Kok et al. performed liver biopsy in all patients. On the contrary, Bolognesi et al. performed biopsy in patients with any signs of liver dysfunction. The current results were consistent with those of Bolognesi et al. because in this study liver biopsy was also performed in patients with any signs of liver dysfunction. Severe portal venulitis and liver parenchyma injury may occur in patients with AR with a biochemical dysfunction in comparison with that in patients without biochemical dysfunction. Other reasons are the etiology of liver disease and the surgical methods. In the study of Kok et al., the etiology of liver disease was very heterogeneous, including not only cirrhosis but also biliary atresia, acute liver failure, and metabolic disorders. On the other hand, in our study, 87% of the patients demonstrated cirrhosis. In patients with cirrhosis, the PVV dramatically increased immediately after transplantation in comparison with those without cirrhosis.[15] Moreover, in the study of Kok et al., most of the patients underwent whole liver transplantation. On the other hand, in our study, all patients underwent partial liver transplantation. In patients with a small liver graft, the PVV dramatically increased immediately after liver transplantation.[21] As a result, the instability of the PVV in patients with cirrhosis who underwent partial liver transplantation may be one of the adverse effects on the hepatic hemodynamics observed in patients with AR.

To elucidate the clinical efficacy of serial quantitative measurement of Doppler ultrasonography, the cutoff value to diagnose clinically relevant AR was determined. A PVV of less than 20.2 cm/second was the effective cutoff value to distinguish between patients with clinically relevant AR and those without clinically relevant AR according to the ROC analysis. Bolognesi et al.[5] reported that absolute values of the portal venous velocity were not useful in distinguishing between patients with AR and those without AR. This difference was attributed to the surgical procedure and specifically the use of LDLT or orthotopic liver transplantation. A relatively small graft in LDLT affects posttransplant hemodynamic changes more than orthotopic liver transplantation.

Core-needle biopsy is the gold standard in the diagnosis of AR. Biopsy specimens may show the following: (1) mixed but predominantly mononuclear portal inflammation containing blastic (activated) lymphocytes, neutrophils, and frequently eosinophils; (2) bile duct inflammation/damage; and (3) subendothelial inflammation of the portal veins or terminal hepatic venules.[3] These pathological findings lead to hepatic microcirculation disturbance and decreased total hepatic blood flow (especially the portal venous flow). Therefore, clinically relevant AR should be suspected when a sharp decreased portal venous flow is seen by serial Doppler measurements, and that should be an indication to perform liver biopsy to correctly diagnose clinically relevant AR.

The hepatic hemodynamic Doppler parameters after LDLT are relatively stable in cases without complications such as AR.[15] In this study, the cutoff value to detect clinically relevant AR by PVV was 20.2 cm/second. However, other variable hepatic hemodynamic abnormalities were also seen in patients with clinically relevant AR. Although the cutoff values could not be determined for these single parameters, they can be measured easily and noninvasively and may also suggest clinically relevant AR. The usefulness of SAPI was reported by Bolognessi et al.[5] In the current study, SAPI was increased at the onset of AR. The usefulness of a damping of the hepatic venous waveform was reported by Harms et al.[11] In the current study, although the early postoperative HVPI was lower in the patients with clinically relevant AR than in those without clinically relevant AR, the HVPI gradually decreased in most patients. We could not determine the cutoff value to detect clinically relevant AR. We speculate that the HVPI in partial liver transplantation might be influenced by postoperative graft swelling caused by relative congestion in comparison with whole liver transplantation. A decreased HVPI may therefore be one of the negative signs in hepatic hemodynamics.

The degree of severity of AR is variable. AR with no biochemical graft dysfunction seems to be safe as long as the graft function is carefully monitored. The rationale for performing protocol biopsies in the absence of biochemical graft dysfunction is questionable.[3] Measuring the hepatic Doppler parameters provides a detailed profile of the hepatic circulation that can be used to carefully monitor graft function after LDLT. Moreover, vascular complications such as portal vein thrombus or hepatic arterial thrombus can be diagnosed by measurement of the Doppler parameters. Graft dysfunction is indicated when abnormal Doppler parameters are seen, such as a decreased PVV, a reciprocally increased HAPSV, an increased SAPI, or a decreased HVPI. In particular, a marked decrease in the PVV, which is defined as a PVV < 20.2 cm/second or a percentage decrease of PVV $< 47\%$ in this study, is a sign of clinically relevant AR. Furthermore, an adequate portal venous flow is a critical factor for achieving good posttransplant liver generation and function; therefore, a markedly decreased PVV is considered to be a negative factor.

In conclusion, serial Doppler measurements of the hepatic parameters in LDLT are useful for the diagnosis of clinically relevant AR. Clinically relevant AR should therefore be suspected when a marked unexpected decrease in the PVV is observed.

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Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation

Nagai S, Ito M, Kamei H, Nakamura T, Ando H, Kiuchi T. Indirect immunohistochemical evaluation of graft fibrosis and interface hepatitis after pediatric liver transplantation.
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Abstract: Fibrosis or IH following pediatric liver transplantation is recognized as major causes of graft loss, but the etiology remains unclear. To determine this issue, we used an indirect immunohistochemistry technique with post-transplant serum samples from recipients and normal human liver tissues from living liver donors, and the association between occult antibody reaction to the liver and the occurrence of fibrosis or IH was evaluated. Forty-three recipients were evaluated, and both hepatocytes and biliary epithelial cells were evaluated for staining intensity. Fibrosis and IH occurred in 13 and six patients, respectively. According to staining results for the hepatocytes and biliary epithelial cells, 18 and 11 patients, respectively, were classified into the positive group. According to log-rank analysis, positive reaction for hepatocytes was associated with increased rates of fibrosis and IH ($p = 0.002$ and 0.048, respectively), while positive reaction for biliary epithelial cells was associated with an increased rate of fibrosis ($p = 0.014$). Multivariate analysis revealed that positive reaction for hepatocytes and biliary epithelial cells was independently associated with fibrosis occurrence ($p = 0.020$ and 0.047, respectively). In conclusion, immune-mediated reactions by occult antibodies may underlie the pathogeneses of fibrosis and IH.

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Key words: liver transplantation – fibrosis – interface hepatitis – immunohistochemistry – late cellular rejection – *de novo* autoimmune hepatitis

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Liver transplant recipients often have abnormal histology findings during long-term follow-up, which may lead to late-onset graft dysfunction (1–8). There are several causes of late-onset graft dysfunction, such as vascular and biliary complications, recurrent primary disease, and infections, and more recently *de novo* AIH and fibrosis have been noted (4–13). *De novo* AIH is characterized by IH, along with the presence of autoantibodies and hypergammaglobulinemia (14–16). The etiology is uncertain, although it is considered to be a kind of rejection (6, 7). Moreover, it was reported that pediatric liver transplant recipients sometimes show chronic hepatitis, which is predominantly portal-based mononuclear inflammation associated with IH, which does not meet the criteria of *de novo* AIH (3, 5). These pathological changes are also considered to lead to graft dysfunction. In contrast, fibrosis is a consequence of a variety of complications, including vascular and biliary complications, viral hepatitis, and immunological morbidity, such as intractable acute cellular rejection, chronic ductopenic rejection, and occult chronic hepatitis (17). Fibrosis sometimes develops without obvious clinical signs, although it was reported that the presence of

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANA, antinuclear antibodies; ASMA, anti-smooth muscle antibody; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; IgG, immunoglobulin G; IH, interface hepatitis; LKM-1, anti-liver kidney microsomal type 1; TB, total bilirubin.

autoantibodies is a risk factor for fibrosis development (1, 3, 5, 9, 12, 13).

We previously reported that recipient serum often contains antibodies against donor T lymphocytes (18, 19), and speculated that occult antibodies in that serum provoke immune reactions to the liver graft, possibly leading to late graft dysfunction. In the present study, we utilized an indirect immunohistochemistry technique to test recipient serum samples. Our method is based on the theory that indirect immunohistochemistry is able to detect unknown antibodies (20, 21), as it has been proposed that immunological reactions of antibodies produced in recipient serum against a normal human liver can be detected (22). Using this method, we investigated the relationship between positive reactions and late-onset graft morbidity.

Materials and methods

Forty-three patients (15 males, 28 females) who underwent living donor liver transplantation between 1992 and 2008 were evaluated. All patients underwent liver biopsy at least once after the LT. Table 1 shows patient's characteristics. Only one patient was an adult (25.3 yr old), but primary diagnosis is biliary atresia. Therefore, this patient was included in this study. Informed consent was obtained from each patient or his/her parents before study entry.

Table 1. Patient characteristics

Patient present age (yr)	11.0 ± 6.5 (median, 8.6; 2.3–27.1)
Patient age at LT (yr)	4.8 ± 5.7 (median, 2.0; 4 months to 25.3)
Gender	
Male	15 (35%)
Female	28 (65%)
Follow-up period (yr)	6.2 ± 3.6 (median, 5.2; 1.1–15.8)
Indication for LT	
Biliary atresia	31 (72%)
Fulminant hepatic failure	6 (14%)
Metabolic disease	4 (9%)
Metastatic solid and cystic tumor of the pancreas	1 (2%)
Congenital extrahepatic portal venous obstruction	1 (2%)
Graft type	
Left lateral segment	32 (74%)
Monosegment	3 (7%)
Left liver	4 (9%)
Right liver	4 (9%)
Blood type compatibility	
Identical	29 (67%)
Compatible	10 (23%)
Incompatible	4 (9%)*

LT, liver transplantation.
*One of four patients was more than one yr old, and additional immunosuppressants were given.

Post-transplant management

Immunosuppression was based on tacrolimus and steroids. Target tacrolimus trough levels ranged from 12 to 15 ng/mL for the first two wk, then around 10 ng/mL from two to four wk after LT, and 5–8 ng/mL from one to six months after LT, after which they decreased gradually to <5 ng/mL more than six months after LT. Intravenous methylprednisolone was administered during the first week after LT, followed by oral prednisolone. Steroids were started during graft reperfusion at a dose of 10 mg/kg, then tapered from 1 to 0.5 mg/kg at one wk after LT, with doses of 0.3 mg/kg from two to four wk after LT, 0.2 mg/kg in the second month, and 0.1 mg/kg in the third month. As a general rule, steroids were withdrawn at the end of the third month. An antiproliferative agent, such as azathioprine or mycophenolate mofetil, was considered as an additional agent, when a patient showed renal dysfunction because of calcineurin inhibitor or had pathological lesions, such as IH or fibrosis. In terms of immunosuppressive regimen for blood type incompatible cases, when a patient is less than one yr old, there is no difference from standard regimen. When a patient is more than one yr old, we check titer of ABO antibody and decrease this $<1:8$ with whole blood change transfusion before LT. Additional immunosuppressants were given to inhibit humoral rejection. About 2 mg/kg/day of methylprednisolone and 0.01 μg/kg/minute of prostaglandin E1 were continuously administered through portal vein catheter for one to two wk postoperatively. In addition, 2 mg/kg/day of cyclophosphamide was provided immediately after LT and was switched to 20 mg/kg/day of mycophenolate mofetil from one month after LT (23). Management of immunosuppression therapy was based on clinical information and the results of indirect immunohistochemistry were not informed to clinical staff.

Indication for a liver biopsy was based on the results of laboratory data (liver function tests and fibrosis markers, such as hyaluronic acid, procollagen type III, and collagen type IV), and/or an ultrasound examination. Recipients were evaluated every month at the outpatient clinic after hospital discharge, and blood tests and ultrasound examinations were routinely performed. In terms of autoantibody detection, ANA, ASMA, and LKM-1 antibody were routinely measured every three months (cut off titer of both ANA and ASMA are $<1:40$ and cut off level of anti LKM-1 is 17.0 index). IgG quantitation was also included in blood tests every three months conducted at the outpatient clinic.

Histopathologic evaluations

Liver biopsies were taken percutaneously with a 16- or 18-gauge biopsy needle, when clinically indicated, and sections were routinely stained with hematoxylin–eosin. Two well-experienced pathologists evaluated all of the samples, with pathological assessments based on published criteria (2, 15, 24). Fibrosis was evaluated based on METAVIR score (25, 26). In this study, METAVIR score was used for the evaluation of fibrosis. This scoring system was specially designed for patients with hepatitis C and originally designed in the context of post-necrotic cirrhosis. It is useful to exclude inter- and intra-observer variation in the assessment of liver biopsy. IH was defined as periportal or periseptal hepatitis with a predominantly lymphoplasmacytic necroinflammatory infiltrate, with or without lobular involvement and portal-portal or central-portal bridging necrosis, and with the formation of liver cell rosettes and nodular regeneration (23).

Indirect immunohistochemistry technique

Indirect immunohistochemistry assessment was performed with post-transplant serum samples from the 43 recipients and normal human liver tissues from two living liver donors (20, 21). Post-transplant serum samples were collected at one certain point during follow-up period, and liver biopsy was performed before collecting these samples. All serum samples were collected between 1.7 and 184.1 months after LT (median, 50.0 months), and stored at −80 °C. Donor liver tissue was routinely collected at the beginning of living liver donor operations, which is called time 0 biopsy. Histologically, normal liver tissues from one male and one female donor with blood type O were selected to avoid the effect of gender specificity and blood type compatibility between sera and tissue. All liver tissues were fixed in 10% buffered formalin and embedded in paraffin.

A recipient serum sample diluted 1:100 was used for raising the primary antibody. Peroxidase labeled rabbit anti-human IgG antibody (Dako, Glostrup, Denmark) diluted at 1:100 was used as a secondary antibody. Paraffin sections were deparaffinized, then, the antigen was retrieved by microwaving in a 0.01 M citrate buffer (pH 7.0). Sections were incubated in 1% normal rabbit serum for blocking non-specific bindings, after which the primary antibody was added and incubated overnight at 4 °C. After washing three times with phosphate-buffered saline for five min to block endogenous peroxidase activity, specimens were incubated with 0.3% hydrogen peroxidase in methanol for 10 min. After washing, the specimens were incubated with the secondary antibody for one h. Color development was performed using diaminobenzidine solution for 10 min and counter-staining was performed with hematoxylin. As a negative control, a serum sample taken from the same donor was used to raise the primary control antibody.

The evaluation of staining intensity and the number of positive cells were performed as a relative assessment. To avoid being subjective, specimens were randomized and coded before analysis, which was conducted by two independent observers, who evaluated all specimens at least twice within a given interval to minimize intra-observer variation. Any inter-observer differences in scoring were discussed with consensus as the outcome. Both hepatocytes and biliary epithelial cells were evaluated for staining intensity and number of positive cells. According to relative evaluations of the specimens, they were classified as negative, doubtful, positive, and strongly positive, respectively (Figs. 1 and 2). According to our preliminary data, immunohistochemistry between serum samples and liver tissues from the same donor was considered to be negative reaction. Therefore, a reaction between liver tissue and the donor serum sample was established as a negative control. On the other hand, we tried to detect the reaction between the occult antibodies and human liver tissue in this study, which means that it was not sure what types of serum samples or which combination showed positive reactions before this analysis. Consequently, after observing all reactions between two liver tissues and 43 serum samples, we considered the most strongly stained sample as a positive control. When strongly positive reactions were observed in both male and female liver tissues, they were scored as 3. When positive reactions were observed in both or when strongly positive reactions were observed in one and positive reactions were observed in another, they were scored as 2. Weakly positive reactions in both or either were regarded as doubtful, and scored as 1. For analysis of outcome in terms of IH and fibrosis, scores of 0 and 1 were considered to be negative and scores of 2 and 3 positive. As a result, only obviously positive reaction in both types of liver tissues was categorized into the positive group for analysis, otherwise they were categorized into the negative group.

Statistical analysis

IH and fibrosis were set as the endpoint, and disease-free time was computed according to the Kaplan–Meier method. In addition, disease-free time was compared using log-rank analysis. For comparison of the means of quantitative variables, an unpaired *t*-test was used. Qualitative variables were evaluated using cross-tables with a chi-square test. A chi-square test for univariate analysis and logistic regression analysis for multivariate analysis were performed to assess

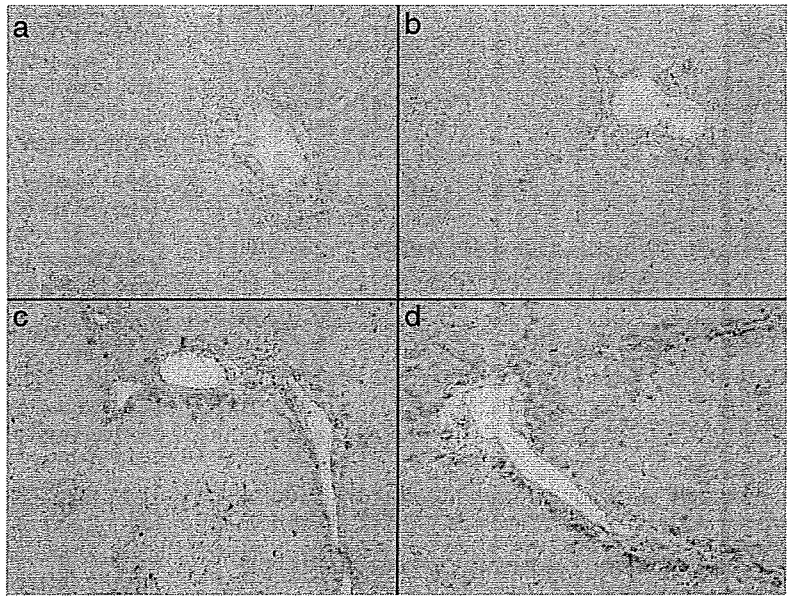

Fig. 1. Classification for staining intensity of indirect immunohistochemistry: Hepatocytes (original amplification ×200). Strongly stained hepatocytes tended to be located in the periportal and/or pericentral area. (a) Negative finding. (b) Doubtful finding (weakly positive reaction). (c) Positive reaction. (d) Strongly positive reaction.

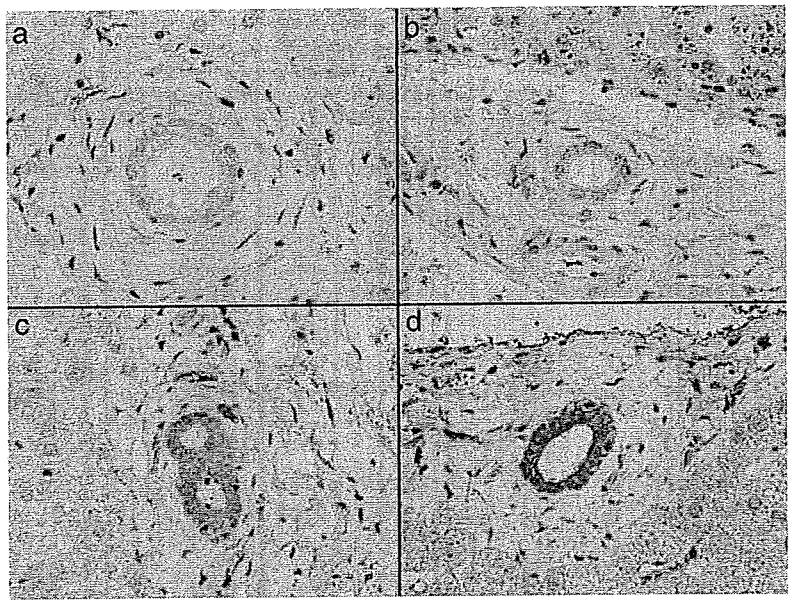

Fig. 2. Classification for staining intensity of indirect immunohistochemistry: Biliary epithelial cells (original amplification ×400). Stained biliary epithelial cells were observed in most of portal areas. (a) Negative finding. (b) Doubtful finding (weakly positive reaction). (c) Positive reaction. (d) Strongly positive reaction.

the risk factors for development of fibrosis and IH. Data are shown as the mean ± standard deviation or median with range. The software package SPSS (version 16.0, SPSS Japan Inc. Tokyo, Japan) was used for statistical analysis, with the level of significance set at $p < 0.05$.

Results

Histological results for post-transplant follow-up biopsy specimens

Fibrosis of grade 1 or more occurred in 13 (30%) of 43 patients. Mean time between LT and initial diagnosis of fibrosis was 4.4 ± 3.6 yr (range, three months to 11.6 yr; median, 5.0 yr). As for the severity of fibrosis, two (5%) cases were F3, four (9%) were F2, and seven (16%) were F1. Two patients classified as F3 showed bridging fibrosis, while in 11 patients classified as F2 and F1, fibrosis was observed in the periportal area. On the other hand, IH was observed in six (14%) of 43 patients. The mean time between LT and initial diagnosis of IH was 6.8 ± 2.7 yr (range 3.7–11.6 yr; median, 6.3 yr). Twenty-one patients (49%) had episodes of late-onset acute cellular rejection, which occurred more than three months after LT.

Results of indirect immunohistochemistry

In terms of evaluation of staining intensity, there was a little discrepancy between observers, but that was subtle and did not influence on the final results of this study. The observers largely concurred on the immunohistochemical findings. Positive hepatocytes were detected in 18 patients, with a score of two given to 12 patients and a score of three to six patients. Strongly stained hepatocytes were generally located in the periportal and/or pericentral area (Fig. 1). Staining pattern of hepatocyte is characterized by diffusely stained cytoplasm. In terms of patient characteristics categorized by indirect immunohistochemistry evaluations, primary diagnosis, recipient gender, recipient age, follow-up period, date of serum sample, and donor age were similar between patients with positive and negative reactions of hepatocytes. In addition, several factors regarding characteristics were shown in Table 2. The antiproliferative agents utilized and steroid therapy tended to be associated with positive reactions for hepatocytes, although those associations were not significant. The presence of autoantibody tended to be higher in the positive group, but not significant. Liver function parameters, including AST, ALT, TB, and GGT, and IgG levels were measured using the same serum samples used for the indirect immunohistochemistry evaluations, and no differences were found between the two groups (data not shown). The occurrence of fibrosis and IH was significantly associated with positive reactions for hepatocytes ($p < 0.001$ and $p = 0.026$, respectively). Furthermore, according to log rank analysis, a positive reactions for hepatocytes were associated with a significantly shorter time to fibrosis (Fig. 3a), as the fibrosis-free period was 6.4 yr (95% CI = 4.3–8.6 yr) in the positive group and 13.8 yr (95% CI = 11.3–16.4 yr) in the negative group ($p = 0.002$). Moreover, a positive reaction for hepatocytes was associated with the occurrence of IH (Fig. 3b). Patients in the positive group had a significantly shorter time to IH, as the disease-free

Table 2. Recipient and donor characteristics based on indirect immunohistochemistry results

	Hepatocytes			Biliary epithelial cells		
	Positive (n = 18)	Negative (n = 25)	p value	Positive (n = 11)	Negative (n = 32)	p value
ABO blood-type compatibility						
Identical	15 (83%)	14 (56%)	0.166	9 (82%)	20 (63%)	0.421
Compatible	2 (11%)	8 (32%)		1 (9%)	9 (28%)	
Incompatible	1 (6%)	3 (12%)		1 (9%)	3 (9%)	
Antiproliferative agent	11 (61%)	9 (36%)	0.103	7 (64%)	13 (41%)	0.187
Azathioprine	7 (39%)	4 (16%)		4 (36%)	7 (22%)	
Mycophenolic acid	4 (22%)	4 (16%)		3 (28%)	5 (16%)	
Mizoribine	0	1 (4%)		0	1 (3%)	
Steroids use						
Ongoing	11 (61%)	8 (32%)	0.058	6 (55%)	13 (41%)	0.423
Withdrawal	7 (39%)	17 (68%)	0.100	5 (45%)	19 (59%)	0.804
History of bolus treatment	15 (83%)	15 (60%)	0.947	8 (73%)	22 (69%)	0.209
Initial withdrawal (days)†	161 ± 150	157 ± 221		112 ± 59	172 ± 216	
R/D gender match						
Mismatch	12 (67%)	11 (44%)	0.142	7 (64%)	16 (50%)	0.434
F to M/M to F	6/6	5/6		6/1	5/11	
No mismatch	6 (33%)	14 (56%)		4 (36%)	16 (50%)	
M to M/F to F	1/5	1/13		1/3	1/15	
Fibrosis (F1/F2/F3)	11 (7/3/1) (61%)	2 (0/1/1) (8%)	**<0.001**	7 (4/2/1) (64%)	6 (3/2/1) (19%)	**0.005**
IH	5 (28%)	1 (4%)	**0.026**	2 (18%)	4 (13%)	0.639
Late-onset ACR*	11 (61%)	10 (40%)	0.172	6 (55%)	15 (47%)	0.661
Presence of autoantibody	8 (44%)	5 (20%)	0.085	6 (55%)	7 (22%)	**0.042**
ANA 1:40	2	3		2	3	
ANA 1:80	1	0		1	0	
ANA 1:2560	1	0		0	1	
ASMA 1:40	2	0		2	0	
LKM	2‡	2§		1¶	3**	
IgG (mg/dL)	1331 ± 425	1346 ± 415	0.911	1337 ± 373	1341 ± 433	0.975

M, male; F, female; LT, liver transplantation; R/D, recipient and donor; M, male; F, female; IH, interface hepatitis; ACR, acute cellular rejection; ANA, anti nuclear antibody; ASMA, anti-smooth muscle antibody; LKM, anti-liver kidney microsomal type 1 antibody; IgG, immunoglobulin G.

*Episode of ACR greater than three months after LT.

†Four patients in the positive group and five in the negative group were treated by steroids throughout the follow-up period.

‡The index levels of anti LKM-1 in two patients were 35.7 and 32.5, respectively (cut off level is 17.0 index).

§The index levels of anti LKM-1 in two patients were 22.5 and 27.5, respectively.

¶The index level of anti LKM-1 in one patient was 35.7.

**The index levels of anti LKM-1 in three patients were 22.5, 27.5, and 32.5, respectively.

period was 8.5 yr (95% CI = 6.0–11.0 yr) in the positive group and 10.3 yr (95% CI = 9.1–11.4 yr) in the negative group (p = 0.048).

On the other hand, positive biliary epithelial cells were detected in 11 patients, of whom a score of 2 was given to 8 and a score of 3 was given to 3 (Fig. 2). In terms of staining pattern of biliary epithelial cells, cytoplasm was diffusely stained. Primary diagnosis, recipient gender, recipient age, follow-up period, date of serum sample, and donor age were similar between patients with positive and negative reactions of hepatocytes. Table 2 also shows patient characteristics categorized by indirect immunohistochemistry evaluations of biliary epithelial cells. The presence of autoantibody was significantly higher in the positive group (p = 0.042). As for liver function tests measured with the same serum samples, there were no differences between the two groups (data not shown). The occurrence of fibrosis was associated with positive reactions for biliary epithelial cells (p = 0.005). According to log rank analysis results, a positive reaction for biliary epithelial cells was associated with a shorter time to fibrosis (Fig. 3c), as the fibrosis-free period was 6.2 yr (95% CI = 3.1–9.3 yr) in the positive group and 11.8 yr (95% CI = 9.3–14.4 yr) in the negative group (p = 0.014). However, this finding was not associated with the occurrence of IH (Fig. 3d), as that disease-free period was 10.2 yr (95% CI = 7.8–13.6 yr) in the positive group and 9.2 yr (95% CI = 7.8–10.6 yr) in the negative group (p = 0.819).

Analysis of risk factors for fibrosis and IH

We investigated the risk factors for development of fibrosis and IH (Table 3). Univariate analysis showed that fibrosis was significantly associated with late-onset acute cellular rejection,

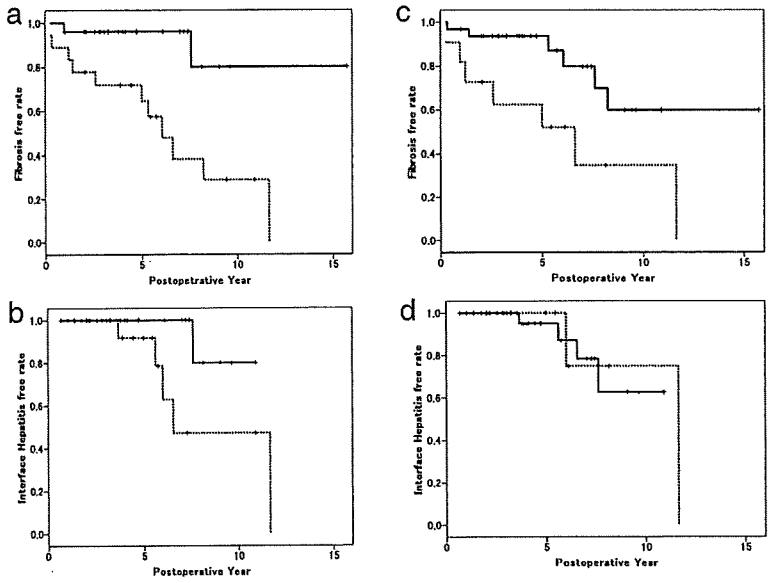

Fig. 3. Comparison of cumulative disease-free rates between the positive and negative group of immunohistochemical assay results. Interrupted and solid lines represent the positive group and the negative group, respectively. (a) Cumulative fibrosis-free rates compared between patients shown positive and negative in indirect immunohistochemistry for hepatocytes. Log rank analysis revealed that a positive reaction for hepatocytes was associated with a significantly shorter time to occurrence of fibrosis ($p = 0.002$). (b) Cumulative IH-free rates compared between patients rated as positive and negative in indirect immunohistochemistry for hepatocytes. A positive reaction was associated with a significantly shorter time to occurrence of IH ($p = 0.048$). (c) Cumulative fibrosis-free rates compared between patients shown positive and negative in indirect immunohistochemistry for biliary epithelial cells. A positive reaction for biliary epithelial cells was associated with a significantly shorter time to occurrence of fibrosis ($p = 0.042$). (d) Cumulative IH-free rates compared between patients rated as positive and negative for biliary epithelial cells. A positive reaction was not associated with occurrence of IH ($p = 0.819$).

development of IH, presence of autoantibodies, and positive reactions for hepatocytes, and biliary epithelial cells. The development of IH was significantly associated with a duration of follow-up more than five yr, donor age < 30 yr old, gender mismatch, episode of late-onset of acute cellular rejection, and positive reactions for hepatocytes. In contrast, the presence of autoantibodies was not associated with the development of IH. In terms of liver function and fibrosis markers, patients with fibrosis showed significantly higher level of procollagen type 3, but other two parameters, hyaluronic acid and collagen type IV, were not significant. ALT was significantly higher in patients with IH. AST also tended to be higher in patients with IH. In addition, primary diagnosis, recipient gender, recipient age, and ABO blood type compatibility were not considered as a risk factor for fibrosis or IH (data not shown).

We entered these variable factors into a logistic regression model for multivariate analysis, which revealed that positive reactions for both hepatocytes and biliary epithelial cells as well as episode of late-onset acute cellular rejection remained independently associated with fibrosis (Table 4). In terms of risk factors for the development of IH, a multivariate analysis could not be performed, because of multicollinearity between the factors (27).

Discussion

The etiology of fibrosis and IH in liver transplant recipients has not been clarified (2, 4, 9, 28–30). In particular, it is not known whether the etiology of fibrosis in pediatric liver recipients is immunological. In terms of the pathogenesis of fibrosis and IH, we hypothesized that recipients might have some antibodies to the transplanted liver that may lead to chronic hepatitis, such as IH, and finally cause fibrosis. Our results suggest that the etiology of IH and fibrosis may be immunological and may be associated with the presence of occult antibodies in recipient's sera. These antibodies reacting to the human liver may be present even before LT or may be produced as a reaction to immune-mediated aggression of transplanted liver. Actually, it remains controversial whether antibodies reactions that were detected in this study cause IH and/or fibrosis. However, there is still a possibility that some antibodies in recipient's sera can react to transplanted liver, which causes IH and/or fibrosis.

Table 3. Risk factors for fibrosis and interface hepatitis

	Fibrosis			Interface hepatitis		
	Positive (n = 13)	Negative (n = 30)	p value†	Positive (n = 6)	Negative (n = 37)	p value†
Follow-up period (yr)						
≤ 5	6 (46%)	17 (57%)	0.526	0	23 (62%)	**0.005**
>5	7 (54%)	13 (43%)		6 (100%)	14 (38%)	
Donor age (yr)						
≤ 30	7 (54%)	7 (23%)	0.050	5 (83%)	9 (24%)	**0.004**
>30	6 (46%)	23 (77%)		1 (17%)	28 (76%)	
R/D gender mismatch	3 (23%)	12 (33%)	0.876	6 (100%)	17 (46%)	**0.014**
Late-onset ACR*	11 (85%)	10 (33%)	**0.002**	6 (100%)	15 (41%)	**0.007**
IH	5 (38%)	1 (3%)	**0.002**	–	–	–
Fibrosis	–	–	–	5 (83%)	8 (22%)	**0.002**
Presence of autoantibodies	7 (54%)	6 (20%)	**0.026**	3 (50%)	10 (27%)	0.256
Indirect immunohistochemistry						
Positive reaction for hepatocytes	11 (85%)	7 (19%)	**<0.001**	5 (83%)	13 (35%)	**0.026**
Positive reaction for biliary epithelial cells	7 (54%)	4 (13%)	**0.005**	2 (33%)	9 (24%)	0.639
Fibrosis marker						
Hyaluronic acid (ng/mL)	46.9 ± 30.3	36.3 ± 28.9	0.217	51.8 ± 50.1	35.9 ± 25.1	0.225
Procollagen type III (units/mL)	1.73 ± 0.94	1.18 ± 0.33	**0.006**	1.25 ± 0.38	1.36 ± 0.66	0.577
Collagen type IV (ng/mL)	217.1 ± 114.1	162.9 ± 78.4	0.137	209.3 ± 145.1	174.4 ± 83.3	0.588
Liver function						
AST (units/L)	33.5 ± 20.2	29.1 ± 9.1	0.469	39.3 ± 29.4	29.0 ± 8.8	0.081
ALT (units/L)	24.5 ± 23.0	20.5 ± 11.5	0.560	37.2 ± 31.0	19.2 ± 10.4	**0.008**
GGT (units/L)	30.0 ± 22.6	34.0 ± 66.6	0.771	22.0 ± 13.4	34.5 ± 60.8	0.278
TB (mg/dL)	0.72 ± 0.27	0.93 ± 0.86	0.241	0.82 ± 0.37	0.88 ± 0.78	0.769
IgG (mg/dL)	1401 ± 437	1313 ± 409	0.545	1671 ± 510	1286 ± 378	0.128

R/D, recipient and donor; LT, liver transplantation; ACR, acute cellular rejection; IH, interface hepatitis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; TB, total bilirubin.
*Episode of ACR greater than three months after LT.
†Chi-square test in univariate analysis.

Table 4. Factors related to development of fibrosis

	Multivariate p value†	Odds ratio	95% CI
Late-onset ACR*	**0.034**	36.3	1.3–1013.6
IH	0.295	6.1	0.2–177.1
Presence of autoantibodies	0.652	1.7	0.2–16.1
Indirect immunohistochemistry			
Positive reaction for hepatocytes	**0.020**	22.1	1.6–302.3
Positive reaction for biliary epithelial cells	**0.047**	20.0	1.0–385.1

ACR, acute cellular rejection; IH, interface hepatitis; CI, confidence interval.
*Episode of ACR greater than three months after LT.
†Logistic regression model in multivariate analysis.

These antibodies probably react to normal human liver tissue as well. As a result, positive reactions can be observed with this indirect immunohistochemistry technique.

In the present study, the development of fibrosis was associated with positive reactions for both hepatocytes and biliary epithelial cells, whereas IH was associated with positive results for hepatocytes but not biliary epithelial cells. It is assumed that fibrosis may be provoked by a variety of antibodies that target hepatocytes as well as biliary epithelial cells. On the other hand, IH may be caused by antibodies that mainly target hepatocytes. These results also indicate that fibrosis is a consequence of a variety of types of immunological morbidity.

The theoretical basis of this indirect immunohistochemistry technique is similar to that of the anti-LKM antibody identification method (31). A previously reported immunohistochemical staining method that utilized paraffin-embedded tissue samples was able to identify specific immunoglobulins reactions without non-specific immunoglobulin staining (32, 33). With this method, the reaction between unknown antibodies in patient serum to the normal liver tissue element could be demonstrated. It is difficult to predict what types of serum samples would show positive reactions before the analysis. Therefore, the most strongly stained specimen was established as positive control in this analysis. On the other hand, although antigen–antibody reactions can be detected with this technique, the subcellular location of the potential autoantigen is still unclear with this technique. This indirect immunohistochemistry technique we used in this study is unable to specify the kinds of antibodies involved and the nature of antigens. A method for detection of the kind of occult antibodies and

the nature of the targeted antigens in liver tissue is considered to be the next step. Moreover, an examination of post-transplant liver allograft biopsy for antibody staining could be helpful to understand the etiology of IH and fibrosis.

It was reported that the presence of autoantibodies in liver transplant recipients is associated with the presence of chronic hepatitis, which leads to chronic hepatitis and progressive fibrosis (3, 7, 10, 12, 13). However, the presence of autoantibodies was not an independent factor in our analysis. Moreover, late-onset acute cellular rejection was shown to be a risk factor for both fibrosis and IH, whereas the presence of autoantibodies was a risk factor only for fibrosis. It is assumed that the presence of autoantibodies is one of the clinical signs that indicate immunologically activated status, and late-onset acute cellular rejection may occur as a consequence of an immunologically activated status. These results suggest that it is difficult to predict immunological activation by monitoring the presence of known autoantibodies and that unknown antibodies may play an important role in the development of fibrosis and IH, as well as late-onset acute cellular rejection.

This study has certain limitations. To confirm the mechanism of the production of occult antibodies by hepatocytes and biliary epithelial cells, another approach will be needed. In our series, the biopsies were taken as clinically indicated and the serum samples were taken shortly after this; therefore, the timepoint was variable. Indirect immunohistochemistry with longitudinally collected serum samples may be useful to indicate when these antibodies are produced. According to preliminary data of longitudinal investigations in one patient who showed positive reaction for hepatocytes in this present study, the result was negative with pretransplant serum sample, and it turned out to be positive with a sample collected on postoperative day 7 and strongly positive with a sample collected on postoperative day 28 in terms of hepatocyte staining. This patient had episodes of late-onset acute cellular rejection, and then showed fibrosis two yr after LT. These results indicate that antibodies to liver tissue may be produced after LT. However, more detailed examination will be needed to clarify this issue.

In conclusion, indirect immunohistochemistry between liver recipient serum between human liver tissue showed antigen–antibody reaction on hepatocytes or biliary epithelial cells, which indicates that there is a possibility that the pathogenesis of fibrosis and/or IH observed in pediatric liver transplant recipients in the chronic phase is immunological. To improve the long-term outcome of liver transplant recipients, an investigation into the mechanisms of late-onset immunological morbidity is crucial. The present results suggest that immune-mediated reactions by occult antibodies underlie the pathogenesis of fibrosis and IH observed in liver transplant recipients.

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