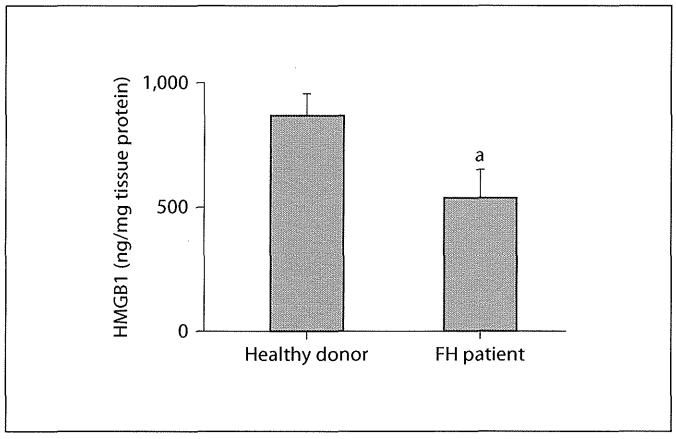

Fig. 5. Hepatic tissue levels of HMGB1 in patients with FH and healthy donors. [a] $p < 0.05$ versus healthy donors. Data represent mean + SD.

Liver Histology and Immunohistochemistry for HMGB1

Histological examination of hematoxylin- and eosin-stained sections from healthy donors showed no evidence of pathological changes (fig. 4a, b). In contrast, panlobular necrosis or extensive liver cell loss with hemorrhagic collapsed stroma and proliferation of ductular-like structures was noted in liver sections from FH patients (fig. 4c, d). Immunohistochemical staining for HMGB1 was strong and clear in the nuclei of hepatocytes in liver sections from healthy donors (fig. 4e, f), but little staining in either nuclei or cytoplasm was evident in specimens from FH patients (fig. 4g, h). Image analysis showed that the average intensity of HMGB1-positive nuclei, the number of HMGB1-positive nuclei, and the total intensity of HMGB1-positive nuclei were all significantly lower in FH livers than in healthy-donor livers (fig. 4i–k).

Hepatic Tissue Levels of HMGB1

The hepatic tissue levels of HMGB1 (ng/mg tissue protein) were 539 ± 116 in patients with FH and 874 ± 81 in healthy donors (fig. 5). There was a significant difference between the levels in patients with FH and healthy donors ($p < 0.05$).

Discussion

Since the first report of HMGB1 as 'death mediator' in 1999 [5], a growing body of research in animals and humans has indicated that HMGB1 is involved in various disease conditions [4–23]. The role of HMGB1 in ALF has not been thoroughly elucidated, though it is very likely that HMGB1 is released from the necrotic liver in ALF. Zhang et al. [12] reported that the plasma levels of HMGB1 were increased in a porcine D-galactosamine-induced ALF model. Our previous study also showed that the plasma levels of HMGB1 were increased in a rat model of drug-induced ALF [11]. However, there is no animal model that is completely analogous with ALF in humans. Therefore, we investigated plasma levels of HMGB1 in patients with ALF. This study showed that the mean HMGB1 levels were higher in patients with liver disease, especially FH-PE– patients, than in the HV group. Moreover, we clearly showed that there was a significant and strong relationship between the mean plasma HMGB1 level and the logarithm of mean AST. Since FH-PE+ patients underwent PE before sample collection, we analyzed the relationship among the diseases either including or excluding FH-PE+, and found a strong positive correlation in both analyses. Based on the findings above, it appears that plasma levels of HMGB1 are increased in liver diseases in proportion with disease severity. We also compared plasma HMGB1 levels before and after liver transplantation or conservative treatment and found that the levels were markedly or significantly decreased after successful treatment. Moreover, plasma HMGB1 levels showed similar changes to AST levels in 1 patient for whom samples were collected serially after liver transplantation. These results also support the possibility that patients have increased plasma HMGB1 levels when hepatic function is impaired.

After confirming that the plasma level of HMGB1 was increased in ALF patients, we assessed hepatic tissue HMGB1 levels to determine whether HMGB1 was released from the liver. Previous studies have suggested that HMGB1 release can occur passively from injured nuclei or actively via secretion from monocytes/macrophages [29–31]. Based on immunohistochemical findings, image analyses, and tissue HMGB1 levels, it was evident that the hepatic tissue level of HMGB1 was lower in FH livers than in healthy-donor livers. These findings were compatible with our previous findings in a rat model of drug-induced liver failure [11]. Although it is still not clear from our studies whether active secretion by macrophages of HMGB1 into the plasma contributes to HMGB1 elevation under ALF conditions, it does appear that passive leakage from injured hepatocyte nuclei is involved. It also remains unclear whether the liver is the only contributor to elevated plasma HMGB1 levels or if other sources are involved. In the rat ALF model, pathological changes in the

lung and kidney were minimal, suggesting that the main origin of plasma HMGB1 was the liver [11]. In the present study, samples of nonhepatic organs, such as the lung and kidney, were not available. Based on physiological and laboratory findings of the patients' respiratory and renal condition (i.e. arterial oxygen saturation, chest X-ray, urinary output, and plasma creatinine level; data not shown), the extent of damage might be minor in the lungs and kidneys compared to the liver. The fact that all patients recovered after liver transplantation also supports the observation that damage to nonhepatic organs was minor. It appears likely that the liver is the main contributor to plasma HMGB1 elevation in ALF patients without multiple organ failure. Since ALF patients are at risk of developing a wide variety of complications including sepsis, and since sepsis increases plasma HMGB1 levels [4, 5, 13, 17], further investigation focusing on the complications is needed to determine exactly which organs or conditions contribute to HMGB1 elevation in ALF patients.

It is also important to determine whether HMGB1 is a prognostic factor for ALF. Previous reports have investigated the prognostic significance of HMGB1 in a variety of diseases in humans. Suda et al. [19] reported that the preoperative serum level of HMGB1 was higher in patients with postoperative complications, suggesting that HMGB1 is a predictor of the clinical course after surgery. Yasuda et al. [20] and Bitto et al. [21] reported that serum HMGB1 levels were significantly increased in patients with either severe acute pancreatitis or lung injury and that the levels were higher in nonsurvivors than in survivors. However, other studies have failed to confirm the prognostic value of HMGB1 [22, 23]. Since most of the patients in the present study survived after the treatments, we could not collect a sufficient number of samples to enable a meaningful analysis. Additional analyses of samples from nonsurvivors are needed to determine the value of plasma HMGB1 levels as a predictor of the clinical outcome in ALF patients. HMGB1 also represents a potential therapeutic target for ALF. In the rat ALF model, various blood, histologic and survival parameters were improved following a single injection of anti-HMGB1 antibody [11]. Bianchi [32] emphasized that HMGB1 forms specific complexes with other signaling molecules such as inflammatory cytokines and that the complexes exert their biological actions via receptors. ALF patients have an inflammatory cytokine storm [26]. Therapies targeting HMGB1 or HMGB1 complexes await validation in further human clinical trials.

In conclusion, we confirmed that plasma HMGB1 levels were increased in patients with ALF. Based on a comparison between the HMGB1 contents in normal and ALF livers, it is very likely that HMGB1 is released from injured liver tissue. Future studies should include a mechanistic investigation to clarify whether HMGB1 is actually involved in the pathophysiology of ALF and whether it can serve as an indicator of disease severity.

Acknowledgements

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ELSEVIER

Pharmacokinetics of Mizoribine in Adult Living Donor Liver Transplantation

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ABSTRACT

We investigated the pharmacokinetics of mizoribine in the acute phase after adult living donor liver transplantation (LDLT). Between February 2004 and October 2009, 16 recipients received immunosuppressive therapy that included mizoribine (100 to 200 mg/d) after undergoing LDLT. We determined the serum levels of mizoribine before (C0) and 3 (C3), 4 (C4), and 10 (C10) hours after administration on postoperative days 3, 7, and 21. We assessed area under the concentration time curve (AUC) (hour · μg/mL), normalized serum concentration (NSC) at C0 [concentration (μg/mL)/dose (mg/kg body weight)], and estimated glomerular filtration rate (eGFR). The mizoribine concentration showed increases at C3 and C4 followed by a decrease at C10 on all days. AUC was 4.3, 5.9, and 8.3 in the 200-mg/d dose group on days 3, 7, and 21, respectively. NSC at C0 increased for 3 weeks after LDLT. There was a significant correlation between the NSC at C0 and eGFR on day 21, but not on days 3 and 7. There were no correlations between the NSC at C0 and either aspartate aminotransferase, total bilirubin, albumin, trough cyclosporine, or trough tacrolimus on any day. The pharmacokinetics of mizoribine in the acute phase after LDLT seems to be affected by postoperative day and renal function.

MIZORIBINE IS AN ORAL IMMUNOSUPPRESsive agent approved in Japan, Korea, and China for the prevention of graft rejection in renal transplantation. Its immunosuppressive potential is promising, and three-drug combination therapy with a calcineurin inhibitor, a steroid, and mizoribine is sometimes used for patients after renal transplantation.[1–3] The application of mizoribine has now been extended to lupus nephritis, chronic rheumatoid arthritis, and nephritic syndrome treatment in Japan.[4] Using antimetabolites as immunosuppressants in combination therapy may be beneficial for reducing the dose and side effects of calcineurin inhibitors or steroids after living donor liver transplantation (LDLT).[5,6] Due to the absence of information on the pharmacokinetics of mizoribine in liver transplantation, the options for secondary and tertiary agents in immunosuppressive combination therapy are limited to azathioprine (AZA) and mycophenolate mofetil (MMF). It is critical to ensure that the concentrations of the immunosuppressive agent used is maintained within an appropriate range, especially in the acute phase after LDLT, because even a minor failure in management postsurgically, when liver graft volume and function are not fully recovered, can be lethal. Since mizoribine is excreted from the kidneys, and since AZA and MMF are metabolized or activated in the liver, it is worthwhile to investigate the pharmacokinetics of these antimetabolites in patients with hepatic dysfunction. In this study, we monitored mizoribine levels on postoperative day 3, 7, and 21 and assessed the pharmacokinetics of mizoribine in the acute phase of LDLT.

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PATIENTS AND METHODS

Patients

Between February 2004 and October 2009, 16 transplant recipients were treated with an immunosuppressive regimen that included mizoribine after undergoing LDLT. The backgrounds of the patients are summarized in Table 1. The immunosuppressive regimen was a three-drug combination therapy. In cases of hepatitis C positivity, basiliximab was used instead of a steroid. In cases of ABO blood-type incompatibility, the three-drug combination therapy and additional regimens were employed; patients were preoperatively administered rituximab twice, and a steroid, prostaglandin El, and gabexate mesilate were administered through the portal vein for 3 weeks postoperatively. Mizoribine was given orally twice a day at a dose of 100 mg/d in the initial three cases, and 200 mg/d in the other 13 cases. Tacrolimus was chosen as the calcineurin inhibitor for the initial cases of hepatitis C positivity and ABO blood type incompatibility, and cyclosporine was used in the other cases.

Serum samples were collected before and 3, 4, and 10 hours after dosing on postoperative days 3, 7, and 21. All serum samples were analyzed to determine the mizoribine concentration. The concentrations before and 3, 4, and 10 hours postadministration were defined as C0, C3, C4, and C10, respectively. Blood samples were centrifuged for 5 minutes at 1500g, and all serum samples were stored at −80°C prior to being assayed. Informed consent was obtained from each patient or family, and the study protocol conformed to the ethical guidelines of Keio University School of Medicine.

Mizoribine Assay

Concentrations of mizoribine in serum were measured by Asahi Kasei Pharma Corporation (Tokyo, Japan) using high-performance liquid chromatography (HPLC). The serum was deproteinized and filtered (Ultra-Free C3LCC, Millipore, Tokyo). Filtrate (10 μL) was injected into an HPLC column (Shim-Pack CLC-NH2 15 cm × 6.0 mm internal diameter, Shimadzu, Kyoto). The mobile phase consisted of 66.7 mmol/L phosphate buffer (pH 2.5) and acetonitrile (27.5:72.5), and the flow rate was set at 1.3 mL/min. The drug was detected at a wavelength of 280 nm using a UV detector, and the detection limit was 0.02 μg/mL.

Table 1. Patient Background

Liver diseases	
Virus-related liver cirrhosis	11
Fulminant hepatitis	2
Primary biliary cirrhosis	2
Budd-Chiari syndrome	1
Sex	
Male	10
Female	6
Age (y)	49.5 ± 9.5
Body weight (kg)	60.6 ± 10.5
ABO blood type compatibility	
Identical	8
Compatible	4
Incompatible	4
Immunosuppressive regimen	
Cyclosporine, steroid, and mizoribine	8
Cyclosporine, basiliximab, and mizoribine	2
Tacrolimus, steroid, and mizoribine	2
Tacrolimus, basiliximab, and mizoribine	3
Cyclosporine to tacrolimus convert on day 14, steroid, and mizoribine	1
Mizoribine dose (minimum to maximum, mg/kg/day)	0.60–4.0

Blood Biochemistry

The serum levels of aspartate aminotransferase (AST), total bilirubin (TB), creatinine, albumin, and trough levels of cyclosporine and tacrolimus were determined by a biochemistry laboratory in our hospital.

Pharmacokinetic Parameters and Estimated Glomerular Filtration Rate

The area under the concentration-time curve (AUC) (hour · μg/mL) was estimated for the 100-mg/d dose patients, 200-mg/d dose patients, and all patients by summing three trapezoidal areas (C0 to C3, C3 to C4, and C4 to C10). Each trapezoid area was calculated by multiplying the concentration (μg/mL) by time (hours). The concentration of mizoribine was normalized according to dose and body weight using the following equation: [normalized serum concentration (NSC)] = [concentration of mizoribine (μg/mL)]/[dose of mizoribine (mg/kg)]. The area under the NSC-time curve [(hour · μg/mL)/(mg/kg)] was estimated for all patients by summing three trapezoidal areas (0 to 3 hours, 3 to 4 hours, and 4 to 10 hours). Each trapezoid area was calculated by multiplying the NSC [(μg/mL)/(mg/kg)] by time (hour). The highest concentration among C0, C3, C4, and C10 was defined as Cmax (μg/mL). The time from C0 to Cmax was defined as Tmax (hours). Clearance of mizoribine (Cl, L/h) was estimated by the following method: (1) elimination rate constant (kel) was calculated using the equation; kel ($hour^{-1}$) = −([natural logarithm of C10] − [natural logarithm of C4])/(10 − 4); (2) C12 was estimated using the equation: $C12 = C10 \times \exp^{(-kel \times 2)}$; (3) the AUC of C0 to C12 was estimated by adding three trapezoidal areas (C0 to C3, C3 to C4, and C4 to C10) and one additional trapezoid (C10 to estimated C12); and (4) Cl was estimated using the equation; Cl (L/h) = [mizoribine dose per intake (mg)]/[estimated AUC of C0 to C12 (hour · μg/mL)]. The glomerular filtration rate (GFR) was estimated using the following equation: estimated GFR (eGFR, mL/min/1.73 m^2) = 194 × serum $creatinine^{-1.094} \times age^{-0.287}$ (if female, × 0.739).[7]

Adverse Events

A diagnosis of acute cellular rejection (ACR) was reached when patients showed elevation of hepatic enzymes and needle liver biopsy results showed more than moderate-grade ACR. Patients were diagnosed as having symptomatic infection if they had prolonged high fever and infection marker positivity (bacteria, cytomegalovirus, etc), or asymptomatic infection if they had the infection marker positivity without high fever. Patients were diagnosed as having hepatic dysfunction if they had reelevation of hepatic enzymes, and as having renal dysfunction if they underwent serum filtration.

Statistical Analysis

Results are expressed as means ± standard deviations (SDs) unless noted otherwise. For parametric data, differences between groups were evaluated using Student *t* test for unpaired data, based on the assumption that the data were derived from populations with equal SDs. Correlations were evaluated using the Spearman rank test. Differences were considered significant at *P* values less than .05.

RESULTS

Serum Mizoribine Concentrations

Figure 1a shows mean mizoribine concentrations in all patients in this study on postoperative days 3, 7, and 21. The levels were increased at C3 and C4 followed by a decrease at C10 on postoperative days 3, 7, and 21. The highest concentrations were 0.40, 0.65, and 0.90 μg/mL at C4 on postoperative days 3, 7, and 21, respectively. The C3/C0 ratios were 1.80 ± 0.73, 2.07 ± 1.68, and 1.94 ± 0.50 on postoperative days 3, 7, and 21; the respective C4/C0 ratios were 2.07 ± 1.33, 1.88 ± 1.14, and 1.79 ± 0.57, and the C10/C0 ratios were 1.54, 1.35, and 0.86. Figures 1b and 1c show mean mizoribine concentrations on postoperative days 3, 7, and 21 in the 200 and 100-mg/d dose groups,

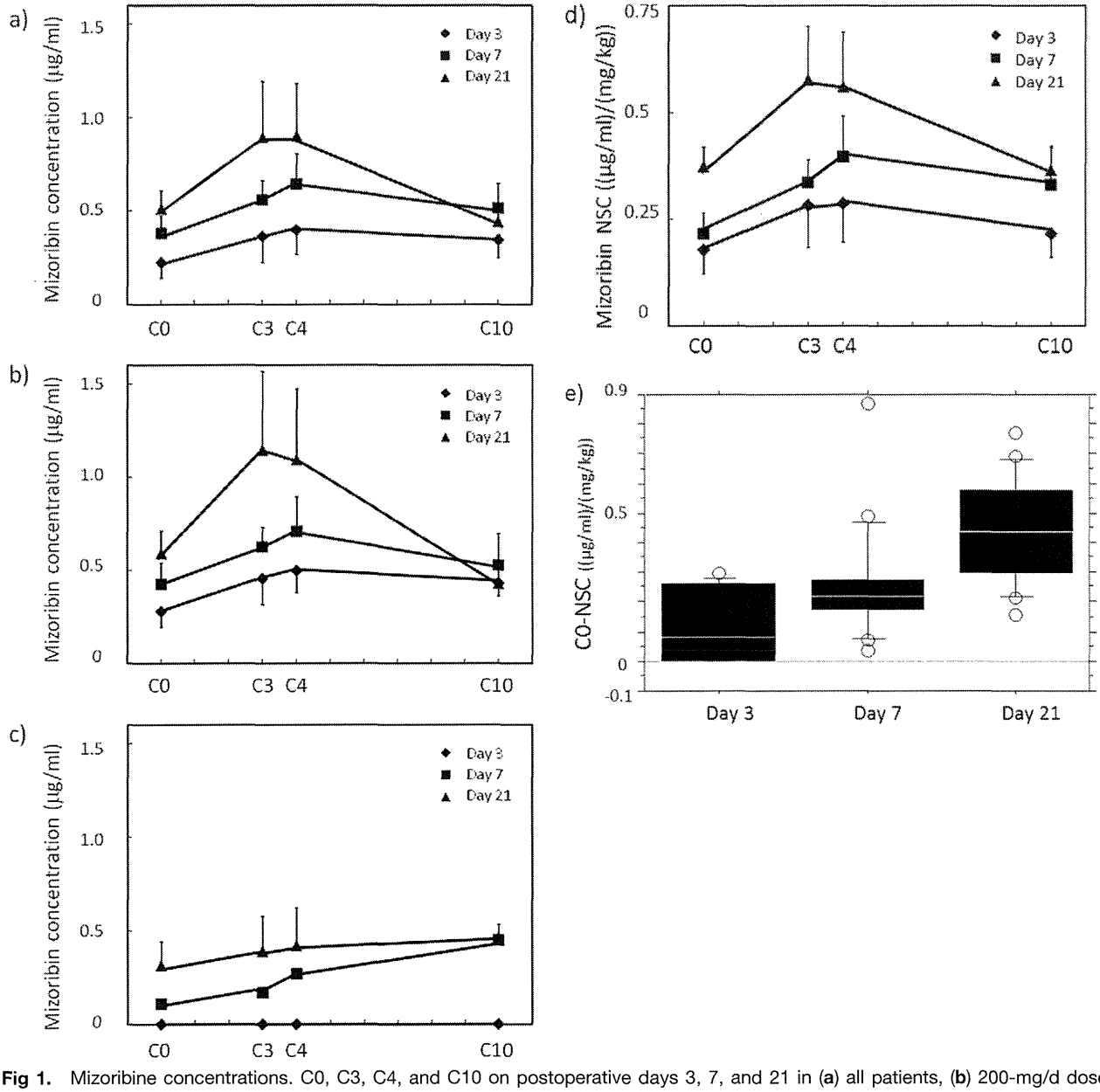

Fig 1. Mizoribine concentrations. C0, C3, C4, and C10 on postoperative days 3, 7, and 21 in (**a**) all patients, (**b**) 200-mg/d dose patients, and (**c**) 100-mg/d dose patients. (**d**) NSC at C0, C3, C4, and C10 on postoperative days 3, 7, and 21 in all patients. Results are expressed as mean + SEM or mean − SEM. C0, C3, C4, and C10; serum mizoribine concentration before and 3, 4, and 10 hours after mizoribine administration, respectively. (**e**) A quantile box plot of NSC at C0 on postoperative days 3, 7, and 21. The box for each day represents the interquartile range (25–75th percentile) and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated as open circles. NSC, normalized serum concentration; SEM, standard error of the mean.

respectively. Figure 1d shows NSC in all patients on postoperative days 3, 7, and 21. The NSC at C0 was increased in a time-dependent manner from day 3 to 21 and was significantly higher on postoperative day 21 compared to days 3 and 7 ($P < .05$; mean ± SD values of NSC at C0: 0.18 ± 0.14, 0.22 ± 0.14, and 0.37 ± 0.13 on days 3, 7, and 21, respectively). Figure 1e shows a quantile box plot of NSC at C0 on postoperative days 3, 7, and 21.

Pharmacokinetic Parameters

AUC, area under the NSC time curve, C0, Cmax, Tmax, and Cl are shown in Table 2. Results are expressed as mean ± SD. Maximum, median, and minimum values are indicated parenthetically in order.

Estimated Glomerular Filtration Rate

A quantile box plot of Fig 2 shows eGFR on postoperative days 3, 7, and 21. The mean ± SD eGFR values were 78 ± 23, 78 ± 31, and 64 ± 32 on days 3, 7, and 21, respectively. There were no significant differences among these values.

Effect of Parameters on Mizoribine Concentration

There was a significant correlation between the NSC at C0 and eGFR on day 21 (Fig 3c, $R^2 = 0.495$, $P < .05$), but not on days 3 and 7 (Fig 3a, 3b). There were no correlations between the NSC at C0 and either AST, TB, albumin, trough cyclosporine, or trough tacrolimus on any day (the R^2 values were extremely low and P values were >.05 in these analyses). There were no differences between the mizoribine trough NSCs at C0 in patients with tacrolimus and cyclosporine. There were no differences between the mizoribine trough NSCs at C0 in patients with and without steroids.

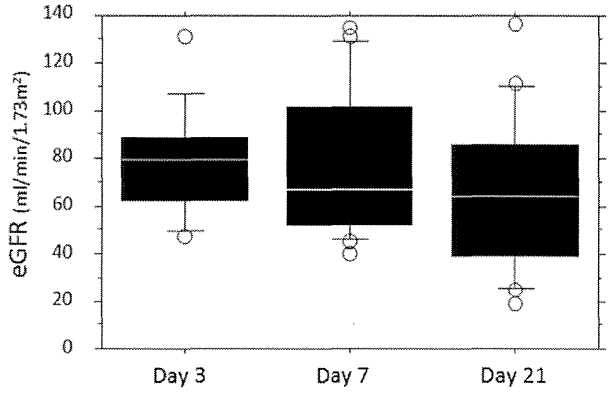

Fig 2. A quantile box plot of eGFR on postoperative days 3, 7, and 21. The box for each day represents the interquartile range (25–75th percentile) and the line within this box is the median value. Bottom and top bars of the whisker indicate the 10th and 90th percentiles, respectively. Outlier values are indicated as open circles. eGFR, estimated glomerular filtration rate.

Table 2. Pharmacokinetic Parameters

	100 mg/d	200 mg/d	All patients
Area under the concentration time curve (h · μg/mL)			
Postoperative day 3	0	4.3	3.4
Postoperative day 7	2.8	5.9	5.4
Postoperative day 21	4.1	8.3	7.0
Area under the NSC time curve [(h · μg/mL)/(mg/kg)]			
Postoperative day 3			2.4
Postoperative day 7			3.3
Postoperative day 21			4.7
C0 (μg/mL)			
Postoperative day 3	0 (0, 0, 0)	0.28 ± 0.19 (0.51, 0.38, 0.36)	0.22 ± 0.20 (0.51, 0.36, 0)
Postoperative day 7	0.18 ± 0.09 (0.24, 0.17, 0.11)	0.43 ± 0.28 (0.89, 0.35, 0.13)	0.36 ± 0.26 (0.89, 0.26, 0.11)
Postoperative day 21	0.34 ± 0.16 (0.47, 0.40, 0.16)	0.60 ± 0.26 (1.07, 0.56, 0.28)	0.52 ± 0.26 (1.07, 0.39, 0.16)
Cmax (μg/mL)			
Postoperative day 3	0 (0, 0, 0)	0.59 ± 0.22 (0.89, 0.61, 0.36)	0.59 ± 0.22 (0.89, 0.61, 0.36)
Postoperative day 7	0.35 ± 0.15 (0.45, 0.34, 0.24)	0.48 ± 0.50 (1.64, 0.59, 0.36)	0.66 ± 0.42 (1.64, 0.58, 0.24)
Postoperative day 21	0.49 ± 0.16 (0.67, 0.42, 0.38)	1.08 ± 0.82 (2.69, 0.83, 0.44)	0.88 ± 0.72 (2.69, 0.67, 0.38)
Tmax (h)			
Postoperative day 3		4.2 ± 4.1 (4, 3, 0)	4.2 ± 4.1 (4, 3, 0)
Postoperative day 7	5.0 ± 7.0 (10, 5, 0)	2.8 ± 1.4 (4, 3, 0)	3.4 ± 2.9 (10, 3, 0)
Postoperative day 21	5.6 ± 3.7 (10, 4, 3)	4.5 ± 2.7 (10, 3.5, 3)	4.8 ± 2.9 (10, 4, 3)
Cl (L/h)			
Postoperative day 3		20.7 ± 6.3 (28.2, 18.7, 12.7)	20.7 ± 6.3 (28.2, 18.7, 12.7)
Postoperative day 7	23.4 ± 14.4 (33.6, 23.4, 13.2)	18.0 ± 9.4 (30.1, 10.7, 7.1)	19.9 ± 9.7 (33.6, 19.2, 7.1)
Postoperative day 21	11.8 ± 4.3 (15.7, 12.6, 7.1)	14.3 ± 9.5 (30.4, 11.8, 5.1)	13.4 ± 7.6 (30.4, 12.2, 5.1)

NSC, normalized serum concentration; C0, serum mizoribine concentration before mizoribine administration; Cmax, highest concentration of serum mizoribine; Tmax, time from mizoribine administration to Cmax; Cl, clearance of mizoribine.

Adverse Events and Patient Outcomes

The incidence of adverse events is shown in Table 3. The seven cases of asymptomatic infection were patients who tested positive for cytomegalovirus infection but did not present with fever. All patients survived more than 3 weeks after operation.

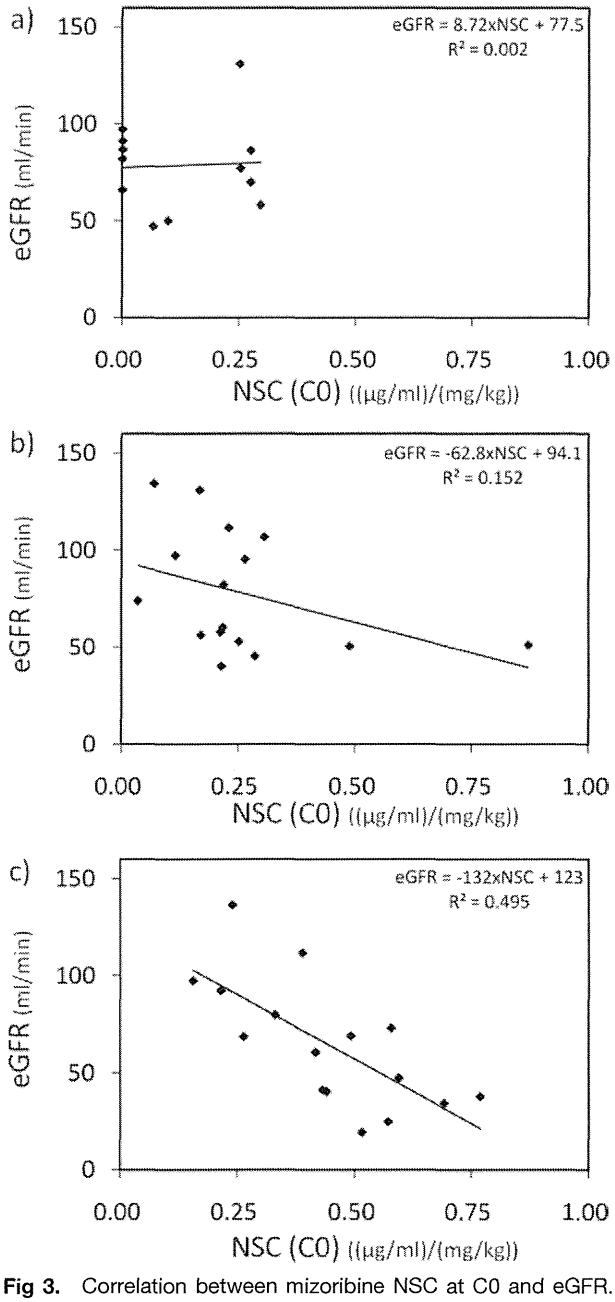

Fig 3. Correlation between mizoribine NSC at C0 and eGFR. Correlation between mizoribine NSC at C0 and eGFR on postoperative days 3 (**a**), 7 (**b**), and 21 (**c**). NSC, normalized serum concentration; eGFR, estimated glomerular filtration rate.

Table 3. Incidences of Adverse Events

	No. of cases
Mortality	0 (0%)
Antibody-mediated rejection	0 (0%)
Acute cellular rejection	1 (6%)
Central nervous disorder	1 (6%)
Hepatic dysfunction	1 (6%)
Pancytopenia	1 (6%)
Symptomatic infection	2 (12%)
Renal dysfunction	3 (18%)
Asymptomatic infection	7 (43%)

DISCUSSION

Our preliminary study in initial cases after LDLT showed that peak concentrations of mizoribine occurred 3 or 4 hours postadministration. Sugitani and colleagues reported that peak drug concentrations were reached approximately 3 hours after intake in patients who were treated with mizoribine more than 1 month after renal transplantation, at which time their condition had stabilized.[3] Therefore, we decided to collect serum samples before and 3, 4, and 10 hours after oral mizoribine administration to assess peak drug levels. The analysis showed that the highest concentration was 0.40, 0.65, and 0.90 μg/mL at C4 on postoperative days 3, 7, and 21, respectively, and the peak level was approximately twice the level at C0 on all days. Sugitani et al studied patients who took higher doses of mizoribine (4 to 6 mg/kg/d) and reported that the peak concentration was 2.87 μg/mL, the peak level was approximately twice the trough level, and there were few adverse events.[3] Our study employed LDLT patients who took lower doses of mizoribine (0.60 to 4.0 mg/kg/d) and showed that the peak concentrations were much lower than those reported by Sugitani et al. The lower concentrations in our study might be a result of not only the lower mizoribine dose used but also differences in intestinal absorption and renal excretion between subject populations. Since the incidence of adverse events in our study and that of Sugitani et al was acceptably low in both cases, a potential alternative mizoribine protocol for LDLT could include a higher dose of mizoribine to achieve higher trough and peak drug levels. However, the optimal serum concentration of mizoribine in organ transplantation patients has never been determined. In a study by Sonda et al, which employed the mixed lymphocyte reaction assay to assess the effects of mizoribine on peripheral lymphocytes from healthy adults, the inhibition rates were 2.4%, 36.4%, 43.8%, 52.6%, 62.2% at mizoribine concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 μg/mL, respectively (8). According to these data, the drug doses used by Sugitani et al and in our study may have had an immunosuppressive effect on lymphocytes. Future clinical studies should determine the optimal serum concentration of mizoribine when it is used as a second or third agent in combination immunosuppressive therapy.

This study also showed that the NSC at C0 increased from postoperative day 3 to 21, suggesting that the NSC at

C0 did not reach a steady state until 3 weeks after the operation. The time to reach steady-state drug levels is generally calculated by multiplying the half-life of the drug by 3 to 5, if the excretion rate is stable. The half-life of mizoribine is 1.6 hours in patients with normal renal function (creatinine clearance > 70 mL/min) and 4.6 hours in patients with severely impaired renal function (creatinine clearance < 40 mL/min),[9] and it can be estimated that steady-state levels of mizoribine were reached within a few days postadministration in LDLT patients. The present finding that the NSC at C0 did not reach to a steady state until 3 weeks postoperatively suggests that the time to reach steady state was markedly prolonged in the LDLT patients. The pharmacokinetics of mizoribine depends on both intestinal absorption and renal excretion.[4] eGFR showed that levels were virtually unchanged on days 3 and 7 and slightly decreased on day 21. It is known that intestinal absorption is impaired in patients who have undergone long-duration laparotomy. Absorption may also be impaired in LDLT, which sometimes takes more than 10 hours to perform and involves major surgical procedures on the intestine. Therefore, we reason that the prolonged time to reach steady-state levels of mizoribine within 3 weeks after LDLT could be mainly attributable to poor absorption due to delayed gastric emptying and reduced intestinal motility following surgery. Recent studies have investigated the potential impact of bile flow[10] and the drug transporter of concentrative nucleoside transporter 1 polymorphisms[11] on mizoribine absorption. These factors might contribute to interindividual differences in the plasma disposition of mizoribine. Assessing the status of cholestasis in the liver and intestine of patients by measuring bile flow from biliary drainage tubes and genotyping for concentrative nucleoside transporter may provide additional insight into postoperative mizoribine absorption.

Because mizoribine is excreted by the kidneys, the serum concentration of mizoribine achieved during therapy should correlate with renal function. In fact, there was a significant relationship between trough NSC at C0 and eGFR on day 21. It is noteworthy that the correlation between NSC at C0 and eGFR was found only on day 21, but not on days 3 and 7. Sonda et al reported that the dose of mizoribine should be adjusted according to renal function in patients after renal transplantation.[8] We assume that this suggestion may not apply in the acute phase (several weeks) after LDLT because it may take time for mizoribine absorption to fully recover. It is reasonable that the serum concentration of mizoribine was independent of hepatic function represented by the hepatic markers of AST or TB, because mizoribine is neither metabolized nor activated in the liver. We investigated other possible factors that could be associated with mizoribine concentrations. One very important aspect and potential confounder in all pharmacokinetic studies after transplantation is the albumin concentration. However, mizoribine does not bind to proteins and, in fact, this study demonstrated that there was no relationship between trough NSC at C0 and albumin concentration on days 3, 7, and 21. Another aspect of interest is the impact of concomitant immunosuppressants such as steroids and calcineurin inhibitors on mizoribine concentrations. Hohage and colleagues reported that cyclosporine withdrawal resulted in a significant increase in the trough levels and AUC of mycophenolic acid in a group of renal transplant recipients with impaired renal function.[12] It is not known whether calcineurin inhibitors have a pharmacological effect on mizoribine concentrations. This study showed that there were no differences between the mizoribine trough NSCs at C0 in patients treated with tacrolimus and cyclosporine. Similarly, there were no differences between the mizoribine trough NSCs at C0 in patients with and without steroids. Therefore, this study did not find any factors associated with mizoribine concentration other than except renal function.

The efficacy and safety of mizoribine when used after LDLT is of great interest. Although mizoribine is now our preferred choice as a third agent in combination therapy, we previously used AZA or MMF as the third immunosuppressive agent together with a calcineurin inhibitor and a steroid in LDLT patients. It cannot be determined from this study if the immunosuppressive effect and incidence of adverse events with mizoribine are equivalent to those with AZA and MMF because the backgrounds of patients in whom mizoribine, AZA, and MMF were used are not comparable. We did not encounter lethal or severe adverse events arising from the use of mizoribine in this study. The finding that there was no relationship between NSC at C0 and AST or TB also provides information about the pharmacodynamics of mizoribine (ie, low-dose mizoribine may have a minimal adverse effect on hepatic function). Although our conclusions are limited by the small sample size in this study and a lack of comparative studies, it appears that low doses of mizoribine may be used safely after liver transplantation.

In the present study, we reported the pharmacokinetics of mizoribine in the acute phase after LDLT. The trend from C0 to C10 clearly showed that there were daily troughs and peaks, as was shown in a past study on renal transplantation by Sugitani and associates.[3] However, in our study both the peak and trough levels were much lower than those reported by Sugitani et al, probably because our protocol employed a relatively low dose of mizoribine. A new finding is that trough level increases for 3 weeks postoperatively. We assume that mizoribine adsorption is poor in the very early postoperative phase and, therefore, it takes 3 weeks until the serum concentration of this agent reaches a steady state. Mizoribine undergoes renal excretion, and its serum concentration should show a correlation with renal function if absorption is stable. It may not be possible to apply this correlation in the initial days following surgery, and a higher dose may be needed to compensate for poor absorption. The dose may need to be adjusted for renal excretion if more than 3 weeks passes after surgery. It is expected that mizoribine would have minimal adverse effects on hepatic function after surgery. Although there is

a period of poor mizoribine absorption in the very early postoperative phase, it could be a valuable alternative to agents that are metabolized or activated hepatically in cases where a renally excreted antimetabolite is preferable.

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Suppressive Effects of Interleukin-18 on Liver Function in Rat Liver Allografts

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Background. **Interleukin-18 (IL-18) is a potent proinflammatory cytokine that augments both innate and acquired immune responses. It is also a crucial regulator of lymphocyte production of interferon-γ (IFN-γ), which can promote acute cellular rejection of transplanted solid organs.**

Methods. **To evaluate the role of IL-18 in liver transplantation, we constructed an adenoviral vector encoding IL-18 binding protein (Adex-IL18bp), which specifically suppressed the biologic activity of IL-18, and examined the effect of this suppression on liver allografts by using a high-responder rat model (ACI to Lewis) of orthotopic liver transplantation (OLTx). Donor rats were given one intravenous injection of Adex-IL18bp or Adex-LacZ (control vector) 2 d before OLTx.**

Results. **Seven days after OLTx, overexpression of IL-18bp resulting from the adenovirus gene transfer was associated with significantly decreased serum alanine aminotransferase levels and less histologic hepatic injury in recipient rats with Adex-IL18bp-pretreated donors compared with Adex-LacZ controls. Adex-IL18bp pretreatment also significantly prolonged rat/allograft survival, inhibited expression of IFN-γ, and reduced levels (*versus* control values) of both CXCL10 and CX3CL1, which can be induced by IFN-γ.**

Conclusion. **These results suggest that IL-18 has an important role in liver allograft rejection through IFN-γ and chemokines and that specific suppression of IL-18 may improve liver function early after transplantation.**

Key Words: **adenovirus vector; interferon-γ; interleukin-18; liver allograft.**

INTRODUCTION

Much progress has been made in identifying the cellular and molecular mechanisms by which cytokines participate in rejection processes after organ transplantation. Although Th1- and Th2-type cytokine profiles have been associated with, respectively, rejection and tolerance, recent studies have suggested that the mechanism of allograft rejection involves not simply Th1/Th2 deviations, but that regulation within the cytokine network is substantially more complicated, with substantial contributions from several cytokines. One cytokine that may affect both Th1 and Th2 responses is interleukin (IL)-18 [1].

IL-18 is produced not only by various immune cells but also by non-immune cells such as intestinal and airway epithelial cells. In collaboration with IL-12, IL-18 stimulates Th1-mediated immune responses, which play a critical role in host defense against intracellular microbes through the induction of interferon-γ (IFN-γ).

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However, overproduction of IL-12 and IL-18 induces severe inflammatory disorders, suggesting that IL-18 is a potent proinflammatory cytokine that is pathophysiologically involved in several inflammatory conditions [2]. Moreover, IL-18 has been found to be significantly up-regulated in both the serum and the alloresponse site in patients with acute rejection of a kidney allograft [1]; therefore, this cytokine may play an important part in acute rejection of transplanted solid organs.

IL-18 binding protein (IL-18bp) was first identified in the urine of healthy persons and purified by using IL-18-coupled beads [3]. IL-18bp is thought to be a soluble decoy receptor because it can specifically block binding of mature IL-18 to its authentic receptor and thereby inhibit IL-18-induced IFN-γ production [2]. Since IL-18 is an early stimulant of Th-1 cells, IL-18bp probably has an important role in regulation of the immune response [2].

Acute rejection of liver allografts is characterized histologically by a mixed portal-tract infiltrate containing mononuclear cells. An accumulation of activated lymphocytes in the allograft is essential to the pathogenesis of tissue injury. The mechanism by which these lymphocytes are recruited to the graft from the circulation is poorly understood, but it probably involves local chemotactic factors that promote migration, positioning, and retention of effector cells in the graft [4, 5]. Chemokines are expressed and secreted by a wide variety of cell types, including lymphocytes [6] and endothelial components of allografts activated during rejection [4, 7-9]. Several studies have shown that both CXCL10 (IFN-γ-inducible protein 10) and CX3CL1 (fractalkine), which are induced by IFN-γ [10], are up-regulated during rejection of murine cardiac [11, 12] and rat liver allografts [5].

The aim of the current study was to assess the role of IL-18 in liver transplantation. We constructed an adenoviral vector encoding IL-18bp, which specifically suppressed the biologic activity of IL-18. We then examined the effects of this inhibition in a high-responder model of rat orthotopic liver transplantation (OLTx).

MATERIALS AND METHODS

Cloning of Rat IL-18bp cDNA and Construction of Adenoviral Vector

Rat IL-18bp cDNA was amplified from the Rat Liver Marathon-Ready cDNA library (Clontech, Tokyo, Japan) by using polymerase chain reaction (PCR) with the following primers: 5′-CATGCAGGTCTCCCATGAGACACTGTGGCTGTGCAGCAG-3′ and 5′-TGGTTGCTGGAGTGGGGCCCCTGGGCCTGCTGATCTGG-3′ (underlining indicates the restriction-enzyme sites of *Bsa*I and *Xho*I, respectively). A plasmid vector, pTriEx-rIL18bpHis, which expresses rat IL-18bp with a C-terminal 6xHis tag, was constructed by inserting the amplified fragment digested with *Bsa*I and *Xho*I into the *Nco*I-*Xho*I site of the plasmid pTriEx1.1 (Novagen, Tokyo). The sequence of the insert was then confirmed.

The replication-defective adenoviral vector containing the CAG promoter, *E coli lac*Z gene, and poly(A) sequence (that is, the Adex-LacZ control) was kindly provided by Dr. I. Saito, Institute of Medical Science, University of Tokyo [13]. Another replication-defective adenoviral vector (Adex-IL18bp), which expresses the rat IL-18bp gene derived from pTriEx-rIL18bpHis, was constructed by using an adenovirus expression vector kit (Takara Bio, Tokyo). The amplified fragment containing the coding region with the primers 5′-CATTGAATTCACAATCAAAGGAGATATACC-3′ and 5′-TGATAGAATTCCTGCACCTGAGGTTAATCAC-3′ from pTriEx-rIL18bp was inserted into the unique *Swa*I site of the adenovirus genome in the cassette cosmid pAxCAwt. After sequencing of the coding region, the cosmid bearing an expression unit was co-transfected into human embryonic kidney 293 cells, together with the adenovirus DNA-terminal protein complex. The cloned recombinant adenoviruses were purified by using cesium chloride ultracentrifugation. Titers were assessed with an Adeno-X Rapid Titer Kit (Clontech) and expressed as plaque-forming units (pfu).

In Vitro Gene Delivery

HeLa cells were infected with Adex-IL18bp or Adex-LacZ (80 MOI) in DMEM with 10% fetal-calf serum (Gibco, Rockville, MD) and incubated for 48 h at 37°C. The supernatant was collected and stored at –70°C until the biologic activity assessment.

In Vitro Assay of Biologic Activity of IL-18bp

An assay of the biologic activity of IL-18bp in the Adex-IL18bp construct was performed. IL-18bp biologic activity was considered to be represented by the ability of IL-18bp to inhibit production of IFN-γ as determined by the following method. Freshly isolated splenocytes from Lewis rats (2.5×10^6 cells/mL) in RPMI with 10% fetal-calf serum were stimulated with recombinant rat IL-18 (10 ng/mL; Pierce Biotechnology, Rockford, IL) with or without supernatant (various volumes) of Adex-IL18bp-infected HeLa cells. Supernatant of Adex-LacZ-infected HeLa cells or uninfected HeLa cells was used as the control. Clarified supernatant samples of splenocytes were assayed for rat IFN-γ by using a sandwich-type enzyme-linked immunosorbent assay (eBioscience, San Diego, CA).

Rats

Adult male Lewis rats ($RT1^l$) weighing 200–300 g were used as liver transplant recipients, and adult male ACI ($RT1^a$) rats weighing 200–250 g were used as donors. All rats were cared for and used in accordance with institutional guidelines of Keio University School of Medicine. Rats were allowed to become acclimated to the animal research laboratory for at least 5 d before they were used in an experiment, and they had free access to food and water before and after the experiment.

In Vivo Gene Delivery

To accomplish gene delivery in donor rats, 1×10^9 pfu of Adex-IL18bp or Adex-LacZ (control) in 2 mL of normal saline was injected through a penile vein 2 d before OLTx. Two days after the viral infection, the whole liver was recovered as a liver graft.

Transplantation Procedure

Operations in both donors and recipients were done with the animals under isoflurane anesthesia. OLTx was carried out by using the technique described by Kamada and Calne [14], without hepatic artery reconstruction. Livers were perfused with 20 mL of lactated Ringer's solution (4°C) through a catheter placed in the abdominal aorta, and excised grafts were stored in lactated Ringer's solution

(4°C). The cold ischemia time was less than 90 min. The anhepatic phase was less than 16 min. No immunosuppressive agents were administered. Previous studies found that the median survival time of liver allografts from ACI donors in Lewis recipients was 10 d [15–18].

Blood Sampling and Tissue Preparation

Seven days after OLTx, whole blood was collected from the infrarenal inferior vena cava of recipient rats immediately after abdominal incision. Centrifugation was used to separate out the serum, which was stored at –70°C until determination of serum alanine aminotransferase (ALT) concentrations. Concurrently, the rats were sacrificed and whole liver tissues were removed. Half the tissues were immediately frozen in liquid nitrogen; the rest were fixed in 10% formalin. Frozen tissue was stored at –70°C until the cytokine and chemokine assays.

Real-time Quantitative PCR Analysis

Total RNA was extracted from rat liver tissue by using Trizol reagent (Invitrogen, Carlsbad, CA) and purified with an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNAs were synthesized from 1 μg total RNA by using oligo-dT primers (Qiagen) and ReverTra Ace RT (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed with 1 μg cDNA, 300 nM primers, and a FastStart Universal SYBER Green Master Mix device (Roche Applied Science, Mannheim, Germany) using a 7500 ABI platform (Applied Biosystems, Foster City, CA). The cycling conditions were as follows: degradation of the preamplified templates at 50°C for 10 min, denaturation at 95°C for 10 min, and 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and amplification at 72°C for 1 min. This was followed by a dissociation step or melting-curve analysis to determine the melting point of the double-stranded DNA products produced.

Histologic and Immunohistochemical Analyses

Liver tissues were studies histologically. From formalin-fixed specimens embedded in paraffin, 5 μm-thick sections were cut and stained with hematoxylin and eosin. To evaluate the expression of IL-18bp in Adex-IL18bp-infected liver grafts, immunohistochemical studies were performed with untreated liver samples and Adex-LacZ-infected liver grafts used as controls. Mouse anti-His-tag monoclonal antibody (MBL, Tokyo, Japan) was used as the primary antibody (1:1,500 dilution; 120 min at room temperature). Alexa Fluor 488 goat anti-mouse antibody (1:1,600 dilution; 60 min at room temperature; Invitrogen, Tokyo, Japan) was used as the secondary antibody. All histologic characteristics were assessed by a pathologist.

Statistical Analysis

Student's *t*-tests were used to compare results (means ± SEM) in two groups. Allograft survival times in the treatment and control groups were compared by using log-rank testing. A P value <0.05 was considered to represent a significant difference.

RESULTS

Adex-IL18bp Vector was Functional *In Vitro*

Supernatant of HeLa cells infected with Adex-IL18bp, which should contain a considerable amount of IL-18bp, decreased IFN-γ levels in the rat splenocyte supernatant in a dose-dependent manner, indicating blockage of the activity of rat recombinant IL-18 (Fig. 1). Supernatant of Adex-LacZ-infected HeLa cells or uninfected HeLa cells had no effect on IL-18 activity (data not shown).

IL-18bp was Overexpressed in Livers of ACI Rats Treated with Adex-IL18bp

Samples of liver tissue obtained from untreated ACI rats and from ACI rats 2 d after one intravenous injection of 1.0×10^9 pfu of Adex-LacZ or Adex-IL18bp were analyzed for IL-18bp mRNA expression by using real-time quantitative PCR assays and for IL-18bp expression by means of immunohistochemical examination.

IL-18bp mRNA levels were higher in the Adex-IL18bp group than in either of the other two groups (Fig. 2). In addition, His-tag immunohistochemical analysis showed strong and clear staining in the cytoplasm of liver cell samples from the Adex-IL18bp group. Liver cell samples from the other two groups showed no staining (Fig. 3).

Expression of IL-18 mRNA was Up-Regulated in Liver Allografts 7 D after OLTx

Samples of liver tissue were obtained from Lewis recipients 7 d after OLTx of livers from ACI donors given an intravenous injection of Adex-LacZ (1.0×10^9 pfu; control); from ACI rats 2 d after one intravenous

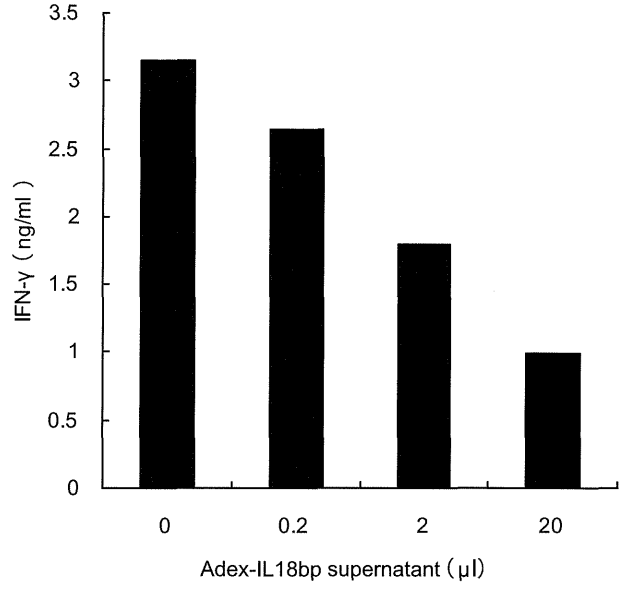

FIG. 1. *In vitro* assay of biologic activity of IL-18bp in Adex-IL18bp construct. HeLa cells were infected with Adex-IL18bp (80 MOI) for 48 h at 37°C. Freshly isolated splenocytes from Lewis rats (2.5×10^6 cells/mL) were stimulated with 10 ng/mL of recombinant rat IL-18 in various volumes of supernatant of Adex-IL18bp-infected cells. The concentration of IFN-γ in the rat splenocyte supernatant was assessed by using an ELISA. The results indicate that supernatant of HeLa cells infected with Adex-IL18bp blocked the activity of rat IL-18 in a dose-dependent manner.

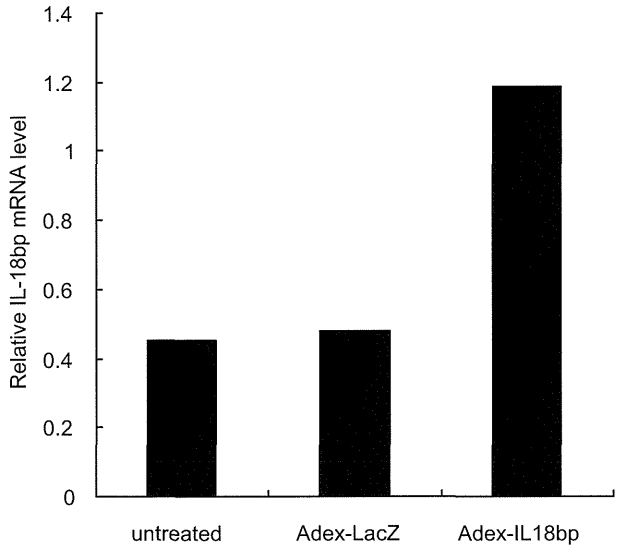

FIG. 2. *In vivo* IL-18bp mRNA assay after administration of Adex-IL18bp. Samples of liver tissue were obtained from untreated ACI rats and from ACI rats 2 d after one intravenous injection of 1.0×10^9 pfu of Adex-IL18bp or Adex-LacZ (control). Expression of IL-18bp mRNA was assessed by using quantitative real-time PCR assays, with normalization according to β-actin (housekeeping gene) expression levels. Samples from rats given Adex-IL18bp had greater IL-18bp mRNA expression than those from untreated or Adex-LacZ-treated rats.

injection of 1.0×10^9 pfu of Adex-LacZ; and from native livers in ACI rats. Levels of IL-18 mRNA in the samples were assessed by using real-time PCR assays. IL-18 mRNA levels were significantly higher in the allograft group than in either of the other two groups (Fig. 4), suggesting that the adenovirus itself did not induce IL-18 mRNA expression.

IL-18bp Overexpression Improved Liver Function after OLTx

To determine whether IL-18bp expression affected liver allograft function, serum levels of ALT were measured in Lewis recipients of ACI livers 7 d after OLTx. Histologic studies of liver allografts were performed at the same time point. As shown in Fig. 5, serum ALT levels in recipients whose donors were pretreated with Adex-IL18bp were significantly lower than those in recipients whose donors were given Adex-LacZ, the control vector (96.5 ± 12.1 *versus* 268.3 ± 99.4 IU/L; $P < 0.05$). Histopathologic analyses showed that allografts from the Adex-IL18bp group had no bridging necrosis (Fig. 6A), whereas those from the Adex-LacZ group did have necrosis (Fig. 6B). The portal vein in allografts after Adex-IL18bp pretreatment of donors was patent (Fig. 6A), whereas that after Adex-LacZ administration was severely stenosed and showed extensive infiltration with inflammatory cells (Fig. 6B).

IL-18bp Overexpression Reduced Levels of Some Chemokines and IFN-γ in Liver Allografts

Total RNA was isolated from liver allografts 7 d after OLTx and analyzed by using real-time quantitative PCR assays. Levels of CXCL10, CX3CL1, and IFN-γ mRNA were significantly lower in allografts after donor pretreatment with Adex-IL18bp compared with Adex-LacZ (Fig. 7A, B).

IL-18bp Overexpression Prolonged Allograft Survival

To investigate the possible overall benefit of IL-18bp gene delivery before OLTx, animal survival after OLTx was evaluated. As shown in Fig. 8, recipients of liver allografts from donors pretreated with Adex-IL18bp survived significantly longer than recipients whose donors were given Adex-LacZ (mean survival time, 13 *versus* 10 d).

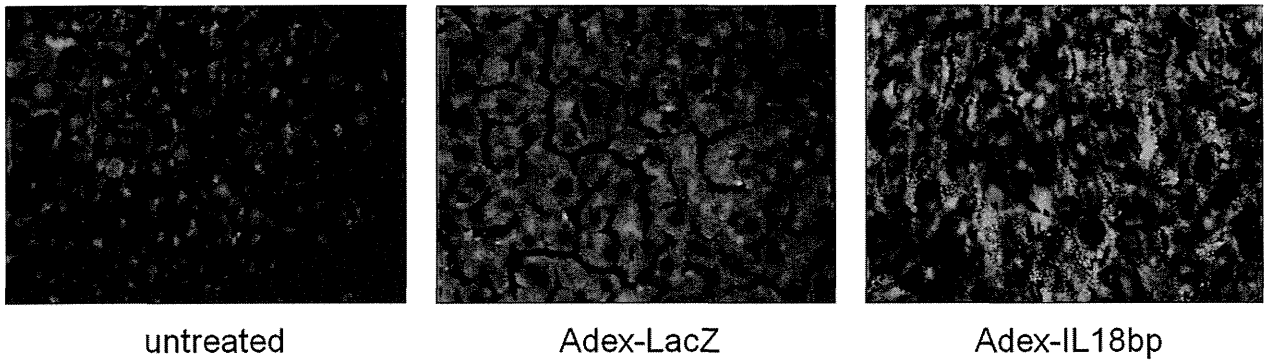

FIG. 3. Results of immunohistochemical analysis for IL-18bp linked to a His-tag in livers of donor ACI rats. Samples of liver tissue were obtained from untreated ACI rats and from ACI rats 2 d after one intravenous injection of 1.0×10^9 pfu of Adex-LacZ (control) or Adex-IL18bp. In samples from rats given Adex-IL18bp, the cytoplasm of hepatocytes was clearly stained, indicating that the Adex-IL18bp injection led to the overexpression of IL-18bp in liver grafts. (Color version of figure is available online.)

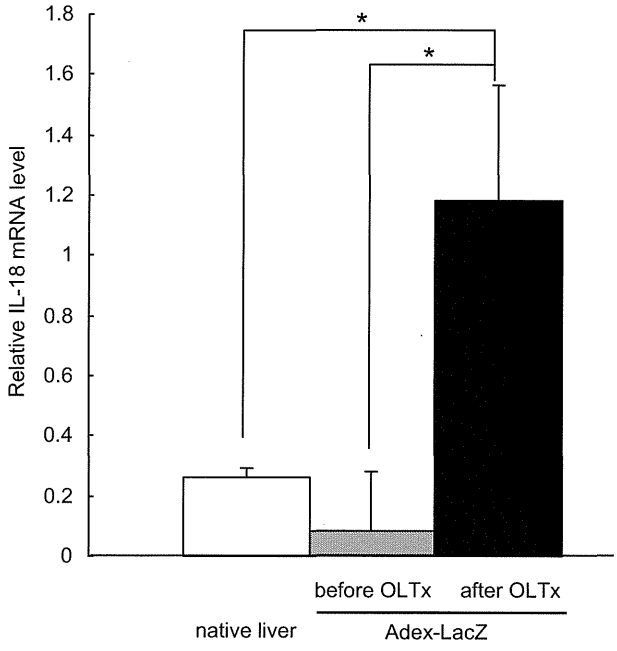

FIG. 4. Expression of IL-18 mRNA in liver allografts. Samples of liver tissue were obtained from Lewis rat recipients 7 d after transplantation of liver allografts from ACI donors given an intravenous injection of 1.0×10^9 pfu of Adex-LacZ; from ACI rats given an intravenous injection of 1.0×10^9 pfu of Adex-LacZ (no transplantation); and from native livers of ACI rats. Expression of IL-18 mRNA was assessed by using quantitative real-time PCR assays, with normalization according to β-actin expression levels. IL-18 mRNA levels were significantly higher in the allograft group than in either of the other two groups. Results represent the mean ± SEM ($n = 3$ or 4 per group); $^*P < 0.05$.

DISCUSSION

IL-18 was first cloned as an IFN-γ-inducing factor that augmented natural killer (NK) activity in splenocytes in mice [19]. Although IL-18 is structurally homologous to IL-1, and its receptor belongs to the IL-1R/Toll-like receptor superfamily, the function of IL-18 is known to be quite different from that of IL-1. Several lines of evidence suggest that IL-18 may be important in the pathogenesis of inflammatory processes. Serum levels of IL-18 have been found to be elevated in patients with postoperative sepsis [20], chronic liver disease [21–25], and acute coronary syndromes [26]. In a murine model of cardiac transplantation, IL-18 production was correlated with a histologic rejection pattern and with induction of IFN-γ [27]. Although in a previous study, levels of IL-18 mRNA in liver allografts (ACI to Lewis) peaked the d 5 after transplantation whereas liver isografts (Lewis to Lewis) had no such peaks [28], the effect of inhibition of IL-18 mRNA on acute cellular rejection (ACR) remains unclear. An investigation in a murine model of acute kidney rejection showed that IL-18 was up-regulated and produced principally by intragraft macrophages [29], and this finding is consistent with results of studies in patients [1] that established a link between kidney rejection and serum IL-18 levels.

To investigate the role of IL-18 in liver transplantation, as well as the possible effects of inhibition of IL-18 on liver allograft rejection, we constructed an adenoviral vector expressing the rat IL-18bp gene with His-tag at the C-terminal, assuming that this construction would specifically suppress IL-18 biologic activity. Gene delivery systems using adenoviral vectors have been described in several reports [30, 31], and we previously demonstrated that protein expression in rat livers after gene delivery with this method continued for more than 14 d [32]. This was sufficiently long for the current study because ACR is generally considered to occur early after OLTx. Our experiments showed that IL-18bp in the Adex-IL18bp construct had biologic activity *in vitro*. Adex-IL18bp also induced mRNA expression of IL-18bp and protein expression of His-tagged IL-18bp *in vivo*. These results indicate that Adex-IL18bp infection provided adequate IL-18bp expression. In addition, in our rat model, expression of IL-18 mRNA was up-regulated in liver allografts

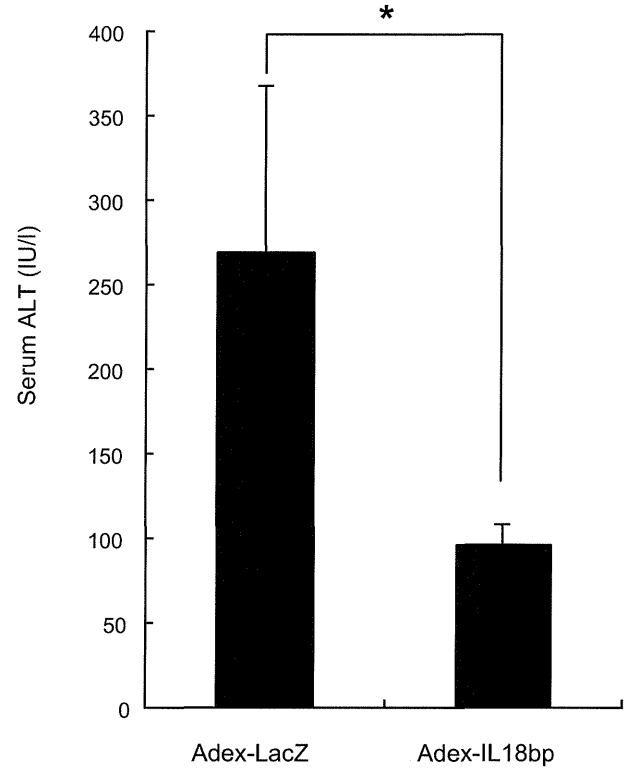

FIG. 5. Serum levels of ALT in recipients of liver allografts from pretreated and control donors. Donor ACI rats were treated with one intravenous injection of 1.0×10^9 pfu of either Adex-IL18bp or Adex-LacZ (control) 2 days before OLTx of their livers into Lewis rat recipients. Seven days after OLTx, serum ALT levels in recipients whose donors were given Adex-IL18bp ($n = 4$) were significantly lower than those in recipients whose donors were given Adex-LacZ ($n = 3$). Results represent the mean ± SEM; $^*P < 0.05$.

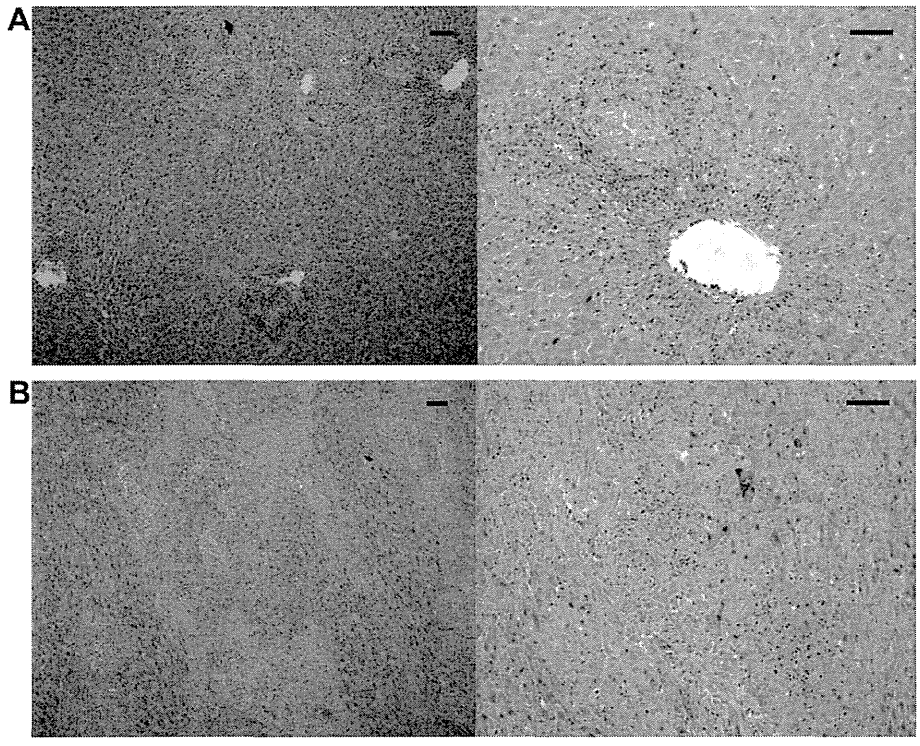

FIG. 6. Histopathologic findings in liver samples obtained from rat transplant recipients 7 d after OLTx. Samples obtained from allograft recipients whose donors were pretreated with Adex-IL18bp showed no bridging necrosis (A, left panel), whereas samples from recipients whose donors were given Adex-LacZ did show necrosis (B, left panel). The portal vein in allografts after Adex-IL18bp pretreatment was patent (A, right panel), whereas that in allografts after Adex-LacZ administration was stenosed and showed extensive infiltration with inflammatory cells (B, right panel). Hematoxylin and eosin stain; scale bar = 100 μm. (Color version of figure is available online.)

during rejection but not in Adex-LacZ-treated livers that were not transplanted. This suggests that adenoviral vector infection alone did not affect IL-18 expression.

IL-18 is known to induce IFN-γ production, and we previously showed that IFN-γ, which is produced by NK cells early after rat OLTx, is a key modulator of ACR [5]. However, a network of many cytokines is involved in ACR, so controlling rejection by inhibiting only one cytokine has been unsuccessful. To our knowledge, only one previous study found that controlling one type of cytokine alone (without any other immunosuppressive agents) suppressed ACR after allogeneic liver transplantation [33]. Our findings suggest that IL-18bp gene delivery to liver allografts may by itself significantly increase graft survival times relative to control values in a high-responder model, even though the mean difference between survival times was only 3 d. Furthermore, the lower serum ALT levels 7 d after OLTx in recipients whose donors were given Adex-IL18bp compared with Adex-LacZ clearly indicate that liver function was better after Adex-IL18bp pretreatment. The existence of this cytoprotective effect was confirmed by histopathologic studies. IFN-γ expression was also significantly decreased in the Adex-IL18bp group relative to the Adex-LacZ group. Because IL-18 is capable of inducing IFN-γ, and IFN-γ is one of the candidates for a key regulator of ACR, the cytoprotective effect resulting from the specific suppression of IL-18 may be produced through the inhibition of IFN-γ. These findings suggest that IL-18 may play an important role in liver allograft rejection. Future studies of this issue should investigate the mechanism of IL-18 in ACR by using a subtherapeutic dose of cyclosporine A with Adex-IL18bp.

To elucidate the mechanism of the effect of IL-18bp gene transfer on liver allografts in our model, we analyzed the levels of several chemokines. Chemokines are important in the trafficking of lymphoid cells to areas of inflammation, including ACR sites. Previously, we found that both CXCL10 and CX3CL1, which are induced by IFN-γ [10], were significantly up-regulated in rat liver allografts compared with syngeneic grafts beginning 3 d after transplantation [5]. These results suggest that chemokines are involved in promoting recruitment of effector cells to allogeneic tissue. Other studies showed that CXCL10 and CX3CL1 are up-regulated during rejection of murine cardiac allografts [11, 12]. Therefore, expression of CXCL10 and CX3CL1 in solid-organ allografts may be important in directing the recruitment of effector cells, including antigen-activated T and NK cells, into those allografts

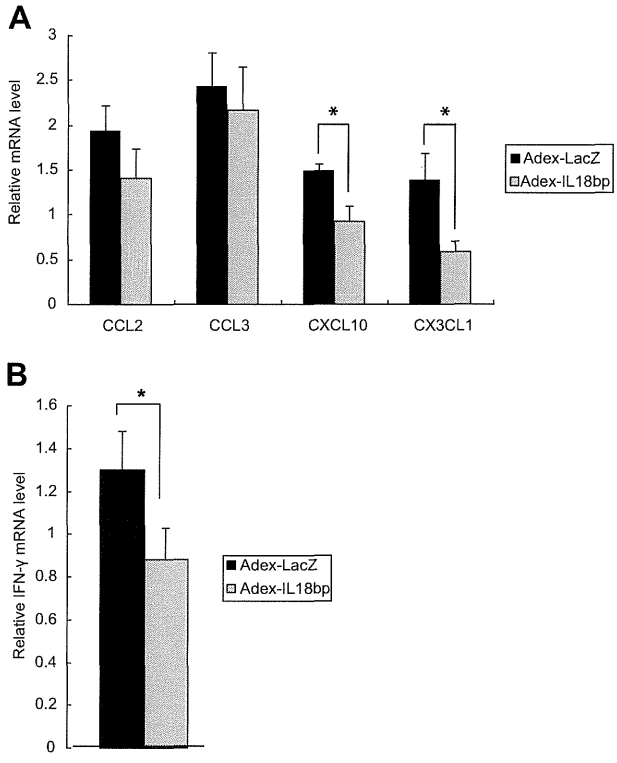

FIG. 7. Expression of chemokines and IFN-γ mRNA in liver samples obtained from rat transplant recipients 7 d after OLTx. Total RNA was isolated from liver allografts obtained from recipients whose donors were pretreated with Adex-IL18bp or Adex-LacZ (control) and was analyzed by using real-time quantitative PCR assays, with normalization according to β-actin mRNA expression levels. (A) Levels of mRNA of four chemokines in the treatment ($n = 4$) and control ($n = 3$) group. Expression of CXCL10 and CXC3L1 was significantly decreased in the Adex-IL18bp group compared with the Adex-LacZ group. (B) Levels of IFN-γ mRNA. IFN-γ expression was significantly reduced in the Adex-IL18bp group ($n = 4$) compared with the Adex-LacZ group ($n = 3$). Results represent the mean ± SEM; $^*P < 0.05$.

during acute rejection. In the current study, levels of both CXCL10 and CX3CL1 in the Adex-IL18bp group were significantly suppressed compared with control values, indicating that specific suppression of IL-18 during allograft rejection may have a crucial role in ACR through these chemokines.

Conversely, in a murine model, Wyburn *et al.* [34] found that IL-18 deficiency and IL-18 neutralization by IL-18bp did not provide significant protection against kidney allograft rejection, although IL-18 pathways appeared to be active in the alloimmune process. Anti-IL-18 strategies alone were apparently inadequate for overriding the T-cell-driven alloimmune response. However, it remains possible that in liver transplantation, a mechanism that works though IL-18 may be important in the rejection process.

There is considerable evidence showing that IL-18 has a major role in the pathogenesis of inflammation or the alloimmune response [1, 20, 21, 26, 35-39]. We previously reported that mitigation of nonspecific

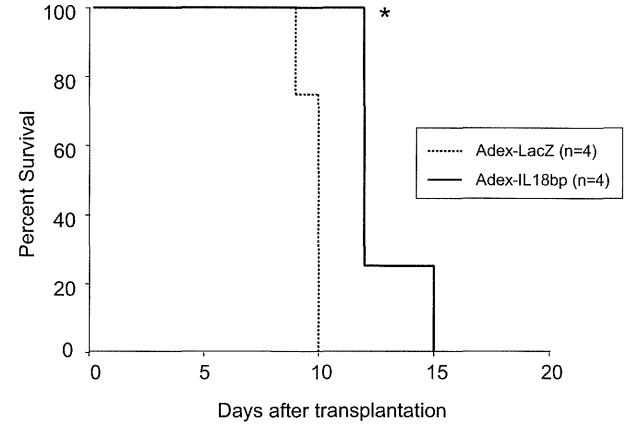

FIG. 8. Allograft survival after OLTx. Recipients of allografts whose donors were pretreated with Adex-IL18bp had significantly prolonged survival compared with those whose donors were given Adex-LacZ ($n = 4$ in each group); $^*P = 0.01$.

inflammation, including ischemia-reperfusion injury, significantly suppresses the alloimmune response [5]. In the current study, although specific inhibition of IL-18 significantly prolonged allograft survival, the difference in survival time was not large. Furthermore, whether this inhibition suppressed nonspecific inflammation or a specific immune response—that is, ACR—is unclear. Additional studies using such methods as combination therapy including Adex-IL18bp and another agent may be needed to clarify the possible cytoprotective effects of specific suppression of IL-18 in liver transplantation.

In the light of our results, we propose a theory regarding the involvement of IL-18 and IL-18bp in liver transplantation. We found that specific suppression of IL-18 significantly prolonged allograft survival, with improvement of liver function, reduced expression of IFN-γ, and several chemokines in our model. These results suggest that IL-18 plays an important role in liver allograft rejection, through IFN-γ and chemokines, and that interventions that induce specific suppression of IL-18 may have the potential to enhance liver function early after transplantation.

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Case Report

Discontinuation of Living Donor Liver Transplantation due to Donor's Intraoperative Latex-Induced Anaphylactic Shock

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We report on a 33-year-old female liver donor candidate who developed intraoperative latex-induced anaphylactic shock during surgery for living donor transplantation. She was the mother of the organ recipient, who was a 9-year-old boy with biliary atresia. We planned extended lateral segmentectomy for her. Although we dissected the ligament around the left lobe, the systolic blood pressure suddenly dropped and her body became flushed and warm. We administered transfusion and an ephedrine injection to recover the blood pressure. Because she recovered after the treatment, we restarted the procedure. However, she went into shock again within a few minutes. We decided to discontinue the operation. Postoperative blood tests revealed an increase in IgE-RAST and basophil activation, suggesting that the anaphylactic shock was induced by latex. Because latex allergy has become a public health problem, this allergy should be kept in mind as a potential donor operation risk.

Key words: Latex allergy – Living donor liver transplantation – Anaphylactic reaction – Shock

Latex is derived from the *Hevea brasiliensis* tree indigenous to the Amazon region of South America. It can be found in many of the items used in the medical-hospital environment, such as tourniquets, catheters, urine collecting bags, tubing, and gloves. These products can be the source of reactions to latex. The first report of anaphylactic reactions linked to latex sensitivity was in 1989[1] and since then the number of case reports of latex-induced anaphylactic reactions has steadily grown. Here, we

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report a case of a living donor for liver transplantation in whom latex-induced anaphylactic shock occurred during the operation, necessitating discontinuation of both the donor and recipient operations.

Case Report

This living donor was a Japanese woman who was 153 cm tall and weighed 56 kg. She was the mother of the organ recipient, who was a 9-year-old boy with biliary atresia. She had undergone cesarean section 4 times in the past and had allergy to pollen and mackerel. Her preoperative evaluation (hemogram, biochemical markers, coagulation profile, hepatitis virus markers, tumor markers, abdominal X-ray, abdominal-enhanced computed tomography [CT], and drip infusion cholangiography CT) revealed no abnormalities, except for positivity to antinuclear antibody. At our facility, intraoperative donor liver biopsy (zero biopsy) is performed routinely at the beginning of living donor liver transplantation (LDLT) to confirm eligibility of the donor liver for LDLT in terms of steatosis and portal zone inflammation.[2] We usually perform lateral segmentectomy and extended segmentectomy through a 10-cm incision using a Thompson retractor (Thompson Surgical Instruments Inc, Traverse City, Michigan). For this donor, we planned a 10-cm laparotomy operation that included zero biopsy and subsequent extended lateral segmentectomy, estimating that the graft weight-to-recipient weight ratio was 1.31%. During the operation, we initially made a 10-cm incision in the donor's upper abdomen and performed zero biopsy of the liver, which revealed no abnormal findings. Although we dissected the ligament around the left lobe through this incision, the systolic blood pressure suddenly dropped to 50 mmHg (Fig. 1) and her face and upper extremities became flushed and warm. We stopped the surgical procedure and administered treatment consisting of transfusion and an ephedrine injection to recover the blood pressure. We knew that she had multiple prior surgical procedures without a complication and did not strongly suspect latex-induced anaphylactic shock. One of the differential diagnoses was mesenteric traction syndrome because the episode occurred while we made traction on the liver. Because her systolic blood pressure recovered to more than 80 mmHg about 20 minutes later, we restarted the procedure avoiding traction on either the liver or mesentery. However, she again went into shock within a couple of minutes. After this second episode, we suspected latex-induced anaphylactic shock, because the use of gloves was a common denominater between the episodes. We switched to latex-free gloves and removed the urinary catheter from the patient's bladder (a latex-free urinary catheter was inserted immediately after the operation). Her systolic blood pressure recovered again within about 20 minutes after a second round of treatment consisting of epinephrine, norepinephrine, chlorpheniramine, and hydrocortisone by injection. Her body looked edematous and her intraoperative chest X-ray revealed mild pulmonary edema. We decided to discontinue the donor operation. Although the recipient operation had already proceeded to laparotomy and detachment of adhesion, it was also discontinued. The only procedure that we completed in the donor was dissection of the ligament around the left lobe. At the end of the operation, the donor's serum albumin level and prothrombin time-international normalized ratio were 1.7 mg/dL and 1.59, respectively. She was admitted to the intensive care unit intubated until the following day. Although she was edematous and drowsy for a few days after she returned to the general ward, her overall condition and blood parameters gradually recovered and she was discharged 7 days after the operation. Postoperatively, the blood level of IgE-RAST (radio-allergosorbent test, BML Laboratories, Inc, Kawagoe, Japan) was elevated (10.0 ARU/mL versus upper limit of 0.34 ARU/mL) and a basophil activation test (CD203c up-regulation; BML Laboratories) showed a strong positive result (23.1% at 1:312.5 dilution; evaluation criteria were negative at <6%, positive in 15% to 20%, and strongly positive at >20%), suggesting that anaphylactic shock was induced by latex. Postoperatively, we asked the patient about her history again, focusing on a specific issue of latex allergy potential and found that she had experienced itching when she used kitchen rubber gloves within the past couple of months. The recipient also gradually recovered and is currently waiting to undergo deceased donor liver transplantation.

Discussion

Latex allergy was unusual until the late 1980s, but as more health care workers began using latex gloves to control infections in the 1990s, the incidence steadily increased thereafter. Anaphylactic reaction linked to latex sensitivity was first reported in 1989,[1] and since there have been numerous case reports of latex-induced anaphylactic reactions in patients with various diseases.[3–5] The number of LDLTs per-