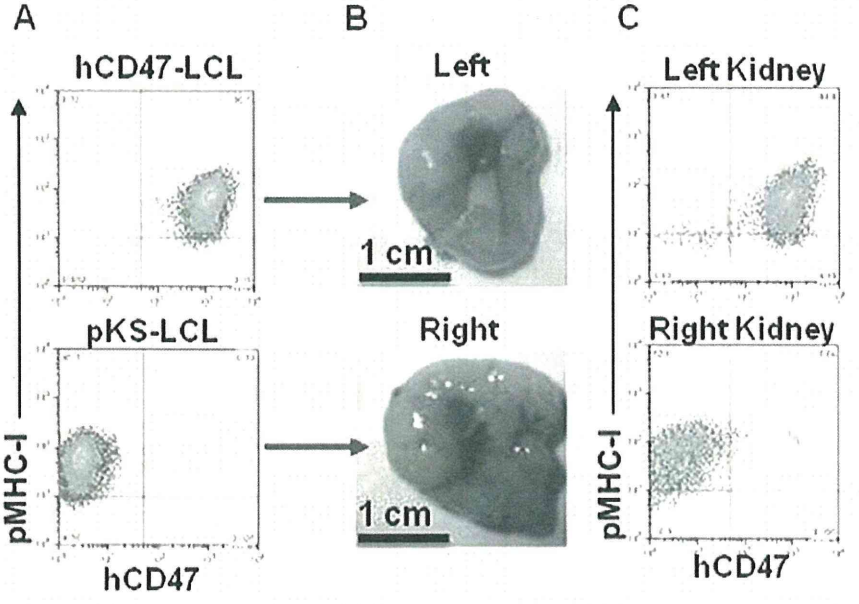

Figure 3. Both hCD47-LCL and pKS-LCL cells can survive and form tumors in macrophage-depleted NOD/SCID mice. Macrophage-depleted NOD/SCID mice ($n = 3$) were injected with hCD47-LCL and pKS-LCL cells into the subcapsular space of left (top panel) and right (bottom panel) kidney (5×10^7 per kidney), respectively. (A) Flow cytometric analysis of hCD47-LCL (top) and pKS-LCL (bottom) cell inocula. (B) Tumors found in left and right kidneys. (C) Flow cytometric analysis of tumor cells from left and right kidneys.

itve (Mac-1$^+$) myeloid cells are significantly more resistant to rejection by macrophages than lymphoid cells (23). In that study, mixed bone marrow chimeras were established by injection of CD47 KO bone marrow cells into sublethally irradiated wild-type mice after transient macrophage depletion. Although initial engraftment of CD47 KO cells was achieved in these mice, the levels of CD47 KO donor chimerism declined rapidly after transplantation. Of note, Mac-1$^+$ CD47 KO cells were rejected at a significantly slower rate than CD47 KO lymphoid cells and remained detectable for approximately 30 weeks.

Genetic intervention has been shown promising in improving xenograft survival. The successful production of viable pigs with homozygous deletion of the α1,3-galactosyltransferase gene was an important advance in xenotransplantation (8,13,15). The use of organs from these pigs successfully avoided both hyperacute and acute humoral xenograft rejection without requiring complement inhibition or antibody absorption (14,27). The addition of thromboregulatory and anti-inflammatory genes to the α1,3-galactosyltransferase-deficient background is expected to further prolong xenograft survival (18). Although studies in pig-to-primate transplant models are needed to reach a firm conclusion, the present study suggests that transgenic pigs expressing human CD47 could be advantageous as donors for clinical xenotransplantation. CD47-SIRPα signaling also plays an important role in the control of dendritic cell maturation and activation (4,23). Our recent studies demonstrated that CD47 expression on donor cells is required to repress recipient dendritic cell activation and suppress allograft rejection after donor-specific transfusion (25). Thus, it is possible that xenografts from human CD47 transgenic pigs may also have a reduced potency to stimulate adaptive xenoimmune responses, and this hypothesis merits further study.

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Impact of Adjuvant Immunotherapy Using Liver Allograft-Derived Lymphocytes on Bacteremia in Living-Donor Liver Transplantation

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Background. Bacteremia is one of the leading causes of mortality in living-donor liver transplant (LDLT) recipients. Lymphocytes, including natural killer cells, are believed to play a role in the first line of defense against invading infectious microbes.

Methods. From January 2004 to December 2009, 114 consecutive LDLT recipients were studied for postoperative bacteremia. The impact of adjuvant immunotherapy using activated liver allograft-derived lymphocytes on bacteremia was retrospectively evaluated by a one-to-one match using propensity score to overcome bias due to the different distribution of covariates for the two groups.

Results. After one-to-one matching, 21 patients who did not receive adjuvant immunotherapy and 21 who did not receive adjuvant immunotherapy had the same preoperative and operative characteristics. Six (28.6%) of the 21 patients who did not receive adjuvant immunotherapy had bacteremia, whereas only one (4.8%) of the 21 patients who received adjuvant immunotherapy had bacteremia; thus, the incidence of bacteremia in patients who had received adjuvant immunotherapy was significantly lower than that in patients who had not received adjuvant immunotherapy ($P=0.038$).

Conclusions. Adjuvant immunotherapy using liver allograft-derived lymphocytes may be a promising modality for reducing the postoperative bacteremia after LDLT.

Keywords: Living-donor liver transplantation, Natural killer cells, Bacteremia, Adjuvant immunotherapy.

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Infection is one of the leading causes of morbidity and mortality in liver transplant patients. The incidence of infection in patients after liver transplantation is higher than that after renal and heart transplantation (*1*). The high incidence of infection in liver transplant patients is likely attributed to the technical complexity of the surgery, latent contamination in the abdominal cavity, and the poor medical condition of the patients. After transplantation, the incidence of bacterial and fungal infection is approximately 50% and 10%, respectively (*2*, *3*). Most infections occur within the first month after liver transplantation and are primarily the result of surgical complications or are nosocomial in origin (*2–5*). Bacteremia has been reported to be the main cause of mortality in liver transplant recipients (*2*, *5*). Associated mortality rates of approximately 30% have been reported for bacteremia (*6–8*).

We have recently developed a novel strategy of using adjuvant immunotherapy to prevent the recurrence of hepatocellular carcinoma (HCC) or hepatitis C virus (HCV) infection after living-donor liver transplantation (LDLT) (*9*, *10*). This immunotherapeutic strategy involves intravenous injection of activated liver allograft-derived lymphocytes into LDLT patients. These lymphocytes can mount an antitumor immune response (*9*) and are known to play a role in the first line of defense against invading microbes.

In this study, we retrospectively investigated whether this adjuvant immunotherapy reduced the incidence of post-transplant bacteremia in LDLT recipients. However, there may be selection bias of liver function and operative characteristics between the liver transplant recipients who received adjuvant immunotherapy and who did not receive it. To overcome selection bias due to the different distribution of clinical characteristics such as severity of liver function impairment between the two groups, a one-to-one match was created using propensity score analysis. Thus, we obtained two groups that were comparable for baseline variables.

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RESULTS

The Adjuvant Immunotherapy Using Activated Liver Allograft-Derived Lymphocytes

We performed ex vivo perfusion of graft livers through the portal vein. The proportion of interleukin-2 (IL-2)-stimulated CD56+CD3-natural killer (NK) cells and CD56+CD3+ natural killer T (NKT) cells extracted from the liver perfusates was 40%±15% and 20%±9%, respectively (n=24). The recipients were administered a single intravenous injection of IL-2-stimulated liver allograft-derived lymphocytes 3 days after liver transplantation ($793\pm350 \times 10^6$ cells injected per patient, n=24). During the follow-up period, no significant adverse effects or rejection episodes were observed.

Clinicopathologic Characteristics and Postoperative Course of the Entire Study Group

Differences in characteristics between patients who received adjuvant immunotherapy and those who did not are listed in Table 1. Specifically, patients who received immunotherapy were older (57 vs. 54; $P=0.03$), more likely to have HCC (87.5% vs. 38.9%; $P<0.001$), and had a lower score of model for end-stage liver disease (MELD) (11 vs. 16; $P<0.0038$). Of 90 patients who did not receive immunotherapy, 26 (28.9%) developed bacteremia, whereas only 1 (4.2%) of the 24 patients who received immunotherapy had bacteremia. The incidence of bacteremia was significantly lower in the adjuvant immunotherapy group than in the nonadjuvant immunotherapy group ($P=0.034$) (Table 2). The 1-year survival rate tended to be higher in the patients who had received adjuvant immunotherapy (100%) than in those who had not (80%) ($P=0.068$; Table 2). The sources and pathogens present in bacteremia are listed in Tables 3 and 4, respectively. Among the 27 recipients with episodes of bacteremia, five (18.5%) had primary bacteremia. The three most common sources of bacteremia were catheter-related infections (5/27 patients, 18.5%), peritonitis (8/27 patients, 29.6%), and cholangitis (5/27 patients, 18.5%). Gram-positive and gram-negative bacteria were detected in 15 (55.6%) and 12 (44.4%) of the 27 patients with bacteremia, respectively. The most common isolated pathogens were methicillin-resistant *Staphylococcus aureus* (MRSA) (n=9), coagulase-negative *staphylococcus* (n=5), *Escherichia coli* (n=5), *Enterobacter spp.* (n=3), and *Pseudomonas aeroginosa* (n=3). There was no difference in postoperative clinical data, including duration of central venous catheterization, hospital stay, and intensive care unit stay between the two groups (Table 5).

Results After Propensity Score Matching

Characteristics after propensity score matching analysis are shown in Table 1. Twenty-one of the 24 patients who received adjuvant immunotherapy were matched with 21 patients who did not receive therapy after covariate adjustment. Therefore, three patients who received immunotherapy and 69 patients who did not receive the therapy were excluded because their propensity scores could not be matched. The study group of 42 patients was well matched; in particular, all covariates that significantly affected overall survival in the entire study group were equally distributed over the two matched groups. Matched patients who received adjuvant immunotherapy had a similar age (56 vs. 57; $P=0.62$), donor age (36 vs. 36; $P=0.51$), graft-to-recipient weight ratio (0.89 vs. 0.85; $P=0.72$), score of MELD (13 vs. 11; $P=0.73$), blood loss (3270 vs. 3420 mL; $P=0.53$), and rate of postoperative bile leakage (14.3% vs. 19.0%; $P=0.67$) to those of matched patients who did not receive adjuvant immunotherapy. Similarly, other clinical variables were similar for patients who did and did not receive the therapy. Of the 21 patients who did not receive immunotherapy, six (28.6%) developed bacteremia. Of the 21 patients who received immunotherapy, one (4.8%) developed bacteremia. The incidence of bacteremia was significantly lower in the adjuvant immunotherapy group than in the nonadjuvant immunotherapy group, respectively ($P=0.038$) (Table 2). The 1-year survival rate tended to be higher in the patients who had received adjuvant immunotherapy (100%) than in those who had not received the therapy (80%) ($P=0.10$; Table 2). The sources and caus-

TABLE 1. Clinical characteristics of whole study and one-to-one matching study using propensity scores

	Whole study			Matched study population		
	IT (−) (n=90)	IT (+) (n=24)	*P*	IT (−) (n=21)	IT (+) (n=21)	*P*
Ageing (median, range)	54 (20–69)	57 (44–68)	0.03	57 (46–69)	56 (44–68)	0.62
Male	54 (60%)	16 (66.7%)	0.55	13 (61.9%)	15 (71.4%)	0.51
Age of donor	36 (17–67)	34 (20–54)	0.33	36 (18–57)	36 (20–54)	0.51
Illness						
HCC (%)	35 (38.9%)	21 (87.5%)	0.00021	16 (76.2%)	19 (90.1%)	0.21
Previous operation (%)	15 (16.7%)	5 (20.8%)	0.63	6 (28.6%)	4 (19.0%)	0.47
GRWR	0.91 (0.62–1.95)	0.87 (0.70–1.22)	0.11	0.85 (0.62–1.35)	0.89 (0.70–1.22)	0.72
MELD	16 (6–55)	11 (7–25)	0.0038	11 (7–25)	13 (7–23)	0.73
Presence of ascites	57 (63%)	15 (62.5%)	0.65	11 (52.4%)	9 (42.9%)	0.75
Blood loss (ml)	3675 (345–14,000)	3120 (1080–8990)	0.64	3420 (1100–8200)	3270 (1200–8990)	0.53
Operating time (min)	738 (530–1167)	706 (535–1009)	0.66	749 (530–930)	702 (535–1009)	0.27
Postoperative biliary leak (%)	14 (15.6%)	3 (12.5%)	0.7	4 (19.0%)	3 (14.3%)	0.67
Reoperation (%)	23 (25.6%)	4 (16.7%)	0.36	3 (14.3%)	5 (23.8%)	0.43

IT, adjuvant immunotherapy; HCC, hepatocellular carcinoma; GRWR, graft recipient weight ratio; MELD, model for end-stage liver disease.

TABLE 2. Outcomes of postoperative bacteremia after living-donor liver transplantation

Infection site	Whole study			Matched study population		
	IT (−) (n=90)	IT (+) (n=24)	*P*	IT (−) (n=21)	IT (+) (n=21)	*P*
Number of patients with bacteremia (%)	26 (28.9%)	1 (4.2%)	0.004	6 (28.6%)	1 (4.8%)	0.038
One-year survival	84%	100%	0.061	80%	100%	0.1

IT, immunotherapy.

TABLE 3. Causes of postoperative bacteremia after living-donor liver transplantation

Infection site	No. of patients with episodes of postoperative bacteremia			
	Whole study		Matched study population	
	IT (−) (n=90)	IT (+) (n=24)	IT (−) (n=21)	IT (+) (n=21)
Abdominal infection				
Intraabdominal abscess	3	0	0	0
Peritonitis	7	1	1	1
Cholangitis	5	0	2	0
Enteritis	1	0	0	0
Catheter infection	5	0	2	0
Primary	5	0	1	0

IT, immunotherapy.

TABLE 4. Etiological organisms of bacteremia after living-donor liver transplantation

Pathogen	No. patients with episodes of bacteremia			
	Whole study		Matched study population	
	IT (−) (n=90)	IT (+) (n=24)	IT (−) (n=21)	IT (+) (n=21)
Bacteria				
Gram (+)				
MRSA	9	0	2	0
Coagulase-negative *staphylococcus*	5	0	1	0
Others	1	0	0	0
Gram (−)				
Escherichia coli	4	1	2	1
Enterobacter sp	3	0	0	0
Pseudomonas aeruginosa	3	0	0	0
Serratia sp	1	0	1	0

IT, immunotherapy; MRSA, methicillin-resistant staphylococcus aureus.

ative pathogens of bacteremia are shown in Tables 3 and 4, respectively, and were as follows: one patient with primary bacteremia (MRSA), two patients with catheter-related infections (*Escherichia coli*, and *Serratia sp*), two patients with peritonitis (MRSA and *Escherichia coli*), and two patients with cholangitis (coagulase-negative *Staphylococcus* and *Escherichia coli*). There was no difference in postoperative clinical data, including duration of central venous catheterization, hospital stay, and intensive care unit stay between the two groups (Table 5).

DISCUSSION

Bacteremia is one of the main complications of liver transplantation, and it is also an important factor influencing the mortality associated with liver transplantation (*6, 7*). Many studies have revealed that the period of hospitalization and stay in the intensive care unit is longer for patients with postoperative bacteremia (*4, 11, 12*).

Adjuvant immunotherapy using liver allograft-derived lymphocytes was principally performed to prevent HCC recurrence. As these lymphocytes may have antibacterial properties, this study was performed as a secondary aim to investigate whether this adjuvant immunotherapy reduced the incidence of posttransplant bacteremia in LDLT recipients. In this one-to-one matching study using propensity score, we have shown that adjuvant immunotherapy using liver allograft-derived lymphocytes significantly reduced the incidence of postoperative bacteremia after LDLT. We have previously shown that stimulation with IL-2 significantly increased the expression of tumor necrosis factor-related apoptosis-inducing ligand on liver NK cells exerting anti-HCV and tumoricidal effects (*9, 10*). We have also confirmed that the number of interferon (IFN)-γ-secreting cells, including NK and NKT cells, in the peripheral blood of the liver transplant patients who received adjuvant immunotherapy was significantly higher than that in the peripheral blood of patients who did not receive the therapy at 14 days after LDLT (*10*). It is expected that the circulating activated NK and NKT cells in the peripheral blood may prevent postoperative bacteremia by exerting an antibacterial and antifungal effect, and the effect for preventing the postoperative infections may last during at least 2 weeks after LDLT. The adjuvant immunotherapy might reduce the number of postoperative infections as it involved the use of IL-2 stimulated NK and NKT cells. NK cell has protective functions for bacterial infection such as *Staphylococcus aureus* and *Pseudomonas aeroginosa* by NK cytotoxicity and augmentation of phagocytosis by macrophages by IFN-γ and tumor necrosis factor production secreted by NK cells (*13, 14*). NKT cells also have protective effects for bacterial infection by IFN-γ production (*15*). In this study, no adverse effects, such as graft-versus-host disease, were observed. To prevent graft-versus-host disease, we added the anti-CD3 monoclonal antibody to the culture medium a day before the inoculation. As the current immunosuppressive regimen following after LDLT reduces the adaptive immune components, adjuvant

TABLE 5. Postoperative clinical data after living-donor liver transplantation

	Whole study			Matched study population		
	IT (−) (n=90)	IT (+) (n=24)	*P*	IT (−) (n=21)	IT (+) (n=21)	*P*
Duration of CV catheter	6 (5–23)	7 (5–11)	0.71	6 (5–16)	7 (5–10)	0.78
ICU stay (day)	4 (3–22)	6 (2–12)	0.56	4 (3–22)	5 (2–12)	0.67
Hospital stay (day)	54 (22–1414)	53 (16–190)	0.76	50 (22–1414)	53 (16–190)	0.95
WBC (mm^3)	7865 (4290–33,740)	7010 (2690–10,930)	0.09	7565 (4290–15,620)	7450 (2690–10,930)	0.21
Lymphocyte count (mm^3)	558 (89–1222)	491 (148–2142)	0.77	521 (89–1020)	545 (148–2142)	0.73
CRP	1.24 (0.15–11.3)	3.5 (0.3–14.1)	0.07	1.01 (0.15–9.6)	2.4 (0.3–14.1)	0.25
BT (°C)	37.1 (36.6–38.8)	37.1 (36.5–38.1)	0.86	37.0 (36.6–38.4)	36.9 (36.5–38.1)	0.89

CRP and BT were measured on day 7 after living-donor liver transplantation. Data were expressed as median (range).

IT, immunotherapy; CV, central venous; ICU, intensive care unit; WBC, white cell count; CRP, C-reactive protein; BT, body temperature; WBC, lymphocyte count.

immunotherapy may be a promising approach for reducing the posttransplant bacteremia.

In this study, we adopted a one-to-one matching study using propensity score analysis to overcome bias due to the different distribution of covariates among patients from the two groups (patients who received adjuvant immunotherapy and those who did not). Bert et al. (*7*) have shown that gender, kidney transplant, intraoperative transfusions, MELD score, return to surgery, retransplantation, and biliary complications are associated with bacteremia. Kim et al. (*8*) have revealed an association between bacteremia and age, intravenous catheterization, United Network for Organ Sharing status IIA, and posttransplant hemodialysis. The clinical characteristics that may influence outcomes tended to differ between the two groups in the whole study (Table 1). The proportion of the older patients with HCC was significantly higher in the adjuvant immunotherapy group than those in no adjuvant immunotherapy group because the liver transplant patients who received adjuvant immunotherapy with IL-2-stimulated lymphocytes were limited to patients with HCC or HCV. The scores of MELD were significantly lower in the adjuvant immunotherapy group than in the nonadjuvant immunotherapy group. A high MELD score is known to indicate a major risk of postoperative infections after liver transplantation (*16*, *17*). However, after matching using propensity scores analysis, there were no differences in clinical variables influencing outcomes such as age, MELD score, underlying liver disease, operative factors, and postoperative operative factors between the two groups. Iida et al. (*6*) have shown that the presence of preoperative massive pleural effusion or ascites requiring drainage were independent risk factors for postoperative bacteremia. In this study, there was no significant difference in the preoperative presence of ascites between the two groups. Although there was a significant difference in the incidence of bacteremia between the two groups, this study was unable to reveal a significant difference in outcomes, likely due to small study size. It is possible that the learning curve of individual surgeons or of the entire institution may be associated with an incidence of septic complications. Second, there was a selection bias confounding our results between the two groups, because adjuvant immunotherapy was selected into only patients with HCC or HCV, thus larger study is required to confirm the positive effect of adjuvant immunotherapy on outcomes of liver transplant recipients.

Bacteremia episodes were predominantly caused by gram-positive bacilli, which accounted for 55.6% of all isolated infections. Methicillin-resistant *staphylococci* were commonly observed among the gram-positive organism, whereas *Escherichia coli* strains were found to be the most prevalent species among the isolated gram-negative rods. During the 1990s, MRSA had become endemic and emerged as a major pathogen in many transplant centers. However, in the late 1990s, the incidence of gram-negative bacterial infection increased, probably because of the use of prophylactic antibiotics (*18*). In contrast, our study has shown that gram-positive bacteria have remained as the major pathogens (*8*, *19*). Our results support recent studies that report coagulase-negative *staphylococcus* as the major cause of bacteremia (*6*). Catheter-related infections were found to occur frequently after surgery. Appropriate management of the catheter, which disrupts the organization of the skin epithelium and the mucosa, and early catheter removal are important for lowering the incidence of catheter-related infections.

In conclusion, we found that adjuvant immunotherapy using liver allograft-derived lymphocytes significantly reduced the incidence of postoperative bloodstream infections after LDLT in the setting of one-to-one matching study using propensity score. As the immunosuppressive regimen currently used after LDLT reduces the adaptive immune components, adjuvant immunotherapy using liver allograft-derived lymphocytes may be a promising approach for reducing posttransplant bacteremia.

MATERIALS AND METHODS

Patients

We retrospectively studied 114 consecutive patients who had undergone LDLT at Hiroshima University Hospital, from April 2004 to December 2009. The LDLTs had been performed after the approval of the Liver Transplantation Ethics Committee of Hiroshima University, and informed consents were obtained from all the patients.

Perioperative Management Strategy

The procedures for donor evaluation, donor surgery, recipient surgery, and perioperative management followed in our hospital have been described in previously published studies (*20–22*). A central venous catheter (triple

lumen catheter, SA series, Arrow Corporation, Japan) was place in the internal jugular vein at induction of general anesthesia.

Antimicrobial prophylaxis consisted of intravenous cefmetazole (1.0 g) administration immediately before surgery and every 6 hr during surgery; thereafter, a dosage of 2 g/day was maintained for 5 days. Vancomycin (0.5 g/day, orally) was administered for 3 days before surgery. Itraconazole (200 mg/day) was orally administered for 7 days before surgery as prophylaxis against fungal infections. Trimethoprim/sulfamethoxazole (80/400 mg/day) was orally administered after surgery as prophylaxis against *Pneumocystis*.

The basic immunosuppression regimen comprised tacrolimus and methylprednisolone. If liver function stabilized, then the patients were weaned off the steroids 2 to 3 months after the operation. Rejection episodes were mainly treated with methylpredonisolone.

The use of adjuvant immunotherapy involving activated liver allograft-derived lymphocytes was approved by the Clinical Institutional Ethical Review Board of Hiroshima University, and the immunotherapy was started from January 2006. Gradient centrifugation with Ficoll-Paque was performed to isolate liver mononuclear cells from the perfusate effluents of liver grafts obtained from healthy donors. Liver mononuclear cells were cultured with human recombinant IL-2 for 3 days. A day before the infusion, the CD3-positive cell fraction was incubated with an anti-CD3 monoclonal antibody for opsonization. The purity of the isolated fractions was assessed by flow cytometric analysis, and the viability of the cells was assessed by dye-exclusion test before injection. The cells were suspended along with 5% human serum albumin in 0.9% sodium chloride; 3 days after liver transplantation, the cell suspension was then injected into patients with HCV or HCC (*10*).

Definitions of Bacteremia

Infections were defined according to the criteria proposed by the Centers for Disease Control (*23*). For this study, we included cases in which the infections had developed within 3 months after surgery and documented the first episode of bacteremia. Isolation of bacteria (other than common skin contaminants) from a single blood culture in the presence of clinical symptoms or signs of infection was considered proof of bacteremia. Bacteremia caused by common skin contaminants was considered significant only if the organism was also isolated from two blood cultures and was associated with clinical signs of infection. The bacteremia source was determined on the basis of clinical criteria and isolation from a clinically significant site of infection of the same organism found in the blood isolate on the basis of species identification and antibiotics susceptibility results. Bacteremia was classified as primary if it was of unknown origin (no physical, radiological, or pathological evidence of a definite infection source). Catheter-related infections were documented when the blood isolate was cultured from the catheter tip and blood cultures obtained by the catheter were found to be the same as the peripheral blood culture. Briefly, blood cultures were taken out by puncture. The blood culture was as follows: a 10-mL blood sample was aseptically inoculated at bedside into each bottle of a set of aerobic and anaerobic blood culture bottles. Bottles were incubated at 37°C for a period of 5 days or until a positive reading was detected by the instrument. Bacteremia was classified as primary if it was of unknown origin.

Statistical Analysis

Continuous variables were compared using the Mann-Whitney test. Categorical data were compared using the χ^2 test or Fisher's exact test, as appropriate. Overall survival analyses were carried out using the Kaplan-Meier method; comparisons between different groups were carried out using the log-rank test. To overcome bias due to the different distribution of covariates among patients from the two groups (patients who received adjuvant immunotherapy and those who did not), a one-to-one match was created using propensity score analysis (*24, 25*). The propensity score represents the probability of each individual patient being assigned to a particular condition in a study given a set of known covariates. Propensity scores are used to reduce selection bias by equating groups on the basis of these covariates and are used to adjust for selection bias in observational studies through matching. Variables entered in the propensity model were age, sex, donor age, the presence of HCC, previous surgery, graft-to-recipient weight ratio, MELD score, operative factors (blood loss during operation and operation time), and postoperative complications (biliary leakage and reoperation). The model was then used to obtain a one-to-one match by using the nearest-neighbor matching method. We used a matching algorithm based on linear predictive values without replacement, until all possible matches had been formed. Initially, matching was performed to five decimal points, followed by 4, 3, 2, and 1 decimal point matching; cases whose propensity score deviated more than 0.10 were considered unmatched (*26, 27*) Patients with unmatchable propensity scores were excluded from further analysis. Once the matched groups were obtained, differences in postoperative infection and prognosis were further analyzed to assess the unbiased influence of adjuvant immunotherapy on postoperative infection. Overall, survival analysis was performed within each matched subgroup to assess the influence of adjuvant immunotherapy on postoperative infection amended from the confounding factors. All analyses were performed using the SPSS 16.0, and P values less than 0.05 were considered statistically significant.

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Research Article

Evidence for the Immunosuppressive Potential of Calcineurin Inhibitor-Sparing Regimens in Liver Transplant Recipients with Impaired Renal Function

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Patients requiring liver transplantation (LT) frequently experience renal insufficiency (RI), which affects their survival. Although calcineurin inhibitor-sparing immunosuppressive regimens (CSRs) are well known to prevent RI, the immune state in recipients receiving CSR remains to be intensively investigated. Among 60 cases of living-donor LT at our institute, 68% of the patients had none to mild RI (non-RI group) and 32% of the patients had moderate to severe RI (RI group). The RI group received a CSR comprising reduced dose of tacrolimus, methylprednisolone, and mycophenolate mofetil, while the non-RI group received a regimen comprising conventional dose of tacrolimus and methylprednisolone. One year after LT, the mean estimated glomerular filtration rate (eGFR) in the RI group had significantly improved, although it was still lower than that of the non-RI group. Serial mixed lymphocyte reaction assays revealed that antidonor T-cell responses were adequately suppressed in both groups. Thus, we provide evidence that CSR leads to improvement of eGFR after LT in patients with RI, while maintaining an appropriate immunosuppressive state.

1. Introduction

Renal insufficiency (RI) has been widely recognized as a serious complication of liver transplantation that significantly compromises patient outcome [1–4]. Since a number of patients already have varying degrees of RI, including hepatorenal syndrome, before undergoing liver transplantation, and since postoperative standard immunosuppression protocols based on calcineurin inhibitors (CNIs) can lead to severe tubular atrophy, interstitial fibrosis, and focal hyalinosis of the small renal arteries and arterioles, a majority of liver recipients develop some degree of RI [5–7]. An analysis of data from the Scientific Registry of Transplant Recipients indicates that the cumulative incidence of stage 4 [estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73 m^2] or stage 5 chronic kidney disease (eGFR < 15 mL/min/1.73 m^2 or need for renal replacement therapy) after liver transplantation is 18% at 5 years [8].

Late renal failure is associated with both pre- and posttransplant factors, including higher concentrations of CNIs both early and late posttransplant and can be predicted by creatinine levels in the first year posttransplant [9, 10]. The recognition of these effects induced interest in strategies using a CNI-sparing immunosuppressive regimen (CSR). Current strategies to overcome CNI toxicity include reduction or withdrawal of CNIs concurrent with switching over to less nephrotoxic drugs like the mammalian target of rapamycin (mTOR) inhibitor or mycophenolate mofetil (MMF) [11–17]. Although these strategies have clearly demonstrated the ability to reduce the incidence of nephrotoxicity in various studies, CSR may result in an increased risk for acute rejection episodes in a subset of patients.

In the present study, we investigated the immune state in liver transplant patients suffering from RI who received a CSR comprising a reduced dose of CNI, methylprednisolone, and MMF. For monitoring the immune-state response to

antidonor allostimulation in these patients, we employed a mixed lymphocyte reaction (MLR) assay using an intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique. By applying the CFSE-based method, the proliferation of viable $CD4^+$ and $CD8^+$ responder T-cells in response to allostimulation could be separately quantified using multiparameter flow cytometry [18]. The technique allowed us to find that antidonor T-cell responses were adequately suppressed in patients with RI who received the CSR and in patients without RI who received a conventional immunosuppressive regimen.

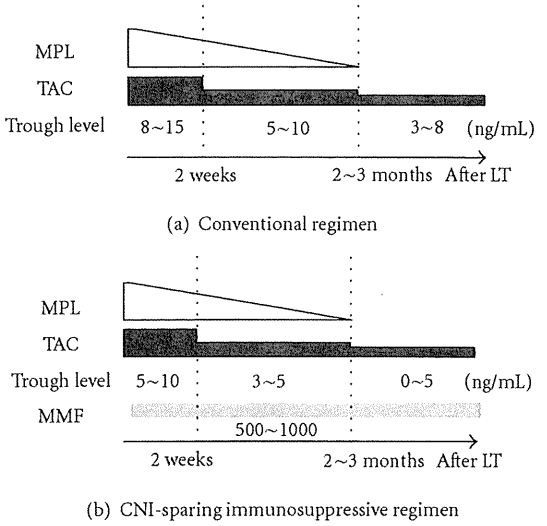
2. Patients and Methods

2.1. Patients. Between January 2003 and December 2009, 122 patients underwent living-donor LTs at Hiroshima University Hospital. Of these, 50 patients infected with hepatitis C virus (HCV) and 12 patients who received liver allografts from ABO-blood group incompatible donors were excluded from the study, because they were treated with the diverse immunosuppressive protocols. For the remaining 60 patients, the relationship between RI prior to LT and the clinical/immunological state after LT was investigated. The following information was collected at the time of the transplant: age, sex, etiology of liver disease, model for end-stage liver disease (MELD) score, and diagnosis of hepatocellular carcinoma (HCC) prior to LT. Renal function was evaluated in each participant by determining eGFR. The eGFR of each participant was calculated from their serum creatinine value (SCr) and their age by using the new Japanese equation [19] as follows:

$$\begin{aligned} &\text{eGFR } (\text{mL/min/1.73 m}^2) \\ &\quad = 194 \times \text{Age} - 0.287 \\ &\qquad \times \text{S} - \text{Cr} - 1.094 \text{ (if female} \times 0.739). \end{aligned} \tag{1}$$

In this study, RI was defined as none to mild (eGFR $\geq$ 60 mL/min/1.73 m^2) and moderate (30–59 mL/min/1.73 m^2) to severe (< 30 mL/min/1.73 m^2). The MELD score was calculated for each patient using the United Network for Organ Sharing (UNOS) formula based on the laboratory values obtained just prior to LT. Patients were monitored for renal function using serum creatinine level and eGFR at 1, 3, 6, and 12 months after LT.

2.2. Immunosuppressive Protocol. The basic immunosuppressive regimen after LT for the non-RI group comprised tacrolimus (TAC) and methylprednisolone, with gradual tapering of doses. Patients with RI received a CSR comprising a reduced dose of TAC, methylprednisolone, and MMF (Figure 1). In the conventional regimen, the trough whole blood levels of TAC were maintained between 8 and 15 ng/mL in the first few postoperative weeks and between 5 and 10 ng/mL thereafter. In the CSR, the trough whole blood levels of TAC were maintained between 5 and 10 ng/mL in the first few postoperative weeks and between 3 and 5 ng/mL thereafter.



(a) Conventional regimen

(b) CNI-sparing immunosuppressive regimen

FIGURE 1: Immunosuppressive protocol after liver transplantation. The basic immunosuppressive regimen comprised tacrolimus (TAC) and methylprednisolone (MPL), with doses gradually being tapered off. The trough whole blood levels of TAC were maintained between 8 and 15 ng/mL in the first few postoperative weeks and between 5 and 10 ng/mL thereafter (a). Renal insufficiency (RI) group received CNI-sparing immunosuppressive regimen (CSR) consisting of TAC reduction and concomitant use of mycophenolat mofetil (MMF) (b).

2.3. Immune Monitoring by an In Vitro MLR Assay. For monitoring the immune state, an in vitro MLR assay was performed at 1, 3, 6, and 12 months after LT. Briefly, peripheral blood mononuclear cells prepared from the blood of the recipients, donors, and healthy volunteers with the same blood type as the donors (third-party control) for use as the stimulator cells were irradiated with 30 Gy, and those obtained from the recipients for use as responder cells were labeled with 5 lm CFSE (Molecular Probes Inc., Eugene, OR, USA), as described previously [18]. The stimulator and responder cells were incubated for 5 days. CFSE stably stains intracellular proteins without causing toxicity, and the fluorescence intensity of each stained cell segregates equally among daughter cells during cell division, resulting in sequential halving of the cellular fluorescence intensity with every successive generation. After culturing for MLR, the harvested cells were stained with either phycoerythrin- (PE-) conjugated antihuman CD4 or PE-conjugated antihuman CD8 monoclonal antibodies and subjected to analysis by flow cytometry. All analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). T-cell proliferation was visualized by the serial-halving of the fluorescence intensity of CFSE. $CD4^+$ and $CD8^+$ T-cell proliferation and stimulation index were quantified using a method described previously [18].

2.4. Statistical Analysis. Quantitative variables were expressed as mean ± standard deviation (SD) or median (range). Categorical variables were presented as values and

Table 1: Patient characteristics at living donor liver transplantation.

(eGFR (mL/min/1.73 m^2))	Non-RI group (n = 41) (94.8 ± 26.9)	RI group (n = 19) (42.5 ± 15.9)	P value
Age at LT (years)	49.2 ± 11.5	52.9 ± 9.0	0.23
Male sex—n (%)	21 (51.2)	13 (68.4)	0.21
Primary diagnosis—n (%)			0.63
HBV	15 (36.6)	9 (47.4)	
Alcoholic	8 (19.5)	5 (26.3)	
AIH	4 (9.8)	1 (5.3)	
Others	14 (34.1)	4 (21.1)	
MELD	16.5 ± 7.1	24.7 ± 10.7	< 0.01
eGFR at 1st year after LT (mL/min/1.73 m^2)	77.2 ± 28.2	60.1 ± 13.5	< 0.01
eGFR > 60 at 1st year after LT—n (%)	26 (72.2)	10 (58.8)	0.33
AR within 1st year—n (%)	10 (24.4)	5 (26.3)	0.87
Bacterial infections—n (%)	13 (31.7)	8 (42.1)	0.43
Fungal infections—n (%)	4 (9.8)	4 (21.1)	0.23
CMV infections—n (%)	10 (24.4)	7 (36.8)	0.32

RI, renal insufficiency; LT, liver transplantation; HBV, hepatitis B virus; AIH, Autoimmune hepatitis; eGFR, estimated glomerular filtration rate; MELD, model for end-stage liver disease; AR, acute rejection; CMV, cytomegalovirus. Data are expressed as means ± standard deviation. Difference with $P < 0.05$ was considered significant.

percentages. Student's t-test, Mann-Whitney test, chi-square test, and Fischer's exact test were used to compare variables between the two groups. Paired t-tests were performed to compare continuous variables throughout the study period. The Kaplan-Meier analyses were used to compare time-to-event variables. P Values < 0.05 were considered statistically significant.

3. Results

The 60 patients included 34 males and 26 females; their ages ranged from 20 to 69 (median 52) years. The primary diseases in these patients included hepatitis B virus-related cirrhosis in 24 patients (of these, 18 patients had HCC), alcoholic cirrhosis in 13 patients (of these, 6 patients had HCC), autoimmune hepatitis in 5 patients (of these, 1 patient had HCC), and other diseases in 18 patients.

Before the LTs, 68% of the patients had none to mild RI (non-RI group; mean eGFR, 94.8 ± 26.9 mL/min/1.73 m^2) and 32% of the patients had moderate to severe RI (RI group; mean eGFR, 42.5 ± 15.9 mL/min/1.73 m^2). The characteristics of these patients are listed in Table 1. There was a difference in MELD score between the groups. Mean TAC trough levels during the first year after LT in the non-RI and RI groups are shown in Figure 2(a). There were differences in mean TAC trough levels during 3 months after LT between the groups. One year after the LDLTs, the mean eGFR in the non-RI group had significantly deteriorated (from 94.8 ± 26.9 to 77.2 ± 28.2 mL/min/1.73 m^2, $P < 0.01$). In contrast, the mean eGFR in the RI group had significantly improved after LT (from 42.5 ± 15.9 to 60.1 ± 13.5 mL/min/1.73 m^2, $P < 0.01$), although it was still lower than that of the non-RI group (Figure 2(b)). Notably, 53% of the patients in the RI group were completely cured of RI by 1 year after LT. None of the patients had severe RI at 1 year after LT nor required chronic hemodialysis during the observation period.

To evaluate the immune status of these patients, we employed a serial MLR assay using a CFSE-labeling technique. Lack of proliferation of both CD4$^+$ and CD8$^+$ T-cells in the antidonor CFSE-MLR assay indicates suppression of the antidonor response, whereas a remarkable proliferation of these T-cells reflects a strong antidonor response. In both groups, limited CD4$^+$ and CD8$^+$ T-cell proliferation was observed in the antidonor responses as compared with the anti-third-party responses through the first year. At 1 month after LT, the average of stimulation index (SI) for CD4$^+$ T-cells in response to anti-third-party stimulation was >2 (the average value in healthy volunteers without any immunosuppressive treatment) that is, there was a normal response in the anti-third-party (Figures 3(a) and 3(b)). At 1 year after LT, the average of SIs for CD4$^+$ and CD8$^+$ T-cells in response to both antidonor and anti-third-party stimulation was <2 (Figures 3(c) and 3(d)). There were no significant differences in acute rejection rates, bacterial, fungal, or cytomegalovirus infection rates and patient survival between the groups (Table 1).

4. Discussion

Chronic RI is a serious complication in liver transplantation that significantly compromises patient survival and outcome. Depending on the criteria applied for a definition of chronic renal insufficiency and the duration of followup, the reported rate of chronic renal insufficiency after liver transplantation may vary from 10% to 80% [1, 20–22]. CNI toxicity has been defined as one of the possible risk factors for renal insufficiency in long-term liver transplant survivors. It has been shown that exposure to CNIs within the first 6 months

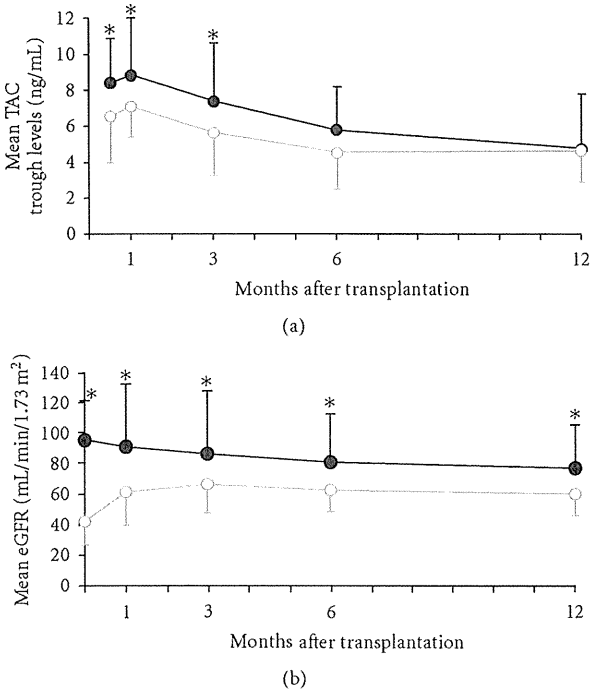


FIGURE 2: Kinetics of mean trough levels of tacrolimus and mean estimated glomerular filtration rate (eGFR) in the RI group and non-RI group during the first year after transplantation. (a) Mean trough levels of tacrolimus in the non-RI group (black line) and RI group (gray line). (b) Mean estimated glomerular filtration rate (eGFR) in the non-RI group (black line) and RI group (gray line). Data are median ± SD of values. $^*P < 0.05$.

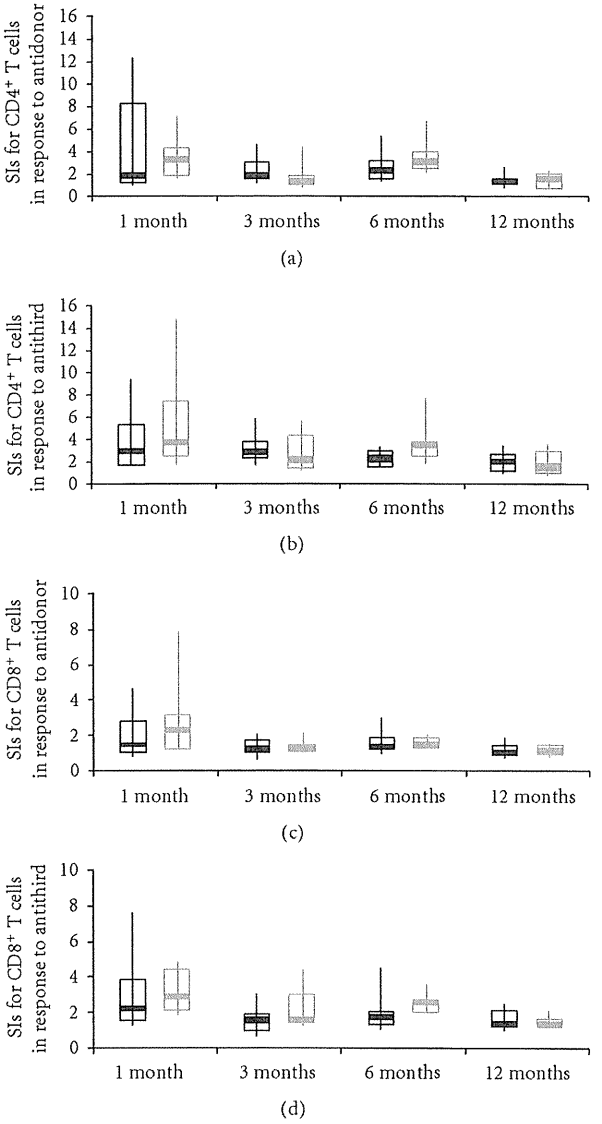


FIGURE 3: Kinetics of stimulation index in the RI group and non-RI group during the first year after transplantation. Stimulation index (SI) of each of the CD4⁺ T-cell (a, b) and CD8⁺ T-cell (c, d) subsets in the antidonor (a, c) and anti-third-party (b, d) MLR in patients in non-RI group (black line) and RI group (gray line). CD4⁺ and CD8⁺ T-cell proliferation and their SIs were quantified as follows. The number of division precursors was extrapolated from the number of daughter cells of each division, and the number of mitotic events in each of the CD4⁺ and CD8⁺ T-cell subsets was calculated. Using these values, the mitotic index was calculated by dividing the total number of mitotic events by the total number of precursors. The SIs of allogeneic combinations were calculated by dividing the mitotic index of a particular allogeneic combination by that of the self-control. The box plot represents the 25th to 75th percentile, the dark line is the median, and the extended bars represent the 10th to the 90th percentile.

after liver transplantation represents a risk factor for renal failure [23]. The GFR at 1 year had a better correlation with later renal function than the pretransplant GFR [24]. The recognition of these facts induced interest in preventing CNI toxicity. It has also reported that the use of adjunctive MMF immediately after LT might protect against CNI nephrotoxicity, potentially without the need for dose reduction or increased risk of adverse events [25]. Therefore, current strategies to overcome CNI toxicity include reduction or withdrawal of CNIs along with switching to mTOR inhibitor or MMF-based regimens [11, 12, 14, 15, 26–28]. These strategies have been documented in several recent and ongoing trials to achieve an improvement in renal function in a large proportion of liver transplant patients.

In our CSR using MMF, wherein our study results agree with the results from previous studies, patients with pre-transplant renal insufficiency were associated with less impairment of renal function without an increased frequency of rejection, infection, or patient survival. In addition to this clinical evidence for the usefulness of the CSR using MMF, the present study provides immunological evidence, by analyzing the data obtained from an MLR assay, that antidonor T-cell responses were adequately suppressed in patients who received the CSR and in patients who received the conventional immunosuppressive regimen. Notably, the individual variations of SIs of CD4⁺ T-cell and CD8⁺ T-cell subsets on antidonor T-cell responses in patients who received the CSR were smaller than those in patients who

received the conventional regimen, although the average values of both were similar. This might be explained by the possibility that the CSR comprising triple immunosuppressive drugs was equally effective in a wide variety of patients.

Several limitations of this study are present. Our sample size was relatively small without long-term followup, and single-center retrospective data are reported. Since the 2 groups of patients are not perfectly comparable as renal impairment can reduce immune responses, we could not rule out a possibility that reduced CNI, without necessarily adding MMF, may be sufficient for the treatment of these patients.

We excluded HCV positive cases and ABO-blood group incompatible cases from the study because of diverse protocol (In brief, in patients with HCV infection, methylprednisolone is not administered, which may be beneficial for preventing enhanced viral replication. Instead, basiliximab and MMF are usually administered to such patients. In ABO-blood group incompatible cases, anti-CD20 monoclonal antibody is administered for eliminating temporarily B cells 2 weeks before transplantation, and simultaneously commencing administration of CNI and MMF.). Hence, the effect of CSR in RI patients with those backgrounds remains to be elucidated. Nevertheless, this first evaluation of the immune state in liver transplant patients suffering from RI received a CSR was essential before to propose an evaluation at a larger scale.

In conclusion, patients with pre-transplant RI receiving CSR under immunological monitoring using an MLR assay were associated with less impairment of renal function without an increased frequency of rejection or patient survival. Antidonor T-cell responses were adequately suppressed in these patients as well as in patients who received the conventional immunosuppressive regimen comprising a standard dose of CNI.

Abbreviations

AIH: Autoimmune hepatitis
AR: acute rejection
CFSE: carboxyfluorescein diacetate succinimidyl ester
CMV: cytomegalovirus
CNI: calcineurin inhibitor
CSR: CNI sparing immunosuppressive regimen
eGFR: estimated glomerular filtration rate
HBV: hepatitis B virus
HCV: hepatitis C virus
LT: liver transplantation
MELD: model for end-stage liver disease
MLR: mixed lymphocyte reaction
MMF: mycophenolate mofetil
mTOR: mammalian target of rapamycin
MPL: methylprednisolone
RI: renal insufficiency
SI: stimulation index
TAC: tacrolimus.

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Therapeutic Potential of Propagated Hepatocyte Transplantation in Liver Failure

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Background. **This study aimed to evaluate the therapeutic potential of intrasplenic transplantation of culture-propagated homologous hepatocytes in rats suffering from acute liver failure (ALF).**

Methods. **ALF was induced in dipeptidyl peptidase IV-negative ($DPPIV^-$) Fischer 344 rats by totally removing the two anterior liver lobes (68% of the liver) and ligating the pedicle of the right lobe (24% of the liver). Hepatocytes isolated from $DPPIV^+$ Fischer 344 rats were cultured for 11 d to propagate 3-fold, and the resulting hepatocytes were dubbed "culture-propagated hepatocytes (CPHEPs)". A total of 1.5×10^7 cells of CPHEPs were transplanted intrasplenically before ALF induction (CPHEP group). Similarly, freshly isolated hepatocytes (FIHEPs) were transplanted as a positive control (FIHEP group), and culture medium (CM) was injected into rats as a negative control (CM group).**

Results. **The survival of the CPHEP group was comparable to that of the FIHEP group and longer than that of the CM group ($P < 0.01$). Both CPHEP and FIHEP transplantation improved blood parameters such as ammonia, total bilirubin, glutamic pyruvic transaminase, and glutamic oxaloacetic transaminase; transplantation also affected liver tissue parameters such as apoptosis rate and bromodeoxyuridine-labeling index.**

Conclusions. **Transplantation of culture-propagated homologous hepatocytes has a remarkable therapeutic potential for ALF in rats.**

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Key Words: **dipeptidyl peptidase IV mutant rats; intrasplenic transplantation; omental lobe; apoptosis; histopathology; hepatectomy.**

INTRODUCTION

Orthotopic liver transplantation (OLT) has been proven to be an effective treatment for acute liver failure (ALF) [1–3]. However, the availability of donor organs for OLT is severely limited. Hepatocyte transplantation, which could provide a solution to donor organ shortages, has potential advantages over OLT [4].

The development of the hepatocyte transplantation technology over the past two decades reflects the progress of basic studies on human hepatocytes. Several patients have received hepatocyte transplantation as treatment for ALF to either give the native liver time to recover or serve as a bridge to liver transplantation [5–7]. However, there is a shortage of human hepatocytes for transplantation, which requires us to develop technology for repeatedly multiplying normal human hepatocytes *in vitro*.

Previously, we devised a new culture method by which adult rat and human hepatocytes could be maintained/propagated for up to at least 1 mo, repeatedly dividing and showing a bipotential differentiation capacity [8–11]. These highly replicative hepatocytes were isolated from liver tissues as "small hepatocytes" and were cultured in a new culture medium (hepatocyte clonal growth medium [HCGM]). The proliferative

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hepatocytes under culture expressed normal differentiated hepatocytic phenotypes and retained normal liver functions, including albumin (Alb) secretion and lidocaine and D-galactose metabolization. We dubbed these hepatocytes propagated *in vitro* as "culture-propagated hepatocytes" (CPHEPs). In the present study, we demonstrate that transplantation of homologous CPHEPs to a rat model of ALF improves its survival.

MATERIAL AND METHODS

Animals

Two types of Fischer 344 rats were used in the present study: wild-type with respect to the dipeptidyl peptidase IV (DPPIV) gene, DPPIV-positive ($DPPIV^+$), and its mutant, DPPIV-negative ($DPPIV^-$). Ten-wk-old wild-type rats, weighing 220 g, were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), and age-matched mutant female rats, weighing 140 g, were obtained from Charles River Japan, Inc. (Kanagawa, Japan). They were housed in accordance with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*, prepared by the National Academy of Science.

Preparation of Cells

Hepatocytes were separated from the rats by the two-step collagenase perfusion method [12, 13] . Their viability, as measured by the trypan blue exclusion test, was more than 90%. The hepatocytes were then suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies Inc., Rockville, MD)—containing 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT), 20 mM/L HEPES (Gibco BRL), 44 mmol/L $NaHCO_3$, and antibiotics (100 IU/mL penicillin G and 100 μg/mL streptomycin; Gibco BRL)—and were used as freshly isolated hepatocytes (FIHEPs) in transplantation experiments.

Aliquots of FIHEPs were inoculated at 8.5×10^3 cells/cm^2 in HCGM; 24 h later, they were cocultured with Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) at a density of 8.5×10^3 cells/cm^2 treated with 10 μg/mL mitomycin C (Sigma-Aldrich, Tokyo, Japan), as reported previously [8–10]. The culture was maintained for 11 d to allow cell proliferation, with medium changes every 3 d for the first 9 d. The resulting cells were used as CPHEPs in transplantation experiments. In the preliminary experiments, we investigated the growth kinetics and viability of the hepatocytes during primary and secondary culture. The hepatocytes progressively expanded and reached the culture confluent state 11 d after commencing the culture. During primary culture, the viability of the expanded hepatocytes was well maintained. After secondary culture, however, the growth of the hepatocytes was rather limited and their viability was not well maintained. Based on these results, we used hepatocytes cultivated for 11 d for treatment in this study. Other aliquots of FIHEPs were suspended in DMEM, subjected to more than three times warming/freezing (liquid nitrogen) cycle, and used as "dead hepatocytes" (DHEPs). Single-passaged syngeneic rat fibroblasts (FBs) were cultured for 10 d and used for transplantation experiments.

Induction of ALF

The surgical animal ALF model [14, 15] was used as the host for the transplantation experiments. After laparotomy, the common pedicle to the right lobes was ligated, and the two anterior liver lobes were removed [16], leaving the omental lobes intact.

Hepatocyte Transplantation

FIHEPs and CPHEPs were each suspended in 0.3 mL DMEM and were individually transplanted into the spleen using a 27-gauge needle (TERUMO, Tokyo, Japan). $DPPIV^-$ rats were used as recipients, and hepatocytes from the wild-type ($DPPIV^+$) counterparts were used as donor cells to distinguish donor cells from host cells [13, 17]. Control group animals were injected with culture medium (CM group). The same numbers of DHEPs and rat FBs were similarly transplanted into the spleen. Thus, in the present study, there were five groups of rats: the FIHEP, CPHEP, DHEP, FB, and CM groups. Each group contained 5 to 17 animals. Their blood and omental lobe were obtained for blood chemistry and histopathology, respectively.

Gene Expression in Hepatocytes

The expression of albumin (Alb), cytochrome P450 (CYP), glutamine synthetase (GS), and glycerol-3-phosphate dehydrogenase (G3PDH) genes was quantified in FIHEPs and CPHEPs by real-time RT-PCR. Total RNAs were periodically extracted from them by using the RNeasy Total RNA System (Qiagen, Tokyo, Japan), 1 μg of which was used as a template to synthesize cDNAs, as reported previously [18]. The abovementioned genes were amplified using the cDNAs as templates in the PRISM 7700 Sequence Detector (Applied Biosystems Inc., Foster City, CA). Primers used were the following: Alb, CAACTACGGTGAACTGGCTGA (5′ primer) and TGCTGCAG GAAACACTCGTT (3′ primer); CYP2C7, GGCATTTTCTACTGTGT (5′ primer) and TGATAGAGGGAAGGGACTTGGAT (3′ primer); GS, CAGATGTTGGACAGGTAGCCAG (5′ primer) and CCTTAAAC TAAGCCCAGGGACA (3′ primer); G3PDH, TGCCATCACTGCCACT CAG (5′ primer) and TGCCCCACGGCCAT (3′ primer). Products under amplification were monitored directly by measuring the increase in dye intensity of SYBR Green I. The expression levels obtained were normalized against those of G3PDH.

Blood Chemistry

Sera were analyzed for concentrations of glucose (Glu), ammonia (NH_3), Alb, and total bilirubin and for glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activity by using the FDC 3500 photometer (FUJIFILM Co. Ltd., Tokyo, Japan).

Growth Assessment of the Omental Lobe

The bromodeoxyuridine (BrdU)-labeling index was determined as follows: 1 h before sacrifice, the rats were intraperitoneally injected with BrdU at a dose of 30 mg/kg body weight and 5-fluoro-2′-deoxyuridine at a dose of 3 mg/kg body weight. After sacrifice, rat liver tissues were processed to obtain 5-μm-thick paraffin sections, and subjected to immunohistochemistry for BrdU using anti-BrdU-mouse mAbs (Dakopatts). BrdU was visualized using the Vectastain ABC Kit. The labeling index was expressed as the ratio of $BrdU^+$ hepatocytes to the total hepatocytes counted. In each liver, hepatocytes in five different photographic fields were counted.

To identify apoptotic hepatocytes, liver tissues were processed to obtain paraffin sections, and subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay using the ApopTag Peroxidase Kit (Intergen Co., Purchase, NY). The apoptotic index was expressed as the mean ratio of $TUNEL^+$ hepatocytes to the total hepatocytes counted in five different microscopic fields for each specimen.

Characterization of Transplanted Hepatocytes

Spleen tissues were obtained from the rats 24 h post-ALF induction and were subjected to cryosectioning for immunohistochemistry and enzyme histochemistry. The cryosections were fixed in acetone at

−20°C for 5 min. Immunostaining for Alb and DPPIV was performed using rabbit anti-rat Abs (Cappel, Durham, NC) and mouse mAbs against rat DPPIV (a gift from Dr. D.C. Hixson) as the primary Ab. The Abs were visualized with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) using DAB, Texas red-conjugated goat anti-rabbit IgG, or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM as a substrate. Nuclei were counterstained with hematoxylin or Hoechst 33258.

Quantification of mRNA in Hepatocyte-Transplanted Spleen

Spleen tissues were excised from the rats 24 h post-ALF induction. Total RNAs were extracted from approximately 250 mg of the tissues with the RNeasy Total RNA System, treated with RNase-free DNase I, and used for quantifying mRNAs of Alb, CYP2C7, and coagulating factor X (F-X) by RT-PCR. The primer of F-X was TGAACCTGAC CCTGAAGACCTC (5′ primer) and CAGAGGTAGTTCGGTTCGCT (3′ primer). Other primers were described previously. Similar measurements were performed for total RNAs extracted from 250 mg of liver tissues isolated from rats as a positive control.

ELISA for TNF-α, TGF-β1, IL-1β, and IL-6

Sera were collected from the rats 24 h post-ALF induction to determine the concentrations of TNF-α (Diaclone, Besançon *Cedex*, France), TGF-β1, IL-1β, and IL-6 (BioSource International, Camarillo, CA) by ELISA.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Statistical significance analysis was performed using the Kaplan-Meier survival test, log-rank test, and Student's *t*- test. A P value of <0.05 was considered statistically significant.

RESULTS

Propagation of Hepatocytes in Culture

As reported previously [10], hepatocytes cocultured with Swiss 3T3 cells in HCGM grew steadily and became confluent at 11 d (Fig. 1A), resulting in a 2.81 ± 0.5-fold increase in their numbers.

The levels of Alb, CYP2C7, and GS mRNAs at 1 d of culture were significantly lower than those of FIHEPs and continued to fall for up to 11 d (Fig. 1B).

Prolongation of Survival of ALF Rats by Hepatocyte Transplantation

To determine the optimal dose of hepatocytes for transplantation, the rats were transplanted with different numbers of FIHEPs (0.5, 1.0, and 1.5×10^7 cells) through the spleen. An upper limit of the injectable volume of cell suspension into the spleen was approximately 300 μL, which made the maximum injectable number of hepatocytes per animal approximately 1.5×10^7 cells. The animals were then subjected to ALF and their survival was observed (Fig. 2A). The rats that received 1.5×10^7 and 1.0×10^7 cells survived significantly longer ($P < 0.01$ and $P < 0.05$, respectively) than the control rats, which received CM alone (CM group); however, the effect of transplanting 0.5×10^7 cells was not significant. In subsequent experiments, the rats were transplanted with 1.5×10^7 FIHEPs.

We next evaluated the therapeutic potential of CPHEP transplantation in ALF. Rats were transplanted with 1.5×10^7 CPHEPs (CPHEP group) and treated for ALF, and their survival time was compared with those receiving the same numbers of FIHEPs (FIHEP group), dead FIHEPs (DHEP group), and FBs (FB group). Approximately 30% of the CPHEP group rats survived for 120 h after ALF, showing survival curves almost identical to those of the FIHEP rats (Fig. 2B). As the CM group, the FB group rats did not survive beyond 40 h, indicating hepatocyte specificity of the rescue effects of cell transplantation on liver failure. DHEP transplantation improved survival rates ($P = 0.07$ *versus* the CM group) far more than FIHEP or CPHEP transplantation. These results indicate that CPHEPs were as effective as FIHEPs in increasing the lifespan of ALF rats.

Engraftment of Hepatocytes in the Spleen

By using the DPPIV positivity of the donor HEPs, we evaluated the engraftment of the transplanted cells in the graft site (spleen) by immunohistochemical analysis. There was an abundance of $DPPIV^+$ clusters of hepatocytes at 24 h post-ALF induction in the FIHEP group, demonstrating their successful engraftment (Fig. 3A–C). These $DPPIV^+$ cells had Hoechst 33258^+ nuclei (Fig. 3C). Similarly, $DPPIV^+$ clusters of hepatocytes were often seen in the CPHEP-transplanted spleen (Fig. 3D). As in the FIHEP group, some of the $DPPIV^+$ cells had Hoechst 33258^+ nuclei (Fig. 3D–F). However, most of them lost the Hoechst 33258^+ nuclei (Fig. 3G–I). These Hoechst 33258^- cells are considered to be dead after the engraftment in the spleen. In contrast, $DPPIV^+$ cells were absent even in the remnant liver lobe of successfully transplanted rats at any time points.

As a measure of the engraftment level of the transplanted hepatocytes, we compared the expression levels of the hepatocyte specific genes (Alb, CYP2C7, and F-X) in the spleen among the FIHEP, CPHEP, and CM groups. These levels were also compared with those of liver tissues. The expression levels in the FIHEP spleen were higher than those in the CPHEP spleen (Fig. 1C). These genes were not expressed in the CM spleen. These results support the histologic observations mentioned above, suggesting that most of the transplanted CPHEPs die soon after the engraftment. The expression levels in the FIHEP spleen were lower than those in the liver.

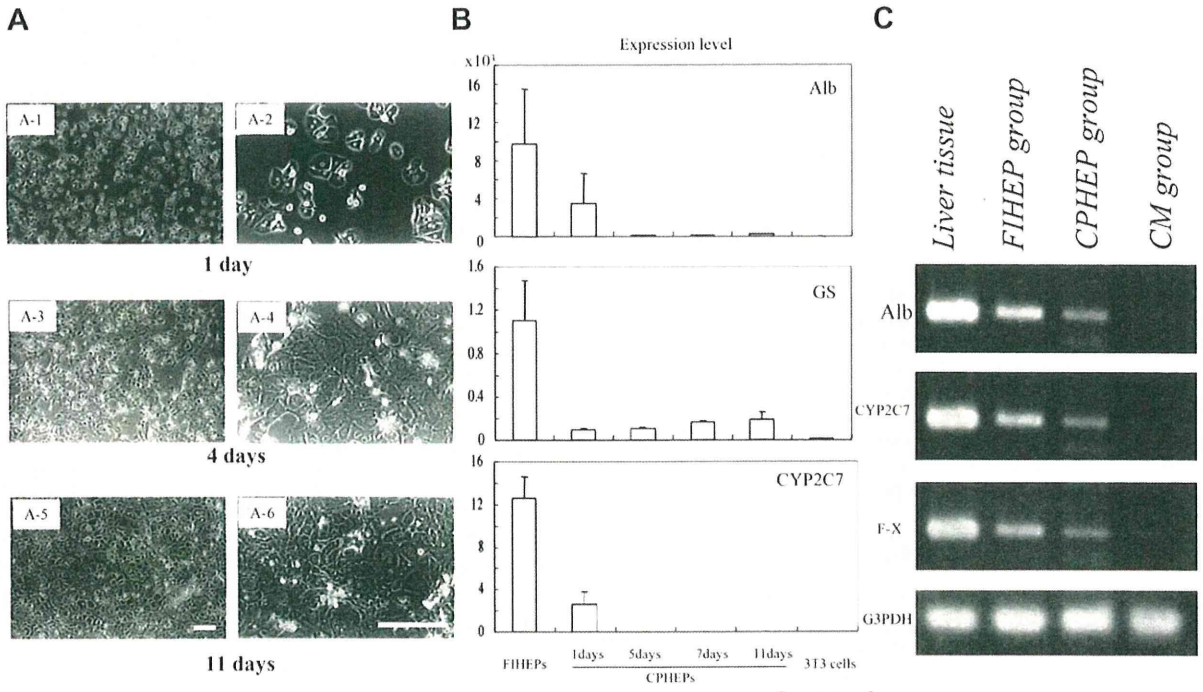

FIG. 1. (A) Phase contrast image of proliferating hepatocytes. Hepatocytes (8.5×10^3 cells/cm^2) were cocultured with Swiss 3T3 cells in HCGM on 15.0-cm dishes. Photographs were taken for the same fields at 1 (A-1, 2), 4 (A-3, 4), and 11 d (A-5, 6) with lower (A-1, 3, 5) and higher (A-2, 4, 6) magnifications. Binuclear and mononuclear hepatocytes were observed at day 1 (A-2). Hepatocytes formed clusters at 4 d (A-3) and became confluent at 11 days (A-5). Bar, 100 μm. (B) Hepatocyte marker gene expression in hepatocytes in culture. Expression of mRNAs of Alb, GS, and CYP2C7 in cultivated hepatocytes is shown. The expression levels (copy numbers) of each gene are normalized with respect to the expression levels (copy numbers) of G3PDH. (C) Hepatocyte-specific gene expression levels in the hepatocyte-transplanted spleen. The rats were transplanted with FIHEPs and CPHEPs and subjected to ALF as in Fig. 2. Control rats were given CM. Spleens were isolated at 24 h to determine the expression levels of Alb, CYP2C7, F-X, and G3PDH mRNAs by RT-PCR. Normal liver tissue was used as a positive control.

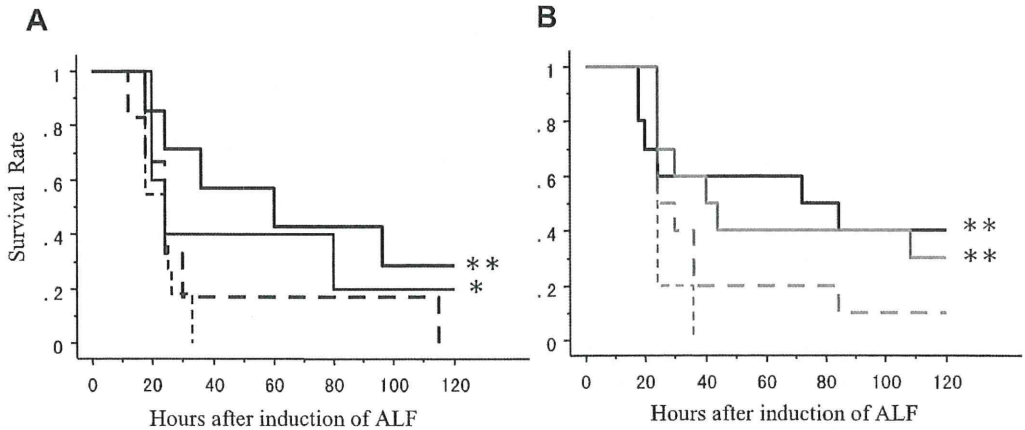

FIG. 2. Survival curves of ALF rats with FIHEP transplantation. The rats were transplanted with HEPs or FBs through the spleen and then subjected to ALF. Some rats were given CM as controls. (A) Rescue of ALF by FIHEP transplantation. The rats were given varying numbers of FIHEPs: 1.5×10^7 cells ($n = 7$, thick solid line), 1.0×10^7 cells ($n = 5$, thin solid line), 0.5×10^7 cells ($n = 6$, thick dotted line). The reference animals were given CM ($n = 11$, thin dotted line) as control. *$P < 0.05$ *versus* the CM group. **$P < 0.01$ *versus* the CM group. (B) Rescue of ALF by CPHEP transplantation. The rats were transplanted with either FIHEPs ($n = 10$, thick solid line), CPHEPs ($n = 10$, thick solid gray line), DHEPs ($n = 10$, thick gray dotted line), or FBs ($n = 5$, thin gray dotted line), 1.5×10^7 cells each, and were subjected to ALF as in (A). Some rats were given CM instead of the cells and served as controls. **$P < 0.01$ *versus* the FB group.

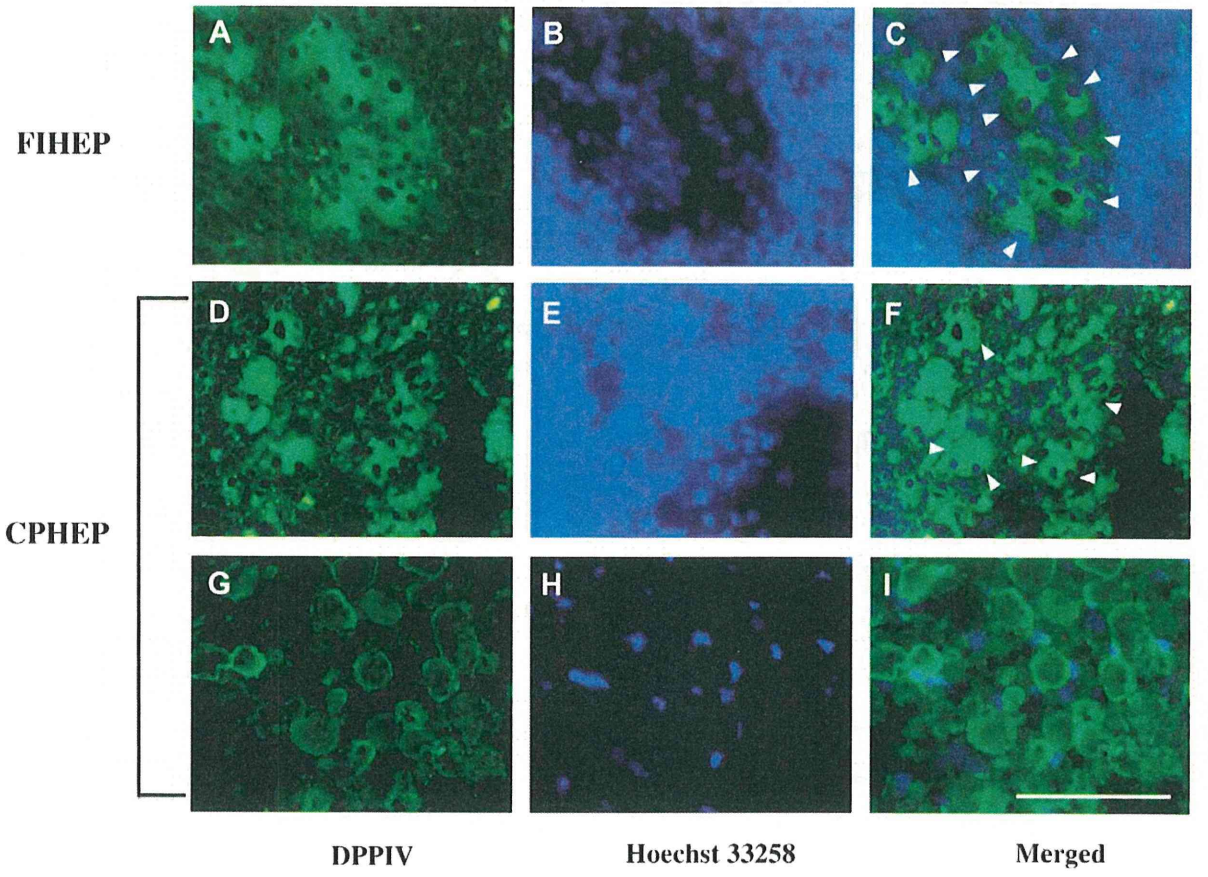

FIG. 3. Engraftment of the transplanted hepatocytes in the spleen of ALF rats. The rats were transplanted with FIHEPs (A)–(C) or CPHEPs (D)–(I) and subjected to ALF as in Figure 2B. Spleens were removed at 24 h after ALF induction and processed to cryosectioning for immunohistochemical analysis to detect DPPIV (green; A, D, G). The sections were counterstained with Hoechst 33258 [blue; (B), (E), (H)]. (A) and (B), (D) and (E), and (G) and (H) were merged into (C), (F), and (I), respectively. The arrowhead indicates $DPPIV^+$/Hoechst 33258^+ viable hepatocytes. Bar, 100 μm.

Blood Chemistry

Hepatocyte transplantation therapy for ALF was evaluated by measuring the blood levels of total bilirubin, GOT, GPT, NH_3, and Glu. The rats in the CM group showed higher levels of total bilirubin, GOT, GPT, and NH_3, and lower levels of Glu, than the hepatocyte-transplanted groups at 24 h post-ALF induction (Fig. 4), indicating that the rats experienced severe liver failure. FIHEP transplantation improved these biochemical data. The CPHEP groups showed improvement to an extent similar to the FIHEP groups. Total bilirubin and NH_3 values improved significantly, which strongly suggests that both engrafted FIHEPs and CPHEPs are functional in cholestasis and NH_3 metabolisms in ALF. However, neither FIHEP nor CPHEP transplantation significantly improved the levels of transaminase, suggesting that the transplanted hepatocytes were not sufficient to prevent ischemic changes induced by ligation of the liver lobes.

Concentrations of inflammatory cytokines in sera were also determined at 24 h post-ALF induction. TGF-β1 measured approximately 7 ng/mL, but IL-1β and IL-6 were not detected in sham-operated rats (Table 1). IL-1β and IL-6 levels in the CM group rose to approximately 300 pg/mL and 4000 pg/mL, respectively. TGF-β1 concentration in the CM group was approximately two times higher than that in sham-operated rats. IL-6 and TGF-β1 concentrations in the FIHEP and CPHEP groups became significantly lower than those in the CM group, although IL-1β concentration did not (Table 1).

Proliferation of the Remnant Liver Hepatocytes Post-ALF Induction

Hepatocyte transplantation increased the host's lifespan, suggesting that the hepatocytes in the remnant liver might be stimulated to proliferate or their cell death rates might decrease despite no gain in liver

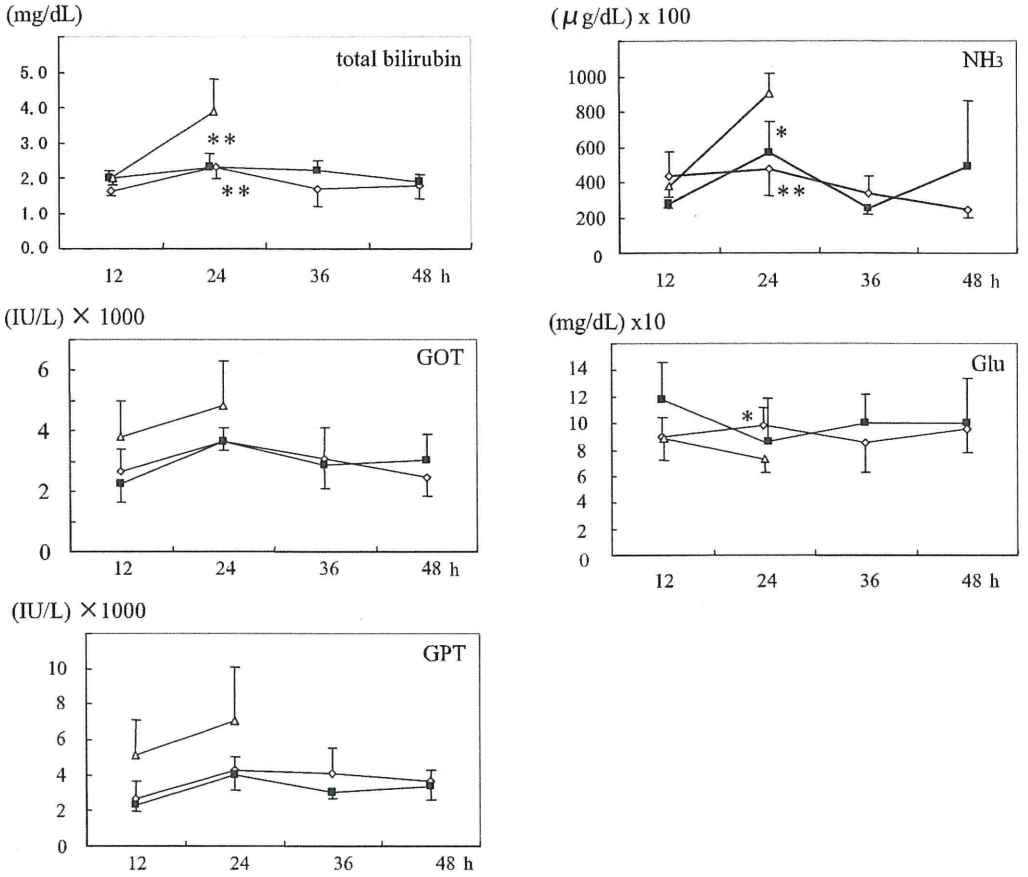

FIG. 4. Biochemical evaluation of hepatocyte transplantation therapy for ALF. The rats were subjected to hepatocyte transplantation and ALF treatment as described in Figure 2. At the indicated time points after ALF treatment, blood was collected for total bilirubin, NH_3, GOT, Glu, and GPT assessment. The mean values of total bilirubin, GOT, GPT, NH_3, and Glu in the normal control rats were 0.3 ± 0.1 (mg/dL), 75 ± 18 (IU/L), 25 ± 6 (IU/L), 151 ± 23 (μg/dL), and 197 ± 26 (mg/dL), respectively. The open diamond, closed rectangle, and open triangle indicate the FIHEP, CPHEP, and CM groups, respectively. *$P < 0.05$ *versus* the CM group. **$P < 0.01$ *versus* the CM group.

weight within the experimental period (up to 5 d). To address this possibility, the BrdU-labeling index and TUNEL activity were determined as a measure of cell proliferation activity and cell death, respectively. BrdU-labeling indexes at 24 h post-ALF in the CM, FIHEP, and CPHEP groups are shown in Figure 5A-1, A-2, and A-3, respectively. $BrdU^+$ nuclei were present in the FIHEP and CPHEP groups but were scarce in the CM group. These $BrdU^+$ hepatocytes were host hepatocytes because they were $DPPIV^-$. The BrdU-labeling indexes are shown in Figure 5A-4. The indexes at 12 h were low (<2%) and not significantly different among the three groups of rats. The indexes of the FIHEP and CPHEP groups at 24 h significantly increased, compared with those of the CM group. At 48 h post-ALF, there was a similarly large increase in the labeling indexes (>10%) in both the FIHEP and CPHEP rat livers, indicating that CPHEP transplantation stimulated the proliferation of the remnant hepatocytes as effectively as FIHEP transplantation. In a parallel experiment, some sections at 24 h post-ALF were stained for TUNEL activity. $TUNEL^+$ hepatocytes were frequently observed in the CM rats (Fig. 5B-1) but decreased substantially in the FIHEP (Fig. 5B-2) and CPHEP (Fig. 5B-3) rats. The ratios of the $TUNEL^+$ hepatocytes to the total hepatocytes are shown in Figure 5B-4 as apoptotic indexes. The apoptotic index

TABLE 1

Comparison of Inflammatory Cytokines 24 h Post-ALF Induction

Exp. group	IL-1β (pg/mL)	IL-6 (pg/mL)	TGF-β1 (ng/mL)
SO	ND	ND	7.27 ± 3.16
FIHEP	382.1 ±107.3	499.8 ± 485.6	10.56 ± 4.21*
CPHEP	418.1 ± 73.8	337.4 ± 150.7*	10.79 ± 1.94*
CM	329.1 ± 32.8	4375.5 ± 5568.9	15.27 ± 2.74

ALF = acute liver failure; SO = sham operation; ND = not detected. FIHEP = freshly isolated hepatocyte; CPHEP = culture-propagated hepatocyte; CM = culture medium

Sham operation indicates laparotomy alone.

*$P < 0.05$ *versus* the CM group.