Statistical analyses were performed using unpaired Student's *t* test and chi-square test with Fisher's exact test. Overall survival and disease-free survival rates were calculated using the Kaplan–Meier method and compared using log-rank test. Disease-free survival was calculated, considering any death or recurrence as an event. A *P* value <0.05 was considered to be statistically significant. Statistical analysis was performed using StatView for Windows (Version 5.0; SAS Institute, Cary, NC, USA).

Results

As shown in Fig. 1, there were 151 patients with initially resectable advanced HCC who did not fulfill MC (i.e., exceeding MC) and 471 patients who met MC.

In the exceeding-MC group, the mean follow-up period for all survivors was 4.1±3.1 years (range, 0.5 to 14.5 years). Table 1 shows the patients' backgrounds. Overall operative mortality and in-hospital mortality rates were the same, i.e., 0.7% (n=1) in both conditions. The incidence of complications that developed after hepatectomy is also shown in Table 1. Thirty of the 151 patients (20%) had postoperative complications (Table 1). Nineteen of the 151 patients (13%) were grade III or more.

Figure 2a shows the survival rates of patients who underwent curative resection of HCC (meeting MC and exceeding MC). The survival rate of the exceeding-MC group was significantly lower than that of the group that met MC (P=0.030). The 3-, 5-, and 10-year survival rates

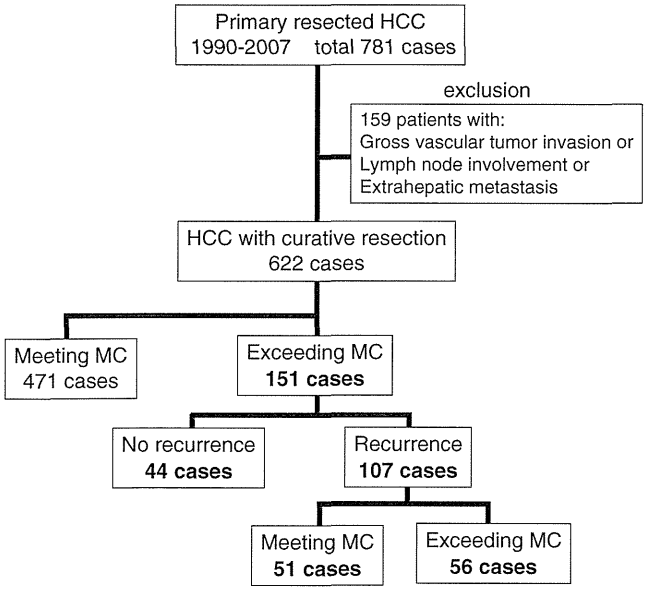

Fig. 1 Overview of outcomes of patients with primary resected hepatocellular carcinoma (HCC). The number of HCC patients who underwent curative resection was 622, subdivided by the Milan criteria (MC)

Table 1 Patients' backgroud

	Number of patients	Percent
Age (year)		
≤60	57	38.4
>60	94	61.6
Gender		
Male	127	84.1
Female	24	15.9
Type of hepatitis virus		
Non-HCV	61	40.4
HCV	90	59.6
Child–Pugh grade		
A	129	85.4
B	22	14.6
Type of hepatectomy		
Limited resection	82	54.3
Segmentectomy or more	69	45.7
Operative mortality: yes	1	0.7
In-hospital mortality: yes	1	0.7
Postoperative complications[a]: yes	30	19.9
Grade I, II	11	7.3
Grade III or more	19	12.6

[a] Postoperative complications was defined as any event satisfying the criteria advocated by Dindo et al.[12]

of the exceeding-MC group were 77%, 55%, and 33% and 86%, 68%, and 37% in those that met MC, respectively. The 3-, 5-, and 10-year disease-free survival rates of the exceeding-MC group were 36%, 30%, and 17% and 47%, 30%, and 13% in those that met MC, respectively (Fig. 2b).

Table 2 summarizes the results of the univariate analysis according to clinicopathological factors. A platelet count $<10^5/mm^3$ (P<0.001), multiple tumors (P=0.012), and cirrhosis of noncancerous tissue (P=0.035) were significant adverse prognostic factors for overall survival. Similarly, a platelet count $<10^5/mm^3$ (P=0.001), multiple tumors (P=0.005), and cirrhosis of noncancerous tissue (P=0.020) were significant adverse prognostic factors for disease-free survival.

By multivariate analysis, a platelet count $<10^5/mm^3$ (P=0.007) was found to be an independent adverse prognostic factor for overall survival (Table 3), and the 3-, 5-, and 10-year overall survival rates of patients with HCC exceeding MC whose platelet count was $\geq 10^5/mm^3$ were 76%, 65%, and 44%, respectively, comparable with the group that met MC (86%, 68%, and 37%, respectively; Fig. 2a). A platelet count $<10^5/mm^3$ (P=0.039) was also an independent adverse factor for disease-free survival (Table 3).

Out of 151, a total of 107 (71%) patients with HCC exceeding MC experienced a recurrence after the initial hepatectomy. Table 4 shows the patterns of cancer recurrence

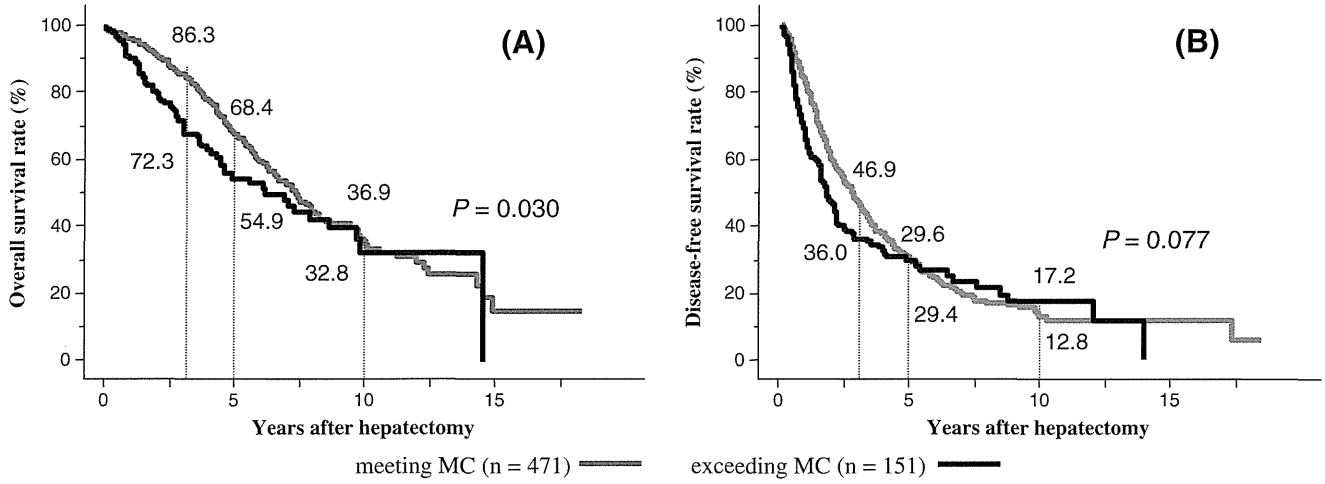

Fig. 2 Survival and disease-free survival curves of patients who received curative resection of HCC that met (471 patients) or were exceeding (151 patients) MC. (A) The 3-, 5-, and 10-y survival rates of patients exceeding MC were 72.3%, 54.9%, and 32.8%, respectively, and 86.3%, 68.4%, and 36.9%, respectively, in those who met MC. (B) The 3-, 5-, and 10-y disease-free survival rates of patients exceeding MC were 36.0%, 29.4%, and 17.2%, respectively, and 46.9%, 29.6%, and 12.8%, respectively, in those who met MC

and compares the consequent treatment details between patients whose platelet counts were $\geq 10^5/mm^3$ and $<10^5/mm^3$. The rate of HCC recurrence was significantly lower in patients whose platelet count was $\geq 10^5/mm^3$; 76 (66%) of such patients experienced a recurrence of HCC after hepatectomy, as compared to 31 (89%) patients whose platelet count was $<10^5/mm^3$ (P=0.009). Further, regarding the pattern of recurrence, the proportion of patients who had a recurrence of HCC that met MC was significantly higher in patients with a platelet count$\geq 10^5/mm^3$ than those with a platelet count of $<10^5/mm^3$ (51% vs. 39%; P<0.001).

The proportion of patients who received curative treatment for the first recurrence, such as repeat hepatectomy and local ablation therapy, had significantly higher platelet counts, i.e., $\geq 10^5/mm^3$ (44% vs. 23%; P=0.047).

Of the 107 patients who experienced a recurrence, 51 (48%) met MC and 56 (52%) were exceeding MC, including extrahepatic recurrence (Fig. 1). The 3- and 5-year survival rates after recurrence were significantly superior in patients with a recurrence that met MC (71% and 40%, respectively) than those exceeding MC (17% and 9%) (P<0.001; Fig. 3).

Table 5 shows the details of the treatments for recurrences after hepatectomy. The proportions of patients who received ablation therapy or repeat hepatectomy after recurrence was higher in patients with a recurrence that met MC than those exceeding MC (P=0.001). Two patients with a recurrence that met MC, who underwent salvage living donor liver transplantation (LDLT), did not have a recurrence after liver transplantation at the 2- and 3-year follow-up, respectively. One patient with a recurrence that was exceeding MC, and who underwent salvage LDLT, experienced a recurrence of HCC within 1.5 years.

Discussion

The ultimate goal of a treatment for HCC is to prolong survival by eradicating malignant legions while preserving hepatic function. Surgical resection, by partial hepatectomy or total hepatectomy followed by OLT, is the standard treatment with a curative intent.[13] The resectability and choice of procedure depend on many factors, including baseline liver function, absence of extrahepatic metastasis, size of residual liver, availability of resources (including liver grafts), and expertise of the surgical team.

Although hepatic resection, ablation therapy, and liver transplantation are accepted, effective treatments for patients with cirrhosis and early HCC, the proper strategy for advanced HCC has not been established. Therefore, we studied HCC patients who were exceeding MC—who are not eligible for OLT as the initial treatment. We investigated the impact of hepatectomy on outcomes of HCC that exceeded MC and examined the rationale of hepatectomy as an initial treatment for HCC exceeding MC.

In our series, the 5- and 10-year survival rates of patients with HCC exceeding MC were 55% and 33%, respectively, comparable with Kamiyama et al.[14] We also identified significant prognostic factors of patients with HCC exceeding MC who underwent hepatectomy: platelet count, tumor number, and cirrhosis. Moreover, our multivariate analysis revealed that platelet count was the sole independent prognostic factor in these HCC patients.

The prognosis of such patients after hepatectomy was clearly stratified by platelet count, which is typically predictable by preoperative laboratory tests. The 3-, 5-, and 10-year overall survival rates of patients with HCC exceeding MC, whose platelet count was $\geq 10^5/mm^3$, were

Table 2 Overall and disease-free survival rates of patients with HCC exceeding MC according to clinicopathological factor

		Overall survival (%)				Disease-free survival (%)			
		3-year	5-year	10-year	*P* value	3-year	5-year	10-year	*P* value
All cases (n=151)		73	55	33		36	30	17	
Age (year)	≤60 (n=57)	69	58	38	0.873	35	28	17	0.977
	>60 (n=94)	74	53	30		37	30	18	
Gender	Male (n=127)	75	55	34	0.647	34	27	15	0.247
	Female (n=24)	61	56			45	45		
Type of hepatitis virus	Non-HCV (n=61)	71	65	36	0.498	46	39	25	0.054
	HCV (n=90)	73	50	32		29	22	12	
Total bilirubin (/mm^3)	<1.0 (n=125)	71	52	32	0.151	37	32	19	0.515
	≥1.0 (n=26)	78	72	36		30	19	9	
Platelet counts (/mm^3)	<10^5 (n=35)	61	27		< 0.001	16	8		0.001
	≥10^5 (n=116)	76	65	44		42	36	21	
ALT (IU/I)	<60 (n=106)	71	49	29	0.08	36	30	15	0.707
	≥60 (n=45)	77	70	45		36	27	18	
Alb (g/dL)	<3.5 (n=37)	73	52	41	0.995	42	31	23	0.55
	≥3.5 (n=114)	73	58	32		35	29	15	
ICG-R15 (%)	<20 (n=111)	72	57	43	0.303	40	32	20	0.467
	≥20 (n=39)	75	52			26	22		
Child–Pugh grade	A (n=129)	73	56	30	0.643	35	29	18	0.645
	B (n=22)	72	54	46		43	32	17	
AFP (ng/mL)	<400 (n=101)	77	56	31	0.905	33	26	16	0.495
	≥400 (n=48)	65	55	41		45	39	22	
Number of tumors	Single (n=60)	79	71	52	0.012	52	41	28	0.005
	Mutiple (n=91)	68	45	23		26	22	12	
Tumor distribution	One section (n=77)	81	56	43	0.083	42	33	28	0.091
	more (n=74)	61	55	25		32	23	8	
Non-cancer tissue	Cirrhosis (n=52)	67	39	29	0.035	23	15	8	0.02
	Others (n=99)	75	65	38		42	36	23	
Preoperative TAE	Yes (n=102)	72	55	30	0.91	35	28	15	0.366
	No (n=45)	73	56	50		40	33	25	
Type of hepatectomy	Limited resection (n=82)	73	51	29	0.743	34	27	8	0.472
	Segmentectomy or more (n=69)	71	60	39		39	33	33	
Transfusion	Yes (n=20)	64	46	0	0.071	25	17	0	0.103
	No (n=131)	74	57	37		38	31	21	
Microscopic vascular invasion	Yes (n=74)	60	48	30	0.089	30	28	17	0.144
	No (n=77)	84	61	35		42	31	18	
Histologic grading	Well or moderate (n=122)	71	55	30	0.718	34	28	18	0.777
	poor (n=26)	74	52	43		42	31	12	
Diabetes mellitus	Yes (n=53)	73	58	34	0.929	39	30	17	0.493
	No (n=95)	72	52	31		33	29	17	
SF criteria	Meeting SF (n=59)	74	52	23	0.704	30	28	15	0.734
	Exceeding SF (n=92)	71	57	38		40	32	19	

HCC hepatocellular carcinoma, *MC* Milan criteria, *ALT* alanine aminotransferase, *ICG-R15* indocianine green retension rate at 15 min, *AFP* alpha-fetoprotein, *SF* San Francisco criteria (1 lesion <6.5 cm, 2–3 lesions each <4.5 cm with total diameter <8 cm)

76%, 65%, and 44%, respectively, comparable with those that met MC (86%, 68%, and 37%, respectively).

Hepatectomy should be the first-line treatment in patients with HCC exceeding MC whose platelet count is >10^5/mm^3.

Table 3 Results of Cox's proportional hazards analysis for overall and disease-free survival after hepatectomy

Variables	*P* value	Relative risk	95% CI
Overall survival			
Plt. Count: $<10^5/mm^3$	0.007	2.155	1.232–3.774
Number of tumors: multiple	0.103	1.65	0.903–3.021
Tumor distribution: more than one section	0.168	1.439	0.858–2.410
Transfusion: Yes	0.13	1.667	0.861–3.228
Microscopic vascular invasion: Yes	0.067	1.596	0.969–2.629
Non-cancer tissue: cirrohsis	0.488	1.207	0.709–2.058
Disease-free survival			
HCV infection: Yes	0.585	1.148	0.699–1.887
Plt. Count: $<10^5/mm^3$	0.039	1.653	1.025–2.667
Number of tumors: multiple	0.202	1.368	0.845–2.221
Tumor distribution: more than one section	0.098	1.412	0.939–2.123
Non cancer tissue: cirrohsis	0.274	1.277	0.824–1.979

In general, platelet count, which reflects the severity of portal hypertension, is a significant predictor of survival. Several studies have shown that platelet count is a risk factor for carcinogenesis from chronic hepatitis and for survival and recurrence of HCC after treatment, including liver resection.[15–18] In fact, we observed that recurrence of HCC after hepatectomy decreased in patients whose platelet count was $\geq 10^5/mm^3$ and that the proportion of patients who experienced a recurrence of HCC that met MC was significantly higher in patients with a platelet count $<10^5/mm^3$. Further, the proportion of patients who underwent repeat hepatectomy or RFA as a curative treatment for a recurrence of HCC was significantly higher in patients whose platelet count was $\geq 10^5/mm^3$.

After resection with curative intent, many patients experience a recurrence, which is a significant cause of late death. In this study, the recurrence rate was high: 70.9% of patients with HCC exceeding MC were diagnosed as having had a recurrence (mean follow-up, 4.1 years). Tumor number was an independent factor of disease-free survival, and the 3-, 5-, and 10-year disease-free survival rates were 51%, 41%, and 28%, respectively, even in patients with a single tumor.

The reported cumulative 5-year recurrence rates range from 50% to 100%.[19–22] In our series, 107 (71%) of 151 patients with HCC exceeding MC experienced a recurrence of HCC, 51 (48%) of whom met MC. These results demonstrate that downstaging a recurrence to within MC was achieved by hepatectomy as an initial treatment for HCC exceeding MC. The proportion of patients who underwent repeat hepatectomy or local ablation therapy as a curative treatment for HCC recurrence was significantly higher in patients with a recurrence of HCC within MC versus exceeding MC. The outcomes after recurrence were significantly better in patients whose recurrence was downstaged to within MC compared with those who did

Table 4 Recurrent pattern and treatment of recurrent HCC after hepatectomy (comparison with platelet counts)

	Platelet counts $>10^5$ (n=116)	Platelet counts $<10^5$ (n=35)	*P* value
Cancer recurrence[a]: yes	76 (66)	31 (89)	0.009[c]
Recurrent pattern[b]			<0.001[c]
Meeting MC	39 (51)	12 (39)	
Exceeding MC or extrahepatic recurrence	37 (49)	19 (61)	
Treatments for recurrence[b]			0.047[c]
Curative treatment	34 (44)	7 (23)	
Non-curative treatment	41 (55)	22 (70)	
Salvage liver transplantation	1 (1)	2 (6)	

Curative treatment included partial hepatectomy, local ablation therapy; non-curative treatment included transarterial chemoembolization, systemic chemotherapy, radiation therapy and conservative

HCC hepatocellular carcinoma, *MC* Milan criteria

[a] Data are expressed as the number of patients (percentage of total patients)

[b] Data are expressed as the number of patients (percentage of patients who had a recurrence)

[c] Statistically significant difference

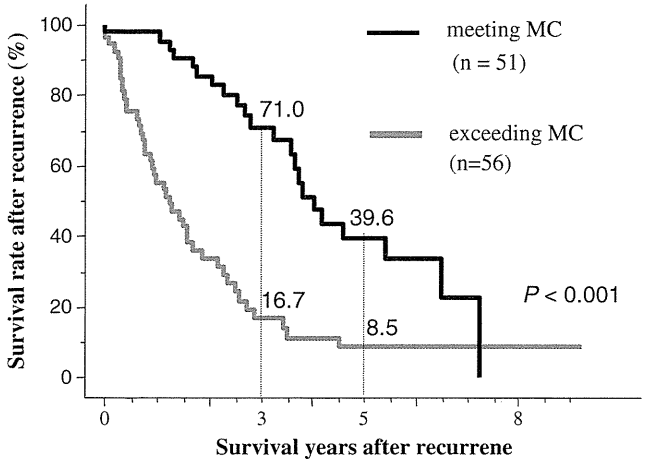

Fig. 3 Comparison of survival curves after recurrence of HCC according to recurrent pattern. The 3-and 5-year survival rates of patients with a recurrence that met MC were 71.0% and 39.6%, respectively, and 16.7% and 8.5%, respectively, in those who exceeding MC including extrahepatic recurrence

not achieve such downstaging. These results indicate that hepatectomy as an initial treatment is an important component of the treatment strategy for HCC exceeding MC.[23]

With regard to the treatment of recurrent HCC patients, we reported that the more hepatectomy was repeated, the shorter the recurrence-free interval became, suggesting a limitation of repeat hepatectomy in curing recurrent HCC.[24] Liver transplantation has been discussed as the next strategy to treat tumor recurrences after initial hepatectomy in patients with advanced HCC. Several studies have reported salvage transplantation for recurrence after hepatectomy,[6,25–27] suggesting that primary hepatectomy and salvage liver transplantation is a feasible and rational strategy for patients with small HCC that preserves liver function. In this series, of the patients who had recurrence after resection for tumors exceeding MC, approximately 48% had recurrent tumors that were within MC. This result also indicates that approximately half of the patients with recurrence would be candidates for salvage liver transplantation after partial hepatectomy performed for downstaging to within MC. Salvage LDLTs were adopted for three patients, two of whom, who had a recurrence that met MC, did not experience a recurrence after salvage LDLT at the 2- and 3-year follow-up, respectively. Yao et al. and Ravaioli et al. reported that locoregional treatments, including RFA, were effective for downstaging prior to liver transplantation.[23,28] In general, RFA was indicated for HCCs with diameters less than 3 cm. Although RFA may be effective for downstaging multiple small HCCs, its effectiveness may be limited in the case of downstaging large HCCs with diameters greater than 3 cm. Further studies are required to clarify the indications for the use of RFA and hepatectomy as downstaging modalities prior to liver transplantation.

A significant proportion of patients with HCC exceeding MC might benefit from liver transplantation. Mazzaferro et al. proposed an expansion of the indications for liver transplantation, using up to seven criteria.[29] Takada et al. demonstrated that LDLT could be safely extended to ≤10 tumors (all ≤5 cm in diameter and PIVA-II≤400 mAU/mL) with acceptable outcomes.[30] Liver transplantation has been proposed as an initial treatment for patients with HCC exceeding MC whose platelet count is $<10^5/mm^3$, although the extension of the indications of liver transplantation is restricted.

Conclusion

Hepatectomy for patients with HCC exceeding MC increases survival rates, especially for patients with sufficiently high platelet counts, although their recurrence rates

Table 5 Treatments for recurrent HCC after initial hepatectomy

Modalities	Recurrent pattern *N* (%)[a]		*P* value
	Meeting MC (*n*=51)	Exceeding MC or extrahepatic (*n*=56)	
Partial hepatectomy	10 (20)	5 (9)	<0.001[b]
Salvage liver transplantation	2 (4)	1 (2)	
Resection of distant metastasis	0	3 (5)	
Percutaneous ablation therapy	18 (35)	8 (14)	
TACE	21 (41)	23 (41)	
Chemotheraphy and/or radiation	0	10 (18)	
Non-treatment	0	6 (11)	

MC Milan criteria, *TACE* transarterial chemoembolization

[a] Data are expressed as the number of patients (percentage of patients who had a recurrence of each group)

[b] Statistically significant difference

after initial hepatectomy are high. Hepatectomy as an initial treatment is an important component of the treatment for HCC exceeding MC to downstage the recurrence to within MC.

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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.

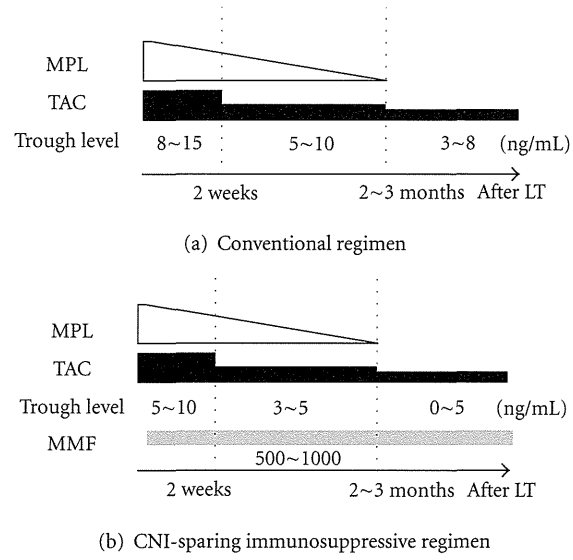

FIGURE 1: Immunosuppressive protocol after liver transplantation. The basic immunosuppressive regimen comprised tacrolimus (TAC) and methylprednisolone (MPL), with doses gradually being tapered off. The trough whole blood levels of TAC were maintained between 8 and 15 ng/mL in the first few postoperative 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

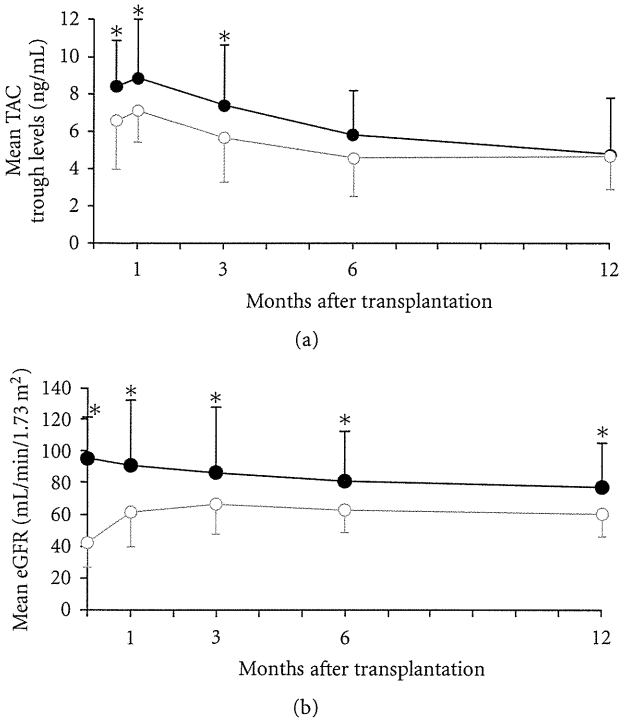

FIGURE 2: Kinetics of mean trough levels of tacrolimus and mean estimated glomerular filtration rate (eGFR) in the RI group and non-RI group during the first year after transplantation. (a) Mean trough levels of tacrolimus in the non-RI group (black line) and RI group (gray line). (b) Mean estimated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

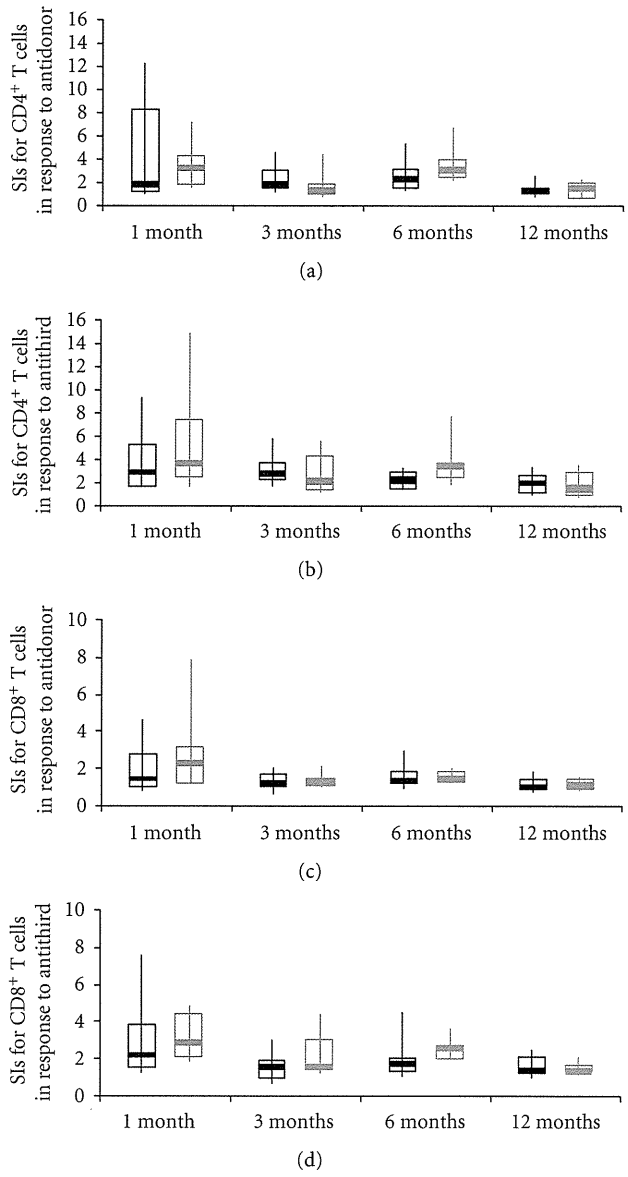

FIGURE 3: Kinetics of stimulation index in the RI group and non-RI group during the first year after transplantation. Stimulation index (SI) of each of the CD4⁺ T-cell (a, b) and CD8⁺ T-cell (c, d) subsets in the antidonor (a, c) and anti-third-party (b, d) MLR in patients in non-RI group (black line) 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.

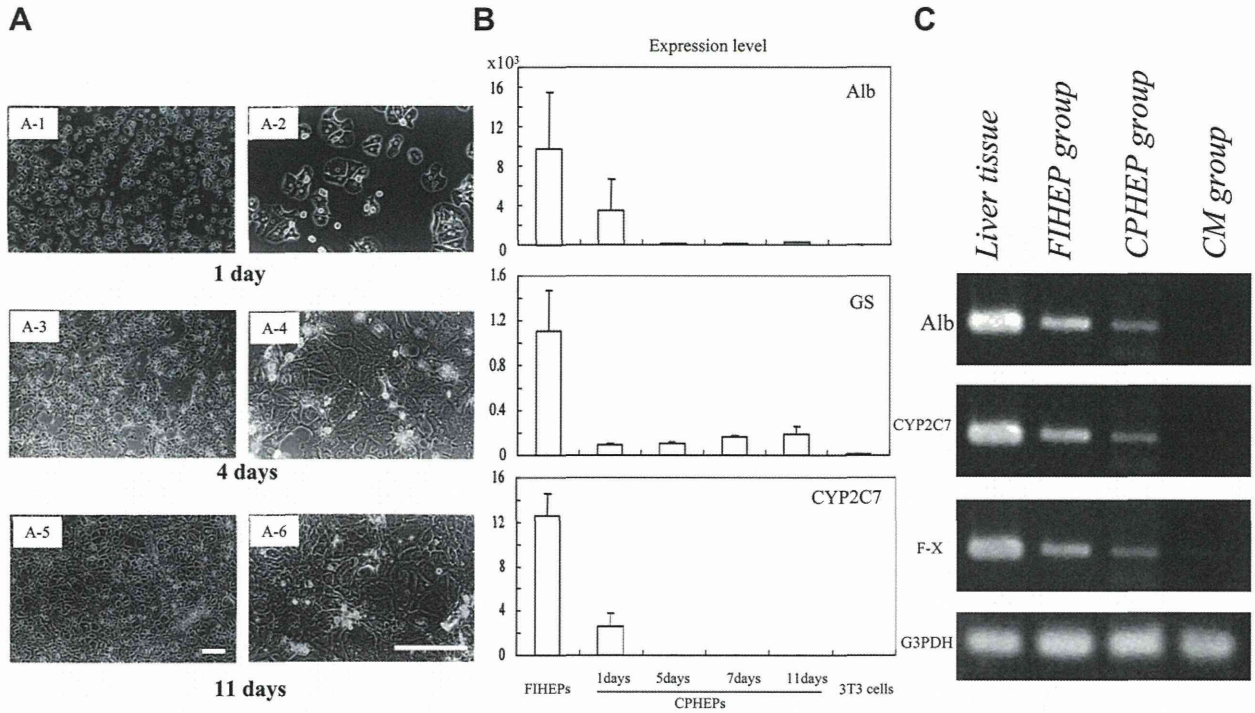

FIG. 1. (A) Phase contrast image of proliferating hepatocytes. Hepatocytes (8.5×10^3 cells/cm^2) were cocultured with Swiss 3T3 cells in HCGM on 15.0-cm dishes. Photographs were taken for the same fields at 1 (A-1, 2), 4 (A-3, 4), and 11 d (A-5, 6) with lower (A-1, 3, 5) and higher (A-2, 4, 6) magnifications. 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.

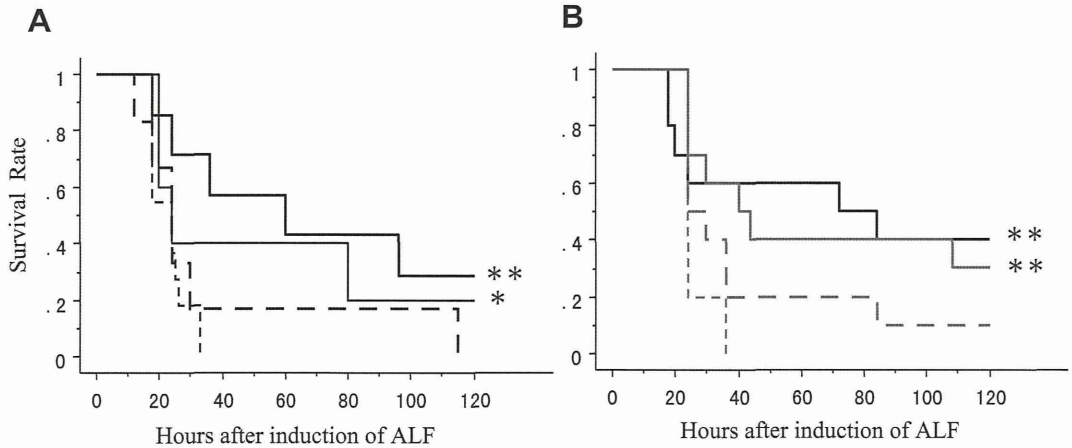

FIG. 2. Survival curves of ALF rats with FIHEP transplantation. The rats were transplanted with HEPs or FBs through the spleen and then subjected to ALF. Some rats were given CM as controls. (A) Rescue of ALF by FIHEP transplantation. The rats were given varying numbers of FIHEPs: 1.5×10^7 cells ($n = 7$, 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.

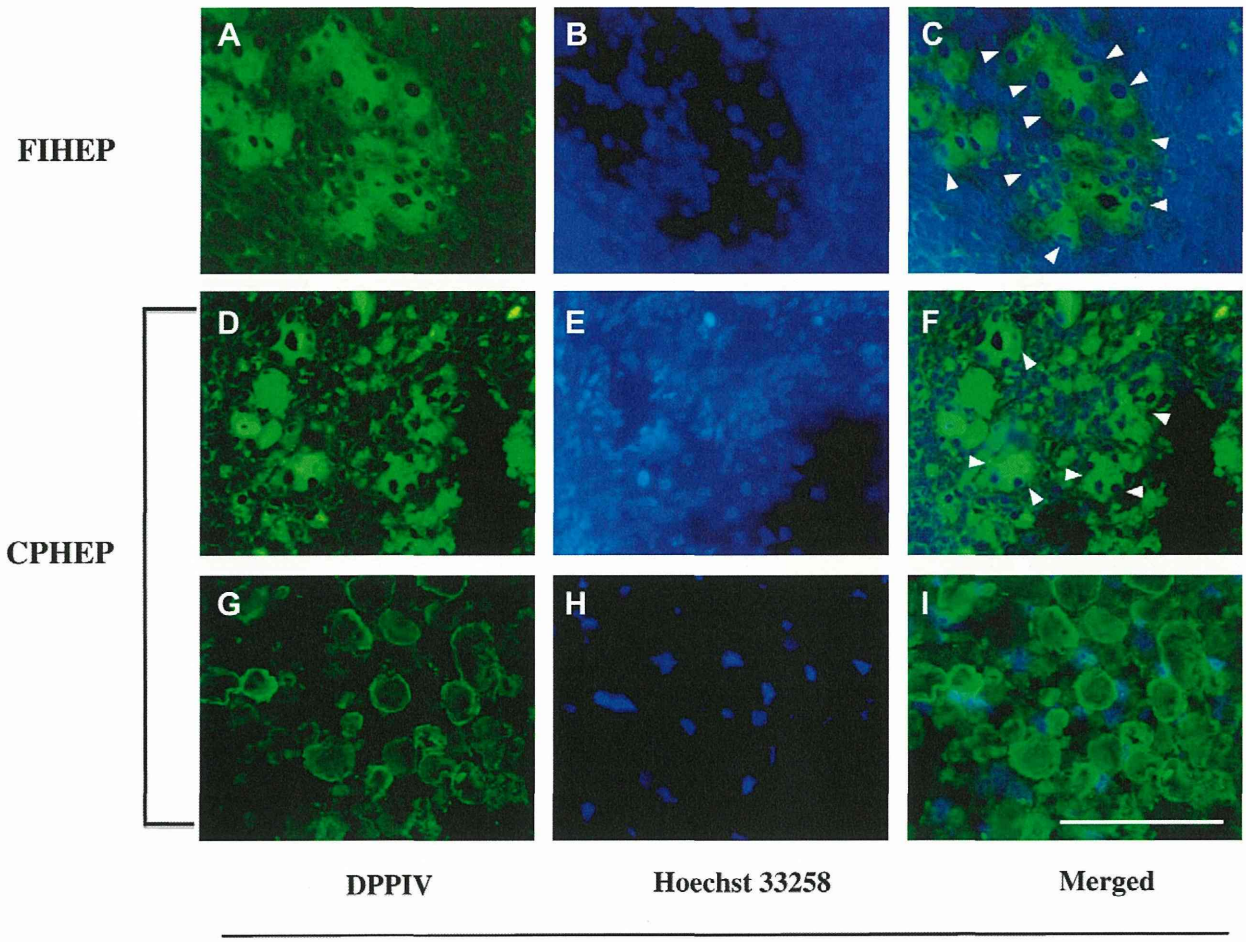

FIG. 3. Engraftment of the transplanted hepatocytes in the spleen of ALF rats. The rats were transplanted with FIHEPs (A)–(C) or CPHEPs (D)–(I) and subjected to ALF as in Figure 2B. Spleens were removed at 24 h after ALF induction and processed to cryosectioning for immunohistochemical analysis to detect 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

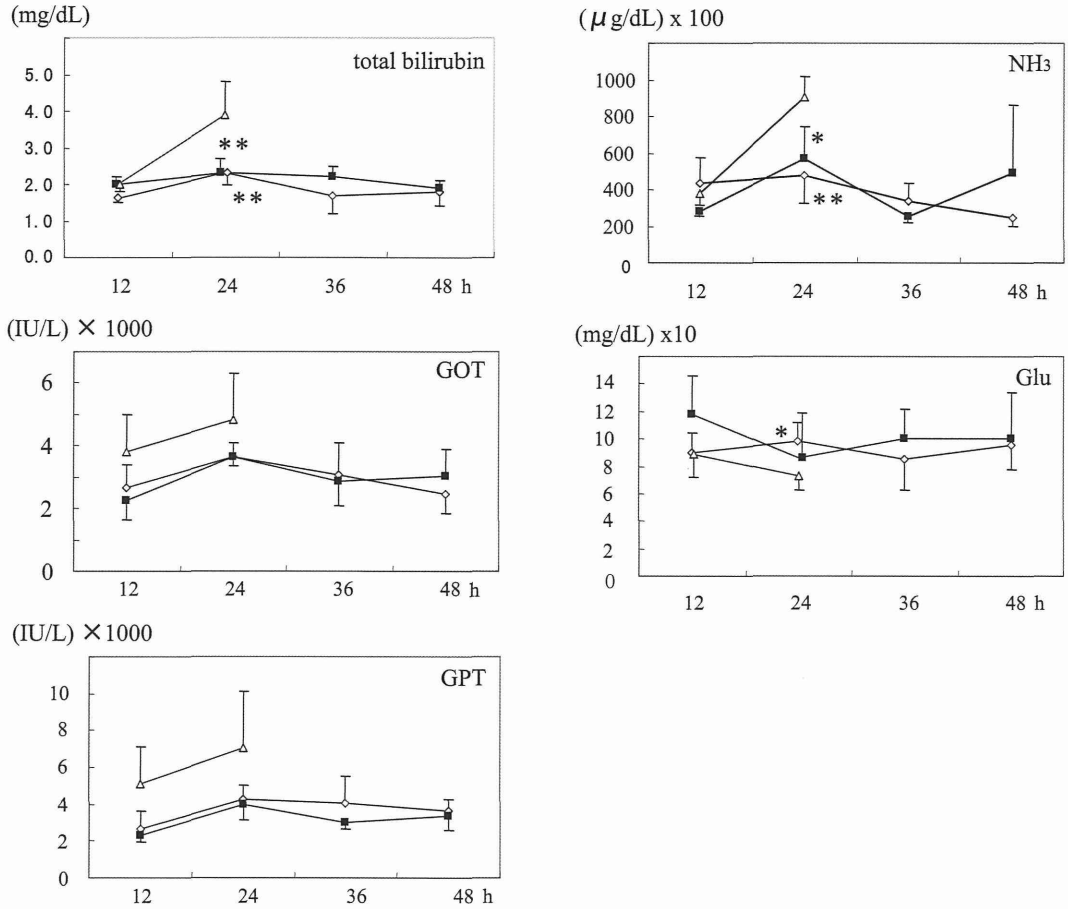

FIG. 4. Biochemical evaluation of hepatocyte transplantation therapy for ALF. The rats were subjected to hepatocyte transplantation and ALF treatment as described in Figure 2. At the indicated time points after ALF treatment, blood was collected for total bilirubin, NH_3, GOT, Glu, and GPT assessment. 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.

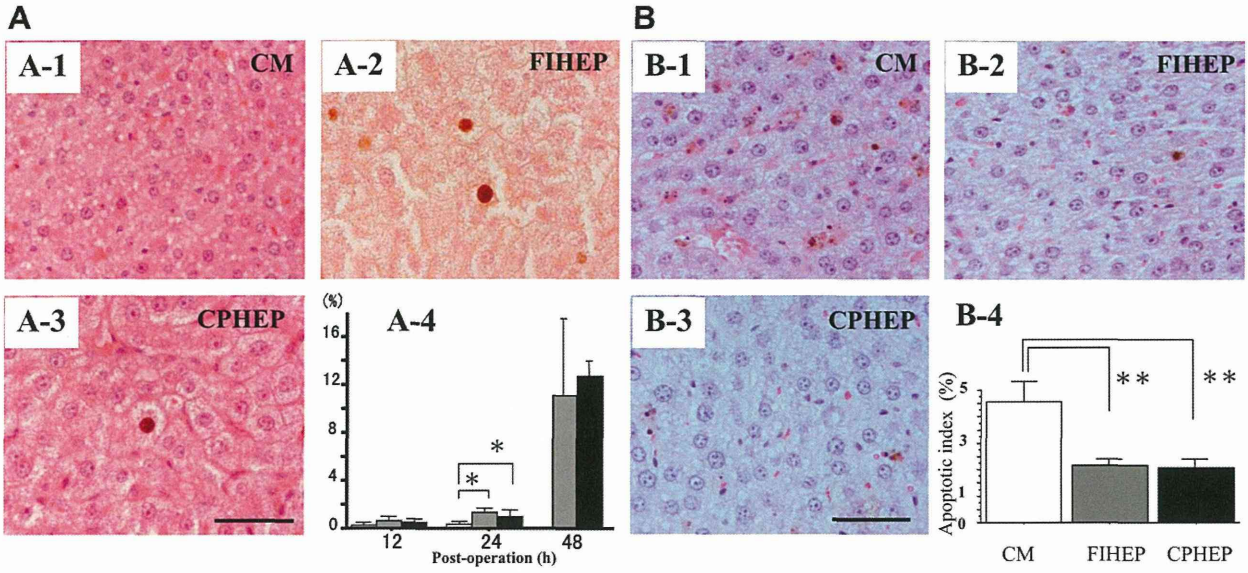

FIG. 5. (A) BrdU-labeling index of hepatocytes in the remnant liver of the hepatocyte- transplanted rat. The rats were injected with CM, transplanted with FIHEPs or CPHEPs, and subjected to ALF as in Fig. 2. The remnant livers (omental lobe) were removed at 12, 24, and 48 h post-induction of ALF and processed to obtain paraffin sections for BrdU staining. (A-1), (A-2), and (A-3) are representative of photos from rats with CM, FIHEPs, and CPHEPs, respectively, taken at 24 h post-ALF. $BrdU^+$ nuclei are brown in color. In (A-4), $BrdU^+$ cells were counted from five microscopic fields of each section from 4 rats in each group at the time points indicated, and the BrdU-labeling index was calculated as the ratio of $BrdU^+$ cells to the total cells in a counted field. The open bar, gray bar, and black bar indicate the CM, FIHEP, and CPHEP groups, respectively. $*P < 0.05$ *versus* the CM group. Bar, 50 μm. (B) Suppression of remnant hepatocyte apoptosis by hepatocyte transplantation. The rats were transplanted with hepatocytes and subjected to ALF as described in Fig. 2. Paraffin sections were prepared from the remnant livers (omental lobes) isolated from the CM (B-1), FIHEP (B-2), and CPHEP groups (B-3) at 24 h post-ALF and were stained for TUNEL activity. $TUNEL^+$ pycnotic nuclei (brown) were frequently observed in the CM group, but less often in the FIHEP and CPHEP groups. Apoptotic cells were counted from five microscopic fields of liver tissue sections from four rats in each group. The ratio of apoptotic cells to total cells in the counted field was expressed as the apoptotic index (B-4). The open bar, gray bar, and black bar indicate the CM, FIHEP, and CPHEP groups, respectively. $**P < 0.01$ *versus* the CM group. Bar, 50 μm.

of the remnant liver in the FIHEP and CPHEP groups decreased to approximately 50% of that in the CM group. These $TUNEL^+$ hepatocytes were host hepatocytes because they were $DPPIV^-$. Thus, CPHEP transplantation suppressed the apoptotic changes in the host hepatocytes as effectively as FIHEP transplantation.

DISCUSSION

Although several studies have supported the effectiveness of hepatocyte transplantation in treating patients with ALF, there is a severe problem in using hepatocyte transplantation therapy as a general clinical treatment for patients with liver failure: owing to the lack of donor organs available for clinical use, hospitals cannot supply sufficient quantities of normal human hepatocytes to such patients. One way to overcome this limitation might be to devise a method of abundantly propagating hepatocytes in culture, starting with a small amount of hepatocytes isolated from small pieces of available liver tissues. However, it does not seem to be a practical solution, because it is well documented that normal hepatocytes show poor multiplication ability *in vitro* despite their remarkable growth potential *in vivo* [19].

We have been engaged in developing a technology to abundantly propagate hepatocytes in culture [8, 9] and previously reported that rat hepatocytes were capable of repeatedly multiplying *in vitro* when cocultured with Swiss 3T3 cells in a medium that we devised [10]. We have now shown that such CPHEPs can be used as a source of hepatocyte transplantation for preventing hepatectomy-induced ALF. Resection of hepatic tumors is currently the gold standard treatment for patients with either primary or secondary liver malignancies. An extended hepatectomy is often necessary to achieve curative resection; however, ALF after massive hepatectomy remains a challenging problem (i.e., the risk of insufficiency of remnant liver volume, leading to unresectability). If we devise a countermeasure to prevent ALF beforehand, aggressive hepatic resection could be safely performed. Seeking to answer this clinical question, we evaluated the prevention efficacy of CPHEP transplantation in a surgical model of hepatectomy-induced ALF.

To estimate the efficacy of transplanting either FIHEPs or CPHEPs in ALF, we employed an experimental ALF model induced by subjecting rats to two-thirds-hepatectomy and ligation of the right-lobe pedicle. This method induces more severe liver failure than