primary HSCs and was upregulated during activation of HSCs.[6]

To gain further insights into the pathogenesis of NASH, we examined the influence of AGEs on HSCs in vitro. We found that AGEs enhanced activation and proliferation of HSCs, which was accompanied by increased generation of intracellular reactive oxygen species (ROS). Furthermore, AGEs increased the production of proinflammatory cytokines from HSCs. These findings suggest a novel molecular mechanism by which AGEs contribute to hepatic fibrosis and inflammation in NASH.

Methods

Chemicals

N-Acetylcysteine (NAC), diphenylene iodonium chloride (DPI), indomethacin, allopurinol, and 2',7'-dichlorofluorescein diacetate (DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium cyanide (KCN) was obtained from Nakalai Tesque (Kyoto, Japan).

Cell cultures

We utilized HSC line LI90 (JCRB0160, Japan Health Science Foundation, Tokyo, Japan), established by Murakami et al.,[7] which exhibits characteristics compatible with normal activated HSCs. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) on uncoated plastic dishes, and then starved in FBS-free media for 48 h before the start of all experiments.

Preparation of AGE proteins

Glycer-AGE was prepared as described previously.[8] Control nonglycated bovine serum albumin (BSA), without reducing sugars, was made under the same conditions as the glycer-AGE.

Western blot analysis

Cells were lysed in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Denver, CO, USA) for 20 min on ice, followed by centrifugation at 13000 *g* for 5 min. The extracted proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 for 90 min, and then incubated overnight at 4°C with primary antibody of rabbit anti-receptor for AGEs (RAGE)[9] at a 1:1000 dilution, or mouse anti-β-actin (Sigma, Tokyo, Japan) at a 1:10000 dilution. Following incubation with a horseradish peroxidase-linked secondary antibody, the bands were visualized using an ECL Plus Western blotting analysis system (Amersham Biosciences, Buckinghamshire, UK).

Measurement of intracellular ROS generation

Intracellular ROS generation[10] was investigated by using the fluorescent probe DCFDA to detect the liberation of dichlorofluorescein (DCF). Subconfluent LI90 cells in 96-well plates were preloaded with 10 μM DCFDA for 30 min and washed twice with phosphate-buffered saline (PBS). Cells were then exposed to 100 μg/ml glycer-AGE for 24 h with either 10 mM NAC or a series of inhibitors of ROS-producing systems: DPI (25 μM), KCN (500 μM), allopurinol (100 μM), and indomethacin (100 μM), as previously reported.[11–13] The fluorescence intensity was measured by a fluorescence spectrophotometer (ARVO sx 1420 Multilabel Counter, Wallac, Waltham, MA, USA) at the wavelength of 490 nm for excitation and 520 nm for emission.

Cell proliferation assay

A methyl tetrazolium salt (MTS) assay (Promega, Madison, MI, USA) was utilized to examine LI90 cell proliferation. LI90 cells (5×10^3 cells/well) were seeded into 96-well culture plates. After 48 h of incubation with 100 μg/ml of glycer-AGE, 20 μL/well of MTS solution was added and cells were incubated for another 2 h. Fluorescent intensity was analyzed by measuring absorbance at 490 nm with a microplate reader (ImmunoMini NJ-2300 Inter Med, Tokyo, Japan).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated with an RNeasy Mini-kit (Qiagen, Hilden, Germany). Single-stranded complementary DNA was synthesized from 1 μg of RNA using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and subjected to the following PCR reactions with specific primer sets (Table 1).[14–17] To examine the mRNA expression of RAGE, we conducted semi-quantitative PCR using a GeneAmp Gold RNA PCR Core Kit (Applied Biosystems). To quantify the mRNA encoding α-smooth muscle actin (α-SMA), TGF-β1, MCP-1, and collagen type Iα2 (COL1A2), real-time PCR was performed with a LightCycler-FastStart DNA Master SYBR Green I kit and LightCycler 5.32 software (Roche Molecular Biochemicals, Mannheim, Germany). The relative expression

Table 1. Polymerase chain reaction primers used in this study

Gene	Primer sequences (sense/antisense)	GenBank accession number	Annealing temperature (°C)
RAGE	5′-ATGGAAACTGAACACAGGCC-3′ 5′-CACACATGTCCCCACCTTAT-3′	NM_001136	59
TGF-β1	5′-GCCCTGGACACCAACTATTGC-3′ 5′-GCTGCACTTGCAGGAGCGCAC-3′	NM_000660	60
α-SMA	5′-ACTGGGACGACATGGAAAAG-3′ 5′-TAGATGGGGACATTGTGGGT-3′	NM_001613	59
COL1A2	5′-ACCTGGTCAAACTGGTCCTG-3′ 5′-GTGTCCCCTAATGCCTTTGA-3′	NM_000089	60
MCP-1	5′-GACCACCTGGACAAGCAAAC-3′ 5′-CTCAAAACATCCCAGGGGTA-3′	NM_002982	55
β-actin	5′-GAGCGGGAAATCGTGCGTGACATT-3′ 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′	NM_001101	55

RAGE, advanced glycation end product receptor; TGF-β1, transforming growth factor β1; α-SMA, α-smooth muscle actin; COL1A2, collagen type Iα2; MCP-1, monocyte chemoattractant protein 1

levels were calculated according to the formula $2^{-\Delta Ct}$, where ΔCt is the difference in threshold cycle (Ct) values between the target and the reference gene, β-*actin*.

Quantification of MCP-1 protein by enzyme-linked immunosorbent assay

To quantify the protein levels of MCP-1 in media, LI90 cells (1×10^5 cells/well) were grown in a six-well dish and stimulated by 100 μg/ml of glycer-AGE with or without 10 mM NAC for 48 h. The supernatant of the conditioned media was subjected to analysis with a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data are expressed as means ± SEM. Statistical comparisons between two groups were performed with the Mann-Whitney *U* test. The level of significance was set at $P < 0.05$ in all cases.

Results

RAGE expression in LI90

We first investigated the expression of RAGE in LI90 cells. RT-PCR identified RAGE mRNA (Fig. 1A), and the presence of RAGE protein was confirmed by Western blot analysis (Fig. 1B).

AGE-induced intracellular ROS generation

Increased ROS formation and resulting oxidative stress in HSCs are considered to contribute to the pathogenesis of various liver diseases.[18] Therefore, we examined whether HSCs generate ROS in response to AGEs. We observed significant ROS induction following exposure of cells to glycer-AGE (Fig. 2). Furthermore, cotreatment with NAC was found to reduce AGE-induced ROS generation. To elucidate the source of ROS, we then examined the inhibitory effect of the following

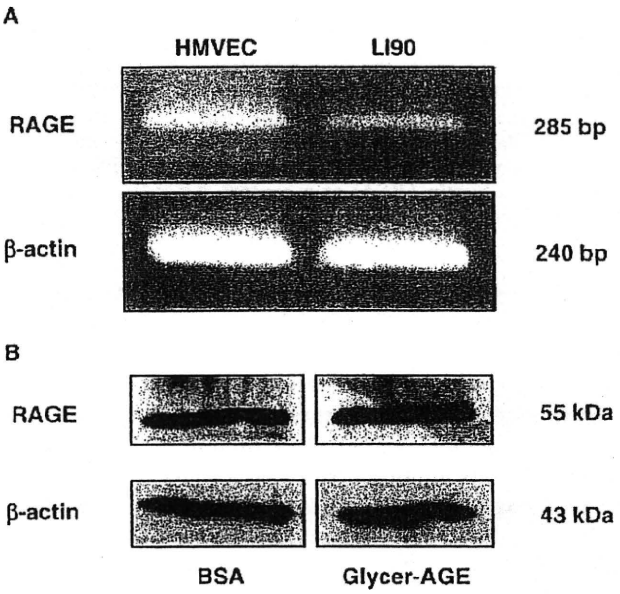

Fig. 1A, B. Expression of advanced glycation end product receptor (*RAGE*) in LI90 cells. **A** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that RAGE mRNA is expressed in LI90 cells. *HMVEC*, human dermal microvascular endothelial cells (positive control). **B** Western blot analysis showed that RAGE protein is expressed in LI90 cells, and exposure to glyceraldehyde-derived advanced glycation end product (*glycer-AGE*) for 24 h did not affect its expression level

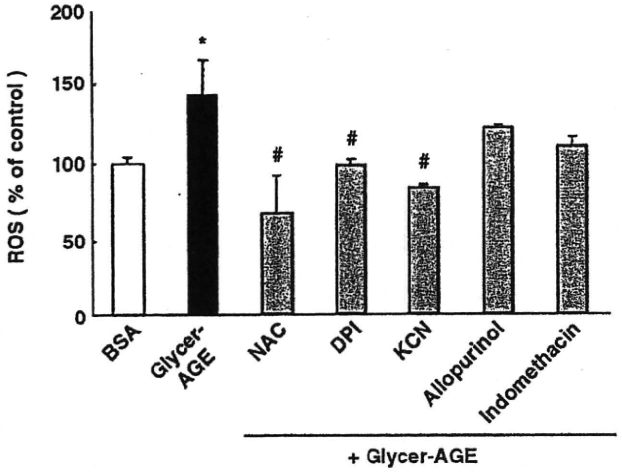

Fig. 2. Effects of advanced glycation end products (AGEs) on intracellular reactive oxygen species (*ROS*) generation Following exposure to glycer-AGE with or without *N*-acetylcysteine (*NAC*) for 24 h, ROS generation was quantified spectrophotometrically with 2′,7′-dichlorofluorescein diacetate (DCFDA). To assess the source of ROS generation, the inhibitory effects of diphenylene iodonium chloride (*DPI*), potassium cyanide (*KCN*), allopurinol, and indomethacin were tested. Data are shown as means ± SEM from six independent experiments. $*P < 0.01$ compared with nonglycated bovine serum albumin (*BSA*) as a control. $\#P < 0.01$ compared with glycer-AGE alone

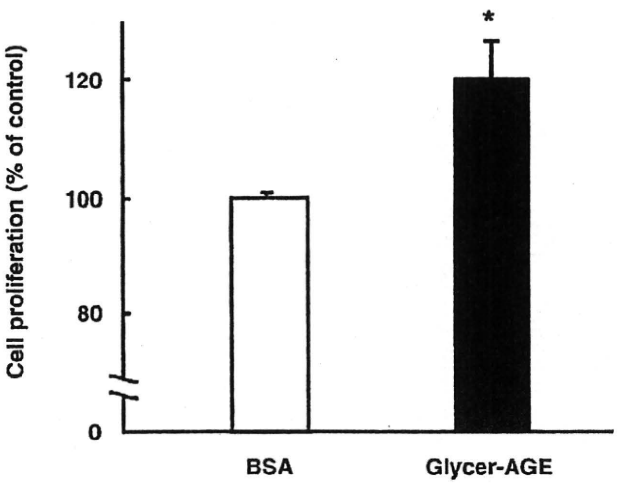

Fig. 3. LI90 cell proliferation following stimulation with AGEs. LI90 cells treated with glycer-AGE for 48 h were subjected to a cell proliferation assay with an MTS assay kit. Data are shown as means ± SEM from four independent experiments. $*P < 0.05$ compared with nonglycated BSA as a control

chemicals: DPI, an NADPH oxidase inhibitor; KCN, an inhibitor of cytochrome oxidase in mitochondria; allopurinol, a xanthine oxidase inhibitor; and indomethacin, a cyclooxygenase inhibitor. These specific inhibitors did not cause morphological changes in LI90 cells, suggesting no cytotoxicities. Among these inhibitors, DPI and KCN significantly inhibited AGE-induced ROS generation, implying that the increased ROS were derived mainly from NADPH oxidase and the mitochondrial respiratory chain system.

Effect of AGEs on HSC proliferation

HSC proliferation is one of the key factors that provoke the progression of hepatic fibrogenesis. The MTS assay showed that stimulation by glycer-AGE significantly increased HSC proliferation, compared with the nonglycated BSA control (Fig. 3).

AGEs stimulate HSCs activation in a redox-sensitive manner

In addition to cell proliferation, activation of HSCs is a key process in hepatic fibrogenesis. Hence, we investigated the effects of AGEs on HSC activation. Transcripts encoding α-SMA and TGF-β1, both of which are established markers for HSC activation, were significantly upregulated by glycer-AGE (Fig. 4A and B). Moreover, mRNA of COL1A2, one of the major components of extracellular matrix, was also intensified by glycer-AGE (Fig. 4C). These changes were prevented by cotreatment with NAC.

Effect of AGEs on MCP-1 expression and secretion

MCP-1 is known to be secreted by HSCs and to amplify hepatic inflammation after liver injury.[19] To assess the possibility that AGEs influence hepatic inflammation through MCP-1 production, we investigated the expression of MCP-1 mRNA and secretion of MCP-1 protein. Glycer-AGE caused upregulation of MCP-1 mRNA (Fig. 5A) and stimulated secretion of MCP-1 protein into culture media (Fig. 5B); this secretion was markedly reduced by NAC.

Discussion

AGEs have been reported to accumulate in multiple tissues in diabetic patient and during normal aging, affecting tissue functions. We previously reported that six distinct AGE structures are elevated in the serum of diabetic patients,[20,21] and that among these, glycer-AGE exhibits neuronal toxicity in vitro.[22] We also have shown that glycer-AGE is prevalent in the serum of NASH patients.[5] In our preliminary experiments, we confirmed higher RAGE expression in liver biopsy tissue from NASH patients compared with tissues from healthy controls or NAFLD patients (data not shown). Hence,

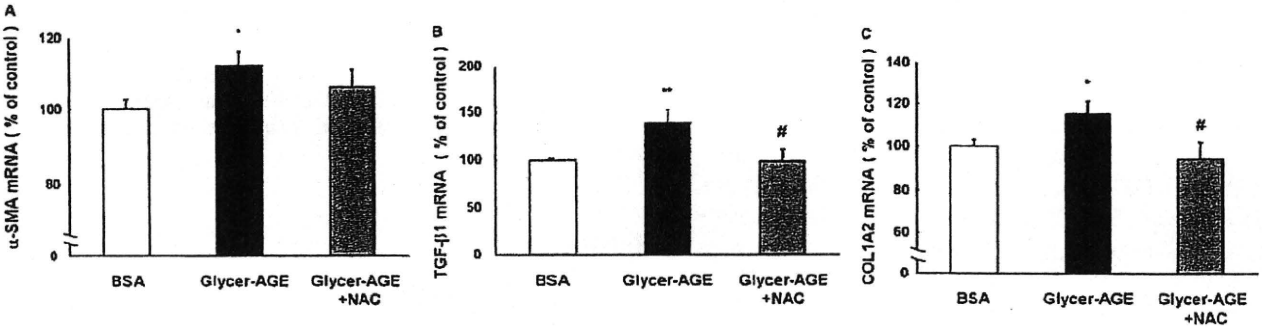

Fig. 4A–C. Effects of AGEs on the expression of mRNAs encoding hepatic fibrogenic markers. LI90 cells were incubated with glycer-AGE for 4h, followed by quantitative real-time PCR measurement of mRNA encoding α-smooth muscle actin (*α-SMA*) (**A**), transforming growth factor β1 (*TGF-β1*) (**B**), and collagen type Iα2 (*COL1A2*) (**C**). Data are shown as means ± SEM from six independent experiments. $*P < 0.01$; $**P < 0.05$ compared with nonglycated BSA as a control; $\#P < 0.01$ compared with glycer-AGE alone

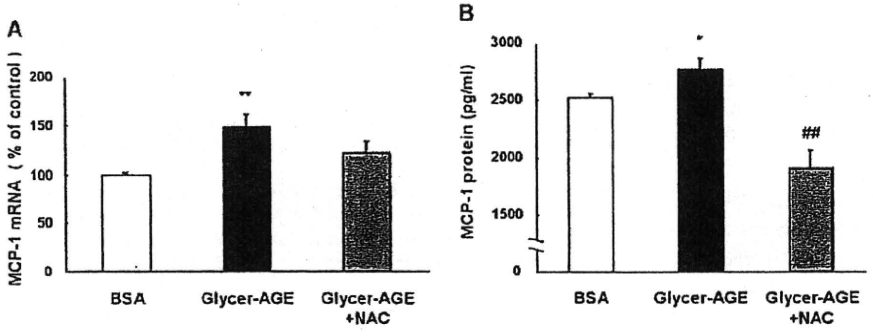

Fig. 5A, B. Monocyte chemoattractant protein 1 (*MCP-1*) production in LI90 cells following exposure to AGEs. **A** LI90 cells were stimulated for 4h with glycer-AGE alone or in the presence of NAC. The level of MCP-1 mRNA was quantified by real-time PCR. **B** LI90 cells were stimulated for 48h with glycer-AGE alone or in the presence of NAC. The level of MCP-1 protein in the conditioned media was quantified by enzyme-linked immunosorbent assay. Data are shown as means ± SEM from six independent experiments. $*P < 0.01$; $**P < 0.05$ compared with nonglycated BSA as a control; $\#\#P < 0.05$ compared with glycer-AGE alone

we hypothesized that glycer-AGE might play an important role in the progression of NASH. In this connection, we examined the biological effect of glycer-AGE on human activated HSC line LI90.

Interestingly, a recent study reported that RAGE is present in rat primary HSCs and is upregulated during their transdifferentiation to myofibroblasts.[6] However, to date, no direct evidence of RAGE expression in human HSCs has been available. The present study demonstrated for the first time the expression of RAGE in human HSC line LI90 at both mRNA and protein levels.

We also provided evidence that treatment of HSCs with AGEs enhances intracellular ROS generation. This result is consistent with previous reports that the AGE–RAGE axis causes pathological conditions via induction of ROS generation in retinal pericytes, vascular endothelial cells, and mesangial cells.[1–3] Since ROS have been shown to stimulate HSC proliferation in vitro,[23] AGE-induced ROS generation might be one mechanism of liver damage in NASH patients. In fact, antioxidants such as betaine, *N*-acetylcysteine, and vitamin E have shown promising results on serum hepatic enzyme levels in patients with NASH.[24–26]

In this study, ROS generation in LI90 cell in response to AGEs was preferentially derived from NADPH oxidase and the mitochondrial respiratory chain system (Fig. 2). These findings are in agreement with the previous report that NADPH oxidase components are functionally expressed in HSCs and are the predominant contributor to angiotensin II-induced ROS generation in HSCs,[27] as well as to platelet-derived growth factor-induced proliferation of HSCs.[13] Furthermore, increased mitochondrial ROS formation in the liver has been

demonstrated in genetically diabetic *ob/ob* mice[28] and in rat NASH models.[29]

The finding in this study that AGEs intensified HSC activation in association with enhanced TGF-β1 adds new information to the mechanism of liver fibrogenesis under glucose intolerance. Recent studies have demonstrated that TGF-β is a potent inducer of both α1(I) and α2(I) collagen genes. In addition, a TGF-β-responsive element has been mapped to the promoter region of α2(I) collagen genes.[30] In keeping with this, the *COL1A2* gene was upregulated by AGE stimulation and accompanied by induction of TGF-β.

MCP-1 is a member of the CC subgroup of chemokines and is a potent chemoattractant of monocytes and T lymphocytes.[31] In addition to macrophages and endothelial cells, MCP-1 is secreted by activated HSCs, and it exhibits great involvement in the pathogenesis of NASH in animal models[10] and humans.[32] In this study, we demonstrated that stimulation of HSCs by AGEs led to upregulation of MCP-1, suggesting that AGE-induced MCP-1 secretion from activated HSCs elicits chronic liver inflammation during the progression of NASH.

Previous studies have indicated that activation of nuclear factor-κB (NF-κB) is induced by ROS in various nonphagocytic cells.[33] It has also been reported that AGEs activate NF-κB via an upstream signaling cascade, such as p38 mitogen-activated protein kinase in macrophages,[34] glomerular mesangial cells,[35] and endothelial cells.[36] Although further investigation is needed, we speculate from these observations that ROS generated by AGE–RAGE signaling may activate a transcription factor such as NF-κB, which results in activation of HSCs.

In this study, we utilized human HSC line LI90, which exhibits the characteristics of activated HSC when cultured in plastic dishes. Although we did not examine whether AGEs initiate the transdifferentiation from the quiescent to the activated phenotype, our findings that treatment with AGEs enhanced fibrotic markers as well as cell proliferation suggest that AGEs may play a role in the development of NASH, probably as a second hit.

In conclusion, we confirmed the expression of RAGE in human HSC line LI90. AGEs enhanced established fibrotic markers and proliferation of HSCs, which was associated with an increase of intracellular ROS generation via NADPH oxidase and the mitochondrial electron transport system. Together with the observation that AGEs are elevated in the serum of patients with NASH, these results suggest AGEs may be a second modulator in the progression of this disease. As antioxidants markedly retrieve the overexpression of fibrotic markers in HSCs, we speculate that the administration of antioxidants may represent a novel treatment strategy for NASH patients.

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References

1. Yamagishi S, Amano S, Inagaki Y, Okamoto T, Koga K, Sasaki N, et al. Advanced glycation end products-induced apoptosis and overexpression of vascular endothelial growth factor in bovine retinal pericytes. Biochem Biophys Res Commun 2002;290: 973–8.
2. Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, et al. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. J Clin Invest 2001;108: 261–8.
3. Yamamoto Y, Yamagishi S, Yonekura H, Doi T, Tsuji H, Kato I, et al. Roles of the AGE–RAGE system in vascular injury in diabetes. Ann N Y Acad Sci 2000;902:163–70; discussion 170–2.
4. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980;55:434–8.
5. Hyogo HYS, Iwamoto K, Arihiro K, Takeuchi M, Sato T, Ochi H, et al. Elevated levels of serum advanced glycation end products in patients with nonalcoholic steatohepatitis. J Gastroenterol Hepatol 2007;22:1114–9.
6. Fehrenbach H, Weiskirchen R, Kasper M, Gressner AM. Upregulated expression of the receptor for advanced glycation end products in cultured rat hepatic stellate cells during transdifferentiation to myofibroblasts. Hepatology 2001;34:943–52.
7. Murakami K, Abe T, Miyazawa M, Yamaguchi M, Masuda T, Matsuura T, et al. Establishment of a new human cell line, LI90, exhibiting characteristics of hepatic Ito (fat-storing) cells. Lab Invest 1995;72:731–9.
8. Takeuchi M, Makita Z, Bucala R, Suzuki T, Koike T, Kameda Y. Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds in vivo. Mol Med 2000;6:114–25.
9. Sasaki N, Takeuchi M, Chowei H, Kikuchi S, Hayashi Y, Nakano N, et al. Advanced glycation end products (AGE) and their receptor (RAGE) in the brain of patients with Creutzfeldt-Jakob disease with prion plaques. Neurosci Lett 2002;326:117–20.
10. Ito M, Suzuki J, Tsujioka S, Sasaki M, Gomori A, Shirakura T, et al. Longitudinal analysis of murine steatohepatitis model induced by chronic exposure to high-fat diet. Hepatol Res 2007; 37:50–7.
11. Vrbacky M, Drahota Z, Mracek T, Vojtiskova A, Jesina P, Stopka P, et al. Respiratory chain components involved in the glycerophosphate dehydrogenase-dependent ROS production by brown adipose tissue mitochondria. Biochim Biophys Acta 2007;1767: 989–97.
12. Lee SB, Bae IH, Bae YS, Um HD. Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death. J Biol Chem 2006;281: 36228–35.
13. Adachi T, Togashi H, Suzuki A, Kasai S, Ito J, Sugahara K, et al. NAD(P)H oxidase plays a crucial role in PDGF-induced proliferation of hepatic stellate cells. Hepatology 2005;41:1272–81.
14. Ueki N, Taguchi T, Takahashi M, Adachi M, Ohkawa T, Amuro Y, et al. Inhibition of hyaluronan synthesis by vesnarinone in cultured human myofibroblasts. Biochim Biophys Acta 2000; 1495:160–7.
15. Von Bubnoff D, Matz H, Cazenave JP, Hanau D, Bieber T, De La Salle H. Kinetics of gene induction after FcepsilonRI ligation

of atopic monocytes identified by suppression subtractive hybridization. J Immunol 2002;169:6170–7.
16. Wang L, Wang J, Wang BE, Xiao PG, Qiao YJ, Tan XH. Effects of herbal compound 861 on human hepatic stellate cell proliferation and activation. World J Gastroenterol 2004;10:2831–5.
17. Yamagishi S, Hsu CC, Taniguchi M, Harada S, Yamamoto Y, Ohsawa K, et al. Receptor-mediated toxicity to pericytes of advanced glycosylation end products: a possible mechanism of pericyte loss in diabetic microangiopathy. Biochem Biophys Res Commun 1995;213:681–7.
18. Loguercio C, Federico A. Oxidative stress in viral and alcoholic hepatitis. Free Radic Biol Med 2003;34:1–10.
19. Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, et al. Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. Hepatology 1999;29:140–8.
20. Takeuchi M, Makita Z. Alternative routes for the formation of immunochemically distinct advanced glycation end-products in vivo. Curr Mol Med 2001;1:305–15.
21. Takeuchi M, Yanase Y, Matsuura N, Yamagishi Si S, Kameda Y, Bucala R, et al. Immunological detection of a novel advanced glycation end-product. Mol Med 2001;7:783–91.
22. Takeuchi M, Yamagishi S. TAGE (toxic AGEs) hypothesis in various chronic diseases. Med Hypotheses 2004;63:449–52.
23. Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, et al. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. Hepatology 2005;41:1074–84.
24. Abdelmalek MF, Angulo P, Jorgensen RA, Sylvestre PB, Lindor KD. Betaine, a promising new agent for patients with nonalcoholic steatohepatitis: results of a pilot study. Am J Gastroenterol 2001;96:2711–7.
25. Gulbahar OKZ, Ersoz G, Akarca US, Musoglu A. Treatment of nonalcoholic steatohepatitis with *N*-acetylcysteine. Gastroenterology 2000;118:A1444 (abstract).
26. Lavine JE. Vitamin E treatment of nonalcoholic steatohepatitis in children: a pilot study. J Pediatr 2000;136:734–8.
27. Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. J Clin Invest 2003;112:1383–94.
28. Yang S, Zhu H, Li Y, Lin H, Gabrielson K, Trush MA, et al. Mitochondrial adaptations to obesity-related oxidant stress. Arch Biochem Biophys 2000;378:259–68.
29. Hensley K, Kotake Y, Sang H, Pye QN, Wallis GL, Kolker LM, et al. Dietary choline restriction causes complex I dysfunction and increased H(2)O(2) generation in liver mitochondria. Carcinogenesis 2000;21:983–9.
30. Inagaki Y, Truter S, Ramirez F. Transforming growth factor-beta stimulates alpha 2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem 1994;269:14828–34.
31. Luster AD. Chemokines—chemotactic cytokines that mediate inflammation. N Engl J Med 1998;338:436–45.
32. Haukeland JW, Damas JK, Konopski Z, Loberg EM, Haaland T, Goverud I, et al. Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. J Hepatol 2006;44:1167–74.
33. Fan X, Subramaniam R, Weiss MF, Monnier VM. Methylglyoxal-bovine serum albumin stimulates tumor necrosis factor alpha secretion in RAW 264.7 cells through activation of mitogen-activating protein kinase, nuclear factor kappaB and intracellular reactive oxygen species formation. Arch Biochem Biophys 2003;409:274–86.
34. Wu CH, Huang CM, Lin CH, Ho YS, Chen CM, Lee HM. Advanced glycosylation end products induce NF-kappaB dependent iNOS expression in RAW 264.7 cells. Mol Cell Endocrinol 2002; 194:9–17.
35. Chang PC, Chen TH, Chang CJ, Hou CC, Chan P, Lee HM. Advanced glycosylation end products induce inducible nitric oxide synthase (iNOS) expression via a p38 MAPK-dependent pathway. Kidney Int 2004;65:1664–75.
36. Bierhaus A, Illmer T, Kasper M, Luther T, Quehenberger P, Tritschler H, et al. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. Circulation 1997;96:2262–71.

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HEPATOLOGY

Prospective study of short-term peginterferon-α-2a monotherapy in patients who had a virological response at 2 weeks after initiation of interferon therapy

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Key words
adverse effects, clinical trial, efficacy, hepatitis C virus, peginterferon-α-2a.

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Abstract

Background and Aims: Long-term interferon (IFN) therapy is effective in eliminating hepatitis C virus (HCV). However, it carries the risk of adverse effects and reduced quality of life. To assess whether short-term IFN therapy effectively eliminates HCV, we performed a prospective pilot study of pegylated (peg)IFN-α-2a therapy for 8 or 24 weeks.

Methods: After excluding patients with high titers of genotype-1, 55 HCV patients received pegIFN-α-2a. Patients who became negative for HCV-RNA at week 2 were allocated to either an 8-week ($n = 19$) or 24-week ($n = 15$) course of IFN. We evaluated the efficacy of and tolerance to IFN therapy.

Results: The sustained virological response rate was excellent in the two groups (8 weeks, 89.5% [17/19]; 24 weeks, 100% [15/15], respectively,). IFN dose reduction was required in one patient of the 8-week group, but in six patients of the 24-week group ($P = 0.028$). Treatment was completed by all patients of the 8-week group, but discontinued in five patients of the 24-week group ($P = 0.011$).

Conclusions: The 8-week IFN therapy is more tolerable than the 24-week therapy and had similar outcomes. Excluding the patients with high titers of genotype-1, we recommend switching to an 8-week course of pegIFN-α monotherapy once patients show an ultra rapid virological response at week 2 from the start of IFN therapy.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease with an estimated 170 million chronic carriers worldwide.[1] Chronic HCV infection is usually associated with liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[2–6] In Japan, 60–70% of patients with HCC or LC are HCV carriers.[7] Antiviral therapy of interferon (IFN) is widely used for the treatment of chronic HCV infection and is assumed to prevent progression to LC and HCC, especially in patients who show a sustained virological response (SVR).

The reported total HCV-RNA elimination rate is approximately 30–40% in patients treated with conventional IFN monotherapy.[8–10] However, better results have been reported when pegylated (peg)IFN-α is used in both naive patients and in those who fail to respond to or relapse after conventional IFN-α monotherapy. In Japan, two kinds of pegIFN are available: pegIFN-α-2a and pegIFN-α-2b. PegIFN-α-2b can be used with ribavirin, a purine nucleoside analog, in naive patients with genotypes 1 and 2 with a high viral load (>100 KIU/mL of HCV-RNA) or patients with any viral load in whom previous IFN treatment did not eliminate HCV-RNA. PegIFN-α-2a has been used in Japan without ribavirin only since December 2003 because of health insurance restrictions. However, ribavirin combination therapy has been covered by public health insurance since March 2007 in Japan. The HCV elimination rate with pegIFN-α-2b plus ribavirin combination therapy is up to 54% in patients with genotype 1.[11] Several investigators have reported that pegIFN and ribavirin combination therapy for a period of 24 or 48 weeks ensures a viral clearance in most patients with HCV genotypes 2 or 3 infection.[12,13] However, ribavirin combination therapy frequently causes anemia and should be carefully used in the elderly, anemic, or pregnant young patients, and in those who require long-term treatment.[14] Apart from patients with a high viral load of genotype 1, IFN monotherapy is also effective in HCV elimination even when used without ribavirin. Previous studies suggest that the SVR achieved with pegIFN-α-2a is similar to that observed with pegIFN-α-2a combined with ribavirin in patients with hepatitis C.[15,16]

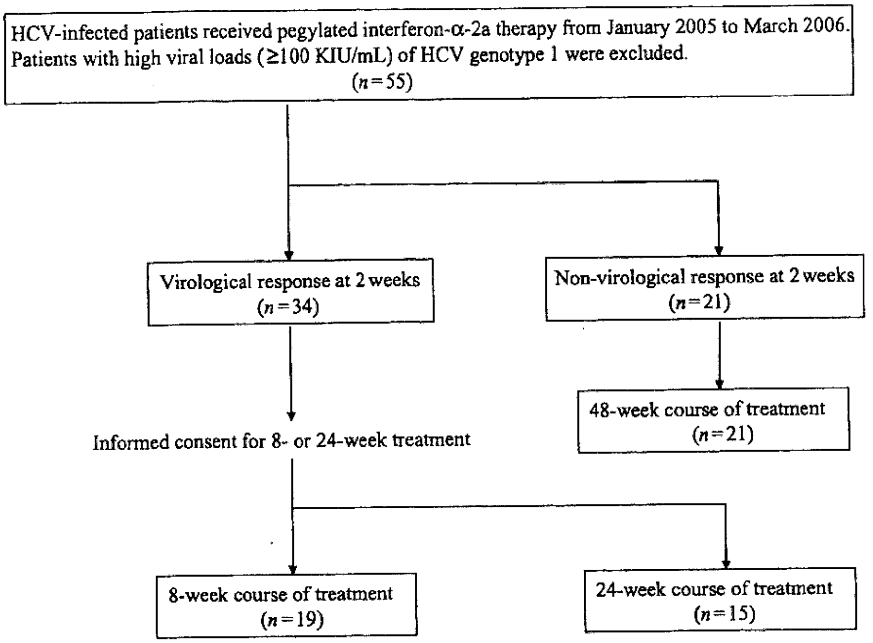

Figure 1 Flow diagram of the clinical trial. HCV, hepatitis C virus.

Although the tolerability of pegIFN is similar to that of the conventional IFN,[15] the 180 μg dose of pegIFN-α-2a therapy for 48 weeks is sometimes not tolerated by some patients. With the exception of those with high viral loads of genotype 1, the above regimen is expected to produce a high viral clearance rate, especially in patients with an early virological response. Several studies report the effectiveness of short-course IFN therapy (<24 weeks) for patients with an early virological response.[17–20] Therefore, a treatment duration of 48 weeks may be too long or more than sufficient for some patients, especially when one considers the undesirable adverse effects or the cost of treatment.

In the present study, we conducted a prospective controlled trial to compare the efficacy of an 8-week versus a 24-week course of pegIFN-α-2a (180 μg/time/week) for patients negative for HCV-RNA at 2 weeks after the initiation of therapy.

Methods

Patients

Between January 2005 and March 2006, a total of 55 HCV-infected patients received pegIFN-α-2a therapy at Hiroshima University Hospital (Hiroshima, Japan) and its associated hospitals in Japan. Patients with high viral loads (≥100 KIU/mL) of HCV genotype 1 were excluded from this study because of their low SVR rate. Among the 55 patients, 34 consecutive patients who showed a rapid virological response at 2 weeks were enrolled in this study (Fig. 1). Eligible patients had antibodies to HCV, were positive for HCV-RNA at study entry, and had not received previous IFN therapy. They included 21 men and 13 women, with a mean age of 53 years (range, 21–71 years). Their HCV genotypes were 1b, 2a, and 2b with variant HCV-RNA (5.1–400 KIU/mL by a reverse transcriptase-polymerase chain reaction [RT-PCR]). All patients underwent liver biopsies within 12 weeks before the start of IFN therapy and were confirmed to have chronic hepatitis by histopathological examination. Patients with any other cause of liver disease including coinfection with hepatitis-B virus or HIV, alcoholic hepatitis, fatty liver, autoimmune hepatitis, or previous organ transplantation were excluded from this study.

Study design

This multicenter prospective controlled study compared the efficacy and safety of 8 weeks versus 24 weeks of pegIFN-α-2a monotherapy in previously untreated patients with chronic hepatitis C who had a virological response at 2 weeks after the start of IFN. Patients with a virological response at 2 weeks were invited to sign a consent form accepting treatment with IFN for 8 weeks only. Those patients who refused consent received a 24-week course of treatment. The primary measure of efficacy was SVR, which was defined as undetectable HCV-RNA in the serum at 24 weeks after the cessation of treatment. All patients agreed to participate in the research protocol, which was approved by the hospital research ethics board, and gave written informed consent. The eligible patients received pegIFN-α-2a (Pegasys, F. Hoffmann-LaRoche, Basel, Switzerland) at 180 μg once per week subcutaneously, either for 8 weeks or 24 weeks, without ribavirin. Other patients who showed no rapid virological response at 2 weeks after the start of pegIFN-α-2a were treated for 24–48 weeks.

All patients were evaluated in an outpatient setting for safety, tolerance, and efficacy every week during the IFN treatment. Blood count was checked just before the IFN injection every week. The qualitative detection of HCV-RNA was performed by a standardized qualitative RT–PCR assay (Amplicor HCV monitor v2.0; Roche diagnostics Co., Tokyo, Japan) at the first 2 weeks and every 4 weeks during and after IFN treatment. The primary efficacy end point for this study was defined as a disappearance of detectable serum HCV-RNA at week 24 after the completion of the IFN treatment.

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Table 1 Patients' characteristics

	8-week group ($n = 19$)	24-week group ($n = 15$)
Age (years)	51† (21–71)	47† (25–58)
Sex (male/female)	14/5	7/8
Height (cm)	169† (147–178)	161† (139–178)
Weight (kg)	64.6† (40.6–85)	59† (47–92.4)
Body mass index (kg/m²)	23.4† (18.0–27.8)	21.5† (18.6–30.5)
Platelet count (×10⁴/μL)	19.5† (9.6–30.7)	18.1† (8.9–31.7)
Alanine aminotransferase (IU/L)	55† (22–152)	60† (21–184)
γ-Glutamyl transpeptidase (IU/L)	25† (9–155)	47† (14–137)
Creatinine (mg/dL)	0.76† (0.6–0.9)	0.68† (0.38–0.85)
Total cholesterol (mg/dL)	160† (116–219)	154† (125–201)
Fasting blood glucose (mg/dL)	90† (72–104)	96† (84–115)
Diabetes mellitus	0	1
Hyaluronic acid (ng/mL)	24† (13–72)	78† (16–191)
HCV genotype (1b/2a/2b)	2/15/2	2/10/3
HCV-RNA (KIU/mL)	45† (5.1–370)	43† (5.3–400)
Fibrosis (F1/F2/F3/F4)	6/8/5/0	5/6/4/0

†Median. HCV, hepatitis C virus.

Statistical analysis

We compared the response to an 8-week course of pegIFN-α-2a with that to a 24-week course of pegIFN-α-2a. The χ^2-test and Fisher's exact test were used for comparisons of categorical variables between groups, while Student's *t*-test and the Wilcoxon test were used for continuous and ordinal variables as appropriate. *P*-values less than 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC, USA) was used for the statistical analysis of data.

Results

Baseline characteristics

Thirty-four patients who became HCV-RNA-negative at week 2 subsequently received either an 8-week course ($n = 19$) or 24-week ($n = 15$) course of 180 μg pegIFN-α-2a. The baseline characteristics of the two groups at the start of the IFN therapy are summarized in Table 1. None of the patients had LC, based on clinical, laboratory, and histopathological findings. Table 2 also shows the data of 21 patients with a non-rapid virological response at 2 weeks after the start of pegIFN-α-2a. The pretreatment viral loads of non-rapid virological responders were significantly higher than those of rapid virological responders ($P < 0.0001$).

Tolerance of IFN therapy and adverse events

Among the 19 patients of the 8-week group, the dose was reduced by 50% (to 90 μg of pegIFN-α-2a) in one patient with SVR at 3 weeks due to a fall in platelet count. However, all other patients were able to complete the full 8-week course without discontinuation. In 15 patients of the 24-week course, the dose was reduced to half (90 μg of pegIFN-α-2a) in six patients due to neutropenia ($n = 2$; one patient at 8 weeks and one patient at 10 weeks), thrombocytopenia ($n = 3$; two patients at 9 weeks and one patient at 10 weeks) and epigastralgia ($n = 1$; at 14 weeks). Furthermore, IFN therapy was withdrawn in another five patients, including two patients at 8 weeks due to thrombocytopenia, two patients at 12 weeks due to generalized fatigue, and one patient at 18 weeks due to various neurological symptoms, such as hand numbness. Thus the proportion of patients who required a dose reduction was lower in the 8-week group than in the 24-week group ($P = 0.028$). Furthermore, the proportion of patients who completed the treatment was significantly higher in the 8-week group than the 24-week group ($P = 0.011$). We concluded that our patients with HCV could tolerate 8 weeks of IFN therapy better than 24 weeks.

Table 2 Characteristics of 21 patients who did not show a rapid virological response

Age (years)	51† (22–76)
Sex (male/female)	11/8
Height (cm)	164.5† (148–175.5)
Weight (kg)	58.5† (42.5–75)
Body mass index (kg/m2)	22.5† (16.9–27.3)
Platelet count (x104/_L)	20.5† (12–28.6)
Alanine aminotransferase (IU/L)	93† (17–157)
γ-Glutamyl transpeptidase (IU/L)	39† (10–145)
Creatinine (mg/dL)	0.58† (0.5–0.96)
Total cholesterol (mg/dL)	158† (111–214)
Fasting blood glucose (mg/dL)	87† (68–119)
Diabetes mellitus	0
Hyaluronic acid (ng/mL)	45.6† (10–100)
HCV genotype (1b/2a/2b)	0/15/6
HCV-RNA (KIU/mL)	660† (40–830)
Fibrosis (F1/F2/F3/F4)	12/6/3/0

†Median. HCV, hepatitis C virus.

Biochemical and virological responses to therapy

With regard to the alanine aminotransferase (ALT) response to IFN therapy, all patients of both groups showed biochemical normalization at the end of treatment and at 6 months after the end of treatment. There was no difference in the sustained ALT response between the 8-week group and 24-week group. With regard to the virological response to IFN therapy, all patients of both groups exhibited a rapid decrease in HCV-RNA, reaching undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 2. All patients had negative HCV-RNA levels at the end of treatment and none showed a null response. There was no significant difference in the rate of fall of the virological load between patients who had a sustained response and those who had a relapse, as discussed later. The proportions of patients who showed a SVR in the 8-week group and 24-week group were not significantly different (89.5% [17/19] and 100% [15/15], respectively [$P = 0.195$]). Two patients of the 8-week group had viral relapse after the end of treatment; one who had HCV genotype 2a with 50 KIU/mL pretreatment viral load relapsed at 12 weeks after the end of the treatment while the other had genotype 2b with 230 KIU/mL pretreatment viral load and relapsed at 8 weeks after the end of the treatment. The non-

rapid virological responders had a lower SVR rate. Eight (38%) patients showed SVR, 11 (52%) patients developed relapse after discontinuation of IFN, and two (10%) patients had no virological response.

Discussion

In Japan, pegIFN-α-2a monotherapy has been covered by public health insurance since December 2003. The standard duration of treatment with pegIFN-α-2a is 24 weeks for patients with low viral loads of genotype HCV-1 and any viral loads of genotype HCV-2 infection. Recent studies have reported that a treatment duration of more than 24 weeks in such cases does not increase the SVR rate.[11,13,21,22] Moreover, patients with early virological response seem to have a high rate of SVR.[23–25] In those patients, to reduce unnecessary exposure to treatment and its potential side-effects and to reduce costs, short-term IFN therapy has been used by several groups.[17–21] However, details of the IFN regimen differ from those of others and there are no studies that use short-term of pegIFN-α-2a treatment. We therefore conducted a prospective pilot study on the efficacies of an 8-week and 24-week pegIFN-α-2a regimen for patients with low viral titers of genotype HCV-1 and any viral titers of genotype HCV-2 who exhibited a virological response at 2 weeks after the initiation of IFN. In our study, patients with a relatively low viral load before the start of the IFN therapy tended to have a very early virological response.

Our results demonstrated that the virological response to the 8-week treatment (89.5% [17/19]) was excellent and was similar to the 24-week course (100% [15/15]). This high SVR rate of 8-week pegIFN-α-2a monotherapy seems as high as that reported in another short course study of 14-week pegIFN plus ribavirin combination treatment for patients with HCV genotype HCV-2 or HCV-3.[26] This high SVR rate of the 8-week course of pegIFN-α-2a may be associated with a rapid viral disappearance. Several studies have indicated that negative HCV-RNA at week 2 after the commencement of IFN is a predictor of SVR.[19,20,27,28] Therefore, for patients with a low HCV-1 viral load or those with HCV-2 infection with any viral load, we recommend switching to an 8-week course of pegIFN-α-2a monotherapy once they show an ultra rapid virological response, that is, negative HCV-RNA at week 2 from the start of IFN therapy. Furthermore, a longer course of IFN therapy with or without ribavirin can be prescribed when HCV-RNA becomes positive after discontinuation of the 8-week course of IFN therapy.

Although in our study all of the patients in the 24-week course showed SVR, it seems that 24 weeks is a long treatment period for those patients who become negative for HCV-RNA by week 2 of treatment to ascertain SVR. Our results showed that all patients in the 8-week course completed the course to the end of treatment. However, 33% of the patients of the 24-week course did not continue their treatment to the end of the course. Because patients tend to adhere to shorter regimens, which are also better tolerated than longer treatment regimens, a shorter exposure will probably translate into a better benefit–risk ratio in patients with early virological response.

Our study identified two relapsers among patients of the 8-week course after discontinuation of pegIFN-α-2a therapy. These two patients had a negative history of exposure to new HCV infection. One patient who had genotype HCV-2b and a high pretreatment viral load (HCV-RNA: 230KIU/mL) relapsed at 8 weeks after the discontinuation of IFN therapy, while the other who had genotype HCV-2a and a low pretreatment viral load (HCV-RNA: 50KIU/mL) relapsed at 12 weeks after the discontinuation of IFN therapy. These two patients could have SVR after additional IFN therapy for 24 weeks (one patient; pegIFN-α-2a monotherapy, one patient; pegIFN-α-2b and ribavirin combination therapy). We could not identify a definite factor associated with SVR or relapse. Although pretreatment factors, like genotype, viral load, and grade of fibrosis can be used to predict the mean treatment outcome for study cohorts, they are often of limited value in individual patients.[29,30]

As mentioned earlier, a short course of pegIFN-α-2a therapy for 8 weeks could be recommended in those patients who show an ultra rapid virological response at week 2 after the initiation of IFN therapy. Although the study by Shiffman *et al.*[31] demonstrates the inferiority of a shorter regimen in a large-scale, randomized, controlled study, the characteristics of their patients were largely different from those of our study, including racial difference (mostly Caucasian patients versus Japanese, a heavier body weight for the Caucasians versus Japanese patients) and differences in pretreatment viral load (variable and higher HCV-RNA level versus relatively low viral load in our patients). Patients' selection was also different between the two studies; our study was carried out only in cases negative for HCV-RNA at 2 weeks after the start of IFN compared to their randomized study, irrespective of a rapid virological response.

In conclusion, patients chronically infected with low titers of HCV-1 and those with HCV-2, regardless of their viral loads, who achieve an ultra rapid virological response, that is, HCV-RNA negativity at week 2, can receive only 8 weeks of pegIFN-α-2a monotherapy without compromising the chance of SVR. The results of our prospective study are encouraging, although the study population was small and was based on non-randomized methodology. The data of the present study are not conclusive for patients with very high pretreatment viremia who might achieve a rapid virological response or for those patients who do not achieve a rapid virological response. Further clinical trials are required to optimize the treatment duration in these patients.

References

1 Alter MJ. Epidemiology of hepatitis C in the West. *Semin. Liver Dis.* 1995; **15**: 5–14.
2 Poynard T, Bedossa P, Opolon P, The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC Groups. Natural history of liver fibrosis progression in patients with chronic hepatitis C. *Lancet* 1997; **349**: 825–32.
3 Kiyosawa K, Sodeyama T, Tanaka E et el. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671–5.
4 Tsukuma H, Hiyama T, Tanaka S *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N. Engl. J. Med.* 1993; **28**: 1797–801.
5 Takano S, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C. A prospective study of 251 patients. *Hepatology* 1995; **21**: 650–5.
6 Shiratori Y, Shiina S, Imamura M *et al.* Characteristic difference of hepatocellular carcinoma between hepatitis-B and C-viral infection in Japan. *Hepatology* 1995; **22**: 1027–33.

7 Tomimatsu M, Ishiguro N, Taniai M *et al.* Hepatitis C virus antibody in patients with primary liver cancer (hepatocellular carcinoma, cholangiocarcinoma, and combined hepatocellular-cholangiocarcinoma) in Japan. *Cancer* 1993; **72**: 683–8.
8 Poynard T, Leroy V, Cohard M *et al.* Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. *Hepatology* 1996; **24**: 778–89.
9 Carithers RL Jr, Emerson SS. Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials. *Hepatology* 1997; **26**: S83–8.
10 Orito E, Mizokami M, Nakano T *et al.* Serum hepatitis C virus RNA level as a predictor of subsequent response to interferon-alpha therapy in Japanese patients with chronic hepatitis C. *J. Med. Virol.* 1994; **44**: 410–14.
11 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001; **358**: 958–65.
12 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* 2002; **347**: 975–82.
13 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.* 2004; **140**: 346–55.
14 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.
15 Zeuzem S, Feinman SV, Rasenack J *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C. *N. Engl. J. Med.* 2000; **343**: 1666–72.
16 Lai MY, Kao JH, Yang PM *et al.* Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. *Gastroenterology* 1996; **111**: 1307–12.
17 Wagner MV, Huber M, Berg T *et al.* Peginterferon-α-2a (40KD) and Ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. *Gastroenterology* 2005; **129**: 522–7.
18 Mangia A, Santoro R, Minerva N *et al.* Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. *N. Engl. J. Med.* 2005; **352**: 2609–17.
19 Nomura H, Tsuchiya Y, Maruyama T, Miki K, Yokota T, Okubo H. Interferon therapy and hepatitis C virus: the effects of a high dose, short course of interferon on hepatitis C. *J. Gastroenterol. Hepatol.* 1999; **14**: 85–9.
20 Sato Y, Tokuue H, Kawamura N *et al.* Short-term interferon therapy for chronic hepatitis C patients with low viral load. *Hepatogastoenterology* 2004; **51**: 968–72.
21 Tabaru A, Narita R, Hiura M, Abe S, Otsuki M. Efficacy of short-term interferon therapy for patients infected with hepatitis C virus genotype 2a. *Am. J. Gastroenterol.* 2005; **100**: 862–7.
22 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients with HCV genotype 2 or 3. *J. Hepatol.* 2004; **40**: 993–9.
23 Bjøro K, Bell H, Hellum KB *et al.* Effect of combined interferon-alpha induction therapy and ribavirin on chronic hepatitis C virus infection: a randomized multicentre study. *Scand. J. Gastroenterol.* 2002; **37**: 226–32.
24 Bekkering FC, Stalgis C, McHutchison JG, Brouwer JT, Perelson AS. Estimation of early hepatitis C viral clearance in patients receiving daily interferon and ribavirin therapy using a mathematical model. *Hepatology* 2001; **33**: 419–23.
25 Zeuzem S. Clinical implications of hepatitis C viral kinetics. *J. Hepatol.* 1999; **31** (Suppl. 1): 61–4.
26 Dalgard O, Bjøro K, Hellum KB *et al.* Treatment with pegylated interferon and ribavirin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. *Hepatology* 2004; **40**: 1260–5.
27 Hino K, Okuda M, Konishi T, Ishiko H, Okita K. Serial assay of hepatitis C virus RNA in serum for predicting response to interferon-alpha therapy. *Dig. Dis. Sci.* 1995; **40**: 14–20.
28 Kakumu S, Aiyama T, Okumura A, Iwata K, Ishikawa T, Yoshioka K. Earlier loss of hepatitis C virus RNA in interferon therapy can predict a long-term response in chronic hepatitis C. *J. Gastroenterol. Hepatol.* 1997; **12**: 468–72.
29 Davis GL, Lau JY. Factors predictive of a beneficial response to therapy of hepatitis C. *Hepatology* 1997; **26**: 122S–127S.
30 Tong MJ, Blatt LM, McHutchison JG, Co RL, Conrad A. Prediction of response during interferon alfa 2b therapy in chronic hepatitis C patients using viral and biochemical characteristics: a comparison. *Hepatology* 1997; **26**: 1640–5.
31 Shiffman ML, Suter F, Bacon BR *et al.* Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N. Engl. J. Med.* 2007; **357**: 124–34.

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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.

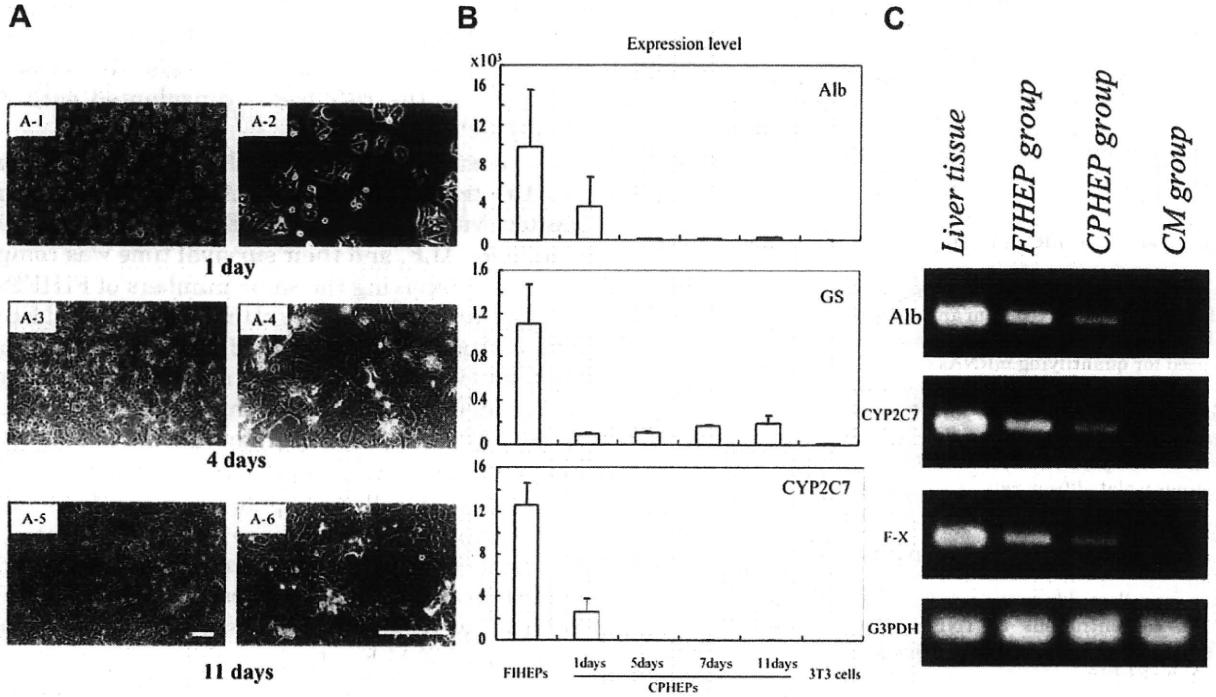

FIG. 1. (A) Phase contrast image of proliferating hepatocytes. Hepatocytes (8.5×10^3 cells/cm^2) were cocultured with Swiss 3T3 cells in HCGM on 15.0-cm dishes. Photographs were taken for the same fields at 1 (A-1, 2), 4 (A-3, 4), and 11 d (A-5, 6) with lower (A-1, 3, 5) and higher (A-2, 4, 6) magnifications.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

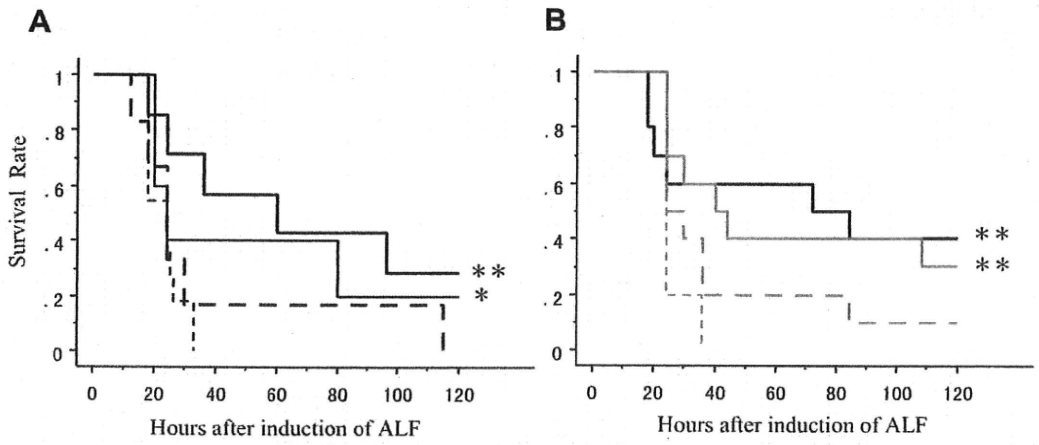

FIG. 2. Survival curves of ALF rats with FIHEP transplantation. The rats were transplanted with HEPs or FBs through the spleen and then subjected to ALF. Some rats were given CM as controls. (A) Rescue of ALF by FIHEP transplantation. The rats were given varying numbers of FIHEPs: 1.5×10^7 cells ($n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

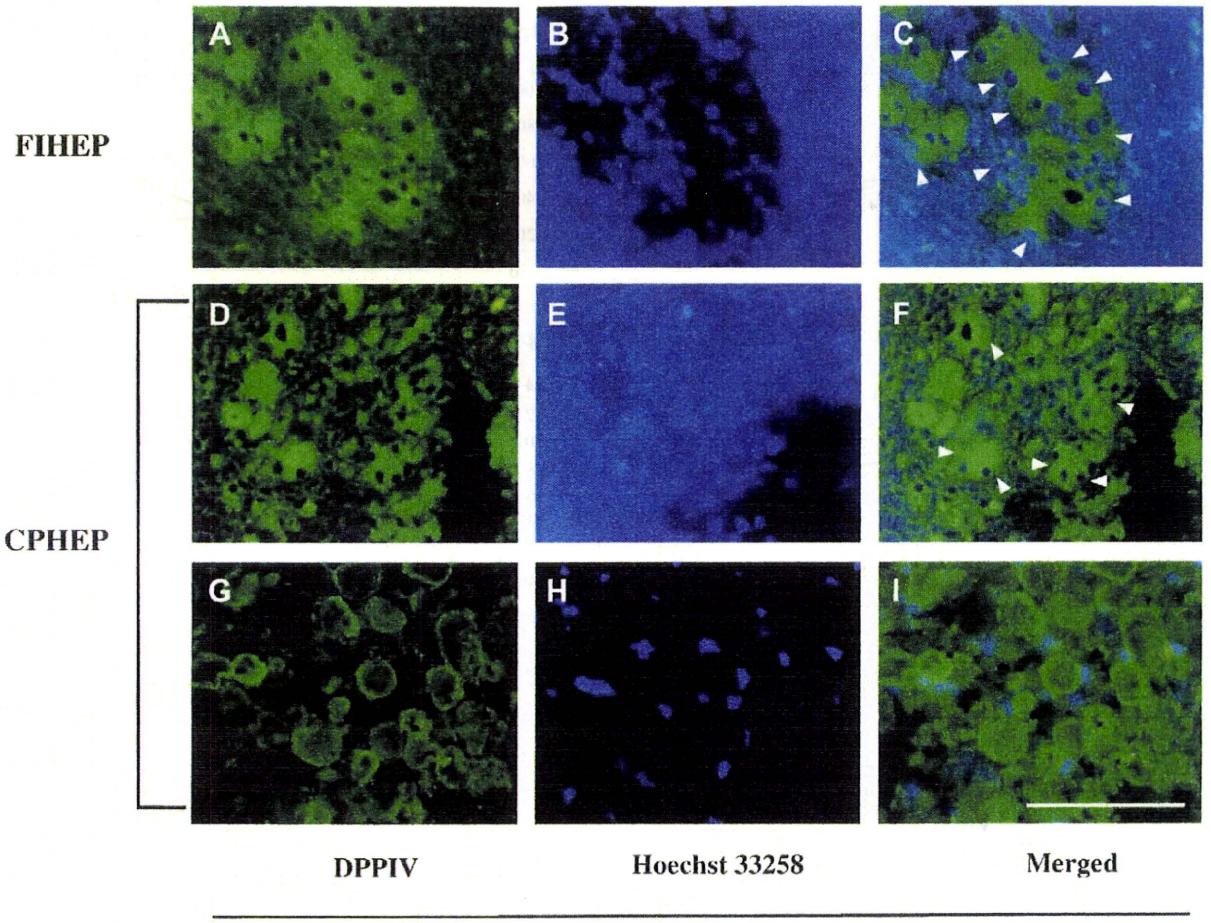

FIG. 3. Engraftment of the transplanted hepatocytes in the spleen of ALF rats. The rats were transplanted with FIHEPs (A)–(C) or CPHEPs (D)–(I) and subjected to ALF as in Figure 2B. Spleens were removed at 24 h after ALF induction and processed to cryosectioning for immunohistochemical analysis to detect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

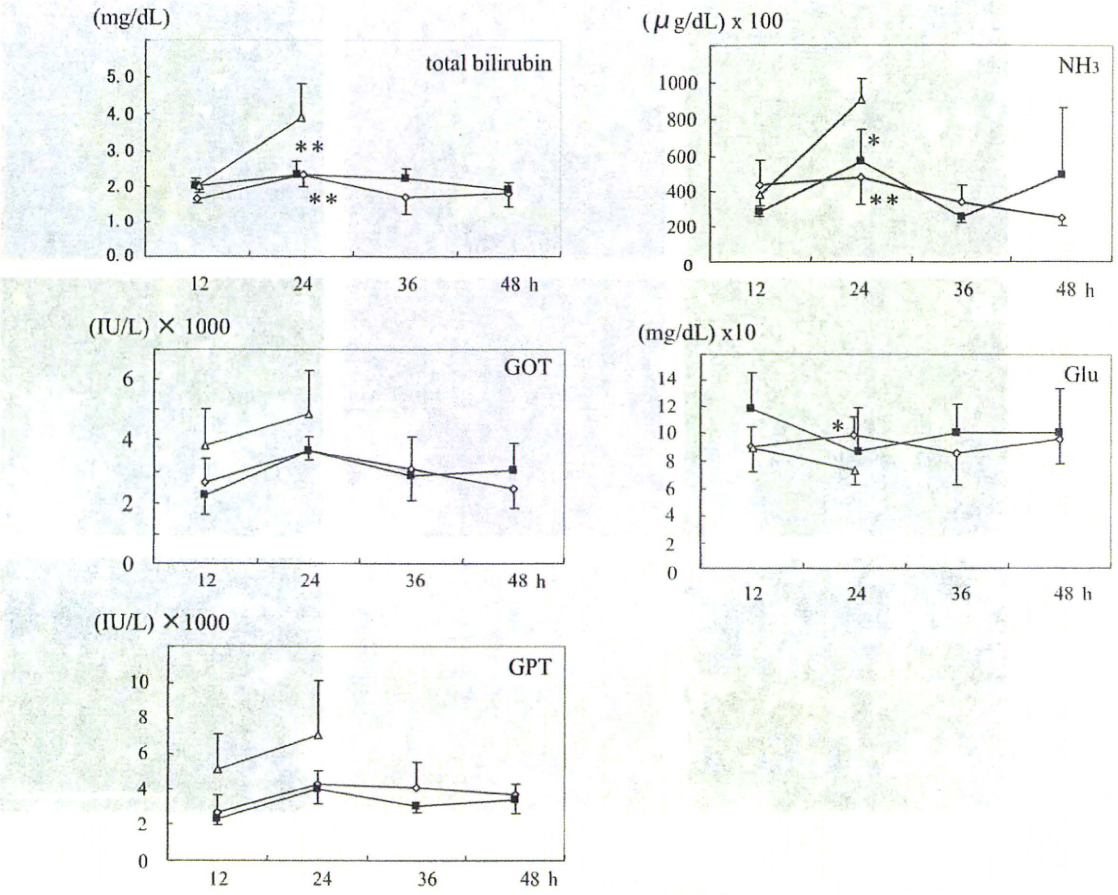

FIG. 4. Biochemical evaluation of hepatocyte transplantation therapy for ALF. The rats were subjected to hepatocyte transplantation and ALF treatment as described in Figure 2. At the indicated time points after ALF treatment, blood was collected for total bilirubin, NH_3, GOT, Glu, and GPT assessment.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

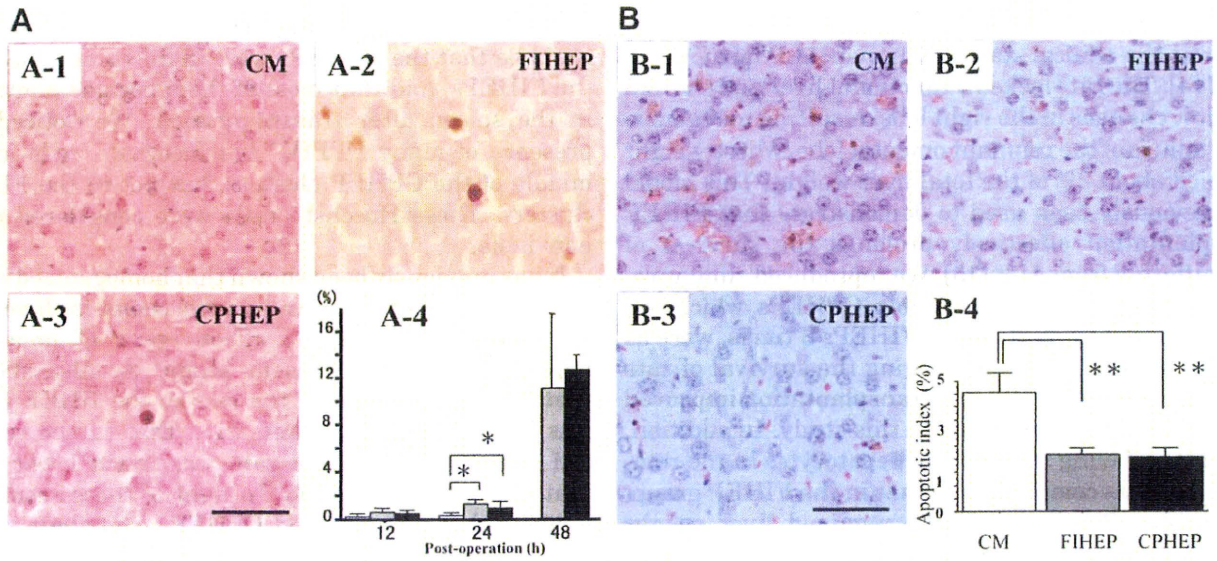

FIG. 5. (A) BrdU-labeling index of hepatocytes in the remnant liver of the hepatocyte- transplanted rat. The rats were injected with CM, transplanted with FIHEPs or CPHEPs, and subjected to ALF as in Fig. 2. The remnant livers (omental lobe) were removed at 12, 24, and 48 h post-induction of ALF and 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

a model induced by 90% hepatectomy and is considered to mimic the clinical status of human ALF fairly faithfully [14]. The rats lacked a functional liver and showed ischemic changes in the right lobe, resulting in regeneration failure of the remnant omental lobe, whose weight occupied about 8% of the total liver weight. This model has previously been used to demonstrate that FIHEP transplantation effectively prolongs the survival of rats suffering from ALF [15]. We reproduced similar results in the present study. Notably, CPHEPs, which had been prepared by multiplying FIHEPs 3 times, were as effective as FIHEPs in prolonging the survival of rats suffering from ALF. CPHEP transplantation improved all the liver functions tested in this study. In addition, the BrdU-labeling index of the hepatocytes in the remnant liver was comparable to that in the FIHEP group. Rats with CPHEPs gradually regained liver weight after ALF induction, as did those with FIHEPs. These results together indicate that both CPHEP and FIHEP could be a source for hepatocyte transplantation to promote regeneration of the remnant liver after ALF induction.

There have been two explanations for lethal hepatic failure after excessive hepatectomy: hepatectomy causes microcirculer disturbances [20] or induces cytotoxic factors such as TNF-α, TGF-β1, and oxidative stress-related factors [21, 22]. In the present study, we did not find any evidence of microvascular disturbances on hematoxylin and eosin (H&E)-stained sections of the remnant lobe in the ALF-induced rats, but we did observe hypercytokinemia of cytokines such as IL-6 and TGF-β1. Apoptotic hepatocytes were frequently seen by TUNEL assay in the remnant liver lobe of the ALF-induced rats. CPHEP and FIHEP transplantation decreased the concentrations of IL-6 and TGF-β1 in sera, as well as the frequency of apoptotic hepatocytes. Therefore, it appears that both CPHEPs and FIHEPs prolonged the survival of ALF-induced rats by suppressing the hepatocytic apoptosis in the remnant liver.

In the present study, we demonstrated the presence of DPPIV$^+$ hepatocytes in the spleen at 24 h after ALF induction, which clearly indicated the engraftment of both transplanted CPHEPs and FIHEPs in the graft site. There were no significant differences in the frequency of DPPIV$^+$ hepatocytes between the FIHEP and CPHEP groups. However, the expression level of hepatocyte-specific mRNAs such as Alb, CYP2C7, and GS in the spleen of the CPHEP rats was considerably lower than that in the FIHEP rats. This might be explained by the fact that CPHEPs showed lower expression levels of these marker genes than FIHEPs at the time of transplantation; this was due to the fact that the CPHEP cells had been cultured for 11 d before transplantation, during which time the expression levels had decreased (Fig. 1B). Another explanation could be that the CPHEPs were more vulnerable than the FIHEPs, and that most of them became nonviable in the spleen after transplantation. We noticed the presence of many DPPIV$^+$ but Hoechst$^-$ cells in the middle of the CPHEP clusters, but not in the FIHEP clusters. These Hoechst$^-$ cells were considered to be nonviable.

It has previously been shown that homogenized hepatocytes were even effective as a treatment for liver failure [23], suggesting the effectiveness of nonviable hepatocytes. In the present study, we also showed that the survival rate of the rats in the DHEP group was better, to some extent, than that in the control CM group, although the rate was much lower than that of the CPHEP group. In light of these results, it is likely that transplanted CPHEPs contribute to the improvement of liver failure by substituting the function of the host liver. They may also provide some growth factors or enzymes to support the regeneration of the remnant liver. It remained to be elucidated whether the cryopreserved CPHEPs also display such beneficial effects. Hepatocytes are known to be very sensitive to freezing damage. Three distinct modes of cryopreservation-induced hepatocyte death have been identified, namely, physical cell rupture, necrosis, and apoptosis [24]. The susceptibility of hepatocytes to such freeze-thaw injury is attributed to the damage to mitochondria, including loss of mitochondrial membrane integrity, increase in membrane permeability, etc. The inhibition of mitochondria damage, for instance, by broad-spectrum caspase-inhibitor, would prevent cryopreservation-induced damage of propagated hepatocytes.

In conclusion, the transplantation of homologous CPHEPs has a remarkable therapeutic potential for ALF in rats. Since we have recently established a culture method that enables us to multiply human hepatocytes 50 to 100 times during 50 d of culture [25], CPHEPs might be a useful source of hepatocytes for transplantation to treat human patients with ALF.

REFERENCES

1. Lee WM. Acute liver failure. N Engl J Med 1993;329:1862.
2. Bismuth H, Samuel D, Casting D, et al. Orthotopic liver transplantation in fulminant and subfulminant hepatitis. Ann Surg 1995;222:109.
3. Moreno GE, Garcia GI, Loinaz SC, et al. Liver transplantation in patients with fulminant hepatic failure. Br J Surg 1995;82:118.
4. Gewartowska M, Olszewski WL. Hepatocyte transplantation-biology and application. Ann Transplant 2007;12:27.
5. Habibullah MC, Syde HI, Qamar A, et al. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation 1994;58:951.
6. Strom SC, Fisher RA, Thompson MT, et al. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation 1997;63:559.

7. Fisher RA, Bu D, Thompson M, et al. Defining hepatocellular chimerism in liver failure patient bridge with hepatocyte infusion. Transplantation 2000;69:303.
8. Tateno C, Yoshizato K. Long-term cultivation of adult rat hepatocytes that undergo multiple cell divisions and express normal parenchymal phenotypes. Am J Pathol 1996;148:383.
9. Tateno C, Takai-Kajihara K, Yamasaki C, et al. Heterogeneity of growth potential of adult rat hepatocytes *in vitro*. Hepatology 2000;31:65.
10. Sato H, Funahashi M, Kristensen DB, et al. Pleiotrophin as a Swiss 3T3 cell-derived potent mitogen for adult rat hepatocytes. Exp Cell Res 1999;246:152.
11. Hino H, Tateno C, Sato H, et al. A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes. Biochem Biophys Res Commun 1999;256:184.
12. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29.
13. Katayama S, Tateno C, Asahara T, et al. Size-dependent *in vivo* growth potential of adult rat hepatocytes. Am J Pathol 2001; 158:97.
14. Eguchi S, Lilja H, Hewitt W, et al. Loss and recovery of liver regeneration in rats with fulminant hepatic failure. J Surg Res 1997;72:112.
15. Eguchi S, Kamolt A, Ljubiova J, et al. Fulminant hepatic failure in rats: Survival and effects on blood chemistry and liver regeneration. Hepatology 1996;24:1452.
16. Higgins GM, Anderson RM. Experimental pathology of the liver. 1. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931;12:186.
17. Gordon GJ, Coleman WB, Hixon DC, et al. Liver regeneration in rats with retrosine-induced hepatocellular injury proceeds through a novel cellular response. Am J Pathol 2000; 156:607.
18. Asahina K, Sato H, Yamasaki C, et al. Pleiotrophin/heparin-binding growth-associated molecule as a mitogen of rat hepatocytes and its role in regeneration and development of liver. Am J Pathol 2002;160:2191.
19. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology 2006;43(2 Suppl. 1):45.
20. Kamimukai N, Togo S, Hasegawa S, et al. Expression of Bcl-2 family reduces apoptotic hepatocytes after excessive hepatectomy. Eur Surg Res 2001;33:8.
21. Leist M, Gantner F, Bohlinger I, et al. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995;146:1220.
22. Oberhammer FA, Pavelka M, Sharma S, et al. Induction of apoptosis in culture hepatocytes and in regressing liver by transforming growth factor β1. Proc Natl Acad Sci U S A 1992; 89:5408.
23. Grundmann R, Koebe HG, Waters W. Transplantation of cryopreserved hepatocytes or liver cytosol injection in the treatment of acute liver failure in rats. Res Exp Med 1986; 186:141.
24. Terry C, Dhawan A, Mitry RR, Hughes RD. Cryopreservation of isolated human hepatocytes for transplantation: State of the art. Cryobiology 2006;53:149.
25. Yamasaki C, Tateno C, Aratani A, et al. Growth and differentiation of colony-forming human hepatocytes in vitro. J Hepatol 2006;44:749.