resistance mutations on viral replication. HCV RNA titer in mice infected with the telaprevir-resistant strain KT9-NS3-A156S was lower than in mice infected with the wildtype strain HCV-KT9-wild (Fig. 4B). HCV NS proteins include proteases for sequential processing of the polyprotein and are thought to be important in viral replication.[28] Our results suggest that differences in viral fitness underlie the differences in viral replication capacity. We analyzed the antiviral efficacy of telaprevir and the sequence of the NS3 region using HCV-infected mice treated with telaprevir. Although telaprevir treatment suppressed serum HCV RNA titer in mice infected with HCV-KT9, the decline of HCV RNA titer was only 0.6 log copy/mL in a mouse infected with KT9-NS3-A156S under the same treatment (Fig. 5A). These results suggest that our genetically engineered HCV-infected mouse model is useful for analyzing HCV escape mutants associated with antiviral drugs. Interestingly, treatment with telaprevir resulted in selection for V36A variants in the NS3 region in an HCV-KT9-infected mouse (Fig. 5B). There are a few controversial reports proposing that resistant variants may already be present at low frequency (<1%) within the quasispecies population in treatment-naïve patients,[29] consistent with their rapid emergence only days after treatment initiation.[26,30] This might well occur, due to the large number of mutated HCV clones. However, our results provide evidence in support of *de novo* emergence of telaprevir resistance induced by viral mutation followed by selection. HCV has both a high replication rate (10^{12} particles per day) and a high mutation rate (10^{-3} to 10^{-4}),[9,10] suggesting that the viral quasispecies population is likely to represent a large and genetically diverse substrate for immune selection.

In summary, we established an infection model of a genotype 1b HCV clone using the human hepatocyte chimeric mouse model. Using this model we demonstrate rapid emergence of *de novo* telaprevir-resistant HCV quasispecies from wildtype HCV.

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Impact of Viral Amino Acid Substitutions and Host Interleukin-28B Polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus

Nobuhiko Hiraga,[1,2] Hiromi Abe,[1,2] Michio Imamura,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] C. Nelson Hayes,[1,2] Hidenori Ochi,[2,3] Chise Tateno,[2,4] Katsutoshi Yoshizato,[2,4] Yusuke Nakamura,[5] Naoyuki Kamatani,[6] and Kazuaki Chayama[1,2,3]

Amino acid (aa) substitutions of core 70 and 91 and in the NS5A (nonstructural protein 5A) interferon sensitivity determining region (ISDR) as well as genetic polymorphisms in the host interleukin-28B (IL28B) locus affect the outcome of interferon (IFN)-based therapies for patients with chronic hepatitis C. The combination of these factors and the quasispecies nature of the virus complicate understanding of the underlying mechanism. Using infectious hepatitis C virus (HCV) genotype 1b clone HCV-KT9, we introduced substitutions at both core aa70 (Arg to Gln) and aa91 (Leu to Met). We also introduced four and nine ISDR aa substitutions into core mutant HCV-KT9. Using human hepatocyte chimeric mice with different IL28B genotypes, we examined the infectivity, replication ability, and susceptibility to IFN of these clones. Although aa substitutions in the ISDR significantly impaired infectivity and replication ability of the virus, core aa70 and 91 substitutions did not. The effect of IFN treatment was similar in core wild-type and mutant viruses. Interestingly, virus titer was significantly higher in mice with the favorable IL28B allele (rs8099917 TT and rs12979860 CC) in the transplanted hepatocytes than in mice with hepatocytes from rs8099917 TG and rs12979860 TT donors ($P < 0.001$). However, the effect of IFN was significantly greater, and intrahepatic expression levels of IFN-stimulated genes were significantly higher in mice with the favorable IL28B allele. *Conclusion:* Our data suggest that HCV replication levels and response to IFN are affected by human hepatocyte IL28B single-nucleotide polymorphism genotype and mutations in the ISDR. The mechanism underlying the clinically observed association of wild-type core protein in eradication-favorable host cells should be investigated further. (HEPATOLOGY 2011;54:764-771)

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.[1,2] Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection, and the most effective currently available therapy is combination therapy with pegylated (PEG)-IFN and ribavirin (RBV).[3-5] Among HCV genotypes, genotype 1 is the most resistant to

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL28B, interleukin-28B; ISDR, interferon-sensitivity-determining region; ISG, interferon-stimulated gene; MxA, myxovirus resistance protein A; NVR, nonvirological response; OAS, oligoadenylate synthetase; PBS, phosphate-buffered saline; PEG, pegylated; PKR, RNA-dependent protein kinase; RBV, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SNP, single-nucleotide polymorphism; SVR, sustained virological response; uPA, urokinase-type plasminogen activator.

From the [1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan; [3]Laboratory for Digestive Diseases, RIKEN Center for Genomic Medicine, Hiroshima, Japan; [4]PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan; [5]Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan; and [6]Laboratory for Statistics, RIKEN Center for Genomic Medicine, Yokohama, Japan.

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Address reprint requests to: Kazuaki Chayama, M.D., Ph.D., Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate school of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp; fax: +81-82-255-6220.

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IFN therapy.[6] The limited success of combination therapy for genotype 1 HCV infection is because of the low response rate during therapy and high relapse rate after therapy.[7]

Recent studies have identified both viral and host factors predictive of IFN therapy. Among the viral factors, amino acid (aa) substitutions in the IFN-sensitivity–determining region (ISDR) (nucleotides 2209-2248 or aa positions 237-276 within the NS5A region) are associated with sustained virological response (SVR) after IFN treatment in HCV genotype 1b patients.[8,9] Akuta et al. reported that substitution of aa70 or 91 in the HCV core region are independent predictors of SVR and nonvirological response (NVR).[10-12] Recently, we[13] and another group[14] also reported that wild-type HCV core aa70 and two or more aa substitutions in the ISDR are effective predictors of SVR in patients with HCV genotype 1b.

Among host factors associated with SVR, many common genetic polymorphisms in the human genome have been identified, including single-nucleotide polymorphisms (SNPs).[15-19] More recently, an association between several linked SNPs in the interleukin-28B (IL28B) locus and the effect of combination therapy has been reported.[20-22]

We recently reported that the core aa wild type is significantly more likely to be found in patients with the eradication-favorable IL28B SNP genotype.[23-25] The underlying mechanism of this association as well as the reason for the differential response to therapy by viruses with core aa substitutions are unknown. This is partly because of the presence of HCV quasispecies in human serum samples and the difficulty of performing infection experiments in a small animal model.

The severe combined immunodeficient (SCID) urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice able to develop HCV viremia after injection of serum samples positive for the virus.[26] We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs, such as IFN-alpha and NS3-4A protease inhibitor.[27,28] We have further improved the replacement levels of the human hepatocytes in this mouse model,[29] which enabled us to perform infection experiments more easily because highly repopulated mice (defined as human serum albumin [HSA] levels well above 1 mg/mL) successfully develop viremia more often than poorly repopulated mice.[30] Using this mouse model, we developed a reverse genetics system for HCV.[31,32] This system is useful for studying characteristics of HCV strains with various substitutions of interest, because the effects of quasispecies can be minimized. Furthermore, as there is no adaptive immune system in this mouse model, we are able to examine the replication of HCV and the effect of therapy while avoiding the influence of the immunological response. In the present study, we investigated effects of viral and host factors on HCV infectivity, replication ability, and IFN susceptibility using genetically engineered genotype 1b HCV-infected mice that underwent transplantation with hepatocytes having eradication-favorable or eradication-unfavorable IL28B SNP genotypes.

Table 1. Characteristics of Donors for Transplanted Human Hepatocytes

Donor	A	B	C	D
Sex	Female	Male	Female	Male
Age	10	2	5	2
Ethnic group	Caucasian	Caucasian	African American	Hispanic
rs8099917	TG	TT	TG	TT
rs8109886	AA	CC	AA	CC
rs12979860	TT	CC	TT	CC
rs11882871	GG	AA	GG	AA
rs73930703	TT	CC	TT	CC
rs8107030	AG	AA	AG	AA
rs28416813	GG	CC	GG	CC
rs8103142	CC	TT	CC	TT
rs11881222	GG	AA	GG	AA
rs4803217	AA	CC	AA	CC

Materials and Methods

Animal Treatment. Generation of the $uPA^{+/+}$/$SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[29] All animal protocols described in this study were performed in accord with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of HSA, which serve as useful markers of the extent of repopulation, were measured as previously described.[29] Mice underwent transplantation with frozen human hepatocytes obtained from four different human donors (Table 1). Genotyping of IL28B SNPs of human hepatocytes was performed using the Invader assay as described previously.[33,34] We used 1000 IU/g/day of IFN-alpha (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for 2 weeks. This dosage was selected based on a previous report showing that this regimen reduced mouse serum

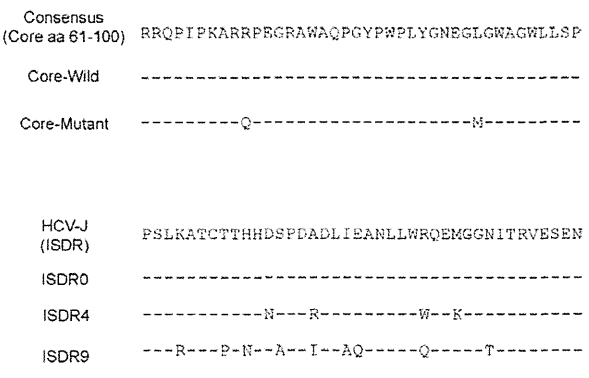

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

HCV RNA levels by 0.5-2 log copies/mL during therapy.[31]

HCV RNA Transcription and Inoculation into Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9, that was obtained from a Japanese patient with severe acute hepatitis (GenBank accession no. AB435162).[32] Ten micrograms of plasmid DNA, linearized by digestion with *Xba*I (Promega, Madison, WI), was transcribed in a 100-μL reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and then analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μL of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.[32] The HCV-KT9 clone has aa substitutions at aa70 and 91 (arginine to glutamine and leucine to methionine, respectively) in the core region (Core-Mutant), compared to the consensus sequence,[10-12] and no aa substitutions in the ISDR (ISDR0),[8] relative to the prototype sequence (HCV-J).[35] Using the original HCV-KT9 clone, we created two additional HCV clones having wild-type core aa70 and 91 (Core-Wild) and four (ISDR4) and nine (ISDR9) aa substitutions in the ISDR, respectively (Fig. 1). To introduce the aa substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Human Serum Samples. Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6 copies/mL) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. Core 70 and 91 aas were Gln and Leu, respectively, and only one aa substitution was present in the ISDR. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Quantitation of HCV RNA and IFN-stimulated gene-expression levels. RNA was extracted from mice serum and liver samples by Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in 8.8 μL of ribonuclease-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in 20 μL of reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostics, Tokyo, Japan) were performed as previously described.[32] Quantitation of IFN-stimulated genes (ISGs) (myxovirus resistance protein A [MxA], oligoadenylate synthetase [OAS], and RNA-dependent protein kinase [PKR]) was performed using real-time PCR Master Mix (Toyobo, Kyoto, Japan) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. ISG messenger RNA expression levels were expressed relative to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis. The HCV infectious ratio of chimeric mice was assessed using the chi-square test. Mice serum HCV RNA titers, HSA concentrations, and ISG expression levels were compared using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

Results

Influence of aa Substitutions in the HCV Core Region and ISDR on HCV Infectivity and Replication Ability. We investigated the influence of aa substitutions in the core region and ISDR on HCV infectivity and replication ability in mice that underwent transplantation with human hepatocytes obtained from donor A (Table 1). Each 30 μg of *in vitro*–transcribed RNA was inoculated into the livers of mice. Six weeks after inoculation, serum HCV RNA titers increased above the detectable limit (1000 copies/mL) in 11 of 12 (92%) mice infected with Core-Wild-ISDR0 and in 14 of 16 (88%) mice with Core-Mutant-ISDR0 (Fig. 2A). HCV RNA titers in Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice increased to the same levels (Fig. 2B). In contrast, serum HCV

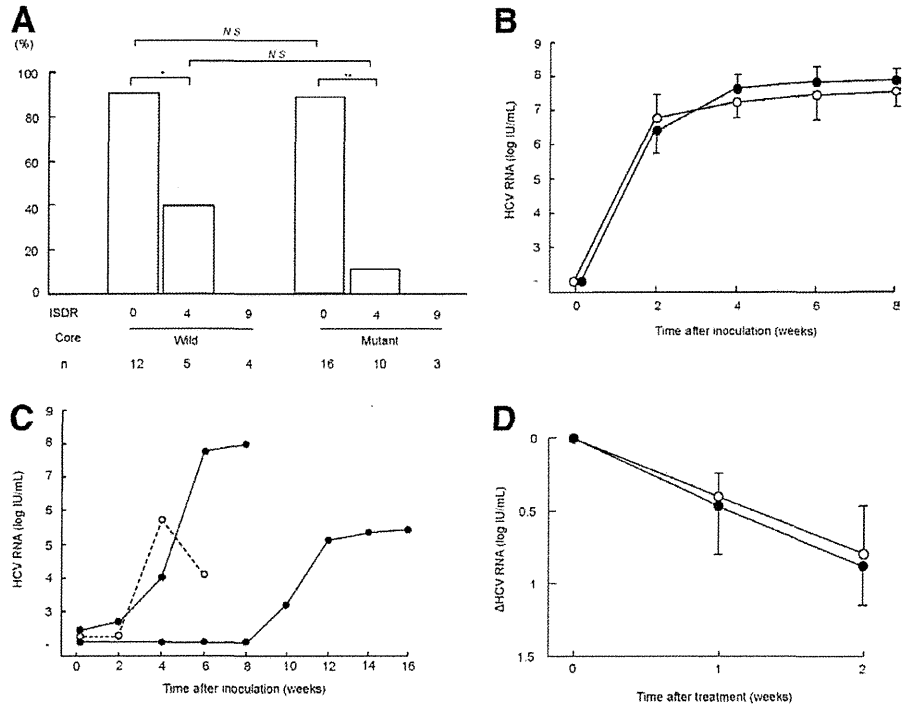

Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μg of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above the detection limit (1000 copies/mL) 6 weeks after inoculation. aa sequences of the core (Wild or Mutant) and number of substitutions in the ISDR are noted below the graph. (B) Time course of serum HCV RNA levels in mice inoculated with either Core-Wild-ISDR0 (closed circles, n = 11) or Core-Mutant-ISDR0 (open circles, n = 14) HCV clones. Data are represented as mean ± standard deviation. (C) Time course of serum HCV RNA levels in two Core-Wild-ISDR4-infected mice (closed circles) and a Core-Mutant-ISDR4-infected mouse (open circles). Serum HCV RNA levels were measured until the mice died. (D) Core-Wild-ISDR0- (closed circles, n = 8) and Core-Mutant-ISDR0 (open circles, n = 4)-infected mice were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Mice serum HCV RNA titers were measured at the indicated times. $*P < 0.05$, $**P < 0.01$; NS, not significant.

RNA titer increased above the detection limit in only two of five (40%) Core-Wild-ISDR4 mice and in only 1 of 10 (10%) Core-Mutant-ISDR4 mice, and the titers in these mice were lower than in mice with ISDR0 (Fig. 2C). HCV RNA titers failed to increase above the detection limit in mice with Core-Wild-ISDR9 and Core-Mutant-ISDR9 (Fig. 2A).

Influence of Core aa Substitutions on the Effect of IFN. To investigate the influence of aa substitutions in the core region on the effect of IFN, Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in a 0.84 ± 0.3 log IU/mL reduction of HCV RNA titer in Core-Wild-ISDR0–infected mice and a 0.79 ± 0.34 log IU/mL reduction in Core-Mutant-ISDR0–infected mice (Fig. 2D).

We also investigated the influence of aa substitutions in the core region on the effect of IFN plus RBV combination therapy. Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha and 20 mg/kg of RBV daily for 2 weeks. The treatment resulted in similar HCV RNA reductions in all treated mice. However, as with IFN monotherapy, there were no significant differences in HCV reductions among mice with different aa substitutions in the core region (data not shown). The dose of ribavirin used was relatively small, however, because of the drug's toxicity in mice.

HCV Infectivity, Replication Levels, and IFN Susceptibility by Core aa Substitutions and Genetic Variation in the IL28B Locus. We investigated the influence of IL28B genotypes on HCV infectivity, replication ability, and IFN susceptibility. *In vitro*–transcribed RNA (30 μg) was inoculated into the livers of mice with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) or donor B (rs8099917 TT and rs12979860 CC). Eight weeks after inoculation, serum HCV RNA titers increased above the detection limit in 22 of 25 (88%) mice with hepatocytes from donor A and in 20 of 23 (87%) mice with hepatocytes from donor B (Fig. 3A). Serum HCV RNA levels were

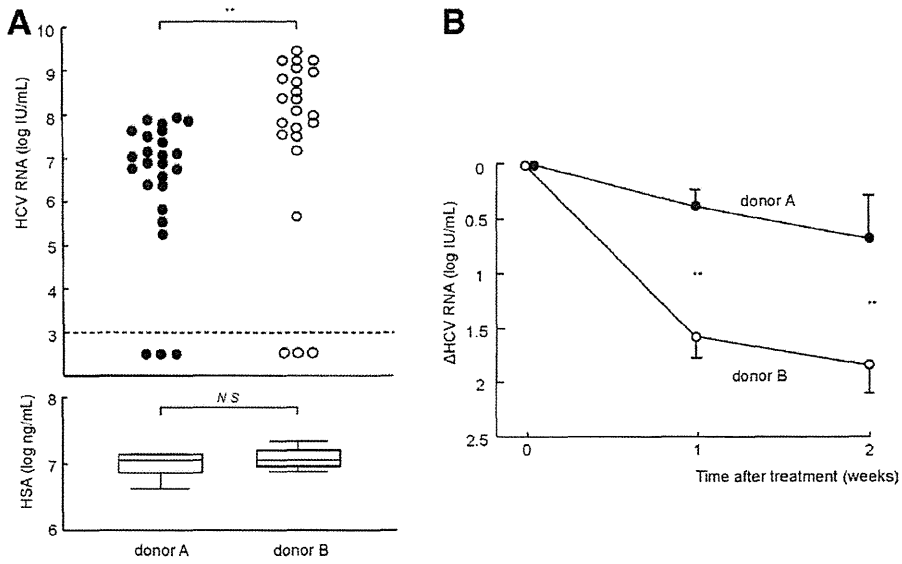

Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, n = 25) or B (rs8099917 TT and rs12979860 CC) (open circles, n = 23) were intrahepatically inoculated with RNA transcribed from either Core-Wild-ISDR0 or Core-Mutant-ISDR0 clones. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B) HCV-infected mice with hepatocytes from donor A (closed circles, n = 12) or B (open circles, n = 8) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. Data are represented as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

significantly higher in mice with hepatocytes from donor B than from donor A ($P < 0.001$). HCV-infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in 0.65 ± 0.38 and 1.84 ± 0.23 log IU/mL reductions in HCV RNA titer in mice with hepatocytes from donors A and B, respectively ($P < 0.01$) (Fig. 3B). Interestingly, despite the higher serum HCV RNA levels, reduction levels of HCV were higher in mice that underwent transplantation with hepatocytes obtained from donor B than in mice that underwent transplantation with hepatocytes obtained from donor A.

To confirm an association between IL28B SNP genotype and HCV RNA titer, we compared HCV RNA titers using mice with hepatocytes from an additional pair of donors with the favorable (donor C) and unfavorable (donor D) SNP genotypes. To determine whether results obtained by clonal infection would be comparable to results obtained using the more natural serum injection, which should have contained more complex viral species, mice were injected with genotype 1b HCV obtained from a human patient with core and ISDR substitutions, as described above. Mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) or donor D (rs8099917 TT and rs12979860 CC) were inoculated intravenously with 10^5 copies of HCV. Eight weeks after inoculation, serum HCV RNA titer increased above the detection limit in 13 of 14 (93%) mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) and in 12 of 12 (100%) mice with hepatocytes from donor D (rs8099917 TT and rs12979860 CC) (Fig. 4A). With results similar to those found for the mice inoculated with transcribed HCV RNA, serum HCV RNA levels were significantly higher in mice with hepatocytes from donor D than from donor C ($P < 0.001$), and the effect of IFN was also greater in donor D mice than in donor C mice (Fig. 4B); however, statistical significance using these donors was only achieved at week 1, probably resulting from fluctuation of HCV RNA titers and the small number of animals analyzed.

Expression Levels of ISGs in Mouse Livers. ISG expression levels in mice livers were measured after 2 weeks of IFN treatment (Fig. 4B). MxA, OAS, and PKR levels were significantly higher in mice with human hepatocytes from donor D than from donor C (Fig. 4C).

Discussion

In this study, we investigated the effect of substitutions at core protein aa70 and 91 and within the

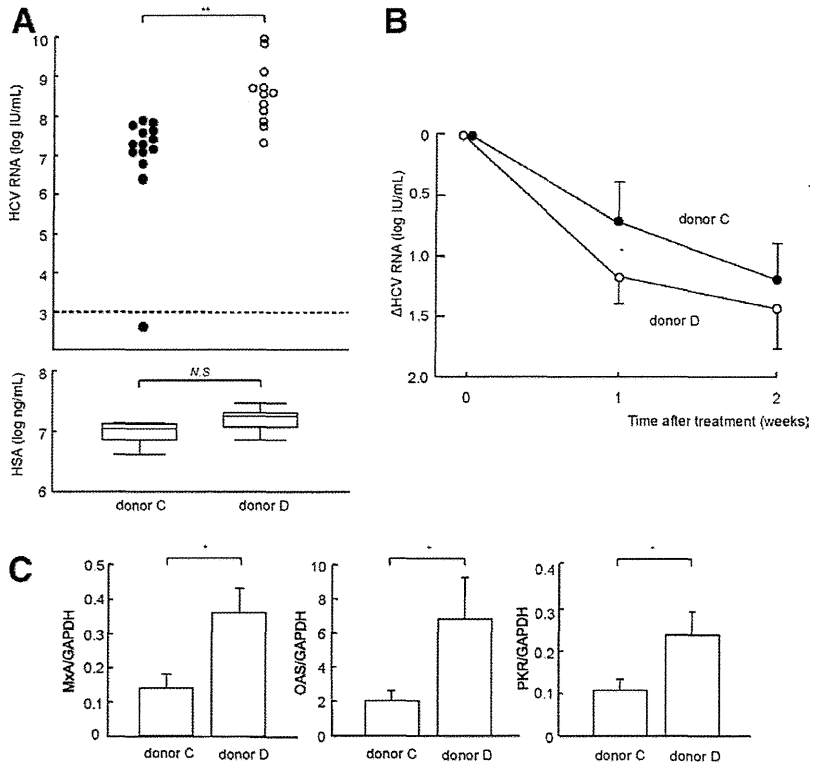

Fig. 4. HCV infectivity, replication ability, and IFN susceptibility in HCV-infected mice. Mice that underwent transplantation with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) (closes circles, n = 14) or D (rs8099917 TT and rs12979860 CC) (open circles, n = 12) were intravenously injected with HCV-infected patient serum samples. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. HCV-infected mice with hepatocytes from donor C (closed circles, n = 5) or D (open circles, n = 4) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. (B) Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. (C) Intrahepatic ISG expression levels in the IFN-treated mice with donor C (n = 4) or D (n = 3) were measured and expressed relative to GAPDH messenger RNA. Data are reported as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

ISDR, which have been reported to be associated with the outcome of IFN plus ribavirin combination therapy.[8-14] Clones with core aa70 and 91 substitutions showed comparable infection and replication abilities, whereas clones with substitutions in the ISDR showed reduced infectivity and replication rates. It has been reported that patients infected with HCV strains with multiple substitutions in the ISDR have lower viral titers than those with wild-type ISDR, and that these patients respond well to IFN therapy.[8,9] We showed, in this study, that infectivity and replication ability of HCV are apparently impaired in ISDR mutants (Fig. 2A,C). This may explain, at least partially, the better effect of IFN therapy in patients with multiple ISDR mutations. However, why aa substitutions in this particular region are associated with the effect of IFN still remains to be elucidated. In contrast, aa substitutions in the core, which more profoundly affect the outcome of combination therapy,[10-13] did not influence the infectivity and replication ability of the virus (Fig. 2A,B). This suggests that aa substitutions in this region affect response to therapy in a way that is independent of the replication level of the virus. A recent report by Eng et al.[36] showed that a mutation in core aa91 results in the production of minicore protein, which might alter the effect of IFN. The presence of minicore protein and its effect on IFN therapy should be further investigated using the chimeric mouse model.

In contrast to these viral substitutions, host IL28B genotype significantly affected viral replication levels (Figs. 3A and 4A). Curiously, replication levels of the virus are higher in mice with human hepatocytes from donors with rs8099917 TT and rs12979860 CC genotypes, even though these genotypes are associated with successful response to the therapy.[20-22] This result is consistent with clinical observation of higher viral loads in patients with the rs12979860 CC genotype.[20] The favorable IL28B genotype is associated not only with successful response to IFN treatment, but also to spontaneous clearance of the virus.[37,38] However, the incidence of HCV infection was similar in mice with hepatocytes from donors with rs8099917 TT and rs8099917 TG (Figs. 3A and 4A), suggesting that spontaneous clearance was rare. The fact that our animal model was immunodeficient suggests that spontaneous clearance of HCV might require the involvement of the adaptive immune system. The wild-type core protein, aa70, is reported to be found more often in patients with the rs8099917 TT genotype,[23,24] even though patients with this genotype are more likely to be able to eradicate the virus without therapy during

the natural course of infection.[37,38] These data suggest that core aa70 wild-type virus can be eradicated more easily in the natural course of infection, especially in patients with rs8099917 TT or rs12979860 CC genotypes; but once the infection is established, core aa70 wild type replicates more effectively than core aa70 mutant strains.

The effect of IFN on reduction of the virus did not differ between core aa70 wild-type and mutant strains, which showed similar replication levels (Fig. 2D). This is in contrast to clinical observations that the effect of therapy on viral reduction is more prominent in patients with wild-type core protein.[13,25] One of the differences between the mouse model and human patients is term of infection. Long-term HCV infection results in alteration of lipid metabolism and accumulation of lipids in hepatocytes.[39] Patients with fatty change of the liver often fail to respond to therapy.[40] We observed no severe fatty change in mouse livers, suggesting that such long-term change might be absent in this mouse model (data not shown).

On the other hand, the effect of IFN was significantly greater in mice with hepatocytes with the eradication-favorable IL28B genotype (rs8099917 TT and rs12979860 CC) (Figs. 3B and 4B), despite the higher replication rate of the virus. This suggests that the IL28B genotype affects the outcome of therapy based on a different mechanism than viral replication. Because of strong linkage disequilibrium, genotypes of the SNPs around the two IL28B landmark SNPs (rs8099917 and rs12979860) were identical between donors A and C as well as between B and D (data not shown). Further study using human hepatocytes with various IL28B SNP genotypes will identify a primary SNP that directly affects the outcome of therapy. Response to IFN was associated with higher expression levels of ISGs, including MxA, OAS, and PKR (Fig. 4C). This is in agreement with previous studies showing that SVR is associated with stronger induction of ISG expression.[41] However, we observed no statistically significant differences in ISG expression levels from the IL28B SNP genotype before therapy (data not shown). This may result from lower ISG expression levels before therapy and the relatively small number of mice examined. Because there is no adaptive immune system in this mouse model, such differences primarily involve individual hepatocytes, although whether the presence of immune cells enhances this difference should be investigated further.

In summary, we demonstrated that viral infectivity and replication ability are associated with hepatocyte IL28B genotype and are not associated with viral substitutions in the core protein or ISDR. Understanding the mechanism underlying the higher, more prolonged expression of antiviral genes in response-favorable hepatocytes will help us to develop improved therapeutic regimens to eradicate HCV more effectively.

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Therapeutic Potential of Propagated Hepatocyte Transplantation in Liver Failure

Hironobu Amano, M.D.,* Hiroshi Hino, M.D.,* Chise Tateno, Ph.D.,† Kentaro Emoto, M.D.,* Yasuhiro Imaoka, M.D.,* Chihiro Yamasaki, Ph.D.,‡ Toshiyuki Itamoto, M.D.,* Hirotaka Tashiro, M.D.,* Toshimasa Asahara, M.D.,* Hideki Ohdan, M.D.,* and Katsutoshi Yoshizato, Ph.D.†,‡,§,[1]

**Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; †PhoenixBio, Co., Ltd., Hiroshima, Japan; ‡Yoshizato Project, CLUSTER, Prefectural Institute of Industrial Science and Technology, Hiroshima, Japan; and §Osaka City University Graduate School of Medicine, Osaka, Japan*

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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.**

[1] To whom correspondence and reprint requests should be addressed at PhoenixBio, Co., Ltd., 3-4-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan. E-mail: katsutoshi.yoshizato@phoenixbio.co.jp.

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.

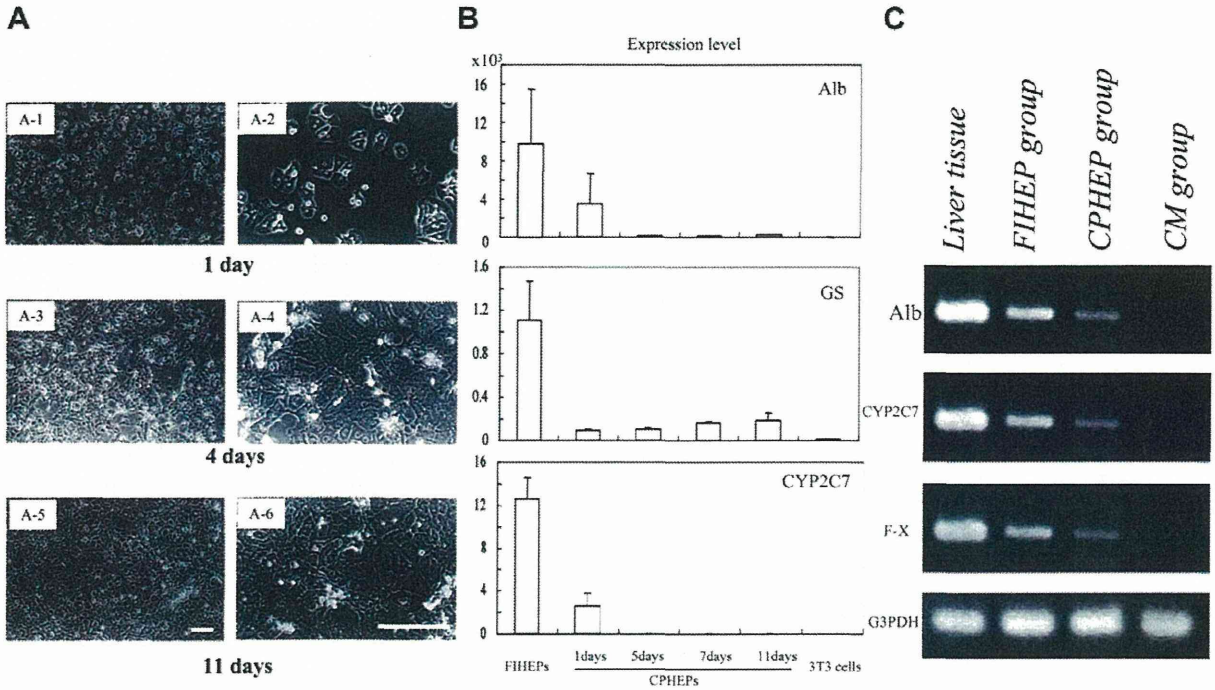

FIG. 1. (A) Phase contrast image of proliferating hepatocytes. Hepatocytes (8.5×10^3 cells/cm^2) were cocultured with Swiss 3T3 cells in HCGM on 15.0-cm dishes. Photographs were taken for the same fields at 1 (A-1, 2), 4 (A-3, 4), and 11 d (A-5, 6) with lower (A-1, 3, 5) and higher (A-2, 4, 6) magnifications.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

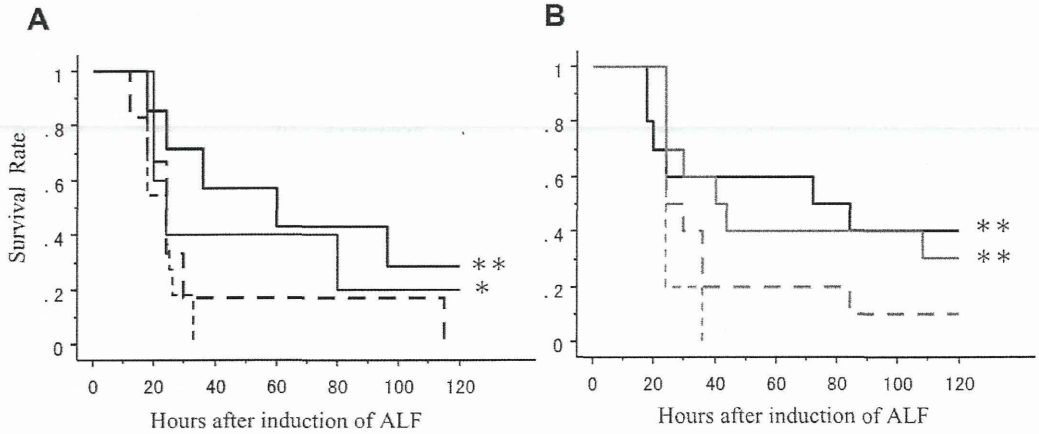

FIG. 2. Survival curves of ALF rats with FIHEP transplantation. The rats were transplanted with HEPs or FBs through the spleen and then subjected to ALF. Some rats were given CM as controls. (A) Rescue of ALF by FIHEP transplantation. The rats were given varying numbers of FIHEPs: 1.5×10^7 cells ($n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

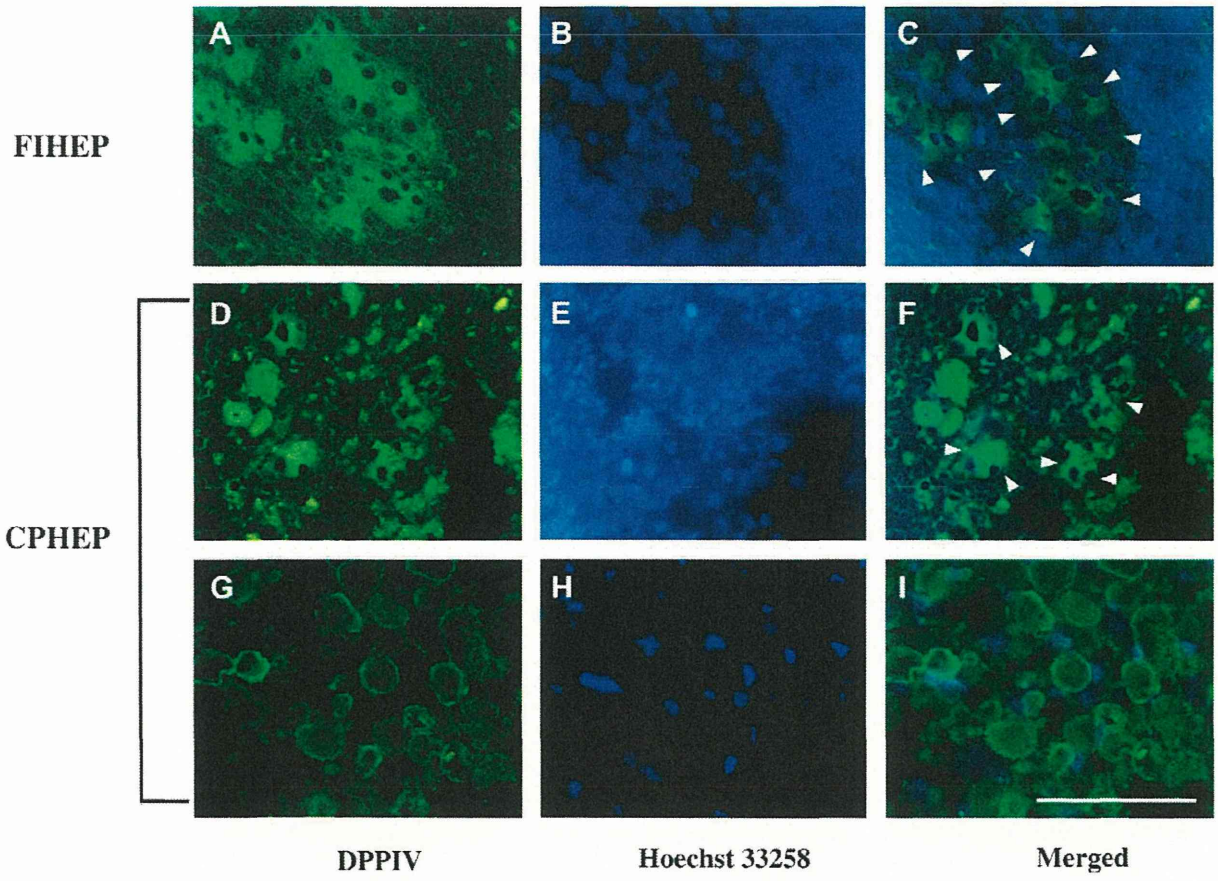

FIG. 3. Engraftment of the transplanted hepatocytes in the spleen of ALF rats. The rats were transplanted with FIHEPs (A)–(C) or CPHEPs (D)–(I) and subjected to ALF as in Figure 2B. Spleens were removed at 24 h after ALF induction and processed to cryosectioning for immunohistochemical analysis to detect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

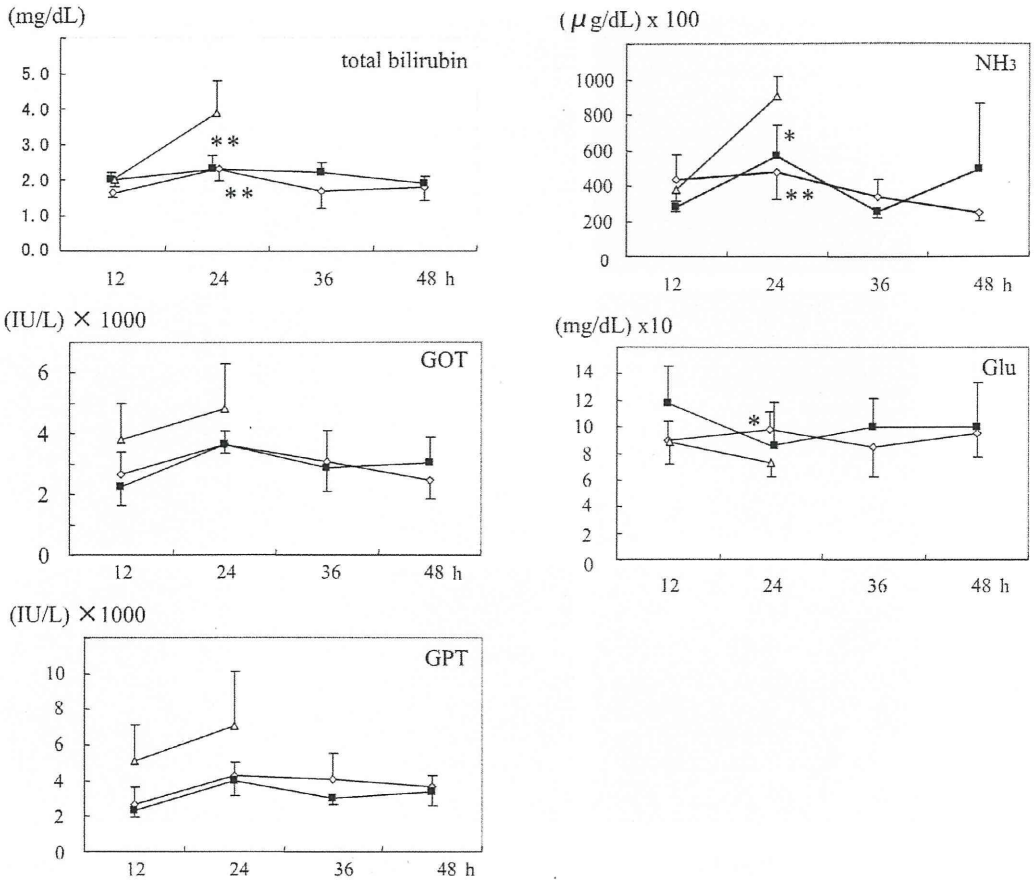

FIG. 4. Biochemical evaluation of hepatocyte transplantation therapy for ALF. The rats were subjected to hepatocyte transplantation and ALF treatment as described in Figure 2. At the indicated time points after ALF treatment, blood was collected for total bilirubin, NH_3, GOT, Glu, and GPT assessment.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

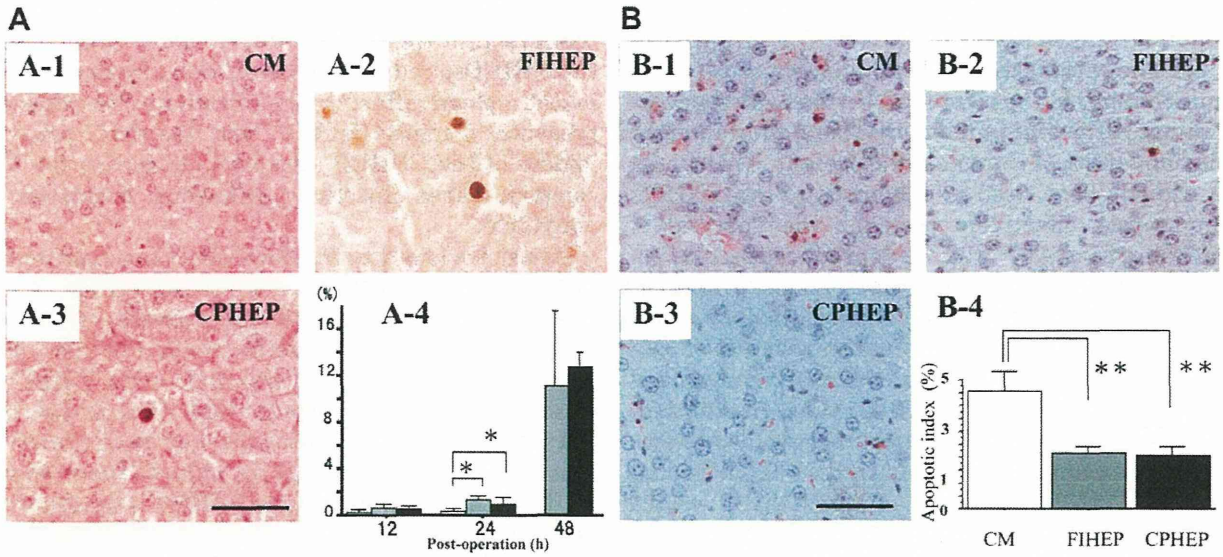

FIG. 5. (A) BrdU-labeling index of hepatocytes in the remnant liver of the hepatocyte- transplanted rat. The rats were injected with CM, transplanted with FIHEPs or CPHEPs, and subjected to ALF as in Fig. 2. The remnant livers (omental lobe) were removed at 12, 24, and 48 h post-induction of ALF and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

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Growth Hormone-Dependent Pathogenesis of Human Hepatic Steatosis in a Novel Mouse Model Bearing a Human Hepatocyte-Repopulated Liver

Chise Tateno, Miho Kataoka, Rie Utoh, Asato Tachibana, Toshiyuki Itamoto, Toshimasa Asahara, Fuyuki Miya, Tatsuhiko Tsunoda, and Katsutoshi Yoshizato

Yoshizato Project (C.T., M.K., R.U., A.T., K.Y.), Hiroshima Prefectural Institute of Industrial Science and Technology, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), and PhoenixBio, Co. Ltd. (C.T., A.T., K.Y.), Higashihirosima, Hiroshima 739-0046, Japan; Hiroshima University Liver Project Research Center (C.T., T.A., K.Y.) and Division of Frontier Medical Science (T.I., T.A.), Department of Surgery, and Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Hiroshima 734-8551, Japan; Laboratory for Medical Informatics (F.M., T.T.), Center for Genomic Medicine, RIKEN, Yokohama, Kanagawa 230-0045, Japan; Developmental Biology Laboratory and Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine (K.Y.), Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Hiroshima 739-8526, Japan; and Departments of Hepatology and Liver Research Center (K.Y.), Graduate School of Medicine, Osaka City University, Osaka 545-8586, Japan

Clinical studies have shown a close association between nonalcoholic fatty liver disease and adult-onset GH deficiency, but the relevant molecular mechanisms are still unclear. No mouse model has been suitable to study the etiological relationship of human nonalcoholic fatty liver disease and human adult-onset GH deficiency under conditions similar to the human liver *in vivo*. We generated human (h-)hepatocyte chimeric mice with livers that were predominantly repopulated with h-hepatocytes in a h-GH-deficient state. The chimeric mouse liver was mostly repopulated with h-hepatocytes about 50 d after transplantation and spontaneously became fatty in the h-hepatocyte regions after about 70 d. Infusion of the chimeric mouse with h-GH drastically decreased steatosis, showing the direct cause of h-GH deficiency in the generation of hepatic steatosis. Using microarray profiles aided by real-time quantitative RT-PCR, comparison between h-hepatocytes from h-GH-untreated and -treated mice identified 14 GH-up-regulated and four GH-down-regulated genes, including *IGF-I*, *SOCS2*, *NNMT*, *IGFLS*, *P4AH1*, *SLC16A1*, *SRD5A1*, *FADS1*, and *AKR1B10*, respectively. These GH-up- and -down-regulated genes were expressed in the chimeric mouse liver at lower and higher levels than in human livers, respectively. Treatment of the chimeric mice with h-GH ameliorated their altered expression. h-Hepatocytes were separated from chimeric mouse livers for testing *in vitro* effects of h-GH or h-IGF-I on gene expression, and results showed that GH directly regulated the expression of *IGF-I*, *SOCS2*, *NNMT*, *IGFALS*, *P4AH1*, *FADS1*, and *AKR1B10*. In conclusion, the chimeric mouse is a novel h-GH-deficient animal model for studying *in vivo* h-GH-dependent human liver dysfunctions. (***Endocrinology*** **152: 1479–1491, 2011)**

To study pathophysiological characteristics of the human liver, we previously generated a humanized (chimeric) mouse whose liver was almost completely repopulated with human (h-)hepatocytes by transplanting h-hepatocytes into immunodeficient and liver-damaged mice, which had been obtained by mating an albumin enhancer/promoter-driven

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Abbreviations: AGHD, Adult-onset GH deficiency; Alb, albumin; CK, cytokeratin; GO, gene ontology; h-, human; m-, mouse; 9MM, 9-month-old male; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ORO, Oil Red O; qRT-PCR, quantitative RT-PCR; RI, replacement index; SCID, severe combined immunodeficient; uPA, urokinase-type plasminogen-activator; 25YF, 25-yr-old female; 61YF, 61-yr-old female; 28YM, 28-yr-old male; 57YM, 57-yr-old male.