

for both hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further analyzed for production of HBV particles. Finally, three cell lines that produced more than 10^5 copies per milliliter of HBV DNA in supernatant were selected and used for further experiments.

For transfection with HCV RNA, we used pJFH1, which contains the complementary DNA of full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In vitro* synthesis of HCV RNA and electroporation into Huh7 cells were performed as described previously [22,23]. Briefly, cells were treated with trypsin, washed twice with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I (Invitrogen, Carlsbad, CA, USA) at a final concentration of 7.5×10^6 cells per milliliter. Then, 10 μ g of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and subjected to an electric pulse (950 μ F and 260 V) using the Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish.

2.2. Generation of human hepatocyte chimeric mice

Generation of the urokinase-type plasminogen activator (uPA)^{+/+} and severe combined immunodeficiency (SCID)^{+/+} mice and transplantation of human hepatocytes were performed as described recently by our group [21,23,24]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. Infection, extraction of serum samples, and euthanasia were performed under ether anesthesia. The concentration of serum human serum albumin, which correlates with the repopulation index [24], was measured in mice as described previously [21]. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.

2.3. Human serum samples

Human serum samples containing high titers of either HBV DNA (5.3×10^6 copies per milliliter) or genotype 1b HCV (2.2×10^6 copies per milliliter) were obtained from patients with chronic hepatitis with a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use. Chimeric mice were injected intravenously with 50 μ L of either HBV- or HCV-positive human serum. Some mice were injected with HBV-positive human serum at 6 weeks after injection of HCV-positive human serum.

2.4. Analysis of HBV and HCV

HBsAg and HBeAg in culture supernatants were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abbott Japan, Osaka, Japan). DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μ L H₂O [21,25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μ L RNase-free H₂O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20- μ L reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY, USA).

2.5. RNA extraction and measurement of mRNAs of interferon-induced genes by quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One nanogram of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random

Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, OAS, and PKR. Amplification and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA). Results were normalized to the transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Statistical analysis

Changes in HBV DNA and HCV RNA in mice sera were compared by Mann-Whitney test and unpaired *t* test. Differences in HBV DNA and HCV core antigen in mice sera and culture supernatants were analyzed by one-way analysis of variance followed by Scheffé's test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Infection of chimeric mouse with HBV and HCV and susceptibility to interferon

To investigate the interference between HBV and HCV and to examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μ L of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies per milliliter (range: 5.3×10^6 – 3.6×10^9 copies per milliliter) at 6 weeks after inoculation (Fig. 1A), similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies per milliliter (range: 1.2×10^6 – 0.8×10^7 copies per milliliter) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g per day of intramuscular IFN- α for 2 weeks. Treatment resulted in a decrease of only 0.3 log in mice serum HBV DNA level compared to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a rapid decrease in HCV RNA to undetectable levels, as confirmed by quantitative polymerase chain reaction (PCR; Fig. 1B).

To investigate the direct interference of the two viruses, we performed double-infection experiments. Ten chimeric mice were first inoculated intravenously with 50 μ L of HCV-positive human serum samples. Six weeks after HCV infection when the mice developed HCV viremia, 50 μ L of HBV-positive human serum samples were inoculated intravenously in 5 of 10 HCV-infected mice. All five mice became positive for both HBV and HCV at 2 weeks after HBV infection. No significant decrease in HCV RNA levels was observed in these superinfected mice before or after the development of HBV viremia (Fig. 2A). After HBV infection, there was no apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in HBV–HCV-coinfected mice was comparable with that of only HBV-infected mice (Fig. 2B). These results sug-

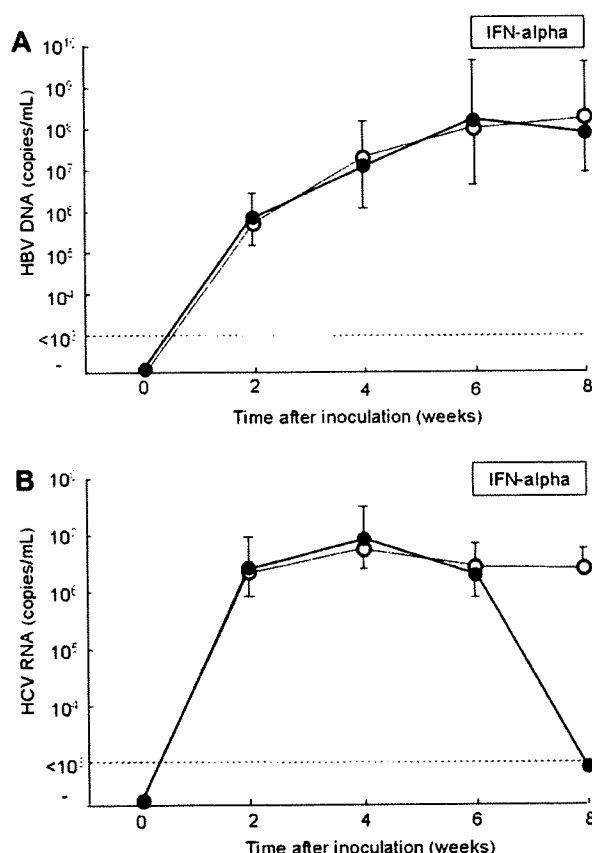


Fig. 1. Changes in serum virus titers in mice inoculated with hepatitis B virus (HBV) – positive or hepatitis C virus (HCV) – positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g per day of interferon-alpha intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean plus or minus standard deviation ($n = 3$). The horizontal dashed line represents the detection limit (10^2 copies per milliliter).

gest no interference between the two viruses in mice, which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the effect of IFN against the other virus, three HBV–HCV-coinfected mice were treated with IFN- α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast, no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B). These results are similar to the reduction of HCV RNA and HBV DNA in mice that were infected with either of these hepatitis viruses. These results indicate that HCV is more susceptible to IFN- α than HBV and that each virus does not alter the effect of IFN on the other virus. Because the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. It is possible,

however, that HBV and HCV replicated in different cells in these mice. Because it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histologic examination, we performed *in vitro* experiments.

3.2. Production of both HBV- and HCV-producing cells and the effect of interferon

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stable HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42, and -53) that produced HBsAg, HBeAg, and HBV DNA into the supernatant were selected (Table 1). These cell lines continuously produced HBV for more than 3 months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after JFH1 transfection (Fig. 4A). In contrast, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than in Huh7 cells, and the level was not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

3.3. Effects of interferon on HBV and HCV *in vitro*

The effects of IFN on virus production in both HBV- and HCV-producing cell lines was examined by adding different amounts of IFN- α (0, 50, and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as Mx α , OAS, and PKR increased in a dose-dependent manner in all three cell lines as well as in parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV–HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN, and the decrease was dose-dependent (Fig. 5C).

4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

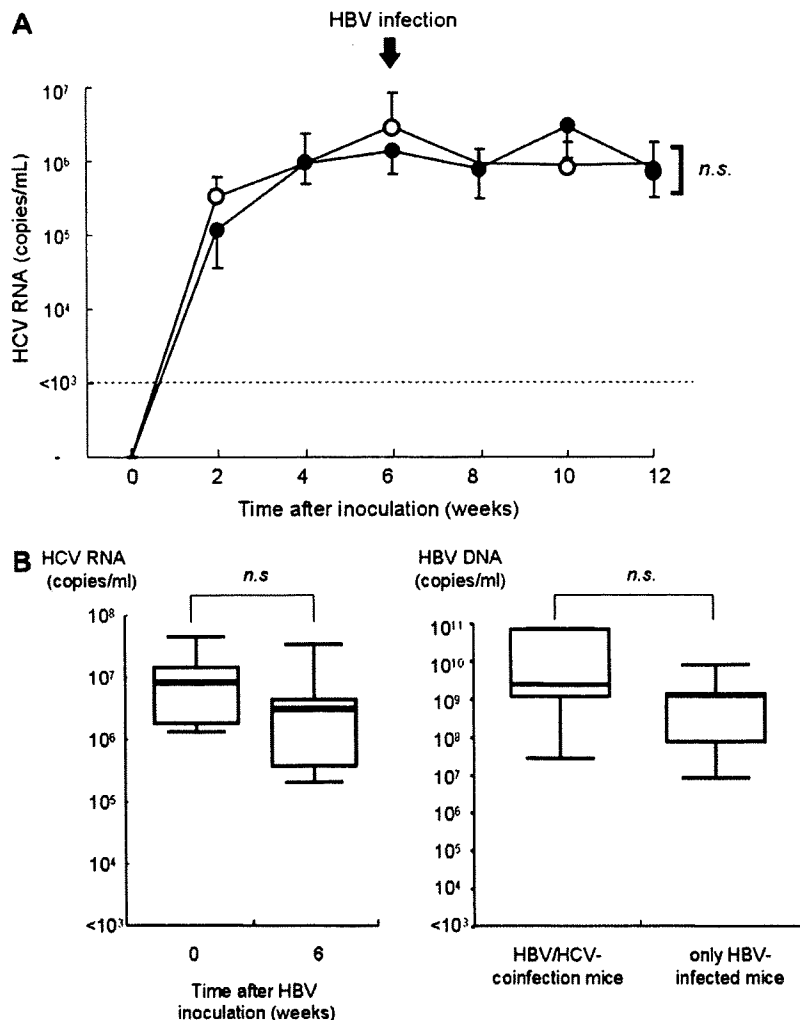


Fig. 2. Comparison of hepatitis C virus (HCV) and hepatitis B virus (HBV) titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining five mice (open circles) did not receive HBV inoculation. Data are mean plus or minus standard deviation ($n = 3$). (B) Serum HCV RNA titers in five mice infected with HCV before and at 6 weeks after HBV superinfection (left panel). Serum HBV DNA titers in five mice coinfecting with HBV and HCV were compared with those of five mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Accumulation of mononuclear cells is usually seen in the livers of infected individuals, in association with the state of inflammation. It is thus difficult to examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte chimeric mouse model is ideal for investigating such interaction. We expected either reduction of HCV after inoculation of HBV in HCV-infected mice or failure to develop HBV viremia or low-level

HBV viremia in these mice due to viral interference; however, no reduction in HCV titers occurred in these mice, and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry or expansion in HBV-infected chimpanzee livers and suggested that HBV was a stealthy virus early in the infection [19]. Because no reduction in HCV was noted during and after the development of high-level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without

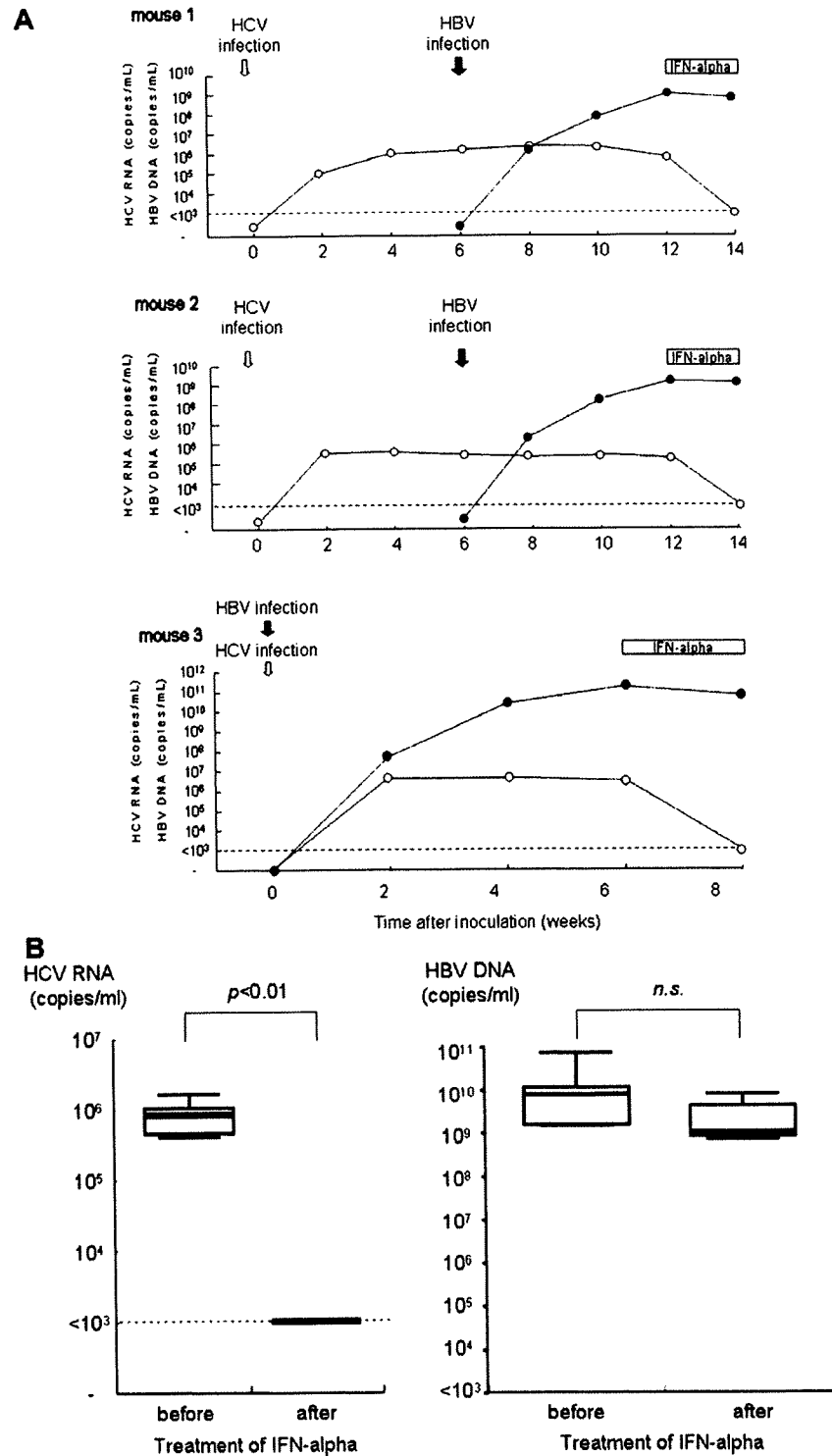


Fig. 3. Changes in serum hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA levels and effects of IFN on HBV–HCV-coinfected mice. Three mice (mouse 1, 2, and 3) were inoculated with both HBV- and HCV-positive human serum samples and treated daily with 7000 IU/g per day of interferon-alpha (IFN- α) intramuscularly for 2 weeks. Mice sera samples were obtained every 2 weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles) were analyzed by quantitative polymerase chain reaction. (A) The horizontal dashed line represents the detectable limit (10^3 copies per milliliter). (B) Serum HCV RNA and HBV DNA titers in mice before and after 2-week IFN- α treatment. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Table 1
Hepatitis B virus (HBV) markers in supernatants of stable HBV-transfected cell lines.

Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies per milliliter)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen.

evoking the IFN production system in liver cells. Further study using double-infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in mice infected with HCV only and in those coinfecting with HBV and HCV (Figs. 1 and 3). This finding indicated that HBV does not disturb the effect of IFN through signal transduction from the IFN receptor through the Jak-STAT pathway. It was, however, considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same liver cell could be infected with both HBV and HCV [20,26], but it was difficult in the present study to confirm that these two viruses replicate in the same liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathologic sections of the mouse liver. To address this issue, we transfected HCV to stable HBV-producing cell lines

(Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stable HBV-producing cells. Presence of the both hepatitis viruses in the same hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments that only HBV-transfected cell lines produced HBV and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are consistent with a recent report [20] that showed that HCV could infect cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV-transfected HBV-producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain machinery to disrupt the innate immune system; however, once exposed a large concentration of IFN, the virus shows high sensitivity, as shown in the replicon system [16,27]. Thus, HCV seems to have a relatively weak ability to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [28]. The fact that HBV does not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts the actions of IFN at IFN-induced antiviral product levels.

Although the culture environment is different from the replicon system, the JFH1 strain seems relatively resistant to IFN [29]. This suggests that the core and envelope proteins, which are absent in the replicon system, might play a role in IFN resistance; however, we could not show any effect for HCV infection on the actions of IFN on HBV replication. This finding sug-

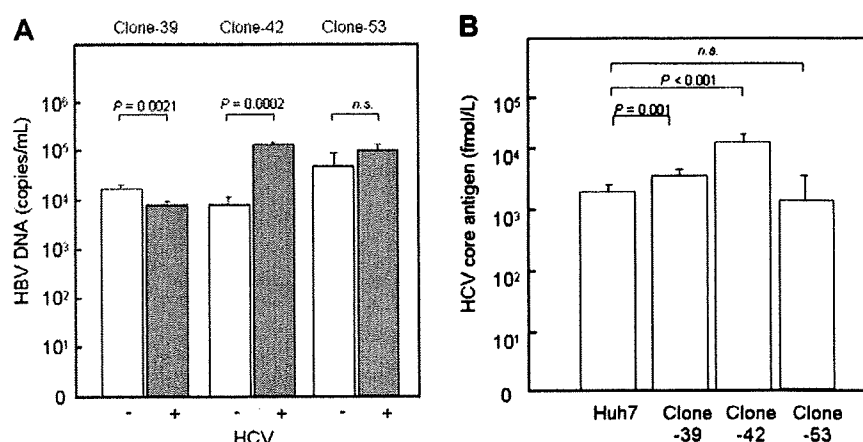


Fig. 4. Virus titers in supernatants of hepatitis B virus (HBV)-transfected or hepatitis C virus (HCV)-transfected cell lines. Huh7 cells were initially stably transfected with 1.4 genome-length HBV DNA. Three cell lines (Clone-39, -42, and -53) producing HBV DNA into the supernatant were selected. (A) HBV DNA levels in supernatants of HBV-producing cell lines 72 hours after transfection with JFH1 RNA (HCV positive) or control plasmid (HCV negative). (B) HCV core antigen levels in the supernatant of parental Huh7 cells and HBV-producing cell lines 72 h after transfection with JFH1 RNA. Data are mean plus or minus standard deviation ($n = 3$).

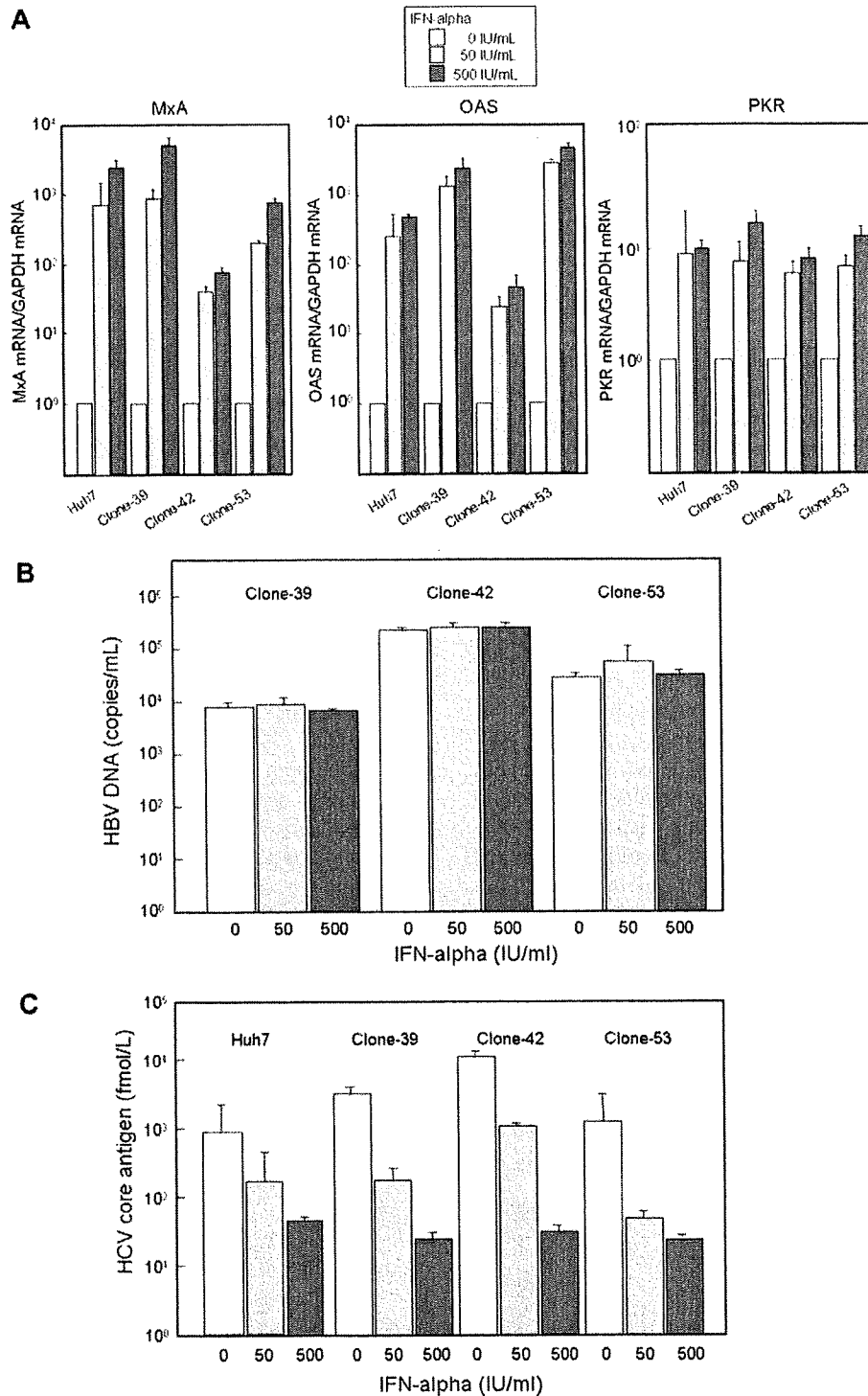


Fig. 5. Effects of interferon (IFN) treatment on hepatitis B virus (HBV) and hepatitis C virus (HCV) *in vitro*. Parental Huh7 cells and three HBV-transfected Huh7 cell lines (Clone-39, -42, and -53) were transfected with JFH1 RNA. Immediately after JFH1 transfection, the cell lines were treated with IFN- α (0, 50, and 500 IU/mL) for 72 h. (A) Intracellular gene expression levels of mixovirus resistance protein A (MxA), 2',5'-oligoadenylate synthetase (OAS), and RNA-dependent protein kinase (PKR) were measured. RNA levels were expressed relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) messenger RNA. (B) HBV DNA and (C) HCV core antigen in supernatants were measured. Data are mean plus or minus standard deviation ($n = 3$).

gests that the core and envelope proteins have only a weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also the case in the cell culture system, as we showed in this study and has been reported in previous studies [20,28]. The mechanism by which hepatitis viruses resist IFN needs to be clarified in order to develop new and effective therapies for eradication of these viruses.

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Hepatocytes From Fibrotic Liver Possess High Growth Potential in Vivo

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Hepatocyte transplantation is effective for treating liver failure, but healthy donors as a source of hepatocytes are quite limited. The livers of patients with hepatic fibrosis could be an alternative source; however, few reports have examined the nature of hepatocytes from fibrotic livers (f-hepatocytes). In this study, we compared the growth of f-hepatocytes and hepatocytes from normal livers (n-hepatocytes). Hepatocytes were isolated from normal and CCl₄-treated wild-type Fischer rats that express dipeptidyl dipeptidase IV (DPP-IV) gene (DPP-IV⁺). The n- and f-hepatocytes proliferated in culture at similar rates. Both types of hepatocytes were transplanted into DPP-IV⁻ mutant Fischer rats that had been treated with retrorsine to injure the liver and were partially hepatectomized (PHx) before transplantation. Both n- and f-DPP-IV⁺-hepatocytes proliferated and formed colonies. The colony sizes of f-hepatocytes 21 days posttransplantation were approximately three times those of n-hepatocytes. The hepatocytes were analyzed using a fluorescence activated cell sorter (FACS). The FACS profile differed between f- and n-hepatocytes: f-hepatocytes were less granular, less autofluorescent, and smaller than n-hepatocytes. These characteristics of f-hepatocytes resembled those reported for small-sized n-hepatocytes (SHs), which are highly proliferative and preferentially express a unique set of 10 SH genes. However, f-hepatocytes preferentially expressed only five of the SH genes. The expression profile of f-hepatocytes was rather similar to that of proliferating n-hepatocytes in the regenerating liver after PHx. The f-hepatocytes were morphologically normal and did not show any preneoplastic phenotype. These normal and proliferative natures of f-hepatocytes in vivo suggest the fibrotic liver as a source of hepatocytes for transplantation.

Key words: Hepatocyte transplantation; Liver regeneration; Hepatocyte proliferation; Hepatectomy; Retrorsine; Carbon tetrachloride

INTRODUCTION

The liver cell transplantation therapy for a damaged liver was first reported in 1993, in which hepatocytes (1–60 × 10⁷ cells) were autotransplanted into the spleen of cirrhosis or chronic hepatitis patients (11). The donor cells lived for up to 11 months. Strom et al. transplanted less than 1.2 × 10⁹ allogeneic hepatocytes into the spleen as a bridge to the ensuing liver transplantation (14). The patients who received hepatocyte transplantation before

liver transplantation had an average survival of 3.8 ± 3.3 days versus 2.8 ± 2.5 days for those without hepatocyte transplantation. Although the survival period did not differ statistically, hepatocyte transplantation improved serum ammonia, cerebral blood flow, and intracranial pressure. Two of 30 patients were able to recover with hepatocyte transplantation alone without usual liver transplantation (14).

Patients with metabolic diseases have been also subjected to hepatocyte transplantation in some cases. For

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example, an ornithine transcarbamoylase (OTC)-deficient 5-year-old patient was transplanted with 1.7×10^9 normal hepatocytes. The patient improved symptomatically but died from pneumonia 43 days posttransplantation (15). When a 10-year-old patient with Crigler-Najjar syndrome was transplanted with 7.5×10^9 hepatocytes, an amount approximately equal to 5% of the entire liver, the patient was able to decrease the serum bilirubin to a half the level before liver transplantation (4). Recently, a patient with glycogen storage disease type Ib caused by a deficiency of glucose-6-phosphate transporter was treated with hepatocyte transplantation, which resulted in normalization of glucose-6-phosphatase activity in the liver, and substantial improvement in quality of life (9).

These previous studies show that the transplantation of hepatocytes equivalent to 1–5% of the total hepatocytes in the liver is therapeutically effective. Cell transplantation is easier, safer, and less expensive than liver transplantation, and can be performed repeatedly. The major challenge to hepatocyte transplantation is the shortage of transplantable cells owing to the limited availability of healthy donors (16). Hepatocytes from fibrotic livers (f-hepatocytes) are a potential source of hepatocytes for transplantation because of their greater availability over hepatocytes from normal livers (n-hepatocytes). Mito et al. isolated n- and f-hepatocytes from rats and transplanted each of them into the syngenic rat spleen (10). Although the yield and viability of the f-hepatocytes were lower than the n-hepatocytes, and their features were different from the normal counterpart in some aspects, they were able to continue to proliferate for 6 months after transplantation. However, the proliferative ability of f-hepatocytes has not been characterized in the liver, which is of prime importance when they are utilized as the cells for transplantation.

This study aimed to characterize the growth of f-hepatocytes using rats as a model animal. The adult rat liver contains a minor population of hepatocytes called small hepatocytes (SHs) (6,17,19). These cells are smaller and have greater replicative potential than typical parenchymal hepatocytes (PHs). SHs have been characterized using an "SH fraction" that contaminated PHs. Previously, we isolated a PH-free SH fraction and SH-free PH fraction from the adult rat liver using a fluorescence-activated cell sorter (FACS) combined with centrifugal elutriation and characterized the hepatocytes in the fraction. These hepatocytes were designated as pure SHs or R3Hs and pure large hepatocytes (LH) or R2Hs in our previous study, respectively (2). For the sake of simplicity, we call pure SHs "SHs" and pure LHs "LHs" in this study and assume that "hepatocytes" consist of LHs and SHs. SHs were mononuclear and of lower ploidy. Previously, we identified 10 genes that are preferentially

expressed in SHs: *p55cdc*, *hydroxysteroid sulfotransferase* (*Sta*), *cytochrome P450 17* (*CYP17*), *prostaglandin E2 receptor EP3 subtype* (*Pge2r*), *pancreatic secretory trypsin inhibitor* (*Psti*), *Cdc2*, *connexin 26* (*Cx26*), *mitotic centromere-associated kinesin* (*Mcak*), *rat EST 207254*, and an unknown gene (*ab088476*) (2). These genes are referred to as the SH-associated genes.

We isolated n- and f-hepatocytes from rats. The f-hepatocytes were characterized in terms of size, FACS profile, in vitro and in vivo proliferation ability, and gene expression, and were compared with n-hepatocytes and hepatocytes in the regenerating liver (r-hepatocytes). As a result, we clearly demonstrated that f-hepatocytes are different from LHs, are similar to SHs in proliferation ability, and are similar to r-hepatocytes in their gene expression profile.

MATERIALS AND METHODS

Treatment of Animals

Dipeptidyl dipeptidase IV-deficient (DPPIV⁻) mutant male Fischer rats aged 9–10 weeks were purchased from Charles River Japan, Inc. (Yokohama, Japan) and their wild-type (DPPIV⁺) counterparts from Japan SLC, Inc. (Shizuoka, Japan). Liver fibrosis was induced by intramuscular injection of DPPIV⁺ rats with CCl₄ at 1 ml/kg body weight, twice a week, for 6 weeks. Hepatocytes were isolated from the rats 6 weeks postinjection. DPPIV⁺ male Fischer rats aged 15–16 weeks were subjected to two thirds partial hepatectomy (PHx). Then, r-hepatocytes were isolated from the rats 36 h after PHx.

Hepatocyte Isolation

DPPIV⁺ male Fischer rats aged 15–16 weeks were divided into three groups, one used for normal controls, second for CCl₄ treatment, and the last for PHx experiment. The n-, f-, and r-hepatocytes were isolated from these rats using the two-step collagenase perfusion method (6), and were collected by centrifugation at $50 \times g$ for 2 min. The pellets were centrifuged through 45% Percoll at $50 \times g$ for 24 min (6). These pellets were centrifuged again at $50 \times g$ for 2 min, and the final pellets were used as hepatocytes, which consisted of LHs and SHs. In our previous study, PHs and SHs were obtained as the pellet and supernatant, respectively, after centrifugation of the original hepatocyte preparations at $50 \times g$ for 1 min (17). The hepatocyte preparation used in the present study corresponds to this previous preparation consisting of PHs and SHs. The viability of the hepatocytes was determined by Trypan blue exclusion. The n-, f-, and r-hepatocytes were subjected to FACS analysis, and to determining the cell diameter (17) and the gene expression levels. In addition, the proliferation ability of n- and f-hepatocytes was assayed in vitro and in vivo.

FACS Analysis

Hepatocytes were analyzed using a cell sorter (FACS Vantage; Becton Dickinson, Mountain View, CA) with a 100- μ m nozzle, as reported previously (17). Fluorescence excited at 488 nm was measured through 530-nm (FL1) and 575-nm (FL2) filters with 4-decade logarithmic amplification. To measure the physical characteristics of the cells, linear amplification was used for the forward scatter, which is a measure of cell size, and 4-decade logarithmic amplification was used for the side scatter (SCC), which is a measure of cytoplasmic complexity. The optical bench was calibrated at a fixed amplitude and photomultiplier voltage using fluorescent polystyrene beads (Fluorospheres Calibration Grade 6- μ m YG microspheres; Polysciences, Warrington, PA), and the instrument was used in the conditions under which these beads fell in the same peak channels. Propidium iodide was added to the cell suspensions to be analyzed, at a concentration of 1 μ g/ml. The cells that excluded the dye were analyzed as viable cells. Data obtained from the FACS experiments were analyzed using Cell Quest software (Becton Dickinson).

Determination of the Growth Potential of Hepatocytes In Vitro

The n- and f-hepatocytes were cocultured with mitomycin C-treated Swiss 3T3 cells on Celldesks in HCGM culture medium (i.e., Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, 30 μ g/ml L-proline, 0.5 μ g/ml insulin, 10^{-7} M dexamethasone, 44 mM NaHCO₃, 10 mM nicotinamide, 10 ng/ml EGF, 0.2 mM L-ascorbic acid 2-phosphate, 100 IU/ml penicillin G, and 100 μ g/ml streptomycin) (1,13, 17–19). They were incubated in a 5% CO₂/95% air atmosphere at 37°C. Swiss 3T3 cells were seeded on the hepatocytes 24 h after the hepatocytes were plated. The growth potential of the hepatocytes was determined at 10 days, as previously shown (17).

Hepatocyte Transplantation

DPPIV⁻ rats weighing 130–140 g were given two intraperitoneal injections of retrorsine at 30 mg/kg body weight, 2 weeks apart. Four weeks after the last injection, a two thirds PHx was performed (6,8). The n- and f-hepatocytes (1.5×10^5) isolated from the DPPIV⁻ rats were transplanted into the retrorsine-treated partial hepatectomized (retrorsine/PHx) DPPIV⁻ rats via the portal vein. Some rats were killed 48 h after transplantation to measure the engraftment rate of the transplanted hepatocytes, the ratio of transplanted cells to cells integrated to the liver plate. Other rats were killed 21 days after transplantation. Tissues from each lobe of the liver were

frozen until use for enzyme-histochemical or immunohistochemical analyses.

Enzyme-Histochemical and Immunohistochemical Staining

DPPIV enzyme histochemistry was performed on 10- μ m-thick cryosections that had been prepared from the liver, fixed in ice-cold acetone for 5 min, air-dried, and washed for 5 min in ice-cold 95% ethanol. The sections were air-dried and incubated for 40–60 min in a substrate reagent consisting of 0.5 mg/ml Gly-Pro-methoxy- β -naphthylamide (Sigma Chemical, St. Louis, MO), 1 mg/ml Fast Blue BB (Sigma Chemical), 100 mmol/L Tris-maleate (pH 6.5), and 100 mmol/L NaCl. Then, the sections were washed with PBS and fixed in 10% formaldehyde. All tissue sections were counterstained with hematoxylin.

Cryosections were fixed in -20°C acetone for 5 min and subjected to immunohistochemistry to detect liver-related proteins. The primary antibodies (Abs) used were as follows: anti-rat DPPIV mouse monoclonal antibody (gift from Dr. D. C. Hixson, Rhode Island Hospital), anti-rat albumin rabbit antiserum (a hepatocyte marker; Cappel, Durham, NC), anti-human α -smooth muscle actin (SMA) mouse monoclonal antibody (an activated stellate cell marker; MBL, Nagoya, Japan), anti-rat glutathione-S-transferase (a preneoplastic hepatocyte marker, GST-P) rabbit antiserum (MBL), OV6 mouse monoclonal antibody (an oval cell marker; gift from Dr. D.C. Hixson) (5), and anti-human cytokeratin 19 monoclonal antibody (CK19; a bile duct cell and oval cell marker; Amersham). The primary Abs were visualized with Alexa 488-labeled goat anti-mouse-IgG or Alexa 594-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

Morphometric Analysis

Livers were isolated from rats 48 h after transplantation, weighed, and processed for cryosectioning and DPPIV staining. The approximate numbers of hepatocytes per gram of liver were calculated from the liver weight using the value $115 \times 10^6 \pm 15 \times 10^6$ hepatocytes/g liver (12). DPPIV⁺ and DPPIV⁻ hepatocytes were counted in arbitrary areas visualized on tissue sections, and the numerical ratio of the former to the latter was calculated as the occupancy rate of engrafted hepatocytes in the liver. The number of engrafted hepatocytes per liver was calculated by multiplying the occupancy rate of the engrafted hepatocytes by the total number of host hepatocytes per liver. The engraftment index (the numerical ratio of engrafted hepatocytes to injected hepatocytes on transplantation) was calculated by dividing the number of engrafted hepatocytes per liver by the number of injected hepatocytes.

A transplanted hepatocyte homes to the liver, starts to replicate there, and forms a colony soon after transplantation. The area of colonies formed by the transplanted hepatocytes was measured using NIH image ver. 1.62, and the data were analyzed using StatView ver. 5.0 (SAS Institute, Cary, NC). The colony size increases as engrafted hepatocytes proliferate. The volume of a colony was estimated using a reported method (6). Briefly, we made 10- μm -thick semiserial sections from liver specimens, at 100- μm intervals. Different parts of a DPPIV⁺ colony were seen in several different serial sections when the diameter of the colony exceeded 100 μm , which is a colony area corresponding to >7,850 μm^2 . We selected the section in which the longest diameter of a particular DPPIV⁺ colony was seen and calculated the colony area using this diameter. For a colony with a diameter <100 μm , we assumed that the diameter measured in a section was the longest diameter and calculated the colony area using this diameter. We only measured the colonies consisting of more than eight DPPIV⁺ transplanted hepatocytes. The colonies were oval or round. To quantify the area of these colonies, 40 portal areas were examined in each animal at 21 days posttransplantation. The volume of a colony was calculated from the area of the colony, assuming that the colony was spherical and that the cross section was made along the maximum diameter of the sphere. To calculate the mean diameter of cells in colonies formed by the transplanted hepatocytes, the area and cell number therein were measured for 10 colonies. The mean cell number in a colony was calculated using the area or volume of the colony and the mean cell diameter of hepatocytes.

Quantification of mRNA

Total RNA was purified from n-, f-, and r-hepatocytes using an RNeasy minikit (QIAGEN K.K., Tokyo, Japan) and was treated with an RNase-free DNase set (QIAGEN). cDNA was synthesized using PowerScript reverse transcriptase (Clontech, Palo Alto, CA) and was amplified with a set of gene-specific primers (2) and SYBR Green PCR mix in a PRISM 7700 Sequence Detector (Applied Biosystems, Tokyo, Japan). A series of diluted plasmid cDNAs containing each gene was used to plot the standard amplification curves. The mRNA copy numbers in cDNA samples were calculated using the standard amplification curves (2).

Statistical Analysis

The area of colonies formed by the transplanted hepatocytes was measured using NIH image ver. 1.62, and the data were analyzed using StatView ver. 5.0 (SAS Institute, Cary, NC). The significance of differences was

analyzed using the Mann-Whitney rank sum test. A value of $p < 0.05$ was considered significant.

RESULTS

Size and FACS Characterization of Hepatocytes

We prepared n- and f-hepatocytes from normal and CCl₄-treated rats, respectively. The yield of the n- and f-hepatocytes was $35.0 \pm 19.0 \times 10^6$ and $2.6 \pm 1.5 \times 10^6$ cells per animal ($n = 3$), respectively, and their viability was $95.5 \pm 0.6\%$ and $69.6 \pm 2.2\%$ ($n = 3$), respectively. Similarly, r-hepatocytes were isolated from rats 36 h after PHx. These cells were placed on nonadhesive dishes to determine their diameters as a measure of cell size. The n-, f-, and r-hepatocytes had diameters of 22.1 ± 0.2 , 20.1 ± 0.2 , and 21.5 ± 1.0 μm , respectively. Previously, we had found diameters of 23.2 ± 0.5 and 17.4 ± 0.0 μm for LHs and SHs, respectively (2).

The shape of contour lines in FACS analysis were variable from experiment to experiment, depending on the axis adjustment of the cell souter that is usually made and fixed in each series of experiment. We always compared the level of FL1 (autofluorescence) and SSC (granularity) of experimental cells (f- and r-hepatocytes in this study) with control cells (n-hepatocytes). It was confirmed that the similar differences of f-hepatocytes over n-hepatocytes and that of r-hepatocytes over n-hepatocytes were reproducibly observed in three independent analyses. The FACS analysis showed that f-hepatocytes were totally different from n-hepatocytes (Fig. 1A). Most of the f-hepatocytes were less granular and less autofluorescent. The FACS profile of r-hepatocytes was similar to that of n-hepatocytes, although the former contained more populations with low granularity and low autofluorescence than the latter (Fig. 1B).

Growth Ability of n- and f-Hepatocytes

The n- and f-hepatocytes were isolated from three normal rats and four CCl₄-treated rats, respectively, and then cultured with Swiss 3T3 cells in HCGM for 10 days. The ratios of the cell number at day 10 to that at day 1 were 3.5 ± 1.0 and 3.3 ± 0.1 for f- and n-hepatocytes ($n = 3$), respectively, clearly indicating similar growth ability between the two types of hepatocytes in vitro.

The growth ability in vivo was compared between n- and f-hepatocytes. DPPIV⁺ n- and f-hepatocytes were transplanted into DPPIV⁻ retrorsine/PHx rats via the portal vein. The livers were harvested 48 h after transplantation. The DPPIV⁺ n- and f-hepatocytes were counted on the host liver sections. The engraftment index of f-hepatocytes obtained by two transplantation experiments was 12.3% and 7.4% (average 9.9%), which was similar to that of n-hepatocytes, 13.0% and 5.6% (9.3%). A previous study revealed that oval cells were not seen

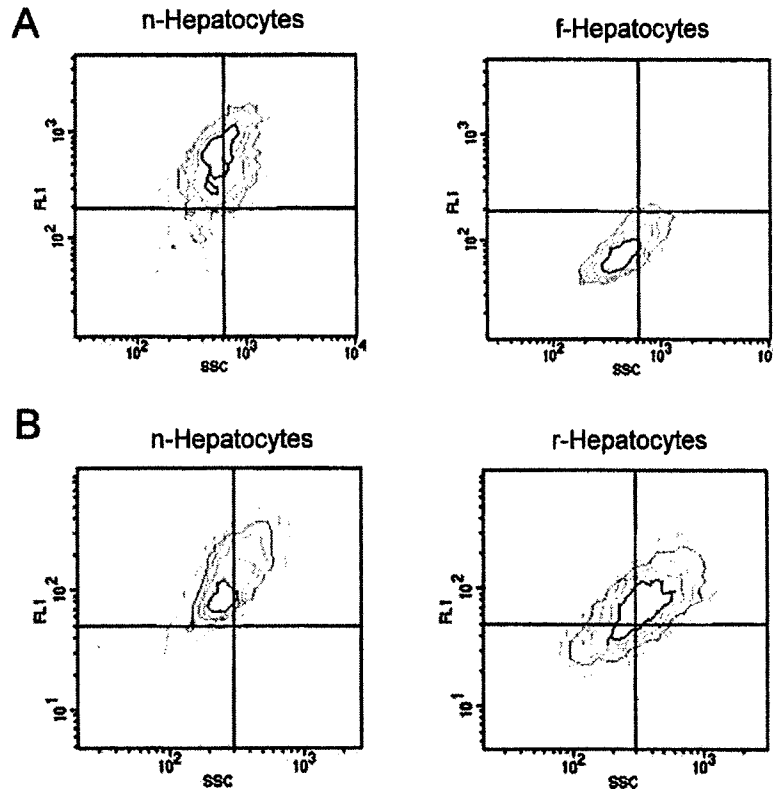


Figure 1. FACS profiles of hepatocytes. Two series (A, B) of experiments were performed in which hepatocytes were analyzed by FACS in terms of the side scatter (SSC) and fluorescence through 530-nm filter (FL1). (A) One series compared the FACS profiles of n- and f-hepatocytes, and (B) the other compared the profiles of n- and r-hepatocytes.

in the livers of CCl_4 -treated rats (3). In the present study, serial liver sections were immunostained for DPPIV and an oval cell marker, OV6. There were no DPPIV and OV6 double-positive cells (data not shown), showing the absence of oval cells in the hepatocyte preparations used in this study.

Retrorsine/PHx DPPIV rats were transplanted with n- and f-hepatocytes that had been isolated from four DPPIV⁺ rats each as described above, killed 21 days after transplantation, and their livers were processed for DPPIV⁺ enzyme histochemistry (Fig. 2). The f-hepatocyte colonies were more heterogeneous in size and appeared larger than the n-hepatocyte colonies, suggesting higher growth potential of the former than the latter. To quantitatively evaluate the growth ability, we measured two parameters on histological sections, the colony area and volume. Area size distribution of the hepatocyte colonies was determined and is shown in Figure 3, which confirmed the above suggestions of higher size heterogeneity and larger colony area of f-hepatocytes than those of n-hepatocytes. The mean colony area was calculated from these graphs and is shown in Table 1. f-

Hepatocytes formed colonies whose areas were approximately 2.4-fold larger (significant at $p < 0.05$) than those of n-hepatocytes. The diameter of the hypothetical sphere formed by the transplanted hepatocytes was measured and also is shown in Table 1. As expected, the colony volume of f-hepatocytes was approximately 3.7-fold larger (significant at $p < 0.05$) than that of n-hepatocytes. From the mean area and volume of colonies formed by the transplanted hepatocytes, we estimated the replication potential of a transplanted cell. To simplify the calculation, we assumed that transplanted hepatocytes were spherical, and we calculated the number of transplanted cells per area and volume of a colony as described in the Materials and Methods. As shown in Table 2, the number of transplanted cells per area and volume of a colony was about 1.9- and 2.6-fold larger than that of n-hepatocytes, respectively. The average numbers of cell divisions for transplanted f- and n-hepatocytes over 21 days were calculated to be 11–13 and 9–11, respectively (Table 2). From these measurements, we concluded that f-hepatocytes have higher growth potential than n-hepatocytes in vivo.

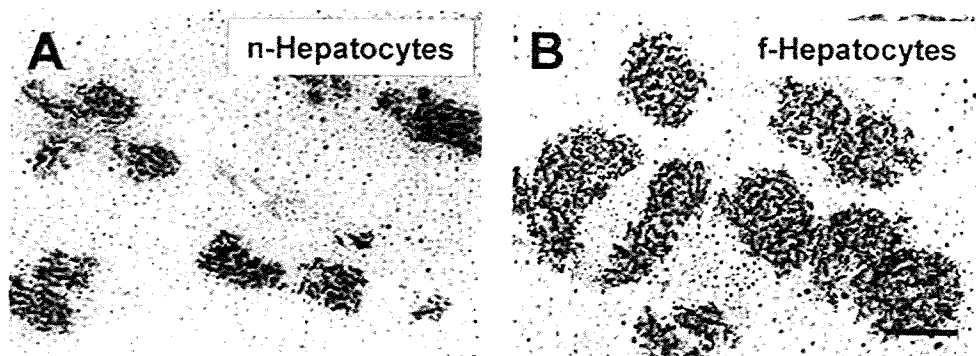


Figure 2. Colonies formed by transplanted hepatocytes. (A) DPPIV⁺ n-hepatocytes and (B) DPPIV⁺ f-hepatocytes were transplanted into the livers of retrorsine/PH DPPIV⁻ rats. Liver sections were prepared from the rats 21 days after transplantation. Cryosections of the livers were subjected to DPPIV histochemical staining. The transplanted hepatocytes were stained black. Scale bar: 200 μ m.

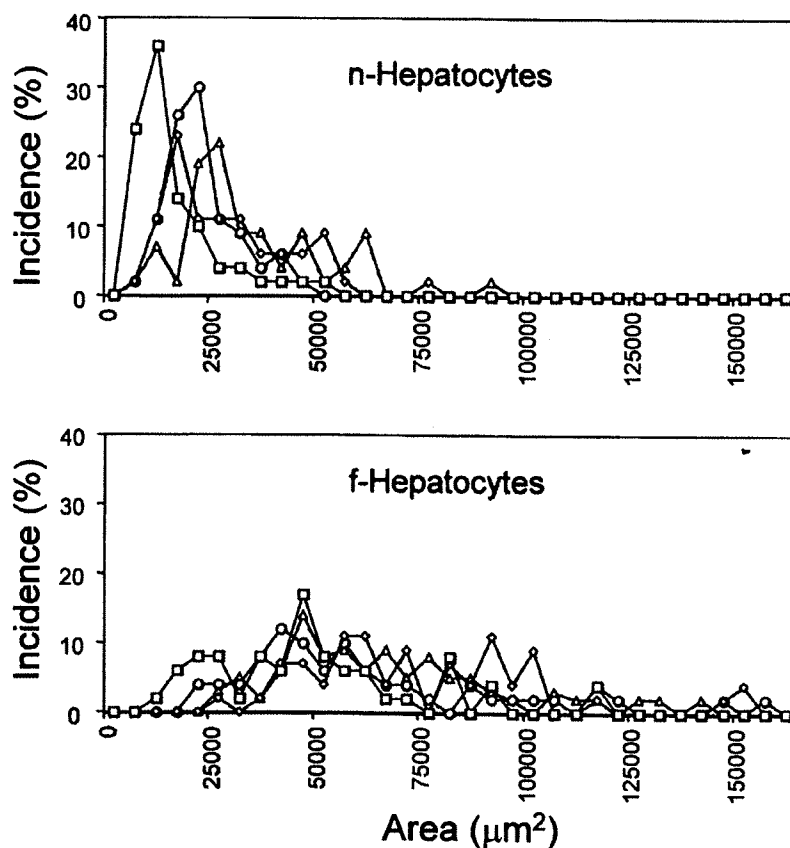


Figure 3. Size distribution of the hepatocyte colonies. Forty regions were selected randomly from DPPIV-stained sections for four individuals as shown in Figure 2 and were photographed. The colony area was measured on the photographs using NIH ver. 1.62. Each line represents the proportion (%) of the area measured for each of the four animals. f-Hepatocytes occupied more area than n-hepatocytes.

Table 1. The Mean Area and Volume of Colonies Formed by Transplanted Hepatocytes

Transplanted Cells	Area (μm^2)	Volume (μm^3)	Number
n-Hepatocytes	$26,387 \pm 1,015$]*	$3,580,888 \pm 212,285$]*	211
f-Hepatocytes	$64,450 \pm 2,016$]*	$13,207,708 \pm 628,909$]*	206

Hepatocytes were transplanted into the livers of rats, which were killed 21 days after transplantation. The areas of colonies were measured for four different rats transplanted with hepatocytes under the identical experimental conditions, as shown in Figure 2. The mean $\pm$ SE of areas and volumes of colonies was calculated from these measurements.

* $p < 0.05$. The p -values were determined by Mann-Whitney rank sum test.

Cell Phenotype in Colonies Formed by Transplanted Hepatocytes

The cells in the colonies of n- and f-hepatocytes were characterized in terms of the expression of four lineage-specific markers of liver cells: albumin, α -SMA, CK19, and GST-P. The cells in colonies of both types of hepatocytes expressed albumin at a high level, comparable to that of the surrounding host hepatocytes (Fig. 4A–D). Neither CK19 nor GST-P was expressed in the cells of colonies formed by both types of hepatocytes (data not shown), supporting the notion that f-hepatocytes do not show preneoplastic or bile duct epithelial cell phenotypes. These results strongly suggest that f-hepatocytes retain the phenotype of normal hepatocytes and maintain the normal phenotype throughout replication. In addition, α -SMA⁺ cells (activated stellate cells) were not observed in the f-hepatocyte-transplanted livers (Fig. 4G, H) as in the n-hepatocyte-transplanted livers (Fig. 4E, F).

Gene Expression in f-Hepatocytes

Previously, we had identified 10 SH-associated genes: *p55cdc*, *Sta*, *CYP17*, *Pge2r*, *Psti*, *Cdc2*, *Cx26*, *Mcak*, *rat EST 207254*, and an unknown gene (*ab088476*) (2). The mRNA expression levels of all of these genes except *rat EST 207254* were determined using real-time RT-PCR in n-, f-, and r-hepatocytes (Fig. 5B, Table 3) and were compared with our previous data, which are shown in Figure 5A. The expression levels of the SH-associated genes were much higher in SHs than in LHs (Fig. 5A). The expression levels of four genes

(*ab088476*, *Pge2r*, *Cx26*, and *Psti*) were similar between n- and f-hepatocytes. By contrast, five genes (*CYP17*, *p55cdc*, *Cdc2*, *Mcak*, and *Sta*) were expressed at much higher levels in f-hepatocytes than in n-hepatocytes. It was noteworthy that the overall expression profile of n-hepatocytes was similar to that of r-hepatocytes (Fig. 5B).

DISCUSSION

Fibrotic livers have been characterized mainly with respect to activated stellate cells, which are known as extracellular matrix (ECM)-producing cells, and little is known about the nature of hepatocytes in the fibrotic liver. Mito et al. reported that hepatocytes from fibrotic liver exhibited greater proliferation than hepatocytes from normal liver when transplanted into the spleen, despite the injection of approximately 1/200 of the amount of normal cells (10). Histological observations revealed that transplanted hepatocytes from cirrhotic livers differentiated into plates two to several cells thick, whereas plates of one-cell thickness prevailed with the transplantation of normal hepatocytes. One interpretation of this observation is that cirrhotic hepatocytes are still in the regenerating phase. Hepatocytes from cirrhotic livers retained normal functions such as glycogenesis and albumin synthesis (10). On electron micrographs, collagen fibers in the space of Disse were observed 1 year after the transplantation of hepatocytes from cirrhotic livers, but were not present with normal hepatocyte transplantation (10). In the present study, we characterized hepatocytes isolated from the fibrotic liver for the first time in

Table 2. Mean Cell Number in a Colony

Transplanted Cells	Cell Diameter (μm)	Cell Number per Colony Area	Cell Number per Colony Volume	Cell Divisions During 21 Days
n-Hepatocytes	22.8 ± 0.5	110 ± 12	$1,217 \pm 206$	9–11
f-Hepatocytes	20.3 ± 0.3	209 ± 21	$3,131 \pm 481$	11–13

Data are expressed as the mean $\pm$ SE.

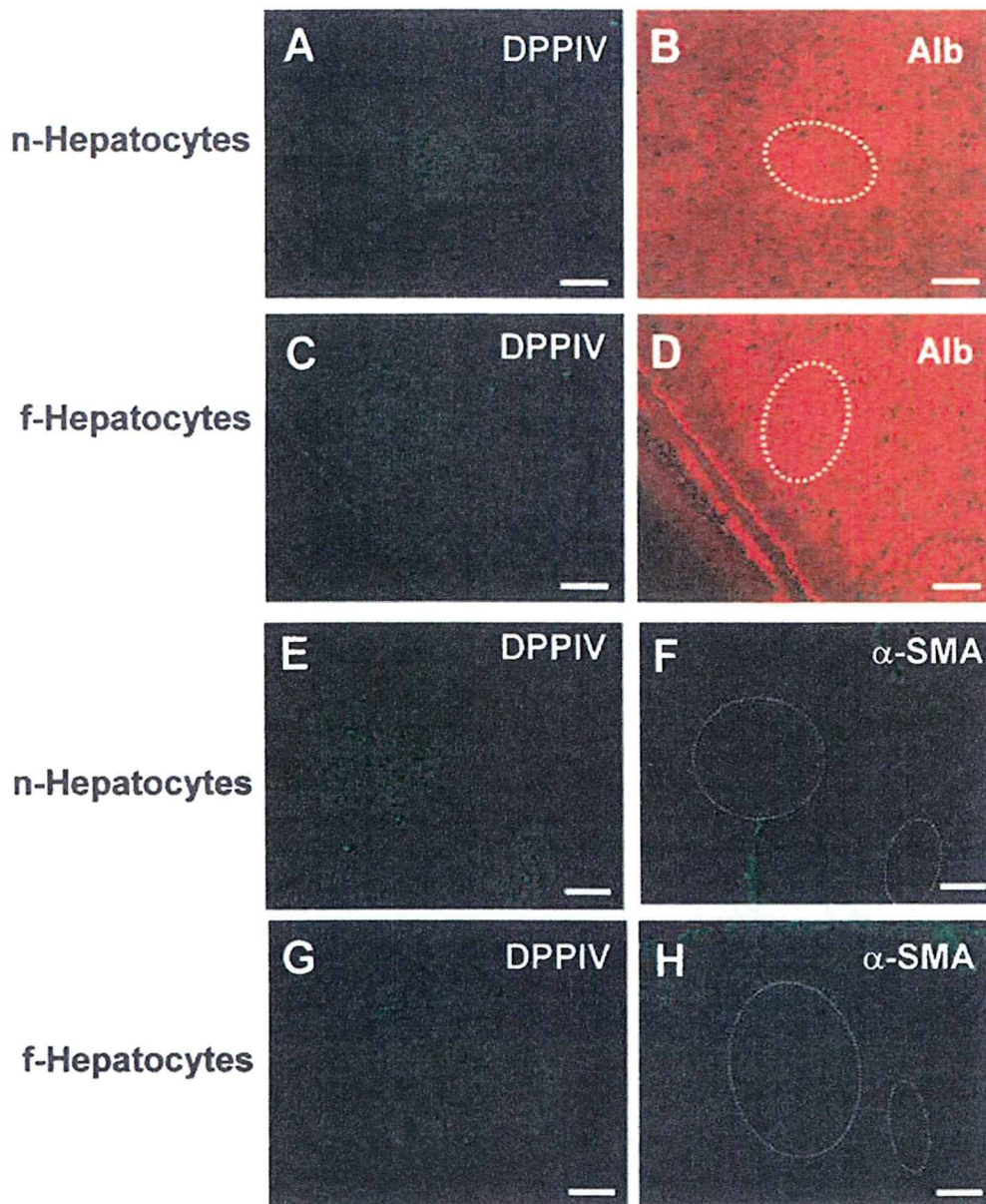


Figure 4. Phenotypes of the cells in the colonies formed by transplanted hepatocytes. DPPIV⁺ n-hepatocytes (A, B, E, and F) and DPPIV⁺ f-hepatocytes (C, D, G, and H) were transplanted into retrorsine/PH-treated DPPIV⁻ rats. Serial liver cryosections were prepared 21 days after transplantation and were stained for DPPIV (A, C, E, and G), albumin (B and D), and α -SMA (F and H). The colonies formed by the transplanted hepatocytes are localized as DPPIV⁺ regions (A, C, E, and G). These colonies are each marked by broken lines on the corresponding immunostained serial sections (B, D, F, and H). The DPPIV⁺ cells were all albumin⁺. There were no α -SMA⁺ cells in the livers transplanted with both n- and f-hepatocytes. Scale bar: 100 μ m.

terms of their growth potential in vitro and in vivo (in the retrorsine/PH rat liver), FACS profile, cell size, growth potential, and gene expression.

The growth potential of hepatocytes is heterogeneous: the hepatocyte with the greatest proliferative ability forms a colony consisting of more than 100 cells

within 10 days, whereas the hepatocyte with the lowest ability does not divide (19). The adult rat liver contains a minor population of hepatocytes called SHs, which have a smaller size and higher replicative potential than LHs (6,17,19). Previously, we had used FACS and centrifugal elutriation to isolate highly proliferative SHs as

cells with low autofluorescence and low granularity (2). In the present study, most of the f-hepatocytes had low autofluorescence and low granularity, similar to SHs. Therefore, we investigated whether f-hepatocytes have

characteristics similar to SHs in terms of size, growth potential in vitro and in vivo, and gene expression.

The average diameter of f-hepatocytes ($20.1 \pm 0.2 \mu\text{m}$) was comparable to that of n-hepatocytes (22.1 ± 0.2

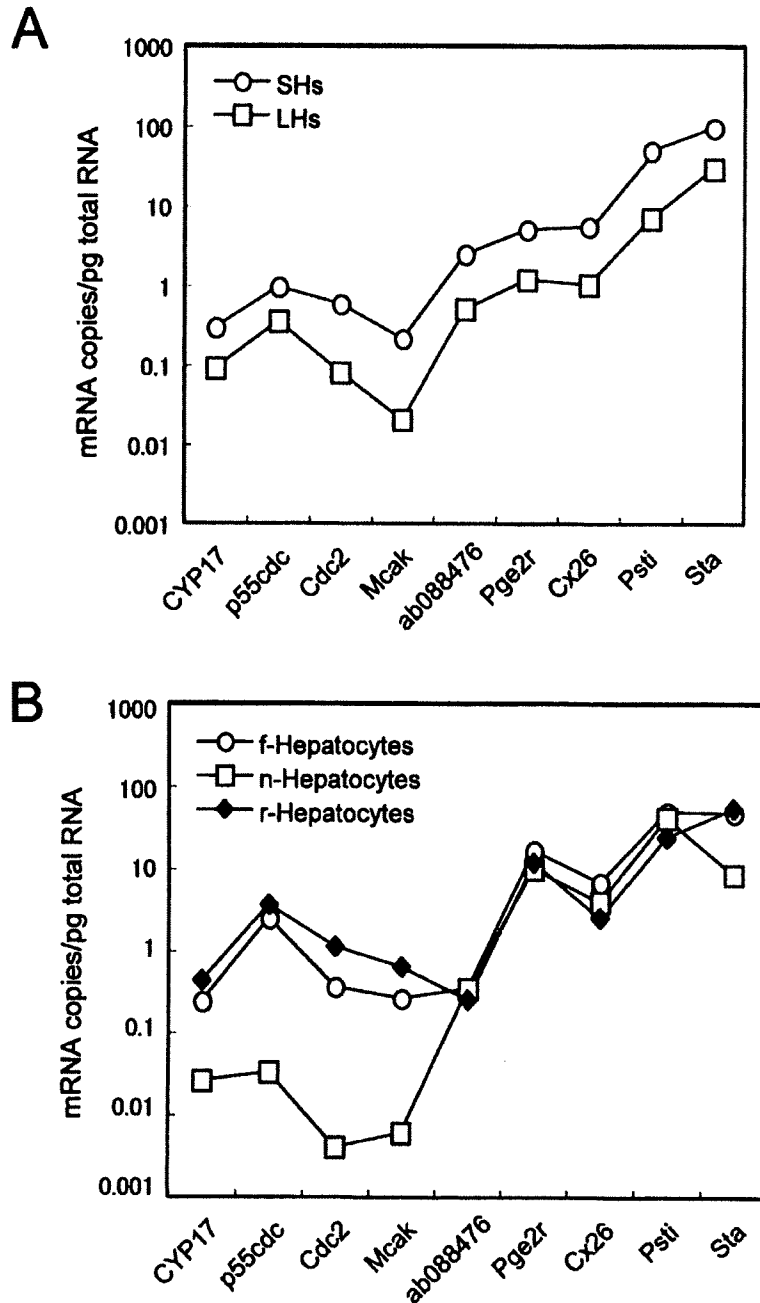


Figure 5. Expression of the SH-associated genes in hepatocytes. (A) Gene expression levels in SHs and LHs. This graph is drawn using the mRNA expression data presented in Tables 4 and 5 of Asahina et al. (2). (B) The gene expression levels in n-, f-, and r-hepatocytes were determined by measuring the mRNA copy number using real-time RT-PCR.

Table 3. Expression Levels of SH-Associated Genes in n-, r-, and f-Hepatocytes

Gene*	mRNA Copies/pg Total RNA		
	n-Hepatocytes	f-Hepatocytes	r-Hepatocytes
<i>CYP17</i>	0.026 ± 0.000	0.239 ± 0.147	0.430 ± 0.216
<i>p55cdc</i>	0.032 ± 0.022	2.443 ± 3.138	3.527 ± 1.044
<i>Cdc2</i>	0.004 ± 0.004	0.360 ± 0.424	1.092 ± 0.397
<i>Mcak</i>	0.006 ± 0.008	0.259 ± 0.308	0.627 ± 0.197
Unknown (<i>ab088476</i>)	0.343 ± 0.566	0.339 ± 0.281	0.252 ± 0.032
<i>Pge2r</i>	9.770 ± 8.698	16.452 ± 11.455	11.557 ± 1.699
<i>Cx26</i>	3.870 ± 0.967	6.690 ± 2.426	2.481 ± 0.267
<i>Psti</i>	40.961 ± 9.499	48.189 ± 11.954	23.374 ± 9.560
<i>Sta</i>	8.429 ± 7.905	46.431 ± 36.094	52.897 ± 21.741

The primers used for real-time RT-PCR have been reported elsewhere (2). Each value represents the mean ± SD of three different rats.

*Genes identified as differentially expressed using a cDNA microarray and representational difference analysis in SHs (2).

μm) but was larger than that of SHs (17.4 ± 0.0 μm). The growth of f-hepatocytes in vitro was compared with that of n-hepatocytes. Previously, we had shown that SH proliferation was about four times that of LHs in vitro (17). The f-hepatocytes showed growth ability similar to that of n-hepatocytes. SHs were also highly proliferative in vivo (about three times compared with LHs), as determined by colony-forming ability after transplantation into the livers of the retrorsine/PH rats (6). Here, we also estimated the growth potential of hepatocytes using this model. The engraftment index obtained in this study at 48 h after transplantation was 9.9% for f-hepatocytes and 9.3% for n-hepatocytes, and did not differ significantly. The colonies formed by transplanted cells at 21 days were examined in all of the residual livers of the recipients and showed that f-hepatocytes were more proliferative (about 2.5 times) than n-hepatocytes.

From the data, we concluded that f-hepatocytes have higher growth ability than n-hepatocytes in vivo but not in vitro. At present, there is no explanation for this apparent discrepancy between the in vivo and in vitro growth abilities of the two types of hepatocytes. However, it seems that the culture conditions used in the present study were not sufficient for f-hepatocytes to exhibit their full proliferation potential.

Ten SH-associated genes had been identified previously (2). We examined whether f-hepatocytes also express the SH-associated genes, and we compared the expression of SH-associated genes between f- and r-hepatocytes. The f-hepatocytes expressed five of the nine SH genes, at similar levels as SHs. Importantly, the overall expression profile of f-hepatocytes resembled that of r-hepatocytes.

The expression of marker proteins specific to hepatocytes, preneoplastic hepatocytes, and bile duct epithelial

cells was characterized in f-hepatocytes. When rats were treated with CCl₄ for more than 7 weeks, morphologically recognizable GST-P⁺ preneoplastic nodules were observed in some of the treated rats (7). In the present study, the cells in colonies formed by transplanted f-hepatocytes were albumin⁺ but CK19⁻ and GST-P⁻, like n-hepatocytes, showing that the cells in the f-hepatocyte colonies expressed hepatocyte markers but not bile duct or preneoplastic markers. In addition, stellate cells did not express α-SMA in the f-hepatocyte colonies. There seems to be a regulatory mechanism in vivo under which f-hepatocytes stably express hepatocytic phenotypes but do not express biliary phenotypes.

In this study, we demonstrated that hepatocytes surrounded by ECM in the fibrotic liver retained high growth potential. f-Hepatocytes can be used instead of n-hepatocytes for hepatocyte transplantation, although the yield of hepatocytes from a fibrotic liver is fewer than that from a normal liver. However, it remains to be determined whether human f-hepatocytes have similar normal and proliferative phenotypes as rat f-hepatocytes, when isolated from the livers of fibrotic patients and transplanted into normal recipients. If they show these phenotypes, fibrotic livers could be a useful source of hepatocytes for the treatment of liver damage using hepatocyte transplantation.

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Expert Opinion

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In vivo modeling of human liver for pharmacological study using humanized mouse

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The liver occupies a central place in the treatment of the substances taken into the body. If we could devise an *in vivo* or *in vitro* model that perfectly mimics the naturally-created human (h) liver, the work required for making effective and safe medicines would become easier and could be undertaken more cost effectively than it is currently. Considering the advantages of *in vivo* modeling over *in vitro* modeling under the current technological state of life sciences research, we have created an experimentally workable *in vivo* h-liver model, a liver-humanized mouse, in which host hepatocytes are largely replaced with healthy normal h-hepatocytes. Xenogenic h-hepatocytes are capable of constructing a histologically normal liver by collaborating with mouse-nonparenchymal cells in an elaborately organized manner. Considering its potential use for drug development, we have extensively characterized the mouse regarding the infectivity toward h-hepatitis viruses, activities of h-enzymes in Phase I and II of drug metabolisms, and h-hepatocyte-related drug transporters. These studies indicate that the humanized mouse liver mimics h-phenotypes at a level appropriate for pharmacological studies, and, thus, can be used not only for developing new medicines, but also for examining biological and pathological mechanisms in the h-liver.

Keywords: human hepatocytes, humanized mouse, immunodeficient mouse, *in vivo* drug metabolism

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1. Introduction

The body takes in and treats natural and artificial substances from the surrounding environment; some of the processed materials are used for life activities, while others are excreted from the body. The liver plays a central role in the processing of internalized substances (metabolism). The nominal functions of the liver are performed by a group of cells called parenchymal cells, or hepatocytes. It is well known that there are interspecies differences in the hepatic metabolic patterns of a given xenobiotic. Thus, the analytical results obtained from experiments using hepatocytes from rodents, such as rats and mice, are not always relevant in predicting the responses of human (h)-hepatocytes, indicating that h-hepatocytes are required to examine the metabolism and toxicity of a given chemical when the purpose of the study is to understand the reactions of the h-liver. However, there are also difficulties in using h-hepatocytes as an analytical tool.

In creating an *in vivo* replica of the liver, a normal, healthy human would be the ideal model; however, humans cannot serve as experimental targets. Perhaps the simple and easiest way to use h-hepatocytes for such purposes is to isolate them from an appropriate source, cultivate them for propagation and utilize them in the desired experiments. However, in doing this there are several problems,

including the limited number of normal h-livers available, the reluctance of h-hepatocytes to replicate *in vitro* and the attenuation of a normal h-hepatocytic phenotype under *in vitro* culture conditions.

Importantly, such *in vitro* characterization of hepatocytes is insufficient to correctly understand hepatocyte function *in situ*, because their biological features such as growth, proliferation and expression of phenotypes are regulated in a complex and intricate manner by other types of liver cells (nonparenchymal cells), including resident vascular endothelial cells, stellate cells and Kupffer cells (macrophages), in addition to non-resident cells, such as immune-responsive cells. These cells are tightly associated, not only structurally, but also functionally, and constitute a well-organized biological entity, the liver. That is, hepatocytes fulfill their given tasks in the context of this specialized and unique community of different cell types. It is clear that even the most advanced modern biotechnological techniques cannot create a suitable *in vitro* environment to enable isolated populations of hepatocytes to function as they would *in vivo*; however, such cells can mimic some of their *in vivo* functions.

We accept that *in vitro* experimentation is superior to its *in vivo* counterpart in terms of understanding complex biological phenomena in that cause and effect relationships among the factors involved can be examined in a point-by-point manner. However, we are still far from the time when such complete *in vitro* experimentation will be possible. Currently, *in vivo* experimentation is considered to be better than *in vitro* experimentation for examining the normal phenotypes of hepatocytes, although the interpretation of results obtained in this way is not always simple because of the complex nature of the interactions between populations of various cell types.

Given these considerations, we sought to develop a small *in vivo* animal model for use in studying the biological and pharmaceutical phenotypes of h-hepatocytes. Additionally, for both *in vivo* and *in vitro* modeling, the development of technologies capable of producing large numbers of normal h-hepatocytes is required because normal h-hepatocytes are not readily available to researchers due to their obviously limited source. Generally, normal hepatocytes are not conducive for replicating *in vitro*, despite their high proliferative potential *in vivo*. If we could create such an animal model, h-hepatocytes could be readily propagated in a host liver.

To accomplish our goal to generate a small animal model with h-liver that can be utilized for investigation of drug metabolism, we utilized an immunodeficient and liver-injured mouse as a host. Such a mouse will be tolerant of receiving h-hepatocytes in its liver. The engrafted h-hepatocytes would be stimulated to actively proliferate in the liver because the host hepatocytes have been injured and they would repopulate the liver by expelling the not-replicable injured mouse (m)-hepatocytes. With this idea, we undertook intensive laboratory works investigating whether the xenogenic h-hepatocytes can actually collaborate with m-nonparenchymal cells in a

proper fashion and reconstruct a normal liver both functionally and histologically. These works resulted in generating a mouse whose hepatocytes are mostly of human origin.

We next undertook works to test whether the chimeric liver is practically humanized and suitable for predicting h-type drug metabolism. These continuous endeavors in collaborations with researchers in pharmacological, pharmaceutical and medical areas have enabled us to launch a 'humanized mouse factory' that produces homogeneous mice in a large scale whose livers are largely repopulated with h-hepatocytes and made them available to researchers on demand for drug testing. In our experience, we are able to conclude that the factory mouse can contribute to the needs for high-throughput predictive models required for drug discovery. In this article, we overview the development in producing liver-humanized mice and their usefulness for research and development (R&D) activities in discovering new medicines that are suitable to humans.

Although this humanized mouse provides an ideal *in vivo* model of h-liver compared to other currently available ones, it has some problems to be solved in the future due to the lack of other types of h-cells and endocrinological h-factors that affect the metabolic activities of hepatocytes. Including these issues, we present our opinions regarding studies that have not been explored enough yet, but are important in making the liver humanized mouse a much better tool for pharmaceutical researches.

2. Ample regenerative capacity of hepatocytes *in vivo*

In adults, the liver is functionally very active, but largely quiescent in terms of proliferation. The turnover rate of h-hepatocytes is around 1 year in rodents [1,2]; however, the same organ is ready for rapid regeneration if the liver mass is reduced. Generally, the weight of an organ is closely related to body weight [3]. The ratio of liver:body weight ($R_{L/B}$) in humans is about 2.4 – 2.6% [4]. If the $R_{L/B}$ falls below this range, residual hepatocytes in the G0 phase of the cell cycle enter G1 and progress to S phase within 24 h. The hepatocytes will continue to replicate until the $R_{L/B}$ reaches 2.4 – 2.6%. This weight loss-induced regeneration is conserved through life, although the capacity decreases somewhat with age.

The exceptionally high regenerative capacity of the liver *in vivo* suggests a means for the abundant propagation of h-hepatocytes, starting with small numbers of h-hepatocytes in the liver of an appropriate model animal. In terms of the best species for this type of experimental design, mice or rats are preferable because they are commonly used in the laboratory. A key requirement for this type of experimental design is the availability of an immunodeficient mouse or rat whose liver is damaged and, thus, whose hepatocytes are in a proliferative phase.

If a rodent model satisfying these requirements is available, we could propagate h-hepatocytes by engrafting them in the animal model liver. The engrafted h-hepatocytes would then