culture have been used to study the toxicity and metabolic pathways of chemical entities. However, the availability of freshly isolated normal h-hepatocytes is limited, and their quality is inconsistent among samples [1,2]. Additionally, hepatocyte functions, especially the metabolic activities on xenobiotics, and their susceptibility to hepatitis B virus (HBV) and C virus (HCV), are known to decline in cultures [3–7].

Previously, we successfully developed an h-hepatocyte chimeric mouse model by transplanting h-hepatocytes into the livers of albumin (Alb) enhancer-/promoter-driven urokinase-type plasminogen activator (uPA)-transgenic mice bearing severe combined immunodeficiency (SCID) gene [8–10]. In this murine liver, a large number (>70%) of h-hepatocytes replaced the host cells, retaining the normal features, such as pharmacological responses and susceptibility to HBV and HCV [8–13]. Thus, these chimeric mice are useful for the prediction of human metabolism of new drugs and the investigation of intrahepatic viral infection. However, their small body size restricted the availability of biological samples, such as blood and bile, for biochemical analyses and also caused difficulties in surgical manipulation.

Rats are medium-sized rodents that are 10 times larger than mice, particularly docile, and easy to handle. Furthermore, abundant pharmacological data have been accumulated in the studies with rats, because they have been traditionally and commonly used in the pre-clinical development of new drugs. These advantages led us to develop a method to utilize rats as larger hosts for h-hepatocyte transplantation. But neither gene-manipulated rat model with injury-induced liver like uPA transgenic mice had been developed nor immunodeficient rats lacking T and B cells, such as SCID, Rag2 knockout, and NOG mice, which have been used in xenotransplantation studies [8,14–16], are available, and we needed to search alternate model rats to achieve our purpose.

Recently, we attempted to produce "immunotolerant" rats with injury-induced liver. Their bone marrow cells (BMCs) were replaced with SCID mouse's BMCs after exposing rats to X-ray to generate immunotolerance [17]. These rats were treated with hepatotoxin retrorsine (RS), a pyrrolizidine alkaloid that cross-links to DNA, to inhibit hepatocyte proliferation, which induces liver injury when the liver is insulted. Actually, h-hepatocytes were able to engraft those livers, when animals were transplanted with h-hepatocytes following partial hepatectomy (PH), which provide a proliferative stimulus to transplanted cells. However, the engrafted h-hepatocytes were few and proliferated rarely. This low abundance of h-hepatocytes might be caused by insufficient growth stimulation: this model was only able to tolerate 40% PH, a low percentage PH compared with 70% PH, which is commonly and effectively used as a method to induce extensive liver repopulation in rats [18,19]. Moreover, several months (up to <10 weeks after birth) were required as a preparatory time period before transplantation to replace BMCs completely and to make liver failed, which may cause the rats to be too old to allow the transplanted cells to sufficiently grow.

In this study, we tried to make chimeric rats bearing h-hepatocytes using infant rats and avoiding time-consuming BMC replacement as a method to generate immunotolerance. Infant liver is considered to provide highly pro-proliferative microenvironments to hepatocytes, because infant hepatocytes can proliferate actively at levels similar to regenerating adult hepatocytes after PH [20]. Use of infant rats also allows us to reduce the required number of transplanted cells. RS was utilized as an inducer of liver damages as in the cited previous study [17]. The rats were treated with an immunosuppressant FK506 to generate immunotolerance after h-hepatocyte transplantation instead of BMC replacement. To improve the engraftment of h-hepatocytes, rats were given clodronate [21] and ascites of monoclonal antibody 3.2.3 [22] after h-hepatocyte transplantation, which are known to deplete Kupffer and natural killer cells, respectively.

Methods

Animals

Male and female dipeptidyl dipeptidase-IV mutant ($DPPIV^-$) Fischer 344 (F344) rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). These rats were allowed to mate and produce infant rats. The litters were kept with their mothers until the infants were weaned (for approximately 3 weeks). R-hepatocytes as donor cells were isolated from 9- to 13-week-old male DPPIV wild ($DPPIV^+$) F344 rats (Japan SLC, Inc., Shizuoka, Japan). $DPPIV^-$ and $DPPIV^+$ rats were used as recipient and donor, respectively, for identifying transplanted donor cells in the host liver. Alb enhancer-/promoter-driven uPA/SCID mice were also used as host animals. All animals were maintained under pathogen-free conditions. Our study received ethical approval from the Ethics Boards of the Hiroshima Prefectural Institute of Industrial

Science and Technology and PhoenixBio Co., Ltd. (Higashihiroshima, Japan).

Treatment of animals and hepatocyte transplantation

Two-week-old rats were given intraperitoneal injections of RS (Sigma, St. Louis, MO, USA) at 10 mg/kg. For syngenic hepatocyte transplantation, r-hepatocytes were isolated using the two-step collagenase perfusion method described previously [23]. Their viability was 93.3% (n = 2). For xenogeneic hepatocyte transplantation, we used cryopreserved h-hepatocytes derived from a 6-yr-old girl (BD Gentest. Woburn, MA), which was the best batch of cryopreserved hepatocytes among several available batches using the chimeric mouse model that we have described in detail [8,24,25]. We could quantitatively estimate the engraftment capacity and proliferative potential of donor hepatocytes by this model. The h-hepatocytes of the batch used in this study exhibited high engraftment levels and growth capacity, reaching >70% of replacement index (RI) in more than half of the tested mice at 12 weeks post-transplantation. The cells were thawed according to the manufacturer's protocol. The hepatocytes showed 75.7 ± 3.3% viability (n = 3) with a 17.9 ± 1.0 μm diameter (n = 3) after thawing and Percoll purification. Intra-batch variations among vials were quite small. Three days after RS treatment, hepatocytes (5.0×10^5 viable cells each) were transplanted into the rats via the portal vein. To deplete Kupffer and natural killer cells, the rats were injected intraperitoneally with 10 ml/kg of liposome-encapsulated clodronate [21] and 30 μl of ascites of monoclonal antibody 3.2.3 [22] 2 days before and 3 days after transplantation, respectively. These procedures improved the engraftment of donor hepatocytes in RS-treated infant rats (data not shown). All rats transplanted with h-hepatocytes were immunosuppressed with daily subcutaneous injections of FK506 (1 mg/kg; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) after transplantation until sacrifice. The same donor cells (1.0×10^6 cells/mice) were transplanted into 3-week-old uPA/SCID mice via spleen in the previous study [24].

Measurement of h-Alb in rat plasma

Plasma samples were collected periodically from the tail vein, and h-Alb levels were determined by enzyme-linked immunosorbent assay (Human Serum Albumin ELISA Quantitation Kit; Bethyl Laboratories, Inc., Montgomery, TX, USA). The antibodies used in this assay were h-specific and were not cross-reactive for rats.

Reverse-transcription (RT) PCR assays

To detect human transcripts, total RNA was extracted from the livers of normal non-transplanted rats, chimeric rats, and from h-hepatocytes (donor cells) using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). cDNA was synthesized using 1 μg of total RNA by PowerScript reverse transcriptase (Clontech Laboratories, Inc., San Jose, CA, USA) and Random Primer oligonucleotides (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The following h-specific or rat and human (r/h) cross-reactive genes were subjected to semiquantitative PCR using the primers listed in Table 1: *Alb, α1-antitrypsin (AAT), glucose-6-phosphatase (G6P), hepatocyte nuclear factor-4 (HNF-4), cytochrome P450 (CYP) 1A2, 2C9, 2D6, 2E1, 3A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).*

Immunohistochemistry and histochemistry

The rat livers were harvested at several time points after transplantation. When necessary, bromodeoxyuridine (BrdU) (50 mg/kg; Sigma) was injected intraperitoneally 1 h before sacrifice. Paraffin and frozen sections of 5 μm thickness were prepared from the liver tissues and were subjected to H&E or immunohistochemical staining using the primary antibodies listed in Table 2. The specificity of the primary antibodies was shown in Table 2. For bright-field immunohistochemistry, the antibodies were visualized with a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) or EnVision+ kit (DakoCytomation, Glostrup, Denmark) using DAB substrates and counterstained with hematoxylin. Fluorescence immunohistochemistry was performed using Alexa 488- or 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). To detect the transplanted $DPPIV^+$ r-hepatocytes, DPPIV

Table 1. Primer sets for RT-PCR

Gene	Forward primer	Reverse primer
h-*Alb*	5'-tgccgaagtggaaaatgatgag-3'	5'-gcaagtctcagcagcagcacg-3'
h-*AAT*	5'-accctttgaagtcaaggacaccg-3'	5'-ccattgctgaagaccttagtgatgc-3'
h-*G6P*	5'-tgggatccagtcaacacattac-3'	5'-caaaacccaccagtatggacgc-3'
h-*HNF-4*	5'-tcacctcccccgtctcc-3'	5'-tgcgatgctggcaatctt-3'
h-*CYP1A2*	5'-gcttctacatccccaagaaat-3'	5'-tcccacttggccaggact-3'
h-*CYP2C9*	5'-ccagatctgcaataatttttctc-3'	5'-caagctttcaatagtaaattcagatg-3'
h-*CYP2D6*	5'-tggatgagctgctaactgag-3'	5'-ggcatgtgagcctggtca-3'
h-*CYP2E1*	5'-cagcacaactctgagatatgggc-3'	5'-gggcatctcttgcctatcctt-3'
h-*CYP3A4*	5'-ctctgccttttttgggaaata-3'	5'-ggctgttgaccatcataaaag-3'
r/h -*GAPDH*	5'-accacagtccatgccatcac-3'	5'-tccaccaccctgttgctgta-3'

h, human-specific; r/h, cross-reactive with rat and human.

Table 2. Antibodies for immunohistochemical analysis

Antibody	Clone (Clone name)	Host	Sections	Supplier
BrdU	Monoclonal (Bu20a)	Mouse	Frozen	DakoCytomation, Glostrup, Denmark
h-Alb	Polyclonal	Goat	Frozen/Paraffin	Bethyl Laboratories, Inc., Montgomery, TX, USA
h-CK8/18	Monoclonal (NCL 5D3)	Mouse	Frozen	MP Biomedicals, Aurora, OH, USA
h-CK8	Monoclonal (35BetaH11)	Mouse	Frozen	NeoMarkers, Fremont, CA, USA
h-AAT	Polyclonal	Rabbit	Frozen	DakoCytomation, Glostrup, Denmark
r/h-HNF-4	Monoclonal (K9218)	Mouse	Frozen	Perseus Proteomics, Tokyo, Japan
h-CYP1A2	Polyclonal	Rabbit	Frozen	AFFINITI Research Products, Ltd., Exeter, UK
h-CYP2C9	Polyclonal	Rabbit	Frozen	Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan
r/h-CYP2D6	Polyclonal	Rabbit	Frozen	Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan
r/h-CYP2E1	Polyclonal	Rabbit	Frozen	AFFINITI Research Products, Ltd., Exeter, UK
h-CYP3A4	Polyclonal	Rabbit	Frozen	AFFINITI Research Products, Ltd., Exeter, UK

h, human-specific; r/h, cross-reactive with rat and human.

histochemical staining was performed as described previously [19].

Replacement index (RI) was calculated as the ratio of the area occupied by donor hepatocytes to the entire area examined in immunohistochemical [human; h-cytokeratin 8/18 (CK8/18)] or histochemical (rat; DPPIV) sections of three major lobes of seven total lobes, as described previously [8]. The ratios of $BrdU^+$-nuclei to h-$CK8/18^+$-hepatocytes were determined by counting cells in five randomly selected visual fields of the liver sections.

Results

Effect of RS treatment on infant rats

To examine whether the RS induces liver injury in the infant $DPPIV^-$ F344 rats, RS was administered to 2-week-old rats at 10 mg/kg. All animals tolerated the RS treatment for 3 weeks, but then some started to die, the survival rate being >83% at 5–6 weeks (n = 6). The livers were examined at 3 and at 5–6 weeks after treatment. The livers of RS-treated rats became atrophic and were smaller compared with those of the untreated animals: the liver weight to body weight ratio at 3 weeks and 5–6 weeks after treatment was 2.6 ± 0.1% (mean ± standard deviation, n = 4) and 2.7 ± 0.4% (n = 5) in RS-treated rats, respectively, vs. 3.5 ± 0.1% (n = 3) and 3.4 ± 0.2% (n = 3) in untreated rats, respectively. Histopathologically, hepatocytes of RS-treated infant rats were megalocytic, with large hyperchromatic nuclei (Fig. 1A, B), as reported previously in the case of those of RS-/PH-treated adult rats [18]. Thus, the RS-treated rats generated in the present study exhibit the two features required for an animal model to test the repopulation capacity of test hepatocytes: first its liver should have pro-proliferative microenvironments and, second, its hepatocytes are defective in replication.

Repopulation of normal rat adult hepatocytes in RS-treated infant liver

We tested the above consideration that the RS-treated infant rat could be suitable as a model for examining a donor hepatocyte's repopulation capacity by transplanting normal $DPPIV^+$ r-hepatocytes into nine RS-treated $DPPIV^-$ rat livers. The rat livers were subjected to DPPIV histochemi-

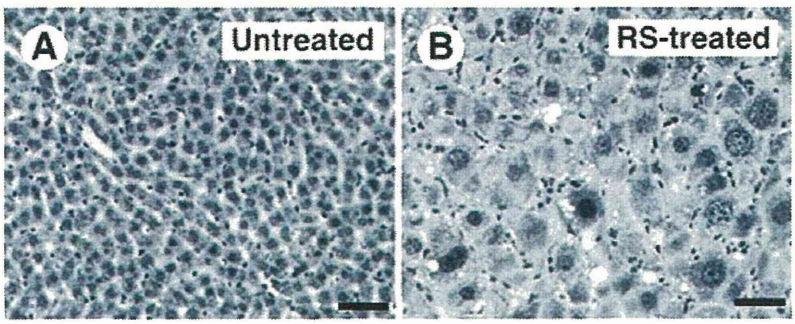

Fig. 1. RS-induced hepatocyte damages in infant rat liver. Two-week-old rats were untreated (A) or treated with RS (B), and the livers were harvested 5 to 6 weeks later for histological examinations by H&E staining, representatives of which are shown herein. Hepatocytes in RS-treated rat livers were much enlarged compared with those in untreated rat livers. Bars indicate 100 μm.

cal staining at appropriate time points post-transplantation. DPPIV$^+$ cells were visible within 1 week after transplantation as single cells or small clusters (n = 3, data not shown). Larger clusters were observed at 3 weeks post-transplantation (Fig. 2A,C), and size of DPPIV$^+$ clusters increased at 6 weeks post-transplantation (Fig. 2B,D). RI calculated on photographs as shown in Fig. 2C and D was 16.4 ± 6.7% ranging 10.9 to 23.8% (n = 3) and 48.3 ± 29.3% ranging 15.5 to 71.7% (n = 3) at 3 and 6 weeks post-transplantation, respectively. Thus, we concluded that our RS-treated infant rats are usable as a rat model to evaluate repopulation capacity of transplanted hepatocytes.

Repopulation of transplanted xenogeneic H-hepatocytes in RS-treated infant rat liver

H-hepatocytes (6-yr-old, 5.0×10^5 cells) were transplanted into five RS-treated infant rats via portal vein under immunosuppressive conditions. The plasma levels of h-Alb in these rats were detectable at 1 week and increased steadily thereafter, resulting 0.4 to 1.4×10^5 ng/ml h-Alb levels were detected at 3 weeks post-transplantation (Fig. 3A). There was variability in growth kinetics among individual hosts. We transplanted the same donor cells (1.0×10^6 cells) into uPA/SCID mice via spleen in the previous study [25]. Blood h-Alb levels were 1.7 to 5.9×10^5 ng/ml (n = 9) at 3 weeks post-transplantation, and >88% of the transplanted mice reached more than 70% RI (>6.0×10^6 ng/ml blood h-Alb) 5 to 8 week post-transplantation [25].

To validate the engraftment and repopulation of h-hepatocytes at the tissue level, the livers were subjected to immunohistochemical staining for h-CK8/18 (Fig. 3B) or h-Alb (Fig. 3D) at 3 weeks post-transplantation together with staining with H&E (Fig. 3C). The photographs shown herein are from the liver of no. 1 rat as representatives. H-CK8/18$^+$ or h-Alb$^+$ cells were visible as small clusters, demonstrating the engraftment and repopulation of h-hepatocytes. RI calculated from photographs shown in Fig. 3B was 2.5 ± 1.5% ranging 1.0 to 5.1% (n = 5).

Morphological features of h-hepatocyte colonies were examined on the serial liver sections stained with anti-h-Alb antibodies (Fig. 3D,E). The h-hepatocytes in colonies were morphologically normal, but their cytoplasm appeared to be "clear" (Fig. 3C), as we previously reported for h-hepatocytes in humanized mice generated with uPA/SCID mice as hosts [8]. No lymphocyte infiltrations were found in the FK506-treated chimeric rat livers.

The evidence that such colony formation was a result of replications of the engrafted h-hepato-

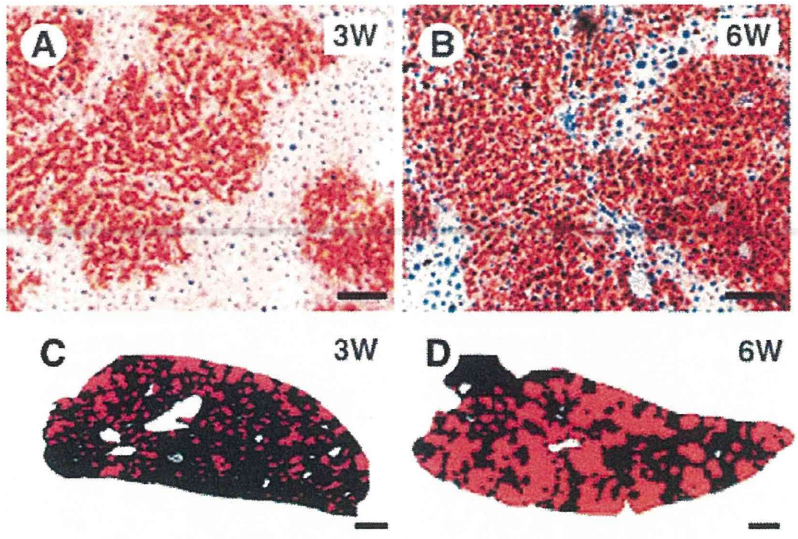

Fig. 2. Repopulation of normal adult r-hepatocytes in RS-induced damaged infant rat liver. Normal DPPIV$^+$ hepatocytes from 9- to 13-week-old F344 rats were transplanted into livers of three 2-week-old DPPIV$^-$-rats that had been treated with RS for 3 days before transplantation. The animals were killed for DPPIV-enzyme histochemistry at 3 (A) and 6 (B) weeks post-transplantation. These photographs are shown as representatives of the stains. RI was calculated using photographs C and D, low-magnification images of photographs A and B, respectively. To distinguish the donor cells clearly form the host cells, DPPIV$^+$ (donor) and DPPIV$^-$ (host) regions were converted to red and black, respectively. The transplanted DPPIV$^+$ hepatocytes were engrafted and repopulated in the recipient DPPIV$^-$ liver. Bars in A and B, and C and D indicate 100 μm and 1 mm, respectively.

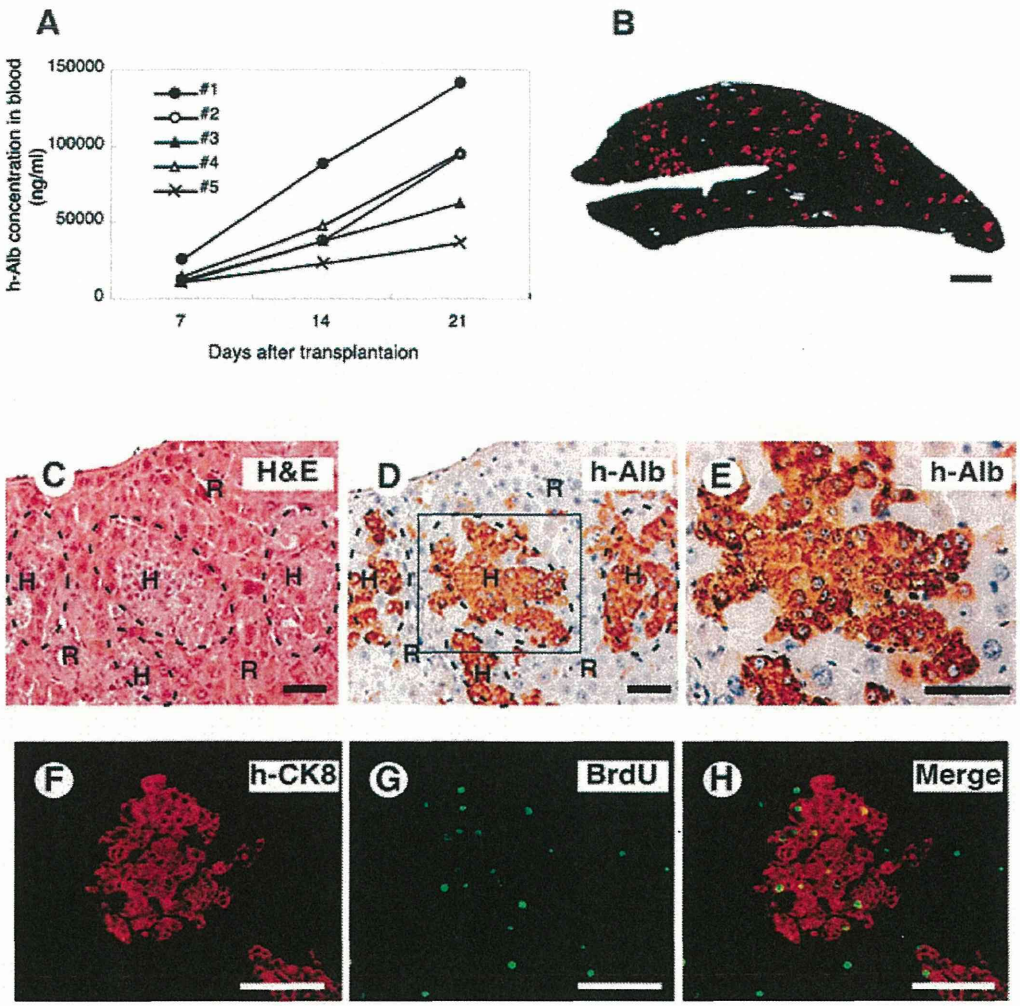

Fig. 3. Growth of h-hepatocytes in RS-treated rat livers. H-hepatocytes from a 6-yr-old donor were transplanted into five immunosuppressed 2-week-old RS-treated rats. The hosts for mitotic frequency experiments were injected with BrdU 1 hour before killing the animals. H-Alb blood levels were individually monitored weekly (A). Liver sections were prepared from these animals at 3 weeks post-transplantation and used for histochemical and immunohistochemical examinations (B through H). The results obtained from no. 1 rat liver are shown herein as representatives. The sections were stained for h-CK8/18 to calculate RIs (B). To distinguish the donor and the host cells clearly, h-CK8/18$^+$ (donor) and h-CK8/18$^-$ (host) regions were converted to red and black, respectively. The transplanted h-hepatocytes were engrafted and repopulated in the recipient rat livers. The liver sections were stained with H&E (C). Colonies of cells with clear cytoplasm were observed. Semi-serial sections of (C) were stained for h-Alb (D, E). The region enclosed by the square in D is magnified and shown in E. H-Alb-positive cells were uniform in size, and most were mononuclear. R and H indicate regions of r-hepatocyte (host) and h-hepatocyte (donor), respectively. Double immunostaining was performed for h-CK8 (F) and BrdU (G). Photographs F and G were merged to create the photograph H. Some h-CK8$^+$ cells were labeled with BrdU. Bars indicate 1 mm (B) and 100 μm (C–H).

cytes was obtained from BrdU-labeling experiments (Fig. 3F–H), in which the presence of BrdU-positive donor cells is clearly shown. BrdU-labeling index for h-hepatocytes in the colonies was 5.9 ± 1.8% (n = 5).

Gene and protein expression profiles of H-hepatocytes in RS-treated infant rat livers

Three h-hepatocyte-transplanted rats (nos. 1, 2, 4) were selected from the rats shown in Fig. 3A at 3 weeks post-transplantation because of their relatively higher levels of blood h-Alb (approximately 1.0×10^5 ng/ml) and were used to extract RNAs from the liver to examine gene-expression profiles by RT-PCR. As a result, it was found that these livers expressed the genes known as hepatocyte-specific or functional markers such as h-*Alb*, h-*AAT*, h-*G6P*, h-*HNF-4*, and h-*CYP 1A2*, *2C9*, *2D6*, *2E1*, and *3A4* (Fig. 4A).

Protein expression in the chimeric rat livers was immunohistochemically investigated using the liver

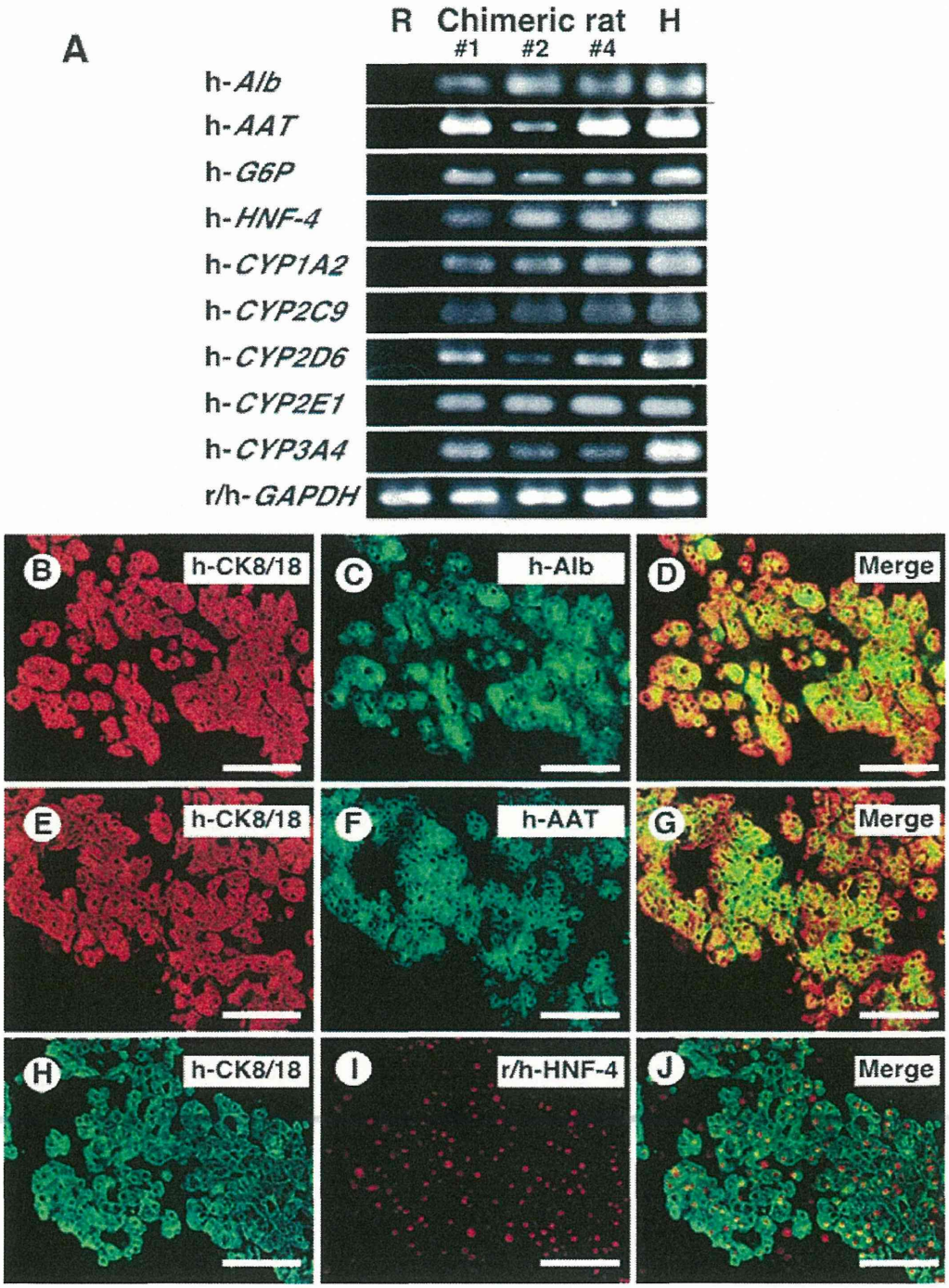

Fig. 4. Profiles of gene- and protein-expressions. Livers were obtained from three rats (nos. 1, 2, 4 shown in Fig. 3A) at 3 weeks after transplantation. A. Gene expressions. Total RNA was extracted from the livers and used as templates for semiquantitative RT-PCR for h-*Alb*, h-*AAT*, h-*G6P*, h-*HNF-4*, and h-*CYP1A2*, *2C9*, *2D6*, *2E1*, and *3A4*. R/h-*GAPDH* was used as an internal control. Liver tissues from DPPIV$^-$ rats that did not receive transplantation (R) were used as negative controls (the most left column) and donor h-hepatocytes (H) as positive controls (the most right column). The tested h-genes were all detected in the three chimeric rat livers except in the untransplanted rat liver. B–J. Histological sections were prepared from the above livers and immunostained for h-CK8/18 (B, E, H), h-Alb (C), h-AAT (F), and r/h-HNF-4 (I). Photographs B and C were merged to create the photograph D. Similarly, photographs of E and F were merged to the photograph G and photographs H and I to create the photograph J. Most h-CK8/18$^+$ cells were h-Alb$^+$ and h-AAT$^+$. About half of the h-CK8/18$^+$ cells were HNF-4$^+$. Because HNF-4 antibodies were cross-reactive with rat and human, some host r-hepatocytes could be also positive. Open bars in B to J indicate 100 μm.

tissues as a measure of functionality of h-hepatocytes in the colonies of the host liver (Figs 4B–J, 5). The photographs shown herein are from the liver of no. 1 rat as representatives. H-CK8/18$^+$ cells shown in Fig. 4B,E, and H all expressed the following tested liver-specific markers: h-Alb (Fig. 4C,D), h-AAT (Fig. 4F,G), and r/h-HNF-4 (Fig. 4I,J), respectively. We also investigated the expressions of five major h-CYP proteins in the humanized rat livers. Some h-CK8/18$^+$ cells located near the central veins shown in Figs. A and M expressed h-CYP1A2 (Fig. 5B and C) and 3A4 (Fig. 5N and O), respectively. Most h-CK8/18$^+$ cells shown in Fig. 5D,G, and J expressed h-CYP2C9 (Fig. 5E,F), r/h-CYP2D6 (Fig. 5H,I), and r/h-CYP2E1 (Fig. 5K,L), respectively. These results all support the notion that these repopulated h-hepatocytes maintain normal functions in the rat-liver tissues.

Survival of the humanized rats

The rats with humanized livers were further observed after 3 weeks post-transplantation regarding their health and survival. A half (3 of 6, 50%) of the animals died within 4 weeks post-transplantation, and the rest died by 6 weeks (0 of 3, 0%). This survival rate and timing of death were similar to those of RS- and FK506-treated rats that did not receive hepatocyte transplantation (3 of 4, 75% at 2 weeks, 0 of 3, 0% at 5 weeks) and were more severe compared with RS single treatment (>83% at 5 to 6 weeks after treatment). However, when the RS- and FK506-treated rats were transplanted with r-hepatocytes, their survival rate within 3 weeks a little improved to 5 of 6 (>83%) and 6 weeks were 1 of 2 (50%). These results may indicate that the combined treatment of infant rats with RS and FK506 was lethal, but the high mortality could be rescued by r-hepatocyte transplantation at some extent. However, h-hepatocyte transplantation did not improve the survival rate of the RS- and FK506-treated rats probably, because the RI in h-hepatocyte transplantation was much lower than that in r-hepatocyte transplantation. Then, we tested the possibility that FK506 suppressed the growth of transplanted donor hepatocytes. The RI of FK506-treated rat livers (20.4 ± 8.8%; n = 3) at 3 weeks post-transplantation was similar to that of livers that were not treated with FK506 (16.4 ± 6.7%; n = 3). Moreover, it should be noted that all infant rats that received daily FK506 treatment (n = 3) survived 6 weeks (100%) when they were not treated with RS. These results suggest that treatment with FK506 did not influence survival rate of host rats nor proliferation of donor cells.

Discussion

Production of h-hepatocyte-repopulated chimeric rats requires the host animal to exhibit at least two features, immunotolerance, and possession of liver that has pro-proliferative microenvironments and its hepatocytes are in replication-defect. Recently, we produced chimeric rats by transplanting h-hepatocytes into PH-livers of RS-treated "SCID" rats, whose BMCs almost had been repopulated with SCID mouse BMCs [17]. H-hepatocytes were able to engraft the rat liver. But the repopulation of h-hepatocytes in this study was considered to be quite low, because blood h-Alb levels were approximately 100 ng/ml at best [17].

Wu et al. [26] produced h-hepatocyte chimeric rats for HBV infection study. H-hepatocytes were injected into the abdominal cavities of fetal rats through the uteri of pregnant rats with the intention to endow fetal rats with immunotolerance. The newborn rats at 24 h after birth were transplanted with h-hepatocytes via the spleen. The authors calculated the extent of chimerism at 16 weeks post-transplantation by dot blotting of h-Alb DNA in the chimeric rat liver, which showed that one h-hepatocyte was present among 6.0×10^3 r-hepatocytes, indicating RI = 0.017% [27].

The humanized chimeric rats generated in the present study seem to be superior to those produced in these preceding studies at least regarding the accomplished RI. In the present study, we utilized RS-treated infant rats as hosts, considering that the former (RS treatment) provides the host with hepatocytes that are in replication-defect, and the latter (infancy) provides the host with the liver that has proliferative microenvironments. Because hepatocytes from an infant (an early growing rat) show a high proliferative capacity: they increase their numbers by approximately 3-fold during 2 to 3 weeks after birth, and this is proportional to the increasing body weight [20]. It is thought that the infant liver provides hepatocytes with pro-proliferative microenvironments composed of auto-, para-, and juxtacrine stimulants, and a comparable situation can be found in the adult liver after PH. With adult RS/PH rats as hosts, we showed that adult rat donor hepatocytes repopulated approximately 10 to 15% and 40 to 50% of the host rat liver at 2 weeks and 1 month after transplantation, respectively [18]. In our RS infant rat model, the adult rat donor hepatocytes repopulated approximately 16 and 49% of the host liver at 3 weeks and 6 weeks post-transplantation, respectively.

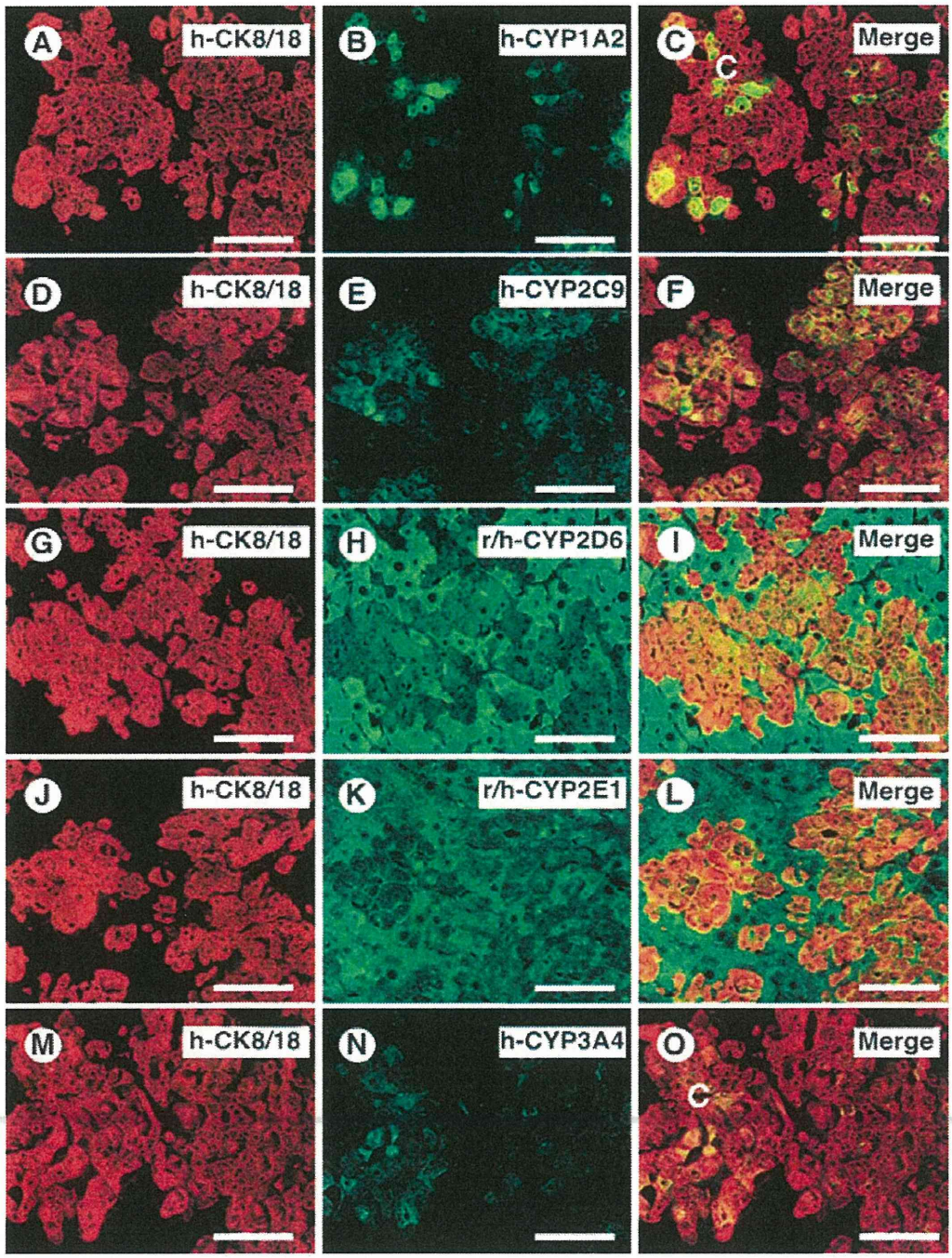

Fig. 5. h-CYP expressions of h-hepatocytes in RS-treated rat livers. Histological sections were prepared from chimeric rat livers at 3 weeks post-transplantation as in Fig. 4B–J. The liver sections were all stained for h-CK8/18 (A, D, G, J, M), which were then for h-CYP1A2 (B), h-CYP2C9 (E), r/h-CYP2D6 (H), r/h-CYP2E1 (K), and h-CYP3A4 (N). H-CYP1A2, 2C9, and 3A4 antibodies were h-specific, and CYP2D6 and 2E1 were not h-specific (r/h). The photographs in the right column (C, F, I, L, O) are obtained by merging the photographs in the left (A, D, G, J, M) and middle columns (B, E, H, K, N) on the corresponding lines. Most h-CK8/18$^+$ cells were also CYP2C9$^+$, 2D6$^+$ and 2E1$^+$. H-CK8/18$^-$ cells near the central veins ("C" in photographs C and O) were positive to CYP1A2 and 3A4. Open bars indicate 100 μm.

Although we cannot make a direct comparison of the proliferative microenvironment of the host liver from these independent studies, our results indicate a distinct similarity between the proliferative microenvironments of the host liver in adults and infants. To compare the pro-proliferative

The combined treatment of infant rats with RS and FK506 was lethal, whereas the singular treatment with RS or FK506 did not influence mortality. Although r-hepatocyte transplantation could improve mortality rates at some extent, h-hepatocytes could not. This may be attributable to the above-mentioned differences in growth kinetics between the h- and r-hepatocytes. We are not able to utilize the presently developed humanized rat model for studies that require long-term observations of the xenograft. The depletion of immune cells by antibodies against immune-responsive cells [32–34] could be an alternative method to the administration of immunosuppressive agents. Recently, X-SCID rats were generated using the zinc-finger nuclease method [35]. X-SCID or SCID rats will be preferable hosts for the production of h-hepatocyte chimeric rats. The RS-treated infant rat model presented in this study should be applicable for X-SCID or SCID rats to develop liver-humanized chimeric rats. In the present study, infant rats were treated with 10 mg/kg of RS before hepatocyte transplantation. Rats younger than approximately 20 days are known to be more sensitive to the hepatotoxic effects of RS than older animals. Gender-based differences in the metabolism of RS were significantly observed at approximately 30 days [36]. In our preliminary study, we treated 1- and 2-week-old infant rats with RS. When r-hepatocytes were transplanted into 1-week-old RS-treated rats, the repopulation index at 3 weeks after transplantation was lower than that at 2 weeks after transplantation. We also attempted 2 RS doses—5 mg/kg and 10 mg/kg—because the LD50 of RS is known to be 10 to 14 mg/kg in 2-week-old rats [36]. When 5 mg/kg of RS was injected into 2-week-old rats, we observed that liver damage was not severe and the liver to body weight ratio did not change. Thus, we selected 10 mg/kg as the final dose. Currently, it is considered that the conditions to treat rats with RS for damaging the rat liver should be optimized to obtain a better host survival rate. Overexpression of uPA in the mouse model has been well accepted as a suitable method to induce host liver injury for repopulation by xenogeneic hepatocytes. This is not the case for RS treatment.

Rodents are widely used as animal models for investigating the physiology and pathology of the liver. However, the liver characteristics of different species are well documented, especially between humans and rodents. This hinders the ability to correlate experimental data from animal models to the human. Therefore, h-hepatocytes are vital tools to investigate liver function, and, as such, it is important to know their physiological and pathological characteristics under experimentally controllable conditions. Our ultimate goal is to generate rats with >70% h-hepatocytes. It is evident that these rats (hepatocyte-humanized rats) greatly contribute to deepening our understanding concerning the characteristics of h-hepatocytes. This rat model is superior to the conventional mouse model because blood samples are more easily accessible and available in larger volumes. These samples are a critical source of understanding the characteristics of h-hepatocytes. This rat model will be useful in pharmacokinetic studies, HBV- or HCV-infection studies, toxicity tests, and carcinogenicity studies for drug development. In addition, this model will prove to be an important tool for xenograft studies with the ultimate goal of enhancing our understanding of clinical hepatocyte transplantation across species.

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Author contributions

AT and CT conceived, designed, and coordinated the study and wrote the draft manuscript. CT and KY revised the manuscript.

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potential of the adult and infant hepatic microenvironment, we calculated the average size of colonies formed by adult rat donor hepatocytes at 3 weeks after transplantation in adult and infant rat models. The average size of a colony is measured in terms of area in the adult RS/PH rat model and the infant RS rat model and was found to be approximately 26 000 μm^2 [28] and 60 000 μm^2 (this study), respectively. This result indicates that the pro-proliferative potential is higher in the infant liver than in its adult counterpart. This model has some inherent advantages such as PH, quite invasive operation to the host, is unnecessary and the amount of transplanted hepatocytes is reducible quite much in comparison with the RS-/PH-treated adult rat model [18,19]. Our RS-treated infant rat model might be useful as host for liver repopulation studies using a limited number of liver stem cells or progenitor cells derived from adult tissues such as induced pluripotent and embryonic stem cells as donors.

To incorporate xenogeneic hepatocytes into this RS-treated infant rat model, we used FK506 to suppress immune reactivity. We did not notice lymphocyte infiltrations in the FK506-treated chimeric rat livers, which led us to conclude that the FK506 treatment successfully inhibited the host rejection reactions. H-hepatocytes engrafted and repopulated the rat liver at an appreciable level of RI (1 to 5% at 3 week post-transplantation), although the level was much lower than that (11 to 24% at the corresponding time point post-transplantation) accomplished in the transplantation of syngeneic r-hepatocytes. However, the RI value by h-hepatocytes (in the highest case, 5% at 3 weeks post-transplantation, corresponding to 1.4×10^5 ng/ml h-Alb in plasma) was quite high in rat liver compared with the previous study [17,27]. These repopulated h-hepatocytes were concluded to be morphologically normal from microscopical observations on histological sections and also biochemically functional because they expressed the representative h-hepatocyte-specific transcripts and proteins. However, there are also some differences between h-hepatocytes in the chimeric rat liver and in the human body liver. For example, h-CYP1A2, 3A4, and 2E1 expressions were reported to show zonation in normal livers in that only hepatocytes located near the central veins express these proteins [29]. In the present study, the former two CYPs were localized near the central veins, but h-CYP2E1 was expressed diffusedly in liver lobules.

The observed difference in RIs between h- and r-hepatocyte transplantation is not likely to have been caused by the immunosuppressive therapy because the RI of r-hepatocytes did not change in the host rats that received FK506 daily. Wu et al. [30] reported that FK506 perturbed neither transplanted cell engraftment nor proliferation in syngeneic r-hepatocyte transplantation using the RS/PH model, which supports our results. Recently, we generated chimeric mice using uPA/SCID mice as hosts and concordant (rat) and disconcordant (human) hepatocytes as donors [25,31]. The h-hepatocytes proliferated more slowly in the mouse liver and required longer periods of time to achieve near-complete repopulation than the r-hepatocytes, suggesting the difference in proliferation kinetics of h- and r-hepatocytes. This difference could explain the lower RIs of h-hepatocytes. In addition, r- and h-hepatocytes might respond differently to the hepatic microenvironments in the host liver. Molecular incompatibilities in a discordant xenogeneic combination (human to mouse) may greatly impair the engraftment and/or growth efficiency compared with those in a concordant xenogeneic combination (rat to mouse) [24,25].

This study was performed by comparing the results and data on the chimeric mouse model. We transplanted 1.0×10^6 human child (6 yrs old) hepatocytes/6 g body weight (1.67×10^5 cells/g) in the mouse model [24]. However, in the rat model, we transplanted the same donor cells, but in lower quantities (5.0×10^5 cells/20 g body weight, 0.25×10^5 cells/g), accounting for 85% less donor cells/g body weight. The transplant was delivered through the spleen in the mouse model, but through the portal vein in the rat model. Irrespective of the fact that only 15% of donor cells in the mouse model were transplanted, h-Alb levels at 3 weeks post-transplantation (0.4 to 1.4×10^5 ng/ml) in the rat model were comparable to those (1.7 to 5.9×10^5 ng/ml) in the mouse model [24]. When child (9 months old) cells were injected, h-Alb levels at 3 weeks post-transplantation were $2.3 \pm 0.6 \times 10^5$ ng/ml ($n = 3$) ($7.4 \pm 4.1\%$ in the repopulation rate) [25] and $0.9 \pm 0.4 \times 10^5$ ng/ml ($n = 5$) ($2.5 \pm 1.5\%$) for the mouse and rat models, respectively. These data showed that engraftment in the host liver and early growth in the rat model took place at comparable levels to the mouse model. This finding indicates that the number of adopted h-hepatocytes was sufficient for repopulation at comparable levels to the mouse model. As such, no problems exist concerning the quality of the donor cells. We also examined donor cell quality prior to transplantation. Taken together, we concluded that h-hepatocytes are able to engraft the host liver and grow well therein, for at least 3 weeks post-transplantation.