virus replicon clone in mouse cells. Virol J 2006;3:89

56. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-6

57. Bissig KD, Wieland SF, Tran P, et al. Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. J Clin Invest 2010;120:924-30

58. Tsuge M, Hiraga N, Takaishi H, et al. Yoshizato K. nfection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology 2005;42(5):1046-54

59. Kimura T, Imamura M, Hiraga N, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. J Gen Virol 2008;89(Pt 9):2108-13

60. Kamiya N, Iwao E, Hiraga N, et al. Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor. J Gen Virol 2010;91(Pt 7):1668-77

61. Ohara E, Hiraga N, Imamura M, et al. Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice. J Hepatol 2011;54(5):872-8

62. Abe H, Imamura M, Hiraga N, et al. ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo. J Hepatol 2011;55(1):11-18

63. Perni RB, Almquist SJ, Byrn RA, et al. Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. Antimicrob Agents Chemother 2006;50:899-909

64. Hiraga N, Imamura M, Abe H, et al. Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. Hepatology 2011;54(3):781-8

65. Song X, Guo Y, Duo S, et al. A mouse model of inducible liver injury caused by tet-on regulated urokinase for studies of hepatocyte transplantation. Am J Pathol 2009;175:1975-83

66. Held WA, Mullins JJ, Kuhn NJ, et al. T antigen expression and tumorigenesis in transgenic mice containing a mouse major urinary protein/SV40 T antigen hybrid gene. EMBO J 1989;8:183-91

67. Weglarz TC, Degen JL, Sandgren EP. Hepatocyte transplantation into diseased mouse liver. Kinetics of parenchymal repopulation and identification of the proliferative capacity of tetraploid and octaploid hepatocytes. Am J Pathol 2000;157:1963-74

68. He Z, Zhang H, Zhang X, et al. Liver xeno-repopulation with human hepatocytes in Fah-/-Rag2-/- mice after pharmacological immunosuppression. Am J Pathol 2010;177:1311-19

69. Escribano O, Fernández-Moreno MD, Piña MJ, et al. Pretreatment with FK506 up-regulates insulin receptors in regenerating rat liver. Hepatology 2002;36:555-61

70. Legrand N, Ploss A, Balling R, et al. Humanized mice for modeling human infectious disease: challenges, progress, and outlook. Cell Host Microbe 2009;6:5-9; Review

71. Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature 2005;436:946-52; Review

72. Washburn ML, Bility MT, Zhang L, et al. A humanized mouse model to study hepatitis C virus infection, immune response, and liver disease. Gastroenterology 2011;140:1334-44

•• **The authors put forward the idea and technology for the second-generation chimeric mice, that is, mice with dual chimerisms, hepatocytes and immune cells.**

73. Di Stasi A, Tey SK, Dotti G, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med 2011;365(18):1673-83

74. Pajvani UB, Trujillo ME, Combs TP, et al. Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipoatrophy. Nat Med 2005;11:797-803

75. Robinet E, Baumert TF. A first step towards a mouse model for hepatitis C virus infection containing a human immune system. J Hepatol 2011;55:718-20

76. Bataller R, Paik YH, Lindquist JN, et al. Hepatitis C virus core and nonstructural proteins induce fibrogenic effects in hepatic stellate cells. Gastroenterology 2004;126:529-40

77. Utoh R, Tateno C, Kataoka M, et al. Hepatic hyperplasia associated with discordant xenogeneic parenchymal-nonparenchymal interactions in human hepatocyte-repopulated mice. Am J Pathol 2010;177:654-65

78. Tsuge M, Fujimoto Y, Hiraga N, et al. Hepatitis C virus infection suppresses the interferon response in the liver of the human hepatocyte chimeric mouse. PLoS ONE 2011;6(8):e23856

79. Okazaki A, Hiraga N, Imamura M, et al. Severe necroinflammatory reaction caused by natural killer cell-mediated Fas/Fas ligand interaction and dendritic cells in human hepatocyte chimeric mouse. Hepatology 2012;56:555-66

80. Turrini P, Monego G, Gonzalez J, et al. Human hepatocytes in mice receiving]g pre-immune injection with human cord blood cells. Biochem Biophys Res Commun 2005;326(1):66-73

81. Cruse JM, Lewis RE Jr. The immune system victorious: selective preservation of self. Immunol Res 1993;12(2):101-14; Review

82. Porteus MH, Carroll D. Gene targeting using zinc finger nucleases. Nat Biotechnol 2005;23(8):967-73

83. Geurts AM, Cost GJ, Freyvert Y, et al. Knockout rats via embryo microinjection of zinc-finger nucleases. Science 2009;325(5939):433

84. Mashimo T, Takizawa A, Kobayashi J, et al. Generation and characterization of severe combined immunodeficiency rats. Cell Rep 2012;2(3):685-94

85. Katayama S, Tateno C, Asahara T, Yoshizato K. Size-dependent in vivo growth potential of adult rat hepatocytes. Am J Pathol 2001;158(1):97-105

86. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126(4):663-76

87. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-20

88. Si-Tayeb K, Noto FK, Nagaoka M, et al. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 2010;51(1):297-305

89. Takayama K, Inamura M, Kawabata K, et al. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1alpha transduction. J Hepatol 2012;57(3):628-36

90. Schwartz RE, Trehan K, Andrus L, et al. Modeling hepatitis C virus infection using human induced pluripotent stem cells. Proc Natl Acad Sci USA 2012;109(7):2544-8

•• **This paper demonstrated that iPS cell-derived hu-hepatocyte-like cells are permissive to HCV infection in culture and suggests their usefulness as a faithful in vitro modeling of HCV-induced hepatic diseases.**

91. Wu X, Robotham JM, Lee E, et al. Productive hepatitis C virus infection of stem cell-derived hepatocytes reveals a critical transition to viral permissiveness during differentiation. PLoS Pathog 2012;8(4):e1002617

92. Marukian S, Andrus L, Sheahan TP, et al. Hepatitis C virus induces interferon-λ and interferon-stimulated genes in primary liver cultures. Hepatology 2011;54(6):1913-23

93. Espejel S, Roll GR, McLaughlin KJ, et al. Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. J Clin Invest 2010;120(9):3120-6

94. Grompe M, Lindstedt S, al-Dhalimy M, et al. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. Nat Genet 1995;10(4):453-60

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XENOTRANSPLANTATION

Original Article

Repopulation of the immunosuppressed retrorsine-treated infant rat liver with human hepatocytes

Tachibana A, Tateno C, Yoshizato K. Repopulation of the immunosuppressed retrorsine-treated infant rat liver with human hepatocytes. Xenotransplantation 2013: 20: 227–238. © 2013 John Wiley & Sons A/S.

Abstract: Background: We previously generated humanized chimeric mice by transplanting h-hepatocytes into the livers of the diseased-liver transgenic mouse model with immunodeficient background. These mice with livers mostly replaced by human (h) hepatocytes have been proved to be useful for research on drug metabolism and toxicity and on intrahepatic pathogens such as hepatitis. However, their small body size prohibited collecting sufficient biological samples and made surgical manipulation difficult, which motivated us to produce humanized larger animal(s) bearing h-hepatocytes.
Methods: Fischer 344 (F344) rats at 2 weeks of age were administrated with hepatotoxin retrorsine (RS) and then transplanted with syngeneic F344 rat (r)- or h-hepatocytes via the portal vein. The hosts were injected daily with FK506 immunosuppressant. The livers were harvested periodically for determining donor-cell replacement ratios and compared with those of the humanized chimeric mice, and liver-specific mRNA and protein expressions by immunohistochemistry and reverse-transcription PCR.
Results: RS treatment of infant rats inhibited hepatocyte proliferation, resulting in decreased liver weight and megalocytic changes in hepatocytes. R-hepatocytes transplanted into RS-treated rats engrafted into and repopulated the liver at ratios of 16.4 ± 6.7% and 48.3 ± 29.3% at 3 and 6 weeks after transplantation, respectively. H-hepatocytes also engrafted into the rat liver and showed a repopulation ratio of 2.5 ± 1.5% at 3 weeks post-transplantation, which was comparable to the ratio in the humanized chimeric mouse model at least until 3 weeks. Propagated h-hepatocytes in the rat liver expressed hepatocyte-specific mRNA and proteins at least 3 weeks after transplantation.
Conclusions: Xenogeneic hepatocytes were able to engraft rat liver and grow well therein for at least 3 weeks post-transplantation in rats when immunosuppression was combined appropriately with liver injury at comparable levels to the well-characterized humanized chimeric mouse model.

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Key words: human hepatocytes – retrorsine – xenotransplantation

Abbreviations: Alb, albumin; AAT, α1-antitrypsin; BMCs, bone marrow cells; BrdU, bromodeoxyuridine; CYP, cytochrome P450; CK, cytokeratin; DPPIV, dipeptidyl dipeptidase-IV; F344, fischer 344; G6P, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBV, hepatitis B virus; HCV, hepatitis C virus; HNF-4, hepatocyte nuclear factor-4; h, human; PH, partial hepatectomy; r, rat; r/h, rat and human; RI, replacement index; RS, retrorsine; RT, reverse transcription; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

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Introduction

Hepatocytes play a major role in a variety of metabolic processes mediated by the reactions of many kinds of enzymes. Because humans and animals differ in isoform composition, expression levels, and catalytic activities of drug-metabolizing enzymes, human (h) hepatocytes under in vitro

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′-acccttttgaagtcaaggacaccg-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-

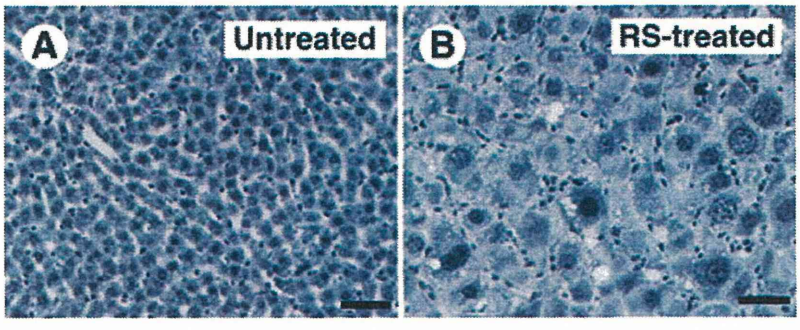

Fig. 1. RS-induced hepatocyte damages in infant rat liver. Two-week-old rats were untreated (A) or treated with RS (B), and the livers were harvested 5 to 6 weeks later for histological examinations by H&E staining, representatives of which are shown herein. Hepatocytes in RS-treated rat livers were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

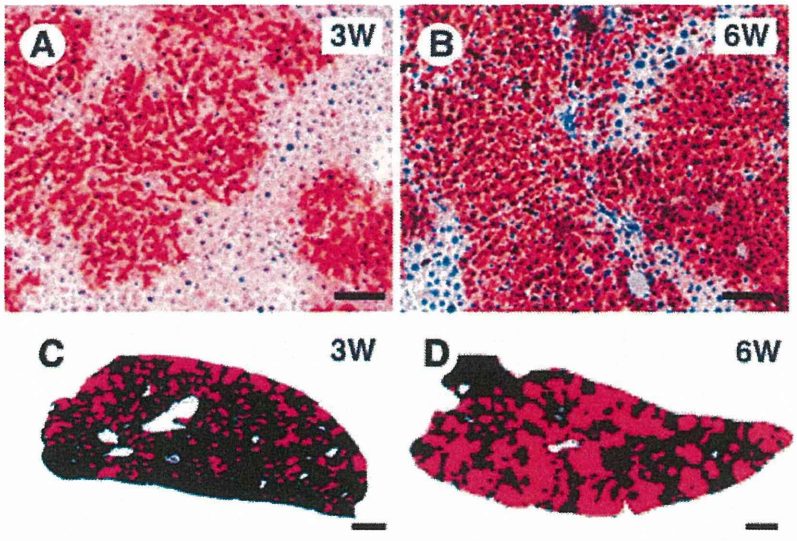

Fig. 2. Repopulation of normal adult r-hepatocytes in RS-induced damaged infant rat liver. Normal DPPIV$^+$ hepatocytes from 9- to 13-week-old F344 rats were transplanted into livers of three 2-week-old DPPIV$^-$-rats that had been treated with RS for 3 days before transplantation. The animals were killed 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.

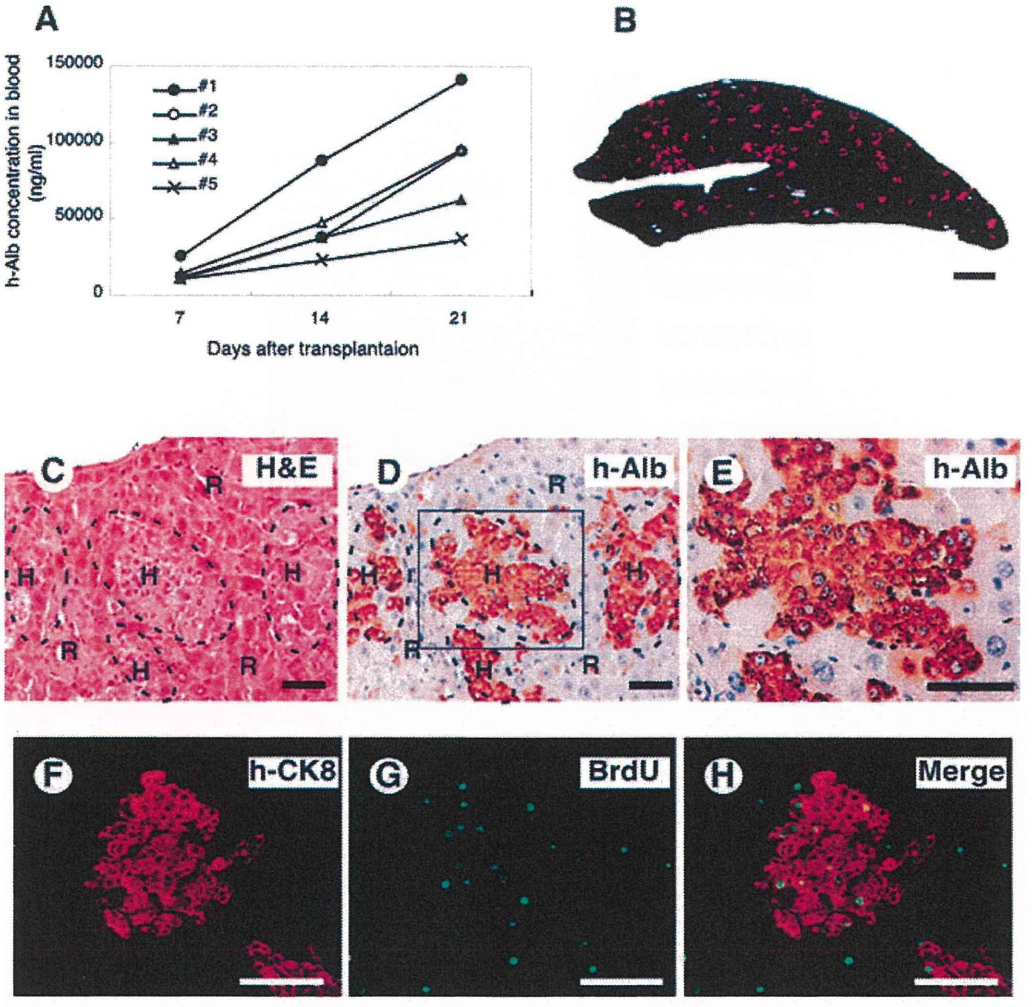

Fig. 3. Growth of h-hepatocytes in RS-treated rat livers. H-hepatocytes from a 6-yr-old donor were transplanted into five immunosuppressed 2-week-old RS-treated rats. The hosts for mitotic frequency experiments were injected with BrdU 1 hour before killing the animals. H-Alb blood levels were individually 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

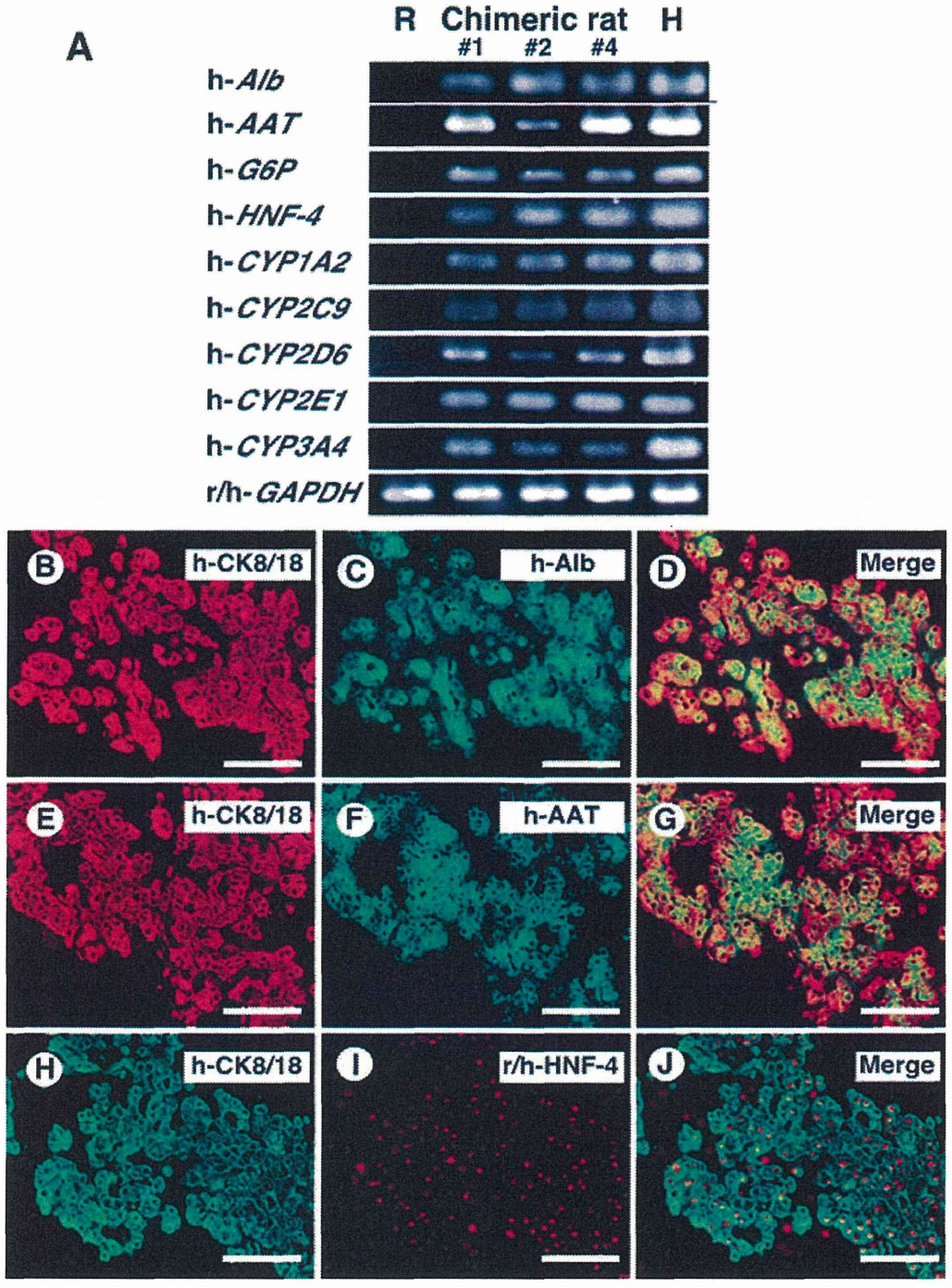

Fig. 4. Profiles of gene- and protein-expressions. Livers were obtained from three rats (nos. 1, 2, 4 shown in Fig. 3A) at 3 weeks after transplantation. A. Gene expressions. Total RNA was extracted from the livers and used as templates for semiquantitative RT-PCR for h-*Alb*, h-*AAT*, h-*G6P*, h-*HNF-4*, 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.